



Office de la Propriété
Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2102115 C 2009/04/07

(11)(21) **2 102 115**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 1992/04/27

(87) Date publication PCT/PCT Publication Date: 1992/11/12

(45) Date de délivrance/Issue Date: 2009/04/07

(85) Entrée phase nationale/National Entry: 1993/10/29

(86) N° demande PCT/PCT Application No.: US 1992/003419

(87) N° publication PCT/PCT Publication No.: 1992/019760

(30) Priorité/Priority: 1991/04/30 (US693,388)

(51) Cl.Int./Int.Cl. *C07K 14/705* (2006.01),
C07K 14/745 (2006.01), *C07K 16/28* (2006.01),
C07K 7/04 (2006.01), *C07K 7/06* (2006.01),
G01N 33/543 (2006.01), *G01N 33/566* (2006.01),
G01N 33/577 (2006.01), *G01N 33/58* (2006.01)

(72) Inventeurs/Inventors:
GINSBERG, MARK H., US;
FRELINGER, ANDREW L, III, US;
PLOW, EDWARD F., US

(73) Propriétaire/Owner:
THE SCRIPPS RESEARCH INSTITUTE, US

(74) Agent: FETHERSTONHAUGH & CO.

(54) Titre : METHODE DE DETERMINATION IN VITRO D'ACCIDENTS THROMBOTIQUES IN VIVO

(54) Title: IN VITRO METHODS FOR DETERMINING IN VIVO THROMBOTIC EVENTS

(57) Abrégé/Abstract:

Diagnostic systems, methods, polypeptides and antibodies for detecting the presence of C-terminal hGP1Ib fragment of the platelet receptor GP1Ib-IIIa in a body fluid sample are disclosed.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/00, G01N 33/53	A1	(11) International Publication Number: WO 92/19760 (43) International Publication Date: 12 November 1992 (12.11.92)
(21) International Application Number: PCT/US92/03419 (22) International Filing Date: 27 April 1992 (27.04.92) (30) Priority data: 693,388 30 April 1991 (30.04.91) US (71) Applicant: THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US). (72) Inventors: GINSBERG, Mark, H. ; 2944 Renault Place, San Diego, CA 92122 (US). FRELINGER, Andrew, L., III ; 7 Westward Circle, North Reading, MA 01864 (US). PLOW, Edward, F. ; 4359 Rous Street, San Diego, CA 92122 (US).		(74) Agents: FITTING, Thomas et al.; The Scripps Research Institute, 10666 North Torrey Pines Road, Mail Drop TPC 8, La Jolla, CA 92037 (US). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), MC (European patent), NL (European pa- tent), SE (European patent). 2102115 Published <i>With international search report.</i>
(54) Title: <i>IN VITRO</i> METHODS FOR DETERMINING <i>IN VIVO</i> THROMBOTIC EVENTS (57) Abstract Diagnostic systems, methods, polypeptides and antibodies for detecting the presence of C-terminal hGPIIb fragment of the platelet receptor GPIIb-IIIa in a body fluid sample are disclosed.		

IN VITRO METHODS FOR DETERMINING
IN VIVO THROMBOTIC EVENTS

DESCRIPTION

5 Technical Field

 The present invention relates to a fragment of the C-terminal region of GPIIb heavy chain (hGPIIb) that is produced in vivo by indigenous protease cleavage of the GPIIb-IIIa platelet glycoprotein receptor, and to in vitro detection of the fragment in body fluids of individuals undergoing thrombotic events. The present invention also relates to polypeptide analogs of the hGPIIb C-terminal fragment, and antibodies that immunoreact with the C-terminal fragment and to diagnostic methods for detecting thrombi and clotting disorders.

Background

 Thrombosis involves cell adhesion of platelets. Platelet cell adhesion generally involves recognition of specific adhesive proteins by the platelet cell surface receptor GPIIb-IIIa.

 GPIIb-IIIa is a noncovalent, Ca^{++} dependent, heterodimer complex comprised of noncovalently associated alpha and beta subunits. Jennings et al., J. Biol. Chem., 257:10458-10466 (1982). The alpha subunit, GPIIb consists of a heavy chain (hGPIIb) having a relative molecular weight of about 120 kilodaltons (KDa), and a light chain (lGPIIb) of about 20 KDa that are linked together by disulfide bonds. The beta subunit, GPIIIa is a single chain polypeptide of about 100 KDa. Phillips et al., J. Biol. Chem., 252:2121-2126 (1977).

 GPIIb-IIIa contributes to platelet function through interactions with RGD-containing proteins such as fibrinogen [Bennett et al., Proc. Natl. Acad.

2102115

2

Sci.USA, 80:2417-2421 (1983)], fibronectin [Ginsberg et al., J. Clin. Invest., 71:619-624 (1983)], and von Willebrand factor [Ruggeri et al., Proc. Natl. Acad. Sci. USA, 79:6038-6041 (1982)], and therefore is a component of the common platelet adhesive protein receptor [Pytela et al., Science, 231:1559-1562 (1986) and Plow et al., J. Biol. Chem., 259:5388-5391 (1984)].

The sites on GPIIb-IIIa that are required to function as an adhesion receptor are not well characterized. Several observations suggest that a functionally significant site on GPIIb-IIIa is near the epitope defined by the monoclonal antibody PMI-1. This antibody binds to the heavy chain of GPIIb [Shadle et al., J. Cell. Biol., 99:2056-2060 (1984)] and defines a region of GPIIb that is associated with several distinct functional activities. First, PMI-1 inhibits adhesion of washed platelets to collagen. Shadle et al., J. Cell. Biol., 99:2056-2060 (1984). Second, the surface orientation of this region is regulated by divalent cations because millimolar (mM) concentrations of calcium or magnesium suppress expression of the PMI-1 epitope. Ginsberg et al., J. Clin. Invest., 78:1103-1111 (1986). Third, abnormal divalent cation regulation of the conformation of this site is associated with a functional thrombasthenic state. Ginsberg et al., J. Clin. Invest., 78:1103-1111 (1986). Fourth, stimulation of platelets with up to 100 micromolar adenosine diphosphate (ADP) or epinephrine, 1 unit per milliliter thrombin, or 50 micrograms per milliliter calf skin collagen does not substantially increase the binding of PMI-1 antibodies to platelets.

Platelet activation has been reported to produce the appearance of antigenic sites on the

platelet surface that are not present in the non-activated platelet, and at least one of such induced sites has been localized to the GPIIb-IIIa receptor complex. Shattil et al, J. Biol. Chem. 260:11107-11114 (1985); Collier, B.S., J. Cell Biol., 103:451-456 (1986).

Numerous assays based on the release of fragments of blood coagulation enzymes or platelet activation have been developed in attempts to identify thrombotic and pre-thrombotic states. From the platelet perspective, initial efforts were focused on release of platelet specific antigens. Ginsberg, et al., In Immunologic Analysis: Recent Progress in Diagnostic Labor. Immunol., Nakamura et al., Eds. Masson Publishing, USA Inc., 199-209 (1982). Recent studies have focused on the use of monoclonal antibodies selective for activated platelets. Frelinger et al., J. Biol. Chem., 263:12397-12402 (1988); Aiken et al., Sem. Thromb. Hemost., 13:307-316 (1987); Berman et al., J. Clin. Invest., 78:130-137 (1986); Hsu-Lin et al., J. Biol. Chem., 259:9121-9126 (1984); George et al., J. Clin. Invest., 78:340-348 (1986); Shattil et al., Blood, 73:150-158 (1989); Shattil et al., J. Biol. Chem., 260:11107-11114 (1985); Adelman et al., Blood, 70:1362-1366 (1987).

Each of the above methods has been plagued by unintentional in vitro activation because it is difficult to obtain blood samples in a manner such that the platelet activation events are not triggered during venipuncture, sample handling or the like. This difficulty has severely limited the clinical utility of such assays. It would be desirable, therefore, to develop a method to detect the occurrence of thrombotic events without relying on platelet activation events. The utility of determining the

2102115

4

elevation in a plasma glycoalbumin, as a potential assay for in vivo platelet activation, has also been reported. Steinberg et al., N. Engl. J. Med., 317:1037-1042 (1987); Collier et al., J. Clin. Invest., 73:794-799 (1984).

It has recently been found that a class of antigenic determinants are expressed when GPIIb-IIIa specifically binds to its ligand. The antigenic determinants are not expressed by either the non-occupied GPIIb-IIIa receptor or the non-bound ligand. One such determinant located on the C-terminus of the GPIIb heavy chain is recognized by the previously described PMI-1 monoclonal antibody. Shadle et al., J. Cell Biol., 99:2056-2060 (1984); Frelinger et al., J. Biol. Chem., 263:12397-12402 (1988).

Brief Summary of the Invention

It has now been found that a portion of the C-terminus of hGPIIb, containing the PMI-1 antigenic determinant, becomes exposed upon receptor-ligand binding and is cleaved by indigenous proteases induced by thrombus formation to form a cleaved fragment. The fragment, referred to as C-terminal hGPIIb fragment, is released into the vascular fluid, and finds its way into other body fluids, such as urine.

Simple activation of platelets with agonists in vitro is not sufficient to release the C-terminal hGPIIb fragment. Treatment with a protease is required. In the thrombus, the platelet receptor is occupied, thereby exposing this region of the molecule, and a variety indigenous proteases, such as plasmin, are generated. Thus, in the present invention, detecting the release of C-terminal hGPIIb fragment by proteases is not subject to the problems

of in vitro activation, and can be performed on a routinely obtained plasma or urine specimen from a patient.

5 The principal utility of the present invention is its ability to identify in vitro thrombotic events ongoing in vivo, and to monitor thrombolysis. Clinical settings include acute thrombotic events such as stroke or coronary thrombosis, or more chronic events such as deep venous
10 thrombosis. In addition, detection of chronic ongoing thrombotic events can serve to identify individuals at risk of developing acute thrombosis.

The present invention is directed to a substantially isolated C-terminal hGPIIb fragment that
15 has a molecular weight of about 3900 daltons, comprising an amino acid residue sequence that corresponds to the carboxy terminal portion of the amino acid residue sequence of hGPIIb shown in SEQ ID NO 1, including the amino acid residue sequence
20 represented by the formula -IHPAHHK-, shown in SEQ ID NO 1 from residue 194 to residue 200. This fragment has the capacity to immunoreact with the monoclonal antibody PMI-1, and preferably has the amino acid residue sequence comprising the amino acid residue
25 sequence shown in SEQ ID NO 1 from about residue 173 to about residue 200.

Also contemplated is a hGPIIb analog comprising a polypeptide of no more than about 200 residues, having an amino acid residue sequence that
30 corresponds to the sequence of hGPIIb shown in SEQ ID NO 1 and includes at least about 7 contiguous amino acid residues from the amino acid residue sequence of hGPIIb shown in SEQ ID NO 1 from residue 173 to residue 200. A preferred analog is a polypeptide
35 comprising an amino acid residue sequence

28395-20

6

corresponding to the formula: -PQPPVNPLK-;
-PQPPVNLPLKVDWGLPIPSPSP-; -PLKVDWGLPIP-;
-PIPSPSPIHPAHHK-; or-IHPAHHK- having a sequence shown
in SEQ ID NO 1 from residue 173 to residue 181, from
5 residue 173 to residue 193, from residue 179 to
residue 189, from residue 187 to residue 200, or from
residue 194 to residue 200.

Further contemplated is an antibody
comprising an antibody molecule or fragment thereof
10 that is capable of immunoreacting specifically with
C-terminal hGPIIb fragment but does not substantially
immunoreact with a polypeptide represented by the
formula REQNSLDSWGPK having the sequence in SEQ ID NO
1 from residue 113 to residue 124. In preferred
15 embodiments, the antibody composition contains a
monoclonal antibody or fragment thereof that is
substantially similar to the antibody, or fragment of
the antibody, produced by hybridoma PMI-1.

The present invention also contemplates a
20 method for assaying the presence of a C-terminal
hGPIIb fragment in a body fluid sample. The method
comprises admixing an aliquot of body fluid with an
antibody of this invention. Contact of the body fluid
and the antibody is maintained for a time period
25 sufficient for an immunoreaction product to form. The
fragment-containing immunoreaction product formed is
then detected, thereby indicating the presence of the
C-terminal hGPIIb fragment in the sample.

A diagnostic system in kit form is further
30 contemplated by the present invention. In one
embodiment is a diagnostic system in kit form for
assaying for the presence of a C-terminal hGPIIb
fragment in a body fluid sample. The system comprises
a package containing, in an amount sufficient to
35 perform at least one assay, an antibody of this

2102115

7

invention. The antibody can be in a liquid solution but in one embodiment is preferably attached to a solid-phase matrix. The system may also contain a label for indicating the presence of the antibody molecules in the immunoreaction product formed.

In a more preferred embodiment, the diagnostic system further contains a solid support comprising a solid matrix having affixed thereto at least one of the set of hGPIIb analog and a polypeptide having an amino acid residue sequence that includes the sequence of a C-terminal hGPIIb fragment, thereby providing a kit for assaying by a competition ELISA a vascular fluid or urine sample for the presence of C-terminal hGPIIb fragment.

In one preferred embodiment, the before-described method is utilized to assess the extent of acute thrombosis by detecting the presence of C-terminal hGPIIb fragment in a body fluid sample and relating the amount of immunoreaction product formed to predetermined standards correlative with the diseased state.

In another preferred embodiment, this method is useful to monitor the course of plasminogen activator-induced thrombolysis by detecting the presence of C-terminal hGPIIb fragment in a body fluid sample and relating the amount to correlative predetermined levels.

In yet another preferred embodiment, this method is contemplated for identifying individuals at risk of developing acute thrombosis, which method determines the extent of chronic thrombosis and relates it to predetermined levels correlating with the risk of acute thrombosis.

28395-20

8

Brief Description of the Drawings

In the Figures forming a portion of the disclosure of this invention:

Figure 1 illustrates the effect of in vitro incubation of platelets at 37C in the presence of 30 uM chymotrypsin as described in Example 1c. One portion of the digested platelet suspension was centrifuged and the supernatant (open diamond) and resuspended pellet (+) were assayed separately. An equal portion of the platelet suspension was assayed comprising total PMI-1 immunoreactive material (open square). Levels of the PMI-1 epitope (Figure 1A) or the Tab epitope (Figure 1B) (a monoclonal antibody with a different GPIIb epitope specificity) were determined by competitive ELISA assays, as described hereinafter. Note the release of material reactive with PMI-1, but not with Tab, into the platelet-free supernatant.

Brief Description of the Sequence Listing

SEQ ID NO 1 listed in the Sequence Listing beginning on page 59 illustrates the nucleotide and corresponding amino acid residue sequence of a cDNA that codes for a portion of GPIIb heavy chain (hGPIIb) wherein the nucleotide sequence is shown from left to right and in the direction of 5' terminus to 3' terminus using the single letter nucleotide base code represented as an uninterrupted linear series of bases from base 1 to base 600. The amino acid residue sequence is shown from left to right and in the direction from amino-terminus to carboxy-terminus using the single letter amino acid residue code represented as an uninterrupted linear series of residues from residue 1 (G) at the amino-terminus to residue 200 (K) at the carboxy-terminus.

28395-20

8a

In one aspect, the invention relates to a substantially isolated C-terminal hGPIIb fragment that has a molecular weight of about 3,900 daltons, comprising an amino acid residue sequence that corresponds to the carboxy terminal portion of the amino acid residue sequence of hGPIIb shown in SEQ ID NO:2, wherein the carboxy terminus of said fragment ends with the amino acid residue sequence represented by the formula -IHPAHHK-, said fragment having the capacity to immunoreact with the monoclonal antibody, PMI-1.

In another aspect, the invention relates to a substantially isolated C-terminal hGPIIb polypeptide that has a molecular weight of about 3,900 daltons and comprises an amino acid residue sequence shown in SEQ ID NO:2 from residue 173 to 200.

In another aspect, the invention relates to a hGPIIb analog (i) comprising a polypeptide which has no more than about 200 residues and which comprises at least 7 contiguous amino acid residues of the amino acid sequence shown in SEQ ID NO:2 from the region from residue 173 to residue 200; and (ii) having the amino acid sequence -IHPAHHK- at the carboxy terminus.

In another aspect, the invention relates to a hGPIIb analog consisting of a polypeptide having an amino acid residue sequence that corresponds to the sequence of hGPIIb selected from the group consisting of the sequence shown in SEQ ID NO:2 from residues 173 to 181, from residues 179 to 189, from residues 187 to 200, and from residues 194 to 200.

In another aspect, the invention relates to a substantially isolated polypeptide having an amino acid

28395-20

8b

residue sequence as shown in SEQ ID NO:2 from residue 173 to residue 193.

In another aspect, the invention relates to an antibody that immunoreacts with the polypeptide as described
5 above, and does not specifically bind with a polypeptide having amino acid residue sequence REQNSLDSWGP set forth in SEQ ID NO:2 from residue 113 to residue 124.

In another aspect, the invention relates to a method for detecting a C-terminal heavy chain platelet
10 glycoprotein receptor (hGPIIb) polypeptide in a body fluid sample, comprising the steps of: (a) admixing an aliquot of the body fluid sample with an antibody to form an immunoreaction admixture, wherein the antibody: (i) immunoreacts with the polypeptide comprising amino acid
15 sequence PQPPVNPLKVDWGLPIPS set forth in SEQ ID NO:2 from residue 173 to residue 193, PQPPVNPLK set forth in SEQ ID NO:2 from residue 173 to residue 181, or PLKVDWGLPIP set forth in SEQ ID NO:2 from residue 179 to residue 189; and (ii) does not specifically immunoreact with the polypeptide
20 comprising amino acid sequence REQNSLDSWGP set forth in SEQ ID NO:2 from residue 113 to residue 124; (b) maintaining the immunoreaction admixture under biological assay conditions for a time period sufficient to form an immunoreaction product; and (c) detecting the immunoreaction product, and
25 thereby the C-terminal hGPIIb polypeptide.

In another aspect, the invention relates to a diagnostic test kit comprising (i) a package containing an antibody and (ii) instructions for using the antibody for assaying for the presence of C-terminal human
30 glycoprotein IIb (hGPIIb) fragments in a body fluid sample, wherein the antibody: a) immunoreacts with at least one polypeptide having an amino acid residue sequence

28395-20

8c

PQPPVNPLKVDWGLPIPSPSP as shown in SEQ ID NO:2 from residue 173 to residue 193, PQPPVNPLK as shown in SEQ ID NO:2 from residue 173 to residue 181, or PLKVDWGLPIP as shown in SEQ ID NO:2 from residue 179 to residue 189; and b) does not immunoreact with a polypeptide having the amino acid residue sequence REQNSLDSWGP, as shown in SEQ ID NO:2 from residue 113 to residue 124.

In another aspect, the invention relates to a diagnostic test kit comprising separate packages containing an antibody and a polypeptide and instructions for using the antibody and the polypeptide for assaying for the presence of C-terminal hGPIIb fragment in a body fluid sample, wherein a) the antibody: (i) immunoreacts with at least one polypeptide having amino acid sequence PQPPVNPLKVDWGLPIPSPSP set forth in SEQ ID NO:2 from residue 173 to residue 193, PQPPVNPLK set forth in SEQ ID NO:2 from residue 173 to residue 181, PLKVDWGLPIP set forth in SEQ ID NO:2 from residue 179 to residue 189, PIPSPSPIHPAHHK set forth in SEQ ID NO:2 from residue 187 to residue 200, or IHPAHHK set forth in SEQ ID NO:2 from residue 194 to residue 200; and (ii) does not specifically immunoreact with the polypeptide having amino acid sequence REQNSLDSWGP set forth in SEQ ID NO:2 from residue 113 to residue 124; and (b) the polypeptide has an amino acid residue sequence comprising the C-terminal portion of SEQ ID NO:2 from residue 173 to residue 200, or a hGPIIb analog consisting of a polypeptide corresponding to the sequence of hGPIIb shown in SEQ ID NO:2 from residues 1 to 200, or a polypeptide or a hGPIIb analog having a sequence corresponding to SEQ ID NO:2 from residues 173 to 193, residues 173 to 181, residues 179 to 189, residues 187 to 200, or residues 194 to 200.

The reading frame is indicated by the placement of the deduced amino acid residue sequence below the nucleotide sequence such that the single letter that represents each amino acid residue is located below the first base in the corresponding codon.

The nucleotide base sequence shown from base 1 through base 600 in SEQ ID NO 1 corresponds to the nucleotide sequence shown in Figure 2 of Poncz et al., J. Biol. Chem., 262:8476-8482(1987), from base 2060 to base 2659. The amino acid residue sequence from residue 1 to residue 200 shown in SEQ ID NO 1 corresponds to the sequence shown in Figure 2 of Poncz et al., supra, from residue 656 to residue 855.

Detailed Description of the Invention

I. Definitions

Amino Acid: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues identified herein are preferably in the natural "L" isomeric-configuration. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of the polypeptide. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3557-59 (1969) and adopted at 37 C.F.R. 1.822(b)(2), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

28395-20

10

TABLE OF CORRESPONDENCE

	<u>SYMBOL</u>		<u>AMINO ACID</u>
	<u>1-Letter</u>	<u>3-Letter</u>	
	Y	Tyr	L-tyrosine
5	G	Gly	glycine
	F	Phe	L-phenylalanine
	M	Met	L-methionine
	A	Ala	L-alanine
	S	Ser	L-serine
10	I	Ile	L-isoleucine
	L	Leu	L-leucine
	T	Thr	L-threonine
	V	Val	L-valine
	P	Pro	L-proline
15	K	Lys	L-lysine
	H	His	L-histidine
	Q	Gln	L-glutamine
	E	Glu	L-glutamic acid
	W	Trp	L-tryptophan
20	R	Arg	L-arginine
	D	Asp	L-aspartic acid
	N	Asn	L-asparagine
	C	Cys	L-cysteine

25 It should be noted that all amino acid residue
sequences are represented herein by have a left to
right orientation in the conventional direction of
amino-terminus to carboxy-terminus. In addition, the
phase "amino acid residue" is broadly defined to
30 include modified and unusual amino acids, such as
those listed in 37 C.F.R. 1.822(b)(4).

Furthermore, it should be
noted that a dash at the beginning or end of an amino
acid residue sequence indicates a peptide bond to a
35 further sequence of one or more amino acid residues or

2102115

11

a covalent bond to an amino-terminal group such as NH_2 or acetyl or to a carboxy-terminal group such as COOH .

Polypeptide and Peptide: Polypeptide and peptide are terms used interchangeably herein to designate a linear series of no more than about 50 amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

Protein: Protein is a term used herein to designate a linear series of greater than 50 amino acid residues connected one to the other as in a polypeptide.

II. Introduction

Platelets play a central role in thrombosis. The invention provides an in vitro means to detect platelet incorporation into thrombi in vivo, and thereby detects thrombotic events.

When platelets are incorporated into a thrombus, occupancy of GPIIb-IIIa receptor by its ligand is believed to expose the C-terminal region of the GPIIb heavy chain. Indigenous proteases, such as plasmin or leukocyte enzymes, generated at the site of the thrombus in vivo cleave the C-terminal portion of hGPIIb, releasing the small peptide fragments described above. Measurement of these fragments in vascular fluid or in urine provides a diagnostic correlate of the extent of the thrombotic process. These measurements are also valuable in monitoring the course of thrombolysis induced by plasminogen activators.

PMI-1, a monoclonal antibody derived from immunization of mice with isolated human platelet plasma membranes, reacts with the carboxy terminal aspect of the heavy chain of the platelet membrane

2102115

12

glycoprotein hGPIIb. Shadle et al., J. Cell Biol., 99:2056-2060 (1984); and Loftus et al., Proc. Natl. Acad. Sci. USA, 840:7114-7118 (1987). In the present invention, an enzyme-linked immunosorbent assay (ELISA) has been developed to measure levels of PMI-1 antigenic material. The principal observation, illustrated in Figure 1, is that in vitro treatment of platelets with protease releases from the carboxy terminus of the GPIIb heavy chain a small (approximately 7 kDa, based on average MW of amino acid residue = 120) fragment that contains the PMI-1 epitope. The fragment is referred to herein as the C-terminal hGPIIb fragment.

A potential site of cleavage of GPIIb-IIIa by indigenous proteases is downstream from the cysteine residue at position 171 of the sequence shown in SEQ ID NO 1. The cysteine at position 171 participates in disulfide bridging to the light chain of GPIIb. Calvette et al., Biochem. J., 261:551-560 (1989). The preferred site for chymotrypsin cleavage of GPIIb-IIIa when present on platelets is between residues 172 and 173 of the sequence shown in SEQ ID NO 1.

The carboxy terminus of hGPIIb has been identified by Loftus et al., J. Biol. Chem. 263:11025 (1988). After initial processing to cleave the GPIIb precursor protein into the heavy and light chain, alternate proteolytic processing results in partial trimming back of the carboxy terminus of hGPIIb. The partial proteolysis produces a polypeptide having either an lysine (K) or arginine (R) at the carboxy terminus, corresponding to residue 200 or 201, respectively, of the sequence shown in SEQ ID NO 1.

The capacity of the PMI-1 monoclonal antibody to inhibit platelet adhesion to collagen,

13 2102115

Shadle et al., J. Cell Biol., 99:2056-2060 (1984),
its upregulation by chelation of divalent cations,
Ginsberg et al., J. Clin. Invest., 78:1103-1111
(1986), and by occupancy of GPIIb-IIIa with adhesive
5 ligands, Frelinger et al., J. Biol. Chem.,
263:12397-12402 (1988), has been previously published
by the applicants and is well known. There have,
however, been no prior publications on the use of an
ELISA technique for detecting this novel hGPIIb
10 fragment as a means of identifying and evaluating
thrombosis.

As discussed before, and reviewed by
Ginsberg et al., in Immunologic Analysis: Recent
Progress in Diagnostic Labor. Immunol., Nakamura et
15 al., Eds. Masson Publishing, USA Inc., 199-209 (1982),
there have been numerous assays designed to quantitate
released platelet granule components in the
circulation as a means for detecting platelet
activation. The present invention, however, does not
20 involve release of a granule component, but rather
involves the detection of a proteolytic attack on a
platelet surface component, and as such is less
susceptible to in vitro artifact, e.g., false positive
results due to in vitro activation of platelets.

25

III. hGPIIb Fragment and Analogs

A. Fragment

In one embodiment, the present invention
contemplates a substantially isolated C-terminal
30 hGPIIb fragment having a molecular weight of about
3900 daltons, comprising an amino acid residue
sequence that corresponds to the carboxy-terminal
portion of the amino acid residue sequence of hGPIIb.
The C-terminal hGPIIb fragment includes as a portion
35 of its amino acid residue sequence the sequence

2102115

14

represented by the formula -IHPAHHK- shown is SEQ ID NO 1 from residue 194 to residue 200, and has the capacity to immunoreact with the monoclonal antibody, PMI-1. Preferably, the C-terminal hGPIIb fragment has the amino acid residue sequence shown in SEQ ID NO 1 from about residue 173 to about residue 200.

A C-terminal hGPIIb fragment can be purified from human urine or plasma using well known immunoaffinity methods, such as described in Example 1c(2). Alternatively, C-terminal GPIIb fragment can be produced in vitro by proteolysis of platelets or by proteolysis of isolated GPIIb-IIIa, and subsequently immunoaffinity purified as described in Example 1. Proteolysis of platelets or isolated GPIIb-IIIa using the protease chymotrypsin is particularly preferred. The latter procedure is preferred due to the availability of platelets and the amount of GPIIb-IIIa contained therein.

C-terminal hGPIIb fragment is substantially isolated if compositions containing the fragment are comprised of greater than 5 percent, by weight, of fragment per total protein in the composition.

C-terminal hGPIIb fragment, once isolated, is useful to produce immunogen for raising antibody specific for the fragment, and for use in the diagnostic methods and systems for detecting the presence and amount of C-terminal hGPIIb fragment in body fluid samples, as disclosed herein.

B. Polypeptide Analogs of the C-Terminal hGPIIb Fragment

One embodiment of the present invention contemplates a polypeptide that includes amino acid residue sequences capable of mimicking antigenic determinants expressed by the C-terminal hGPIIb

fragment. The hGPIIb fragment-mimicking polypeptide is also referred to herein as an analog, or hGPIIb analog. A hGPIIb polypeptide analog of the present invention comprises an amino acid residue sequence that corresponds, and preferably is identical, to a portion of the hGPIIb sequence shown in SEQ ID NO 1 from residue 1 to residue 200. The hGPIIb analog is from about 7 to about 200 amino acid residues in length, preferably less than about 50, and more preferably less than 20 residues in length, and includes a minimum of about 7 contiguous residues of the hGPIIb amino acid residue sequence shown in SEQ ID NO 1 from residue 173 to residue 200.

In one preferred embodiment, a hGPIIb analog includes at least the following amino acid residue sequence:

-IHPAHHK-,

representing the amino acid residues 194-200 shown in SEQ ID NO 1. In this embodiment, a hGPIIb analog is also capable of immunoreacting with the monoclonal antibody, PMI-1. The antibody PMI-1 is known to be immunospecific for (i.e., immunoreacts with) the minimum epitope -IHPAHHK- on hGPIIb.

In another embodiment, a preferred hGPIIb analog is a polypeptide that comprises an amino acid residue sequence corresponding to one of the following amino acid sequences:

-PQPPVNPLK-,

-PLKVDWGLPIP-,

-PIPSPSPIHPAHHK-, or

-IHPAHHK-,

having an amino acid residue sequence shown in SEQ ID NO 1 from residue 173 to residue 181, from residue 179 to residue 189, from residue 187 to residue 200, or from residue 194 to residue 200, respectively.

2102115

16

Particularly preferred hGPIIb analogs are contemplated having an amino acid residue sequence as shown in Table 1 below.

Table 1

5	<u>Designation</u>	<u>Amino Acid Residue Sequence</u>
	p173-193	PQPPVNPLKVDWGLPIPSPP
	p173-181	PQPPVNPLK
	p179-189	PLKVDWGLPIP
	p187-200	PIPSPSPIHPAHHK
10	p194-200	IHPAHHK

* The designation of each polypeptide represents the included amino acid residue sequence as shown in SEQ ID NO 1.

15 One contemplated hGPIIb analog has an amino acid residue sequence that corresponds to the sequence shown in SEQ ID NO 1 from residue 1 to residue 200. More preferred is a hGPIIb analog that corresponds in sequence to a C-terminal hGPIIb fragment and has a
20 sequence shown in SEQ ID NO 1 from residue 173 to residue 200.

The polypeptides shown in Table 1 are further characterized by their ability to neutralize (competitively inhibit) the binding of antibody
25 molecules to their respective antigenic determinants on the native hGPIIb molecule, described hereinbelow.

It should be understood that a hGPIIb analog of the present invention need not be identical to the amino acid residue sequence of hGPIIb, so long
30 as it has sufficient similarity with (i.e., corresponds to) portions of hGPIIb that the analog is able to competitively inhibit the immunoreaction of specific antibody molecules, elicited as described hereinbelow. Therefore, a hGPIIb analog can be subject
35 to various changes, such as insertions, deletions and

2102115

17

substitutions, either conservative or non-conservative, where such changes provide for certain advantages in their use.

Conservative substitutions are those where one amino acid residue is replaced by another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue, such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as between arginine and lysine, between glutamic and aspartic acids or between glutamine and asparagine and the like. The term "conservative substitution" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that such a polypeptide also displays the requisite immunoreaction activity.

When a polypeptide of the present invention has a sequence that is not identical to the corresponding sequence of hGPIIb because one or more conservative or non-conservative substitutions have been made, usually no more than about 20% and preferably no more than 10% of the amino acid residues are substituted, except where additional residues have been added at either terminus for the purpose of providing a "linker" by which the polypeptides of this invention can be conveniently affixed to a label or solid matrix, or antigenic carrier. Labels, solid matrices and carriers that can be used with the polypeptides of this invention are described hereinafter.

Amino acid residue linkers are usually at least one residue and can be 40 or more residues, more often 1 to 10 residues. Typical amino acid residues used for linking are tyrosine, cysteine, lysine,

2102115

18

glutamic and aspartic acid, or the like. In addition,
a polypeptide sequence of this invention can differ
from the natural sequence by the sequence being
modified by terminal-NH₂ acylation, e.g., acetylation,
5 or thioglycolic acid amidation,
terminal-carboxylamidation, e.g., ammonia,
methylaniline, etc.

When coupled to a carrier via a linker to
form what is known in the art as a carrier-hapten
10 conjugate, a hGPIIb analog of the present invention is
capable of inducing formation of antibodies that
immunoreact with the C-terminal fragment of hGPIIb
when present as a free-floating fragment in vascular
fluid or urine.

15 In view of the well established principle of
immunologic cross-reactivity, the present invention
therefore contemplates antigenically related variants
of the polypeptides described before. An
"antigenically related variant" is a polypeptide that
20 is capable of inducing formation of antibody molecules
that immunoreact with a hGPIIb analog and with the
C-terminal hGPIIb fragment when present in body
fluids.

Any hGPIIb analog of the present invention
25 can be synthesized by one of many techniques known to
those skilled in the polypeptide art, including
recombinant DNA techniques. Synthetic chemistry
techniques, such as a solid-phase Merrifield-type
synthesis, are preferred for reasons of purity,
30 antigenic specificity, freedom from undesired side
products, ease of production and the like. An
excellent summary of the many techniques available can
be found in J.M. Steward and J.D. Young, "Solid Phase
Peptide Synthesis", W.H. Freeman Co., San Francisco
35 (1969); Bodansky et al., "Peptide Synthesis", John

28395-20

19

Wiley & Sons, 2nd Ed., (1976); and Meienhofer,
"Hormonal Proteins and Peptides", 2:46, Academic
Press, NY (1983) for solid phase peptide synthesis;
and Schroder et al., "The Peptides", Vol. 1, Academic
5 Press, NY (1965) for classical solution synthesis.

Appropriate protective groups usable in such synthesis
are described in the above texts and in McOmie,
"Protective Groups in Organic Chemistry", Plenum
10 Press, NY (1973).

In general, the solid-phase synthesis
methods contemplated comprise the sequential addition
of one or more amino acid residues or suitably
15 protected amino acid residues to a growing peptide
chain. Normally, either the amino or carboxyl group of
the first amino acid residue is protected by a
suitable, selectively removable protecting group. A
different, selectively removable protecting group is
20 utilized for amino acids containing a reactive side
group such as lysine.

Using a solid-phase synthesis as exemplary,
the protected or derivatized amino acid is attached to
an inert solid support through its unprotected
25 carboxyl or amino group. The protecting group of the
amino or carboxyl group is then selectively removed
and the next amino acid in the sequence having the
complimentary (amino or carboxyl) group suitably
protected is admixed and reacted under conditions
30 suitable for forming the amide linkage with the
residue already attached to the solid support. The
protecting groups (and solid support) are removed
sequentially or concurrently, to afford the final
polypeptide.

2102115 20

5 A hGPIIb analog can be used, inter alia, in the diagnostic methods and systems of the present invention to detect C-terminal hGPIIb fragment present in a body sample, or can be used to prepare an inoculum as described herein for the preparation of antibodies that immunoreact with the fragment.

IV. Antibodies and Antibody Compositions

10 The term "antibody" in its various grammatical forms is used herein to refer to immunoglobulin molecules and fragments thereof, namely immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Illustrative antibody
15 molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')₂ and F(v).

20 The term "antibody combining site" refers to that structural portion of an antibody molecule, comprised of a heavy and light chain variable and hypervariable regions, that specifically binds (immunoreacts with) an antigen. The term
25 "immunoreact" in its various forms is used herein to refer to binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof.

30 The term "antigenic determinant" refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site. The term is also used interchangeably with "epitope".

35 As used herein, the term "specifically bound" refers to a non-random binding reaction between

an antibody and an antigen.

A. Antibody Compositions

5 An antibody composition of the present invention is characterized as containing antibody molecules that immunoreact with a C-terminal hGPIIb fragment or hGPIIb analog as described herein.

10 An antibody of this invention, i.e., an anti-C-terminal hGPIIb fragment antibody, does not immunoreact with other portions of the hGPIIb molecule, such as antigenic determinants defined by polypeptides corresponding in sequence to regions of hGPIIb that do not include residues 173 through 200 of the sequence shown in SEQ ID NO 1. Thus, for example,
15 a contemplated antibody does not immunoreact with a polypeptide having a sequence represented by the formula REQNSLDSWGPK, shown in SEQ ID NO 1 from residue 113 to residue 124. That is, the antibody is specific for the C-terminal hGPIIb fragment identified
20 and defined herein.

Thus an antibody, in its various embodiments, immunoreacts with an epitope present on a C-terminal hGPIIb fragment. Exemplary epitopes are defined by the C-terminal hGPIIb analogs designated
25 p173-181, p179-189, p187-200 or p194-200. Additional epitopes can reside within the hGPIIb C-terminal fragment that are useful for defining the immunoreactivity of an antibody of this invention. Thus other embodiments contemplate an antibody that
30 immunoreacts with a polypeptide defining regions other than the p194-200 portion of hGPIIb C-terminal fragment. Exemplary antibodies immunoreact with the polypeptide p173-193, shown in SEQ ID NO 1 from residues 173 to 193.

2102115

22

Such a contemplated antibody composition is typically produced by immunizing a mammal with an inoculum containing a C-terminal hGPIIb fragment of this invention, or a polypeptide analog, thereby inducing production in the mammal of antibody molecules having immunospecificity for the C-terminal hGPIIb fragment. The antibody molecules are then collected from the mammal and isolated to the extent desired by well known techniques such as, for example, by using DEAE Sephadex to obtain the IgG fraction. To enhance the specificity of the antibody, the antibodies may be purified by immunoaffinity chromatography using solid phase-affixed immunizing polypeptide or C-terminal hGPIIb fragment. The antibody is contacted with the solid phase-affixed immunizing polypeptide or fragment (immunogen) for a period of time sufficient for the immunogen to immunoreact with the antibody molecules to form a solid phase-affixed immunocomplex. The bound antibodies are separated from the complex by standard techniques.

Preferred and exemplary are the methods to produce and isolate antibodies described in Example 3.

The antibody composition so produced can be used inter alia, in the diagnostic methods and systems of the present invention to detect the presence and/or amount of C-terminal hGPIIb fragment in a body sample.

A preferred antibody in an antibody composition of this invention is a monoclonal antibody.

B. Inocula

The word "inoculum" in its various grammatical forms is used herein to describe a composition containing a polypeptide or the C-terminal

hGPIIb fragment of this invention as an active ingredient used for the preparation of antibodies that immunoreact with the C-terminal hGPIIb fragment. The inoculum also contains a pharmaceutically acceptable aqueous diluent such that, when administered by immunization, is capable of eliciting antibodies that immunoreact with the hGPIIb C-terminal fragment.

When a polypeptide is used to induce antibodies it is to be understood that the polypeptide can be used alone, or linked to a carrier as a conjugate, or as a polypeptide polymer, but for ease of expression, the various embodiments of the polypeptides of this invention are collectively referred to herein by the term "polypeptide", and its various grammatical forms.

For a polypeptide that contains fewer than about 35 amino acid residues, it is preferable to use the peptide bound to a carrier for the purpose of inducing the production of antibodies as already noted.

As discussed before, one or more additional amino acid residues can be added to the amino- or carboxy-termini of the polypeptide to assist in binding the polypeptide to a carrier. Cysteine residues added to the amino- or carboxy-termini of the polypeptide have been found to be particularly useful for forming conjugates via disulfide bonds. However, other methods well known in the art for preparing conjugates can also be used. Exemplary additional linking procedures include the use of Michael addition reaction products, di-aldehydes such as glutaraldehyde, Klipstein et al., J. Infect. Dis., 147, 318-326 (1983) and the like, or the use of carbodiimide technology, as in the use of a water-soluble carbodiimide to form amide links to the

2102115

24

carrier. For a review of protein conjugation or coupling through activated functional groups, see Avrameas, et al., Scand. J. Immunol., 1:7-23 (1978).

5 Useful carriers are well known in the art, and are usually proteins themselves. Examples of such carriers are keyhole limpet hemocyanin (KLH), edestin, thyroglobulin, albumins, such as bovine serum albumin (BSA) or human serum albumin (HSA), red blood cells, such as sheep erythrocytes (SRBC), tetanus toxoid, 10 cholera toxoid as well as polyamino acids, such as poly (D-lysine: D-glutamic acid), and the like.

The choice of carrier is dependent upon the ultimate use of the inoculum and is based upon criteria not particularly involved in the present 15 invention. For example, a carrier that does not generate an undesired reaction in the particular animal to be inoculated should be selected.

The inoculum contemplated herein contains an effective immunogenic amount of an immunogen of this 20 invention, e.g., an hGPIIb analog or a C-terminal hGPIIb fragment, typically as a conjugate linked to a carrier. The effective amount of polypeptide or protein per unit dose sufficient to induce an immune response to the immunogen depends, among other things, 25 on the species of animal inoculated, the body weight of the animal and the chosen inoculation regimen as is well known in the art. Inocula typically contain polypeptide or protein concentrations of about 10 micrograms to about 500 milligrams per inoculation (dose), preferably about 50 micrograms to about 50 30 milligrams per dose.

The term "unit dose" as it pertains to the inocula of the present invention refers to physically discrete units suitable as unitary dosages for 35 animals, each unit containing a predetermined quantity

of active material calculated to produce the desired immunogenic effect in association with the required diluent; i.e., carrier, or vehicle. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are directly dependent on (a) the unique characteristics of the active material and the particular immunologic effect to be achieved, and (b) the limitations inherent in the art of compounding such active material for immunologic use in animals, as disclosed in detail herein, these being features of the present invention.

Inocula are typically prepared from the dried solid polypeptide-conjugate by dispersing the polypeptide-conjugate in a physiologically tolerable (acceptable) diluent or vehicle such as water, saline or phosphate-buffered saline to form an aqueous composition. Such diluents are well known in the art and are discussed, for example, in Remington's Pharmaceutical Sciences, 16th Ed., Mack Publishing Company, Easton, PA (1980) at pages 1465-1467.

Inocula can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

C. Monoclonal Antibody Compositions

The phrase "monoclonal antibody composition" in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody composition thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody

28395-20

26

composition is typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces) but one kind of antibody molecule. The hybridoma cell is formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. Such antibodies were first described by Kohler and Milstein, Nature 256:495-497 (1975).

Detailed preparation of a monoclonal antibody composition is described further herein.

Thus, a monoclonal antibody of this invention immunoreacts with a C-terminal hGPIIb fragment or hGPIIb analog of this invention, but does not substantially immunoreact with a polypeptide having a sequence represented by the formula REQNSLDSWGPK shown in SEQ ID NO 1 from residue 113 to residue 124. That is, the antibody is specific for the C-terminal hGPIIb fragment.

Particularly preferred is a monoclonal antibody that immunoreacts with a hGPIIb analog of this invention, particularly the polypeptide IHPAHHK or polypeptides that contain the sequence -IHPAHHK- shown in SEQ ID NO 1 from residues 194 to 200. Exemplary of this preferred embodiment is the monoclonal antibody PMI-1, available from the American Type Culture Collection (ATCC, Rockville, MD) and having ATCC accession number HB 9476, that has been shown to be immunospecific for an epitope on hGPIIb defined by the polypeptide IHPAHHK and present on a C-terminal hGPIIb fragment.

In a related embodiment a monoclonal antibody is contemplated that immunoreacts with a hGPIIb analog defined by a polypeptide comprising a sequence of amino acid residues shown in SEQ ID NO 1 from residues 173 to 193, particularly a polypeptide

2102115

27

having the sequence shown in SEQ ID NO 1 from residues 173 to 181, from residues 179 to 189, or from residues 179 to 193.

5 V. Methods for Producing Monoclonal Antibody
 Compositions

10 The present invention contemplates a method of forming a monoclonal antibody molecule that (a) immunoreacts with an antigenic determinant (epitope) on the C-terminal hGPIIb fragment, said fragment including an amino acid residue sequence as shown in SEQ ID NO 1 from residues 173 to 200, from residues 173 to 181, from residues 173 to 193, from residues 179 to 189, from residues 179 to 193, or from residues 15 194 to 200, but (b) does not substantially immunoreact with a polypeptide represented by the formula REQNSLDSWGPK shown in SEQ ID NO 1 from residues 113 to 124.

20 A preferred method comprises the steps of:

25 (a) Immunizing an animal with an inoculum containing a C-terminal hGPIIb fragment or a polypeptide hGPIIb analog as described herein as the immunogen. Preferably, the immunogen is complexed with a carrier or immunopotentiator such as an ISCOM particle, liposome or the like. The inoculum is administered to an immunologically competent mammal in an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a 30 time period sufficient for it to produce cells secreting antibody molecules that immunoreact with the C-terminal hGPIIb fragment.

35 (b) Preparing a suspension of immune cells. This is typically accomplished by removing the spleen of the mammal and mechanically separating the

2102115

28

individual spleen cells in a physiologically tolerable medium using methods well known in the art.

(c) Treating the suspended antibody-producing cells with a transforming agent capable of producing a transformed ("immortalized") cell line.. Transforming agents and their use to produce immortalized cell lines are well known in the art and include DNA viruses such as Epstein Bar Virus (EBV), Simian Virus 40 (SV40), Polyoma Virus and the like, RNA viruses such as Moloney Murine Leukemia Virus (Mo-MuLV), Rous Sarcoma Virus and the like, myeloma cells such as P3X63-Ag8.653, Sp2/O-Ag14, that are available from the ATCC under the designations CRL 1580 and CRL 1581, respectively, and the like.

In preferred embodiments, treatment with the transforming agent results in the production of a hybridoma by means of fusing the suspended spleen cells with mouse myeloma cells from a suitable cell line by the use of a suitable fusion promoter. The preferred ratio is about 5 spleen cells per myeloma cell in a suspension containing about 10^8 splenocytes. A preferred fusion promoter is polyethylene glycol having an average molecule weight from about 1000 to about 4000 (commercially available as PEG 1000, etc.); however, other fusion promoters known in the art can be employed.

The cell line used should preferably be of the so-called "drug resistant" type, so that unused myeloma cells will die in a selective medium, whereas hybrids will survive. The most cell lines used for this purpose are 8-azaguanine resistant. These lines lack the enzyme hypoxanthine-guanine phosphoribosyl transferase and hence will not be supported by HAT (hypoxanthine, aminopterin, and thymidine) medium. It is also generally preferred that the myeloma cell line

2102115

29

used be of the so-called "non-secreting" type, i.e., does not itself produce antibody molecules. In certain cases, however, secreting myeloma lines may be preferred.

5 (d) Cloning the transformed cells, preferably to monoclonality. The cloning is preferably performed in a tissue culture medium that will not sustain (support) non-transformed cells. When the transformed cells are hybridomas, this is
10 typically performed by diluting and culturing in separate containers the mixture of unused spleen cells, unused myeloma cells, and fused cells (hybridomas) in a selective medium which will not sustain the unused myeloma cells. The cells are
15 cultured in this medium for a time sufficient to cause death of the unused cells (about one week). The dilution can be a limiting dilution, in which the volume of diluent is statistically calculated to isolate a certain number of cells (e.g., 1-4) in each
20 separate container (e.g., each well of a microtiter plate). The medium is one (e.g., HAT medium) that will not sustain the drug-resistant (e.g., 8-azaguanine resistant) unused myeloma cell line.

(e) Screening the tissue culture
25 medium of the cloned transformants to detect the presence of secreted antibody molecules as discussed in Example 4a. Preferably, the medium is monitored using well known immunological techniques for the appearance of antibody molecules that immunoreact with
30 C-terminal hGPIIb fragment.

(f) Selecting a desired transformant and growing it in an appropriate tissue culture medium for a suitable length of time, and harvesting the antibody from the culture supernatant by well known
35 techniques. The suitable medium and suitable length of

2102115

30

culturing time are also well known or are readily determined.

5 A monoclonal antibody of the present invention can also be produced by preparing a culture containing a monoclonal hybridoma that secretes antibody molecules of the appropriate polypeptide specificity. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well known techniques.

10 Representative and preferred methods for producing monoclonal antibody compositions of this invention are described in Example 3c.

15 To produce a much greater concentration of slightly less pure monoclonal antibody, the selected hybridoma can be transferred by injection into mice, preferably syngeneic or semisyngeneic mice. The hybridoma will cause formation of antibody-producing ascites tumors after a suitable incubation time, which will result in a high concentration of the desired antibody (about 5-20 mg/ml) in the bloodstream and peritoneal exudate of the host mouse.

20 Media and animals useful for the preparation of these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. One commonly used synthetic medium is Dulbecco's minimal essential medium [DMEM; Dulbecco et al., Virology 8:396 (1959)] supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal calf serum. A typical inbred mouse strain is the Balb/c.

25 The monoclonal antibody compositions produced by the above method can be used in the same

manner as disclosed herein for antibodies of this invention, and are preferred.

For example, the monoclonal antibody can be used in the diagnostic methods and systems disclosed herein where formation of a C-terminal hGPIIb fragment-containing immunoreaction product is desired.

VI. Hybridomas

Hybridomas of the present invention are those characterized as having the capacity to produce an anti-GPIIb C-terminal fragment monoclonal antibody as described before.

Representative preferred hybridomas are prepared as described in Example 3. Particularly preferred is the hybridoma culture designated PMI-1.

Hybridoma culture PMI-1 has been deposited pursuant to Budapest Treaty requirements with the American Type Culture Collection (ATCC), Rockville, MD, on May 17, 1989, and was assigned accession number HB 9476.

Hybridoma HB 9476 was deposited in a depository affording permanence of the deposit and ready accessibility thereto by the public upon the issuance of a patent, under conditions which assure that access to the hybridoma will be available during the pending of the patent application to those deemed by the Commissioner to be entitled to such access, and that all restrictions on the availability to the public of the hybridoma as deposited will be irrevocably removed upon the granting of the patent. The deposited hybridoma will be maintained by the ATCC for the term of the patent or 30 years from the date of deposit, whichever is longer, and in all events for at least five years after the date of the last request for access. All maintenance fees have been paid.

28395-20

32

Methods for producing hybridomas secreting antibody molecules having a desired immunospecificity, i.e., having the ability to immunoreact with a particular protein, an identifiable epitope on a particular protein and/or a polypeptide, are well known in the art and are described further herein. Particularly applicable is the hybridoma technology described by Niman et al., Proc. Natl. Acad. Sci. USA, 80:4949-4953 (1983), and by Galfre et al., Meth. Enzymol., 73:3-46 (1981).

Other methods of producing a monoclonal antibody, a hybridoma cell, or a hybridoma cell culture are also well known. See, for example, the method of isolating monoclonal antibodies from an immunological repertoire as described by Sastry, et al., Proc. Natl. Acad. Sci., 86:5728-5732 (1989); and Huse et al., Science, 246:1275-1281 (1989).

Also contemplated by this invention is the hybridoma cell, and cultures containing a hybridoma cell that produce a monoclonal antibody of this invention.

VII. Diagnostic Systems

A diagnostic system of the present invention in kit form includes, in an amount sufficient to perform at least one assay, a composition containing polyclonal or monoclonal antibody of this invention or fragments thereof, as a separately packaged reagent. Instructions for use of the packaged reagent are also typically included.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to

be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like. Also included, in one form or another, may be charts, graphs and the like that demonstrate predetermined concentration levels correlating specific physiological conditions to thrombotic events.

A diagnostic system is contemplated for assaying for the presence, and preferably amount, of C-terminal hGPIIb fragment in a body fluid sample, such as blood, plasma or urine according to the diagnostic methods described herein.

Preferably, the antibody is present as a monoclonal antibody composition, comprising a monoclonal antibody as described herein.

A diagnostic system of the present invention typically also includes a label or indicating means capable of signaling the formation of a specifically bound complex containing an antibody molecule of the present invention.

As used herein, the terms "label" and "indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex. Any label or indicating means can be linked to or incorporated in an antibody molecule that is part of an antibody or monoclonal antibody composition of the present invention, or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel methods and/or systems.

28395-20

34

The labeling means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyanate (FITC), 5-dimethylamine-1-naphthalenesulfonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine, rhodamine 8200 sulphonyl chloride (RB200 SC) and the like. A description of immunofluorescence analysis techniques is found in DeLuca "Immunofluorescence Analysis", in Antibody As A Tool, Marchalonis et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982).

In preferred embodiments, the indicating group is an enzyme, such as horseradish peroxidase (HRO), glucose oxidase, or the like. In such cases where the principal indicating group is an enzyme such as HRP or glucose oxidase, additional reagents are required to visualize the fact that a receptor-ligand complex (immunoreactant) has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine. An additional reagent useful with glucose oxidase is 2,2'-azino-di-(3-ethyl-benzthiazoline-G-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling agents and are used illustratively herein. An exemplary radiolabelling agent is a radioactive element that produces gamma ray emissions. Elements which themselves emit gamma rays, such as ^{124}I , ^{125}I , ^{126}I , ^{132}I and ^{51}Cr represent one class of gamma ray emission-producing radioactive element indicating

2102115

35

groups. Particularly preferred is ^{125}I . Another group of useful labelling means are those elements such as ^{11}C , ^{18}F , ^{15}O and ^{13}N which themselves emit positrons. The positrons so emitted produce gamma rays upon encounters with electrons present in the animal's body. Also useful is a beta emitter, such as ^{111}In of ^3H .

The linking of labels, i.e., labeling of, polypeptides and proteins is well known in the art. For instance, antibody molecules produced by a hybridoma can be labeled by metabolic incorporation of radioisotope-containing amino acids provided as a component in the culture medium. See, for example, Galfre et al., Meth. Enzymol., 73:3-46 (1981). The techniques of protein conjugation or coupling through activated functional groups are particularly applicable. See, for example, Avrameas, et al., Scand. J. Immunol., Vol. 8, Suppl. 7:7-23 (1978), Rodwell et al., Biotech., 3:889-894 (1984), and U.S. Pat. No. 4,493,795.

The diagnostic systems can also include, preferably as a separate package, a specific binding agent. A "specific binding agent" is a molecular entity capable of selectively binding a reagent species of the present invention but is not itself an antibody molecule of the present invention. Exemplary specific binding agents are antibody molecules, complement proteins or fragments thereof, protein A and the like. Preferably, the specific binding agent can bind the antibody molecule of this invention when it is present as part of a complex.

In preferred embodiments the specific binding agent is labeled. However, when the diagnostic system includes a specific binding agent that is not labeled, the agent is typically used as an

28395-20

36

amplifying means or reagent. In these embodiments, the labeled specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to a reagent species-containing complex.

The diagnostic kits of the present invention can be used in an "ELISA" format to detect, for example, the presence or quantity of C-terminal hGPIIb fragment in a body fluid sample such as serum, plasma or urine. "ELISA" refers to an enzyme-linked immunosorbent assay that employs an antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of an antigen present in a sample. A description of the ELISA technique is found in Chapter 22 of the 4th Edition of Basic and Clinical Immunology by D.P. Sites et al., published by Lange Medical Publications of Los Altos, CA in 1982 and in U.S. Patents No. 3,654,090; No. 3,850,752; and No. 4,016,043.

An exemplary ELISA protocol is described in Example 4.

Thus, in preferred embodiments, the antibody or antigen reagent component can be affixed (operatively linked) to a solid matrix to form a solid support that is separately packaged in the subject diagnostic systems. The reagent is typically affixed to the solid matrix by adsorption from an aqueous medium, although other modes of affixation, well known to those skilled in the art, can be used. Exemplary affixation methods are described in Example 4.

Particularly preferred are embodiments suitable for competition ELISA assays wherein the antibody is in the liquid phase together with a sample containing an unknown amount of C-terminal hGPIIb

fragment and the antigen is in the solid phase in an amount sufficient to compete with liquid phase antigen for immunoreaction with the liquid phase antibody. In this embodiment, which is described in Example 4, the
5 antigen can be hGPIIb analog, C-terminal hGPIIb fragment, or isolated GPIIb-IIIa.

Useful solid matrices are well known in the art. Such materials include the cross-linked dextran available under the trademark SEPHADEX from Pharmacia
10 Fine Chemicals (Piscataway, NJ); agarose; polystyrene beads about 1 micron to about 5 millimeters in diameter available from Abbott Laboratories of North Chicago, IL; polyvinyl chloride, polystyrene, cross-linked polyacrylamide, nitrocellulose- or
15 nylon-based webs such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from polystyrene or polyvinylchloride.

Thus, in particularly preferred embodiments, a diagnostic kit further contains, in a separate
20 package, an antigen as described above for use in a the competitive ELISA assay in the form of a solid phase antigen as defined above.

The reagent species, labeled specific binding agent or amplifying reagent of any diagnostic
25 system described herein can be provided in solution, as a liquid dispersion or as a substantially dry power, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a
30 system. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

The packages discussed herein in relation to
35 diagnostic systems are those customarily utilized in

2102115

38

diagnostic systems. Such packages include glass and plastic (e.g., polyethylene, polypropylene and polycarbonate) bottles, vials, plastic and plastic-foil laminated envelopes and the like.

5

VIII. Assay Methods

The present invention contemplates any diagnostic method that results in detecting C-terminal hGPIIb fragment in a body fluid sample using an hGPIIb analog, a substantially isolated C-terminal hGPIIb fragment or an antibody of this invention as a reagent to form an immunoreaction product whose amount relates, either directly or indirectly, to the presence, and preferably amount, of C-terminal hGPIIb fragment in the sample.

Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures in which an immunochemical reagent of this invention can be used to form an immunoreaction product whose amount relates to the amount of C-terminal hGPIIb fragment present in a body sample. Thus, while exemplary assay methods are described herein, the invention is not so limited.

Various heterogeneous and homogeneous protocols, either competitive or noncompetitive, can be employed in performing an assay method of this invention.

Generally, to detect the presence of a C-terminal hGPIIb fragment in a patient, an aliquot (i.e., a predetermined amount) of a body fluid sample, such as urine or a vascular fluid, namely blood, plasma or serum from the patient is contacted by admixture (admixed), with an antibody composition of the present invention to form an immunoreaction admixture. The admixture is then maintained under

2102115

39

biological assay conditions for a period of time
sufficient for the C-terminal hGPIIb fragment present
in the sample to immunoreact with (immunologically
bind) a portion of the antibody combining sites
5 present in the antibody composition to form a
C-terminal.hGPIIb fragment-antibody molecule
immunoreaction product (immunocomplex). The complex
can then be detected as described herein. The
presence of the complex is indicative of C-terminal
10 hGPIIb fragment in the sample.

Maintenance time periods sufficient for
immunoreaction are well known and are typically from
about 10 minutes to about 16-20 hours at a temperature
of about 4C to about 45C, with the time and
15 temperature typically being inversely related. For
example, longer maintenance times are utilized at
lower temperatures, such as 16 hours at 4C, and
shorter times for higher temperatures, such as 1 hour
at room temperature.

20 Biological assay conditions are those that
maintain the biological activity of the immunochemical
reagents of this invention and the C-terminal hGPIIb
fragment sought to be assayed such that the reagents
retain their ability to form an immunoreaction
25 product. Those conditions include a temperature range
of about 4C to about 45C, a pH value of about 5 to
about 9 and an ionic strength varying from that of
distilled water to that of about one molar sodium
chloride. Methods for optimizing such maintenance
30 time periods and biological assay conditions are well
known in the art.

Preferred maintenance times and assay
conditions sufficient for immunoreaction to occur are
described in Example 4.

2102115

40

According to the discoveries described herein, thrombosis is accompanied by the cleavage of the carboxy terminus of hGPIIb by indigenous proteases, to form soluble C-terminal hGPIIb fragment in body fluid samples. Thus, in one embodiment, the detection of C-terminal hGPIIb fragment in a body sample is utilized as a means to monitor the presence of an in vivo thrombotic event in a patient (thrombosis) using an antibody molecule specific for the C-terminal hGPIIb fragment as disclosed herein.

In preferred embodiments, immunoassay of C-terminal hGPIIb fragment is conducted using a cell free body fluid sample. By cell free is meant that the sample does not contain detectable amounts of cells, tissue or other macroscopic biological materials normally present in a body fluid such as blood. A body fluid sample typically contains cells as a normal component, or as a contaminant, and can be rendered cell-free by a variety of biochemical procedures including centrifugation, filtration and chromatography, so long as the retained fluid sample contains substantially all of the soluble protein initially present in the sample prior to removal of the cells. Particularly preferred are centrifugation steps such as described in Example 4.

Thus a body fluid sample, such as blood, known to contain both platelets having GPIIb-IIIa thereon and soluble C-terminal hGPIIb fragment, is preferably treated, prior to admixing, to a manipulation that results in forming a cell-free body fluid sample, ie, separating the cells present in the sample away from the body fluid sample to form a cell-free body fluid sample. An aliquot of the cell-free body fluid sample is then admixed with an antibody of the invention to form an immunoreaction admixture.

Alternatively, the separation of C-terminal hGPIIb fragment from cells having GPIIb, such as platelets, may be accomplished at other stages of the assay methods disclosed herein. For example, the immunoreaction product containing C-terminal hGPIIb fragment can be separated from platelets present in the body fluid sample by centrifugation after the maintenance step under conditions that separate cells, i.e., platelets, from protein complexes such as an immunocomplex between C-terminal hGPIIb fragment and an antibody of this invention.

Determining the presence or amount of C-terminal hGPIIb fragment-containing immunoreaction product formed by the above maintenance step, either directly or indirectly, can be accomplished by assay techniques well known in the art, and typically depend on the type of indicating means used.

In a preferred competition assay method, the immunoreaction admixture described above further contains a solid phase having affixed thereto a solid phase antigen comprising a hGPIIb analog or a polypeptide having an amino acid residue sequence that includes the sequence of a C-terminal hGPIIb fragment of this invention. Thus, in this embodiment, the assay comprises the steps of:

(a) admixing a body fluid sample with 1) an antibody composition of this invention and 2) a solid support having affixed thereto (operatively linked) a hGPIIb analog or a polypeptide having an amino acid residue sequence that includes the sequence of a C-terminal hGPIIb fragment of this invention, or both, to form an immunoreaction admixture having both a liquid phase and a solid phase;

(b) maintaining said immunoreaction admixture under biological assay conditions for a time

period sufficient to form an immunoreaction product in the solid phase; and

5 (c) detecting the presence, and preferably amount, of the immunoreaction product formed in the solid phase in step (b), and thereby the amount of presence/amount of C-terminal hGPIIb fragment in the body fluid sample.

10 Where an hGPIIb analog is used in the solid phase, the antibody composition contains antibody molecules that immunoreact with the hGPIIb analog. Preferably, the solid phase antigen is isolated GPIIb-IIIa, prepared as described in Example 1b, or substantially isolated C-terminal hGPIIb fragment, prepared as described in Example 1c.

15 Preferably, the body fluid sample is a cell free body fluid sample such as urine or platelet poor plasma.

More preferably, detecting in step (c) is performed by the steps of:

20 (i) admixing the immunoreaction product formed in step (b) with an indicating means to form a second reaction admixture;

25 (ii) maintaining the second reaction admixture for a time period sufficient for said indication means to bind the immunoreaction product formed in step (b) and form a second reaction product; and,

30 (iii) determining the presence and/or amount of indicating means in the second reaction product, and thereby the presence of the immunoreaction product formed in step (b). Particularly preferred is the use of a labeled second antibody, immunospecific for the first antibody, as the indicating means, and preferably the label is horseradish peroxidase. In
35 one embodiment, it is particularly preferred to use

(1) isolated GPIIb-IIIa as the solid phase antigen,
(2) PMI-1 monoclonal antibody in the antibody
composition, and (3) goat anti-mouse IgG antibodies
labeled with horseradish peroxidase as the indicating
5 means. Exemplary is the competition ELISA format
described in Example 4.

In another competition assay format the
immunoreaction admixture contains (1) a body fluid
sample, preferably cell free, (2) an antibody of this
10 invention and (3) a labeled hGPIIb analog or labeled
C-terminal hGPIIb fragment, wherein the antibody is
present in the solid phase, being affixed to a solid
support, to form a liquid and a solid phase. In this
embodiment, the admixed body fluid sample competes
15 with the labeled reagent for immunoreaction with the
solid phase antibody to form a solid phase
immunoreaction product. Thereafter, the detection of
label in the solid phase correlates with the amount of
C-terminal hGPIIb fragment in the admixed fluid
20 sample.

The following examples are intended to
illustrate, but not limit, the present invention.

Examples

25 1. Preparation of Substantially Isolated C-Terminal hGPIIb Fragment

a. Platelet Isolation

One unit of whole human blood was collected
in ACD (0.065 M citric acid, 0.085 M sodium citrate,
2% dextrose) containing hirudin (Sigma Chemical Co.,
30 St. Louis, MO) at a final concentration of 0.06 units
per milliliter (U/ml) and centrifuged for 15 minutes
(min) at 120 x g. The resulting supernatant,
designated platelet-rich plasma (PRP), was recovered,
isolated and further centrifuged for 15 min at 1200 x
35 g to form a pellet of isolated platelets. The

2102115

44

supernatant formed was collected and designated platelet-poor plasma (PPP).

b. GPIIb-IIIa Isolation from Platelets

5 A platelet pellet prepared as in Example 1a, was resuspended in 5 ml TBS (0.15 M NaCl, 0.2 M Tris, pH 7.4, 5×10^{-4} M CaCl_2 , 10^{-5} M leupeptin) and sonicated on ice for 10 min at a maximum setting using a Model W-375 sonicator (Heat Systems Ultrasonics, Plainview, NY). The sonicated suspension was twice frozen and thawed using a dry ice-methanol ice bath and stored at minus 20 degrees C (20 C). The frozen-thawed platelet sonicate was layered on top of 5 ml of a sucrose solution (40% v/v in TBS), and centrifuged at 4 degrees C for one hour at 38,000 rotations per minute (RPM) in a SW41 centrifuge rotor (Beckman Instruments, Fullerton, CA) to form a milky colored infranatant. The milky-infranatant was then recovered and centrifuged at 43,000 RPM in a SW50.1 centrifuge rotor (Beckman) at 4 C for one hour. The resulting pellet was resuspended in typically 1-2 ml TBS to form a platelet membrane solution, the protein concentration of which was determined to be in the range of 10-25 mg/ml, typically using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA) according to the manufacturer's instructions.

 The platelet membrane solution was again centrifuged in a SW50.1 centrifuge rotor as above and the resulting pellet was resuspended in 2 ml of extraction buffer (0.03 M Tris, pH 7.4, 1×10^{-5} M leupeptin, 200 mM n-octyl-beta-D-glucopyranoside; Calbiochem-Behring, La Jolla, CA). The platelet membrane extract thus formed was admixed thoroughly by vortexing and then maintained at room temperature for 30 min. The extract was thereafter centrifuged at

28395-20

45

45,000 rpm in a SW50.1 centrifuge rotor for 1 hour at 4 C and the platelet membrane extract supernatant thus formed was recovered.

The recovered supernatant was applied to a LKB Ultrogel^{*} Aca 34 gel filtration column (3 x 97 cm, LKB Instruments, Gaithersburg, MD) that had been equilibrated with 1 liter of column buffer (0.03 M Tris, pH 7.4, 0.1 mM CaCl₂, 0.1% n-octyl-beta-D-glucopyranoside) and 5 ml fractions were collected from the resulting column effluent. The optical density at 280 nanometers of each fraction was determined and fractions around the several peaks were combined to form a pool for each peak. Samples from each pool were analyzed by electrophoresis in 6% polyacrylamide slab gels using the reducing buffers and procedures described by Laemmli, Nature (London), 227:680-685 (1970), and low molecular weight protein standards ranging in size from 14.4 kilodaltons (kDa) to 92.5 kDa (Bio-Rad, Richmond, CA). The pool containing predominantly two protein species having molecular weights corresponding to GPIIb and GPIIIa, i.e., 120 kDa and 100 kDa, respectively was recovered and is designated as a composition containing isolated GPIIb-IIIa. The protein concentration of the isolated GPIIb-IIIa containing-composition thus prepared was typically determined using the Bio-Rad Protein Assay Kits to be in the range of 0.3 to 0.8 mg/ml.

c. Isolation of Carboxy-Terminal
Fragment of hGPIIb from Platelets

(1) Digestion with Proteases

A platelet pellet prepared as in Example 1a was resuspended in Tyrode's solution (Sigma; 137 mM NaCl, 2 mM KCl, 12 mM NaHCO₃, 0.3 mM NaH₂PO₄, 2 mM CaCl₂, 1mM MgCl₂, 5.5 mM glucose, 5 mM HEPES, pH 7.4) supplemented with 100 nM PGE1 (Sigma; prostaglandin

*Trade-mark

2102115

46

inhibitor of activation), apyrase (Sigma) (primary suspension contained 7.5 ug/ml of PRP; washes contained 25 ug/ml), and BSA, 3.5 mg/ml. After two centrifugations in supplemented Tyrode's, the platelet concentration was adjusted to 1×10^9 per ml in supplemented Tyrode's. Alpha-chymotrypsin (Sigma) was added to produce a final concentration of 0.75 mg/ml and the solution was incubated for 60 min at 37 degrees C. The reaction was then stopped by adding a 200 mM stock solution of phenylmethylsulfonyl fluoride (PMSF) to form a final concentration of 1.0 mM PMSF in the platelet suspension.

During the time course of the 1 hour chymotrypsin incubation of the platelet suspension, aliquots of 0.2 ml containing 2×10^8 platelets were taken and processed further. A first aliquot (0.2 ml) of platelet suspension was centrifuged at $15,000 \times g$ for 5 min at room temperature to form a supernatant and a pellet. The supernatant was collected to form a supernatant sample. The pellet was resuspended in 0.2 ml of supplemented Tyrode's containing 1 mM PMSF to form a resuspended pellet sample for use in the ELISA assay described in Example 4b. A second aliquot (0.2 ml) of platelet suspension was used directly as a total sample in the ELISA assay of Example 4b. The presence, if any, of C-terminal hGPIIb fragment was then measured in the three samples (supernatant sample, resuspended pellet sample, and total sample extract) using the competition ELISA assay described in Example 4b.

The results are shown in Figure 1, and indicate the levels of the PMI-1 epitope (Figure 1A) and the levels of the TAB epitope (Figure 1B) in the three fractions. Monoclonal antibody PMI-1 immunoreacts with the PMI-1 epitope on hGPIIb defined

28395-20

47

by the polypeptide IHPAHHK (SEQ ID NO 1, from residue 194 to residue 200). Monoclonal antibody TAB immunoreacts with the TAB epitope located on a portion of hGPIIb other than the C-terminal hGPIIb fragment, because the material immunoreactive with TAB does not become released into the supernatant after chymotrypsin digestion.

(2) Isolation of C-Terminal hGPIIb Fragment

Isolated PMI-1 antibody molecules prepared as described in Example 3c, are covalently linked to Sepharose beads using cyanogen bromide-activated Sepharose* (Pharmacia) according to the manufacturer's instructions to form PMI-1 Sepharose having about 20 mg of antibody protein per ml of packed beads.

Five ml of chymotrypsin digested platelet suspension, prepared as in Example 1c(1), is admixed with 5 ml of packed PMI-1 Sepharose beads and the resulting admixture is maintained at 4 C for 12-18 hours to allow any C-terminal hGPIIb fragment in the suspension to bind to the PMI-1 Sepharose. The PMI-1 Sepharose is then transferred to a column containing a scintered glass support, and washed with 25 ml of wash buffer (described in Example 4), also containing 50 mM octylglucoside. Thereafter wash buffer containing 50 mM octylglucoside is added to the column and 1.25 ml fractions are collected as the added buffer comes off (elutes from) the column to form eluted fractions.

Fifty microliters (ul) of each eluted fraction is then analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) using a 10 - 20% acrylamide gel gradient containing 3% SDS and 5% 2-mercaptoethanol according to the methods of Laemmli, Nature, 227:680-685 (1970). Proteins contained in the eluted fractions so analyzed are

*Trade-mark

2102115

48

transferred from the SDS-PAGE gels to nitrocellulose and visualized by autoradiography according to the method of Johnson et al, Gen. Anal. Techn., 1:3-8 (1984) using ¹²⁵I-labeled PMI-1. The apparent molecular weight of the visualized proteins is determined by comparison to protein molecular weight markers also analyzed on the gel. All molecular weights are in units of one thousand daltons.

Fractions containing eluted proteins that reacted with PMI-1 antibody are collected and pooled to form an isolated C-terminal hGPIIb fragment-containing solution, i.e., substantially isolated hGPIIb fragment. The isolated protein exhibited an apparent weight of about 3,900 daltons when analyzed by SDS-PAGE, and constituted the major protein component in the isolated fragment-containing solution.

The amino acid residue sequence of the C-terminal hGPIIb fragment identified herein after in vitro proteolysis of platelets is shown in SEQ ID NO 1 from residue 173 to residue 200.

2. Preparation of Polypeptide Analogs

Based on the amino acid residue sequence deduced from the nucleotide sequence of hGPIIb, polypeptides corresponding to the various hGPIIb C-terminal regions utilized herein are chemically synthesized on an Applied Biosystems Model 430A Peptide Synthesizer using the symmetrical anhydride method of Hagenmaier, et al., Hoppe-Seyler's Z. Physiol. Chem., 353:1973 (1982).

The amino acid residue sequences of the various polypeptide analogs synthesized are shown in Table 1.

28395-20

49

3. Antibody Production

a. Preparation of Immunogen

The term "immunogen", as used herein, describes an entity that induces antibody production in the host animal. In some instances, the antigen and immunogen are the same entity, while in other instances, the two entities are different. Immunogens used to elicit antibodies of this invention are whole platelets, platelet membranes, isolated GPIIb-IIIa, isolated C-terminal hGPIIb fragment or hGPIIb polypeptide analogs as described above.

Polyclonal anti-peptide antibodies are produced by immunizing rabbits with a peptide prepared in Example 2 above and coupled to carrier protein. Coupling of the peptide with glutaraldehyde to thyroglobulin as antigenic carrier (Bovine Type I, Sigma Chemical Co., St. Louis, MO) is conducted as described by J.G. Dockray, Regulatory Peptides, 1:169-186 (1980) to form thyroglobulin coupled peptide.

b. Preparation of Polyclonal Antibody Compositions

Immunizations to produce a polyclonal antibody composition are done according to standard protocols. Briefly, a rabbit is immunized intradermally at one week intervals with 400 micrograms (ug) of immunogen consisting of thyroglobulin coupled peptide at 3 mg/ml. The immunogen is diluted 1:1 in Complete Freund's Adjuvant for the first immunization, in Incomplete Freund's Adjuvant for the second and third immunizations, and in normal saline for the fourth and fifth.

Injectons are administered with 100 ug distributed over 4 sites. Thereafter, the antibody is harvested and tested according to the assay of Example

28395-20

50

4a. In that assay, polyclonal antibody produced using a peptide of Table 1 immunoreacts with the carboxy terminal hGPIIb fragment and with the immunizing peptide, but does not immunoreact with the polypeptide REQNSLDSWGPK (SEQ ID NO 1, from residue 113 to residue 124).

c. Preparation of Monoclonal Antibody Compositions

Monoclonal antibodies that immunoreact with C-terminal hGPIIb fragment were produced using standard hybridoma technology with exceptions as noted. Balb C mice were immunized biweekly with 50 ug of platelet membranes prepared as described by Barber et al., J. Biol. Chem., 245:6357-65 (1970),

emulsified

alternately in complete and incomplete Freund's adjuvant. Mice were bled from the retro-orbital plexus, and sera were screened for antibodies by solid-phase radioimmunoassay as described in Shadle et al., J. Cell Biol., 99:2056-2060 (1984). Three days after the fifth immunization, about 1×10^8 lymphocytes were isolated from the spleens of mice, admixed into a suspension and fused with 5×10^7 P3X63AG8.053 mouse myeloma cells using 50% PEG 4000 as the cell fusion promoter. The resultant transformed (fused) antibody-producing cells (hybridomas) were initially transferred to 96-well microtiter plates at a density of about 1×10^6 cells per well and cultured in selective HAT medium.

Tissue culture supernatant appearing to contain viable HAT resistant hybridoma cells after 8 days of culturing were screened in the ELISA assay described in Example 4a for the presence of antibody molecules that immunoreact with GPIIb-IIIa prepared as described in Example 1b. Hybridoma cultures were

2102115

51

identified that produced GPIIb-IIIa-immunoreacting antibody molecules. The isolated hybridomas were then subcloned twice at limiting dilutions to provide about 1 cell per well and 24 of the resultant hybridoma cultures were shown to be of monoclonal origin on the basis of three criteria: (1) each supernatant was from a single cell focus and immunoreacted with GPIIb-IIIa in the ELISA screen, (2) each supernatant showed a single homogeneous band when analyzed by cellulose acetate gel electrophoresis according to the method described in Monoclonal Antibodies: Principles and Practice, J. W. Goding, ed., Academic Press, Inc., Orlando, Florida, 1983, and (3) each supernatant contained a single isotype of immunoglobulin when analyzed using the Mouse Ig Screening and Isotyping Kit according to the instructions provided by the manufacturer, Boehringer-Mannheim Biochemicals, Indianapolis, Indiana.

One monoclonal antibody producing hybridoma, PMI-1, isolated by the above procedure using an inoculum containing platelet membranes is available from the ATCC and has an accession number HB 9476.

A culture of hybridoma cells capable of secreting PMI-1 monoclonal antibodies were introduced into a mouse and the ascites fluid was then harvested as is well known. PMI-1 monoclonal antibody molecules present in the harvested ascites were isolated using protein-A Sepharose (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions to form isolated PMI-1 antibody molecules.

The protein concentration of isolated antibody molecules in a liquid composition was determined, as needed, using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA) according to the manufacturer's instructions.

2102115

52

To prepare a monoclonal antibody composition containing ^{125}I -labeled antibody molecules, 350 microliters (ul) of PBS (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.09) containing 1 milligram per milliliter (mg/ml) of the above isolated PMI-1 antibody molecules were admixed with 40 micrograms (ug) of chloramine-T and 1 milliCurie (mCi) of carrier-free Na^{125}I (Amersham, Arlington Heights, IL). The resulting admixture was maintained for 5 minutes at about 20 C and then admixed with 20 ul of a 2 mg/ml sodium metabisulfite solution (2 mg/ml) and 20 ul of a potassium iodide solution. Thereafter, 800 ul of PBS containing 1% BSA were admixed followed by further admixture of diisopropylfluorophosphate to a final concentration of 10 mM. The resulting admixture was maintained for 60 minutes at 22 C and then dialyzed against PBS. The specific activity of the resulting ^{125}I -labeled antibody molecules was about 4.5 microCurie (uCi) per ug.

Compositions containing Fab fragments were prepared by digesting isolated PMI-1 antibody molecules with papain (200:1 weight per weight of Ig to papain) for 6 hours at 37°C following the methods of Mage et al., Methods in Enzymology, 70:142-150 (1980). Undigested Ig and Fc fragments were removed by chromatography on protein A-Sepharose. The resulting Fab fragments-containing compositions were then ready for use, or were ^{125}I -labeled, as needed, using the same procedures as described above for monoclonal antibody compositions.

4. Diagnostic Assays

a. ELISA To Screen Monoclonal Antibodies

Antibody molecules contained in hybridoma culture supernatant are examined for their ability to

28395-20

53

immunoreact with hGPIIb polypeptide analogs. Fifty microliters (ul) of coating solution (0.1M NaHCO₃, pH 8.0, 0.1% NaN₃) containing 10 ug/ml of polypeptide analogs prepared in Example 2 are admixed into the wells of flat-bottom 96-well microtiter plates (Immulon 2;^{*} Dynatech Laboratories, Chantilly, VA). The plates are then maintained for 60 minutes at 37°C to permit the GPIIb-IIIa to adsorb onto the walls of the wells. The coating solution is removed by shaking, the wells are rinsed twice with wash buffer (10 mM Tris at pH 7.4, 0.05% (v/v) TWEEN-20,^{*} 0.15 M NaCl, and 200 mg/ml merthiolate), and then 200 ul of blocking solution [5% bovine serum albumin (BSA;w/v) in coating solution] are admixed into each well (solid support) to block excess protein sites.

The wells are maintained for 60 minutes at about 37°C and then the blocking solution is removed. About 50 ul of hybridoma culture supernatant diluted 1:1 in dilution buffer consisting of 0.1% (w/v) BSA in wash buffer is added to each well to form an immunoreaction admixture. The resulting solid/liquid phase immunoreaction admixtures are maintained at room temperature for 60 minutes to permit formation of a first solid phase-bound immunoreaction product between the solid phase-bound polypeptide analog and admixed antibodies. The solid and liquid phases are then separated, the wells are rinsed twice with wash buffer, and excess liquid is removed by shaking.

Fifty ul of a solution containing horseradish peroxidase labeled goat anti-mouse IgG (Tago Inc., Burlingame, CA), diluted 1:1000 in dilution buffer is admixed into each well to form a second solid/liquid phase immunoreaction admixture (labeling immunoreaction admixture). The wells are maintained for 60 minutes at room temperature to

*Trade-mark

2102115

54

5 permit formation of a second immunoreaction product between the labeled antibody and any solid phase-bound antibody of the first immunoreaction product and then rinsed twice with wash buffer to isolate the solid phase-bound label-containing immunoreaction products. Excess liquid is then removed from the wells.

10 Fifty ul of freshly prepared chromogenic substrate solution containing 4.0 mg/ml 0-phenylenediamine and 0.012% (v/v) hydrogen peroxide in CP buffer (243 ml of 0.1 M citric acid and 250 ml of 0.2 M dibasic sodium phosphate per liter H₂O, pH 5.0) are then admixed into each well to form a color developing-reaction admixture. After maintaining the color developing-reaction admixture for 10 minutes at 15 about 20 C, 50 ul of 2 N H₂SO₄ are admixed into each well to stop the developing-reaction, and the resulting solutions are assayed for absorbance at 490 nanometers (nm) light wavelength using a Model 310 ELISA plate reader (Bio-Tek Instruments, Winooski, 20 VT).

Antibody molecule compositions are considered to contain antibody molecules immunoreactive with C-terminal hGPIIb fragment if the measured absorbance at 490 nm (A₄₉₀) is at least 6 25 times above background i.e., above about 0.3 optical density units when measured at A₄₉₀.

b. Competition ELISA Assays to Detect C-Terminal GPIIb Heavy Chain Fragment in Body Fluids

30 Assays to detect C-terminal hGPIIb fragment can be performed on "platelet-poor" plasma, serum or urine samples without concentration. Urine (serum and plasma are used in similar fashion) samples were centrifuged at 15,000 x g for 5 min at room temp, and 35 the resulting supernatants were collected to form cell

free urine samples. The resulting cell-free urine samples are substantially free of platelets and therefore any hGPIIb fragment present in the sample is in the form of soluble, cell-free, hGPIIb fragment.

5 Fifty microliters (ul) of coating solution (0.1M NaHCO₃, pH 8.0, 0.1% NaN₃) containing 10 ug/ml of isolated GPIIb-IIIa prepared as described in Example 1b were admixed into the wells of flat-bottom 96-well microtiter plates (Immulon 2; Dynatech Laboratories, Chantilly, VA). The plates were then maintained for 10 60 minutes at 37°C to permit the GPIIb-IIIa to adsorb onto the walls of the wells. The coating solution was removed by shaking and 200 ul of blocking solution [5% bovine serum albumin (BSA;w/v) in coating solution] 15 were admixed into each well (solid support) to block excess protein sites.

The wells were maintained for 60 minutes at about 37°C and then the blocking solution was removed by rinsing the wells thrice with wash buffer (10 mM 20 Tris at pH 7.4, 0.05% (v/v) TWEEN-20, 0.15 M NaCl, and 200 mg/ml merthiolate). Isolated monoclonal antibody PMI-1 prepared in Example 2c or a control monoclonal antibody (TAB), that immunoreacts with hGPIIb but does not immunoreact with C-terminal hGPIIb fragment, were 25 used in the competition ELISA. TAB was obtained from Dr. R. McEver, University of Texas (San Antonio, TX). About 50 ul of monoclonal antibody, diluted 1:1 in incubation buffer consisting of 0.1% (w/v) BSA in wash buffer, was admixed, along with the sample to be 30 tested, to each well to form an immunoreaction admixture. The proportions were as follows:

(a) 20 ul incubation buffer + 10 ul of 20 mM EDTA in incubation buffer + 20 ul of sample prepared in Example 1c(i); and

35 (b) 50 ul PMI-1 or TAB monoclonal antibody

28395-20

56

[1:1000 of 4.5 mg/ml IgG (purified on Staph. A columns)].

One set of immunoreaction admixtures was prepared using a test sample, and a second set of immunoreaction admixtures was prepared in which the sample is replaced with a reference standard comprised of a predetermined amount of either isolated GPIIb-IIIa, if the antibody was TAB, or polypeptide V43 (PSPSPIHPAHHKRRDQQ) (SEQ ID NO 3), prepared as in Example 2, if the antibody was PMI-1.

Each immunoreaction admixture was incubated overnight at room temperature to permit formation of a first solid phase-bound immunoreaction product containing the solid phase-bound GPIIb-IIIa and the admixed antibodies.

The solid and liquid phases were then separated, the wells were rinsed thrice with washing buffer, and excess liquid was removed by shaking. Fifty ul of a solution containing horseradish peroxidase labeled goat anti-mouse IgG (Tago Inc., Burlingame, CA), diluted 1:1000 in incubation buffer was admixed into each well to form a second solid/liquid phase immunoreaction admixture (labeling immunoreaction admixture). The wells were maintained for 60 minutes at room temperature to permit formation of a second immunoreaction product between the labeled antibody and any solid phase-bound antibody of the first immunoreaction product and then rinsed thrice with wash buffer to isolate the solid phase-bound label-containing immunoreaction products. Excess liquid was then removed from the wells.

Fifty ul of freshly prepared chromogenic substrate solution containing 4.0 mg/ml 0-phenylenediamine and 0.012% (v/v) hydrogen peroxide in CP buffer (243 ml of 0.1 M citric acid and 250 ml

2102115

57

of 0.2 M dibasic sodium phosphate per liter H₂O, pH 5.0) were then admixed into each well to form a color developing-reaction admixture. After maintaining the color developing-reaction admixture for 10 minutes at about 20 C, 50 ul of 2 N H₂SO₄ were admixed into each well to stop the developing-reaction, and the resulting solutions were assayed for absorbance at 490 nanometers (nm) light wavelength using a Model 310 ELISA plate reader (Bio-Tek Instruments, Winooski, VT).

Absorbance measurements obtained using standard were plotted against micromolar (uM) concentration of standard in the immunoreaction admixture to produce a standard curve. Absorbance measurements obtained using samples were compared to the standard curve to produce a value expressed as equivalents of standard.

Figure 1 shows results of the above competition ELISA using samples containing C-terminal hGPIIb fragment that were formed as described in Example 1c(1). The data is expressed as equivalents of standard (in micromoles) detectable in the sample during the time course of the chymotrypsin digestion. Using polypeptide V43 as a standard in combination with antibody PMI-1, antigenic material that corresponds to C-terminal hGPIIb fragment was released into the supernatant during the course of the digest (Figure 1A). In contrast, no detectable antigens were released in the supernatant that immunoreact with the TAB antibody (Figure 1B).

Urine samples from a normal donor in the form of cell free urine samples as described above were tested in the above competition ELISA using various dilutions of urine in incubation buffer from undiluted to 1 part urine to 7 parts buffer (1:8).

2102115 58

Equivalents of peptide V43 detectable in the urine using PMI-1 were 1.5 uM at 1:8, 3.8 uM at 1:4 and 1.5 uM at 1:2. From the data, a normalized value of C-terminal hGPIIb fragment in undiluted urine was 14.4 uM for the normal donor's urine. Thus levels of C-terminal hGPIIb fragment in urine that are significantly greater than about 15 uM, such as a 10% increase over normal values, is indicative of thrombotic activity above normal levels.

Anti-peptide antibodies prepared as described in Example 3c using the polypeptides shown in Table 1 are used in the competition ELISA in place of monoclonal antibody PMI-1, and using the antibody's corresponding polypeptide rather than isolated GPIIb-IIIa as the solid-phase antigen. In this assay, samples containing competing antigen, i.e., C-terminal hGPIIb fragment, or containing standard comprised of the same corresponding peptide, are measured as above for the presence and amount of C-terminal hGPIIb fragment. By this competition ELISA assay, anti-peptide antibodies that immunoreact with a polypeptide shown in Table 2 are shown to also immunoreact with isolated GPIIb/IIIa and with C-terminal hGPIIb fragment prepared in Example 1c(2)

Although the present invention has now been described in terms of certain preferred embodiments, and exemplified with respect thereto, one skilled in the art will readily appreciate that various modifications, changes, omissions and substitutions may be made without departing from the spirit thereof. It is intended, therefore, that the present invention be limited solely by the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ginsberg, Mark H.
Frelinger III, Andrew L.
Plow, Edward F.

(ii) TITLE OF INVENTION: IN VITRO METHODS FOR DETERMINING IN VIVO
THROMBOTIC EVENTS

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Office of Patent Counsel, The Scripps Research Institute
(B) STREET: 10666 North Torrey Pines Road, Mail Drop TPC8
(C) CITY: La Jolla
(D) STATE: California
(E) COUNTRY: United States
(F) ZIP: 92037

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US92/
(B) FILING DATE: 30-APR-1992
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/693,388
(B) FILING DATE: 30-APR-1991

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Fitting, Thomas
(B) REGISTRATION NUMBER: 34,163
(C) REFERENCE/DOCKET NUMBER: SCR0787P

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 619-554-2937

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 600 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

2102115

60

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..600

(D) OTHER INFORMATION: /standard_name= "Portion of GPIIb heavy chain"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGC GCC CAC TAC ATG CGG GCC CTA AGC AAT GTC GAG GGC TTT GAG AGA	48
Gly Ala His Tyr Met Arg Ala Leu Ser Asn Val Glu Gly Phe Glu Arg	
1 5 10 15	
CTC ATC TGT AAT CAG AAG AAG GAG AAT GAG ACC AGG GTG GTG CTG TGT	96
Leu Ile Cys Asn Gln Lys Lys Glu Asn Glu Thr Arg Val Val Leu Cys	
20 25 30	
GAG CTG GGC AAC CCC ATG AAG AAG AAC GCC CAG ATA GGA ATC GCG ATG	144
Glu Leu Gly Asn Pro Met Lys Lys Asn Ala Gln Ile Gly Ile Ala Met	
35 40 45	
TTG GTG AGC GTG GGG AAT CTG GAA GAG GCT GGG GAG TCT GTG TCC TTC	192
Leu Val Ser Val Gly Asn Leu Glu Glu Ala Gly Glu Ser Val Ser Phe	
50 55 60	
CAG CTG CAG ATA CGG AGC AAG AAC AGC CAG AAT CCA AAC AGC AAG ATT	240
Gln Leu Gln Ile Arg Ser Lys Asn Ser Gln Asn Pro Asn Ser Lys Ile	
65 70 75 80	
GTG CTG CTG GAC GTG CCG GTC CGG GCA GAG GCC CAA GTG GAG CTG CGA	288
Val Leu Leu Asp Val Pro Val Arg Ala Glu Ala Gln Val Glu Leu Arg	
85 90 95	
GGG AAC TCC TTT CCA GCC TCC CTG GTG GTG GCA GCA GAA GAA GGT GAG	336
Gly Asn Ser Phe Pro Ala Ser Leu Val Val Ala Ala Glu Glu Gly Glu	
100 105 110	
AGG GAG CAG AAC AGC TTG GAC AGC TGG GGA CCC AAA GTG GAG CAC ACC	384
Arg Glu Gln Asn Ser Leu Asp Ser Trp Gly Pro Lys Val Glu His Thr	
115 120 125	
TAT GAG CTC CAC AAC ACT GGC CCT GGG ACT GTG AAT GGT CTT CAC CTC	432
Tyr Glu Leu His Asn Thr Gly Pro Gly Thr Val Asn Gly Leu His Leu	

61

130	135	140	
AGC ATC CAC CTT CCG GGA CAG TCC CAG CCC TCC GAC CTG CTC TAC ATC			480
Ser Ile His Leu Pro Gly Gln Ser Gln Pro Ser Asp Leu Leu Tyr Ile			
145	150	155	160
CTG GAT ATA CAG CCC CAG GGG GCG CTT CAG TGC TTC CCA CAG CCT CCT			528
Leu Asp Ile Gln Pro Gln Gly Ala Leu Gln Cys Phe Pro Gln Pro Pro			
	165	170	175
GTC AAT CCT CTC AAG GTG GAC TGG GGG CTG CCC ATC CCC AGC CCC TCC			576
Val Asn Pro Leu Lys Val Asp Trp Gly Leu Pro Ile Pro Ser Pro Ser			
	180	185	190
CCC ATT CAC CCG GCC CAT CAC AAG			600
Pro Ile His Pro Ala His His Lys			
	195	200	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 200 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Ala His Tyr Met Arg Ala Leu Ser Asn Val Glu Gly Phe Glu Arg
 1 5 10 15
 Leu Ile Cys Asn Gln Lys Lys Glu Asn Glu Thr Arg Val Val Leu Cys
 20 25 30
 Glu Leu Gly Asn Pro Met Lys Lys Asn Ala Gln Ile Gly Ile Ala Met
 35 40 45
 Leu Val Ser Val Gly Asn Leu Glu Glu Ala Gly Glu Ser Val Ser Phe
 50 55 60
 Gln Leu Gln Ile Arg Ser Lys Asn Ser Gln Asn Pro Asn Ser Lys Ile
 65 70 75 80
 Val Leu Leu Asp Val Pro Val Arg Ala Glu Ala Gln Val Glu Leu Arg
 85 90 95
 Gly Asn Ser Phe Pro Ala Ser Leu Val Val Ala Ala Glu Glu Gly Glu
 100 105 110
 Arg Glu Gln Asn Ser Leu Asp Ser Trp Gly Pro Lys Val Glu His Thr

2102115

62

115		120		125											
Tyr	Glu	Leu	His	Asn	Thr	Gly	Pro	Gly	Thr	Val	Asn	Gly	Leu	His	Leu
130						135					140				
Ser	Ile	His	Leu	Pro	Gly	Gln	Ser	Gln	Pro	Ser	Asp	Leu	Leu	Tyr	Ile
145					150					155					160
Leu	Asp	Ile	Gln	Pro	Gln	Gly	Ala	Leu	Gln	Cys	Phe	Pro	Gln	Pro	Pro
				165					170					175	
Val	Asn	Pro	Leu	Lys	Val	Asp	Trp	Gly	Leu	Pro	Ile	Pro	Ser	Pro	Ser
			180					185					190		
Pro	Ile	His	Pro	Ala	His	His	Lys								
			195				200								

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:

- (A) NAME/KEY: Region
- (B) LOCATION: 1..17
- (D) OTHER INFORMATION: /note= "Polypeptide V43"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro	Ser	Pro	Ser	Pro	Ile	His	Pro	Ala	His	His	Lys	Arg	Asp	Arg	Arg
1				5					10					15	

Gln

28395-20

63

CLAIMS:

1. A substantially isolated C-terminal hGPIIb fragment that has a molecular weight of about 3,900 daltons, comprising an amino acid residue sequence that corresponds
5 to the carboxy terminal portion of the amino acid residue sequence of hGPIIb shown in SEQ ID NO:2, wherein the carboxy terminus of said fragment ends with the amino acid residue sequence represented by the formula -IHPAHHK-, said fragment having the capacity to immunoreact with the monoclonal
10 antibody, PMI-1.
2. The hGPIIb fragment of claim 1 wherein said amino acid residue sequence comprises the amino acid residue sequence shown in SEQ ID NO:2 from about residue 173 to residue 200.
- 15 3. A substantially isolated C-terminal hGPIIb polypeptide that has a molecular weight of about 3,900 daltons and comprises an amino acid residue sequence shown in SEQ ID NO:2 from residue 173 to 200.
4. A hGPIIb analog (i) comprising a polypeptide which
20 has no more than about 200 residues and which comprises at least 7 contiguous amino acid residues of the amino acid sequence shown in SEQ ID NO:2 from the region from residue 173 to residue 200; and (ii) having the amino acid sequence -IHPAHHK- at the carboxy terminus.
- 25 5. The analog of claim 4 wherein the at least 7 contiguous amino acid residues comprise amino acid residue sequence PQPPVNPLK set forth in SEQ ID NO:2 from residue 173 to residue 181, PLKVDWGLPIP set forth in SEQ ID NO:2 from residue 179 to residue 189, PIPSPSPIHPAHHK set forth in SEQ
30 ID NO:2 from residue 187 to residue 200, or IHPAHHK set

28395-20

64

forth in SEQ ID NO:2 from residue 194 to residue 200.

6. A hGPIIb analog consisting of a polypeptide having an amino acid residue sequence that corresponds to the
5 sequence of hGPIIb selected from the group consisting of the sequence shown in SEQ ID NO:2 from residues 173 to 181, from residues 179 to 189, from residues 187 to 200, and from residues 194 to 200.

7. The analog of claim 4 wherein said polypeptide has
10 an amino acid residue sequence that corresponds to the amino acid residue sequence shown in SEQ ID NO:2 from residue 1 to about residue 200.

8. A substantially isolated polypeptide having an amino acid residue sequence as shown in SEQ ID NO:2 from
15 residue 173 to residue 193.

9. An antibody that immunoreacts with the polypeptide of claim 8, and does not specifically bind with a polypeptide having amino acid residue sequence REQNSLDSWGPK set forth in SEQ ID NO:2 from residue 113 to residue 124.

20 10. A method for detecting a C-terminal heavy chain platelet glycoprotein receptor (hGPIIb) polypeptide in a body fluid sample, comprising the steps of:

(a) admixing an aliquot of the body fluid sample with an antibody to form an immunoreaction admixture,
25 wherein the antibody:

(i) immunoreacts with the polypeptide comprising amino acid sequence PQPPVNPLKVDWGLPIPSPP set forth in SEQ ID NO:2 from residue 173 to residue 193, PQPPVNPLK set forth in SEQ ID NO:2 from residue 173 to residue 181, or

28395-20

65

PLKVDWGLPIP set forth in SEQ ID NO:2 from residue 179 to residue 189; and

(ii) does not specifically immunoreact with the polypeptide comprising amino acid sequence REQNSLDSWGPK set forth in SEQ ID NO:2 from residue 113 to residue 124;

(b) maintaining the immunoreaction admixture under biological assay conditions for a time period sufficient to form an immunoreaction product; and

(c) detecting the immunoreaction product, and thereby the C-terminal hGPIIb polypeptide.

11. The method of claim 10, further comprising the step of removing cells from the body fluid sample to form a cell-free body fluid sample.

12. The method of claim 11, wherein the body fluid comprises vascular fluid and the cells comprise platelets.

13. The method of claim 11, wherein the body fluid is urine.

14. The method of any one of claims 10 to 13, wherein the antibody is immunoreactive with a polypeptide comprising the sequence:

PQPPVNPLK, or

PLKVDWGLPIP,

shown in SEQ ID NO:2 from residues 173 to 181, or from residues 179 to 189, respectively.

15. The method of claim 14 wherein the antibody is immunoreactive with a polypeptide comprising the sequence PQPPVNPLK as shown in SEQ ID NO:2 from residues 173 to 181.

28395-20

66

16. The method of claim 14 wherein the antibody is immunoreactive with a polypeptide comprising the sequence PLKVDWGLPIP as shown in SEQ ID NO:2 from residues 179 to 189.

5 17. The method of any one of claims 10 to 13, wherein said antibody is present in a composition further comprising monoclonal antibody PMI-1, produced by a hybridoma having ATCC accession number HB 9476.

18. The method of any one of claims 10 to 17, wherein
10 said admixing in step (a) further comprises admixing the body fluid sample and the antibody with a solid matrix having affixed thereto a polypeptide sequence including the C-terminal hGPIIb polypeptide to form a competition immunoreaction admixture having a liquid phase and a solid
15 phase, and said immunoreaction product formed in step (b) is in the solid phase.

19. The method of claim 18 wherein the polypeptide affixed to the solid matrix has an amino acid residue sequence shown in SEQ ID NO:2 from residue 173 to
20 residue 200.

20. The method of claim 18 wherein the polypeptide affixed to the solid matrix is the platelet glycoprotein GPIIb-IIIa.

21. The method of any one of claims 10 to 17, wherein
25 said admixing in step (a) further comprises admixing the body fluid sample and the antibody with a solid matrix having affixed thereto an hGPIIb analog to form a competition immunoreaction admixture having a liquid phase and a solid phase, and said immunoreaction product formed in
30 step (b) is in the solid phase.

28395-20

67

22. The method of any one of claims 10 to 21 wherein the antibody comprises a label affixed thereto.

23. The method of claim 22 wherein the detecting in step (c) comprises determining the presence of the label in
5 the solid phase immunoreaction product.

24. The method of claim 23, wherein the detecting in step (c) further comprises measuring a quantity of the label in the solid phase immunoreaction product and determining an amount of the C-terminal hGPIIb polypeptide in the body
10 fluid sample.

25. The method of any one of claims 10 to 22, wherein the detecting step comprises measuring a quantity of the immunoreaction product and determining an amount of the C-terminal hGPIIb in the body fluid sample.

15 26. The method of any one of claims 10 to 21, wherein the detecting in step (c) further includes the steps of:

(i) admixing the immunoreaction product with an indicating means to form a reaction admixture;

(ii) maintaining the reaction admixture under
20 conditions sufficient for the indicating means to bind to the immunoreaction product to form a second immunoreaction product; and

(iii) determining the presence of the indicating means in the second immunoreaction product, thereby
25 indicating the presence of the first immunoreaction product.

27. The method of claim 26 wherein said indicating means comprises an antibody having a label affixed thereto.

28. A diagnostic test kit comprising (i) a package containing an antibody and (ii) instructions for using the

28395-20

68

antibody for assaying for the presence of C-terminal human glycoprotein IIb (hGPIIb) fragments in a body fluid sample, wherein the antibody:

a) immunoreacts with at least one polypeptide
5 having an amino acid residue sequence PQPPVNPLKVDWGLPIPSPSP as shown in SEQ ID NO:2 from residue 173 to residue 193, PQPPVNPLK as shown in SEQ ID NO:2 from residue 173 to residue 181, or PLKVDWGLPIP as shown in SEQ ID NO:2 from residue 179 to residue 189; and

10 b) does not immunoreact with a polypeptide having the amino acid residue sequence REQNSLDSWGPK, as shown in SEQ ID NO:2 from residue 113 to residue 124.

29. The diagnostic test kit of claim 28 wherein the antibody immunoreacts with a polypeptide having an amino
15 acid residue sequence PQPPVNPLKVDWGLPIPSPSP as shown in SEQ ID NO:2 from residue 173 to residue 193.

30. The diagnostic test kit of claim 28 wherein the antibody immunoreacts with a polypeptide having an amino acid residue sequence PQPPVNPLK as shown in SEQ ID NO:2 from
20 residue 173 to residue 181.

31. The diagnostic test kit of claim 28 wherein the antibody immunoreacts with a polypeptide having an amino acid residue sequence PLKVDWGLPIP as shown in SEQ ID NO:2 from residue 179 to residue 189.

25 32. The diagnostic test kit of any one of claims 28 to 31 wherein said antibody is affixed to a solid matrix.

33. The diagnostic test kit of any one of claims 28 to 31 further comprising a solid support consisting of a solid matrix having affixed thereto a polypeptide having an
30 amino acid residue sequence comprising the C-terminal

28395-20

69

portion of SEQ ID NO:2 from residue 173 to residue 200, or a hGPIIb analog consisting of a polypeptide having an amino acid residue sequence that corresponds to the sequence of hGPIIb shown in SEQ ID NO:2 from residue 1 to residue 200.

5 34. The diagnostic test kit of any one of claims 28 to 31 further comprising a solid support consisting of a solid matrix having affixed thereto a hGPIIb polypeptide or a hGPIIb analog having a sequence corresponding to:

(i) an amino acid residue sequence

10 PQPPVNPLKVDWGLPIPSPSP as shown in SEQ ID NO:2 from residue 173 to residue 193,

(ii) an amino acid residue sequence PQPPVNPLK as shown in SEQ ID NO:2 from residue 173 to residue 181,

(iii) an amino acid residue sequence PLKVDWGLPIP
15 as shown in SEQ ID NO:2 from residue 179 to residue 189,

(iv) an amino acid residue sequence PIPSPSPIHPAHHK as shown in SEQ ID NO:2 from residue 187 to residue 200, or

(v) an amino acid residue sequence IHPAHHK as shown in SEQ ID NO:2 from residue 194 to residue 200.

20 35. The diagnostic test kit of any one of claims 28 to 34 further comprising, in a separate package, a labeled specific binding agent for signaling the presence of an immunoreaction product on the solid matrix.

36. A diagnostic test kit comprising separate packages
25 containing an antibody and a polypeptide and instructions for using the antibody and the polypeptide for assaying for the presence of C-terminal hGPIIb fragment in a body fluid sample, wherein

a) the antibody:

28395-20

70

(i) immunoreacts with at least one polypeptide having amino acid sequence PQPPVNPLKVDWGLPIPSPSP set forth in SEQ ID NO:2 from residue 173 to residue 193, PQPPVNPLK set forth in SEQ ID NO:2 from residue 173 to residue 181,
5 PLKVDWGLPIP set forth in SEQ ID NO:2 from residue 179 to residue 189, PIPSPSPIHPAHHK set forth in SEQ ID NO:2 from residue 187 to residue 200, or IHPAHHK set forth in SEQ ID NO:2 from residue 194 to residue 200; and

(ii) does not specifically immunoreact with the
10 polypeptide having amino acid sequence REQNSLDSWGP set forth in SEQ ID NO:2 from residue 113 to residue 124; and

(b) the polypeptide has an amino acid residue sequence comprising the C-terminal portion of SEQ ID NO:2 from residue 173 to residue 200, or a hGPIIb analog
15 consisting of a polypeptide corresponding to the sequence of hGPIIb shown in SEQ ID NO:2 from residues 1 to 200, or a polypeptide or a hGPIIb analog having a sequence corresponding to SEQ ID NO:2 from residues 173 to 193, residues 173 to 181, residues 179 to 189, residues 187
20 to 200, or residues 194 to 200.

37. The diagnostic test kit of claim 36 wherein the polypeptide or analog of (b) is affixed to a solid matrix.

FETHERSTONHAUGH & CO.

OTTAWA, CANADA

PATENT AGENTS

1/2

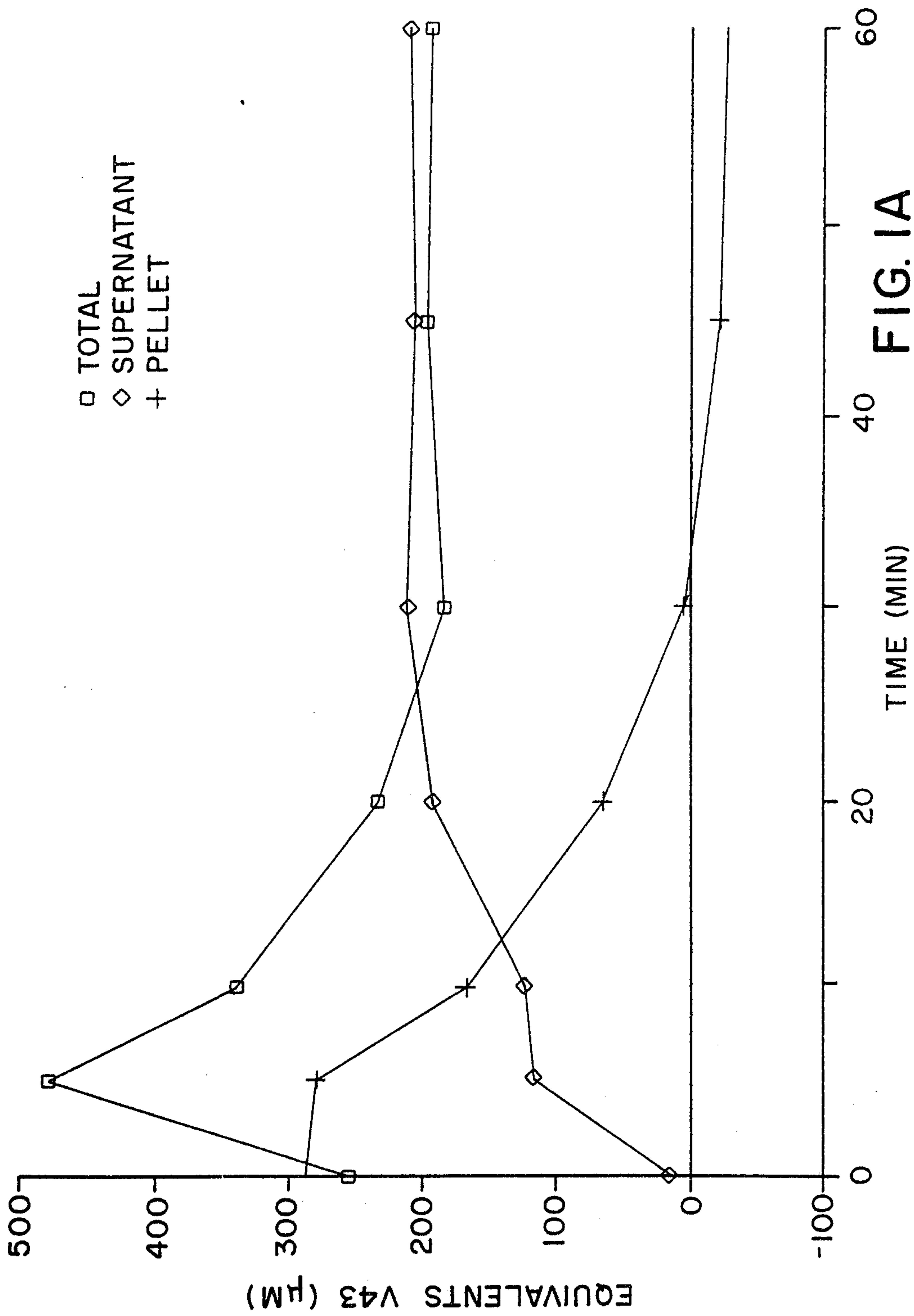
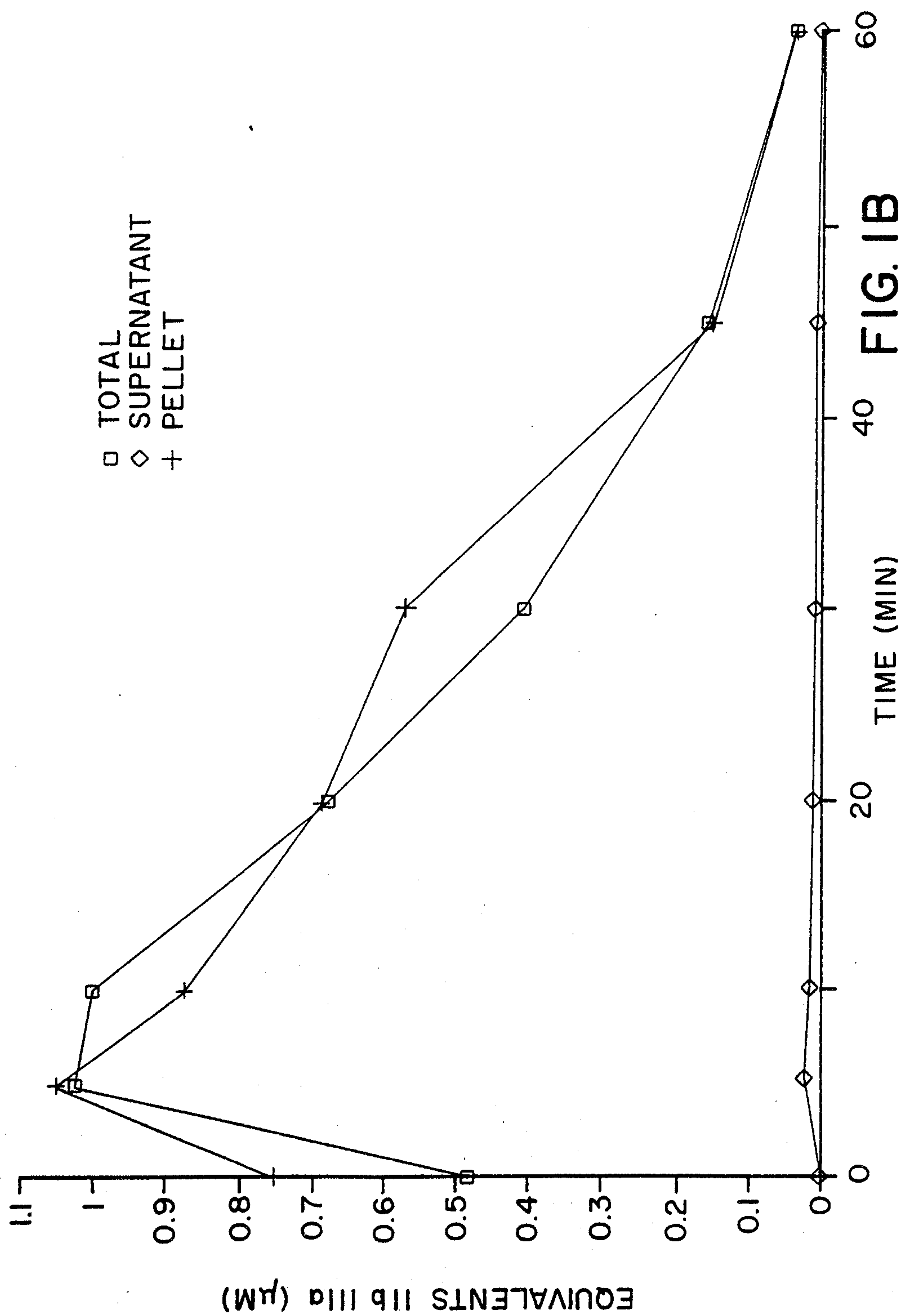


FIG. 1A

SUBSTITUTE SHEET

2102115

2/2



SUBSTITUTE SHEET