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(54)【発明の名称】血小板／白血球相互作用アッセイ及びその試薬

(57)【要約】
固相刺激剤（例えば、血小板、白血球又はその両方に直接相互作用する一以上のリガンドをその表面に結合した磁性粒子、非磁性粒子又はその混合物）を用いた血小板／白血球相互作用アッセイ及びその試薬を提供する。これによって、迅速で信頼性の高い血小板／白血球相互作用のポイント・オブ・ケア評価が提供される。

【特許請求の範囲】
【請求項１】
血小板／白血球相互作用を評価する方法であって、
全血又は血液由来のサンプルを固相刺激剤に接触させる段階と、
一以上の血小板／白血球／固相刺激剤複合体の形成を検出する段階とを含み、
前記固相刺激剤は、血小板又は白血球に選択的に結合するリガンドをその表面に結合している方法。
【請求項２】
血小板／白血球相互作用を評価する方法であって、
全血又は血液由来のサンプルを固相刺激剤に接触させる段階と、
一以上の血小板／白血球／固相刺激剤複合体の形成を検出する段階とを含み、
前記固相刺激剤は、血小板又は白血球に選択的に結合するリガンドをその表面に結合している微粒子を含む方法。
【請求項３】
血小板又は白血球に選択的に結合する前記リガンドは、血漿タンパク質、血漿タンパク質フラグメント、細胞外マトリックスタンパク質、細胞外マトリックスタンパク質フラグメント及びこれらを組み合わせたものからなる群から選択されるものである、請求項２に記載の方法。
【請求項４】
微粒子は不規則若しくは規則的な形状又は球状である、請求項２に記載の方法。
【請求項５】
前記全血又は血液由来のサンプルは哺乳類から得られるものである、請求項２に記載の方法。
【請求項６】
前記哺乳類はヒトである、請求項５に記載の方法。
【請求項７】
微粒子が、ポリスチレン、ラテックス、ポリカーボネート、アクリロニトリル、カルボキシレート、テフロン（登録商標）、ガラス、ナイロン、デキストラン、アガロース、アクリルアミド、シリカ、花粉、微生物、酸化鉄、非磁性金属、常磁性酸化鉄、金、白金及びパラジウムからなる群から選択される一以上の材料から作られる粒子を含む、請求項２に記載の方法。
【請求項８】
前記接触させる段階は、攪拌、振とう、吸引、電磁場の印加、超音波、せん断力及びこれらを組み合わせたものからなる群から選択される方法によって実施される、請求項２に記載の方法。
【請求項９】
前記血小板又は白血球に選択的に結合するリガンドは、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸、前記タンパク質類のフラグメント、白血球選択性抗体及びこれらを組み合わせたものからなる群から選択されるものである、請求項３に記載の方法。
【請求項１０】
血小板又は白血球に選択的に結合するリガンドは、受動的吸着により又は架橋分子に結合することにより、微粒子に共有的に接着される、請求項２に記載の方法。
【請求項１１】
前記血漿タンパク質フラグメント又は細胞外タンパク質フラグメントは、組換え技術、酵素的切断又は非酵素化学的手段を用いたアミノ酸の連結によって、ペプチドの形成、ペプチド擬似体（ｍｉｍｅｔｉｃｓ）の形成又はペプチドミモトープ（ｍｉｍｏｔｏｐｅｓ）

の形成により調製される、請求項３に記載の方法。
【請求項１２】
前記全血又は血液由来のサンプルが抗凝固処理されていない全血である、請求項２に記載の方法。
【請求項１３】
前記全血又は血液由来のサンプルが抗凝固処理された全血である、請求項２に記載の方法。
【請求項１４】
前記全血又は血液由来のサンプルが、軟膜に含まれる細胞である、請求項２に記載の方法。
【請求項１５】
前記全血又は血液由来のサンプルが、輸血の目的で採取された血液製剤である、請求項２に記載の方法。
【請求項１６】
輸血の目的で採取された前記血液製剤は、不特定ドナーの血小板（ｒａｎｄｏｍ ｄｏｎｏｒ ｐｌａｔｅｌｅｔｓ）、アファレーシス血小板（ａｐｈｅｒｅｓｉｓ ｐｌａｔｅｌｅｔｓ）、軟膜及びパックされた（ｐａｃｋｅｄ）赤血球からなる群から選択される特定の血液成分を分離するように設計された一以上の処理に更に付されたものである、請求項１５に記載の方法。
【請求項１７】
全血又は血液由来のサンプルは抗凝固処理されていない全血、抗凝固処理された全血及び軟膜からなる群から選択されるものであり、全血又は血液由来のサンプルは本方法における使用前に、インビボで人工的表面に接触して置かれていたものである、請求項２に記載の方法。
【請求項１８】
全血又は血液由来のサンプルは抗凝固処理されていない全血、抗凝固処理された全血及び軟膜からなる群から選択されるものであり、全血又は血液由来のサンプルは本方法における使用前に、エクスビボで人工的表面に接触して置かれていたものである、請求項２に記載の方法。
【請求項１９】
全血又は血液由来のサンプルは抗凝固処理されていない全血、抗凝固処理された全血及び軟膜からなる群から選択されるものであり、全血又は血液由来のサンプルは本方法における使用前に、インビトロで人工的表面に接触して置かれていたものである、請求項２に記載の方法。
【請求項２０】
前記検出する段階は、フローサイトメトリー、細胞計数法、顕微鏡法、フォトマイクロスコピー、透過型電子顕微鏡法、走査型電子顕微鏡法、共焦点顕微鏡法、ビデオ顕微鏡法、エンザイムリンクドイムノソルベントアッセイ（ＥＬＩＳＡ）、ラジオイムノアッセイ（ＲＩＡ）、イムノラジオメトリックアッセイ（ＩＲＭＡ）、ゲル排除クロマトグラフィー、アフィニティークロマトグラフィー、組織化学的解析、免疫化学的解析、ポリメラーゼ連鎖反応、蛍光ｉｎ－ｓｉｔｕハイブリッド形成法、サザンブロット法、ウェスタンブロット法、レーザ走査サイトメトリー、濁度測定、凝集能測定、細胞内イオン流動測定、細胞外イオン流動測定、細胞遊離物の測定、固相刺激剤／血小板／白血球凝集体サイズの測定、固相刺激剤／血小板／白血球複合体形成速度の測定及びラテックスビーズ凝集からなる群から選択される方法により実施される、請求項２に記載の方法。
【請求項２１】
全血又は血液由来のサンプルは、血小板／白血球相互作用に影響を与える治療剤を用いる治療コースを受けている又は受けようとしている哺乳類から得るものであり、更に該方法は、複数の合一された懸濁液（各懸濁液は、治療中の血小板／白血球相互作用を評価するために治療コース前又は治療コース中に一定の時間間隔で哺乳類から得たサンプルを含む

）の血小板／白血球相互作用の程度を測定して治療の有効性をモニターする段階を含む、請求項２記載の方法。
【請求項２２】
前記接触させる段階の中に、更に、前記全血又は血液由来のサンプル及び固相刺激剤と一定の選択された時間の間血小板／白血球相互作用に影響を与える一以上の剤を合一する段階と、前記一以上の剤を添加する前及び後の血小板／白血球相互作用の程度を測定するする段階を含む、請求項２に記載の方法。
【請求項２３】
血小板／白血球相互作用アッセイ試薬であって
磁性粒子と非磁性粒子の混合物と、
白血球マーカー化合物とを含み、
前記磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記非磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第二のリガンドを結合させており、前記第一のリガンド及び前記第二のリガンドは互いに同一である又は互いに異なることができる血小板／白血球相互作用アッセイ試薬。
【請求項２４】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項２３に記載の血小板／白血球相互作用アッセイ試薬。
【請求項２５】
前記第一のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項２４に記載の血小板／白血球相互作用アッセイ試薬。
【請求項２６】
前記第二のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項２３に記載の血小板／白血球相互作用アッセイ試薬。
【請求項２７】
前記第二のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項２６に記載の血小板／白血球相互作用アッセイ試薬。
【請求項２８】
前記第一のリガンド及び前記第二のリガンドが互いに同一である、請求項２３に記載の血小板／白血球相互作用アッセイ試薬。
【請求項２９】
前記第一のリガンド及び前記第二のリガンドがそれぞれｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項２８に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３０】
前記第一のリガンド及び前記第二のリガンドが互いに異なる、請求項２３に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３１】
前記第一のリガンド及び前記第二のリガンドの一方が、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因

子又はその活性フラグメントである、請求項３０に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３２】
血小板／白血球相互作用アッセイ試薬であって、
磁性粒子と非磁性粒子の混合物
を含み、
前記磁性粒子か前記非磁性粒子のどちらかはその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記磁性粒子か非磁性粒子の他方はその外表面に白血球との直接相互作用に親和性を有する一定量の第二のリガンドを結合させている試薬。
【請求項３３】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項３２に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３４】
前記第一のリガンドは前記非磁性粒子に結合しており、前記第二のリガンドは前記磁性粒子に結合している、請求項３２に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３５】
前記第一のリガンドは前記磁性粒子に結合しており、前記第二のリガンドは前記非磁性粒子に結合している、請求項３２に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３６】
前記試薬は白血球マーカー化合物を更に含む、請求項３５に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３７】
前記白血球マーカー化合物が蛍光化合物である、請求項３６に記載の血小板／白血球相互作用アッセイ試薬。
【請求項３８】
血小板／白血球相互作用アッセイ方法であって、
全血又は血液由来のサンプルと血小板／白血球相互作用アッセイ試薬を振動磁界又は回転磁界の存在下接触させる段階と、
振動磁界又は回転磁界に応答した磁性粒子の運動をモニターして、全血又は血液由来のサンプル内の血小板／白血球相互作用機能の存在又は非存在、血小板／白血球相互作用のレベル又はその両方を測定する段階とを含み、
前記血小板機能アッセイ試薬は、磁性粒子と非磁性粒子の混合物と、白血球マーカー化合物とを含み、前記磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記非磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第二のリガンドを結合させており、前記第一のリガンド及び前記第二のリガンドは互いに同一である又は互いに異なることができる、血小板／白血球相互作用アッセイ方法。
【請求項３９】
前記サンプルが全血である、請求項３８に記載の方法。
【請求項４０】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オ

ステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項３８に記載の方法。
【請求項４１】
前記第一のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項４０に記載の方法。
【請求項４２】
前記第二のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項３８に記載の方法。
【請求項４３】
前記第二のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項４２に記載の方法。
【請求項４４】
前記第一のリガンド及び前記第二のリガンドが互いに同一である、請求項３８に記載の方法。
【請求項４５】
前記第一のリガンド及び前記第二のリガンドがそれぞれｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項４４に記載の方法。
【請求項４６】
前記第一のリガンド及び前記第二のリガンドが互いに異なる、請求項３８に記載の方法。
【請求項４７】
前記第一のリガンド及び前記第二のリガンドの一方が、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項４６に記載の方法。
【請求項４８】
前記接触は回転磁界の存在下で起こる、請求項３８に記載の方法。
【請求項４９】
前記回転磁界は２０００～２５００ｒｐｍで回転する、請求項４８に記載の方法。
【請求項５０】
血小板／白血球相互作用アッセイ方法であって、
全血又は血液由来のサンプルと血小板／白血球相互作用アッセイ試薬を振動磁界又は回転磁界の存在下接触させる段階と、
全血又は血液由来のサンプル内の血小板／白血球相互作用機能の存在又は非存在、血小板／白血球相互作用のレベル又はその両方を検出する段階とを含み、
前記血小板機能アッセイ試薬は磁性粒子と非磁性粒子の混合物を含み、前記磁性粒子か前記非磁性粒子のどちらかはその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記磁性粒子か非磁性粒子の他方はその外表面に白血球との直接相互作用に親和性を有する一定量の第二のリガンドを結合させている、血小板／白血球相互作用アッセイ方法。
【請求項５１】
前記サンプルが全血である、請求項５０に記載の方法。
【請求項５２】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項５０に記載の方法。

【請求項５３】
前記第一のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項５２に記載の方法。
【請求項５４】
前記第二のリガンドは、白血球選択性抗体、ＶＣＡＭ－１、フィブロネクチン、ラミニン、ＩＣＡＭ－１、ＩＣＡＭ－２、ＩＣＡＭ－３、コラーゲン、オステオポンチン、ｖＷｆ、ビトロネクチン、トロンボスポンジン、粘膜アドレシン細胞接着分子１（ＭａｄＣＡＭ－１）、Ｐ－セレクチン、Ｌ－セレクチン及びＥ－セレクチンからなる群から選択されるものである、請求項５０に記載の方法。
【請求項５５】
前記第一のリガンドは前記非磁性粒子に結合しており、前記第二のリガンドは前記磁性粒子に結合している、請求項５０に記載の方法。
【請求項５６】
前記第一のリガンドは前記磁性粒子に結合しており、前記第二のリガンドは前記非磁性粒子に結合している、請求項５０に記載の方法。
【請求項５７】
前記血小板／白血球相互作用試薬は白血球マーカー化合物を更に含む、請求項５６に記載の方法。
【請求項５８】
前記白血球マーカー化合物が蛍光マーカー化合物である、請求項５７に記載の方法。
【請求項５９】
前記検出は、前記振動磁界又は回転磁界に応答した前記磁性粒子の運動をモニターすることにより実施される、請求項５５に記載の方法。
【請求項６０】
前記検出は、前記振動磁界又は回転磁界に応答した前記磁性粒子の運動をモニターすることにより実施される、請求項５６に記載の方法。
【請求項６１】
前記検出は前記サンプルの凝集後の前記サンプルにおける前記白血球マーカー化合物の濃度差の検出、定量又はその両方により実施される、請求項５７に記載の方法。
【請求項６２】
前記接触は回転磁界の存在下で起こる、請求項５０に記載の方法。
【請求項６３】
前記回転磁界は２０００～２５００ｒｐｍで回転する、請求項６２に記載の方法。
【請求項６４】
血小板／白血球相互作用を惹き起こす病態の存在を検出する方法であって、
血小板／白血球相互作用を惹き起こす病態を有する疑いのある患者から得た全血又は血液由来のサンプルと血小板／白血球相互作用アッセイ試薬を振動磁界又は回転磁界の存在下接触させる段階と、
振動磁界又は回転磁界に応答した磁性粒子の運動をモニターして、全血又は血液由来のサンプル内の血小板／白血球相互作用機能の存在又は非存在、血小板／白血球相互作用のレベル又はその両方を測定する段階とを含み、
前記血小板機能アッセイ試薬は、磁性粒子と非磁性粒子の混合物と、白血球マーカー化合物とを含み、前記磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記非磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第二のリガンドを結合させており、前記第一のリガンド及び前記第二のリガンドは互いに同一である又は互いに異なることができる方法。
【請求項６５】
前記サンプルが全血である、請求項６４に記載の方法。
【請求項６６】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブ

ロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項６４に記載の方法。

【請求項６７】

前記第一のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項６６に記載の方法。

【請求項６８】

前記第二のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項６４に記載の方法。

【請求項６９】

前記第二のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項６８に記載の方法。

【請求項７０】

前記第一のリガンド及び前記第二のリガンドが互いに同一である、請求項６４に記載の方法。

【請求項７１】

前記第一のリガンド及び前記第二のリガンドがそれぞれｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項７０に記載の方法。

【請求項７２】

前記第一のリガンド及び前記第二のリガンドが互いに異なる、請求項６４に記載の方法。

【請求項７３】

前記第一のリガンドと前記第二のリガンドの内の一方が、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項７２に記載の方法。

【請求項７４】

前記接触は回転磁界の存在下で起こる、請求項６４に記載の方法。

【請求項７５】

前記回転磁界は２０００～２５００ｒｐｍで回転する、請求項７４に記載の方法。

【請求項７６】

血小板／白血球相互作用を惹き起こす前記病態は、脳血管発作（ＣＶＡ）、一過性虚血性発作（ＴＩＡ）、不安定狭心症、冠動脈疾患（ＣＡＤ）、急性心筋梗塞（ＡＭＩ）及び炎症からなる群から選択されるものである、請求項６４に記載の方法。

【請求項７７】

血小板／白血球相互作用を惹き起こす病態の存在を検出する方法であって、

血小板／白血球相互作用を惹き起こす病態を有する疑いのある患者から得た全血又は血液由来のサンプルと血小板／白血球相互作用アッセイ試薬を振動磁界又は回転磁界の存在下で接触させる段階と、

全血又は血液由来のサンプル内の血小板／白血球相互作用機能の存在又は非存在、血小板／白血球相互作用のレベル又はその両方を検出する段階とを含み、

前記血小板機能アッセイ試薬は磁性粒子と非磁性粒子の混合物を含み、前記磁性粒子か前記非磁性粒子のどちらかはその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記磁性粒子か非磁性粒子の他方はその外表面に白血球との直接相互作用に親和性を有する一定量の第二のリガンドを結合させている方法。

【請求項７８】

前記サンプルが全血である、請求項７７に記載の方法。

【請求項７９】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項７７に記載の方法。
【請求項８０】
前記第一のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項７９に記載の方法。
【請求項８１】
前記第二のリガンドは、白血球選択性抗体、ＶＣＡＭ－１、フィブロネクチン、ラミニン、ＩＣＡＭ－１、ＩＣＡＭ－２、ＩＣＡＭ－３、コラーゲン、オステオポンチン、ｖＷｆ、ビトロネクチン、トロンボスポンジン、粘膜アドレシン細胞接着分子１（ＭａｄＣＡＭ－１）、Ｐ－セレクチン、Ｌ－セレクチン及びＥ－セレクチンからなる群から選択されるものである、請求項７７に記載の方法。
【請求項８２】
前記第一のリガンドは前記非磁性粒子に結合しており、前記第二のリガンドは前記磁性粒子に結合している、請求項７７に記載の方法。
【請求項８３】
前記第一のリガンドは前記磁性粒子に結合しており、前記第二のリガンドは前記非磁性粒子に結合している、請求項７７に記載の方法。
【請求項８４】
前記血小板／白血球相互作用試薬は白血球マーカー化合物を更に含む、請求項８３に記載の方法。
【請求項８５】
前記白血球マーカー化合物が蛍光マーカー化合物である、請求項８４に記載の方法。
【請求項８６】
前記検出は、前記振動磁界又は回転磁界に応答した前記磁性粒子の運動をモニターすることにより実施される、請求項８２に記載の方法。
【請求項８７】
前記検出は、前記振動磁界又は回転磁界に応答した前記磁性粒子の運動をモニターすることにより実施される、請求項８３に記載の方法。
【請求項８８】
前記検出は前記サンプルの凝集後の前記サンプルにおける前記白血球マーカー化合物の濃度差の検出、定量又はその両方により実施される、請求項８４に記載の方法。
【請求項８９】
前記接触は回転磁界の存在下で起こる、請求項７７に記載の方法。
【請求項９０】
前記回転磁界は２０００～２５００ｒｐｍで回転する、請求項８９に記載の方法。
【請求項９１】
血小板／白血球相互作用を惹き起こす前記病態は、脳血管発作（ＣＶＡ）、一過性虚血性発作（ＴＩＡ）、不安定狭心症、冠動脈疾患（ＣＡＤ）、急性心筋梗塞（ＡＭＩ）及び炎症からなる群から選択されるものである、請求項７７に記載の方法。
【請求項９２】
患者が血小板／白血球相互作用を惹き起こす病態に関する素因を有するか否かを決定する方法であって、
患者から得た全血又は血液由来のサンプルと血小板／白血球相互作用アッセイ試薬を振動磁界又は回転磁界の存在下で接触させる段階と、
振動磁界又は回転磁界に応答した磁性粒子の運動をモニターして、全血又は血液由来のサ

ンプル内の血小板／白血球相互作用機能の存在又は非存在、血小板／白血球相互作用のレベル又はその両方を測定する段階とを含み、
前記血小板機能アッセイ試薬は、磁性粒子と非磁性粒子の混合物と、白血球マーカー化合物とを含み、前記磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記非磁性粒子はその外表面に血小板との直接相互作用に親和性を有する一定量の第二のリガンドを結合させており、前記第一のリガンド及び前記第二のリガンドは互いに同一である又は互いに異なることができる方法。
【請求項９３】
前記サンプルが全血である、請求項９２に記載の方法。
【請求項９４】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項９２に記載の方法。
【請求項９５】
前記第一のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項９４に記載の方法。
【請求項９６】
前記第二のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項９２に記載の方法。
【請求項９７】
前記第二のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項９６に記載の方法。
【請求項９８】
前記第一のリガンド及び前記第二のリガンドが互いに同一である、請求項９２に記載の方法。
【請求項９９】
前記第一のリガンド及び前記第二のリガンドがそれぞれｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項９８に記載の方法。
【請求項１００】
前記第一のリガンド及び前記第二のリガンドが互いに異なる、請求項９２に記載の方法。
【請求項１０１】
前記第一のリガンドと前記第二のリガンドの内の一方が、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項１００に記載の方法。
【請求項１０２】
前記接触は回転磁界の存在下で起こる、請求項９２に記載の方法。
【請求項１０３】
前記回転磁界は２０００～２５００ｒｐｍで回転する、請求項１０２に記載の方法。
【請求項１０４】
血小板／白血球相互作用を惹き起こす前記病態は、脳血管発作（ＣＶＡ）、一過性虚血性発作（ＴＩＡ）、不安定狭心症、冠動脈疾患（ＣＡＤ）、急性心筋梗塞（ＡＭＩ）及び炎症からなる群から選択されるものである、請求項９２に記載の方法。
【請求項１０５】
血小板／白血球相互作用を惹き起こす病態の存在を検出する方法であって、

血小板／白血球相互作用を惹き起こす病態を有する疑いのある患者から得た全血又は血液由来のサンプルと血小板／白血球相互作用アッセイ試薬を振動磁界又は回転磁界の存在下で接触させる段階と、
全血又は血液由来のサンプル内の血小板／白血球相互作用機能の存在又は非存在、血小板／白血球相互作用のレベル又はその両方を検出する段階とを含み、
前記血小板機能アッセイ試薬は磁性粒子と非磁性粒子の混合物を含み、前記磁性粒子か前記非磁性粒子のどちらかはその外表面に血小板との直接相互作用に親和性を有する一定量の第一のリガンドを結合させており、前記磁性粒子か非磁性粒子の他方はその外表面に白血球との直接相互作用に親和性を有する一定量の第二のリガンドを結合させている方法。
【請求項１０６】
前記サンプルが全血である、請求項１０５に記載の方法。
【請求項１０７】
前記第一のリガンドが、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸及びこれらの活性フラグメントからなる群から選択されるものである、請求項１０５に記載の方法。
【請求項１０８】
前記第一のリガンドがｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子又はその活性フラグメントである、請求項１０７に記載の方法。
【請求項１０９】
前記第二のリガンドは、白血球選択性抗体、ＶＣＡＭ－１、フィブロネクチン、ラミニン、ＩＣＡＭ－１、ＩＣＡＭ－２、ＩＣＡＭ－３、コラーゲン、オステオポンチン、ｖＷｆ、ビトロネクチン、トロンボスポンジン、粘膜アドレシン細胞接着分子１（ＭａｄＣＡＭ－１）、Ｐ－セレクチン、Ｌ－セレクチン及びＥ－セレクチンからなる群から選択されるものである、請求項１０５に記載の方法。
【請求項１１０】
前記第一のリガンドは前記非磁性粒子に結合しており、前記第二のリガンドは前記磁性粒子に結合している、請求項１０５に記載の方法。
【請求項１１１】
前記第一のリガンドは前記磁性粒子に結合しており、前記第二のリガンドは前記非磁性粒子に結合している、請求項１０５に記載の方法。
【請求項１１２】
前記血小板／白血球相互作用試薬は白血球マーカー化合物を更に含む、請求項１１１に記載の方法。
【請求項１１３】
前記白血球マーカー化合物が蛍光マーカー化合物である、請求項１１２に記載の方法。
【請求項１１４】
前記検出は、前記振動磁界又は回転磁界に応答した前記磁性粒子の運動をモニターすることにより実施される、請求項１１０に記載の方法。
【請求項１１５】
前記検出は、前記振動磁界又は回転磁界に応答した前記磁性粒子の運動をモニターすることにより実施される、請求項１１１に記載の方法。
【請求項１１６】
前記検出は前記サンプルの凝集後の前記サンプルにおける前記白血球マーカー化合物の濃度差の検出、定量又はその両方により実施される、請求項１１２に記載の方法。
【請求項１１７】
前記接触は回転磁界の存在下で起こる、請求項１０５に記載の方法。
【請求項１１８】

前記回転磁界は２０００～２５００ｒｐｍで回転する、請求項１１７に記載の方法。
【請求項１１９】
血小板／白血球相互作用を惹き起こす前記病態は、脳血管発作（ＣＶＡ）、一過性虚血性発作（ＴＩＡ）、不安定狭心症、冠動脈疾患（ＣＡＤ）、急性心筋梗塞（ＡＭＩ）及び炎症からなる群から選択されるものである、請求項１０５に記載の方法。
【発明の詳細な説明】
【０００１】
【発明の属する技術分野】
本発明は、血小板と白血球との相互作用のポイント・オブ・ケア評価を可能とするための血小板／白血球相互作用アッセイ及びその試薬に関する。
【０００２】
【従来の技術】
血小板は白血球と相互作用することが知られているが、この相互作用は次の両方の結果として起こるものである。すなわち、正常血流下での血小板と白血球との接触（ＳｔｏｎｅとＮａｓｈ、Ｂｒｉｔｉｓｈ Ｊｏｕｒｎａｌ ｏｆ Ｈａｅｍａｔｏｌｏｇｙ，１０５：５１４－２２，１９９９；Ｌｏｒｅｎｚら、Ｂｌｏｏｄ Ｃｏａｇｕｌａｔｉｏｎ ａｎｄ Ｆｉｂｒｉｎｏｌｙｓｉｓ，９：Ｓ４９－５９，１９９８）及び様々な病的プロセス（Ｒｉｎｄｅｒら、Ｊｏｕｒｎａｌ ｏｆ Ｃａｒｄｉｏｖａｓｃｕｌａｒ Ｓｕｒｇｅｒｙ，１１８：４６０－６，１９９９；Ｐｅｙｔｏｎら、Ｊｏｕｒｎａｌ ｏｆ Ｖａｓｃｕｌａｒ Ｓｕｒｇｅｒｙ，２７：１１０９－１５，１９９８；Ｓｔｕａｒｄら、Ｉｎｔｅｒｎａｔｉｏｎａｌ Ｊｏｕｒｎａｌ ｏｆ Ａｒｔｉｆｉｃｉａｌ Ｏｒｇａｎｓ，２１：７５－８２，１９９８；Ｇａｗａｚら、Ｅｕｒｏｐｅａｎ Ｊｏｕｒｎａｌ ｏｆ Ｃｌｉｎｉｃａｌ Ｉｎｖｅｓｔｉｇａｔｉｏｎ，２５：８４３－５１，１９９５）。
【０００３】
不安定狭心症や冠動脈疾患（ＣＡＤ）、脳卒中等の病態は、血小板及び白血球の高レベルの活性化を特徴とする。血小板／白血球相互作用を測定することによって、とりわけ、他の診断的因子と併用することによって、これら病態を予測することができる。また、血小板／白血球相互作用の測定は、血小板及び／又は白血球の機能を変化させるための治療をモニタリングする手段として用いることができる。
【０００４】
流血が人工的表面に曝露されると血小板／白血球相互作用が高まることが示されている。関与する細胞型及びこの相互作用の程度は、血液に接触する人工的表面の組成により異なる（Ｇａｗａｚら、Ａｒｔｉｆｉｃｉａｌ Ｏｒｇａｎｓ，２３：２９－３６，１９９９）。
【０００５】
血小板／白血球相互作用は各種技法を用いて定量化されてきた（Ｈｅｎｄｒｉｃｋｓら、米国特許第５５０３９８２号；Ｒｉｎｄｅｒら、Ｂｌｏｏｄ，７８：１７６０，１９９１；）が、この相互作用の評価は循環する血小板／白血球複合体を測定することによって行なわれてきた。現在まで、この測定は血液サンプル中に既に存在する血小板／白血球間の相互作用を評価する形をとってきた。
【０００６】
一部の病態（例えば、急性心筋梗塞（ＡＭＩ）、経皮的冠動脈拡張術（ＰＴＣＡ）等）においては、血小板／白血球複合体形成は、損傷した内皮下層との直接的な（プラーク形成）あるいは間接的な（ＩＣＡＭ－１等の生化学的マーカーの放出、Ｈｅｎｄｒｉｃｋｓら、米国特許第５５０３９８２号参照）相互作用を伴う。
【０００７】
血小板／白血球相互作用の評価に用いる現在のアッセイシステムは、例えば、Ｈｅｎｄｒｉｃｋｓらによるシステム（米国特許第５５０３９８２号）によって示されるように、既に存在する（循環する）血小板／白血球複合体が評価されるが、これらシステムは血管内

皮下層（即ち、細胞外マトリックス）を代表する成分や他の固相刺激剤を用いていない。更に、個々の血小板と白血球が相互作用する閾値は、試験時のこれら細胞の活性化状態に依存して異なり得る。当該技術分野において、血小板及び／又は白血球の活性化が血小板／白血球結合の必要条件であることは知られている。また、当該技術分野においては、ある病態が血小板及び／又は白血球の活性のアップレギュレーションに関連づけられることも知られている。しかし、病的プロセスに関連する細胞活性におけるアップレギュレーションは、付加的刺激なしには血小板／白血球複合体形成をサポートするには不十分な場合があり、更に、このアップレギュレーションは、従来のシステムを用いて検出することができない場合がある。従来のシステムは、血小板／白血球複合体が維持され得る安定化固相支持体を有していない。固相刺激剤は、既に存在する血小板／白血球複合体を局在化する手段及び／又は複合体の形成及び局在化を誘発する傾向のある細胞内でその形成及び局在化しやすくする手段として用いられる。

【０００８】

固定化した内皮下／細胞外マトリックス等の固相成分の使用を組み込むように設計されたアッセイシステムが、簡便で、迅速、且つ手ごろな価格で入手可能で、臨床現場での血小板／白血球相互作用の検出に有用であることは望ましい。

【０００９】

本発明は、血漿タンパク質及び／又は細胞外マトリックスタンパク質で被覆された様々な組成の微粒子を単独で又は組み合わせて用い、血小板／白血球結合の迅速な評価を容易にすることによって従来の方法や技術における欠点に対処するものである。

【００１０】

血小板は様々なメカニズムによって白血球と相互作用し得るが、例えば、正常血流下で血小板と白血球との接触によってその相互作用が起こり得る（Ｌｏｒｅｎｚら、Ｂｌｏｏｄ Ｃｏａｇｕｌａｔｉｏｎ ａｎｄ Ｆｉｂｒｉｎｏｌｙｓｉｓ，９：Ｓ４９－Ｓ５９，１９９８）。あるいは、血小板の機能亢進に関連した病的プロセスの結果としてその相互作用が起こり得る（Ｓｐａｎｇｅｎｂｅｒｇ、Ｔｈｒｏｍｂｏｓｉｓ Ｒｅｓｅａｒｃｈ，７４：Ｓ３５－Ｓ４４，１９９４；Ｒｉｎｄｅｒら、Ｊｏｕｒｎａｌ ｏｆ Ｃａｒｄｉｏｖａｓｃｕｌａｒ Ｓｕｒｇｅｒｙ，１１８：４６０－６，１９９９）。あるいは、炎症プロセスに起因してその相互作用が起こり得る（Ｇａｗａｚら、Ｅｕｒｏｐｅａｎ Ｊｏｕｒｎａｌ ｏｆ Ｃｌｉｎｉｃａｌ Ｉｎｖｅｓｔｉｇａｔｉｏｎ，２５：８４３－５１，１９９５）。血小板表面に見出されるレセプターは、様々な白血球上に見出されるレセプターと直接的な架橋（ｂｒｉｄｇｉｎｇ）又は媒介分子を介した間接的な連結によって相互作用する（ＷｅｂｅｒとＳｐｒｉｎｇｅｒ、Ｊｏｕｒｎａｌ ｏｆ Ｃｌｉｎｉｃａｌ Ｉｎｖｅｓｔｉｇａｔｉｏｎ，１００：２０８５－９３，１９９７）。血小板及び／又は白血球の活性のアップレギュレーションによって、血小板／白血球相互作用が高められる（Ｒｉｎｄｅｒら、１９９９；ＳｔｏｎｅとＮａｓｈ、Ｂｒｉｔｉｓｈ Ｊｏｕｒｎａｌ ｏｆ Ｈａｅｍａｔｏｌｏｇｙ，１０５：５１４－２２，１９９９；Ｋｏｎｓｔａｎｔｏｐｏｕｌｏｓら、１９９８；Ｇａｗａｚら、１９９５；Ｓｐａｎｅｎｂｅｒｇ、１９９４）。

【００１１】

冠動脈疾患（ＣＡＤ）、糖尿病あるいは脳血管虚血の患者は、血小板機能亢進及び進行中の炎症プロセスの両方を示す（ＭｉｃｈｅｌｓｏｎとＦｕｒｍａｎ、Ｃｕｒｒｅｎｔ Ｏｐｉｎｉｏｎ ｉｎ Ｈｅｍａｔｏｌｏｇｙ，６：３４２－８，１９９９）。ＣＡＤ患者の治療には抗血小板剤及び抗炎症薬が用いられてきた（Ｖｏｒｃｈｈｅｉｍｅｒら、ＪＡＭＡ ２８１：１４０７－１４，１９９９；Ｍａｎｎａｉｏｎｉら、Ｉｎｆｌａｍｍａｔｉｏｎ Ｒｅｓｅａｒｃｈ，４６：４－１８，１９９７）。

【００１２】

血小板／単球相互作用（Ｈｅｎｄｒｉｃｋｓら、米国特許第５５０３９８２号）及び血小板／好中球相互作用（Ｇａｗａｚら、Ｅｕｒｏｐｅａｎ Ｊｏｕｒｎａｌ ｏｆ Ｃｌｉｎｉｃａｌ Ｉｎｖｅｓｔｉｇａｔｉｏｎ，２５：８４３－５１，１９９５）はそれぞれ

、急性心筋梗塞（ＡＭＩ）及び炎症を予測するものとして示唆されてきた。様々な病態における血小板／白血球相互作用には、例えばプラーク形成や局所的炎症反応における血管壁が関与していると推定される。

【００１３】

固定化した細胞外マトリックスタンパク質を用いる血小板機能評価は、ＳｈａｗとＳｔｅｗａｒｔによって記載されている（米国特許第５４２７９１３号）。この著者は、ポリスチレンビーズに固定化したｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子（ＶＷＦ）が、血小板を活性化し、血小板機能欠損症の患者から得た血小板の機能状態を判断するのに用い得ることを実証した。更に、この著者は、血小板の機能を変化させるようにデザインされた剤の効果を、ビーズに固定化したＶＷＦを刺激剤として用いることによりモニターし得ることも実証した。これら研究の結果は、正常な血液学的相互作用を評価すること、あるいは血管壁の成分に類似した剤の存在下での病態の血液学的結果を評価することの重要性を強調している。

【００１４】

ＳｈａｗとＳｔｅｗａｒｔは血小板の機能を判断する方法及び組成物について記載しているが、血小板／白血球相互作用を評価するために彼らの方法を用いることについての記載や示唆はない。

【００１５】

ＣＶＤＩのＴＡＳ^{TM}アナライザは、患者の血液サンプルにおける凝固経路の活性化に続くフィブリン重合速度を測定するものである。ＴＡＳ^{TM}アナライザ（使い捨て可能）は、ポイント・オブ・ケアの場において、全血を対象として分析するように設計された。ＴＡＳ^{TM}アナライザ用に開発された各試験のための検出システムでは、常磁性酸化鉄粒子（ＰＩＯＰ）が必須成分である。ＰＩＯＰと特定の試験用の他の凍結乾燥した各種成分とを、ＴＡＳテストカード（使い捨て可能）の浅い反応チャンバに置く。ＰＩＯＰの他に、テスト試薬は、バッファー、安定剤、フィラー及び特定の凝固経路活性剤や各種剤を含み得る。ドライ・ケミストリーテストカードをＴＡＳ^{TM}アナライザのスロットに挿入するとテストが開始されるテストカードは、電磁石の上の反応チャンバに自動的に置かれる。このチャンバに、発光ダイオードからの赤外光が照射される。この機器は、テストカード表面から反射された赤外光を固体フォトダイオード検出器によって測定する。血液あるいは血漿がテストカードのサンプルウェルに添加され、それが毛管現象によって反応チャンバに引き寄せられると、アナライザの光検出器が反射光の強度の変化を測定し、これによりテストは自動的に開始される。反応チャンバ内に存在するアクチベータは、患者のサンプル中の凝集カスケードを刺激してトロンビンを生成し、トロンビンはフィブリンクロットの形成を触媒する。

【００１６】

凝固試験の間、ＴＡＳ^{TM}アナライザの電磁石は、毎秒オンとオフを交互に繰り返す。電磁石がオンの時には磁性粒子は立ち上がり、検出器に入る反射光を増加させ、オフの時には横たわり、検出される光を減少させる。このようなＰＩＯＰの運動により、光検出器から交流電流（ＡＣ）信号が生成する。テストが進むにつれ、フィブリン重合が更に進行し、ＰＩＯＰの運動は減少する。アナライザは、所定のアルゴリズムに従ってＰＩＯＰの相対的な運動により生成される信号を解析し、各テストにおける適切なエンドポイント（凝固時間）を出力する。

【００１７】

ＰＩＯＰはＴＡＳ^{TM}検出システムにとって不可欠な要素であるが、凝固カスケードやフィブリン重合の活性化には直接関与しない。テストカードの反応チャンバ内におけるＰＩＯＰとアクチベータとの間の好ましくない相互作用を防ぐために、ウシ血清アルブミン（ＢＳＡ）でＰＩＯＰを被覆あるいはブロックする。ＢＳＡは、テスト用の表面成分と活性成分との間の不要な相互作用を避けるための、アッセイ開発の当業者に一般に用いられるタンパク質である。ＴＡＳ^{TM}システムは、血小板と白血球との相互作用ではなく、フィブリン重合をモニターするように設計されたものである。

【００１８】
【発明が解決しようとする課題】
従って、本発明の一目的は、全血等の様々な血液製剤をサンプルとして用いることができ、検出が容易な血小板／白血球相互作用のためのアッセイを提供することである。
【００１９】
本発明の他の目的は、血小板／白血球相互作用を迅速に測定するために、ＴＡＳ^{TM}システムにおいて用いることができる血小板／白血球相互作用のためのアッセイを提供することである。
【００２０】
本発明の更に他の目的は、好ましくは本発明のアッセイにおいて用いることができる乾式テストカード形式における血小板／白血球相互作用アッセイ試薬を提供することである。
【００２１】
本発明の更に他の目的は、本発明のアッセイを用いて、血小板／白血球相互作用を引き起こす病態を診断するための方法を提供することである。
【００２２】
本発明の更に他の目的は、本発明のアッセイを用いて、血小板及び／又は白血球の機能亢進を測定するための方法を提供することである。
【００２３】
【課題を解決するための手段】
本発明のこれら及び他の目的は、血小板／白血球相互作用を評価する方法であって、
全血又は血液由来のサンプルを固相刺激剤に接触させる段階と、
一以上の血小板／白血球／固相刺激剤複合体の形成を検出する段階とを含み、
前記固相刺激剤は、血小板又は白血球に選択的に結合するリガンドをその表面に結合している方法；該方法を行うための試薬；並びに、被験者の様々な病態及び被験者のこれら病態に対する素因によって引き起こされる血小板／白血球相互作用の発生を検出のための該方法の使用とを見出すことによって達成された。
【００２４】
【発明の実施の形態】
本発明のより完全な理解及び本発明に付随する利益の多くは、添付図面と共に以下の詳細な説明を参照し本発明の理解が進むに従って容易に分るであろう。
本発明は、血小板／白血球相互作用アッセイ及び該アッセイに用いる試薬に関する。本発明のアッセイは、血液あるいは血液由来サンプルにおける血小板と白血球との相互作用をモニターする。本アッセイは、湿式（ｗｅｔ　ｃｈｅｍｉｓｔｒｙ）及び乾式（ｄｒｙ　ｃｈｅｍｉｓｔｒｙ）のいずれのアッセイ形式においても行うことができる。
【００２５】
本発明において、用語「白血球」及びそのフォームは通常の医学的意味を有するものである。白血球には、顆粒球やリンパ球、単球が挙げられるが、これらに限定されない。顆粒球サブグループには、好中球、好塩基球及び好酸球がある。本発明は、最も好ましくは、血小板と顆粒球及び単球との相互作用を検出するために用いる。しかし、全てのタイプの血小板／白血球相互作用も本発明アッセイの範囲に入る。
【００２６】
本発明は、懸濁液中、固相刺激剤の存在下で血小板／白血球相互作用を評価し定量化するための方法及び組成物に関する。本発明における固相刺激剤は、微粒子に単独であるいは組み合わせて固定化した、血漿タンパク質及び／又は細胞外マトリックスタンパク質あるいはこれらのフラグメントから成る。これらのタンパク質は、受動的にあるいは共有結合及び／又は架橋分子によって微粒子に接着させることができる。微粒子は単一のタイプでもよく、また、ある実施形態においては、二以上の異なるタイプの微粒子を含むことができる。
【００２７】
本発明の好ましい実施形態においては、固相支持体を被覆するために用いるタンパク質は

、ｖｏｎ Ｗｉｌｌｅｂｒａｎｄ因子、フィブリノーゲン、コラーゲン、フィブロネクチン、ビトロネクチン、トロンボスポンジン、ラミニン、オステオポンチン、凝固因子（活性又は不活性型）、フィブリリン、コンドロイチン硫酸及びヘパリン硫酸からなる群より選択される。これらのタンパク質又はそのフラグメントは、単独で又は組み合わせて、受動的に又は共有結合によって固相支持体に固定される。固相粒子状支持体へのタンパク質の接着は、スペーサー分子を用いて行うこともできるが、これは当業者にとっては容易に明らかであろう。

【００２８】

固相刺激剤を、懸濁液中、所定の時間、所定の強制力付与条件（ｆｏｒｃｅ ｃｏｎｄｉｔｉｏｎｓ）下で血小板及び白血球のソースと混合する。強制力付与条件は幅広い反応（ａ ｒａｎｇｅ ｏｆ ｒｅａｃｔｉｖｉｔｉｅｓ）が可能なように設定される。これらの反応は、例えば、単に血小板／白血球複合体を固相刺激剤に接触させこの複合体の結合をもたらす反応から、高い強制力付与条件（例えば高せん断力又は乱流）下で血小板及び／又は白血球を活性化し、懸濁固相刺激剤への血小板／白血球複合体の形成を導く反応に及ぶ。混合条件は、攪拌、振とう、吸引、電磁場及び／又はビームの適用、超音波、コーンプレート粘度計等の装置を用いたせん断力の適用又は所定寸法の導管を通る懸濁液のフローの形をとり得る。導管は、ガラスあるいはプラスチックのチューブや、マイクロチップに形成されたチャンネルや哺乳類由来の血管、反応テストカードに設けられた導管、Ｏｂｅｒｈａｒｄｔの米国特許第５１１０７２７号に記載の導管の形をとり得る。この米国特許明細書を、本明細書の一部を構成するものとしてここに援用する。アッセイサンプルは、通常、血小板及び白血球を含む懸濁液であり、血液あるいは血液由来のサンプルである。アッセイサンプルは、好ましくは、指（ｆｉｎｇｅｒ ｓｔｉｃｋ）から採取した全血、希釈全血、抗凝固処理された全血、洗浄細胞、軟膜又は血小板リッチの血漿である。血小板／白血球懸濁液は、被験者から直接採取するか、あるいは元々この被験者から採取し研究や輸血の目的で保存していた血液製剤から採取する。

【００２９】

本発明の好ましい実施形態においては、被験対象（ｓｕｂｊｅｃｔ）は哺乳類であり、最も好ましくはヒトである。

【００３０】

血小板と白血球との相互作用の評価には、この相互作用を定性的及び／又は定量的に解析できるように、所定の強制力付与条件の適用によってこれら細胞を固相支持体に接着させることを含む。

【００３１】

定性的解析は、本アッセイ時に形成される血小板／白血球／固相支持体複合体を検出することが可能ないずれの方法を用いることによっても行うことができる。このような方法の適切な例としては、巨視的検査（肉眼による）や顕微鏡法、フォトマイクロスコピー、電子顕微鏡法（透過型又は走査型）、共焦点顕微鏡法、ビデオ顕微鏡法が挙げられるが、これらに限定されない。定性的解析は、二者択一的に（又は共存的に）組織化学的解析や免疫組織化学的解析、遺伝学的解析（ＰＣＲ、ＦＩＳＨ、サザンブロット法）、ウェスタンブロット法の形をとり得る。

【００３２】

本アッセイは、血小板－白血球相互作用の定量的あるいは半定量的測定にも用いることができる。このような解析は、アッセイされるサンプル中に存在する血小板／白血球／固相支持体複合体を検出し、その複合体の数をカウントすることが可能ないずれの方法を用いることによっても行うことができる。適切な定量的あるいは半定量的解析方法としては、細胞計数法やフローサイトメトリー、スタティックサイトメトリー、レーザ走査サイトメトリー、濁度測定、吸光度測定、比色測定、エンザイムイムノアッセイ（ＥＬＩＳＡ）、ラジオイムノアッセイ、イムノラジオメトリックアッセイ、ゲル排除クロマトグラフィー、アフィニティークロマトグラフィー、細胞内あるいは細胞外イオン流動測定、細胞遊離物（ｃｅｌｌｕｌａｒ ｒｅｌｅａｓａｔｅｓ）の測定、固相／血小板／白血球凝集体サ

イズの測定、ラテックス凝集アッセイが挙げられるが、これらに限定されない。このような定量的測定のための好ましい方法は、細胞計数法及びサイトメトリー（フローサイトメトリーとスタティックサイトメトリーの両方）である。適切なサイトメーターとしては、ＣｏｍｐｕＣｙｔｅから入手可能なＯＮＣＹＴＥ（登録商標）及びＬＳＣ（登録商標）スタティックサイトメーターや、Ｂｅｃｔｏｎ　Ｄｉｃｋｉｎｓｏｎから入手可能なＦＡＣＳＣａｎ（登録商標）及びＦＡＣＳＣａｌｉｂｕｒ（登録商標）フローサイトメーター、Ｃｏｕｌｔｅｒから入手可能なＥＸＣＥＬ（登録商標）フローサイトメーターが挙げられるが、これらに限定されない。

【００３３】

血小板／白血球相互作用の定性的あるいは定量的評価を他のアッセイと組み合わせて行い、病態をより明確に同定し得る情報及び／又は適切な治療計画を可能とする情報をユーザーに提供することができる。一例として、（Ｍａｈａｎらの米国特許仮出願第６０／２０２６３８号に記載のように（この仮出願の明細書を本明細書の一部を構成するものとしてここに援用する）、あるいは従来の血小板機能テストによって）個人が血小板機能を測定したい場合や、血小板／白血球相互作用の評価に関連づけて心臓マーカー酵素を測定しＡＭＩの発症をより明確にする場合が挙げられる。このようなアッセイの組合せは、単一デバイスにおいて並行してあるいは同時に行えるよう構成され得る。所定の病態のための望ましいアッセイの他の組合せは、当業者には明白であり、本明細書に記載したものに限定されない。

【００３４】

本発明における固相刺激剤は、血小板／白血球コンジュゲートを局在化し、血小板／白血球相互作用の程度を迅速に評価することを可能にする手段を提供する。また、この固相刺激剤は、血小板及び／又は白血球の活性化を誘発し、固相刺激剤上での血小板／白血球複合体の形成を容易にすることもできる。

【００３５】

本発明の好ましい実施形態においては、粒子を血漿タンパク質ソースあるいはそのフラグメントで被覆する。血漿タンパク質の例としては、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子やフィブリノーゲン、フィブロネクチン、ＩＩ因子、ＩＩａ因子、Ｖ因子、Ｖａ因子、ＶＩＩＩ因子、ＶＩＩＩａ因子、ＩＸ因子、ＩＸａ因子、Ｘ因子、Ｘａ因子、ＸＩ因子、ＸＩａ因子、ＸＩＩ因子、ＸＩＩａ因子、ＸＩＩＩ因子、ＸＩＩＩａ因子等の血液凝固因子（活性型又は不活性型、即ちチモーゲン型）、コラーゲン、ビトロネクチン、ラミニン、オステオポンチン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸、これらを組合わせたものが挙げられるが、これらに限定されない。

【００３６】

本発明の好ましい実施形態においては、細胞外マトリックスタンパク質あるいはそのフラグメントを単独で又は組み合わせて用いて粒子を被覆する。細胞外マトリックスタンパク質の例としては、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子やフィブロネクチン、コラーゲン、オステオポンチン、ラミニン、トロンボスポンジン、フィブリリン、コンドロイチン硫酸、ヘパリン硫酸、これらを組み合わせたものが挙げられるが、これらに限定されない。

【００３７】

また、白血球選択性抗体や、白血球膜成分に結合するタンパク質あるいはそのフラグメント等の白血球結合リガンドで粒子の一部を被覆することもできる。このようなタンパク質の例としては、ＶＣＡＭ－１やフィブロネクチン、ラミニン、ＩＣＡＭ－１、ＩＣＡＭ－２、ＩＣＡＭ－３、コラーゲン、オステオポンチン、ｖＷｆ、ビトロネクチン、トロンボスポンジン、粘膜アドレシン細胞接着分子１（ＭａｄＣＡＭ－１）、Ｐ－セレクチン、Ｌ－セレクチン、Ｅ－セレクチンが挙げられる。

【００３８】

タンパク質によって被覆され固相刺激剤を形成する微粒子は、いかなる形状をも取り得る。特に、微小球体あるいは不定形微粒子の形状を取り得る。この微小球体あるいは不定形微粒子は、一以上の上記タンパク質又はそのフラグメントが直接的にあるいは間接的に結

合することのできるいかなる材料からも形成することができる。これらの微小球体あるいは不定形微粒子は、いかなる所望の粒径を有することができ、好ましくは、後述のＰＩＯＰと同じオーダーの粒径を有し、より好ましくは、１～２０ミクロンの粒径を有する。微小球体あるいは不定形微粒子を構成する材料の好ましい例としては、ポリスチレン及び／又はラテックスやポリカーボネート、アクリロニトリル、カルボキシレート、テフロン（登録商標）、ガラス、ナイロン、デキストラン、アガロース、アクリルアミド、シリカ、花粉、微生物（生存可能又は生存不能）、酸化鉄、常磁性酸化鉄、常磁性粒子、非磁性金属ビーズ、金、白金、パラジウムが挙げられるが、これらに限定されない。最も好ましい材料は、Ｓｔｅｗａｒｔら、Ｂｒｉｔｉｓｈ Ｊ．Ｈａｅｍａｔｏｌｏｇｙ，９７，３２１－３２９（１９９７）やＳｈａｗら、米国特許第５９５２１８４号に記載されているように、ポリスチレンビーズである。これら文献の各々を本明細書の一部を構成するものとしてここに援用する。微小球体あるいは不定形微粒子は、元来常磁性であり、及び／又は蛍光標識され、及び／又は適切な基質及び／又は化学薬品の存在下で光反応あるいは呈色反応を引き出すのに適した酵素で標識される。

【００３９】

本発明の好ましい一実施形態においては、微粒子はポリスチレンを含み、球状を呈し、ヒト由来のｖｏｎ Ｗｉｌｌｅｂｒａｎｄ因子で被覆される。

【００４０】

本発明の最も好ましい実施形態においては、血小板／白血球複合体を捕捉するかｖｏｎ Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体上の血小板／白血球複合体の形成を誘発するのに充分な所定の強制力付与条件下で、所定の時間、ｖｏｎ Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体を全血（非抗凝固又は抗凝固）と混合し、引き続き、この複合体形成の存在及び／又はその量を測定する。

【００４１】

更なる好ましい実施形態においては、二種の中心的試薬要素を含む試薬を用いて本発明のアッセイを行う。第一の要素は磁性粒子、好ましくは常磁性酸化鉄（ＰＩＯＰ）であり、例えば現在ＴＡＳ^{TM}アナライザに用いられているもの等である（米国特許第４８４９３４０号、５１１０７２７号、５３５０６７６号、５６０１９９１号、５６７０３２９号及び５６７７２３３号に記載、これら特許の各々の明細書を本明細書の一部を構成するものとしてここに援用する）。これら磁性粒子は、（１）白血球と直接相互作用可能かあるいは（２）血小板と相互作用可能なリガンドを粒子表面に結合させることによって修飾されたものである。様々な磁性粒子を用いることができるが、米国特許第５１１０７２７号に記載されているように、好ましい磁性粒子はＰＩＯＰである。従って、以下の記載においては便宜上ＰＩＯＰについて言及するが、特記しない限り、ＰＩＯＰという用語はいかなる種類の磁性粒子をも表すと了解されるであろう。従来のＴＡＳでの用途におけるのと同様、ＰＩＯＰはまた、アッセイモニタリング及び検出システムにおいて中心的な役割を果たし、それによって、運動磁界に応答する修飾ＰＩＯＰの運動をモニターし、アッセイのエンドポイントを決定する。

【００４２】

この好ましい実施形態における第二の中心的試薬要素は、血小板と直接相互作用可能なリガンドで被覆した非磁性ビーズあるいは微小球体である。これらの非磁性ビーズあるいは微小球体はいかなる所望の粒径であってもよいが、好ましくは、ＰＩＯＰと同じオーダーの粒径であり、より好ましくは１～２０ミクロンの粒径を有する。非磁性ビーズは、その表面にリガンドを結合させることができるものであればどのような非磁性材料からも作ることができる。非磁性ビーズを調製するための好ましい材料としては、上掲した微小球体あるいは不定形粒子用材料、例えばポリスチレンビーズ、ポリオレフィンビーズ、ガラスビーズ、更には非磁性金属ビーズ等が挙げられるが、これらに限定されるものではない。最も好ましい材料は、Ｓｔｅｗａｒｔら、Ｂｒｉｔｉｓｈ Ｊ．Ｈａｅｍａｔｏｌｏｇｙ，９７，３２１－３２９（１９９７）やＳｈａｗら、米国特許第５９５２１８４号に記載されているようにポリスチレンビーズであり、これら文献の各々を本明細書の一部を構成

するものとしてここに援用する。Ｏｂｅｒｈａｒｄｔ及びＳｈａｗの特許に記載されているように、機能上の安定性、試薬の乾燥、サンプル添加の際の材料の再水和を向上させるものとして、当業者に知られている他の試薬（抗凝固剤、バッファー等）もまたテスト組成物に添加することもできる。

【００４３】

リガンドは、リガンド活性が損なわれない限り、粒子に直接的に又はスペーサーを介して間接的に結合することができる。直接的な結合は共有的に又は非共有的に起こり得る。間接的な結合はスペーサーを介して起こり得るが、そのスペーサーの例としてはペプチドスペーサー、抗体スペーサー又は炭水化物スペーサーが挙げられるがこれらに限定されるものではない。これらのスペーサーは通常は粒子とリガンドを架橋する働きをを有するだけであるが、リガンド／レセプター相互作用の効果を変えるために用いることもできる。例えば、７個のアミノ酸からなるペプチドによる架橋によって、ｖＷｆを粒子に結合させると、ｖＷｆと血小板レセプターとの相互作用は減少する場合がある。しかし、ｖＷｆの活性フラグメントを同じ７個のアミノ酸からなるペプチドによる架橋で粒子と結合させて用いると、ｖＷｆフラグメント／レセプター相互作用のアップレギュレーションとなるであろう。他のタイプにおける同様の改善はＢｅｅｒらによっても報告されている（Ｂｌｏｏｄ，７９，１１７－１２８，１９９２）。

【００４４】

本発明のアッセイの一実施形態において、振動磁界を用いる場合には、試薬は磁性粒子のみを含む。しかし、後述するように、回転磁界を用いる場合には、好ましい試薬は、磁性粒子及び非磁性粒子の両方（これら粒子は双方に結合したリガンドを有する）を含む。

【００４５】

本実施形態における非磁性粒子上の血小板と相互作用するリガンドは、その機能を果たすことが可能であり、その結果、血小板が活性化されるものであればいかなる化合物であってもよい。適切なリガンドとしては、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子（ｖＷｆ）、コラーゲン、トロンビン及びこれらのフラグメント（ミメトープとしても知られる。Ｍｉｌｌｅｒにより米国特許第５８７７１５５号に記載されている。この米国特許明細書を本明細書の一部を構成するものとして援用する）等が挙げられるが、これらに限定されない。用いるリガンドとして最も好ましいものはｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子あるいはそのフラグメントである。

【００４６】

非磁性粒子及び磁性粒子上で用いるリガンドは、同じリガンドであっても異なるリガンドであってもよい。両方のタイプの粒子上で同じリガンドを用いる場合、試薬内に白血球マーカーを供給することが更に必要となる。白血球マーカーは、白血球の存在を確認するための従来知られているいずれのマーカー（蛍光マーカー等）であってもよい。適切な白血球マーカーの例としては、ＣＤ４５、ＣＤ１８、ＣＤ１１ａ／ＣＤ１８（ＬＦＡ－１）、ＣＤ１１ｂ／ＣＤ１８（Ｍａｃ－１）、ＣＤ１１ｃ／ＣＤ１８、Ｐ－セレクチンリガンド（ＰＳＧＬ－１）及びＣＤ３４が挙げられる。両方のタイプの粒子上で同じリガンドを用いる場合、最も好ましくは、このリガンドはｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子あるいはそのフラグメントである。血小板との結合を提供するのに、そして血小板を活性化するのに十分な量のリガンドが、磁性及び非磁性粒子の表面上に存在するべきであって、そのような十分量のリガンドによって十分な数の血小板が活性化され、１～２０分、好ましくは２～４分でアッセイのエンドポイントに達する。例えば下に述べる回転磁界の場合、初めに形成された回転ＰＩＯＰリングが固体状の円盤やドットに潰縮（ｃｏｌｌａｐｓｅ）する時にエンドポイントとなる。

【００４７】

あるいは、二種類の粒子に結合するリガンドは異なっていてもよい。これらリガンドは両方とも血小板に直接相互作用するリガンドであり得るが、互いに異なっている。このような場合、上述のように同じタイプの白血球マーカーが必要となる。しかし、別の実施形態においては、一方のリガンドは血小板に直接（及び選択的に）相互作用するリガンドとす

ることができ、他方のリガンドが白血球に直接（及び選択的に）相互作用する。このような白血球選択性リガンドの例としては、白血球選択性抗体やＶＣＡＭ－１、フィブロネクチン、ラミニン、ＩＣＡＭ－１、ＩＣＡＭ－２、ＩＣＡＭ－３、コラーゲン、オステオポンチン、ｖＷｆ、ビトロネクチン、トロンボスポンジン、粘膜アドレシン細胞接着分子１（ＭａｄＣＡＭ－１）、Ｐ－セレクチン、Ｌ－セレクチン、Ｅ－セレクチンが挙げられる（これら全ては、ＦＩＴＣやフィコエリトリン等の蛍光タグによって標識されているかあるいは標識されていない）。このような場合、血小板／白血球／粒子複合体の形成によって、回転ＰＩＯＰリングの潰縮のみが起こり、血小板と白血球との相互作用を示す。これにより、血小板／白血球相互作用の定性的測定が提供されるが、本実施形態では、上記のような白血球マーカーを用いることによって、あるいはＰＩＯＰリングの潰縮時間を既知の血小板／白血球相互作用活性を有する一以上のスタンダードと比較し相関させることによって、この相互作用の定量的測定も提供される。

【００４８】

本発明のアッセイにおいては、血小板機能亢進又は白血球機能亢進のいずれかが存在する場合に、血小板／白血球相互作用が観察される。しかし、両方の活性が正常以下である場合、通常、この相互作用は観察されない。しかし、上述のように、血小板及び／又は白血球を活性化させるための高い強制力付与条件を適用することによって、この相互作用を強制的に起こさせることができる。高い強制力付与条件は、被験者の血小板／白血球相互作用に対する感受性を判断することに用いることができ、従って、上述のように、特にこの強制力付与条件を特定の病態用の他の診断ツールと組み合わせることによって、この相互作用を伴う様々な病態への傾向や素因を判断するための診断ツールを提供することができる。

【００４９】

本発明のアッセイは、湿式（ｗｅｔ　ｃｈｅｍｉｓｔｒｙ）又は乾式（ｄｒｙ　ｃｈｅｍｉｓｔｒｙ）のいずれの形式をとることもできる。いずれの形式においても、比較的平坦な反応表面上で、好ましくは、上のＯｂｅｒｈａｒｄｔの米国特許に記載されているスライド等の反応でテストを行う。最も好ましくは、米国特許第５１１０７２７号に記載の反応スライド又はカードを用いて乾式で行う（この米国特許の内容を、本明細書の一部を構成するものとしてここに援用する）。本アッセイは、所望のサイズの試薬チャンバを備えた、使い捨て形式のものとすることもできる。

【００５０】

本発明の血小板／白血球相互作用アッセイの好ましい実施形態を行うためには、米国特許第５１１０７２７号（先に本明細書の一部を構成するものとして援用した）に記載されている磁界等の振動磁界、あるいは米国特許第５６７０３２９号（本明細書の一部を構成するものとしてここに援用する）に記載されている磁界等の回転磁界の中に反応チャンバを置く必要がある。最も好ましくは、アッセイを回転磁界の存在下で行う。磁石（振動磁界型、回転磁界型いずれか）は、反応チャンバ中に存在するＰＩＯＰすべてに実質的な影響を与えることができるように設計されなければならない。回転磁界の好ましい例においては、反応チャンバがＴＡＳテストカードに存在するチャンバである時、磁極同士はは０．５～２．５ｃｍ離間していることができる。磁石は、磁界が回転している時、ＰＩＯＰの運動をもたらすよう、反応チャンバに十分に接近して置かれる。システム中の磁性粒子の円運動を維持することのできる頻度で、回転磁界を回転させることができ、回転数は好ましくは２０００～２５００ｒｐｍである。回転磁界は、中心軸の周りに永久磁石が回転することによって提供（米国特許第５６７０３２９号に記載あり）されるか、あるいは円形に配置された一連の電磁石を次々に活性化することによって生成される（米国特許第５６７０３２９号）。

【００５１】

本アッセイにおける回転磁界を提供する磁石のデザインとして、直径約３．４ｃｍの金属ディスク上に取り付けられた２セットのボタン磁石を用いることができる。金属ディスクの基板は、その中心でＤＣ電気モータのシャフトに取付けられている。各ボタン磁石アッ

センブリは、直径約１ｃｍの容易に入手可能なボタン磁石を３個含む。ボタン磁石は、金属基板上に互いにきっちりと対向するように置かれる。各ボタン磁石アッセンブリの上には、半径９ｍｍで厚さ約３ｍｍの第２の金属ディスク（準円、半円）の半分を置く。２つのディスクの直線状の淵は互いに向かい合い、約１．５ｃｍ離れている。全アッセンブリは、テストカードの反応チャンバの約２～４ｍｍ下に置く。

【００５２】

全血サンプル又は血小板リッチ血漿サンプルを、上述の試薬を含み、且つ上述の磁石上に置かれる反応チャンバに添加することによって、アッセイが開始される。その磁石は、好ましくは回転磁界を生成する磁石、最も好ましくは２５００ｒｐｍで回転する磁石である。乾式形式の場合の最も好ましい形態においては、試薬をサンプルにより再水和し、磁性粒子を解放し、回転磁界によって運動し始めるようにする。回転磁界の存在下で、磁性粒子は反応領域の外縁に沿って動く濃色物質のリングあるいはバンドを形成する。リングの中心は、初めは透明かやや灰色である（即ち、実質的にＰＩＯＰをほとんど含まない）。好ましくは、非磁性粒子は検出システムの検出限界以下であるように選択する。

【００５３】

通常の非阻害サンプルでは、ＰＩＯＰのバンドは数分かかって次第に縮小し、リングの中心がＰＩＯＰで充填され、反応領域の中心に中実状のドットを形成する。非磁性ビーズ自体は、好ましくはシステム中において容易に視認できるものではなく、アッセイのエンドポイントを決定するのに関与しない。テストのエンドポイントは試薬組成物中に存在するＰＩＯＰの位置及び運動によって定まる。回転磁界により与えられる、反応領域でのＰＩＯＰの運動は、ＰＩＯＰと非磁性ビーズの双方の上に存在する固相作動剤（即ちリガンド）と接触することによって血小板を活性化することを必要とする。２つの固相の凝集は、固相へ血小板が接着すること及びその後に血小板同士が結合すること（血小板凝集）を導く血小板の活性化により、特に両タイプの粒子上の血小板リガンドを用いる場合に起こり得る。このような場合、血小板／白血球相互作用の検出は、白血球マーカー（蛍光マーカー等）を試薬混合物に含めることにより実施され得る。アッセイにより血小板／白血球相互作用が生じると、マーカーは周囲のメディアよりも凝集した粒子の中に一層行き渡るようになる。血小板／白血球相互作用が起こらなければ、マーカーは、周囲のメディアに比べ顕著に凝集粒子内に存在することはないであろう。血小板と白血球の相互作用のレベルは、凝集した粒子内のマーカーの存在の定量的測定により判断できる。凝集は血小板非存在下や阻害剤の存在下では起こらない。

【００５４】

あるいは、一方の粒子が血小板リガンドを有し、他方の粒子が白血球リガンドを有する場合、血小板／白血球相互作用の存在は直接的にあるいは間接的に観察することができる。白血球リガンドが磁性粒子又はＰＩＯＰ粒子に結合している場合、血小板と白血球との相互作用の存在は直接的に観察することができるが、これはＰＩＯＰリングの潰縮がこの相互作用なしでは起こらないからである。一方、白血球リガンドが非磁性粒子に結合している場合、血小板リガンドで被覆したＰＩＯＰの血小板／血小板相互作用によって、非常にゆっくりではあるがＰＩＯＰリングのドットへの潰縮はなお起こり得る。本実施形態における血小板／白血球相互作用の確実な判断は、上述の蛍光マーカー等のマーカーを用いることによって最良になし得るであろう。

【００５５】

本発明の本実施形態のアッセイにおいて、結合したリガンドが血小板及び／又は白血球と相互作用すると、サンプル中に自然に存在するフリーなフィブリノーゲンが活性化した血小板と相互作用し、血小板同士の凝集が起こる。例えば心筋梗塞や脳卒中患者の場合等のように、血小板及び／又は白血球の機能が亢進すると、血小板／白血球相互作用も起こる。血小板の凝集（白血球有りあるいは無しで）が進むと、ＰＩＯＰの周囲の凝集体の有効質量が増大し、粒子によって形成される外側のリングからより重い凝集体が回転磁界の中心へと移動する。アッセイの進行とともに、このリングは最終的に一個の円状ドットへと潰縮し、この円状ドットは回転磁界中心の回りを回転し続ける。

【００５６】
本アッセイのエンドポイントは、ＴＡＳアナライザで用いているのと同様な反射赤外光を利用してモニターできる。これが可能であるのは、反応チャンバの濃色リングで覆われたエリアは、一体に収束したドットのエリアよりも大きいからである。上述のように、この信号は、定性的なＹＥＳ／ＮＯを応答することも定量的な応答も可能である。
【００５７】
反応エリアの中心部分を反射材料で形成した小さなスポットで覆うと、リングから得られる信号とドットから得られる信号の差を大きくすることができる。この場合、ＰＩＯＰの濃色リングはこの反射材料の陰にかくれ、信号が増加する。また、アッセイのエンドポイントはビデオカメラや赤外線カメラでモニターできる。カメラからの出力をデジタル化した後、画像を分析し、リング及びドット構造の形成を判定する。
【００５８】
図１は、米国特許第５１１０７２７号と同様のアッセイテストカードと回転磁界を用いた本発明アッセイの実施に係る図を示す。本図においては、ＰＩＯＰは血液サンプルを加えることにより運動可能となり、反応チャンバ（２０）内にＰＩＯＰの回転リング（１０）を形成する。
【００５９】
図２は、ＰＩＯＰの回転リング（１０）が、回転磁界の軸である中心点（３０）へ向かって潰縮し始めたことを示す。また、図３は、アッセイのエンドポイントを示し、ＰＩＯＰはドット構造（４０）へと完全に潰縮している。このエンドポイントはドラマチックであり、装置を用いても視覚的にも容易に検出可能である。視覚的な検出は、上述の定量的な情報を得る迅速簡便な方法であり、ＴＡＳアナライザ等のシステムを用いた装置による検出では（好ましくは既知の血小板／白血球相互作用レベルを有する複数のサンプルを用いて作成した標準カーブと比較することにより）得られた信号を分析でき、レセプターブロックの定量的な測定が可能である。
【００６０】
振動磁界を用いた場合は、得られる信号はＯｂｅｒｈａｒｄｔの米国特許第５１１０７２７号及び第４８４９３４０号と同様の方法で、粒子の振動をモニターすることで得られた減衰カーブを分析することにより分析される。
【００６１】
本発明の概略を説明したが、更なる理解のために具体例を挙げて本発明を説明する。特段の記載のない限り、本発明はこれらに限定されるものではない。
【００６２】
【実施例】
ｖＷｆ被覆ＰＩＯＰの調製　ＩＳＫ　Ｍａｇｎｅｔｉｃｓ（インディアナ州、バルパライソ）より得たマグネタイト（１０ｇ）を密閉フラスコ内の５０ｍＭトリス（ｐＨ７．４、９０ｍＬ）に添加し、窒素で５分間パージした。その後ＰＩＯＰ懸濁液をモデル７００ＰｏｗｅｒＧｅｎホモジナイザ（Ｆｉｓｈｅｒ　Ｓｃｉｅｎｔｉｆｉｃ）で、直径７ｍｍのロータ・ステータを用い、セッティングを６として５分間ホモジナイズした。ホモジナイズしたＰＩＯＰのアリコート（０．７ｍＬ）を約１０μｇ／ｍＬのｖＷｆ（０．３ｍＬ）に添加し、室温で３０分間インキュベートした。
【００６３】
ｖＷｆ被覆ポリスチレンビーズの調製　Ｐｏｌｙｓｃｉｅｎｃｅｓ　Ｃｏｒｐｏｒａｔｉｏｎ（ペンシルバニア州、ワーリントン）より得たポリスチレンビーズ（４μｍ）は、使用前に０．２ｍｏｌ／Ｌの炭酸塩バッファー（ｐＨ９．３５）で３回洗浄した。ｖＷｆを０．２ｍｏｌ／Ｌの炭酸塩バッファー（ｐＨ９．３５）で２Ｕ／ｍＬに希釈し（ｖＷｆ１ユニットはプールされた正常血漿１ｍＬ中の量で定義される）、炭酸塩バッファー中で予め平衡化したポリスチレンビーズと混合した後、４℃で一晩インキュベートした。
【００６４】
実施例１

健常ボランティアから抗凝固剤としてのクエン酸塩に採取した全血を、ヒトｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆したポリスチレン微小球体と混合した。全血（１００μＬ）をマイクロウェル中の微小球体（約５×１０^{5}、直径４．５μｍ）（５μＬ）に添加し、ロータリーシェーカーを用いて５００ｒｐｍで１～１０分間振とうした。マイクロウェルからアリコートを取り、顕微鏡法によって検査した。反応は、ビデオ顕微鏡法、位相差顕微鏡法及び固定塗抹標本上での分染法によって評価した。血小板が機能亢進を示した場合、又は血小板及び／又は白血球を反復遠沈（８００×ｇ、１０分間）等の機械的ストレスに付した場合、若しくは試験前に２１ゲージニードルを用いて全血を数回強制的にせん断に付した場合にのみ、白血球と血小板との複合体がｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体と結合することが観察された。図４は、血小板／白血球／ｖＷｆ被覆ポリスチレンビーズ複合体の一例であり、ｖＷｆ被覆ビーズに結合した血小板に多形核白血球が結合している様子を示すものである。

【００６５】

実施例２

健常ボランティアから採取しヘパリン添加あるいはクエン酸添加を行った全血に対し、自動セルカウンタを用いてディファレンシャル・セルカウントを行った。全血（１００μＬ）をマイクロウェル中のｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体（約５×１０^{5}、直径４．５μｍ）（５μＬ）に添加し、ロータリーシェーカーを用いて５００ｒｐｍで１～１０分間振とうした。その後、第二のカウントを反応懸濁液に対して行った。ビーズ試薬の希釈効果を考慮に入れ、白血球数の減少は、反応後のカウントと反応前のカウントとの比を計算することによって決定し、得られた結果を１００倍して白血球数の減少率を得た。付随して、被覆していない初期の微小球体を、抗凝固した全血と同様の方法で混合し、上述のように評価した。カウントデータを図５の表に示す。

【００６６】

血小板が機能亢進を示した場合、又は血小板及び／又は白血球を反復遠沈（８００×ｇ、１０分間）等の機械的ストレスに付した場合、若しくは試験前に２１ゲージニードルを用いて全血を数回強制的にせん断に付した場合にのみ、抗凝固した全血をｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体と混合した後、白血球数の減少が見られた。これに対し、被覆していない微小球体を用いた試験では、白血球数の減少は見られなかった。位相差顕微鏡法によって、白血球／血小板複合体はｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体と結合して凝集体を形成し、被覆していない微小球体とは結合しないことが確認された。白血球、血小板及びｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体によって形成された凝集体はサイズが大きいため、ディファレンシャル・セルカウンタでは白血球としてカウントできないことがわかった。

【００６７】

実施例３

冠動脈バイパス移植（ＣＡＢＧ）手術を受ける予定の被験者（ｎ＝３）に対し、本発明の方法を用いて外科手術前及び外科手術時に試験を行った。外科手術時の各時間ポイント（手術前、バイパス移植時（ｏｎ　ｂｙｐａｓｓ）、プロタミン投与後、集中治療室、術後２４時間後）において、クエン酸添加全血に対しディファレンシャル・セルカウントを行った。更に、各時間ポイントで得たクエン酸添加全血（１００μＬ）を、ｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体（約５×１０^{5}、直径４．５μｍ）（５μＬ）を含むマイクロウェルに添加した。このマイクロウェルをロータリーシェーカーを用いて５００ｒｐｍで１～１０分間振とうした。第二のディファレンシャル・セルカウントをマイクロウェルから得た血液に対して行った。ビーズ試薬の希釈効果を考慮に入れ、白血球数の減少は、反応後のカウントと反応前のカウントとの比を計算することによって決定し、得られた結果を１００倍して白血球数の減少率を得た。全ての被験者の手術前のサンプルにおいて、ディファレンシャル・セルカウント法で測定した白血球数に減少が見られた。顕微鏡法によって、ＶＷＦ被覆微小球体表面上での血小板／白血球複合体形成が確認された。バイパス移植時に患者から採取した血液サンプル中では、ＶＷＦ被覆微小球体の存在

下で血小板／白血球複合体の形成が確認された。しかし、いずれの被験者についても、術後２４時間後に採取した血液サンプル中では、ＶＷＦ被覆微小球体存在下での血小板／白血球複合体の形成は見られなかった。

【００６８】

実施例４

健常ボランティアから採取した全血を、クエン酸バキュテイナー採血管、ＥＤＴＡバキュテイナー採血管及びヘパリンバキュテイナー採血管へ注入した。各採血管からの血液滴を、ＶＷＦ被覆ポリスチレンビーズとＶＷＦ被覆常磁性酸化鉄粒子（ＶＷＦ－ＰＩＯＰ）との混合物を含む３個の独立した反応カードを有する反応ウェルに添加し、得られた懸濁液を５分間しっかりと混合した。全血のアリコート（５μＬ）を各カードから除去し、微視的ウェットマウント観察（位相差）及び染色塗抹（ヘマ－３染色、Ｆｉｓｈｅｒ　Ｓｃｉｅｎｔｉｆｉｃ）評価に付した。ＥＤＴＡ添加血液においては、ＶＷＦビーズへの血小板の弱い接着と共に大部分の血小板が結合せずに残っていることが確認された。また、ＥＤＴＡ添加血液においては、血小板はＶＷＦ－ ＰＩＯＰに結合しなかった。クエン酸添加血液サンプル及びヘパリン添加血液サンプルの両方においては、多数の血小板がＶＷＦビーズに結合し、引き続きＶＷＦ－ＰＩＯＰに結合し大型の複合体を形成することが示された。結合せずに残った血小板は殆ど無かった。これらの大型のＶＷＦビーズ／血小板／ＶＷＦ－ＰＩＯＰ複合体に対する（又は複合体内での）白血球の結合は、ウェットマウント及び染色塗抹のいずれにおいても観察されなかった。血小板が機能亢進を示した場合、又は血小板及び／又は白血球を反復遠沈（８００×ｇ、１０分間）等の機械的ストレスに付した場合にのみ、クエン酸添加サンプル及びヘパリン添加サンプルにおいて、白血球と血小板との複合体がｖｏｎ　Ｗｉｌｌｅｂｒａｎｄ因子で被覆した微小球体及びＶＷＦ－ＰＩＯＰと結合することが観察された。機械的ストレスよって、血小板とＶＷＦビーズ又はＶＷＦ－ＰＩＯＰとの結合が増大することはなく、また、ＥＤＴＡ血液サンプル中で白血球／血小板複合体の形成が促進されることもなかった。

【００６９】

テストカードの調製　Ｏｂｅｒｈａｒｄｔの米国特許第５１１０７２７号に記載されているような、約３０μＬの反応チャンバを有するテストカードに、上記の磁性粒子及びｖＷｆで被覆した非磁性粒子を含む試薬組成物を入れる（試薬組成物は、１ｍＬ当り被覆ＰＩＯＰ粒子を１～２ｍｇと2×10^{6}～8×10^{6}個のポリスチレン粒子を含む）。また、反応チャンバには、検出可能なシグナルを提供するのに充分な量の白血球マーカー（ＦＩＴＣ標識抗ＣＤ４５等）を入れる。一旦反応チャンバがサンプルで満たされると、次いで、上記のＯｂｅｒｈａｒｄｔの特許でテストカードの調製について記載されているように、サンプルを凍結し凍結乾燥する。

【００７０】

しかし、被覆非磁性粒子に対する被覆磁性粒子の比は限定されず、回転リングを形成しディスクあるいはドットに潰縮するのに充分な磁性粒子が存在する限りいかなる比も取り得る。

【００７１】

血小板／白血球相互作用テスト　上記の試薬を含むテストカード（使い捨て可能）を回転磁石上方のプラットフォームに置く。全血（又は他の血液由来の）サンプルをウェルに添加すると、サンプルは毛管現象によって反応チャンバに引き寄せられる。磁性粒子と非磁性粒子が遊離される時に、磁性粒子は反応チャンバの中心部の周りに回転リングを形成する。反応が進むにつれて、回転リングの内縁部は中心に向かって移動し、最終エンドポイントにおいては、内縁部は中心点に完全に潰縮しディスクあるいはドットを形成する。総経過時間は約１～２０分であり、通常２～４分の範囲である。

【００７２】

上記のｖＷｆ被覆ＰＩＯＰ及びｖＷｆ被覆ポリスチレン粒子を用いる場合、血小板／白血球相互作用の存在は、元の試薬組成物に存在する白血球マーカーを検出することによって判断される。あるいは、ＰＩＯＰが（ｖＷｆの代わりに）白血球リガンドで被覆されてい

る場合、血小板／白血球相互作用の発生は、ＰＩＯＰリング自身の潰縮によって検出される。

【００７３】

前述の基本的発明思想に基づき、本発明は他の各種改良物及び変形物とすることができることは明らかである。従って、添付の請求の範囲内で、上で述べた実施形態以外の態様でも本発明を実施できるものと理解されるべきである。

【００７４】

本出願は、１９９９年１１月１５日に出願した米国仮出願第６０／１６５４６２号に基づくものであり、その全内容を本明細書の一部を構成するものとしてここに援用する。

【図面の簡単な説明】

【図１】

回転磁界を用いた本発明のアッセイにおいて形成されたＰＩＯＰリングを示す。

【図２】

本発明のアッセイにおいて形成されたＰＩＯＰリングの中心点に向かって潰縮し始めた段階を示す。

【図３】

回転磁界の存在下におけるＰＩＯＰリングの完全な潰縮により形成されたディスクあるいはドットを示す。

【図４】

血小板／白血球／微粒子複合体の形成を示す顕微鏡写真を示す図である。

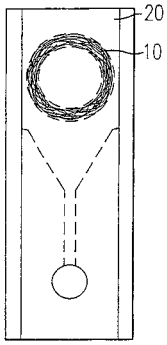
【図５】

上述した実施例において得られたカウントデータを表にまとめたものである。

【符号の説明】

１０　回転リング
２０　反応チャンバ
３０　中心点
４０　ドット構造

【図１】



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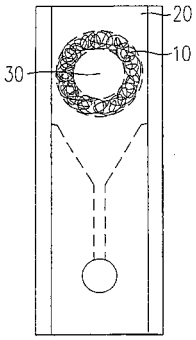

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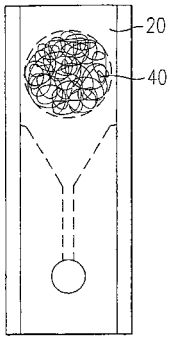

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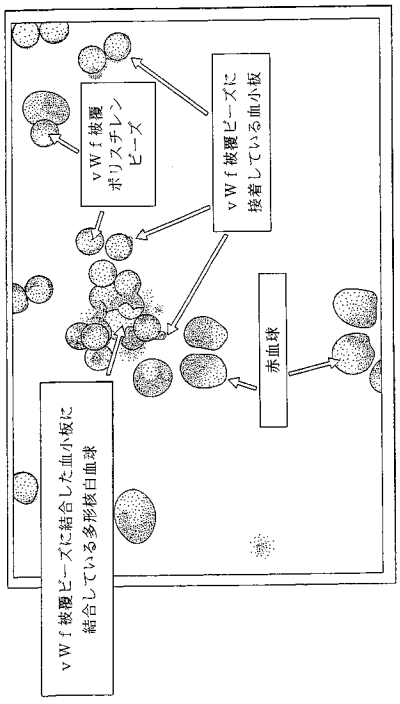

【図５】

脳卒中／ＴＩＡ患者－血小板／白血球相互作用

患者	血小板			単球			顆粒球		
	前	後	%RPC	前	後	減少%	前	後	減少%
1	183	25	14	0.4	0.4	0	8.6	3.1	64
2	141	41	29	0.3	0.3	0	6.2	5.8	6
3	151	2	1	0.4	0.2	50	2.6	2.4	8
4	123	17	14	0.2	0.3	−50	5.1	5	2
5	148	9	6	0.2	0.3	−50	1.5	3	−100
6	239	10	4	0.3	0.3	0	3.6	3.7	−3
7	204	32	16	0.2	0.3	−50	5.8	5.5	5
8	156	4	3	0.3	0.3	0	4.6	3.5	24
9	205	14	7	0.4	0.1	75	4.8	3.8	21
10	134	14	10	0.4	0.2	50	2.9	3.2	−10
11	192	4	2	0.4	0.1	75	2.8	1.3	54
12	172	3	2	0.3	0.2	33	5.2	4.7	10
13	269	11	4	0.6	0.3	50	4.2	2.9	31
14	82	20	24	0.3	0.3	0	2.1	1.85	12
15	203	117	58	0.5	0.7	−40	4.2	3.55	15
16	164	96	59	0.4	0.45	−13	5.9	4.85	18
17	184	105	57	0.5	0.55	−10	5.7	5.95	−4
18	244	61	25	0.2	0.5	−150	5.1	6.05	−19
19	150	24	16	0.3	0.6	−100	6.2	6.5	−5
20	145	39	27	0.4	0.2	50	3	2.95	2
		Mean	18.9		#>9	7		#>9	9
		SD	18.93						
		#<30	17						
		#>30	3						

＊試験を行った脳卒中／ＴＩＡ患者２０名の内１７名の残存血小板カウント（ＲＰＣ）は３０％未満であった。

＊試験を行った脳卒中／ＴＩＡ患者２０名の内７名が、全血をｖＷｆ被覆ビーズと反応させた後に単球カウントの減少を示した。

＊試験を行った脳卒中／ＴＩＡ患者２０名の内８名が、全血をｖＷｆ被覆ビーズと反応させた後に顆粒球カウントの減少を示した。

＊試験を行った脳卒中／ＴＩＡ患者２０名の内１２名が、全血をｖＷｆ被覆ビーズと反応させた後に単球カウント又は顆粒球カウントの減少を示した。

＊＊試験を行った６３の対照サンプルの内５サンプルが、全血をｖＷｆ被覆ビーズと反応させた後に単球カウントの減少を示した。

＊＊試験を行った６３の対照サンプルの内１２サンプルが、ｖＷｆ被覆ビーズとの反応後に顆粒球カウントの減少を示した。

＊＊試験を行った６３の対照サンプルの内１３サンプルが、ｖＷｆ被覆ビーズとの反応後に単球カウント又は顆粒球カウントの減少を示した。

＊＊ｖＷｆ被覆ビーズとの反応後に単球カウント又は顆粒球カウントの減少を示した１３対照サンプルの内８サンプルの残存血小板カウントが３０％未満であった。

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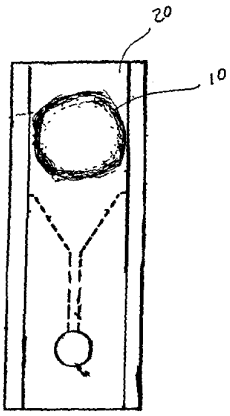
[Continued on next page]

(54) Title: PLATELET/LEUKOCYTE INTERACTION ASSAY AND REAGENT THEREFOR

(57) Abstract: A platelet/leukocyte interaction assay method and reagent therefor are provide using the presence of a solid-phase stimulus, such as magnetic or non-magnetic particles or mixtures thereof, having bound to the surface thereof one or more ligands that interact directly with platelets, leukocytes or both, for providing a fast, reliable point-of-care assessment of platelet/leukocyte interaction.



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PLATELET/LEUKOCYTE INTERACTION ASSAY AND REAGENT THEREFOR

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a platelet/leukocyte interaction assay allowing for point of care assessment of interaction of platelets and leukocytes, and the reagents therefor.

Discussion of the Background

Platelets are known to interact with leukocytes both as a consequence of contact during normal blood flow (Stone and Nash, *British Journal of Haematology*, **105**:514-22, 1999; Lorenz et al, *Blood Coagulation and Fibrinolysis*, **9**:S49-59, 1998) and as a consequence of various pathological processes (Rinder et al, *Journal of Cardiovascular Surgery*, **118**:460-6, 1999; Peyton et al, *Journal of Vascular Surgery*, **27**:1109-15, 1998; Stuard et al, *International Journal of Artificial Organs*, **21**:75-82, 1998; Gawaz et al, *European Journal of Clinical Investigation*, **25**:843-51, 1995).

Pathological conditions such as Unstable Angina, Coronary Artery Disease (CAD), and Stroke are characterized by high levels of platelet and leukocyte activity. Measurement of platelet/leukocyte interaction can be predictive of these pathological states, particularly in combination with other diagnostic factors. Also, measurement of platelet/leukocyte interaction can be used as a means of monitoring therapy directed toward altering platelet and/or leukocyte function.

Exposure of flowing blood to artificial surfaces has been shown to enhance platelet/leukocyte interaction. The cell types involved and the extent of the interaction vary with the composition of the artificial surface in contact with the blood (Gawaz et al, *Artificial Organs*, **23**:29-36, 1999).

Although platelet/leukocyte interactions have been quantified using various techniques (Hendricks et al, US patent 5503982; Rinder et al, *Blood*, **78**:1760, 1991;), assessment of the interaction has relied upon measurement of *circulating* platelet/leukocyte complexes. Measurements, to date, have taken the form of evaluating pre-existing platelet/leukocyte interactions in a blood sample.

In some pathological conditions (e.g. Acute Myocardial Infarction, AMI; post Angioplasty, PTCA; etc.) platelet/leukocyte complex formation is associated with interaction with damaged subendothelium, whether directly (plaque formation) or indirectly (release of biochemical markers such as ICAM-1, see Hendricks et al, US patent 5503982).

Current assay systems used to assess platelet/leukocyte interactions, as exemplified by Hendricks et al (US patent 5503982), evaluate pre-existing (circulating) platelet/leukocyte complexes and do not utilize a component representative of the vessel subendothelium (i.e. extracellular matrix) or other solid-phase stimulus. Moreover, the threshold at which discrete platelets and leukocytes interact could vary depending on the activation status of these cells at the time of testing. It is known in the art that platelet and/or leukocyte activation is a necessary prerequisite of platelet/leukocyte binding. It is also known in the art that certain pathological conditions are associated with upregulation of platelet and/or leukocyte activity. However, the upregulation in cellular activity associated with the pathological process may be insufficient to support platelet/leukocyte complex formation without additional stimulation and moreover, may not be detectable using conventional systems, which lack a stabilizing solid-phase support upon which the platelet/leukocyte complex could be maintained. [A solid-phase stimulus could be used as a means of localizing pre-existing platelet/leukocyte complexes and/or inducing complex formation and localization in cells predisposed to do so.]

It is desirable that assay systems designed to incorporate the use of a solid-phase component such as immobilized subendothelial/extracellular matrix be facile, rapid and of reasonable cost to be useful in detecting platelet/leukocyte interaction in a clinical setting.

The present invention addresses shortcomings of previous methods and technologies by using microparticles of various compositions coated with plasma proteins and/or extracellular matrix proteins, either singly or in combination, to facilitate rapid assessment of platelet/leukocyte binding.

Platelets can interact with leukocytes through various mechanisms, such as contact during normal blood flow (Lorenz et al, *Blood Coagulation and Fibrinolysis*, **9**:S49-S59, 1998), or as a consequence of a pathological process associated with platelet hyperactivity (Spangenberg, *Thrombosis Research*, **74**:S35-S44, 1994; Rinder et al, *Journal of Cardiovascular Surgery*, **118**:460-6, 1999) or due to an inflammatory process (Gawaz et al, *European Journal of Clinical Investigation*, **25**:843-51, 1995). Receptors found on the platelet surface interact with receptors

found on various leukocytes through direct bridging or through an indirect linkage involving intermediary molecules (Weber and Springer, *Journal of Clinical Investigation*, **100**:2085-93, 1997). Upregulation of platelet and/or leukocyte activity favors enhanced platelet/leukocyte interaction (Rinder et al, 1999; Stone and Nash, *British Journal of Haematology*, **105**:514-22, 1999; Konstantopoulos et al, 1998; Gawaz et al, 1995; Spanenberg, 1994).

Individuals with Coronary Artery Disease (CAD), Diabetes or Cerebrovascular Ischemia demonstrate both platelet hyperactivity and an ongoing inflammatory process (Michelson and Furman, *Current Opinion in Hematology*, **6**:342-8, 1999). Treatment of patients with CAD has involved the use of anti-platelet agents and anti-inflammatory medications (Vorchheimer et al, *JAMA* **281**:1407-14, 1999; Mannaioni et al, *Inflammation Research*, **46**:4-18, 1997).

Platelet/monocyte (Hendricks et al, US patent 5503982) and platelet/neutrophil (Gawaz et al, *European Journal of Clinical Investigation*, **25**:843-51, 1995) interaction have been suggested to be predictive of acute myocardial infarction (AMI) and inflammation, respectively. A corollary to platelet/leukocyte interaction in various pathological conditions is involvement of the vessel wall, whether in plaque formation for example, or a localized inflammatory reaction.

Platelet function assessment using immobilized extracellular matrix proteins has been described by Shaw and Stewart (US patent 5,427,913). The authors demonstrated that von Willebrand factor (VWF) immobilized on polystyrene beads could be used to activate platelets and thereby determine the functional status of platelets from patients with platelet function defects. In addition, the authors also demonstrated that the effects of agents designed to alter platelet function could be monitored using bead-immobilized VWF as a stimulus. The results of these studies underline the importance of evaluating a normal hematological interaction or the hematologic consequence of a pathological state in the presence of an agent that mimics components of the vessel wall.

Although Shaw and Stewart describe methods and compositions of determining platelet function, there is no description or suggestion of using their method for evaluating platelet/leukocyte interaction.

CVDI's TAS™ analyzer measures the kinetics of fibrin polymerization following activation of the coagulation pathway in a patient's blood sample. The TAS™ analyzer and disposable were designed for use with whole blood in a point-of-care setting. Paramagnetic iron

oxide particles (PIOP) are an essential component of the detection system for each of the tests developed for the TAS™ analyzer. The PIOP and other lyophilized ingredients for a particular test are located in the shallow reaction chamber of the TAS test card disposable. In addition to PIOP, the test reagent may contain buffers, stabilizers, fillers and specific coagulation pathway activator or agents. A test is initiated by insertion of a dry-chemistry test card into a slot of the TAS™ analyzer that automatically positions the test card reaction chamber above an electromagnet. This chamber is also illuminated with infrared light from a light emitting diode. The instrument measures reflected infrared light from the surface of the test card by means of a solid state photodiode detector. A test is automatically initiated when the analyzer photodetector measures a change in reflected light intensity when blood or plasma is added to the sample well of the test card and, through capillary action, is pulled into the reaction chamber. The activators present in the reaction chambers stimulate the coagulation cascade in the patient's sample to produce thrombin, which in turn catalyzes the formation of the fibrin clot.

During a clotting test the TAS™ analyzer electromagnet oscillates on and off every second. The magnetic particles stand up when the electromagnet is on, causing more light to be reflected to the detector, and fall down when it is off, causing less light to be detected. This movement of PIOP produces an alternating current (AC) signal from the photodetector. As the test proceeds, more and more fibrin polymerization occurs and the PIOP movement is less. The analyzer in accordance with predetermined algorithms interprets the signal produced by the relative movement of the PIOP and reports an endpoint (clotting time) appropriate for each test.

Although PIOP is an integral component of the TAS™ detection system, it does not participate directly in activation of the coagulation cascade or fibrin polymerization. To prevent undesired interactions between PIOP and activators within the reaction chamber of a test card, the PIOP is coated or blocked with bovine serum albumin (BSA). BSA is a protein commonly used by those skilled in assay development to prevent unwanted interactions between surface components of a test and its active ingredients. The TAS™ system was designed to monitor fibrin polymerization and not interactions between platelets and leukocytes.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide an assay for platelet/leukocyte interaction that can be used with a variety of blood products as the sample,

including whole blood, with ease of detection.

A further object of the present invention is to provide an assay for platelet/leukocyte interaction that can be used on the TAS™ system for rapid determination of platelet/leukocyte interactions.

A further object of the present invention is to provide a platelet/leukocyte interaction assay reagent, preferably in a dry chemistry test card format that can be used in the assay of the present invention.

A further object of the present invention is to provide a method for diagnosis of conditions resulting in platelet/leukocyte interactions using the assay of the present invention.

A further object of the present invention is to provide a method for determination of platelet and/or leukocyte hyperactivity using the assay of the present invention.

These and other objects of the present invention have been satisfied by the discovery of a method of assessing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample with a solid-phase stimulus, wherein said solid-phase stimulus has bound to a surface thereof a ligand selective for binding platelets or leukocytes, and

detecting formation of one or more platelet/leukocyte/solid-phase stimulus complexes, and the reagents for performing the method, as well as the use of the method for detection of the occurrence of platelet/leukocyte interaction caused by various disease conditions and the predisposition of subjects to those conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein;

Fig. 1a is a representation of the PIOP ring formed during the assay of the present invention using a rotating magnetic field.

Fig. 1b is a representation of the PIOP ring formed during the assay as it begins to collapse toward the center point of the ring.

Fig. 1c is a representation of the disc or dot formed from complete collapse of the

PIOP ring in the presence of a rotating magnetic field.

Fig. 2 is a photomicrograph showing the formation of a platelet/leukocyte/microparticle complex.

Fig. 3 is a tabular representation of count data obtained in the Examples described below.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to a platelet/leukocyte interaction assay and a reagent for use in the assay. The assay of the present invention monitors interaction between platelets and leukocytes in the blood or blood-derived sample. The assay can be performed either in a wet chemistry format or a dry chemistry format.

Within the context of the present invention, the term "leukocyte" and forms thereof is to be given its normal medical meaning. Leukocytes include, but are not limited to, granulocytes, lymphocytes and monocytes. Within the granulocyte subgroup, there are neutrophils, basophils and eosinophils. The present invention most preferably is used to detect interaction of platelets with granulocytes and monocytes. However, platelet/leukocyte interactions of all types are also within the scope of the present invention assay.

The present invention relates to methods and compositions for evaluating and quantifying platelet/leukocyte interaction in the presence of a solid phase stimulus, in suspension. The solid phase stimulus of the present invention would consist of plasma proteins and/or extracellular matrix proteins, or fragments thereof, in single or in combination immobilized on microparticles. These proteins could be attached to the microparticles passively or through a covalent linkage and/or through bridging molecules. The microparticles may be of a single type or, in certain embodiments, can include two or more differing types of microparticles.

In a preferred embodiment of the invention the proteins used to coat the solid phase support would be chosen from the group of von Willebrand factor, fibrinogen, collagen, fibronectin, vitronectin, thrombospondin, laminin, osteopontin, coagulation factors (in their active or inactive forms), fibrillin, chondroitin sulfate, or heparin sulfate. These proteins or fragments thereof, would be immobilized onto the solid phase support either in single or in combination, either passively or through a covalent linkage. Attachment of the proteins to the solid-phase particulate support could also be accomplished using spacer molecules, as

would be readily apparent to those skilled in the art.

The solid phase stimulus would be mixed in suspension with a source of platelets and leukocytes for a given period of time under defined force conditions. The force conditions could be such that a range of reactivities could be possible; from simple contact of the platelet/leukocyte complexes with the solid-phase stimulus to effect binding of the complexes, through to activation of the platelets and/or leukocytes under high force conditions (e.g. high shear or turbulent flow) to induce platelet/leukocyte complex formation on the suspended solid-phase stimulus. The mixing conditions could take the form of stirring, shaking, aspiration, application of electromagnetic fields and/or beams, ultrasound, or the application of shear through the use of an apparatus such as a cone-plate viscometer or flow of the suspension through conduits of predefined dimensions. The conduits could take the form of glass or plastic tubing; channels formed in microchips or blood vessels derived from mammals, or conduits on reaction test cards, such as those described in Oberhardt, U.S. Patent 5,110,727, hereby incorporated by reference. The assay sample is typically a suspension containing the platelets and leukocytes and is blood or a blood derived sample. The sample can preferably be whole blood from a finger stick, diluted whole blood, anticoagulated whole blood, washed cells, buffy-coat or platelet rich plasma. The platelet/leukocyte suspensions could be obtained either directly from a subject to be tested or from blood products that have been stored for research or transfusion purposes, originally collected from the subject.

In a preferred embodiment of the invention, the subject is a mammal, most preferably a human.

Evaluation of the interaction between the platelets and leukocytes comprises attachment of the cells to the solid-phase support through the application of a predefined set of force conditions, such that either qualitative and/or quantitative analysis of the interaction can be accomplished.

Qualitative analysis can be performed by any method capable of detecting the platelet/leukocyte/solid-phase support complexes formed during the assay. Suitable examples of such methods include, but are not limited to, macroscopic examination (by eye), microscopy, photo-microscopy, electron microscopy (transmission or scanning), confocal microscopy or video microscopy. Qualitative analysis could alternatively (or concomitantly)

take the form of histochemical analysis, immuno-histochemical analysis, genetic analysis (PCR, FISH, Southern Blotting) or Western Blotting.

The assay can also be used for quantitative or semi-quantitative determination of the platelet-leukocyte interaction. Such analysis can be performed using any method capable of detecting and counting the number of platelet/leukocyte/solid-phase support complexes present in the assayed sample. Suitable quantitative or semi-quantitative analysis methods include, but are not limited to, cell counting, flow cytometry, static cytometry, laser-scanning cytometry, turbidity measurement, absorbance measurement, colorimetric measurement, enzyme-linked immunosorbent assay (ELISA), radio-immunoassay, immunoradiometric assay, gel exclusion chromatography, affinity chromatography, intra-cellular or extra-cellular ion flux measurement, measurement of cellular releasates, measurement of solid-phase/platelet/leukocyte aggregate size, or latex agglutination assays. Preferred methods for such quantitative determination are cell counting and cytometry (both flow and static). Suitable cytometers include, but are not limited to, the ONCYTE® and LSC® static cytometers available from CompuCyte, the FACSCan® and FACSCalibur® flow cytometers available from Becton Dickinson and the EXCEL® flow cytometer available from Coulter.

Qualitative or quantitative assessment of platelet/leukocyte interaction can be performed in combination with other assays providing the user with information to allow a more definitive identification of a pathological condition and/or the appropriate treatment regimen. As an example an individual may wish to determine platelet function (such as described in Mahan et al, U.S. Provisional Application 60/202,638, hereby incorporated by reference; or by a conventional platelet function test), and cardiac marker enzymes in conjunction with assessment of platelet/leukocyte interactions to more clearly define the occurrence of AMI. Such combinations of assays could be constructed to run concurrently or simultaneously on a single device. Other combinations of desirable assays for defined pathological conditions are readily evident to those skilled in the art and should not be limited to those mentioned, herein.

The solid-phase stimulus of the present invention provides a means of localizing platelet/leukocyte conjugates enabling rapid assessment of the degree of platelet/leukocyte interaction. The solid-phase stimulus can also induce platelet and/or leukocyte activation facilitating platelet/leukocyte complex formation on the solid-phase stimulus.

In a preferred embodiment of the invention, the particles are coated with a source of plasma protein(s), or fragments thereof. Exemplary plasma proteins include, but are not limited to, von Willebrand factor, fibrinogen, fibronectin, or blood coagulation factors (in their active or inactive, i.e. zymogen forms), such as Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate and combinations thereof.

In a preferred embodiment of the invention the particles are coated with extracellular matrix protein(s), or fragments thereof, in single or in combination. Exemplary extracellular matrix proteins include, but are not limited to, von Willebrand factor, fibronectin, collagen, osteopontin, laminin, thrombospondin, fibrillin, chondroitin sulfate, or heparin sulfate or combinations thereof.

It is also possible to coat some of the particles with a leukocyte binding ligand, such as leukocyte selective antibodies, or proteins or fragments thereof that bind to leukocyte membrane components. Examples of such proteins include VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen, osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin and E-selectin.

The microparticles to which the proteins are coated to form the solid phase stimulus can be any shape. In particular, they can take the form of microspheres or irregularly shaped microparticles. The microspheres or irregularly-shaped microparticles can be made of any material to which it is capable to bind, either directly or indirectly, one or more of the above-noted proteins or fragments thereof. These microspheres or irregularly-shaped microparticles can be any desired particle size, preferably on the same order of size as the PIOP described below, more preferably having a particle size of from 1 to 20 microns. Preferred examples of materials comprising the microspheres or irregularly-shaped microparticles include, but are not limited to, polystyrene and/or latex, polycarbonate, acrylonitrile, carboxylate, teflon, glass, nylon, dextran, agarose, acrylamide, silica, pollen, micro-organisms (viable or non-viable), iron oxide, paramagnetic iron oxide, paramagnetic particles, non-magnetic metal beads, gold, platinum, or palladium. Most preferred are the polystyrene beads as described by Stewart et al, *British J. Haematology*, **97**, 321-329 (1997) and Shaw et al, U.S. Patent

5,952,184, each of which are hereby incorporated by reference. The microspheres or irregularly shaped microparticles could also be paramagnetic in nature and/or fluorescently tagged and/or tagged with an enzyme suitable to elicit a light or color reaction in the presence of the appropriate substrate and/or chemicals.

In one preferred embodiment of the invention the microparticles comprise polystyrene, are spherical in nature, and are coated with von Willebrand factor of human origin.

In a most preferred embodiment of the invention, the von Willebrand factor coated microspheres are mixed with whole blood (unanticoagulated or anticoagulated) for a predefined length of time under predefined force conditions, sufficient to capture platelet/leukocyte complexes or to induce platelet/leukocyte complex formation on the von Willebrand factor coated microspheres, following which the existence and/or amount of such complex formation is determined.

In a further preferred embodiment, the assay of the present invention is run using a reagent comprising two central reagent elements. The first is magnetic particles, preferably paramagnetic iron oxide particles (PIOP), such as those currently employed in the TAS™ analyzer (described in U.S. Patents 4,849,340; 5,110,727; 5,350,676; 5,601,991; 5,670,329; and 5,677,233, each of which is hereby incorporated by reference), which have been modified by binding a ligand to their surface that can either (1) interact directly with the leukocytes or (2) interact with the blood platelets. While a variety of magnetic particles can be used, as described in U.S. Patent 5,110,727, the preferred magnetic particles are PIOP. Accordingly, the following description will refer to PIOP for convenience, but it is to be understood that unless otherwise indicated, the term PIOP can refer to any magnetic particles type. The PIOP, as in the conventional TAS applications, also plays a central role in the assay monitoring and detection system, whereby movement of the modified PIOP in response to a moving magnetic field is monitored to determine the endpoint of the assay.

The second central reagent element in this preferred embodiment is non-magnetic beads or microspheres coated with a ligand that can interact directly with blood platelets. These non-magnetic beads or microspheres can be any desired particle size, preferably on the same order of size as the PIOP, more preferably having a particle size of from 1 to 20 microns. The non-magnetic beads can be made from any non-magnetic material that is

capable of having a ligand bound to its surface. Preferred materials for preparation of the non-magnetic beads include, but are not limited to, those listed above for the microspheres or irregularly-shaped particles, such as polystyrene beads, polyolefin beads, glass beads, and even non-magnetic metal beads. Most preferred are the polystyrene beads as described by Stewart et al, *British J. Haematology*, **97**, 321-329 (1997) and Shaw et al, U.S. Patent 5,952,184, each of which are hereby incorporated by reference. Other reagents known to those skilled in the art to enhance functional stability, reagent drying and material rehydration on sample addition may also be added to the test formulation, such as anticoagulants, buffers, etc, and as described in the Oberhardt, and Shaw patents described herein.

The ligand can be bound to the particles directly or indirectly through a spacer, so long as the activity of the ligand is not impaired. The direct binding can occur either covalently or non-covalently. Indirect binding can occur through spacers, including but not limited to peptide spacers, antibody spacers or carbohydrate spacers. These spacers normally act only as bridges between the particle and the ligand, but could be used in order to alter the effectiveness of the ligand/receptor interaction. For example, coupling vWf to the particle through a 7 amino acid peptide bridge could decrease the interaction of vWf with the platelet receptor. However, use of the active segment of vWf, coupled to the particle through the same 7 amino acid peptide bridge could result in upregulation of the vWf fragment/receptor interaction. Similar enhancements of other types have been seen by Beer et al, *Blood*, **79**, 117-128 (1992).

In one embodiment of the present invention assay, the reagent can contain only the magnetic particles when the oscillating magnetic field is used. However, as described below, when the rotating field is used, a preferred reagent contains both magnetic and non-magnetic particles having ligands bound to both types of particles.

The ligand that interacts with blood platelets on the non-magnetic particles of this embodiment can be any compound capable of performing that function, that results in activation of the platelets. Suitable ligands include, but are not limited to, von Willebrand factor (vWf), collagen and thrombin, as well as fragments (also known as mimetopes, such as those described by Miller, U.S. Patent 5,877,155, hereby incorporated by reference) thereof. Most preferred is the use of von Willebrand factor or a fragment thereof as the ligand.

The ligand used on the non-magnetic particles and the magnetic particles can be the

same ligand or different ligands. When the ligand is the same on both types of particles, it is further necessary to provide a leukocyte marker in the reagent. The leukocyte marker can be any conventional marker known to identify the presence of leukocytes, such as flourescent markers. Examples of suitable leukocyte markers include CD45, CD18, CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18, P-selectin ligand (PSGL-1), and CD34. In the case where the ligand is the same on both types of particles, the ligand is most preferably von Willebrand factor or a fragment thereof. The ligand should be present on the surface of the magnetic and non-magnetic particles in an amount sufficient to provide binding to, and activation of, the platelets, such that a sufficient number of platelets are activated to result in an assay endpoint within a time period of from 1 to 20 minutes, preferably from 2-4 minutes. For example, in the case of the rotating magnetic field as described below, the endpoint is reached when the initially formed rotating PIOP ring collapses into a solid disc or dot.

Alternatively, the ligands bound to the two types of particles can be different. The ligands can both be ligands that interact directly with platelets, while being different from one another. In such case, the same type of leukocyte marker would be needed as noted above. However, in a further embodiment, one ligand can be a ligand that interacts directly (and selectively) with platelets, while the other ligand interacts directly (and selectively) with leukocytes. Examples of such leukocyte selective ligands include leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin (all unlabeled or labeled with a fluorescent tag such as FITC or phycoerythrin). In such a case, collapse of the rotating PIOP ring would only occur through formation of the platelet/leukocyte/particles complex, thus signalling the interaction of platelets and leukocytes. While this provides a qualitative determination of platelet/leukocyte interaction, this embodiment can also provide a quantitative determination of the interaction by use of a leukocyte marker such as described above or by comparison and correlation of the collapse time of the PIOP ring with one or more standards having known platelet/leukocyte interaction activities.

In the assay of the present invention, platelet/leukocyte interaction will be observed when there is either platelet hyperactivity or leukocyte hyperactivity. However, when both activities are normal or low, the interaction will not normally be observed. This can be

forced to occur, however, by the application of high force conditions to activate the platelets and/or leukocytes, as described above. High force conditions can be used to determine the susceptibility of a subject for platelet/leukocyte interaction and hence can provide a diagnostic tool for determining propensity or predisposition to the various disease states involving such interactions, as noted above, particularly when combined with other diagnostic tools for the specific disease state.

The assay of the present invention can be either in a wet chemistry or dry chemistry format. In either format, the test can be performed on a relatively flat reaction surface, preferably in a reaction slide such as that described in the above-noted Oberhardt U.S. Patents. Most preferably, the assay is performed in a dry chemistry format using a reaction slide or card as described in U.S. Patent 5,110,727, the contents of which are hereby incorporated by reference. The present assay can also be adapted for use in disposables with reagent chambers of any desired size and shape.

To perform a preferred embodiment of the platelet/leukocyte interaction assay of the present invention, it is necessary to place the reaction chamber into an oscillating magnetic field, such as that described by U.S. Patent 5,110,727 (previously incorporated by reference) or into a rotating magnetic field, such as that described in U.S. Patent 5,670,329 (hereby incorporated by reference). Most preferably, the assay is performed in the presence of the rotating magnetic field. The magnet (either oscillating field type or rotating field type) must be designed such that the field can influence substantially all of the PIOP present in the reaction chamber. In the preferred example of the rotating magnetic field, when the reaction chamber is that present in a TAS test card, the separation between the magnetic poles may range from 0.5 to 2.5 cm. The magnet must be positioned sufficiently close to the reaction chamber to cause movement of PIOP when the magnetic field is rotating. The rotating magnetic field can rotate at any frequency capable of sustaining the circular movement of the magnetic particles of the system, with the rotational frequency being preferably from 2,000 to 2,500 rpm. The rotating magnetic field can be provided by the rotation of a permanent magnet about a central axis as described in U.S. Patent 5,670,329, or can be generated by sequential activation of a series of electromagnets in a circular arrangement, as also described in U.S. Patent 5,670,329.

One design for the magnet for providing a rotating magnetic field in the present assay

comprises two sets of button magnets mounted on a metal disk approximately 3.4 cm in diameter. The metal disk base is attached at its center to the shaft of a DC electric motor. Each button magnet assembly contains three readily available button magnets of approximately 1 cm in diameter. The button magnets are positioned directly opposite of one another on the metal base. On the top of each button magnet assembly is placed one-half of a second metal disk (semi-circle, half of a circle) approximately 3 mm thick with a radius of 9 mm. The straight edges of the two disks face one another and are separated by approximately 1.5 cm. The entire assembly is positioned approximately 2-4 mm beneath the reaction chamber of a test card.

The assay is initiated by addition of a whole blood or platelet rich plasma sample to a reaction chamber containing the reagents described, positioned above the magnet, preferably generating a rotating magnetic field most preferably at a rotational frequency of 2500 rpm. In the most preferred embodiment using a dry chemistry format, the reagent is re-hydrated by the sample, freeing the magnetic particles and allowing them to begin to move in response to the rotating magnetic field. In the presence of the rotating field, the magnetic particles organize as a ring or band of dark material traveling along the outer edges of the reaction area. The center of the ring initially is clear or slightly gray (i.e. contains substantially fewer PIOP). The non-magnetic particles are preferably chosen so as to be invisible to the detection system.

In a normal uninhibited sample the band of PIOP becomes smaller over a period of a few minutes and the center of the ring fills in with PIOP to form a solid dot in the center of the reaction area. The non-magnetic beads themselves are preferably not readily visible in the system and do not participate in determining an end point for the assay. The end point of the test is established by the position and mobility of the PIOP present in the reagent formulation. Motion of the PIOP in the reaction area imparted by the rotating magnetic field is required to activate the platelets through contact with the solid phase agonist (i.e. the ligand) present on both the PIOP and non-magnetic beads. The aggregation of the two solid phases can occur through platelet adhesion to the solid phase and then platelet activation leading to platelet/platelet binding (platelet aggregation), particularly in the case of using a platelet ligand on both types of particles. In such a case, the detection of platelet/leukocyte interaction can be performed by inclusion of a leukocyte marker, such as a fluorescent

marker, in the reagent mixture. If platelet/leukocyte interaction results from the assay, then the marker will be more prevalent in the aggregated particles than in the surrounding medium. If no platelet/leukocyte interaction occurs, the marker will not be present in the aggregated particles to any significant extent, compared to the surrounding medium. The level of interaction of platelets and leukocytes can be determined by quantitative measurement of the marker's presence in the aggregated particles. Aggregation does not occur in the absence of platelets or in the presence of an inhibitor.

Alternatively, when one type of particle bears the platelet ligand and the other type of particle bears the leukocyte ligand, the presence of platelet/leukocyte interaction can be observed either directly or indirectly. If the leukocyte ligand is bound to the magnetic or PIOP particles, the presence of the interaction of platelets and leukocytes can be directly observed, since collapse of the PIOP ring will not occur without such interaction. On the other hand, if the leukocyte ligand is bound to the non-magnetic particles, the collapse of the PIOP ring into the dot may still occur through platelet/platelet interaction of platelet ligand coated PIOP, albeit much more slowly. Definitive determination of platelet/leukocyte interaction is this embodiment would be best done through use of a marker, such as the fluorescent markers noted above.

In the assay of this embodiment of the present invention, once the bound ligand has interacted with the platelets and/or leukocytes, the free fibrinogen naturally in the sample interacts with the activated platelets, causing platelet/platelet aggregation. If the platelets and/or leukocytes are hyperactivated, such as in the case of a myocardial infarction or stroke victim, platelet/leukocyte interaction also occurs. As the platelets (with or without leukocytes) aggregate, this increases the effective mass of the aggregate around the PIOP, causing the heavier aggregates to migrate inward from the outer ring of particles toward the center of the rotating magnetic field. As the assay progresses, the ring eventually collapses into a circular dot, which continues to rotate about the center of the rotating field.

The end point of the assay can be monitored by using reflected infra red light similar to that of the TAS analyzer. This is possible because the area of the reaction chamber covered by the dark ring is much greater than that of the solid dot. The signal can provide both a qualitative YES/NO response and a quantitative response, as described above.

The difference in signal produced by a ring versus a dot can be enhanced if a small

spot of reflective material covers the very center of the reaction area. In this situation the dark ring of PIOP will disappear behind the reflecting material, thereby increasing the signal. Alternately the end point of the assay could be monitored by a video or infra red camera. The output of the camera can be digitized and the image subsequently analyzed to determine the formation of ring and dot structures.

Fig. 1a provides a representation of the assay of the present invention in operation using a rotating magnetic field with an assay test card such as that from U.S. Patent 5,110,727. In this Figure, the PIOP have been freed by the addition of the blood sample, resulting in the formation of a rotating ring (10) of PIOP within the reaction chamber (20).

Fig. 1b shows the rotating ring (10) of PIOP has started to collapse toward the center point (30) representing the axis of the rotating field. Fig 1c shows the endpoint of the assay in which the PIOP have completely collapsed into a dot structure (40). This endpoint is dramatic and readily detectable both instrumentally and visually. Visual detection provides a quick and easy method for obtaining the qualitative information discussed above, while instrumental detection using a system such as the TAS analyzer, provides the ability to analyze the signal obtained and provide quantitative measures of receptor blockage, preferably by comparison to a standard curve generated using samples of known platelet/leukocyte interaction levels.

When an oscillating magnetic field is used, the signal produced is analyzed in the same manner as in Oberhardt U.S. Patents 5,110,727 and 4,849,340, by analysis of the decay curve produced by monitoring the oscillation of the particles.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Preparation of vWf-coated PIOP. 10 g of Magnetite obtained from ISK Magnetics (Valparaiso, IN) was added to 90 mL of 50 mM Tris pH 7.4 in a sealed flask and purged with nitrogen for 5 minutes. The PIOP suspension was then homogenized by a model 700 PowerGen Homogenizer

(Fisher Scientific) for 5 minutes at a setting of 6 using a 7 mm diameter rotor-Stator. A 0.7 mL aliquot of the homogenized PIOP was added to 0.3 mL of approximatly 10 ug/mL vWf and allowed to incubate for 30 minutes at room temperature.

Preparation of vWf-coated polystyrene beads. Polystyrene beads (4 um) obtained from Polysciences Corporation (Warrington, Pa.) were washed three times with 0.2 mol/l carbonate buffer (pH 9.35) prior to use. vWf was diluted in 0.2 mol/l carbonate buffer (pH 9.35) to 2 U/mL (a unit of vWf is defined as the amount found in 1 mL of pooled normal plasma) and mixed with polystyrene beads pre-equilibrated in carbonate buffer prior to incubation overnight at 4°C.

Example 1

Whole blood from healthy volunteers collected into citrate as an anticoagulant was mixed with polystyrene microspheres coated with human von Willebrand factor. 100 μl of whole blood was added to 5 μl (approximately 5 x 10^5, 4.5 μm diameter) microspheres in microwells and shaken on a rotary shaker at 500 rpm for 1 to 10 minutes. Aliquots were removed from the microwells and examined by microscopy. The reaction was assessed using video microscopy, phase contrast microscopy and by differential staining on fixed smears. Leukocytes and platelets in complex were observed to be associated with the von Willebrand factor coated microspheres, only when the platelets displayed hyperactivity or when the platelets and/or leukocytes were subjected to mechanical stress such as repeated centrifugation (800 x g, 10 minutes), or shearing by forcing the blood through a 21 gauge needle several times prior to testing. Fig. 2 shows an example of the platelet/leukocyte/vWf coated polystyrene bead complex, showing the polymorphonuclear leukocyte binding to platelets bound to vWf-coated beads.

Example 2

A differential cell count was performed on heparinized or citrated whole blood from healthy volunteers using an automated cell counter. 100 μl of whole blood was added to 5 μl (approximately 5 x 10^5, 4.5 μm diameter) von Willebrand factor coated microspheres in microwells and shaken on a rotary shaker at 500 rpm for 1 to 10 minutes. A second count was then performed on the reaction suspension. Taking into account the dilution effect of the bead reagent, the decrease in the number of leukocytes was determined by calculating the ratio

between the post reaction counts and the pre-reaction counts and multiplying the result by 100 to obtain the percent decrease in leukocyte count. Concomitantly, uncoated nascent microspheres were mixed in a like manner with the anticoagulated whole blood and evaluated as described. The count data are tabulated in Fig. 3.

Leukocyte counts were seen to decrease after mixing the anticoagulated whole blood with the von Willebrand factor coated microspheres only when the platelets displayed hyperactivity or when the platelets and/or leukocytes were subjected to mechanical stress such as repeated centrifugation (800 x g, 10 minutes), or shearing by forcing the blood through a 21 gauge needle several times prior to testing. In contrast, testing with uncoated microspheres showed no decrease in leukocyte counts. Phase contrast microscopy confirmed leukocyte/platelet complex association with the von Willebrand factor coated microspheres in aggregates and no association with the uncoated microspheres. The aggregates formed by the leukocytes, platelets and von Willebrand factor coated microspheres were noted to be too large to be counted as leukocytes, by the differential cell counter.

Example 3

Study subjects (n = 3) destined for Coronary Artery Bypass Graft (CABG) surgery were tested prior to, and throughout the surgical procedure, using the method of the present invention. A differential cell count was performed on citrated whole blood at each time point (pre-surgery, on bypass, post-protamine, intensive care unit, 24 hours post-surgery) throughout the surgical procedure. In addition, 100 μl of citrated whole blood from each time point was added to microwells containing 5 μl (approximately 5 x 105, 4.5 μm diameter) von Willebrand factor coated microspheres. The microwells were shaken on a rotary shaker at 500 rpm for 1 to 10 minutes. A second differential cell count was performed on the blood from the microwells. Taking into account the dilution effect of the bead reagent, the decrease in the number of leukocytes was determined by calculating the ratio between the post reaction counts and the pre-reaction counts and multiplying the result by 100 to obtain the percent decrease in leukocyte count. All study subjects showed loss of leukocytes in the pre-surgical sample as determined by the differential cell counting technique. Microscopy confirmed platelet/leukocyte complex formation on the surface of the VWF coated microspheres. Platelet/leukocyte complex formation

in the presence of the VWF coated microspheres was noted in the blood samples collected while the patients were on bypass, however platelet/leukocyte complex formation in the presence of the VWF coated microspheres was not seen in blood samples collected 24 hours post-surgery, in any of the study subjects.

Example 4

Whole blood from a healthy volunteer was drawn into a citrate vacutainer tube, an EDTA vacutainer tube and a heparin vacutainer tube. A drop of blood from each tube was added to the reaction well of three separate reaction cards containing a mixture of VWF-coated polystyrene beads and VWF-coated paramagnetic iron oxide particles (VWF-PIOP) and the suspension mixed vigorously for 5 minutes. Aliquots of whole blood (5 μl) were removed from each card for microscopic wet-mount observation (phase contrast) and stained smear (Hema-3 stain, Fisher Scientific) evaluation. Weak platelet adhesion to the VWF beads was noted with the EDTA blood, with the vast majority of platelets left unbound. Platelets did not associate with VWF-PIOP in the EDTA blood. Both the citrate blood and the heparin blood samples showed extensive binding of platelets to VWF beads with subsequent binding of VWF-PIOP to form large complexes. Few platelets were left unbound. Binding of leukocytes to (or within) these large VWF bead/platelet/VWF-PIOP complexes was not observed either by wet mount or stained smear. Leukocytes and platelets in complex were observed to be associated with the von Willebrand factor coated microspheres and VWF-PIOP in the citrate and heparin samples only when the platelets displayed hyperactivity or when the platelets and/or leukocytes were subjected to mechanical stress such as repeated centrifugation (800 x g, 10 minutes). Mechanical stress did not augment platelet association with the VWF Beads or VWF-PIOP, nor did it promote leukocyte/platelet complex formation in the EDTA blood sample.

Preparation of test card - Into a test card, such as that in Oberhardt, U.S. Patent 5,110,727, having a reaction chamber of approximately 30 µl, is placed a reagent composition containing the above noted magnetic and non-magnetic particles coated with vWf, in amounts such that the reagent composition comprises 1-2 mg of coated PIOP particles per ml and from 2 x 106 to 8 x 106 polystyrene particles per ml. Also placed into the reaction chamber is an amount of a

leukocyte marker, such as FITC-labeled anti-CD45, sufficient to provide a detectable signal. Once the reaction chamber is filled, the sample is then frozen and lyophilized, as described for the preparation of test cards in the above noted Oberhardt patents.

However, the ratio of coated magnetic particles to coated non-magnetic particles is not limited and can be any ratio so long as there are sufficient magnetic particles to form the rotating ring and collapse to the disc or dot.

Platelet/Leukocyte Interaction Test. A disposable or test card containing the reagents described above is placed on a platform above a rotating magnet. Whole blood (or other blood-derived) sample is added to a well, which is pulled by capillary action into the reaction chamber. At that time the magnetic particles and non-magnetic particles are freed, with the magnetic particles forming a rotating ring around the central portion of the reaction chamber. As the reaction progressed, the inner edge of the rotating ring migrated toward the center, with the final endpoint providing a full collapse of the inner edge to the central point to form a disc or dot. The total time elapsed is approximately 1 to 20 minutes, typically in the 2-4 minute range.

When the above noted vWf coated PIOP and vWf coated polystyrene particles are used, the presence of platelet/leukocyte interaction is determined by detection of a leukocyte marker present in the original reagent formulation. Alternatively, if the PIOP are coated with a leukocyte ligand (instead of vWf) the occurrence of the platelet/leukocyte interaction is detected by collapse of the PIOP ring itself.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

* * * * * * *

The present application is based on U.S. Provisional Application 60/165,462, filed November 15, 1999, the entire contents of which are hereby incorporated by reference.

Claims

1. A method of assessing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample with a solid-phase stimulus, wherein said solid-phase stimulus has bound to a surface thereof a ligand selective for binding platelets or leukocytes, and

detecting formation of one or more platelet/leukocyte/solid-phase stimulus complexes.

2. A method of assessing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample with a solid-phase stimulus, wherein said solid-phase stimulus comprises microparticles having bound to a surface thereof a ligand selective for binding platelets or leukocytes, and

detecting formation of one or more platelet/leukocyte/solid-phase stimulus complexes.

3. The method of claim 2, wherein said ligand selective for binding platelets or leukocytes is a member selected from the group consisting of plasma proteins, plasma protein fragments, extracellular matrix proteins, extracellular matrix protein fragments and mixtures thereof.

4. The method of claim 2, wherein the microparticles are of irregular or regular shape or spherical.

5. The method of claim 2, wherein said whole blood or blood-derived sample is obtained from a mammal.

6. The method of claim 5, wherein said mammal is a human.

7. The method of claim 2, wherein the microparticles comprise particles made from one or more materials selected from the group consisting of polystyrene, latex, polycarbonate, acrylonitrile, carboxylate, teflon, glass, nylon, dextran, agarose, acrylamide, silica, pollen, micro-organisms, iron oxide, non-magnetic metals, paramagnetic iron oxide, gold, platinum, and palladium.

8. The method of claim 2, wherein said contacting step is performed by a process selected from the group consisting of stirring, shaking, aspiration, application of electromagnetic fields, ultrasound, shear and combinations thereof.

9. The method of claim 3, wherein said ligand selective for binding platelets or leukocytes is a member selected from the group consisting of von Willebrand factor, fibrinogen,

fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, fragments of said proteins, leukocyte selective antibodies, and combinations thereof.

10. The method of claim 2, wherein the ligands that selective bind platelets or leukocytes are attached to the microparticles covalently, through passive adsorption or through binding to bridging molecules.

11. The method of claim 3, wherein said plasma protein fragments or extracellular protein fragments are prepared by formation of peptides, formation of peptide mimetics or formation of peptide mimotopes either through recombinant technology or enzymatic cleavage or by linkage of amino acids by non-enzymatic chemical means.

12. The method of claim 2, wherein said whole blood or blood-derived sample is unanticoagulated whole blood.

13. The method of claim 2, wherein said whole blood or blood-derived sample is anticoagulated whole blood.

14. The method of claim 2, wherein said whole blood or blood-derived sample is cells contained within the buffy coat.

15. The method of claim 2, wherein said whole blood or blood-derived sample is a blood product collected for transfusion purposes.

16. The method of claim 15, wherein said blood product collected for transfusion purposes has been further subjected to one or more procedures designed to isolate specific blood components selected from the group consisting of random donor platelets, apheresis platelets, buffy coat and packed red cells.

17. The method of claim 2, wherein the whole blood or blood-derived sample is a member selected from the group consisting of unanticoagulated whole blood, anticoagulated whole blood and buffy coat, wherein the whole blood or blood-derived sample has been placed in contact with an artificial surface, *in vivo*, prior to use in the method.

18. The method of claim 2, wherein the whole blood or blood-derived sample is a member selected from the group consisting of unanticoagulated whole blood, anticoagulated whole blood and buffy coat, wherein the whole blood or blood-derived sample has been placed in contact with an artificial surface, *ex vivo*, prior to use in the method.

19. The method of claim 2, wherein the whole blood or blood-derived sample is a member selected from the group consisting of unanticoagulated whole blood, anticoagulated whole blood and buffy coat, wherein the whole blood or blood-derived sample has been placed in contact with an artificial surface, *in vitro*, prior to use in the method.

20. The method of claim 2, wherein said detecting step is performed by a method selected from the group consisting of flow cytometry, cell counting, microscopy, photo-microscopy, transmission electron microscopy, scanning electron microscopy, confocal microscopy, video microscopy, enzyme-linked immuno-sorbant assay (ELISA), radio-immunoassay (RIA), immuno-radiometric assay (IRMA), gel exclusion chromatography, affinity chromatography, histochemical analysis, immuno-chemical analysis, polymerase chain reaction, fluorescence in-situ hybridization, Southern Blotting, Western Blotting, laser-scanning cytometry, turbidity measurement, aggregometry, intra-cellular ion flux measurement, extracellular ion flux measurement, measurement of cellular releasates, measurement of solid-phase-stimulus/platelet/leukocyte aggregate size, measurement of rate of formation of solid-phase-stimulus/platelet/leukocyte complexes, and latex bead agglutination.

21. The method of claim 2, wherein the whole blood or blood-derived sample is obtained from a mammal that is undergoing or about to undergo a course of treatment with a therapeutic agent which affects platelet/leukocyte interaction and the method further comprises determining the extent of platelet/leukocyte interaction of a plurality of combined suspensions, each comprising a sample obtained from a mammal at a preset time interval prior to or during the course of treatment in order to assess platelet/leukocyte interaction during treatment and thereby monitor efficacy of treatment.

22. The method of claim 2, further comprising, during said contacting step, combining said whole blood or blood-derived sample and solid-phase stimulus with one or more agents that affect platelet/leukocyte interaction for a selected period of time, and determining extent of platelet/leukocyte interaction prior to and after addition of said one or more agents.

23. A platelet/leukocyte interaction assay reagent, comprising:

a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood

platelets, wherein said first ligand and said second ligand can be the same or different; and

a leukocyte marker compound.

24. The platelet/leukocyte interaction assay reagent of claim 23, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

25. The platelet/leukocyte interaction assay reagent of claim 24, wherein said first ligand is von Willebrand factor or an active fragment thereof.

26. The platelet/leukocyte interaction assay reagent of claim 23, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

27. The platelet/leukocyte interaction assay reagent of claim 26, wherein said second ligand is von Willebrand factor or an active fragment thereof.

28. The platelet/leukocyte interaction assay reagent of claim 23, wherein said first ligand and said second ligand are the same.

29. The platelet/leukocyte interaction assay reagent of claim 28, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

30. The platelet/leukocyte interaction assay reagent of claim 23, wherein said first ligand and said second ligand are different from one another.

31. The platelet/leukocyte interaction assay reagent of claim 30, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

32. A platelet/leukocyte interaction assay reagent, comprising:

a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second

ligand having an affinity for direct interaction with leukocytes.

33. The platelet/leukocyte interaction assay reagent of claim 32, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

34. The platelet/leukocyte interaction assay reagent of claim 32, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

35. The platelet/leukocyte interaction assay reagent of claim 32, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

36. The platelet/leukocyte interaction assay reagent of claim 35, wherein said reagent further comprises a leukocyte marker compound.

37. The platelet/leukocyte interaction assay reagent of claim 36, wherein said leukocyte marker compound is a fluorescent compound.

38. A platelet/leukocyte interaction assay method, comprising:

contacting a whole blood or blood-derived sample with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood platelets, wherein said first ligand and said second ligand can be the same or different; and a leukocyte marker compound; and

monitoring movement of the magnetic particles in response to the oscillating or rotating magnetic field, to determine the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

39. The method of claim 38, wherein said sample is whole blood.

40. The method of claim 38, wherein said first ligand is a member selected from the

group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

41. The method of claim 40, wherein said first ligand is von Willebrand factor or an active fragment thereof.

42. The method of claim 38, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

43. The method of claim 42, wherein said second ligand is von Willebrand factor or an active fragment thereof.

44. The method of claim 38, wherein said first ligand and said second ligand are the same.

45. The method of claim 44, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

46. The method of claim 38, wherein said first ligand and said second ligand are different from one another.

47. The method of claim 46, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

48. The method of claim 38, wherein said contacting occurs in the presence of a rotating magnetic field.

49. The method of claim 48, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

50. A platelet/leukocyte interaction assay method, comprising:

contacting a whole blood or blood-derived sample with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other

of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with leukocytes; and

detecting the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

51. The method of claim 50, wherein said sample is whole blood.

52. The method of claim 50, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

53. The method of claim 52, wherein said first ligand is von Willebrand factor or an active fragment thereof.

54. The method of claim 50, wherein said second ligand is a member selected from the group consisting of leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin.

55. The method of claim 50, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

56. The method of claim 50, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

57. The method of claim 56, wherein said platelet/leukocyte interaction reagent further comprises a leukocyte marker compound.

58. The method of claim 57, wherein said leukocyte marker compound is a fluorescent marker compound.

59. The method of claim 55, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

60. The method of claim 56, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

61. The method of claim 57, wherein said detecting is performed by detection or quantitation, or both, of differences in concentration of said leukocyte marker compound in said sample after coagulation of said sample.

62. The method of claim 50, wherein said contacting occurs in the presence of a rotating magnetic field.

63. The method of claim 62, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

64. A method for detecting the presence of a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample, obtained from a subject suspected of having a condition causing platelet/leukocyte interaction, with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood platelets, wherein said first ligand and said second ligand can be the same or different; and a leukocyte marker compound; and

monitoring movement of the magnetic particles in response to the oscillating or rotating magnetic field, to determine the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

65. The method of claim 64, wherein said sample is whole blood.

66. The method of claim 64, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

67. The method of claim 66, wherein said first ligand is von Willebrand factor or an active fragment thereof.

68. The method of claim 64, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

69. The method of claim 68, wherein said second ligand is von Willebrand factor or an active fragment thereof.

70. The method of claim 64, wherein said first ligand and said second ligand are the same.

71. The method of claim 70, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

72. The method of claim 64, wherein said first ligand and said second ligand are different from one another.

73. The method of claim 72, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

74. The method of claim 64, wherein said contacting occurs in the presence of a rotating magnetic field.

75. The method of claim 74, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

76. The method of claim 64, wherein said condition causing platelet/leukocyte interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute myocardial infarction (AMI), and inflammation.

77. A method for detecting the presence of a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample, obtained from a subject suspected of having a condition causing platelet/leukocyte interaction, with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with leukocytes; and

detecting the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

78. The method of claim 77, wherein said sample is whole blood.

79. The method of claim 77, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

80. The method of claim 79, wherein said first ligand is von Willebrand factor or an active fragment thereof.

81. The method of claim 77, wherein said second ligand is a member selected from the group consisting of leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin.

82. The method of claim 77, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

83. The method of claim 77, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

84. The method of claim 83, wherein said platelet/leukocyte interaction reagent further comprises a leukocyte marker compound.

85. The method of claim 84, wherein said leukocyte marker compound is a fluorescent marker compound.

86. The method of claim 82, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

87. The method of claim 83, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

88. The method of claim 84, wherein said detecting is performed by detection or quantitation, or both, of differences in concentration of said leukocyte marker compound in said sample after coagulation of said sample.

89. The method of claim 77, wherein said contacting occurs in the presence of a rotating magnetic field.

90. The method of claim 89, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

91. The method of claim 77, wherein said condition causing platelet/leukocyte

interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute myocardial infarction (AMI), and inflammation.

92. A method for determining whether a subject has a predisposition for a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample obtained from the subject with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood platelets, wherein said first ligand and said second ligand can be the same or different; and a leukocyte marker compound; and

monitoring movement of the magnetic particles in response to the oscillating or rotating magnetic field, to determine the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

93. The method of claim 92, wherein said sample is whole blood.

94. The method of claim 92, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

95. The method of claim 94, wherein said first ligand is von Willebrand factor or an active fragment thereof.

96. The method of claim 92, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

97. The method of claim 96, wherein said second ligand is von Willebrand factor or an active fragment thereof.

98. The method of claim 92, wherein said first ligand and said second ligand are the same.

99. The method of claim 98, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

100. The method of claim 92, wherein said first ligand and said second ligand are different from one another.

101. The method of claim 100, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

102. The method of claim 92, wherein said contacting occurs in the presence of a rotating magnetic field.

103. The method of claim 102, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

104. The method of claim 92, wherein said condition causing platelet/leukocyte interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute myocardial infarction (AMI), and inflammation.

105. A method for detecting the presence of a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample, obtained from a subject suspected of having a condition causing platelet/leukocyte interaction, with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with leukocytes; and

detecting the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

106. The method of claim 105, wherein said sample is whole blood.

107. The method of claim 105, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor

V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

108. The method of claim 107, wherein said first ligand is von Willebrand factor or an active fragment thereof.

109. The method of claim 105, wherein said second ligand is a member selected from the group consisting of leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin.

110. The method of claim 105, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

111. The method of claim 105, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

112. The method of claim 111, wherein said platelet/leukocyte interaction reagent further comprises a leukocyte marker compound.

113. The method of claim 112, wherein said leukocyte marker compound is a fluorescent marker compound.

114. The method of claim 110, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

115. The method of claim 111, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

116. The method of claim 112, wherein said detecting is performed by detection or quantitation, or both, of differences in concentration of said leukocyte marker compound in said sample after coagulation of said sample.

117. The method of claim 105, wherein said contacting occurs in the presence of a rotating magnetic field.

118. The method of claim 117, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

119. The method of claim 105, wherein said condition causing platelet/leukocyte interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute

myocardial infarction (AMI), and inflammation.

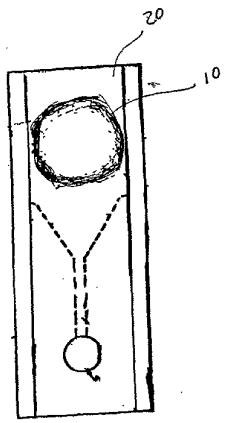

Fig. 1a

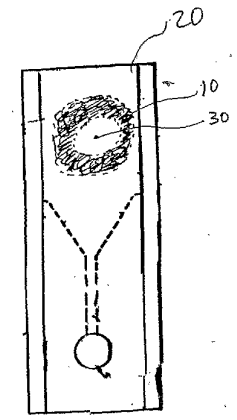

Fig. 1b

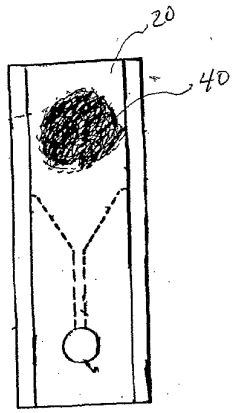

Fig. 1c

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(54) Title: PLATELET/LEUKOCYTE INTERACTION ASSAY AND REAGENT THEREFOR

(57) Abstract: A platelet/leukocyte interaction assay method and reagent therefor are provide using the presence of a solid-phase stimulus, such as magnetic or non-magnetic particles or mixtures thereof, having bound to the surface thereof one or more ligands that interact directly with platelets, leukocytes or both, for providing a fast, reliable point-of-care assessment of platelet/leukocyte interaction.

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(54) Title: PLATELET/LEUKOCYTE INTERACTION ASSAY AND REAGENT THEREFOR

(57) Abstract: A platelet/leukocyte interaction assay method and reagent therefor are provide using the presence of a solid-phase stimulus, such as magnetic or non-magnetic particles or mixtures thereof, having bound to the surface thereof one or more ligands that interact directly with platelets, leukocytes or both, for providing a fast, reliable point-of-care assessment of platelet/leukocyte interaction.

PLATELET/LEUKOCYTE INTERACTION ASSAY AND REAGENT THEREFOR

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a platelet/leukocyte interaction assay allowing for point of care assessment of interaction of platelets and leukocytes, and the reagents therefor.

Discussion of the Background

Platelets are known to interact with leukocytes both as a consequence of contact during normal blood flow (Stone and Nash, *British Journal of Haematology*, **105**:514-22, 1999; Lorenz et al, *Blood Coagulation and Fibrinolysis*, **9**:S49-59, 1998) and as a consequence of various pathological processes (Rinder et al, *Journal of Cardiovascular Surgery*, **118**:460-6, 1999; Peyton et al, *Journal of Vascular Surgery*, **27**:1109-15, 1998; Stuard et al, *International Journal of Artificial Organs*, **21**:75-82, 1998; Gawaz et al, *European Journal of Clinical Investigation*, **25**:843-51, 1995).

Pathological conditions such as Unstable Angina, Coronary Artery Disease (CAD), and Stroke are characterized by high levels of platelet and leukocyte activity. Measurement of platelet/leukocyte interaction can be predictive of these pathological states, particularly in combination with other diagnostic factors. Also, measurement of platelet/leukocyte interaction can be used as a means of monitoring therapy directed toward altering platelet and/or leukocyte function.

Exposure of flowing blood to artificial surfaces has been shown to enhance platelet/leukocyte interaction. The cell types involved and the extent of the interaction vary with the composition of the artificial surface in contact with the blood (Gawaz et al, *Artificial Organs*, **23**:29-36, 1999).

Although platelet/leukocyte interactions have been quantified using various techniques (Hendricks et al, US patent 5503982; Rinder et al, *Blood*, **78**:1760, 1991;), assessment of the interaction has relied upon measurement of *circulating* platelet/leukocyte complexes. Measurements, to date, have taken the form of evaluating pre-existing platelet/leukocyte interactions in a blood sample.

In some pathological conditions (e.g. Acute Myocardial Infarction, AMI; post Angioplasty, PTCA; etc.) platelet/leukocyte complex formation is associated with interaction with damaged subendothelium, whether directly (plaque formation) or indirectly (release of biochemical markers such as ICAM-1, see Hendricks et al, US patent 5503982).

Current assay systems used to assess platelet/leukocyte interactions, as exemplified by Hendricks et al (US patent 5503982), evaluate pre-existing (circulating) platelet/leukocyte complexes and do not utilize a component representative of the vessel subendothelium (i.e. extracellular matrix) or other solid-phase stimulus. Moreover, the threshold at which discrete platelets and leukocytes interact could vary depending on the activation status of these cells at the time of testing. It is known in the art that platelet and/or leukocyte activation is a necessary prerequisite of platelet/leukocyte binding. It is also known in the art that certain pathological conditions are associated with upregulation of platelet and/or leukocyte activity. However, the upregulation in cellular activity associated with the pathological process may be insufficient to support platelet/leukocyte complex formation without additional stimulation and moreover, may not be detectable using conventional systems, which lack a stabilizing solid-phase support upon which the platelet/leukocyte complex could be maintained. [A solid-phase stimulus could be used as a means of localizing pre-existing platelet/leukocyte complexes and/or inducing complex formation and localization in cells predisposed to do so.]

It is desirable that assay systems designed to incorporate the use of a solid-phase component such as immobilized subendothelial/extracellular matrix be facile, rapid and of reasonable cost to be useful in detecting platelet/leukocyte interaction in a clinical setting.

The present invention addresses shortcomings of previous methods and technologies by using microparticles of various compositions coated with plasma proteins and/or extracellular matrix proteins, either singly or in combination, to facilitate rapid assessment of platelet/leukocyte binding.

Platelets can interact with leukocytes through various mechanisms, such as contact during normal blood flow (Lorenz et al, *Blood Coagulation and Fibrinolysis*, **9**:S49-S59, 1998), or as a consequence of a pathological process associated with platelet hyperactivity (Spangenberg, *Thrombosis Research*, **74**:S35-S44, 1994; Rinder et al, *Journal of Cardiovascular Surgery*, **118**:460-6, 1999) or due to an inflammatory process (Gawaz et al, *European Journal of Clinical Investigation*, **25**:843-51, 1995). Receptors found on the platelet surface interact with receptors

found on various leukocytes through direct bridging or through an indirect linkage involving intermediary molecules (Weber and Springer, *Journal of Clinical Investigation*, **100**:2085-93, 1997). Upregulation of platelet and/or leukocyte activity favors enhanced platelet/leukocyte interaction (Rinder et al, 1999; Stone and Nash, *British Journal of Haematology*, **105**:514-22, 1999; Konstantopoulos et al, 1998; Gawaz et al, 1995; Spanenberg, 1994).

Individuals with Coronary Artery Disease (CAD), Diabetes or Cerebrovascular Ischemia demonstrate both platelet hyperactivity and an ongoing inflammatory process (Michelson and Furman, *Current Opinion in Hematology*, **6**:342-8, 1999). Treatment of patients with CAD has involved the use of anti-platelet agents and anti-inflammatory medications (Vorchheimer et al, *JAMA* **281**:1407-14, 1999; Mannaioni et al, *Inflammation Research*, **46**:4-18, 1997).

Platelet/monocyte (Hendricks et al, US patent 5503982) and platelet/neutrophil (Gawaz et al, *European Journal of Clinical Investigation*, **25**:843-51, 1995) interaction have been suggested to be predictive of acute myocardial infarction (AMI) and inflammation, respectively. A corollary to platelet/leukocyte interaction in various pathological conditions is involvement of the vessel wall, whether in plaque formation for example, or a localized inflammatory reaction.

Platelet function assessment using immobilized extracellular matrix proteins has been described by Shaw and Stewart (US patent 5,427,913). The authors demonstrated that von Willebrand factor (VWF) immobilized on polystyrene beads could be used to activate platelets and thereby determine the functional status of platelets from patients with platelet function defects. In addition, the authors also demonstrated that the effects of agents designed to alter platelet function could be monitored using bead-immobilized VWF as a stimulus. The results of these studies underline the importance of evaluating a normal hematological interaction or the hematologic consequence of a pathological state in the presence of an agent that mimics components of the vessel wall.

Although Shaw and Stewart describe methods and compositions of determining platelet function, there is no description or suggestion of using their method for evaluating platelet/leukocyte interaction.

CVDI's TAS™ analyzer measures the kinetics of fibrin polymerization following activation of the coagulation pathway in a patient's blood sample. The TAS™ analyzer and disposable were designed for use with whole blood in a point-of-care setting. Paramagnetic iron

oxide particles (PIOP) are an essential component of the detection system for each of the tests developed for the TAS™ analyzer. The PIOP and other lyophilized ingredients for a particular test are located in the shallow reaction chamber of the TAS test card disposable. In addition to PIOP, the test reagent may contain buffers, stabilizers, fillers and specific coagulation pathway activator or agents. A test is initiated by insertion of a dry-chemistry test card into a slot of the TAS™ analyzer that automatically positions the test card reaction chamber above an electromagnet. This chamber is also illuminated with infrared light from a light emitting diode. The instrument measures reflected infrared light from the surface of the test card by means of a solid state photodiode detector. A test is automatically initiated when the analyzer photodetector measures a change in reflected light intensity when blood or plasma is added to the sample well of the test card and, through capillary action, is pulled into the reaction chamber. The activators present in the reaction chambers stimulate the coagulation cascade in the patient's sample to produce thrombin, which in turn catalyzes the formation of the fibrin clot.

During a clotting test the TAS™ analyzer electromagnet oscillates on and off every second. The magnetic particles stand up when the electromagnet is on, causing more light to be reflected to the detector, and fall down when it is off, causing less light to be detected. This movement of PIOP produces an alternating current (AC) signal from the photodetector. As the test proceeds, more and more fibrin polymerization occurs and the PIOP movement is less. The analyzer in accordance with predetermined algorithms interprets the signal produced by the relative movement of the PIOP and reports an endpoint (clotting time) appropriate for each test.

Although PIOP is an integral component of the TAS™ detection system, it does not participate directly in activation of the coagulation cascade or fibrin polymerization. To prevent undesired interactions between PIOP and activators within the reaction chamber of a test card, the PIOP is coated or blocked with bovine serum albumin (BSA). BSA is a protein commonly used by those skilled in assay development to prevent unwanted interactions between surface components of a test and its active ingredients. The TAS™ system was designed to monitor fibrin polymerization and not interactions between platelets and leukocytes.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide an assay for platelet/leukocyte interaction that can be used with a variety of blood products as the sample,

including whole blood, with ease of detection.

A further object of the present invention is to provide an assay for platelet/leukocyte interaction that can be used on the TAS™ system for rapid determination of platelet/leukocyte interactions.

A further object of the present invention is to provide a platelet/leukocyte interaction assay reagent, preferably in a dry chemistry test card format that can be used in the assay of the present invention.

A further object of the present invention is to provide a method for diagnosis of conditions resulting in platelet/leukocyte interactions using the assay of the present invention.

A further object of the present invention is to provide a method for determination of platelet and/or leukocyte hyperactivity using the assay of the present invention.

These and other objects of the present invention have been satisfied by the discovery of a method of assessing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample with a solid-phase stimulus, wherein said solid-phase stimulus has bound to a surface thereof a ligand selective for binding platelets or leukocytes, and

detecting formation of one or more platelet/leukocyte/solid-phase stimulus complexes, and the reagents for performing the method, as well as the use of the method for detection of the occurrence of platelet/leukocyte interaction caused by various disease conditions and the predisposition of subjects to those conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein;

Fig. 1a is a representation of the PIOP ring formed during the assay of the present invention using a rotating magnetic field.

Fig. 1b is a representation of the PIOP ring formed during the assay as it begins to collapse toward the center point of the ring.

Fig. 1c is a representation of the disc or dot formed from complete collapse of the

PIOP ring in the presence of a rotating magnetic field.

Fig. 2 is a photomicrograph showing the formation of a platelet/leukocyte/microparticle complex.

Fig. 3 is a tabular representation of count data obtained in the Examples described below.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to a platelet/leukocyte interaction assay and a reagent for use in the assay. The assay of the present invention monitors interaction between platelets and leukocytes in the blood or blood-derived sample. The assay can be performed either in a wet chemistry format or a dry chemistry format.

Within the context of the present invention, the term "leukocyte" and forms thereof is to be given its normal medical meaning. Leukocytes include, but are not limited to, granulocytes, lymphocytes and monocytes. Within the granulocyte subgroup, there are neutrophils, basophils and eosinophils. The present invention most preferably is used to detect interaction of platelets with granulocytes and monocytes. However, platelet/leukocyte interactions of all types are also within the scope of the present invention assay.

The present invention relates to methods and compositions for evaluating and quantifying platelet/leukocyte interaction in the presence of a solid phase stimulus, in suspension. The solid phase stimulus of the present invention would consist of plasma proteins and/or extracellular matrix proteins, or fragments thereof, in single or in combination immobilized on microparticles. These proteins could be attached to the microparticles passively or through a covalent linkage and/or through bridging molecules. The microparticles may be of a single type or, in certain embodiments, can include two or more differing types of microparticles.

In a preferred embodiment of the invention the proteins used to coat the solid phase support would be chosen from the group of von Willebrand factor, fibrinogen, collagen, fibronectin, vitronectin, thrombospondin, laminin, osteopontin, coagulation factors (in their active or inactive forms), fibrillin, chondroitin sulfate, or heparin sulfate. These proteins or fragments thereof, would be immobilized onto the solid phase support either in single or in combination, either passively or through a covalent linkage. Attachment of the proteins to the solid-phase particulate support could also be accomplished using spacer molecules, as

would be readily apparent to those skilled in the art.

The solid phase stimulus would be mixed in suspension with a source of platelets and leukocytes for a given period of time under defined force conditions. The force conditions could be such that a range of reactivities could be possible; from simple contact of the platelet/leukocyte complexes with the solid-phase stimulus to effect binding of the complexes, through to activation of the platelets and/or leukocytes under high force conditions (e.g. high shear or turbulent flow) to induce platelet/leukocyte complex formation on the suspended solid-phase stimulus. The mixing conditions could take the form of stirring, shaking, aspiration, application of electromagnetic fields and/or beams, ultrasound, or the application of shear through the use of an apparatus such as a cone-plate viscometer or flow of the suspension through conduits of predefined dimensions. The conduits could take the form of glass or plastic tubing; channels formed in microchips or blood vessels derived from mammals, or conduits on reaction test cards, such as those described in Oberhardt, U.S. Patent 5,110,727, hereby incorporated by reference. The assay sample is typically a suspension containing the platelets and leukocytes and is blood or a blood derived sample. The sample can preferably be whole blood from a finger stick, diluted whole blood, anticoagulated whole blood, washed cells, buffy-coat or platelet rich plasma. The platelet/leukocyte suspensions could be obtained either directly from a subject to be tested or from blood products that have been stored for research or transfusion purposes, originally collected from the subject.

In a preferred embodiment of the invention, the subject is a mammal, most preferably a human.

Evaluation of the interaction between the platelets and leukocytes comprises attachment of the cells to the solid-phase support through the application of a predefined set of force conditions, such that either qualitative and/or quantitative analysis of the interaction can be accomplished.

Qualitative analysis can be performed by any method capable of detecting the platelet/leukocyte/solid-phase support complexes formed during the assay. Suitable examples of such methods include, but are not limited to, macroscopic examination (by eye), microscopy, photo-microscopy, electron microscopy (transmission or scanning), confocal microscopy or video microscopy. Qualitative analysis could alternatively (or concomitantly)

take the form of histochemical analysis, immuno-histochemical analysis, genetic analysis (PCR, FISH, Southern Blotting) or Western Blotting.

The assay can also be used for quantitative or semi-quantitative determination of the platelet-leukocyte interaction. Such analysis can be performed using any method capable of detecting and counting the number of platelet/leukocyte/solid-phase support complexes present in the assayed sample. Suitable quantitative or semi-quantitative analysis methods include, but are not limited to, cell counting, flow cytometry, static cytometry, laser-scanning cytometry, turbidity measurement, absorbance measurement, colorimetric measurement, enzyme-linked immunosorbent assay (ELISA), radio-immunoassay, immunoradiometric assay, gel exclusion chromatography, affinity chromatography, intra-cellular or extra-cellular ion flux measurement, measurement of cellular releasates, measurement of solid-phase/platelet/leukocyte aggregate size, or latex agglutination assays. Preferred methods for such quantitative determination are cell counting and cytometry (both flow and static). Suitable cytometers include, but are not limited to, the ONCYTE® and LSC® static cytometers available from CompuCyte, the FACSCan® and FACSCalibur® flow cytometers available from Becton Dickinson and the EXCEL® flow cytometer available from Coulter.

Qualitative or quantitative assessment of platelet/leukocyte interaction can be performed in combination with other assays providing the user with information to allow a more definitive identification of a pathological condition and/or the appropriate treatment regimen. As an example an individual may wish to determine platelet function (such as described in Mahan et al, U.S. Provisional Application 60/202,638, hereby incorporated by reference; or by a conventional platelet function test), and cardiac marker enzymes in conjunction with assessment of platelet/leukocyte interactions to more clearly define the occurrence of AMI. Such combinations of assays could be constructed to run concurrently or simultaneously on a single device. Other combinations of desirable assays for defined pathological conditions are readily evident to those skilled in the art and should not be limited to those mentioned, herein.

The solid-phase stimulus of the present invention provides a means of localizing platelet/leukocyte conjugates enabling rapid assessment of the degree of platelet/leukocyte interaction. The solid-phase stimulus can also induce platelet and/or leukocyte activation facilitating platelet/leukocyte complex formation on the solid-phase stimulus.

In a preferred embodiment of the invention, the particles are coated with a source of plasma protein(s), or fragments thereof. Exemplary plasma proteins include, but are not limited to, von Willebrand factor, fibrinogen, fibronectin, or blood coagulation factors (in their active or inactive, i.e. zymogen forms), such as Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate and combinations thereof.

In a preferred embodiment of the invention the particles are coated with extracellular matrix protein(s), or fragments thereof, in single or in combination. Exemplary extracellular matrix proteins include, but are not limited to, von Willebrand factor, fibronectin, collagen, osteopontin, laminin, thrombospondin, fibrillin, chondroitin sulfate, or heparin sulfate or combinations thereof.

It is also possible to coat some of the particles with a leukocyte binding ligand, such as leukocyte selective antibodies, or proteins or fragments thereof that bind to leukocyte membrane components. Examples of such proteins include VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen, osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin and E-selectin.

The microparticles to which the proteins are coated to form the solid phase stimulus can be any shape. In particular, they can take the form of microspheres or irregularly shaped microparticles. The microspheres or irregularly-shaped microparticles can be made of any material to which it is capable to bind, either directly or indirectly, one or more of the above-noted proteins or fragments thereof. These microspheres or irregularly-shaped microparticles can be any desired particle size, preferably on the same order of size as the PIOP described below, more preferably having a particle size of from 1 to 20 microns. Preferred examples of materials comprising the microspheres or irregularly-shaped microparticles include, but are not limited to, polystyrene and/or latex, polycarbonate, acrylonitrile, carboxylate, teflon, glass, nylon, dextran, agarose, acrylamide, silica, pollen, micro-organisms (viable or non-viable), iron oxide, paramagnetic iron oxide, paramagnetic particles, non-magnetic metal beads, gold, platinum, or palladium. Most preferred are the polystyrene beads as described by Stewart et al, *British J. Haematology*, **97**, 321-329 (1997) and Shaw et al, U.S. Patent

5,952,184, each of which are hereby incorporated by reference. The microspheres or irregularly shaped microparticles could also be paramagnetic in nature and/or fluorescently tagged and/or tagged with an enzyme suitable to elicit a light or color reaction in the presence of the appropriate substrate and/or chemicals.

In one preferred embodiment of the invention the microparticles comprise polystyrene, are spherical in nature, and are coated with von Willebrand factor of human origin.

In a most preferred embodiment of the invention, the von Willebrand factor coated microspheres are mixed with whole blood (unanticoagulated or anticoagulated) for a predefined length of time under predefined force conditions, sufficient to capture platelet/leukocyte complexes or to induce platelet/leukocyte complex formation on the von Willebrand factor coated microspheres, following which the existence and/or amount of such complex formation is determined.

In a further preferred embodiment, the assay of the present invention is run using a reagent comprising two central reagent elements. The first is magnetic particles, preferably paramagnetic iron oxide particles (PIOP), such as those currently employed in the TAS™ analyzer (described in U.S. Patents 4,849,340; 5,110,727; 5,350,676; 5,601,991; 5,670,329; and 5,677,233, each of which is hereby incorporated by reference), which have been modified by binding a ligand to their surface that can either (1) interact directly with the leukocytes or (2) interact with the blood platelets. While a variety of magnetic particles can be used, as described in U.S. Patent 5,110,727, the preferred magnetic particles are PIOP. Accordingly, the following description will refer to PIOP for convenience, but it is to be understood that unless otherwise indicated, the term PIOP can refer to any magnetic particles type. The PIOP, as in the conventional TAS applications, also plays a central role in the assay monitoring and detection system, whereby movement of the modified PIOP in response to a moving magnetic field is monitored to determine the endpoint of the assay.

The second central reagent element in this preferred embodiment is non-magnetic beads or microspheres coated with a ligand that can interact directly with blood platelets. These non-magnetic beads or microspheres can be any desired particle size, preferably on the same order of size as the PIOP, more preferably having a particle size of from 1 to 20 microns. The non-magnetic beads can be made from any non-magnetic material that is

capable of having a ligand bound to its surface. Preferred materials for preparation of the non-magnetic beads include, but are not limited to, those listed above for the microspheres or irregularly-shaped particles, such as polystyrene beads, polyolefin beads, glass beads, and even non-magnetic metal beads. Most preferred are the polystyrene beads as described by Stewart et al, *British J. Haematology*, **97**, 321-329 (1997) and Shaw et al, U.S. Patent 5,952,184, each of which are hereby incorporated by reference. Other reagents known to those skilled in the art to enhance functional stability, reagent drying and material rehydration on sample addition may also be added to the test formulation, such as anticoagulants, buffers, etc, and as described in the Oberhardt, and Shaw patents described herein.

The ligand can be bound to the particles directly or indirectly through a spacer, so long as the activity of the ligand is not impaired. The direct binding can occur either covalently or non-covalently. Indirect binding can occur through spacers, including but not limited to peptide spacers, antibody spacers or carbohydrate spacers. These spacers normally act only as bridges between the particle and the ligand, but could be used in order to alter the effectiveness of the ligand/receptor interaction. For example, coupling vWf to the particle through a 7 amino acid peptide bridge could decrease the interaction of vWf with the platelet receptor. However, use of the active segment of vWf, coupled to the particle through the same 7 amino acid peptide bridge could result in upregulation of the vWf fragment/receptor interaction. Similar enhancements of other types have been seen by Beer et al, *Blood*, **79**, 117-128 (1992).

In one embodiment of the present invention assay, the reagent can contain only the magnetic particles when the oscillating magnetic field is used. However, as described below, when the rotating field is used, a preferred reagent contains both magnetic and non-magnetic particles having ligands bound to both types of particles.

The ligand that interacts with blood platelets on the non-magnetic particles of this embodiment can be any compound capable of performing that function, that results in activation of the platelets. Suitable ligands include, but are not limited to, von Willebrand factor (vWf), collagen and thrombin, as well as fragments (also known as mimetopes, such as those described by Miller, U.S. Patent 5,877,155, hereby incorporated by reference) thereof. Most preferred is the use of von Willebrand factor or a fragment thereof as the ligand.

The ligand used on the non-magnetic particles and the magnetic particles can be the

same ligand or different ligands. When the ligand is the same on both types of particles, it is further necessary to provide a leukocyte marker in the reagent. The leukocyte marker can be any conventional marker known to identify the presence of leukocytes, such as flourescent markers. Examples of suitable leukocyte markers include CD45, CD18, CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18, P-selectin ligand (PSGL-1), and CD34. In the case where the ligand is the same on both types of particles, the ligand is most preferably von Willebrand factor or a fragment thereof. The ligand should be present on the surface of the magnetic and non-magnetic particles in an amount sufficient to provide binding to, and activation of, the platelets, such that a sufficient number of platelets are activated to result in an assay endpoint within a time period of from 1 to 20 minutes, preferably from 2-4 minutes. For example, in the case of the rotating magnetic field as described below, the endpoint is reached when the initially formed rotating PIOP ring collapses into a solid disc or dot.

Alternatively, the ligands bound to the two types of particles can be different. The ligands can both be ligands that interact directly with platelets, while being different from one another. In such case, the same type of leukocyte marker would be needed as noted above. However, in a further embodiment, one ligand can be a ligand that interacts directly (and selectively) with platelets, while the other ligand interacts directly (and selectively) with leukocytes. Examples of such leukocyte selective ligands include leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin (all unlabeled or labeled with a fluorescent tag such as FITC or phycoerythrin). In such a case, collapse of the rotating PIOP ring would only occur through formation of the platelet/leukocyte/particles complex, thus signalling the interaction of platelets and leukocytes. While this provides a qualitative determination of platelet/leukocyte interaction, this embodiment can also provide a quantitative determination of the interaction by use of a leukocyte marker such as described above or by comparison and correlation of the collapse time of the PIOP ring with one or more standards having known platelet/leukocyte interaction activities.

In the assay of the present invention, platelet/leukocyte interaction will be observed when there is either platelet hyperactivity or leukocyte hyperactivity. However, when both activities are normal or low, the interaction will not normally be observed. This can be

forced to occur, however, by the application of high force conditions to activate the platelets and/or leukocytes, as described above. High force conditions can be used to determine the susceptibility of a subject for platelet/leukocyte interaction and hence can provide a diagnostic tool for determining propensity or predisposition to the various disease states involving such interactions, as noted above, particularly when combined with other diagnostic tools for the specific disease state.

The assay of the present invention can be either in a wet chemistry or dry chemistry format. In either format, the test can be performed on a relatively flat reaction surface, preferably in a reaction slide such as that described in the above-noted Oberhardt U.S. Patents. Most preferably, the assay is performed in a dry chemistry format using a reaction slide or card as described in U.S. Patent 5,110,727, the contents of which are hereby incorporated by reference. The present assay can also be adapted for use in disposables with reagent chambers of any desired size and shape.

To perform a preferred embodiment of the platelet/leukocyte interaction assay of the present invention, it is necessary to place the reaction chamber into an oscillating magnetic field, such as that described by U.S. Patent 5,110,727 (previously incorporated by reference) or into a rotating magnetic field, such as that described in U.S. Patent 5,670,329 (hereby incorporated by reference). Most preferably, the assay is performed in the presence of the rotating magnetic field. The magnet (either oscillating field type or rotating field type) must be designed such that the field can influence substantially all of the PIOP present in the reaction chamber. In the preferred example of the rotating magnetic field, when the reaction chamber is that present in a TAS test card, the separation between the magnetic poles may range from 0.5 to 2.5 cm. The magnet must be positioned sufficiently close to the reaction chamber to cause movement of PIOP when the magnetic field is rotating. The rotating magnetic field can rotate at any frequency capable of sustaining the circular movement of the magnetic particles of the system, with the rotational frequency being preferably from 2,000 to 2,500 rpm. The rotating magnetic field can be provided by the rotation of a permanent magnet about a central axis as described in U.S. Patent 5,670,329, or can be generated by sequential activation of a series of electromagnets in a circular arrangement, as also described in U.S. Patent 5,670,329.

One design for the magnet for providing a rotating magnetic field in the present assay

comprises two sets of button magnets mounted on a metal disk approximately 3.4 cm in diameter. The metal disk base is attached at its center to the shaft of a DC electric motor. Each button magnet assembly contains three readily available button magnets of approximately 1 cm in diameter. The button magnets are positioned directly opposite of one another on the metal base. On the top of each button magnet assembly is placed one-half of a second metal disk (semi-circle, half of a circle) approximately 3 mm thick with a radius of 9 mm. The straight edges of the two disks face one another and are separated by approximately 1.5 cm. The entire assembly is positioned approximately 2-4 mm beneath the reaction chamber of a test card.

The assay is initiated by addition of a whole blood or platelet rich plasma sample to a reaction chamber containing the reagents described, positioned above the magnet, preferably generating a rotating magnetic field most preferably at a rotational frequency of 2500 rpm. In the most preferred embodiment using a dry chemistry format, the reagent is re-hydrated by the sample, freeing the magnetic particles and allowing them to begin to move in response to the rotating magnetic field. In the presence of the rotating field, the magnetic particles organize as a ring or band of dark material traveling along the outer edges of the reaction area. The center of the ring initially is clear or slightly gray (i.e. contains substantially fewer PIOP). The non-magnetic particles are preferably chosen so as to be invisible to the detection system.

In a normal uninhibited sample the band of PIOP becomes smaller over a period of a few minutes and the center of the ring fills in with PIOP to form a solid dot in the center of the reaction area. The non-magnetic beads themselves are preferably not readily visible in the system and do not participate in determining an end point for the assay. The end point of the test is established by the position and mobility of the PIOP present in the reagent formulation. Motion of the PIOP in the reaction area imparted by the rotating magnetic field is required to activate the platelets through contact with the solid phase agonist (i.e. the ligand) present on both the PIOP and non-magnetic beads. The aggregation of the two solid phases can occur through platelet adhesion to the solid phase and then platelet activation leading to platelet/platelet binding (platelet aggregation), particularly in the case of using a platelet ligand on both types of particles. In such a case, the detection of platelet/leukocyte interaction can be performed by inclusion of a leukocyte marker, such as a fluorescent

marker, in the reagent mixture. If platelet/leukocyte interaction results from the assay, then the marker will be more prevalent in the aggregated particles than in the surrounding medium. If no platelet/leukocyte interaction occurs, the marker will not be present in the aggregated particles to any significant extent, compared to the surrounding medium. The level of interaction of platelets and leukocytes can be determined by quantitative measurement of the marker's presence in the aggregated particles. Aggregation does not occur in the absence of platelets or in the presence of an inhibitor.

Alternatively, when one type of particle bears the platelet ligand and the other type of particle bears the leukocyte ligand, the presence of platelet/leukocyte interaction can be observed either directly or indirectly. If the leukocyte ligand is bound to the magnetic or PIOP particles, the presence of the interaction of platelets and leukocytes can be directly observed, since collapse of the PIOP ring will not occur without such interaction. On the other hand, if the leukocyte ligand is bound to the non-magnetic particles, the collapse of the PIOP ring into the dot may still occur through platelet/platelet interaction of platelet ligand coated PIOP, albeit much more slowly. Definitive determination of platelet/leukocyte interaction is this embodiment would be best done through use of a marker, such as the fluorescent markers noted above.

In the assay of this embodiment of the present invention, once the bound ligand has interacted with the platelets and/or leukocytes, the free fibrinogen naturally in the sample interacts with the activated platelets, causing platelet/platelet aggregation. If the platelets and/or leukocytes are hyperactivated, such as in the case of a myocardial infarction or stroke victim, platelet/leukocyte interaction also occurs. As the platelets (with or without leukocytes) aggregate, this increases the effective mass of the aggregate around the PIOP, causing the heavier aggregates to migrate inward from the outer ring of particles toward the center of the rotating magnetic field. As the assay progresses, the ring eventually collapses into a circular dot, which continues to rotate about the center of the rotating field.

The end point of the assay can be monitored by using reflected infra red light similar to that of the TAS analyzer. This is possible because the area of the reaction chamber covered by the dark ring is much greater than that of the solid dot. The signal can provide both a qualitative YES/NO response and a quantitative response, as described above.

The difference in signal produced by a ring versus a dot can be enhanced if a small

spot of reflective material covers the very center of the reaction area. In this situation the dark ring of PIOP will disappear behind the reflecting material, thereby increasing the signal. Alternately the end point of the assay could be monitored by a video or infra red camera. The output of the camera can be digitized and the image subsequently analyzed to determine the formation of ring and dot structures.

Fig. 1a provides a representation of the assay of the present invention in operation using a rotating magnetic field with an assay test card such as that from U.S. Patent 5,110,727. In this Figure, the PIOP have been freed by the addition of the blood sample, resulting in the formation of a rotating ring (10) of PIOP within the reaction chamber (20).

Fig. 1b shows the rotating ring (10) of PIOP has started to collapse toward the center point (30) representing the axis of the rotating field. Fig 1c shows the endpoint of the assay in which the PIOP have completely collapsed into a dot structure (40). This endpoint is dramatic and readily detectable both instrumentally and visually. Visual detection provides a quick and easy method for obtaining the qualitative information discussed above, while instrumental detection using a system such as the TAS analyzer, provides the ability to analyze the signal obtained and provide quantitative measures of receptor blockage, preferably by comparison to a standard curve generated using samples of known platelet/leukocyte interaction levels.

When an oscillating magnetic field is used, the signal produced is analyzed in the same manner as in Oberhardt U.S. Patents 5,110,727 and 4,849,340, by analysis of the decay curve produced by monitoring the oscillation of the particles.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Preparation of vWf-coated PIOP. 10 g of Magnetite obtained from ISK Magnetics (Valparaiso, IN) was added to 90 mL of 50 mM Tris pH 7.4 in a sealed flask and purged with nitrogen for 5 minutes. The PIOP suspension was then homogenized by a model 700 PowerGen Homogenizer

(Fisher Scientific) for 5 minutes at a setting of 6 using a 7 mm diameter rotor-Stator. A 0.7 mL aliquot of the homogenized PIOP was added to 0.3 mL of approximatly 10 ug/mL vWf and allowed to incubate for 30 minutes at room temperature.

Preparation of vWf-coated polystyrene beads. Polystyrene beads (4 um) obtained from Polysciences Corporation (Warrington, Pa.) were washed three times with 0.2 mol/l carbonate buffer (pH 9.35) prior to use. vWf was diluted in 0.2 mol/l carbonate buffer (pH 9.35) to 2 U/mL (a unit of vWf is defined as the amount found in 1 mL of pooled normal plasma) and mixed with polystyrene beads pre-equilibrated in carbonate buffer prior to incubation overnight at 4°C.

Example 1

Whole blood from healthy volunteers collected into citrate as an anticoagulant was mixed with polystyrene microspheres coated with human von Willebrand factor. 100 μl of whole blood was added to 5 μl (approximately 5 x 10^5, 4.5 μm diameter) microspheres in microwells and shaken on a rotary shaker at 500 rpm for 1 to 10 minutes. Aliquots were removed from the microwells and examined by microscopy. The reaction was assessed using video microscopy, phase contrast microscopy and by differential staining on fixed smears. Leukocytes and platelets in complex were observed to be associated with the von Willebrand factor coated microspheres, only when the platelets displayed hyperactivity or when the platelets and/or leukocytes were subjected to mechanical stress such as repeated centrifugation (800 x g, 10 minutes), or shearing by forcing the blood through a 21 gauge needle several times prior to testing. Fig. 2 shows an example of the platelet/leukocyte/vWf coated polystyrene bead complex, showing the polymorphonuclear leukocyte binding to platelets bound to vWf-coated beads.

Example 2

A differential cell count was performed on heparinized or citrated whole blood from healthy volunteers using an automated cell counter. 100 μl of whole blood was added to 5 μl (approximately 5 x 10^5, 4.5 μm diameter) von Willebrand factor coated microspheres in microwells and shaken on a rotary shaker at 500 rpm for 1 to 10 minutes. A second count was then performed on the reaction suspension. Taking into account the dilution effect of the bead reagent, the decrease in the number of leukocytes was determined by calculating the ratio

between the post reaction counts and the pre-reaction counts and multiplying the result by 100 to obtain the percent decrease in leukocyte count. Concomitantly, uncoated nascent microspheres were mixed in a like manner with the anticoagulated whole blood and evaluated as described. The count data are tabulated in Fig. 3.

Leukocyte counts were seen to decrease after mixing the anticoagulated whole blood with the von Willebrand factor coated microspheres only when the platelets displayed hyperactivity or when the platelets and/or leukocytes were subjected to mechanical stress such as repeated centrifugation (800 x g, 10 minutes), or shearing by forcing the blood through a 21 gauge needle several times prior to testing. In contrast, testing with uncoated microspheres showed no decrease in leukocyte counts. Phase contrast microscopy confirmed leukocyte/platelet complex association with the von Willebrand factor coated microspheres in aggregates and no association with the uncoated microspheres. The aggregates formed by the leukocytes, platelets and von Willebrand factor coated microspheres were noted to be too large to be counted as leukocytes, by the differential cell counter.

Example 3

Study subjects (n = 3) destined for Coronary Artery Bypass Graft (CABG) surgery were tested prior to, and throughout the surgical procedure, using the method of the present invention. A differential cell count was performed on citrated whole blood at each time point (pre-surgery, on bypass, post-protamine, intensive care unit, 24 hours post-surgery) throughout the surgical procedure. In addition, 100 μl of citrated whole blood from each time point was added to microwells containing 5 μl (approximately 5 x 105, 4.5 μm diameter) von Willebrand factor coated microspheres. The microwells were shaken on a rotary shaker at 500 rpm for 1 to 10 minutes. A second differential cell count was performed on the blood from the microwells. Taking into account the dilution effect of the bead reagent, the decrease in the number of leukocytes was determined by calculating the ratio between the post reaction counts and the pre-reaction counts and multiplying the result by 100 to obtain the percent decrease in leukocyte count. All study subjects showed loss of leukocytes in the pre-surgical sample as determined by the differential cell counting technique. Microscopy confirmed platelet/leukocyte complex formation on the surface of the VWF coated microspheres. Platelet/leukocyte complex formation

in the presence of the VWF coated microspheres was noted in the blood samples collected while the patients were on bypass, however platelet/leukocyte complex formation in the presence of the VWF coated microspheres was not seen in blood samples collected 24 hours post-surgery, in any of the study subjects.

Example 4

Whole blood from a healthy volunteer was drawn into a citrate vacutainer tube, an EDTA vacutainer tube and a heparin vacutainer tube. A drop of blood from each tube was added to the reaction well of three separate reaction cards containing a mixture of VWF-coated polystyrene beads and VWF-coated paramagnetic iron oxide particles (VWF-PIOP) and the suspension mixed vigorously for 5 minutes. Aliquots of whole blood (5 μl) were removed from each card for microscopic wet-mount observation (phase contrast) and stained smear (Hema-3 stain, Fisher Scientific) evaluation. Weak platelet adhesion to the VWF beads was noted with the EDTA blood, with the vast majority of platelets left unbound. Platelets did not associate with VWF-PIOP in the EDTA blood. Both the citrate blood and the heparin blood samples showed extensive binding of platelets to VWF beads with subsequent binding of VWF-PIOP to form large complexes. Few platelets were left unbound. Binding of leukocytes to (or within) these large VWF bead/platelet/VWF-PIOP complexes was not observed either by wet mount or stained smear. Leukocytes and platelets in complex were observed to be associated with the von Willebrand factor coated microspheres and VWF-PIOP in the citrate and heparin samples only when the platelets displayed hyperactivity or when the platelets and/or leukocytes were subjected to mechanical stress such as repeated centrifugation (800 x g, 10 minutes). Mechanical stress did not augment platelet association with the VWF Beads or VWF-PIOP, nor did it promote leukocyte/platelet complex formation in the EDTA blood sample.

Preparation of test card - Into a test card, such as that in Oberhardt, U.S. Patent 5,110,727, having a reaction chamber of approximately 30 µl, is placed a reagent composition containing the above noted magnetic and non-magnetic particles coated with vWf, in amounts such that the reagent composition comprises 1-2 mg of coated PIOP particles per ml and from 2 x 106 to 8 x 106 polystyrene particles per ml. Also placed into the reaction chamber is an amount of a

leukocyte marker, such as FITC-labeled anti-CD45, sufficient to provide a detectable signal. Once the reaction chamber is filled, the sample is then frozen and lyophilized, as described for the preparation of test cards in the above noted Oberhardt patents.

However, the ratio of coated magnetic particles to coated non-magnetic particles is not limited and can be any ratio so long as there are sufficient magnetic particles to form the rotating ring and collapse to the disc or dot.

Platelet/Leukocyte Interaction Test. A disposable or test card containing the reagents described above is placed on a platform above a rotating magnet. Whole blood (or other blood-derived) sample is added to a well, which is pulled by capillary action into the reaction chamber. At that time the magnetic particles and non-magnetic particles are freed, with the magnetic particles forming a rotating ring around the central portion of the reaction chamber. As the reaction progressed, the inner edge of the rotating ring migrated toward the center, with the final endpoint providing a full collapse of the inner edge to the central point to form a disc or dot. The total time elapsed is approximately 1 to 20 minutes, typically in the 2-4 minute range.

When the above noted vWf coated PIOP and vWf coated polystyrene particles are used, the presence of platelet/leukocyte interaction is determined by detection of a leukocyte marker present in the original reagent formulation. Alternatively, if the PIOP are coated with a leukocyte ligand (instead of vWf) the occurrence of the platelet/leukocyte interaction is detected by collapse of the PIOP ring itself.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

* * * * * * *

The present application is based on U.S. Provisional Application 60/165,462, filed November 15, 1999, the entire contents of which are hereby incorporated by reference.

Claims

1. A method of assessing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample with a solid-phase stimulus, wherein said solid-phase stimulus has bound to a surface thereof a ligand selective for binding platelets or leukocytes, and

detecting formation of one or more platelet/leukocyte/solid-phase stimulus complexes.

2. A method of assessing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample with a solid-phase stimulus, wherein said solid-phase stimulus comprises microparticles having bound to a surface thereof a ligand selective for binding platelets or leukocytes, and

detecting formation of one or more platelet/leukocyte/solid-phase stimulus complexes.

3. The method of claim 2, wherein said ligand selective for binding platelets or leukocytes is a member selected from the group consisting of plasma proteins, plasma protein fragments, extracellular matrix proteins, extracellular matrix protein fragments and mixtures thereof.

4. The method of claim 2, wherein the microparticles are of irregular or regular shape or spherical.

5. The method of claim 2, wherein said whole blood or blood-derived sample is obtained from a mammal.

6. The method of claim 5, wherein said mammal is a human.

7. The method of claim 2, wherein the microparticles comprise particles made from one or more materials selected from the group consisting of polystyrene, latex, polycarbonate, acrylonitrile, carboxylate, teflon, glass, nylon, dextran, agarose, acrylamide, silica, pollen, micro-organisms, iron oxide, non-magnetic metals, paramagnetic iron oxide, gold, platinum, and palladium.

8. The method of claim 2, wherein said contacting step is performed by a process selected from the group consisting of stirring, shaking, aspiration, application of electromagnetic fields, ultrasound, shear and combinations thereof.

9. The method of claim 3, wherein said ligand selective for binding platelets or leukocytes is a member selected from the group consisting of von Willebrand factor, fibrinogen,

fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, fragments of said proteins, leukocyte selective antibodies, and combinations thereof.

10. The method of claim 2, wherein the ligands that selective bind platelets or leukocytes are attached to the microparticles covalently, through passive adsorption or through binding to bridging molecules.

11. The method of claim 3, wherein said plasma protein fragments or extracellular protein fragments are prepared by formation of peptides, formation of peptide mimetics or formation of peptide mimotopes either through recombinant technology or enzymatic cleavage or by linkage of amino acids by non-enzymatic chemical means.

12. The method of claim 2, wherein said whole blood or blood-derived sample is unanticoagulated whole blood.

13. The method of claim 2, wherein said whole blood or blood-derived sample is anticoagulated whole blood.

14. The method of claim 2, wherein said whole blood or blood-derived sample is cells contained within the buffy coat.

15. The method of claim 2, wherein said whole blood or blood-derived sample is a blood product collected for transfusion purposes.

16. The method of claim 15, wherein said blood product collected for transfusion purposes has been further subjected to one or more procedures designed to isolate specific blood components selected from the group consisting of random donor platelets, apheresis platelets, buffy coat and packed red cells.

17. The method of claim 2, wherein the whole blood or blood-derived sample is a member selected from the group consisting of unanticoagulated whole blood, anticoagulated whole blood and buffy coat, wherein the whole blood or blood-derived sample has been placed in contact with an artificial surface, *in vivo*, prior to use in the method.

18. The method of claim 2, wherein the whole blood or blood-derived sample is a member selected from the group consisting of unanticoagulated whole blood, anticoagulated whole blood and buffy coat, wherein the whole blood or blood-derived sample has been placed in contact with an artificial surface, *ex vivo*, prior to use in the method.

19. The method of claim 2, wherein the whole blood or blood-derived sample is a member selected from the group consisting of unanticoagulated whole blood, anticoagulated whole blood and buffy coat, wherein the whole blood or blood-derived sample has been placed in contact with an artificial surface, *in vitro*, prior to use in the method.

20. The method of claim 2, wherein said detecting step is performed by a method selected from the group consisting of flow cytometry, cell counting, microscopy, photo-microscopy, transmission electron microscopy, scanning electron microscopy, confocal microscopy, video microscopy, enzyme-linked immuno-sorbant assay (ELISA), radio-immunoassay (RIA), immuno-radiometric assay (IRMA), gel exclusion chromatography, affinity chromatography, histochemical analysis, immuno-chemical analysis, polymerase chain reaction, fluorescence in-situ hybridization, Southern Blotting, Western Blotting, laser-scanning cytometry, turbidity measurement, aggregometry, intra-cellular ion flux measurement, extracellular ion flux measurement, measurement of cellular releasates, measurement of solid-phase-stimulus/platelet/leukocyte aggregate size, measurement of rate of formation of solid-phase-stimulus/platelet/leukocyte complexes, and latex bead agglutination.

21. The method of claim 2, wherein the whole blood or blood-derived sample is obtained from a mammal that is undergoing or about to undergo a course of treatment with a therapeutic agent which affects platelet/leukocyte interaction and the method further comprises determining the extent of platelet/leukocyte interaction of a plurality of combined suspensions, each comprising a sample obtained from a mammal at a preset time interval prior to or during the course of treatment in order to assess platelet/leukocyte interaction during treatment and thereby monitor efficacy of treatment.

22. The method of claim 2, further comprising, during said contacting step, combining said whole blood or blood-derived sample and solid-phase stimulus with one or more agents that affect platelet/leukocyte interaction for a selected period of time, and determining extent of platelet/leukocyte interaction prior to and after addition of said one or more agents.

23. A platelet/leukocyte interaction assay reagent, comprising:

a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood

platelets, wherein said first ligand and said second ligand can be the same or different; and

a leukocyte marker compound.

24. The platelet/leukocyte interaction assay reagent of claim 23, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

25. The platelet/leukocyte interaction assay reagent of claim 24, wherein said first ligand is von Willebrand factor or an active fragment thereof.

26. The platelet/leukocyte interaction assay reagent of claim 23, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

27. The platelet/leukocyte interaction assay reagent of claim 26, wherein said second ligand is von Willebrand factor or an active fragment thereof.

28. The platelet/leukocyte interaction assay reagent of claim 23, wherein said first ligand and said second ligand are the same.

29. The platelet/leukocyte interaction assay reagent of claim 28, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

30. The platelet/leukocyte interaction assay reagent of claim 23, wherein said first ligand and said second ligand are different from one another.

31. The platelet/leukocyte interaction assay reagent of claim 30, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

32. A platelet/leukocyte interaction assay reagent, comprising:

a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second

ligand having an affinity for direct interaction with leukocytes.

33. The platelet/leukocyte interaction assay reagent of claim 32, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

34. The platelet/leukocyte interaction assay reagent of claim 32, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

35. The platelet/leukocyte interaction assay reagent of claim 32, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

36. The platelet/leukocyte interaction assay reagent of claim 35, wherein said reagent further comprises a leukocyte marker compound.

37. The platelet/leukocyte interaction assay reagent of claim 36, wherein said leukocyte marker compound is a fluorescent compound.

38. A platelet/leukocyte interaction assay method, comprising:

contacting a whole blood or blood-derived sample with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood platelets, wherein said first ligand and said second ligand can be the same or different; and a leukocyte marker compound; and

monitoring movement of the magnetic particles in response to the oscillating or rotating magnetic field, to determine the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

39. The method of claim 38, wherein said sample is whole blood.

40. The method of claim 38, wherein said first ligand is a member selected from the

group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

41. The method of claim 40, wherein said first ligand is von Willebrand factor or an active fragment thereof.

42. The method of claim 38, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

43. The method of claim 42, wherein said second ligand is von Willebrand factor or an active fragment thereof.

44. The method of claim 38, wherein said first ligand and said second ligand are the same.

45. The method of claim 44, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

46. The method of claim 38, wherein said first ligand and said second ligand are different from one another.

47. The method of claim 46, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

48. The method of claim 38, wherein said contacting occurs in the presence of a rotating magnetic field.

49. The method of claim 48, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

50. A platelet/leukocyte interaction assay method, comprising:

contacting a whole blood or blood-derived sample with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other

of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with leukocytes; and

detecting the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

51. The method of claim 50, wherein said sample is whole blood.

52. The method of claim 50, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

53. The method of claim 52, wherein said first ligand is von Willebrand factor or an active fragment thereof.

54. The method of claim 50, wherein said second ligand is a member selected from the group consisting of leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin.

55. The method of claim 50, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

56. The method of claim 50, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

57. The method of claim 56, wherein said platelet/leukocyte interaction reagent further comprises a leukocyte marker compound.

58. The method of claim 57, wherein said leukocyte marker compound is a fluorescent marker compound.

59. The method of claim 55, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

60. The method of claim 56, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

61. The method of claim 57, wherein said detecting is performed by detection or quantitation, or both, of differences in concentration of said leukocyte marker compound in said sample after coagulation of said sample.

62. The method of claim 50, wherein said contacting occurs in the presence of a rotating magnetic field.

63. The method of claim 62, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

64. A method for detecting the presence of a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample, obtained from a subject suspected of having a condition causing platelet/leukocyte interaction, with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood platelets, wherein said first ligand and said second ligand can be the same or different; and a leukocyte marker compound; and

monitoring movement of the magnetic particles in response to the oscillating or rotating magnetic field, to determine the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

65. The method of claim 64, wherein said sample is whole blood.

66. The method of claim 64, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

67. The method of claim 66, wherein said first ligand is von Willebrand factor or an active fragment thereof.

68. The method of claim 64, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

69. The method of claim 68, wherein said second ligand is von Willebrand factor or an active fragment thereof.

70. The method of claim 64, wherein said first ligand and said second ligand are the same.

71. The method of claim 70, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

72. The method of claim 64, wherein said first ligand and said second ligand are different from one another.

73. The method of claim 72, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

74. The method of claim 64, wherein said contacting occurs in the presence of a rotating magnetic field.

75. The method of claim 74, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

76. The method of claim 64, wherein said condition causing platelet/leukocyte interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute myocardial infarction (AMI), and inflammation.

77. A method for detecting the presence of a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample, obtained from a subject suspected of having a condition causing platelet/leukocyte interaction, with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with leukocytes; and

detecting the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

78. The method of claim 77, wherein said sample is whole blood.

79. The method of claim 77, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

80. The method of claim 79, wherein said first ligand is von Willebrand factor or an active fragment thereof.

81. The method of claim 77, wherein said second ligand is a member selected from the group consisting of leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin.

82. The method of claim 77, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

83. The method of claim 77, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

84. The method of claim 83, wherein said platelet/leukocyte interaction reagent further comprises a leukocyte marker compound.

85. The method of claim 84, wherein said leukocyte marker compound is a fluorescent marker compound.

86. The method of claim 82, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

87. The method of claim 83, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

88. The method of claim 84, wherein said detecting is performed by detection or quantitation, or both, of differences in concentration of said leukocyte marker compound in said sample after coagulation of said sample.

89. The method of claim 77, wherein said contacting occurs in the presence of a rotating magnetic field.

90. The method of claim 89, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

91. The method of claim 77, wherein said condition causing platelet/leukocyte

interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute myocardial infarction (AMI), and inflammation.

92. A method for determining whether a subject has a predisposition for a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample obtained from the subject with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein said magnetic particles have bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and wherein said non-magnetic particles have bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with blood platelets, wherein said first ligand and said second ligand can be the same or different; and a leukocyte marker compound; and

monitoring movement of the magnetic particles in response to the oscillating or rotating magnetic field, to determine the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

93. The method of claim 92, wherein said sample is whole blood.

94. The method of claim 92, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

95. The method of claim 94, wherein said first ligand is von Willebrand factor or an active fragment thereof.

96. The method of claim 92, wherein said second ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

97. The method of claim 96, wherein said second ligand is von Willebrand factor or an active fragment thereof.

98. The method of claim 92, wherein said first ligand and said second ligand are the same.

99. The method of claim 98, wherein said first ligand and said second ligand are each von Willebrand factor or an active fragment thereof.

100. The method of claim 92, wherein said first ligand and said second ligand are different from one another.

101. The method of claim 100, wherein one of said first ligand or said second ligand is von Willebrand factor or an active fragment thereof.

102. The method of claim 92, wherein said contacting occurs in the presence of a rotating magnetic field.

103. The method of claim 102, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

104. The method of claim 92, wherein said condition causing platelet/leukocyte interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute myocardial infarction (AMI), and inflammation.

105. A method for detecting the presence of a condition causing platelet/leukocyte interaction, comprising:

contacting a whole blood or blood-derived sample, obtained from a subject suspected of having a condition causing platelet/leukocyte interaction, with a platelet/leukocyte interaction assay reagent in the presence of an oscillating or rotating magnetic field, said platelet function assay reagent comprising a mixture of magnetic and non-magnetic particles, wherein either said magnetic particles or said non-magnetic particles has bound to an outer surface thereof an amount of a first ligand having an affinity for direct interaction with blood platelets and the other of said magnetic particles or non-magnetic particles has bound to an outer surface thereof an amount of a second ligand having an affinity for direct interaction with leukocytes; and

detecting the presence or absence of platelet/leukocyte interaction function, the level of platelet/leukocyte interaction, or both, in the whole blood or blood derived sample.

106. The method of claim 105, wherein said sample is whole blood.

107. The method of claim 105, wherein said first ligand is a member selected from the group consisting of von Willebrand factor, fibrinogen, fibronectin, Factor II, Factor IIa, Factor

V, Factor Va, Factor VIII, Factor VIIIa, Factor IX, Factor IXa, Factor X, Factor Xa, Factor XI, Factor XIa, Factor XII, Factor XIIa, Factor XIII, Factor XIIIa, collagen, vitronectin, laminin, osteopontin, fibrillin, chondroitin sulfate, heparin sulfate, and active fragments thereof.

108. The method of claim 107, wherein said first ligand is von Willebrand factor or an active fragment thereof.

109. The method of claim 105, wherein said second ligand is a member selected from the group consisting of leukocyte selective antibodies, VCAM-1, fibronectin, laminin, ICAM-1, ICAM-2, ICAM-3, collagen osteopontin, vWf, vitronectin, thrombospondin, mucosal addressin cell adhesion molecule 1 (MadCAM-1), P-selectin, L-selectin, and E-selectin.

110. The method of claim 105, wherein said first ligand is bound to said non-magnetic particles and said second ligand is bound to said magnetic particles.

111. The method of claim 105, wherein said first ligand is bound to said magnetic particles and said second ligand is bound to said non-magnetic particles.

112. The method of claim 111, wherein said platelet/leukocyte interaction reagent further comprises a leukocyte marker compound.

113. The method of claim 112, wherein said leukocyte marker compound is a fluorescent marker compound.

114. The method of claim 110, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

115. The method of claim 111, wherein said detecting is performed by monitoring movement of said magnetic particles in response to said oscillating or rotating magnetic field.

116. The method of claim 112, wherein said detecting is performed by detection or quantitation, or both, of differences in concentration of said leukocyte marker compound in said sample after coagulation of said sample.

117. The method of claim 105, wherein said contacting occurs in the presence of a rotating magnetic field.

118. The method of claim 117, wherein said rotating magnetic field is rotating at a frequency of from 2000-2500 rpm.

119. The method of claim 105, wherein said condition causing platelet/leukocyte interaction is a member selected from the group consisting of cerebral vascular accidents (CVA), transient ischemic attack (TIA), unstable angina, coronary artery disease (CAD), acute

myocardial infarction (AMI), and inflammation.

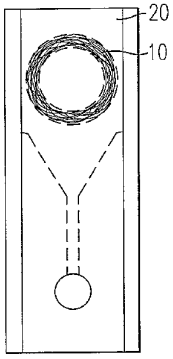

FIG. 1a

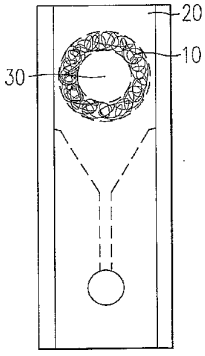

FIG. 1b

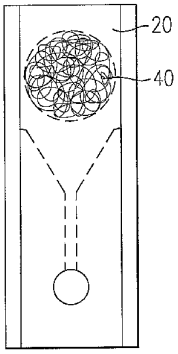

FIG. 1c

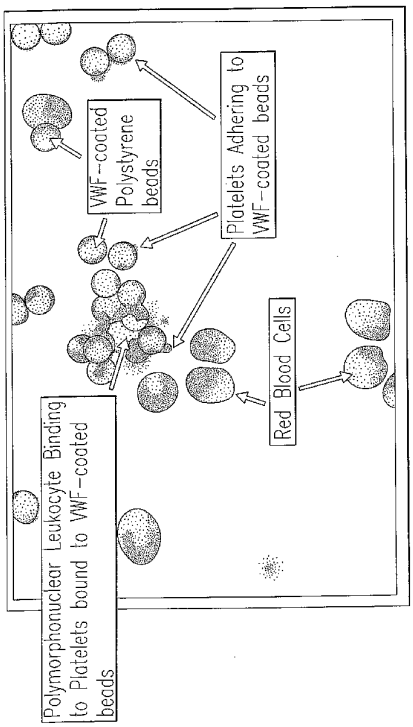
VWF-coated Polystyrene beads
Platelets Adhering to VWF-coated beads
Red Blood Cells
Polymorphonuclear Leukocyte Binding to Platelets bound to VWF-coated beads

FIG. 2

Stroke/TIA Patients–Platelet/Leukocyte Interaction

Patient	Platelets			Monocytes			Granulocytes		
	Pre	Post	%RPC	Pre	Post	%drop	Pre	Post	%drop
1	183	25	14	0.4	0.4	0	8.6	3.1	64
2	141	41	29	0.3	0.3	0	6.2	5.8	6
3	151	2	1	0.4	0.2	50	2.6	2.4	8
4	123	17	14	0.2	0.3	−50	5.1	5	2
5	148	9	6	0.2	0.3	−50	1.5	3	−100
6	239	10	4	0.3	0.3	0	3.6	3.7	−3
7	204	32	16	0.2	0.3	−50	5.8	5.5	5
8	156	4	3	0.3	0.3	0	4.6	3.5	24
9	205	14	7	0.4	0.1	75	4.8	3.8	21
10	134	14	10	0.4	0.2	50	2.9	3.2	−10
11	192	4	2	0.4	0.1	75	2.8	1.3	54
12	172	3	2	0.3	0.2	33	5.2	4.7	10
13	269	11	4	0.6	0.3	50	4.2	2.9	31
14	82	20	24	0.3	0.3	0	2.1	1.85	12
15	203	117	58	0.5	0.7	−40	4.2	3.55	15
16	164	96	59	0.4	0.45	−13	5.9	4.85	18
17	184	105	57	0.5	0.55	−10	5.7	5.95	−4
18	244	61	25	0.2	0.5	−150	5.1	6.05	−19
19	150	24	16	0.3	0.6	−100	6.2	6.5	−5
20	145	39	27	0.4	0.2	50	3	2.95	2
		Mean	18.9		#>9	7		#>9	9
		SD	18.93						
		#<30	17						
		#>30	3						

* 17 OF 20 Stroke/TIA patients tested demonstrated residual platelet counts less than 30%.
* 7 of 20 Stroke/TIA patients tested showed a decrease in monocyte counts after reacting the whole blood with VWF-coated beads
* 8 of 20 Stroke/TIA patients tested showed a decrease in granulocyte counts after reacting the whole blood with VWF-coated beads.
* 12 of 20 Stroke/TIA patients tested showed a decrease in monocyte or granulocyte counts after reacting the whole blood with VWF-coated beads.
** Of 63 control samples tested, 5 samples demonstrated a decrease in the monocyte counts after reacting the whole blood with the VWF-coated beads.
** Of 63 control samples tested, 12 samples demonstrated a decrease in the granulocyte counts after reacting with the VWF-coated beads.
** Of 63 control samples tested, 13 samples showed a decrease in either monocyte or granulocyte counts after reacting with the VWF-coated beads.
** Of the 13 control samples showing a decrease in either monocyte or granulocyte counts after reacting with the VWF-coated beads, 8 samples exhibited residual platelet counts of less than 30%.

FIG. 3

【国際調査報告】

(L)60301180177

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/42946

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : G01N 33/567
US CL. : 435/7.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.21

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,145,784 A (COX et al) 08 SEPTEMBER 1992 (08.09.1992), column 4, lines 1-20.	1-119
A	US 5,427,913 A (SHAW et al) 27 JUNE 1995 (27.06.1995), column 3, lines 4-8.	1-119
A	US 5,503,982 A (HENDRICKS et al) 02 APRIL 1996 (02.04.1996) column 5, lines 10-63.	1-119
A	EP 0 625 267 B1 (PARTON et al) 11 NOVEMBER 1998 (11.11.1998) column 1, lines 10-38 and column 3, lines 33-46.	1-119

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 March 2002 (15.03.2002)	10 May 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Long Le Telephone No. (703) 308-0196

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专利名称(译)	血小板/白细胞相互作用测定及其试剂		
公开(公告)号	JP2004537029A	公开(公告)日	2004-12-09
申请号	JP2002542324	申请日	2001-11-15
[标]申请(专利权)人(译)	PHARMANETICS		
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发明人	マーハン,ドナルド,イー. スチュワート,マイケル,ダブリュー.		
IPC分类号	G01N33/53 A61K G01N33/543 G01N33/553 G01N33/566 G01N33/567 G01N33/569 G01N33/86		
CPC分类号	G01N33/56972 G01N33/86 G01N2800/52 Y10S436/823		
FI分类号	G01N33/53.L G01N33/53.K G01N33/543.581.D G01N33/543.585 G01N33/553 G01N33/566		
代理人(译)	村田正树		
优先权	09/712165 2000-11-15 US		
外部链接	Espacenet		

摘要(译)

使用存在固相刺激物（例如磁性或非磁性颗粒或其混合物）来提供血小板/白细胞相互作用测定方法及其试剂，所述固相刺激物具有与其表面结合的一种或多种直接与血小板相互作用的配体，白细胞或两者，用于提供血小板/白细胞相互作用的快速，可靠的护理点评估。

脳卒中／ＴＩＡ患者－血小板／白血球相互作用

患者	血小板			単球			顆粒球		
	前	後	%RPC	前	後	減少%	前	後	減少%
1	183	25	14	0.4	0.4	0	8.6	3.1	64
2	141	41	29	0.3	0.3	0	6.2	5.8	6
3	151	2	1	0.4	0.2	50	2.6	2.4	8
4	123	17	14	0.2	0.3	−50	5.1	5	2
5	148	9	6	0.2	0.3	−50	1.5	3	−100
6	239	10	4	0.3	0.3	0	3.6	3.7	−3
7	204	32	16	0.2	0.3	−50	5.8	5.5	5
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9	205	14	7	0.4	0.1	75	4.8	3.8	21
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11	192	4	2	0.4	0.1	75	2.8	1.3	54
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14	82	20	24	0.3	0.3	0	2.1	1.85	12
15	203	117	58	0.5	0.7	−40	4.2	3.55	15
16	164	96	59	0.4	0.45	−13	5.9	4.85	18
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18	244	61	25	0.2	0.5	−150	5.1	6.05	−19
19	150	24	16	0.3	0.6	−100	6.2	6.5	−5
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		Mean	18.9		#>9	7		#>9	9
		SD	18.93						
		#<30	17						
		#>30	3						