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(54)【発明の名称】循環血液からの好ましくない分子の一段階除去

(57)【要約】
本発明は、一段階プロセスを利用してホストの血液から好ましくない分子を除去するための方法及びそれによって生じる生成物を提供するものである。好ましくない分子は異なるＡＢＯ血液型を有するソースから移植された器官或いは組織のホスト拒絶を生じさせるであろうところの抗Ａ血液タンパク及び／もしくはＢ血液タンパク抗体であり得る。好ましくない分子はまた罹病したホスト中に存在する過剰抗体やビリオンであり得る。

【特許請求の範囲】
【請求項１】
ホストの血液中における好ましくない分子の存在を一段階で減少させるための方法であって、
ａ）ホストから血液を抽出し；
ｂ）抽出した血液を、囲まれた経路に沿って連続的或いは一時的に断続した流れの中で移動させ、ここで前記経路は該経路に沿って付着した、好ましくない分子に対する特定の固定化結合分子を有し、該結合分子はホストの血液中の好ましくない分子に対して結合可能であり、そして
ｃ）血液をホストの内部循環系に戻す、
方法。
【請求項２】
前記の好ましくない分子は移植された器官もしくは組織中に存在する外来ドナー抗原に対する特定の抗体である請求項１の方法。
【請求項３】
前記の好ましくない分子はビリオンかその副粒子である請求項１の方法。
【請求項４】
前記の好ましくない分子は血液中に存在する抗体の過剰を含む疾患状態に関連する抗体である請求項１の方法。
【請求項５】
前記経路はフラットダイアライザ、半透膜、半透過性中空ファイバ、コイル、透析膜、血漿返還採血フィルタ及びそれらの組合せからなるグループから選択される請求項１の方法。
【請求項６】
前記経路は望ましくない分子の混合及び結合を強めるために、少なくとも部分的にくぼみが設けられ、或いはねじられ、さもなくば変形されている請求項５の方法。
【請求項７】
前記経路はニトロセルロース、セルロース、ナイロン、プラスチック、ゴム、ポリアクリルアミド、アガロース、ポリ（ビニルアルコール－エチレン共重合体）及びそれらの組合せからなるグループから選択された材料からなる請求項５の方法。
【請求項８】
前記特定の結合分子は抗原、抗体、抗－抗体、リガンド、受容体、それらの結合部分及びそれらの組合せから選択される請求項１の方法。
【請求項９】
前記抗原はＡ血液型抗原、Ｂ血液型抗原、タンパクＡ分子、タンパクＧ分子、大部分の組織適合複合分子、それらの結合部分、及びそれらの組合せからなるグループから選択される請求項８の方法。
【請求項１０】
前記抗原は化学的変形、共有結合、強イオン結合、水素結合及びリンカーの利用からなるグループから選択される方法によって、半透過性経路に沿って付着される請求項１の方法。
【請求項１１】
前記化学的変形は臭化シアン、過酸化水素、過酸化ナトリウム、エピクロロヒドリン、１，４－ブタンジオールジグリシドールエーテル、塩化シアヌリル、カルボニルジイミダゾール、置換された塩化スルホニル、及びフルオロメチルピリジニウム塩からなるグループから選択された化合物で処理することによってなされる請求項１０の方法。
【請求項１２】
前記抗原はアビジンもしくはビオチンリンカーによって、囲まれた経路に沿って付着される請求項１の方法。
【請求項１３】

前記特定の抗原は半透過性中空ファイバの壁に付着される請求項５の方法。
【請求項１４】
前記中空ファイバは複数の囲まれた平行膜に連結されている請求項１３の方法。
【請求項１５】
前記複数の囲まれた平行膜は前記中空ファイバと直角に配置されている請求項１４の方法。
【請求項１６】
前記複数の囲まれた平行膜は前記中空ファイバの内部かつその長さに沿って長手方向で配置されている請求項１４の方法。
【請求項１７】
前記複数の平行膜には抗原が付着している請求項１４の方法。
【請求項１８】
血液は囲まれた経路に沿って容器の内外に動くものであり、前記容器は入口及び出口を除いて閉じられており、また前記容器は、
ａ）固定化結合パートナーを有するスラリーを有し、前記固定化結合パートナーがスラリー粒子に付着しており、
ｂ）少なくとも一つのフラットな半透膜を有し、該膜は流動血液から前記スラリーを隔てており、
前記血液成分は前記膜を通じてスラリーと交換できる請求項１の方法。
【請求項１９】
前記フラットな半透膜はニトロセルロース、セルロース、ナイロン、プラスチック、ポリアクリルアミド、アガロース、ポリ（ビニルアルコール－エチレン共重合体）及びゴムからなるグループから選択された材料から形成されている請求項１８の方法。
【請求項２０】
血液が流れる前記容器の部分に少なくとも一つの付加的な膜が存在しており、この付加的な膜は血液の流れに対して直角か或いは水平に配列されている請求項１８の方法。
【請求項２１】
好ましくない分子をほぼ含まない循環血液であって、前記分子は結合パートナーに対する特定或いは非特定結合が可能である循環血液。
【請求項２２】
前記好ましくない分子は抗Ａ血液タンパク及び抗Ｂ血液タンパク抗体である請求項２１の循環血液。
【発明の詳細な説明】
【０００１】
【発明の分野】
本発明は、好ましくない分子の存在を、ホストの血液から除去する技術に関連する。好ましくない分子は、疾患状態に関連する分子や移植臓器や組織の拒絶反応において発生する分子を含んでいる。特に、この発明は、抗Ａ及び抗Ｂ抗体のような不適な分子の存在を一段階の除去プロセスで減少させる方法及びシステムを開示する。また、本発明は、好ましくない抗体、抗原などを実質的に含まない血液も開示する。
【０００２】
【発明の背景】
伝統的に、臓器あるいは組織移植は、移植片の拒絶を回避するために、ＡＢＯ血液型の適合性を要求している。一般的に、ホストの血液は、異種の血液型抗原に対する循環抗体を含有している。ＡＢＯ血液型間の移植は、最初の２４時間以内に移植片の超急性拒絶反応を引き起こす（Ｋｕｂｙ Ｊ：Ｉｍｍｕｎｏｌｏｇｙ．Ｎｅｗ Ｙｏｒｋ，Ｗ．Ｈ．Ｆｒｅｅｍａｎ ａｎｄ Ｃｏｎｐａｎｙ，１９９７）。循環抗体は、移植臓器や組織に見出される赤血球細胞、上皮細胞、及び内皮細胞中に存在する血液抗原に結合する。このような、抗体－抗原複合体は、ホストの補体系を活性化させ、結果として、移植された臓器や組織へ好中球の侵入を生じる。

好中球は、移植片の内皮細胞を破壊する分解酵素を放出し、血小板が付着できる傷害された組織表面を供給する。移植片の毛細血管内部で、塊状の血餅が生成し、このような全体の炎症反応が、血管新生を妨げる。

【０００３】

拒絶反応を低減させる最近の処置としては、移植手術の前後に、複数の免疫抑制剤を投与する方法がある。ＡＢＯ抗原に対する特異的抗体を除去する方法については、研究がなされてきている。これらの方法は、移植された臓器あるいは組織の超急性拒絶反応を低減することについての有益な効果を示している。これらの方法は、ドナー／アクセプターのＡＢＯ型適合の要求性を緩和し、次いで、生存するドナーと死体臓器や組織のプールとを顕著に拡大できる可能性がある点で重要である。

【０００４】

ＡＢＯ抗体を除去する近年の技術としては、溶解性のＡＢＯ抗原の血管投与と組み合わせた血漿交換がある（Ａｌｅｘａｎｄｒｅ ＧＰＪ，ｅｔ ａｌ．，Ｎｅｔｈ Ｊ Ｍｅｄ ２８：２３１－２３４，１９８５）。すなわち、遠心分離あるいは濃縮赤血球細胞を使用する免疫吸着を伴う二重ろ過血漿搬出（ｄｏｕｂｌｅ ｆｉｌｔｒａｔｉｏｎ ｐｌａｓｍａｐｈｅｒｅｓｉｓ）（ＤＦＦＰ）による全血液からの血漿の分離（Ｓｌａｐａｋ Ｍ，ｅｔ ａｌ．，Ｔｒａｎｓｐｌａｎｔａｔｉｏｎ ３１：４－７，１９８１）や、シリカビーズに結合させたＡ抗原及びＢ抗原を用いた抗Ａ及びＢ抗体のカラム免疫吸着を伴うＤＦＰＰ（Ｔａｎａｂｅ Ｋ ｅｔ ａｌ．，Ｔｒａｎｓｐｌａｎｔａｔｉｏｎ Ｐｒｏｃｅｅｄｉｎｇｓ，２７（１）１０２０－１０２３，１９９５）がある。

【０００５】

これらの先行する技術は、標準的治療としての適合性を妨げるという重大な問題を備えている。第１は、感染のリスクである。遠心分離による血漿交換は、血漿タンパク溶液の置換を必要とするため、ウイルス感染のリスクがある。さらに、既述した技術は、最初に全血液からの血漿を分離することと、その後、ＡＢＯ抗体を血漿から除去するという追加の工程とを要する。分離された血漿は、その後、シリカビーズに結合させたＡＢＯ抗原によりカラム上で免疫吸着することにより抗Ａ及びＢ抗体が除去される。

【０００６】

腎臓移植についての一つの研究は、１あるいは２セッションのＤＦＰＰ及び３あるいは４セッションのカラム免疫吸着を受容したＡＢＯ不適合の移植患者が、ＡＢＯ適合移植片を受容した患者と比較して生存率について大きな差がないことを示している（Ｔａｎａｂｅ，ｓｕｐｒａ）。さらに、突発的なＡＢＯ不適合腎臓移植に伴う超急性拒絶反応が、赤血球細胞による免疫吸着を伴う血漿搬出によって回復したケースが報告されている（Ｓｌａｐａｋ，ｓｕｐｒａ）。

【０００７】

【発明の要旨】

この発明は、非自己の臓器あるいは組織表面あるいは内部の異種抗原の存在によって引き起こされる、これらの臓器あるいは組織の移植片によるホストの拒絶反応を低減する方法及びシステムを提供する。この発明は、異種抗原を指向するホスト血液中の抗体の一段階除去のための方法を提供することによって達成される。例えば、ＡＢＯ間の拒絶反応は、ホストの血液中から、一段階で、抗Ａ抗体および／または抗Ｂ抗体を除去することによって、消去されうる。これは、ホストから抽出された血液を、必要に応じて半透過性であり、この経路に固定され、抗Ａ抗体及び抗Ｂ抗体に結合するような抗原のような前記抗体に特異的な抗原を備える経路に沿って移動させ、その後、血液をホストの内部循環系に返すことによってなされる。

【０００８】

他の実施形態では、この発明は、ある種の疾患状態時に存在するような過剰の抗体を、ホストから抽出した血液を、一つの経路、必要に応じて半透過性あって、この経路に固定化され、好ましくない抗体に特異的な抗原や抗－抗体を含有する経路に沿って移動させ、この血液をホストの内部循環系に返すことによって、ホストの血液から、一段階で除去する

方法を提供する。
【０００９】
さらに、他の実施形態では、ホストから抽出した血液を、一つの経路、必要に応じて半透過性であって、この経路に固定化され、前記抗原に特異的な抗体を含有する経路を移動させ、そして、その血液をホストの内部循環系に返すことによって、ホストの血液から好ましくない抗原が除去される。
【００１０】
他の実施形態では、この発明は、抗Ａ抗体や抗Ｂ抗体のような好ましくない分子を実質的に含まない血液を提供する。なお、Ａ及びＢは、血液型抗原である。好ましくない分子は、血液中に過剰に存在する抗体を含む疾患状態に関連する抗体、ウイルス、その他の好ましくない抗原でありうる。
【００１１】
本発明の好ましい実施態様では、中空状ファイバーが、血流からＡ及びＢ抗原に特異的な抗体を分離可能なＡ血液型抗原及びＢ血液型抗原を固定している。他の好ましい実施態様では、抗原を付着させた中空状ファイバーが、同時に、血液を透析あるいは血漿分離を許容する、半透過性の中空部を有している。さらに好ましい実施態様では、この中空状ファイバーは、抗原を固定した直立状の膜が組み合わされている。また、この複数個の膜は、ファイバー内にその長さにわたって、長手方向に沿って配置されることもできる。最も好ましい実施態様では、前記抗原が、中空状ファイバーの壁部に沿って固定されている。さらに好ましい実施例では、中空状ファイバーは、血液が流れあるいは通過することのできる密閉容器内の平坦な膜によって置換することができる。この実施態様では、半透過性膜が、当該膜を介して交換しようとする、抗体のような血液成分を誘導するスラリーから血流を分離するために備えられている。
【００１２】
この発明は、また、ホストの血液から、移植された臓器あるいは組織中に存在する異種抗原に特異的な抗体を一段階で除去することにより、移植に利用可能な臓器や組織のプールを増大させる方法を提供する。
【００１３】
また、この発明は、血液から抗原に特異的な抗体を一段階で除去するためのワンステップシステムを提供する。
【００１４】
また、他の実施態様では、この発明は、好ましくない分子を実質的に含有しない循環血液を提供する。これらの分子は、特異的にあるいは非特異的に、一つの経路に固定化可能な結合パートナーに対して結合可能である。特に、この発明は抗Ａ血液タンパク及び抗Ｂ血液タンパク抗体を実質的に含有しない循環血液を提供する。
【００１５】
【好ましい実施形態の詳細な説明】
この発明は、ホストの血液から移植された器官又は組織内に存在する異種抗原に対して特異的な抗体を一段階で除去する方法及びシステムを提供する。これは、宿主から抽出された血液を結合すなわち固定化された特異抗原を有する中空のファイバ又は平板状の透析器などの閉鎖通路に沿って移動させ、その血液をホストの内部循環に戻すことによって行われる。血液成分は通路の膜を通して透析される一方、同時に、抗体は固定化抗原に結合するすることによって血液から除去される。結合は、抗原が抗体の特異的な結合パートナーとなるように選択されている場合には、特異的であってもよく、タンパクＡ又はタンパクＧのような一般的な結合分子が抗体との結合に使用される場合には、非特異的であってもよい。
【００１６】
このようにして、抗体は、望ましくない小さい分子（尿素、クレアチニン、アンモニア）といっしょに、ホストの血液から除去される。さらに、これらの抗体はそれらをそれらの結合パートナーから解放することによって収集可能である。

【００１７】
この技術についてさらに詳しく述べると、この発明はホストの血液から他の不要な分子を除去する手段も提供する。たとえば、宿主のウィルス感染によって血液中に存在するビリオンの除去がそのビリオンに対する固定化抗体（モノクローナル抗体又はポリクローナル抗体のいずれか）を用いることによって可能である。
【００１８】
材料
閉鎖通路
この発明は閉鎖通路を有する。この通路は血液の流れを許容し、抗体と抗原などの結合対の結合パートナーの一方を捕捉するものである。この装置は種々の物質から形成できる。これらの物質には、限定的ではないが、ニトロセルロース、セルロース、ナイロン、プラスチック、ゴム、ポリアクリルアミド、寒天、ビニルアルコール－エチレンコポリマー（ｐｏｌｙ（ｖｉｎｙｌａｌｃｏｈｏｌ－ｃｏ－ｅｔｈｙｌｅｎｅ））等、及びそれらの組み合わせが含まれる。この物質としては、微小分子を通路の外へ透過させることのできる半透性のものが好ましい。
【００１９】
この装置は種々の形状に形成できる。その形状としては、限定的ではないが、平板状の透析器、半透膜、半透性の中空ファイバ、コイル、透析膜、血漿交換フィルタ、それらの複合体及び組み合わせが含まれる。
図１に示される好ましい実施の形態は商業透析法のための半透性の中空ファイバ１を使用している。このファイバはそのチューブの壁４に抗原３が取り付けられている。チューブの壁は相互に結合しているリンカー分子（ｌｉｎｋｅｒ ｍｏｌｅｃｕｌｅ）、たとえば、ＰＥＧ（ポリエチレングリコール）を有する場合もそうでない場合もある。付着した抗原を有する透析膜の使用は特異抗体５の直接的な膜免疫吸着を許容し、同時に血漿交換が行われる。
【００２０】
また、固定化結合パートナーのための別のアンカーが単独又は組み合わせで使用できる。たとえば、中空のファイバ１は複数の平板状の膜９を有することが可能である。これらの膜はファイバの長手方向に沿って配置されるか（図３）、又は、ファイバに直交するように配置される（図２）。これら複数の膜９に非拡散状にリンクされる抗原３は、特異抗体５が中空のファイバ１に沿って通過するときに、それらを血液から隔離する。膜９、好ましくは、高流量（ｈｉ－ｆｌｕｘ）膜は血液の細胞及び成分を透過させ、目詰まりを発生させない。チューブそれ自体には凹凸、ねじり、その他の変形を付与して、病原体及び抗原の混合性及び結合性を向上させることもできる。
【００２１】
図４は中空のファイバ１の別の実施の形態を示している。この場合、抗原は複数の膜９の間に配置された自在浮動性の透過性の球体１１にリンクされる。これらの球体は、それらの寸法のために、高流量膜の間に捕捉されている。球体１１上の抗原３は特異抗体５を隔離し、それによって、それらを血液から除去する。さらに混合及び結合させるために、空気又はその他の無毒性ガスを微細気泡として低い位置から添加することができる。そのガスはその後標準的な気泡トラップを用いて高い位置から除去することができる（図示せず）。ガスで誘起された混合はシェル側又はチューブ（管腔）側で起こる。
【００２２】
図５はこの発明の別の実施の形態を示している。この場合、抗原３は中空のファイバの代わりの平板状のプレート透析器１５の平板状の半透膜１３に取り付けられている。血液の血漿（下向きの矢印１７で示されている）は対流によって膜を通過するけれども、特異抗体は膜に保持される。血液は、連続的に又は一時的な中断を伴って、図示の左から右へ通路に沿って移動する。
【００２３】
結合対

この発明は結合対とともに使用可能である。結合対としては、限定的ではないが、抗原及び抗体、受容体及びリガンド、抗抗体及び抗体、又はこれらの分子の結合部分が含まれる。「結合部分」という用語は、パートナー分子に対して、特異的又は非特異的に、結合して血液から除去されるか、又は血液から結合パートナーを除去することのできる分子の一部分を意味する。

【００２４】

この発明の好ましい実施の形態においては、ＡＢＯ血液型の抗原は管腔面に結合され、それらに対応する抗体を血液から除去する。抗原／抗体結合対は逆転可能であり、その場合、抗体が管腔面に結合され、抗原が血液から除去される。主要な組織適合性複合体（ＭＨＣ）分子又はこれらの分子の部分などのような他の抗体、抗抗体及び抗原は、これらの分子に対して特異的な抗体を捕捉するのに使用できる。抗原／抗体対は、さらに、特異的親和性を有する複数組の結合対のメンバーと交換可能である。例としては、病原体に対する何らかの特異性を有するリガンド及び受容体がある。

【００２５】

物質Ａ及びＢ抗原はスイスのデイド・インターナショナル（Ｄａｄｅ　Ｉｎｔｅｒｎａｔｉｏｎａｌ）から調達（商標名：Ｎｅｕｔｒ－ＡＢ）できる。物質Ａ及びＢ抗原のこの混合物は各種の天然供給源から調達することができる。その供給源としては、限定的ではないが、ウシ、ブタ、ウマ及びヒトが含まれる。これらの抗原はそれらの最大還元形態の三糖類の状態で人工的に製造することもできる。抗原が抗体の産生をもたらす元の抗原に適合する場合には、その抗原に対する高い親和性が存在する。同様に、抗原が精製されればされるほど、反応性は高くなる。

【００２６】

より多くの抗原が存在し、管腔面に直接固定されたり、閉鎖通路内の結合分子によって取り付けられたりしていると、それだけ多くの特異抗体が流動血液から除去可能である。同様に、コーティングされた膜の表面積が大きくなればなるほど、所望の抗体に結合する能力も高くなる。たとえば、中空のファイバに非拡散的にリンクされた１００ｍｇの抗原は平均値からそれより高い範囲の力価の３００から４００ｍｌの血液の抗Ａ及び抗Ｂ力価を著しく低減することができる。力価は標準的な血球凝集法を用いることによって測定される。このことは、膜結合抗原は抗Ａ及び抗Ｂ抗体を特異的に除去可能であることを示すとともに、この除去が、元の力価に関わらず、最初の１５分の流動（膜に対する約３回の血液の通過）において行われることを示している。また、物質Ａ又はＢなどの一つの抗原タイプを使用することができる。

【００２７】

図７は抗Ａ抗体に対するＡ抗原で修飾されたフィルタの能力を示している。図８は抗Ｂ抗体に対するＢ抗原を用いた場合の同様な能力を示している。血液の連続的なサンプルは膜に通され、その膜が飽和状態になるまで続けられた。その時点では、血液サンプル中の抗体の力価はもはや膜に通しても減少しなかった。抗Ａコーティングされた膜はおよそ３００－４００ｍｌの平均値からそれより高い範囲の力価の血液に対応する能力を有していた。抗Ｂコーティングされた膜はおよそ６００ｍｌに対応する能力を有していた。

【００２８】

標準抗原をさらに精製することによって、抗原１ｍｇ当たりの抗Ａ及び抗Ｂ抗体を除去する能力は少なくとも６倍増大した。精製は、透析によって、市販の抗原溶液から１２，０００ドルトン未満の分子量を有する成分を除去することによって行われた。たとえば、約４０ｍｇの精製抗原で修飾された透析フィルタの抗Ａ抗体能力は６個の１５０ｍｌの血液サンプルそれぞれの抗Ａ力価を２又はそれ以下まで低下させるものであった。標準的な未精製抗原で修飾されたフィルタは最初のサンプルの抗Ａ力価を３２から８へ低下させたが、他の５つのサンプルの力価を低下させることはなかった。この結果は抗Ｂ抗体にについても同様であった。したがって、１００ｍｇの精製された抗原で修飾された透析フィルタは１．８から２．４Ｌの平均値からそれより高い範囲の力価の血液の抗Ａ及び抗Ｂ力価を著しく低下させることができることが予想される。

【００２９】
閉鎖通路への結合パートナーの結合
抗原、抗体、結合対膜、リガンド、又はそれらの結合部分は、各種の一般的な結合方法によって閉鎖通路に結合可能である。結合方法としては、限定的ではないが、化学的修飾、共有結合、強イオン又は水素結合、リンカーの使用などが含まれる。好ましい方法は標準的な臭化シアン（ＣＮＢｒ）結合を使用している。この結合は閉鎖通路をＣＮＢｒで処理した後、抗原及び修飾された通路をインキュベーションすることによって開始する。抗原プロテインのＮ－末端はＣＮＢｒリンカーに共有結合によって結合する。閉鎖通路を処理するための別の化合物としては、限定的ではないが、過酸化水素、過ヨウ素酸ナトリウム、エピクロロヒドリン、１，４－ブタンジオールジグリシドールエーテル、塩化シアヌル、カルボニルジイミダゾール、置換塩化スルホニル、又はフルオロメチルピリジニウム塩、及び同様な方法で適合された抗原が含まれる。アビジン及びビオチンなどの標準的な化学リンカーも使用可能である。
【００３０】
プロセス
濾過
血液からの好ましくない分子の濾過は、一方の腕から血液を採ってそれを他方の腕に戻す標準的な腎臓透析タイプの設備を利用して達成することができる。これに代えて、患者に二箇所で連結されて一箇所から血液を抜いてそれを他方に戻すいかなるポンプシステムでも有効に働く。血液は固定化結合パートナーを有する囲まれた経路を通って流れる。結合パートナーは好ましくない分子をそれらが移動するに伴って結合させる。特定の好ましくない分子を完全に除去するには血液を経路に沿って何回も通過させることが必要であろう。
【００３１】
経路を通って動く血液の流速は凝固を防止するに十分速くなければならないが、血液細胞を損傷するほど速いものであってはならない。この範囲の例としては、毎分血液が約１０～約１０００ｍｌであり、好ましくは約５０及び約７５０ｍｌ／ｍｉｎであり、最も好ましくは、本発明を利用した抗体の除去のための流速は約１００～約５００ｍｌ／ｍｉｎである。凝固を防止するために血液にヘパリンを添加できる。ホスト血液の全体量（～５Ｌ）の処理には、血液から抗体或いはその他の好ましくない分子の完全な除去を達成するために、約２．５時間必要であろう。
【００３２】
流れは連続的であり得る。それに代えて、好ましくない分子とそれらの固定化結合パートナーとの相互作用を強めるために流れを断続させることもできる。同様に、固定化結合パートナーを有する装置の形状は、或る程度の渦及び逆流をもたらして好ましくない分子と固定化結合パートナーとの相互作用時間を増加させるようなものであり得る。
【００３３】
用途
本発明は移植された器官或いは組織において見出される外来抗原に対する特定の抗体を除去し、そして、これらの抗体がほぼない循環血液を提供することによって、器官や組織の移植拒絶を減少させるのに利用できる。本発明は血液中に見出される特定の抗体についての定量分析の一部としても利用できる。例えば、抗Ａ及び抗Ｂ抗体の力価測定濃度についての全体的な体分析を行うことができる。第１に、上記の濾過によって血液から抗体が除去できる。第２に、結合した抗体が自由浮遊抗原或いはその他の結合を防止するための非常に低いイオン強度バッファと競合させることによって解放される。第３に、解放された抗体を血球凝集分析のような方法を利用して力価測定できる。
【００３４】
本発明はまた、血漿瀉血の要なく血液から特定の抗体を予め取り除くのに利用できる。その段階は上記の定量分析と同様である。
【００３５】

さらに、本発明は血液中に存在する過剰な量の抗体を除去するのに利用できる。

【００３６】

また、本発明はビリオンやリガンドのような結合パートナーを有する他の分子を識別し、定量化し、及び／もしくはホストの血液から除去するのに利用できる。

【図面の簡単な説明】

【図１】

本発明の第１の実施形態に基づく抗体除去システムの長手方向に沿う断面図である。

【図２】

本発明の第２の実施形態に基づく抗体除去システムの長手方向に沿う透視図である。

【図３】

本発明の第３の実施形態に基づく抗体除去システムの長手方向に沿う斜視図である。

【図４】

本発明の第４の実施形態に基づく抗体除去システムの長手方向に沿う斜視図である。

【図５】

本発明の第５の実施形態に基づく抗体除去システムの上部斜視図である。

【図６】

抗Ａ抗体及び抗Ｂ抗体を血液から除去するための本発明の方法を使用した場合のアッセイ結果を示す。

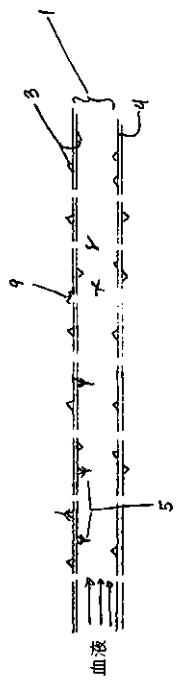
【図７】

抗Ａ抗体を血液から除去するための本発明の方法を使用した場合のアッセイ結果を示す。本発明に係る製品の高い能力を示している。

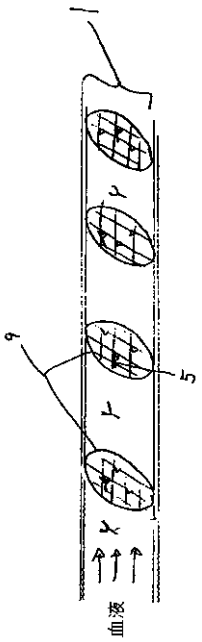
【図８】

抗Ｂ抗体を血液から除去するための本発明の方法を使用した場合のアッセイ結果を示す。本発明に係る製品の高い能力を示している。

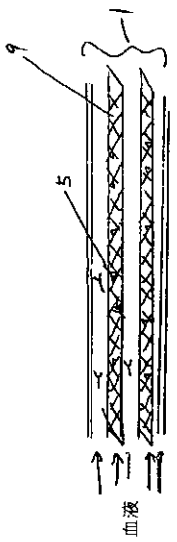
【図１】



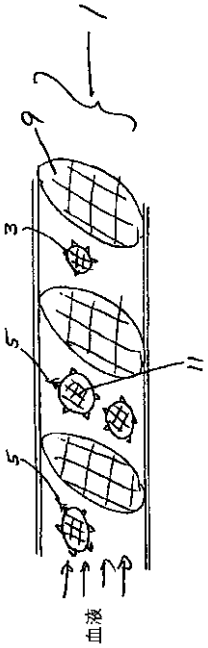
【図２】



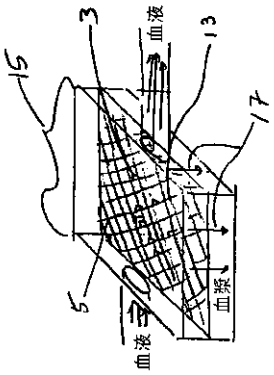
【図３】


1
9
5
血液

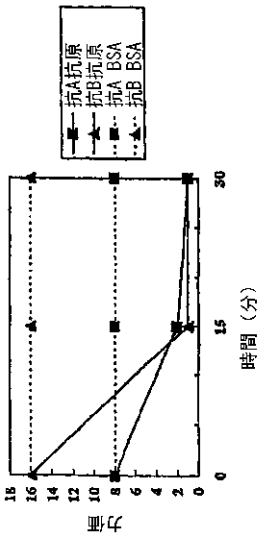
【図４】


1
9
3
5
11
5
血液

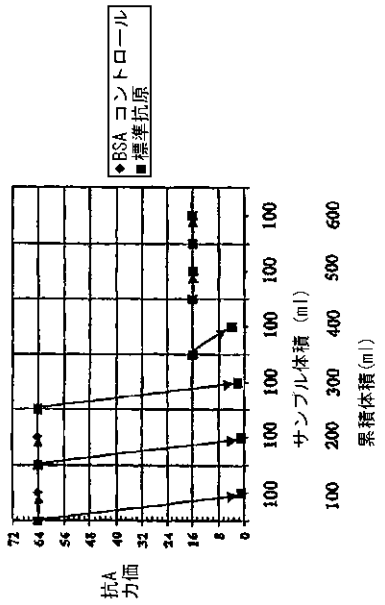
【図５】


15
3
血液
13
17
5
血漿
血液

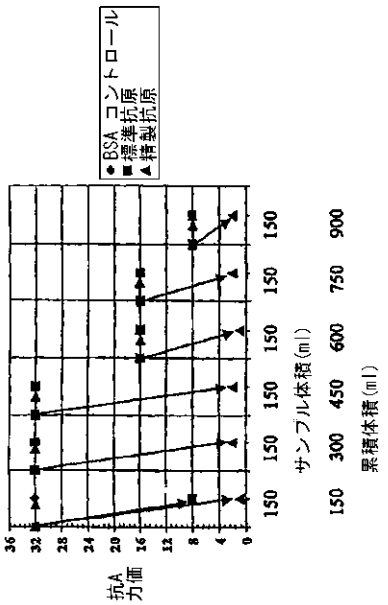
【図６】


抗A抗原
抗B抗原
抗A BSA
抗B BSA
0
2
4
6
8
10
12
14
16
18
0
15
30
時間（分）
力価

【図７】


◆BSA コントロール
■標準抗原
抗A力価
72
64
56
48
40
32
24
16
8
0
100 100 100 100 100 100 100
サンプル体積（ml）
100 200 300 400 500 600
累積体積(ml)

【図８】


◆BSA コントロール
■標準抗原
▲精製抗原
抗A力価
36
32
28
24
20
16
12
8
4
0
150 150 150 150 150 150
サンプル体積(ml)
150 300 450 600 750 900
累積体積(ml)

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(54) Title: ONE STEP REMOVAL OF UNWANTED MOLECULES FROM CIRCULATING BLOOD

(57) Abstract: The present invention provides a method, and resulting product, for the removal of unwanted molecules from a host's blood using a one-step procedure. The unwanted molecules may be anti-A blood protein and/or anti-B blood protein antibodies that would otherwise cause host rejection of transplanted organs or tissues from a source having a different ABO blood type. The unwanted molecules may also be excess antibodies, or virions, present in a diseased host.

DESCRIPTION

One Step Removal of Unwanted Molecules From Circulating Blood

Field Of Invention

This invention relates to technology for reducing the presence of unwanted molecules, including those related to disease states and those involved in rejection of transplanted organs and tissue, from a host's blood. In particular, the invention discloses a method and a system for reducing the presence of unwanted molecules, such as anti-A and anti-B antibodies, by a one step removal process. It also discloses blood substantially free of unwanted antibodies, antigens, and the like.

Background Of The Invention

Traditionally, organ or tissue transplantation requires ABO blood type compatibility in order to prevent graft rejection. Normally, the host's blood contains circulating antibodies against foreign blood type antigens. Transplantation across these ABO blood groups leads to hyperacute rejection of the graft within the first 24 hours (Kuby J: Immunology. New York, W.H. Freeman and Company, 1997). Circulating antibodies bind to blood antigens present in red blood cells, epithelial cells and endothelial cells found in the graft organ or tissue. These antibody-antigen complexes activate the complement system of the host, resulting in infiltration of neutrophils into the graft organ or tissue. The neutrophils release lytic enzymes that destroy the graft endothelial cells, providing a surface of injured tissue to which platelets can adhere. Massive blood clots form within the graft capillaries, and this whole inflammatory reaction prevents vascularization.

Current treatments to reduce rejection include administering a regimen of immunosuppressant drugs before and after the transplantation surgery. Studies have been performed on methods that remove antibodies specific to ABO antigens. These methods have also shown beneficial effects in reducing hyperacute rejection of the transplanted organ or tissue. These methods are important because they may lead to a method which will relax the requirement of donor/recipient ABO compatibility, which in turn can greatly expand both the living donor and cadaver organ or tissue pools.

Current techniques to remove the ABO antibodies include plasma exchange combined with intravenous administration of soluble ABO antigens (Alexandre GPJ, et.al., Neth J Med 28:231-234, 1985); separating plasma from the whole blood by either centrifugation or double filtration plasmapheresis (DFPP) followed by immunoadsorption

using concentrated red blood cells (Slapak M, et.al., Transplantation 31:4-7,1981); and DFPP followed by column immunoadsorption of anti-A and B antibody using A and B antigen bound to silica beads (Tanabe K, et al., Transplantation Proceedings, 27(1) 1020-1023, 1995).

These prior art methods have serious problems which have prevented their adoption as the standard of care. First, there is the risk of infection. Because plasma exchange by centrifugation requires replacement by plasma protein solution, risk of viral transmission is present. Moreover, these techniques described above involve first separation of plasma from whole blood then an additional procedure to remove ABO antibodies from the plasma. Separated plasma can then be stripped of pre-existing anti-A and B antibodies by immunoadsorption with ABO antigens linked to silica beads on a column.

A study on renal transplantation has shown that ABO-incompatible grafted patients who received one or two sessions of DFPP and three or four sessions of column immunoadsorption showed no significant difference in survival rates when compared to patients who received an ABO compatible graft (Tanabe, *supra)*. Additionally, one case has been reported in which hyperacute rejection following accidental ABO-incompatible renal transplant was reversed using plasmapheresis followed by immunoadsorption with red blood cells (Slapak, *supra*).

Summary Of The Invention

This invention provides a method and system for reduction of a host's rejection of a non-autologous organ or tissue transplant caused by the presence of foreign antigens in and on the organ or tissue. This is accomplished by providing a method for one-step removal of antibodies in the host's blood that are directed to the foreign antigens. For example, cross-ABO rejection can be eliminated by removing anti-A and/or anti-B antibodies, in one step, from the host's blood. This is done by moving blood extracted from the host along a pathway, which is optionally semi-permeable, having antigen specific to the antibodies, such as antigens that bind to anti-A and anti-B antibodies, attached to the pathway, and returning the blood to the host internal circulation.

In another embodiment, this invention provides a method for removing, in one step, excess antibodies, such as are present in certain disease states, from a host's blood by moving the blood extracted from the host on a pathway, optionally semi-permeable, having antigens or anti-antibodies specific to the unwanted antibodies immobilized in the pathway, and returning the blood to the host's internal circulation.

In yet another embodiment, unwanted antigen is removed from a host's blood in one step by moving the blood extracted from the host on a pathway, optionally semi-permeable, having antibodies specific to the antigen immobilized in the pathway, and returning the blood to the host's internal circulation.

In another embodiment, this invention provides blood that is substantially free of undesired molecules, such as anti-A and anti-B antibodies, wherein A and B are blood type antigens. The undesired molecules may also be antibodies associated with a disease state comprising an excess of antibodies in the blood, virions, and other undesired antigens.

In the preferred practice of the invention, a hollow fiber has attached A and B blood type antigens that are capable of sequestering the antibodies specific to A and B antigens from the flowing blood. In another preferred practice of the invention, the hollow fiber with attached antigen has semi-permeable pores that allow dialysis or plasmapheresis of the blood to occur at the same time. In a further preferred practice of the invention, the hollow fiber is coupled to a plurality of perpendicular membranes having attached antigen. Alternatively, this plurality of membranes can also be longitudinally placed inside and along the length of the fiber. In the most preferred embodiment, the antigens are attached to the wall of the hollow fiber. In further practice of the invention, the hollow fiber can be replaced by a flat membrane in a closed container that the blood can flow along or pass through. In this embodiment, an optional semi-permeable membrane is present to divide the flowing blood from a slurry that will induce the blood components, such as antibodies, to exchange across the membrane.

The invention also provides a method to increase the organ or tissue pools available for transplant by removing in one step, from the host's blood, antibodies specific to foreign antigens present in the transplanted organ or tissue

The invention also provides a one-step system for removing antibodies to specific antigens from blood in one step.

The invention also provides a one-step system for harvesting antibodies to specific antigens from blood.

In another embodiment, the present invention provides circulating blood that is substantially free of unwanted molecules, wherein these molecules are capable of binding, either specifically or non-specifically, to a binding partner capable of being immobilized on a pathway. In particular, this invention provides circulating blood that is substantially free of anti-A blood protein and anti-B blood protein antibodies.

Brief Description Of The Drawings

Fig. 1 is a longitudinal cross section view of an antibody removal system in accordance with a first embodiment of the present invention.

Fig. 2 is a longitudinal perspective section view of an antibody removal system in accordance with a second embodiment of the present invention.

Fig. 3 is a longitudinal perspective section view of an antibody removal system in accordance with a third embodiment of the present invention.

Fig. 4 is longitudinal perspective section view of an antibody removal system in accordance with a fourth embodiment of the present invention.

Fig. 5 is a top perspective view of an antibody removal system in accordance with a fifth embodiment of the present invention.

Fig. 6 shows the results of an assay using the method of this invention to remove anti-A and anti-B antibodies from blood.

Fig. 7 shows the results of an assay using the method of this invention to remove anti-A antibodies from blood, showing the high capacity of the product.

Fig. 8 shows the results of an assay using the method of this invention to remove anti-B antibodies from blood showing the high capacity of the product.

Detailed Description Of The Preferred Embodiments

This invention provides a method and a system for one step removal, from the host's blood, of antibody specific to foreign antigens present in a transplanted organ or tissue. This is done by moving blood extracted from the host along an enclosed pathway such as a hollow fiber or flat dialyzer comprising bound or immobilized specific antigen, and returning the blood to the host internal circulation. The blood components are dialyzed across the membrane of the pathway, while at the same time antibodies are removed from the blood through binding to the immobilized antigen. The binding can be specific, as when the antigens are chosen to be the specific binding partners of the antibodies, or nonspecific, as when a general binding molecule such as protein A or protein G is used to bind the antibodies.

The antibodies, along with undesired small molecules (urea, creatinine, ammonia), are thus removed from the host's blood. Additionally, these antibodies can be collected by releasing them from their binding partners.

Expanding on this technique, the invention also provides a means for removing other unwanted molecules from a host's blood. For example, virions present in the blood due to a viral infection of the host can be removed by utilizing immobilized antibodies, either monoclonal or polyclonal, to the virion.

Materials:

Enclosed pathway

The present invention comprises an enclosed pathway that allows the flow of blood and the trapping of one of the binding partners of a binding pair, such as an antibody and an antigen. The device can be made out of a variety of substances, including but not limited to nitrocellulose, cellulose, nylon, plastic, rubber, polyacrylamide, agarose, poly(vinylalcohol-co-ethylene), and the like, and combinations thereof. The material is preferably semi-permeable to allow the passage of small molecules out of the pathway.

The device can be formed in a variety of shapes, including but not limited to a flat dialyzer, a semi-permeable membrane, a semi-permeable hollow fiber, a coil, a dialysis membrane, a plasmapheresis filter, and multiples and combinations thereof.

The preferred embodiment as shown in Fig. 1 uses a semi-permeable hollow fiber **1** for commercial dialysis with the antigen **3** attached to the wall **4** of the tubing with or without a linker molecule, for example PEG (polyethylene glycol), connecting one to the other. Use of dialysis membranes with attached antigen allows direct membrane immunoadsorption of the specific antibody **5** and plasmapheresis to occur at the same time.

Alternatively, other anchors for the immobilized binding partner can be used alone or in combination. For example, the hollow fiber **1** can have a plurality of flat membranes **9** that are placed longitudinally along the fiber length (Fig. 3) or perpendicular to the fiber (Fig. 2). The antigens **3** which are non-diffusively linked to this plurality of membranes **9**, sequester the specific antibodies **5** from the blood as they pass along the hollow fiber **1**. The membranes **9**, preferably hi-flux membranes, allow blood cells and components to pass through so that no clogging occurs. The tubes themselves may be dimpled, twisted, or otherwise modified to increase mixing and binding of pathogen and antigen.

Fig. 4 shows another embodiment of the hollow fiber **1** where the antigens are linked to free floating permeable spheres **11** located inbetween the plurality of membranes **9**. These spheres are trapped between the hi-flux membranes because of their size. The antigens **3** on the spheres **11** sequester the specific antibodies **5**, thus removing them from the blood. Air or other non-toxic gas may be added at a lower elevation as small bubbles to further mixing and binding, and then the gas can be removed with a standard bubble trap at a higher elevation (not shown). The gas-induced mixing can occur on either the shell side or the tube (lumen) side.

Fig. 5 shows another embodiment of the invention, where the antigens **3** are attached to flat semi-permeable membranes **13** of a flat plate dialyzer **15** instead of a

hollow fiber. Blood plasma (as shown by downward arrows 17) passes through the membrane by convection but the specific antibodies are retained at the membrane. The blood travels along the pathway, continuously or temporarily interrupted, from left to right in the figure.

Binding Pair

This invention can be used with any binding pair, including but not limited to an antigen and an antibody, a receptor and ligand, an anti-antibody and an antibody, or binding portions of these molecules. By the term "binding portions" is meant any portion of the molecule that is capable of binding, either specifically or non-specifically, to a partner molecule so as either to be removed or to remove the binding partner from the blood.

In the preferred embodiment of the invention, the ABO blood group antigens are bound to the lumenal surface to remove their corresponding antibodies from blood. The antigen/antibody binding pair can be reversed wherein the antibody is bound to the lumenal surface and the antigen is removed from the blood. Other antibodies, anti-antibodies, and antigens, such as major histocompatibility complex (MHC) molecules, or parts of these molecules, can be used to trap antibodies specific to these molecules. The antigen/antibody pair can further be replaced with any members of sets of binding pairs that would have specific affinities. Examples are ligands and receptors with some specificity to a pathogen.

Substance A and B antigens can be procured from Dade International in Switzerland (trade name: Neutr-AB). This mixture of Substance A and B antigen] can be from a variety of natural sources, including but not limited to cows, pigs, horses and humans. These antigens, in their most reduced form trisaccharides, can also be made synthetically. A higher affinity for the antigen will exist when the antigen matches the original antigen to which the antibodies were produced. Likewise, the more purified the antigen is, the stronger the reaction.

The more antigen is present, immobilized directly on the lumenal surface or attached by a linking molecule in the enclosed pathway, the more specific antibody can be removed from the flowing blood. Likewise, the larger the surface area of the coated membrane, the higher the capacity for binding the desired antibody. For instance, 100mg of antigen non-diffusively linked to a hollow fiber can significantly reduce the anti-A and anti-B titers of 300 to 400ml of blood with from average to high titer. Fig. 6 shows the capacity of a modified hollow fiber to sequentially process 100ml of banked human blood. Titer is determined by using a standard hemagglutination assay. This shows that

membrane-bound antigen can specifically remove anti-A and anti-B antibodies, and that this removal takes place in the first 15 minutes of flow (about 3 passages of the blood over the membrane), regardless of original titer. Alternatively, one antigen type, such as Substance A or B, can be used.

Figure 7 shows the capacity of filters modified with A antigen for anti-A antibodies. Figure 8 shows the same using B antigen for anti-B antibodies. Consecutive samples of blood were passed over the membrane until the membrane was saturated. At this point the titer of antibody in the blood samples no longer decreased upon passage over the membrane. The anti-A coated membrane had a capacity of around 300-400 ml. of average to high titer blood. The anti-B coated membrane had a capacity of around 600 ml.

Further purification of the standard antigens leads to at least a six fold increase in capacity to remove anti-A and anti-B antibodies per mg of antigen. Purification is achieved by removing components having molecular weight below 12,000 daltons from the commercially available antigen solution by dialysis. For example, the anti-A antibody capacity of a dialysis filter modified with approximately 40 mg of purified antigen reduced the anti-A titer of each of six 150 ml blood samples to 2 or below. The standard non-purified antigen-modified filter reduced the anti-A titer of the first sample from 32 to 8, and caused no titer reduction of the other five samples. The results were similar for the anti-B antibodies. Hence we expect that a dialysis filter modified with 100 mg of purified may be able to significantly reduce the anti-A and anti-B titers of 1.8 to 2.4 L of average to high titer blood.

Linking of binding partner to the enclosed pathway

The antigen, antibody, binding pair member, ligand, or binding parts thereof, can be linked to the enclosed pathway by a variety of standard linking techniques, including but not limited to chemical modifications, covalent bonding, strong ionic or hydrogen bonding, use of a linker, etc. The preferred method uses standard cyanogen bromide (CNBr) linking which starts by treating the enclosed pathway with CNBr followed by incubation of the antigen and the modified pathway. The N-terminus of the antigen protein will covalently attach to the CNBr linker. Other compounds for treating the enclosed pathway include, but are not limited to, hydrogen peroxide, sodium periodate, epichlorohydrin, 1,4-butanedioldiglycidol ether, cyanuric chloride, carbonyldiimidazole, substituted sulfonyl chloride, or fluoromethyl pyridinium salts, and antigen applied in the same way. Standard chemical linkers such as avidin and biotin can also be used.

Process

Filtration

Filtration of unwanted molecules from blood can be achieved using standard kidney dialysis type equipment which removes blood from one arm and returns it to the other. Alternatively, any pumping system connected to the patient at two sites, so as to draw blood from one site and return it to the other, will work. The blood is passed through the enclosed pathway having immobilized binding partners. The binding partners sequester the unwanted molecules as they move along. Several passes of the blood along the pathway might be required to completely remove the specific unwanted molecules.

The flow rate of blood moving through the pathway must be fast enough to prevent coagulation, yet not so fast as to damage the blood cells. Examples of ranges are from about 10 to about 1000 ml of blood per min., preferably between about 50 and about 750 ml/min., and most preferably the flow rate for removal of antibodies using the invention is between about 100 and about 500ml/min. Heparin can also be added to the blood to prevent coagulation. Processing of an entire host's blood volume (~5L) would require approximately 2.5 hours to achieve complete removal of antibodies or other undesired molecules from the blood.

The flow can be continuous. Alternatively, the flow can be interrupted to increase the interaction of the unwanted molecules with their immobilized binding partners. Likewise, the shape of the device having the immobilized binding partners can be such that it will encourage some swirling and/or backflow to increase the interaction time between the unwanted molecules and the immobilized binding partners.

Uses

The current invention can be used in reducing organ or tissue transplant rejection by removing specific antibodies against foreign antigens found in the transplanted organ or tissue and providing circulating blood substantially free of these antibodies. The invention can also be used as part of a quantitative assay for specific antibodies found in the blood. For example, a whole body assay for the titer of anti-A and anti-B antibodies can be performed. First, the antibodies from the blood can be removed by the filtration described above. Second, the bound antibodies are released by competing with free floating antigens or with other very low ionic strength buffers to prevent binding. Third, released antibodies can be titered using a method such as a hemagglutination assay.

Also, the invention can be used to preparatively purify specific antibodies from the blood without the need to plasmapherese. The steps are similar to the quantification assay described above.

Additionally, the invention can be used to remove excess amounts of antibodies present in the blood.

Further, the invention can be used to identify, quantify and/or remove other molecules having binding partners, such as virions or ligands, from the host's blood.

Claims

1. A method for reducing the presence of unwanted molecules in a host's blood in a single step, comprising:

a) extracting blood from the host;

b) moving the extracted blood in a continuous or temporarily interrupted flow along an enclosed pathway, wherein the pathway has immobilized binding molecules specific to the unwanted molecules attached along the pathway, and the binding molecules are capable of binding to the unwanted molecules in the blood of the host; and

c) returning the blood to the host's internal circulation.

2. A method in accordance with claim 1 wherein the unwanted molecules are antibodies specific to foreign donor antigens present in a transplanted organ or tissue.

3. A method in accordance with claim 1 wherein the unwanted molecules are virions or sub-particles thereof.

4. A method in accordance with claim 1 wherein the unwanted molecules are antibodies associated with a disease state comprising an excess of antibodies present in the blood.

5. A method in accordance with claim 1 wherein the pathway is selected from the group consisting of a flat dialyzer, a semi-permeable membrane, a semi-permeable hollow fiber, a coil, dialysis membrane, a plasmapheresis filter, and combinations thereof.

6. A method in accordance with claim 5 wherein the pathway is at least partially dimpled, twisted, or otherwise modified to increase mixing and binding of unwanted molecules.

7. A method in accordance with claim 5 wherein the pathway is composed of a material selected from the group consisting of nitrocellulose, cellulose, nylon, plastic, rubber, polyacrylamide, agarose, poly(vinylalcohol-co-ethylene), and combinations thereof.

8. A method in accordance with claim 1 wherein the specific binding molecules are selected from antigens, antibodies, anti-antibodies, ligands, receptors, binding portions thereof, and combinations thereof.

9. A method in accordance with claim 8 wherein the antigens are selected from the group consisting of A blood type antigens, B blood type antigens, protein A molecules, protein G molecules, major histocompatibility complex molecules, binding portions thereof, and combinations thereof.

10. A method in accordance with claim 1 wherein the antigens are attached along the semi-permeable pathway by a process selected from the group consisting of chemical modifications, covalent bonding, strong ionic bonding, hydrogen bonding, and use of a linker.

11. A method in accordance with claim 10 wherein the chemical modification is accomplished by treatment with a compound selected from the group consisting of cyanogen bromide, hydrogen peroxide, sodium periodate, epichlorohydrin, 1,4-butanedioldiglycidol ether, cyanuric chloride, carbonyldiimidazole, substituted sulfonyl chloride, and fluoromethyl pyridinium salts.

12. A method in accordance with claim 1 wherein the antigens are attached along the enclosed pathway by avidin or biotin linkers.

13. A method in accordance with claim 5 wherein the specific antigens are attached to the wall of a semi-permeable hollow fiber.

14. A method in accordance with claim 13 wherein the hollow fiber is coupled with a plurality of enclosed parallel membranes.

15. A method in accordance with claim 14 wherein the plurality of enclosed parallel membranes are arranged perpendicular to the hollow fiber.

16. A method in accordance with claim 14 wherein the plurality of enclosed parallel membranes are longitudinally arranged inside and along the length of the hollow fiber.

17. A method in accordance with claim 14 wherein the plurality of parallel membranes have antigens attached to them.

18. A method in accordance with claim 1 wherein the blood is moved into, along an enclosed pathway within, and out of a container, wherein the container is closed except for entrance and exit openings, and wherein the container comprises

a) a slurry having immobilized binding partners attached to the slurry particles, and

b) at least one flat semi-permeable membrane, the membrane dividing the flowing blood from the slurry,

wherein the blood components can exchange with the slurry across the membrane.

19. A method in accordance with claim 18 wherein the flat semi-permeable membrane is made of a material selected from the group consisting of nitrocellulose, cellulose, nylon, plastic, polyacrylamide, agarose, poly(vinylalcohol-co-ethylene), and rubber.

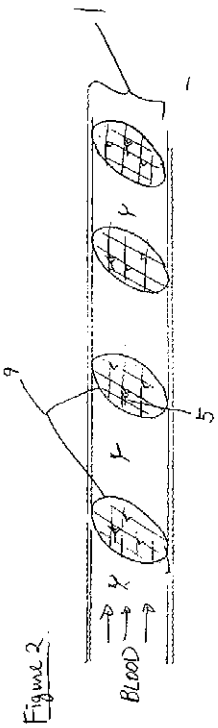
20. A method in accordance with claim 18 wherein at least one additional membrane is present in the portion of the container where the blood flows, and wherein the additional membrane or membranes are arrayed either perpendicularly or horizontally to the blood flow.

21. Circulating blood substantially free of unwanted molecules, wherein the molecules are capable of specific or non-specific binding to a binding partner.

22. The circulating blood in accordance with claim 21 wherein the unwanted molecules are anti-A blood protein and anti-B blood protein antibodies.

Figure 1.

Figure 2



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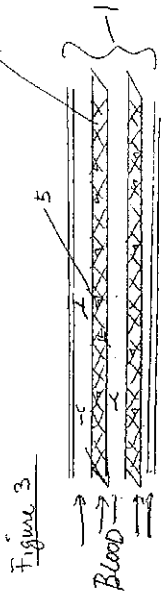


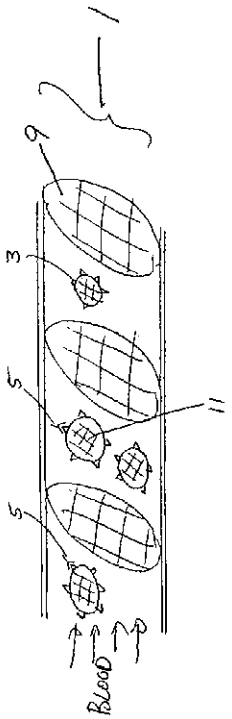
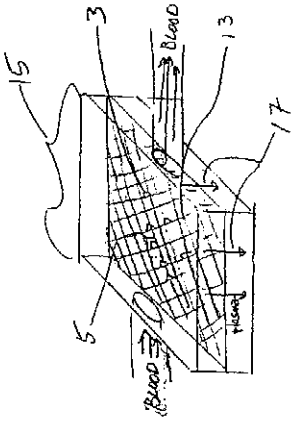


Figure 4



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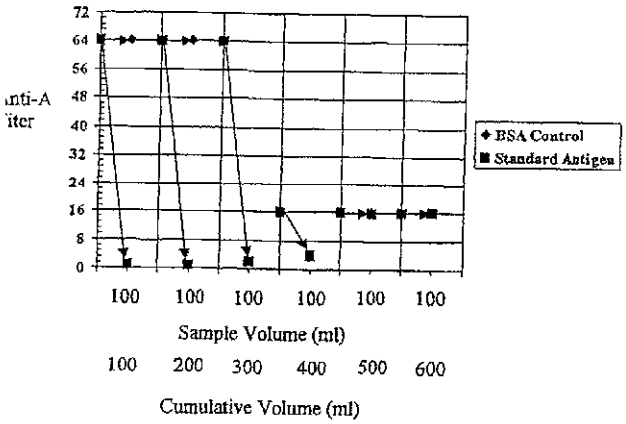
anti-A
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64
56
48
40
32
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BSA Control
Standard Antigen
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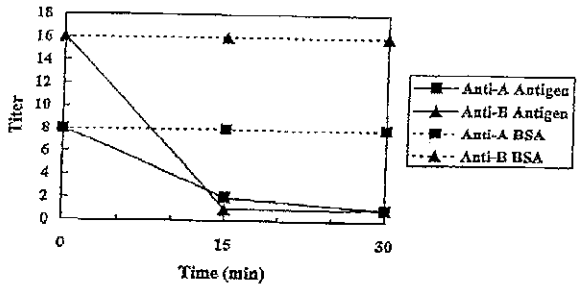
Cumulative Volume (ml)

FIGURE 7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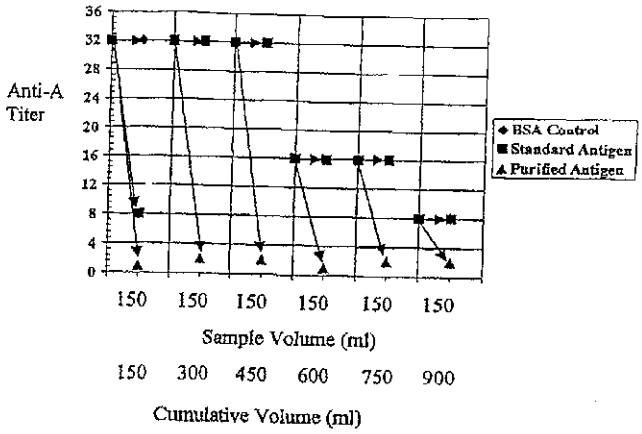
Anti-A BSA
Anti-B BSA

FIGURE 6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Sample Volume (ml)
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Cumulative Volume (ml)

FIGURE 8

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(54) Title: ONE STEP REMOVAL OF UNWANTED MOLECULES FROM CIRCULATING BLOOD

(57) Abstract: The present invention provides a method, and resulting product, for the removal of unwanted molecules from a host's blood using a one step procedure. The unwanted molecules may be anti A blood protein and/or anti B blood protein antibodies that would otherwise cause host rejection of transplanted organs or tissues from a source having a different ABO blood type. The unwanted molecules may also be excess antibodies, or virions, present in a diseased host.

DESCRIPTION

One Step Removal of Unwanted Molecules From Circulating Blood

Field Of Invention

This invention relates to technology for reducing the presence of unwanted molecules, including those related to disease states and those involved in rejection of transplanted organs and tissue, from a host's blood. In particular, the invention discloses a method and a system for reducing the presence of unwanted molecules, such as anti-A and anti-B antibodies, by a one step removal process. It also discloses blood substantially free of unwanted antibodies, antigens, and the like.

Background Of The Invention

Traditionally, organ or tissue transplantation requires ABO blood type compatibility in order to prevent graft rejection. Normally, the host's blood contains circulating antibodies against foreign blood type antigens. Transplantation across these ABO blood groups leads to hyperacute rejection of the graft within the first 24 hours (Kuby J: Immunology. New York, W.H. Freeman and Company, 1997). Circulating antibodies bind to blood antigens present in red blood cells, epithelial cells and endothelial cells found in the graft organ or tissue. These antibody-antigen complexes activate the complement system of the host, resulting in infiltration of neutrophils into the graft organ or tissue. The neutrophils release lytic enzymes that destroy the graft endothelial cells, providing a surface of injured tissue to which platelets can adhere. Massive blood clots form within the graft capillaries, and this whole inflammatory reaction prevents vascularization.

Current treatments to reduce rejection include administering a regimen of immunosuppressant drugs before and after the transplantation surgery. Studies have been performed on methods that remove antibodies specific to ABO antigens. These methods have also shown beneficial effects in reducing hyperacute rejection of the transplanted organ or tissue. These methods are important because they may lead to a method which will relax the requirement of donor/recipient ABO compatibility, which in turn can greatly expand both the living donor and cadaver organ or tissue pools.

Current techniques to remove the ABO antibodies include plasma exchange combined with intravenous administration of soluble ABO antigens (Alexandre GPJ, et.al., Neth J Med 28:231-234, 1985); separating plasma from the whole blood by either centrifugation or double filtration plasmapheresis (DFPP) followed by immunoadsorption

using concentrated red blood cells (Slapak M, et.al., Transplantation 31:4-7,1981); and DFPP followed by column immunoadsorption of anti-A and B antibody using A and B antigen bound to silica beads (Tanabe K, et al., Transplantation Proceedings, 27(1) 1020-1023, 1995).

These prior art methods have serious problems which have prevented their adoption as the standard of care. First, there is the risk of infection. Because plasma exchange by centrifugation requires replacement by plasma protein solution, risk of viral transmission is present. Moreover, these techniques described above involve first separation of plasma from whole blood then an additional procedure to remove ABO antibodies from the plasma. Separated plasma can then be stripped of pre-existing anti-A and B antibodies by immunoadsorption with ABO antigens linked to silica beads on a column.

A study on renal transplantation has shown that ABO-incompatible grafted patients who received one or two sessions of DFPP and three or four sessions of column immunoadsorption showed no significant difference in survival rates when compared to patients who received an ABO compatible graft (Tanabe, *supra*). Additionally, one case has been reported in which hyperacute rejection following accidental ABO-incompatible renal transplant was reversed using plasmapheresis followed by immunoadsorption with red blood cells (Slapak, *supra*).

Summary Of The Invention

This invention provides a method and system for reduction of a host's rejection of a non-autologous organ or tissue transplant caused by the presence of foreign antigens in and on the organ or tissue. This is accomplished by providing a method for one-step removal of antibodies in the host's blood that are directed to the foreign antigens. For example, cross-ABO rejection can be eliminated by removing anti-A and/or anti-B antibodies, in one step, from the host's blood. This is done by moving blood extracted from the host along a pathway, which is optionally semi-permeable, having antigen specific to the antibodies, such as antigens that bind to anti-A and anti-B antibodies, attached to the pathway, and returning the blood to the host internal circulation.

In another embodiment, this invention provides a method for removing, in one step, excess antibodies, such as are present in certain disease states, from a host's blood by moving the blood extracted from the host on a pathway, optionally semi-permeable, having antigens or anti-antibodies specific to the unwanted antibodies immobilized in the pathway, and returning the blood to the host's internal circulation.

In yet another embodiment, unwanted antigen is removed from a host's blood in one step by moving the blood extracted from the host on a pathway, optionally semi-permeable, having antibodies specific to the antigen immobilized in the pathway, and returning the blood to the host's internal circulation.

In another embodiment, this invention provides blood that is substantially free of undesired molecules, such as anti-A and anti-B antibodies, wherein A and B are blood type antigens. The undesired molecules may also be antibodies associated with a disease state comprising an excess of antibodies in the blood, virions, and other undesired antigens.

In the preferred practice of the invention, a hollow fiber has attached A and B blood type antigens that are capable of sequestering the antibodies specific to A and B antigens from the flowing blood. In another preferred practice of the invention, the hollow fiber with attached antigen has semi-permeable pores that allow dialysis or plasmapheresis of the blood to occur at the same time. In a further preferred practice of the invention, the hollow fiber is coupled to a plurality of perpendicular membranes having attached antigen. Alternatively, this plurality of membranes can also be longitudinally placed inside and along the length of the fiber. In the most preferred embodiment, the antigens are attached to the wall of the hollow fiber. In further practice of the invention, the hollow fiber can be replaced by a flat membrane in a closed container that the blood can flow along or pass through. In this embodiment, an optional semi-permeable membrane is present to divide the flowing blood from a slurry that will induce the blood components, such as antibodies, to exchange across the membrane.

The invention also provides a method to increase the organ or tissue pools available for transplant by removing in one step, from the host's blood, antibodies specific to foreign antigens present in the transplanted organ or tissue

The invention also provides a one-step system for removing antibodies to specific antigens from blood in one step.

The invention also provides a one-step system for harvesting antibodies to specific antigens from blood.

In another embodiment, the present invention provides circulating blood that is substantially free of unwanted molecules, wherein these molecules are capable of binding, either specifically or non-specifically, to a binding partner capable of being immobilized on a pathway. In particular, this invention provides circulating blood that is substantially free of anti-A blood protein and anti-B blood protein antibodies.

Brief Description Of The Drawings

Fig. 1 is a longitudinal cross section view of an antibody removal system in accordance with a first embodiment of the present invention.

Fig. 2 is a longitudinal perspective section view of an antibody removal system in accordance with a second embodiment of the present invention.

Fig. 3 is a longitudinal perspective section view of an antibody removal system in accordance with a third embodiment of the present invention.

Fig. 4 is longitudinal perspective section view of an antibody removal system in accordance with a fourth embodiment of the present invention.

Fig. 5 is a top perspective view of an antibody removal system in accordance with a fifth embodiment of the present invention.

Fig. 6 shows the results of an assay using the method of this invention to remove anti-A and anti-B antibodies from blood.

Fig. 7 shows the results of an assay using the method of this invention to remove anti-A antibodies from blood, showing the high capacity of the product.

Fig. 8 shows the results of an assay using the method of this invention to remove anti-B antibodies from blood showing the high capacity of the product.

Detailed Description Of The Preferred Embodiments

This invention provides a method and a system for one step removal, from the host's blood, of antibody specific to foreign antigens present in a transplanted organ or tissue. This is done by moving blood extracted from the host along an enclosed pathway such as a hollow fiber or flat dialyzer comprising bound or immobilized specific antigen, and returning the blood to the host internal circulation. The blood components are dialyzed across the membrane of the pathway, while at the same time antibodies are removed from the blood through binding to the immobilized antigen. The binding can be specific, as when the antigens are chosen to be the specific binding partners of the antibodies, or nonspecific, as when a general binding molecule such as protein A or protein G is used to bind the antibodies.

The antibodies, along with undesired small molecules (urea, creatinine, ammonia), are thus removed from the host's blood. Additionally, these antibodies can be collected by releasing them from their binding partners.

Expanding on this technique, the invention also provides a means for removing other unwanted molecules from a host's blood. For example, virions present in the blood due to a viral infection of the host can be removed by utilizing immobilized antibodies, either monoclonal or polyclonal, to the virion.

Materials:

Enclosed pathway

The present invention comprises an enclosed pathway that allows the flow of blood and the trapping of one of the binding partners of a binding pair, such as an antibody and an antigen. The device can be made out of a variety of substances, including but not limited to nitrocellulose, cellulose, nylon, plastic, rubber, polyacrylamide, agarose, poly(vinylalcohol-co-ethylene), and the like, and combinations thereof. The material is preferably semi-permeable to allow the passage of small molecules out of the pathway.

The device can be formed in a variety of shapes, including but not limited to a flat dialyzer, a semi-permeable membrane, a semi-permeable hollow fiber, a coil, a dialysis membrane, a plasmapheresis filter, and multiples and combinations thereof.

The preferred embodiment as shown in Fig. 1 uses a semi-permeable hollow fiber **1** for commercial dialysis with the antigen **3** attached to the wall **4** of the tubing with or without a linker molecule, for example PEG (polyethylene glycol), connecting one to the other. Use of dialysis membranes with attached antigen allows direct membrane immunoadsorption of the specific antibody **5** and plasmapheresis to occur at the same time.

Alternatively, other anchors for the immobilized binding partner can be used alone or in combination. For example, the hollow fiber **1** can have a plurality of flat membranes **9** that are placed longitudinally along the fiber length (Fig. 3) or perpendicular to the fiber (Fig. 2). The antigens **3** which are non-diffusively linked to this plurality of membranes **9**, sequester the specific antibodies **5** from the blood as they pass along the hollow fiber **1**. The membranes **9**, preferably hi-flux membranes, allow blood cells and components to pass through so that no clogging occurs. The tubes themselves may be dimpled, twisted, or otherwise modified to increase mixing and binding of pathogen and antigen.

Fig. 4 shows another embodiment of the hollow fiber **1** where the antigens are linked to free floating permeable spheres **11** located inbetween the plurality of membranes **9**. These spheres are trapped between the hi-flux membranes because of their size. The antigens **3** on the spheres **11** sequester the specific antibodies **5**, thus removing them from the blood. Air or other non-toxic gas may be added at a lower elevation as small bubbles to further mixing and binding, and then the gas can be removed with a standard bubble trap at a higher elevation (not shown). The gas-induced mixing can occur on either the shell side or the tube (lumen) side.

Fig. 5 shows another embodiment of the invention, where the antigens **3** are attached to flat semi-permeable membranes **13** of a flat plate dialyzer **15** instead of a

hollow fiber. Blood plasma (as shown by downward arrows 17) passes through the membrane by convection but the specific antibodies are retained at the membrane. The blood travels along the pathway, continuously or temporarily interrupted, from left to right in the figure.

Binding Pair

This invention can be used with any binding pair, including but not limited to an antigen and an antibody, a receptor and ligand, an anti-antibody and an antibody, or binding portions of these molecules. By the term "binding portions" is meant any portion of the molecule that is capable of binding, either specifically or non-specifically, to a partner molecule so as either to be removed or to remove the binding partner from the blood.

In the preferred embodiment of the invention, the ABO blood group antigens are bound to the lumenal surface to remove their corresponding antibodies from blood. The antigen/antibody binding pair can be reversed wherein the antibody is bound to the lumenal surface and the antigen is removed from the blood. Other antibodies, anti-antibodies, and antigens, such as major histocompatibility complex (MHC) molecules, or parts of these molecules, can be used to trap antibodies specific to these molecules. The antigen/antibody pair can further be replaced with any members of sets of binding pairs that would have specific affinities. Examples are ligands and receptors with some specificity to a pathogen.

Substance A and B antigens can be procured from Dade International in Switzerland (trade name: Neutr-AB). This mixture of Substance A and B antigen] can be from a variety of natural sources, including but not limited to cows, pigs, horses and humans. These antigens, in their most reduced form trisaccharides, can also be made synthetically. A higher affinity for the antigen will exist when the antigen matches the original antigen to which the antibodies were produced. Likewise, the more purified the antigen is, the stronger the reaction.

The more antigen is present, immobilized directly on the lumenal surface or attached by a linking molecule in the enclosed pathway, the more specific antibody can be removed from the flowing blood. Likewise, the larger the surface area of the coated membrane, the higher the capacity for binding the desired antibody. For instance, 100mg of antigen non-diffusively linked to a hollow fiber can significantly reduce the anti-A and anti-B titers of 300 to 400ml of blood with from average to high titer. Fig. 6 shows the capacity of a modified hollow fiber to sequentially process 100ml of banked human blood. Titer is determined by using a standard hemagglutination assay. This shows that

membrane-bound antigen can specifically remove anti-A and anti-B antibodies, and that this removal takes place in the first 15 minutes of flow (about 3 passages of the blood over the membrane), regardless of original titer. Alternatively, one antigen type, such as Substance A or B, can be used.

Figure 7 shows the capacity of filters modified with A antigen for anti-A antibodies. Figure 8 shows the same using B antigen for anti-B antibodies. Consecutive samples of blood were passed over the membrane until the membrane was saturated. At this point the titer of antibody in the blood samples no longer decreased upon passage over the membrane. The anti-A coated membrane had a capacity of around 300-400 ml. of average to high titer blood. The anti-B coated membrane had a capacity of around 600 ml.

Further purification of the standard antigens leads to at least a six fold increase in capacity to remove anti-A and anti-B antibodies per mg of antigen. Purification is achieved by removing components having molecular weight below 12,000 daltons from the commercially available antigen solution by dialysis. For example, the anti-A antibody capacity of a dialysis filter modified with approximately 40 mg of purified antigen reduced the anti-A titer of each of six 150 ml blood samples to 2 or below. The standard non-purified antigen-modified filter reduced the anti-A titer of the first sample from 32 to 8, and caused no titer reduction of the other five samples. The results were similar for the anti-B antibodies. Hence we expect that a dialysis filter modified with 100 mg of purified may be able to significantly reduce the anti-A and anti-B titers of 1.8 to 2.4 L of average to high titer blood.

Linking of binding partner to the enclosed pathway

The antigen, antibody, binding pair member, ligand, or binding parts thereof, can be linked to the enclosed pathway by a variety of standard linking techniques, including but not limited to chemical modifications, covalent bonding, strong ionic or hydrogen bonding, use of a linker, etc. The preferred method uses standard cyanogen bromide (CNBr) linking which starts by treating the enclosed pathway with CNBr followed by incubation of the antigen and the modified pathway. The N-terminus of the antigen protein will covalently attach to the CNBr linker. Other compounds for treating the enclosed pathway include, but are not limited to, hydrogen peroxide, sodium periodate, epichlorohydrin, 1,4-butanedioldiglycidol ether, cyanuric chloride, carbonyldiimidazole, substituted sulfonyl chloride, or fluoromethyl pyridinium salts, and antigen applied in the same way. Standard chemical linkers such as avidin and biotin can also be used.

Process

Filtration

Filtration of unwanted molecules from blood can be achieved using standard kidney dialysis type equipment which removes blood from one arm and returns it to the other. Alternatively, any pumping system connected to the patient at two sites, so as to draw blood from one site and return it to the other, will work. The blood is passed through the enclosed pathway having immobilized binding partners. The binding partners sequester the unwanted molecules as they move along. Several passes of the blood along the pathway might be required to completely remove the specific unwanted molecules.

The flow rate of blood moving through the pathway must be fast enough to prevent coagulation, yet not so fast as to damage the blood cells. Examples of ranges are from about 10 to about 1000 ml of blood per min., preferably between about 50 and about 750 ml/min., and most preferably the flow rate for removal of antibodies using the invention is between about 100 and about 500ml/min. Heparin can also be added to the blood to prevent coagulation. Processing of an entire host's blood volume (~5L) would require approximately 2.5 hours to achieve complete removal of antibodies or other undesired molecules from the blood.

The flow can be continuous. Alternatively, the flow can be interrupted to increase the interaction of the unwanted molecules with their immobilized binding partners. Likewise, the shape of the device having the immobilized binding partners can be such that it will encourage some swirling and/or backflow to increase the interaction time between the unwanted molecules and the immobilized binding partners.

Uses

The current invention can be used in reducing organ or tissue transplant rejection by removing specific antibodies against foreign antigens found in the transplanted organ or tissue and providing circulating blood substantially free of these antibodies. The invention can also be used as part of a quantitative assay for specific antibodies found in the blood. For example, a whole body assay for the titer of anti-A and anti-B antibodies can be performed. First, the antibodies from the blood can be removed by the filtration described above. Second, the bound antibodies are released by competing with free floating antigens or with other very low ionic strength buffers to prevent binding. Third, released antibodies can be titered using a method such as a hemagglutination assay.

Also, the invention can be used to preparatively purify specific antibodies from the blood without the need to plasmapheresc. The steps are similar to the quantification assay described above.

Additionally, the invention can be used to remove excess amounts of antibodies present in the blood.

Further, the invention can be used to identify, quantify and/or remove other molecules having binding partners, such as virions or ligands, from the host's blood.

Claims

1. A method for reducing the presence of unwanted molecules in a host's blood in a single step, comprising:

a) extracting blood from the host;

b) moving the extracted blood in a continuous or temporarily interrupted flow along an enclosed pathway, wherein the pathway has immobilized binding molecules specific to the unwanted molecules attached along the pathway, and the binding molecules are capable of binding to the unwanted molecules in the blood of the host; and

c) returning the blood to the host's internal circulation.

2. A method in accordance with claim 1 wherein the unwanted molecules are antibodies specific to foreign donor antigens present in a transplanted organ or tissue.

3. A method in accordance with claim 1 wherein the unwanted molecules are virions or sub-particles thereof.

4. A method in accordance with claim 1 wherein the unwanted molecules are antibodies associated with a disease state comprising an excess of antibodies present in the blood.

5. A method in accordance with claim 1 wherein the pathway is selected from the group consisting of a flat dialyzer, a semi-permeable membrane, a semi-permeable hollow fiber, a coil, dialysis membrane, a plasmapheresis filter, and combinations thereof.

6. A method in accordance with claim 5 wherein the pathway is at least partially dimpled, twisted, or otherwise modified to increase mixing and binding of unwanted molecules.

7. A method in accordance with claim 5 wherein the pathway is composed of a material selected from the group consisting of nitrocellulose, cellulose, nylon, plastic, rubber, polyacrylamide, agarose, poly(vinylalcohol-co-ethylene), and combinations thereof.

8. A method in accordance with claim 1 wherein the specific binding molecules are selected from antigens, antibodies, anti-antibodies, ligands, receptors, binding portions thereof, and combinations thereof.

9. A method in accordance with claim 8 wherein the antigens are selected from the group consisting of A blood type antigens, B blood type antigens, protein A molecules, protein G molecules, major histocompatibility complex molecules, binding portions thereof, and combinations thereof.

10. A method in accordance with claim 1 wherein the antigens are attached along the semi-permeable pathway by a process selected from the group consisting of chemical modifications, covalent bonding, strong ionic bonding, hydrogen bonding, and use of a linker.

11. A method in accordance with claim 10 wherein the chemical modification is accomplished by treatment with a compound selected from the group consisting of cyanogen bromide, hydrogen peroxide, sodium periodate, epichlorohydrin, 1,4-butanedioldiglycidol ether, cyanuric chloride, carbonyldiimidazole, substituted sulfonyl chloride, and fluoromethyl pyridinium salts.

12. A method in accordance with claim 1 wherein the antigens are attached along the enclosed pathway by avidin or biotin linkers.

13. A method in accordance with claim 5 wherein the specific antigens are attached to the wall of a semi-permeable hollow fiber.

14. A method in accordance with claim 13 wherein the hollow fiber is coupled with a plurality of enclosed parallel membranes.

15. A method in accordance with claim 14 wherein the plurality of enclosed parallel membranes are arranged perpendicular to the hollow fiber.

16. A method in accordance with claim 14 wherein the plurality of enclosed parallel membranes are longitudinally arranged inside and along the length of the hollow fiber.

17. A method in accordance with claim 14 wherein the plurality of parallel membranes have antigens attached to them.

18. A method in accordance with claim 1 wherein the blood is moved into, along an enclosed pathway within, and out of a container, wherein the container is closed except for entrance and exit openings, and wherein the container comprises

a) a slurry having immobilized binding partners attached to the slurry particles, and

b) at least one flat semi-permeable membrane, the membrane dividing the flowing blood from the slurry,

wherein the blood components can exchange with the slurry across the membrane.

19. A method in accordance with claim 18 wherein the flat semi-permeable membrane is made of a material selected from the group consisting of nitrocellulose, cellulose, nylon, plastic, polyacrylamide, agarose, poly(vinylalcohol-co-ethylene), and rubber.

20. A method in accordance with claim 18 wherein at least one additional membrane is present in the portion of the container where the blood flows, and wherein the additional membrane or membranes are arrayed either perpendicularly or horizontally to the blood flow.

21. Circulating blood substantially free of unwanted molecules, wherein the molecules are capable of specific or non-specific binding to a binding partner.

22. The circulating blood in accordance with claim 21 wherein the unwanted molecules are anti-A blood protein and anti-B blood protein antibodies.

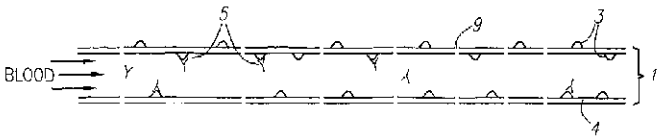


FIG. 1

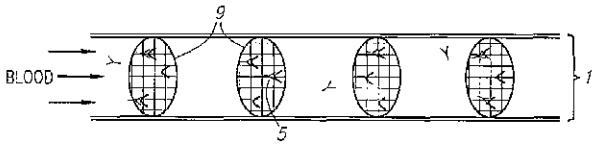
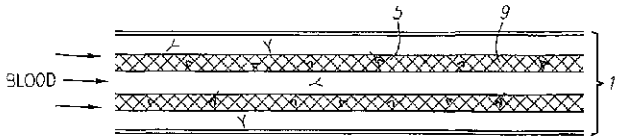


FIG. 2



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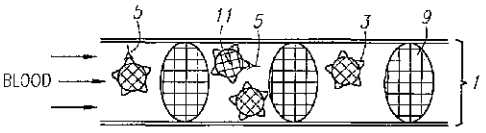

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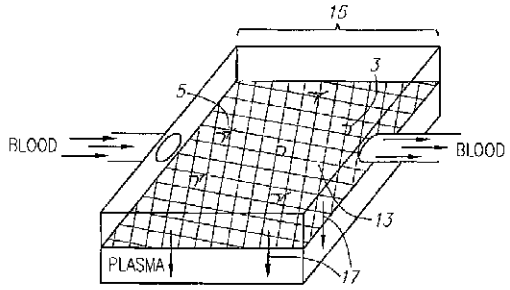

FIG. 5

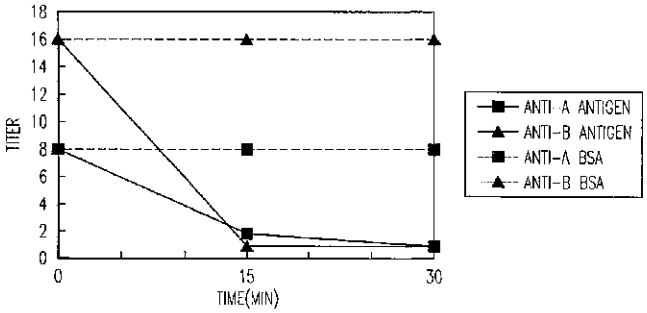
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FIG. 6

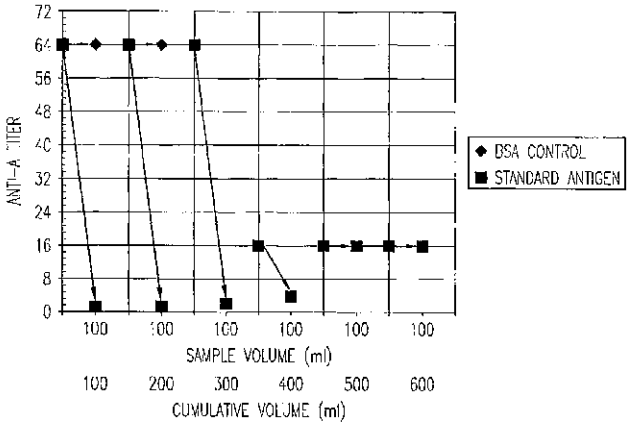
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FIG. 7

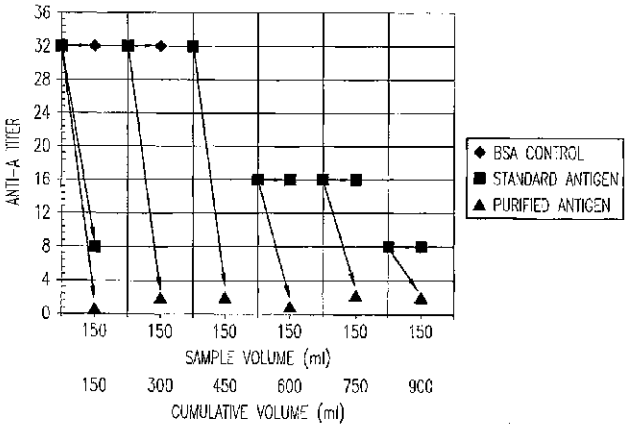
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FIG. 8

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/40049

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :B01D 61/00; A61M 1/36, 1/38
US CL. :Please See Extra Sheet.
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. : Please See Extra Sheet.

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
X -- Y	US 4,787,974 A (AMBRUS et al) 29 November 1988 (29.11.88), see entire document.	1-5, 7-9, 13, 21 ---------- 10-11
X -- Y	US 5,753,227 A (STRAHILEVITZ) 19 May 1998 (19.05.98), see entire document.	1-2, 5-6, 8, 10-11, 18, 21 ---------- 14-17, 20
X -- Y	US 5,871,649 A (OFSTHUN et al) 16 February 1999 (16.02.99), see entire document.	1-5, 7-13, 21 ---------- 14, 19, 22

☐ 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 document 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
31 JULY 2000	22 AUG 2000
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 SUN UK KIM Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/40049

A. CLASSIFICATION OF SUBJECT MATTER:
US CL .

210/195.2, 321.75, 321.78, 321.79, 321.8, 321.84, 321.87, 321.88, 321.89, 500.23, 502.1, 645, 646, 650, 651; 435/2, 7.1, 174; 436/528, 531, 532, 535; 530/413, 811, 812, 815, 816, 817; 604/4.01, 5.01, 5.02, 5.03, 5.04, 644

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

210/195.2, 321.75, 321.78, 321.79, 321.8, 321.84, 321.87, 321.88, 321.89, 500.23, 502.1, 645, 646, 650, 651; 435/2, 7.1, 174; 436/528, 531, 532, 535; 530/413, 811, 812, 815, 816, 817; 604/4.01, 5.01, 5.02, 5.03, 5.04, 644

Form PCT/ISA/210 (extra sheet) (July 1998)•

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Ｂ０１Ｄ 71/20	Ｂ０１Ｄ 61/24		
Ｂ０１Ｄ 71/24	Ｂ０１Ｄ 71/08		
Ｂ０１Ｄ 71/38	Ｂ０１Ｄ 71/10		
Ｂ０１Ｄ 71/40	Ｂ０１Ｄ 71/20		
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	Ｂ０１Ｄ 71/38		
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