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(54)【発明の名称】子宮内膜症の診断アッセイ

(57)【要約】
本発明は、患者の子宮内膜症の検出法を提供し、これは浸入性で高価な外科的処置の改良である。本方法は、トムセン－フリーデンライヒ抗原（Ｔｆ）と反応する血清サンプル中の自己抗体を検出する免疫アッセイを使用する。Ｔｆ様抗原に結合する患者由来の血清サンプル中の自己抗体レベルの増加は、患者の子宮内膜症の指標である。

【特許請求の範囲】
【請求項１】
（ａ）患者から血清サンプルを得て、
（ｂ）前記血清サンプルとトムセン－フリーデンライヒ（Ｔｆ）様抗原をインキュベートし、
（ｃ）前記サンプル中のＴｆ様抗原との自己抗原反応性を検出し、
（ｄ）前記血清サンプル中のＴｆ様抗原に対する自己抗原反応性レベルの増加を前記患者の子宮内膜症診断に相関させる、
という諸段階を含む、子宮内膜症の診断法。
【請求項２】
前記自己抗体反応性を免疫アッセイによって決定する、請求項１に記載の方法。
【請求項３】
前記免疫アッセイが免疫測定アッセイである、請求項２に記載の方法。
【請求項４】
前記免疫アッセイが競合免疫アッセイである、請求項２に記載の方法。
【請求項５】
（ａ）固体支持体上のＴｆ様抗原を固定し、
（ｂ）患者由来の血清サンプルの分取を前記固体支持体に結合させたＴｆ様抗原に添加し、インキュベートし、
（ｃ）前記固体支持体に標識抗ヒト免疫グロブリンを添加、ここで前記抗ヒト免疫グロブリンはシグナル発生系の一部である、
（ｄ）結合抗体から遊離の標識抗体を分離し、
（ｅ）前記固体支持体を含む溶液によって発生したシグナルを測定し、
（ｆ）患者の子宮内膜症診断にシグナル強度の増加を相関させる、
という諸段階を含む、請求項３に記載の方法。
【請求項６】
（ａ）一定量のＴｆ様抗原に結合した標識抗体と異なる濃度の患者由来の血清サンプルとのインキュベーションによって反応混合物を調製、ここで前記標識抗体はシグナル発生系の一部である、
（ｂ）遊離自己抗体から結合標識抗体を分離し、
（ｃ）前記反応混合物中の標識抗体によって発生したシグナルを測定し、
（ｄ）患者の子宮内膜症診断に患者由来の血清サンプルの添加後のシグナル強度の減少を相関させる、
という諸段階を含む、請求項４に記載の競合免疫アッセイ。
【請求項７】
（ａ）Ｔｆ様抗原に結合した第１の抗体と、異なる濃度の患者由来の血清サンプルとのインキュベーションによって反応混合物を調製し、
（ｂ）前記反応混合物に一定量の二次抗体を添加、ここで前記二次抗体は前記第１の抗体の重鎖の定常領域を認識し、前記二次抗体は標識されており、且つシグナル発生系の一部である、
（ｃ）結合抗体から遊離の標識二次抗体を分離し、
（ｄ）前記反応混合物中の前記二次抗体によって発生したシグナルを測定し、
（ｅ）患者の子宮内膜症診断にシグナル強度レベルの減少を相関させる、
という諸段階を含む、請求項４に記載の競合免疫アッセイ。
【請求項８】
前記抗体が酵素標識されており、追加の段階として前記酵素標識抗ヒト免疫グロブリンと反応する固体支持体に基質を添加する段階とその後で行うインキュベーションとが（ｄ）の段階と（ｅ）の段階との間にある、請求項５に記載の方法。
【請求項９】
前記抗体が酵素標識されており、追加の段階として前記酵素標識抗ヒト免疫グロブリンと

反応する固体支持体に基質を添加する段階とその後で行うインキュベーションとが（ｂ）の段階と（ｃ）の段階との間にある、請求項６に記載の方法。
【請求項１０】
前記二次抗体が酵素標識されており、追加の段階として前記酵素標識抗ヒト免疫グロブリンと反応する固体支持体に基質を添加する段階とその後で行うインキュベーションとが（ｃ）の段階と（ｄ）の段階との間にある、請求項７に記載の方法。
【請求項１１】
前記標識抗体が放射標識されている、請求項５、請求項６、または請求項７のいずれかに記載の方法。
【請求項１２】
前記標識抗体がビオチン標識されている、請求項５、請求項６、または請求項７のいずれかに記載の方法。
【発明の詳細な説明】
【０００１】
（発明の背景）
子宮内膜症は、子宮外（異所性）部位での子宮内膜細胞の成長によって特徴付けられる、珍らしくない疾患である。これは、生殖年齢の女性の１０％が罹患し得る、通常の疾患である（１）。子宮内膜症の病因が謎であるにもかかわらず、明らかに細胞性および体液性免疫機能の変化が発症した疾患の特徴である（２～４）。
【０００２】
子宮内膜抗原に対する自己抗体および補体成分の沈積が多数の研究に記載されており（（２）に概説）、多数の血清、腹腔内貯留液、および子宮内膜抗原が記載されている。おそらく、これまでのところ記載された最良に特徴付けられた組織抗原は、ヒト絨毛性ゴナドトロピン受容体（５）および炭酸脱水酵素６－８のイソ型ＩおよびＩＩである。トランスフェリンおよびα$_2$－ヘーレマンス・シュミット糖タンパク質（α$_2$－ＨＳＧ）に対する抗体もまた記載されており、診断マーカー[9、10]として提案されている。子宮内膜症、生殖疾患、および他の自己免疫疾患におけるこれら抗体の発現率の測定に関して多数の研究が行われてきたが、関連するエピトープの性質についてはほとんど注目されていなかった。同定された抗原は、全て糖タンパク質である。たった１つの明白な例外（５）を除いて、これらのタンパク質上の炭水化物抗原は評価されていなかった。
【０００３】
本発明によれば、驚いたことに、共通の炭水化物部分が異なる上記の子宮内膜抗原上に存在することが見出された。共通の炭水化物部分は、Ｔｆ抗原またはＴｆ様抗原とも呼ばれているトムセン－フリーデンライヒ関連抗原、Ｇａｌβ１－３ＧａｌＮＡｃである。本明細書中では、「Ｔｆ様抗原」は、Ｔｆ抗原をも含むものとする。Ｔｆ抗原はシアル酸によってマスクされた、潜在性二糖類構造である。シアル酸部分は、ノイラミニダーゼなどのシアリダーゼによって除去することができる。Ｔｆ抗原は、ヒト赤血球上に存在し、上皮組織中の腫瘍関連抗原である。
【０００４】
本発明は、Ｔｆ様抗原との自己抗体反応性に基づいた診断法を提供する。本診断法は、患者の子宮内膜症の存在判定の一助となり、現在の侵入性の診断法よりも改善されている。
【０００５】
（発明の要約）
本発明は、患者の子宮内膜症の診断法を提供する。本発明の１つの実施形態では、患者の子宮内膜症の診断法は、
（ａ）前記患者から血清サンプルを得て、
（ｂ）前記血清サンプルとトムセン－フリーデンライヒ（Ｔｆ）様抗原をインキュベートし、
（ｃ）前記サンプル中のＴｆ様抗原との自己抗原反応性を検出し、
（ｄ）前記血清サンプル中のＴｆ様抗原に対する自己抗原反応性レベルの増加を前記患者

の子宮内膜症診断に相関させる、という諸段階を含む。
【０００６】
抗体反応性は免疫測定アッセイまたは競合アッセイなどの免疫アッセイによって判定することができる。
【０００７】
本発明の１つの実施形態では、免疫測定アッセイは、
（ａ）固体支持体上のＴｆ様抗原を固定化し、
（ｂ）患者由来の血清サンプルの一定量を前記固体支持体に結合させたＴｆ様抗原に添加し、インキュベートし、
（ｃ）前記固体支持体に標識抗ヒト免疫グロブリンを添加し、ここで前記抗ヒト免疫グロブリンはシグナル発生系の一部であり、
（ｄ）結合抗体から遊離の標識抗体を分離し、
（ｅ）前記固体支持体を含む溶液によって発生したシグナルを測定し、
（ｆ）シグナル強度の増加を患者の子宮内膜症診断に相関させる、という諸段階を含む。
【０００８】
本発明の別の実施形態では、競合免疫アッセイは、
（ａ）一定量のＴｆ様抗原に結合した標識抗体と異なる濃度の患者由来の血清サンプルとのインキュベーションによって反応混合物を調製し、ここで前記標識抗体はシグナル発生系の一部であり、
（ｂ）遊離自己抗体から結合標識抗体を分離し、
（ｃ）前記反応混合物中の標識抗体によって発生したシグナルを測定し、
（ｄ）患者由来の血清サンプルの添加後のシグナル強度の減少を患者の子宮内膜症診断に相関させる、という諸段階を含む。
【０００９】
本発明の別の実施形態では、競合免疫アッセイは、
（ａ）Ｔｆ様抗原に結合した第１の抗体と、異なる濃度の患者由来の血清サンプルとのインキュベーションによって反応混合物を調製し、
（ｂ）前記反応混合物に一定量の第二の抗体を添加し、ここで前記第二の抗体は前記第１の抗体の重鎖の定常領域を認識し、前記第二の抗体は標識化されており、且つシグナル発生系の一部であり、
（ｃ）結合抗体から遊離の標識第二抗体を分離し、
（ｄ）前記反応混合物中の前記第二抗体によって発生したシグナルを測定し、
（ｅ）シグナル強度レベルの減少を患者の子宮内膜症診断に相関させる、という諸段階を含む。
【００１０】
抗体が酵素標識されている場合には、追加の段階として酵素標識抗体と反応する固体支持体に基質を添加しさらにインキュベーションを行う段階を、発生したシグナルの測定前に追加する。
【００１１】
（発明の詳細な説明）
本発明は、患者の子宮内膜症を検出するための診断法を提供する。本診断法は、トムセンーフリーデンライヒ（Ｔｆ）抗原（Ｔｆ様抗原をも含む）と反応する患者の血清中の自己抗体の存在を検出する免疫学的検定法に基づく。本明細書中では、「Ｔｆ様抗原」は、Ｔｆ抗原をも含むものとする。Ｔｆ様抗原に結合する自己抗体の正常な血清レベルと比較する場合、Ｔｆ様抗原に結合する血清サンプル中の自己抗体レベルの増加は、患者の子宮内膜症の診断と相関する。
【００１２】
血液サンプルは、静脈穿刺または他の適切な手段によって、都合よく採取することができる。血清サンプルは、周知の方法を使用して血液サンプルから調製することができる。
【００１３】

本発明の方法に使用することができる多数の異なる型の免疫アッセイが存在する。周知の免疫アッセイ（例えば、酵素結合免疫吸収アッセイ（ＥＬＩＳＡ）、蛍光免疫吸着アッセイ（ＦＩＡ）、化学結合免疫吸着アッセイ（ＣＬＩＡ）、放射免疫アッセイ（ＲＩＡ）、および免疫ブロット法など）を、Ｔｆ様抗原と反応する血清サンプル中の自己抗体レベルの検出に適応させることができる。使用することができる異なる免疫アッセイの概説については、「免疫アッセイハンドブック」、Ｄａｖｉｄ　Ｗｉｌｄ編、Ｓｔｏｃｋｔｏｎ　Ｐｒｅｓｓ、Ｎｅｗ　Ｙｏｒｋ、１９９４を参照のこと。好ましくは、固相分離を使用する競合免疫アッセイまたは抗体試験用の免疫測定アッセイを使用する。「免疫アッセイハンドブック」、第２章を参照のこと。

【００１４】

典型的なアッセイでは、試薬には、患者由来の血清サンプル、検出すべき自己抗体（血清サンプル中に含まれる）、Ｔｆ様抗原、および検出可能なシグナル発生手段が含まれる。

【００１５】

したがって、本発明の１つの実施形態では、患者の子宮内膜症の診断法は、抗体試験のための免疫測定アッセイを使用する。この実施形態では、Ｔｆ様抗原を、ビーズ、プレート、スライド、またはマイクロタイター皿などの固体支持体または表面に固定する。患者由来の血清サンプルの一定量を固体支持体に添加し、液相中でＴｆ様抗原とインキュベートする。Ｔｆ様抗原に反応する血清中に存在するヒト自己抗体中の定常領域を認識する抗体を添加する。この抗体は、抗ヒト免疫グロブリンであり、シグナル発生系の一部でもある。ＩｇＡ、ＩｇＧ、またはＩｇＭ重鎖定常領域に特異的な抗ヒト免疫グロブリンを使用することができる。液相からの固体支持体の分離後、支持相を検出可能なシグナルの有無を試験する。固体支持体上のシグナルの存在は、血清サンプル中に存在するＴｆ様抗原に対する自己抗体が固体支持体上のＴｆ様抗原に結合したことを示す。

【００１６】

シグナル発生系は、１つまたは複数の成分からなり、その少なくとも１つの成分は標識であり、この標識は、結合および／または非結合標識の量、すなわちＴｆ様抗原に結合または非結合している標識の量をあらわす検出可能なシグナルを発生する。標識は、シグナルを発生するか、誘発されてシグナルを発生できる分子である。標識の例には、蛍光発生物質、酵素、化学発光物質、光増感物質、または懸濁可能な粒子が含まれる。シグナルは、酵素活性、発光、または光吸収によって検出し、またこれによって測定することもできる。放射標識もまた使用することができ、シンチレーションカウンターを使用して放射能レベルを検出および測定をすることができる。

【００１７】

抗ヒト免疫グロブリンの標識に使用することができる酵素の例には、β－Ｄ－ガラクトシダーゼ、ホースラディッシュ・ペルオキシダーゼ、アルカリホスファターゼ、およびグルコース－６－リン酸脱水素酵素（「Ｇ６ＰＤＨ」）が含まれる。抗ヒト免疫グロブリンの標識に使用することができる蛍光物質の例には、フルオレセイン、イソチオシアネート、ローダミン化合物、フィコエリトリン、フィコシアニン、アロフィコシアニン、ｏ－フタルアルデヒド、およびフルオレスカミンが含まれる。化学発光物質には、例えば、イソルミノールが含まれる。

【００１８】

遊離標識抗体を結合抗体から分離し、必要ならば、標識（例えば酵素）が反応する適切な基質を添加して、インキュベートする。

【００１９】

好ましい実施形態では、抗ヒト免疫グロブリンは、ホースラディッシュ・ペルオキシダーゼまたはアルカリホスファターゼのいずれかで標識した酵素である。

【００２０】

反応の際に現れる色彩、蛍光、発光、または放射能の量（使用したシグナル発生系に依存する）は、Ｔｆ様抗原と反応する患者の血清中の自己抗体の量に比例する。光学密度の定量化は、分光光度法を使用して行うことができる。放射標識シグナルの定量化は、シンチ

レーション計数法によって行いうる。

【００２１】

正常な血清レベルを超えるＴｆ様抗原に反応する自己抗体レベルの増加は、患者の子宮内膜症診断に相関する。

【００２２】

本発明の別の実施形態では、患者の子宮内膜症の診断法は、既知の抗体と患者の自己抗体とがＴｆ結合を競合する、競合免疫アッセイを使用する。この実施形態では、Ｔｆ様抗原に結合することが知られている一定量の標識抗体を、異なる濃度の患者由来の血清サンプルとインキュベートする。例えば、Ｔｆに結合することが公知のマウスモノクローナル抗体４９Ｈ．８（Ｒａｈｍａｎ ａｎｄ Ｌｏｎｇｅｎｅｃｋｅｒ、１９８２、Ｊ．Ｉｍｍｕｎ．，１２９（５）：２０２１～４）を使用しうる。Ｔｆ様抗原に結合し、競合免疫アッセイに使用することができる他のモノクローナル抗体には、１５５Ｈ７および１７０Ｈ８２（Ｌｏｎｇｅｎｅｃｋｅｒ等、１９８７、Ｊ．Ｎａｔ．Ｃａｎｃｅｒ Ｉｎｓｔ．，７８（３）：４８９～９６）、Ａ７８－Ｇ／Ａ７（Ｋａｒｓｔｅｎ等、１９９５、Ｈｙｂｒｉｄｏｍａ，１４（１）：３７～４４）、ＨＢ－Ｔ１（ＤＡＫＯ Ｃｏ．）、ＲＳ１－１１４およびＡＨＢ－２５Ｂ（Ｓｔｅｉｎ等、１９８９、Ｃａｎｃｅｒ Ｒｅｓ．，４９（１）：３２～７）、ＨＴ８（Ｍｅｔｃａｌｆｅ等、１９８４、Ｂｒ．Ｊ．Ｃａｎｃｅｒ，４９（３）：３３７～４２）、１６１Ｈ４（Ｌｏｎｇｅｎｅｃｋｅｒ等、１９８７）、ＨＨ８（Ｃｌａｕｓｅｎ等、１９８８）、ならびにＢＷ８３５（Ｈａｎｉｓｈ等、１９９５、Ｃａｎｃｅｒ Ｒｅｓ．，５５（１８）：４０３６～４０）が含まれる。上記のように、抗体を、蛍光発生物質、酵素、化学発光物質、光増感物質、懸濁可能な粒子、または放射性同位元素で標識することができる。好ましくは、公知の抗体は酵素標識されている。インキュベーション後、結合した標識抗体を、遊離の自己抗体から分離する。使用するシグナル発生系によっては、必要ならば、標識抗体と反応する適切な基質を添加してインキュベートする。次いで、サンプルが発生したシグナルを測定する。血清サンプルの添加前と添加後、または実験サンプルと対照サンプルとの間の添加による、光学密度または放射能の減少は、血清サンプル中の自己抗体がＴｆに結合したことを示す。健常者由来の実験血清サンプルと比較した場合の光学密度または放射標識シグナルの減少は、患者の子宮内膜症診断に相関する。

【００２３】

競合免疫アッセイの好ましい実施形態では、２つの抗体を使用する間接的方法を使用する。第１の抗体は、標識されていないこと以外は、上の段落に記載のＴｆ様抗原特異的抗体である。第１の抗体を、種々異なる濃度の患者由来の血清サンプルとインキュベートする。一定量の第２の抗体を次にサンプルに添加する。第２の抗体は、第１の抗体の重鎖の定常領域を認識する。例えば、第２の抗体は、Ｔｆ様抗原に反応するマウス免疫グロブリン（抗マウス免疫グロブリン）の重鎖の定常領域を認識する抗体であり得る。第２の抗体を、上記の蛍光標識体、化学発光体、または放射性同位元素で標識する。遊離の第２の抗体を、結合抗体から分離する。酵素標識抗体を使用する場合、酵素標識に反応する適切な基質を添加してインキュベートする。血清サンプルの添加前と添加後、または添加による実験サンプルを対照サンプルと比較しての、光学密度または放射能の減少は、血清サンプル中の自己抗体がＴｆに結合したことを示す。健常者由来の実験血清サンプルと比較した場合の光学密度または放射能の減少は、患者の子宮内膜症診断に相関する。

【００２４】

周知の方法を使用した本発明の方法のために、酵素をＴｆ様抗原反応性抗体に共有結合することができる。多数の周知の結合法が存在する。例えば、グルタルアルデヒドを使用して、アルカリホスファターゼおよびホースラディッシュ・ペルオキシダーゼを抗体に結合させることができる。ホースラディッシュ・ペルオキシダーゼを、過ヨウ素酸塩を使用して結合させることもできる。酵素結合抗体用の市販のキットも広範に販売されている。酵素結合抗ヒトおよび抗マウス免疫グロブリン特異的抗体は、複数の販売者から市販されている。

【００２５】
酵素結合抗体の代わりに、ビオチン標識抗体を使用することができる。このような場合、市販のストレプトアビジン－ホースラディッシュ・ペルオキシダーゼ検出システムを使用して、結合抗体を検出する。
【００２６】
酵素標識抗体は、基質の種類によってそれぞれ異なるシグナル発生源をつくる。シグナル発生には、反応混合物への基質の添加を行う。通常のペルオキシダーゼ基質としては、ＡＢＴＳ（登録商標）（２，２’－アジノビス（エチルベンゾチアゾリン－６－スルホナート））、ＯＰＤ（Ｏ－フェニレンジアミン）およびＴＭＢ（３，３’、５，５’－テトラメチルベンジジン）が含められる。これらの基質は、過酸化水素の存在が必要である。ｐ－ニトロフェニルホスフェートは、普通に使用されるアルカリホスファターゼ基質である。インキュベーション中、酵素は基質の一部を徐々にその最終生成物に変化させる。インキュベーション後、酵素活性を停止させる停止試薬が添加される。シグナル強度は、通常分光光度計を使用して光学密度の測定によって決定される。
【００２７】
アルカリホスファターゼ標識抗体は、蛍光測定法によっても測定することができる。したがって、本発明の免疫アッセイでは、基質４－メチルウンベリフェリルホスフェート（４－ＵＭＰ）を使用することができる。４－ＵＭＰはアルカリホスファターゼによる脱リン酸化により、４－メチルウンベリフェロン（４－ＭＵ）という蛍光標識体が形成される。入射光は３６５ｎｍであり、放射光は４４８ｎｍである。
【００２８】
本発明の方法で使用するために、Ｔｆ様抗原を、種々の供給源から得ることができる。例えばＴｆ様抗原は、アメリカン・タイプ・カルチャー・コレクション（ＡＴＣＣ）から入手可能な腺癌細胞株ＬＳ１７４Ｔなどの腫瘍細胞株の培養に使用した、馴化培養培地から精製することができる。移行性上皮癌株細胞もまた、Ｔｆ様抗原の供給源としての機能を果たしうる。Ｔｆ様抗原は、ＭＡｂ４９Ｈ．８－ＣｎＢｒ活性化セファロースカラムを使用したアフィニティークロマトグラフィーによって、このような馴化培養培地から精製することができる。それ以上に精製するにはゲル濾過を使用することもできる。Ｔｆ様抗原をまたそれ以上に精製するには不溶化落花生凝集素（ＰＮＡ）またはその他のレクチンを使用したレクチンアフィニティークロマトグラフィーを使用することができる。ＰＮＡは、カリフォルニア州サンマテオのＥ－Ｙ　Ｌａｂｏｒａｔｏｒｉｅｓから入手することができる。
【００２９】
ＩｇＡ、ヘモペキシン、およびα－２－ヘーレマンス・シュミットなどのＴｆ様抗原を含む精製血清タンパク質もまた、抗原として使用することができる。Ｔｆ様抗原の好ましい供給源は、ウシ血清アルブミンと共有結合した市販の合成Ｔｆ様抗原である。最近Ｄａｈｌｅｎｂｏｒｇ等による、１９９７、Ｉｎ．Ｊ．Ｃａｎｃｅｒ　７０：６３～７１に記載のように、ＢｉｏＣａｒｂなどのＴｆ様抗原およびその糖形態が市販されている。あるいはＴｆ様抗原は、市販業者からカスタム合成によって得ることができる。Ｔｆ様抗原と反応するモノクローナル抗体を容易に利用することができる。
【００３０】
ＭＡｂ４９．Ｈ（イソ型ＩｇＭ）を、Ｒａｈｍａｎ等、（１９８２）Ｊ．Ｉｍｍｕｎｏｌ．，１２９：２０２１～２０２４およびＬｏｎｇｅｎｅｃｋｅｒ等、（１９８４）Ｉｎｔ．Ｊ．Ｃａｎｃｅｒ　３３：１２３～１２９によって報告されているように、調製および精製することができる。これらの論文および本出願で引用した他の論文の開示は、それらが全文提示されていると同様にみなして本明細書中の一部とみなすべきである。
【００３１】
本発明の方法を実施するために、関連する免疫アッセイを標準化しなければならない。Ｔｆ様抗原は通常、糖タンパク質と会合しているので、天然では不均一である。このような不均一性の原因としては、シアリル化を含み得る。このために、１アンプルの国際標準（

ＩＳ）または国際基準調製（ＩＲＰ）を得るべきである。国立生物学的製剤研究所（ＮＩＢＳＣ、Ｂｌａｎｃｈｅ Ｌａｎｅ、Ｓｏｕｔｈ Ｍｉｍｍｕｓ、Ｐｏｔｔｅｒｓ Ｂａｒ、Ｈｅｒｔｓ ＥＮ６３ＱＧ）は、このようなＴｆ様抗原のサンプルを調製し、その国際単位（ＩＵ）を指定することができる。また今後の多数のキャリブレータに標準値を与えるために、複数の二次標準のセットも調製しなければならない。

【００３２】

本発明の免疫アッセイは、未知のサンプルに数値または濃度を指定するためのキャリブレータの使用を必要とする。通常は未知のサンプルをプロットする前に、約６つのキャリブレータの組をプロットして、検量線を作成する。未知サンプルの濃度は補間法により決定する。最良の方法としてはコンピュータプログラムによる補間を行う。較正に関する考察については、「免疫アッセイハンドブック」、第２章を参照のこと。

【００３３】

本発明はまた、患者の子宮内膜症を緩和、予防、および／または回復させるための治療を提供する。本発明のこの実施形態では、Ｔｆ様抗原またはそのアナログを患者に注射する。注射したＴｆ様抗原またはそのアナログは、自己抗体結合で、子宮内膜症患者に存在するＴｆ様抗原と競合する。

【００３４】

以下の実施例により、本発明をさらに例示する。

【００３５】

（実施例１）

材料と方法

組織ホモジネートの調製

新鮮な異所性および正常の子宮内膜、腹腔内貯留液、および血清を、インフォームドコンセントを行った子宮摘出患者から得た。新鮮な組織（１～２グラム）に、完全プロテアーゼ阻害剤カクテル（Ｂｏｅｈｒｉｎｇｅｒ Ｍａｎｎｈｅｉｍ社、インディアナ州インディアナポリス）を含む１０ｍｌの氷冷ＰＢＳを添加し、調製中全体を通じて４℃に維持した。組織をポリトロンホモジナイザー（Ｂｒｉｎｋｍａｎ社製、スイス ルツェルン）で１分間ホモジナイズし、続いて１３０００ｇで１０分間遠心分離した。上清を回収し、Ｂｒａｎｓｏｎ２５０ソニフィアー（コネチカット州ダンベリー）を使用して、超音波処理した。組織ホモジネートを、０．２２ｍｍで濾過し、２０℃で保存した。

【００３６】

血清の調製

末梢静脈血（６０ｍｌ）を健常なボランティアから、ガラス製のバキュテイナーチューブ（添加剤なし）に採取し、４時間室温で凝固させた。次いで、血清を滅菌チューブに取り出し、４℃で３０分間１２０００ｇの遠心分離により脱脂した。

【００３７】

組織自己抗原の精製

ホモジナイズ後、組織抗原をプロテインＧ ＦＰＬＣカラム（Ａｍｅｒｓｈａｍ Ｐｈａｒｍａｃｉａ社、ニュージャージー州ピスカタウェイ）に通し、ＩｇＧを除去した。プロテインＧカラムに保持されなかったタンパク質を、５０ｍＭリン酸ナトリウム（ｐＨ８．０）で平衡化した陰イオン交換カラム（ＭｏｎｏＱ ＦＰＬＣ、Ａｍｅｒｓｈａｍ Ｐｈａｒｍａｃｉａ社、ニュージャージー州ピスカタウェイ）にかけた。結合したタンパク質は、０～０．５Ｍ ＮａＣｌの線形勾配をかけた同一の緩衝液中で、その後１Ｍ ＮａＣｌまでの段階勾配をかけた同一の緩衝液を使用し、カラムから溶出させた。目的のタンパク質は全て１Ｍ ＮａＣｌ勾配の前に溶出した。

【００３８】

血清自己抗原の精製

α_2－ＨＳＧを、上記の組織抗原について記載の陰イオン交換クロマトグラフィーにより、ＨｉＰｒｅｐ１６／１０ＱＸＬカラムを使用して、正常な全ヒト血清から精製した。この部分精製α_2－ＨＳＧは、いくつかの実験で試験中に示される通りに使用された。さら

なる精製を第２の陰イオン交換カラム（ＭｏｎｏＱ）にて、２０ｍＭピペラジン緩衝液（ｐＨ５．０）で平衡化し、上記のＮａＣｌ勾配を使用した同一の緩衝液で溶出して行った。最終的なα$_2$－ＨＳＧの均一調製物は、ＦＰＬＣスーパーロース１２カラム（Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ社、ニュージャージー州ピスカタウェイ）でのゲル濾過の後に得られた。α$_2$－ＨＳＧ濃度は、ＥＬＩＳＡでモニターした。

【００３９】

ＩｇＡ１は、既に記載されているように、陰イオン交換クロマトグラフィーおよびジャカリンアガロースアフィニティークロマトグラフィーにより、全血清から精製した（１１）。トランスフェリンおよびヘモペキシンは、Zn^{2+}イオンで荷電したキレート化セファロースＦａｓｔ　Ｆｌｏｗ（Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ社、ニュージャージー州ピスカタウェイ）を封入したカラムでの金属キレートクロマトグラフィーにより、全血清から精製した。カラムおよび血清は、１５０ｍＭ　ＮａＣｌ含有２０ｍＭリン酸ナトリウム緩衝液で予め平衡化した。亜鉛結合タンパク質は、０～０．５Ｍイミダゾール線形勾配をかけた同一の緩衝液を使用し、溶出させた。得られたトランスフェリンおよびヘモペキシン含有画分（ウェスタンブロット分析によって決定）を個別にプールし、上記のＭｏｎｏＱ陰イオン交換カラム上、ｐＨ８．０でさらに精製した。本プロトコールによって、電気泳動上純粋なヘモペキシン調製物が得られた。トランスフェリンはしかし、トランスフェリン調製物中のヘモペキシンから分離されなかった。

【００４０】

糖タンパク質による炭水化物－エピトープの修飾

末端シアル酸部分を、アガロース結合ノイラミニダーゼでの処理により、糖タンパク質から除去した。０．５ｍｌ中１ｍｇのタンパク質を、５０ｍＭ酢酸ナトリウム、１５０ｍＭ塩化ナトリウム、４ｍＭ塩化カルシウム（ｐＨ５．５）中、５ユニットのノイラミニダーゼ（Ｓｉｇｍａ、セントルイス、ミズーリ州）で、３７℃下一晩インキュベートした。４０００ｇ５分間の遠心分離によって、アガロースビーズを除去した。上清を取り出し、４℃で保存した。

【００４１】

中心の炭水化物基を、エンドグリコシダーゼＦ（ＥｎｄｏＦ）およびペプチド－Ｎ－グリコシダーゼＦ（ＰＮＧアーゼＦ）での処理によって糖タンパク質から除去した。これは、Ｇｌｙｋｏ（カリフォルニア州ノヴァト）からの市販のキットにより得られた。４０ｍｇのノイラミニダーゼ処理タンパク質を、２０ｍＭリン酸ナトリウムｐＨ７．５、５０ｍＭ　ＥＤＴＡ、０．１％ｖ／ｖＳＤＳ、０．５％ｂ－メルカプトエタノールの存在下、１００℃で２分間の加熱によって変性した。ＰＮＧアーゼＦのＳＤＳによる阻害を防止するため、冷却後に０．１％Ｔｗｅｅｎ－２０を変性サンプルに添加した。次いで、変性タンパク質を、６６７脱グリコシル化単位（ＤＧＵ）のＥｎｄｏ　Ｆ／ＰＮＧアーゼＦ混合物に、３７℃１８時間インキュベートした。

【００４２】

ジャカリン反応性糖タンパク質のサンプルからの除去は、ジャカリン結合アガロースビーズ（Ｖｅｃｔｏｒ、カリフォルニア州バーリンゲーム）での予備吸着によって達成した。サンプルを、ジャカリン結合アガロースで室温３０分間インキュベートし、４０００ｇ５分間遠心分離した。上清を除去し、４℃で保存した。

【００４３】

抗体および糖タンパク質

ヒツジ抗ヒトα$_2$－ＨＳＧおよびヒツジ抗ヒトトランスフェリンの免疫グロブリン画分は、Ｂｉｏｄｅｓｉｇｎ　Ｉｎｔｅｒｎａｔｉｏｎａｌ（メイン州ケネバンク）から得た。ヤギ抗ヒトヘモペキシン抗血清は、Ｋｅｎｔ　Ｌａｂｏｒａｔｏｒｉｅｓ（ワシントン州レドモンド）から得た。ＨＲＰ結合ウサギ抗ヒツジＩｇＧおよびＨＲＰ結合ウサギ抗ヤギＩｇＧは、Ｊａｃｋｓｏｎ　Ｉｍｍｕｎｏｒｅｓｅａｒｃｈ　Ｌａｂｏｒａｔｏｒｉｅｓ（ペンシルベニア州ウェストグローブ）から得た。ＨＲＰ結合ヤギ抗ヒトＩｇＡ（α鎖特異的）およびＨＲＰ結合ヤギ抗ヒトＩｇＧ（γ鎖特異的）は、（Ｓｉｇｍａ）から購入し

た。市販のα$_2$－ＨＳＧは、Ｃａｌｂｉｏｃｈｅｍ－Ｎｏｖａｂｉｏｃｈｅｍ（カリフォルニア州ラ・ホーヤ）から得た。

【００４４】

ＳＤＳ－ＰＡＧＥ

ＳＤＳ－ＰＡＧＥはＬａｅｍｍｌｉの方法（１２）にしたがって行った。手短に述べると、同体積の２×ゲルローディング緩衝液をタンパク質サンプルに添加し、５分間煮沸する。適切な場合には、タンパク質を還元条件下（ゲルローディング緩衝液中、５％ｖ／ｖのｂ－メルカプトエタノール）で溶解した。還元タンパク質の再会合は、煮沸後のヨードアセトアミド（最終濃度６０ｍＭ）の添加により防止した。タンパク質サンプルは、４％～１５％勾配ゲル（Ｂｉｏｒａｄ、カリフォルニア州ハーキュリーズ）を使用して分離した。タンパク質を直接可視可するために、ゲルを７．５％ｖ／ｖ酢酸溶液中の０．０２％ｖ／ｖ　ＳＹＰＲＯ－オレンジ（Ｂｉｏｒａｄ、カリフォルニア州ハーキュリーズ）内に３０分間置いた。次いで、ゲルを７．５％酢酸でリンスし、ゲル画像処理装置（Ａｌｐｈａ　Ｉｎｎｏｔｅｃｈ　Ｃｏｒｐｏｒａｔｉｏｎ、カリフォルニア州サンリアンドロ）上で観察した。

【００４５】

免疫ブロット法

Ｂｉｏｒａｄ　Ｔｒａｎｓｂｌｏｔ　ＳＤセミドライ・ブロッターを用い、タンパク質をＳＤＳゲルからニトロセルロース膜に転移した。ポンソー・レッド染色で、ニトロセルロース膜へのタンパク質の転移を確認した。膜は、４℃のＰＢＳ溶液中５％ｗ／ｖ脱脂粉乳、０．５％Ｔｗｅｅｎ－２０で一晩ブロックした。一次抗体または血清をブロッキング溶液に添加し、室温で２時間インキュベートした。次いで膜を、１回あたり５分間かけて６回ＰＢＳ中で洗浄した。ＨＲＰ結合二次抗体を、ＰＢＳ溶液中０．０５％ｖ／ｖ　Ｔｗｅｅｎ－２０を含む３％ｗ／ｖ脱脂粉乳で希釈した。記載のように５分間６回洗浄する前、膜を室温で２時間インキュベートした。次いで、タンパク質を増強化学発光（ＥＣＬ）（Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ社、ニュージャージー州ピスカタウェイ）により検出した。必要に応じてニトロセルロース膜を剥離し、１００ｍＭグリシン－ＨＣｌ（ｐＨ１．５）中室温で３０分間の撹拌の前に、１回あたり５分間、０．９％ｗ／ｖ　ＮａＣｌ中での洗浄を２回行うことによって再プローブした。次いで、膜を、１回あたり５分間、ＰＢＳ（ｐＨ７．４）で３回洗浄した。膜をブロックし、記載のように抗体で探索した。

【００４６】

酵素免疫測定法（ＥＬＩＳＡ）

Ｆａｌｃｏｎ　Ｍｉｃｒｏｔｅｓｔ　ＩＩＩマイクロタイタープレート（Ｂｅｃｔｏｎ　Ｄｉｃｋｉｎｓｏｎ、カリフォルニア州オックスナード）のウェルを、炭酸ナトリウム緩衝液（ｐＨ９．２）で１００μｌまで希釈した１０μｌのタンパク質にて４℃で一晩コーティングした。プレートは０．０５％ｖ／ｖ　Ｔｗｅｅｎ２０を含むＰＢＳ中で３回洗浄した。ウェルは、３回洗浄の前に、３％ｗ／ｖ　ＢＳＡのＰＢＳ／Ｔｗｅｅｎ２０（ブロッキング緩衝液）にて３７℃で１時間ブロックした。一次抗体または試験血清を、ブロッキング緩衝液で適切に希釈し、１００μｌを各ウェルに添加した。プレートを３７℃で２時間インキュベートし、ＰＢＳ／Ｔｗｅｅｎ－２０で３回洗浄した。ＨＲＰ結合二次抗体をブロッキング緩衝液で希釈し、１００μｌを各ウェルに添加した。プレートを、次いで３回洗浄した。１００ｍｌの比色定量ＨＲＰ基質であるＡＢＴを各ウェルに添加し、室温で３０分間インキュベートした。プレートを４０５ｎｍについてＤｙｎａｔｅｃｈプレートリーダー（バージニア州シャンティ）で読み取ったデータをＧｒａｐｈｐａｄ　Ｐｒｉｓｍソフトウェアを用いて分析した。

【００４７】

（実施例２）

異所性子宮内膜、正常子宮内膜、腹腔内貯留液、および血清の自己抗原の同定　可溶性子宮内膜タンパク質の調製物を、「材料と方法」の節に記載の子宮摘出組織から調製し、プ

ロテインＧクロマトグラフィーに供してＩｇＧを除去した。得られた無ＩｇＧ調製物を、陰イオン交換クロマトグラフィーに供した（図１ａ）。次いで、このカラム由来の画分を、還元条件下でのＳＤＳ－ＰＡＧＥによって分析した（図１ｂ）。同一のゲル上のタンパク質バンドを、ウェスタンブロット分析のためにニトロセルロース膜に転写した。一次抗体供給源としてプールした子宮内膜症患者およびプールした健常男性ドナー由来の血清を使用して、ウェスタンブロットの発色を行った。図１ｃは、患者（Ｗ１３４５）由来の増殖期の正常子宮内膜中で同定された抗原の典型的な例を示す。健常男性ドナー由来の対照血清（示さず）で探索した同一のブロットと比較して、自己反応性ＩｇＧは子宮内膜症患者の血清（図１ｃ）に限定された。

【００４８】

対照血清を用いたＭｏｎｏＱカラムの画分２４に対してＩｇＧのｇ鎖結合が検出されたが、他の画分（示さず）では検出されなかった。興味深いことに、画分２４に対する類似の反応性は、子宮内膜症血清を使用して発色したブロットには現れなかった。プールした子宮内膜症血清を使用して検出された反応性タンパク質の分子量は、他の研究者達[6、10]により記述された分子量と十分に相関する。プールした子宮内膜症血清で探索したウェスタンブロットによって、全部で１０本の自己反応性バンドが検出された。ＩｇＡα鎖特異的およびＩｇＧγ鎖特異的二次抗体の両方を含む画分２３で７２ｋＤａのバンドが検出された（図１ｃおよび１ｄ）。このバンドは、分子量およびＭｏｎｏＱカラムから溶出されたＮａＣｌ濃度に基づいてトランスフェリンとして最初に同定された（この位置でのトランスフェリンの溶出を、ヒツジ抗トランスフェリン抗体を使用したウェスタンブロット分析によって確認した（示さず））。この画分中にＩｇＧγ鎖特異的抗体を含む５４ｋＤａのタンパク質もまた検出されたが、ＩｇＡα鎖特異的を含むものは検出されなかった。このタンパク質は、部分的に脱シアリル化されたα$_2$－ＨＳＧと同一の位置に溶出される（データ示さず）。α$_2$－ＨＳＧは、子宮内膜症患者の自己抗原として以前に同定されている（１０）。画分２６および２９で認められた５９ｋＤａのＩｇＧ反応性バンドはＩｇＡのα鎖であり、溶出位置は、これらの作動条件では、それぞれ単量体および二量体ＩｇＡの既知の溶出位置に対応する。このタンパク質がＩｇＡα鎖であることを、ブロットを剥離して抗α鎖特異的抗体で再探索することによって確認した（示さず）。このブロットはまた、画分２３（トランスフェリン含有画分）中の抗α鎖反応性バンドの存在を示した。この画分中のＩｇＡ抗原の存在は、おそらく他の血清タンパク質[13、14]と共有結合的に複合体形成したＩｇＡに由来している。画分２６はまた、７２ｋＤａに反応性バンドを含み、画分２８および２９は、６９ｋＤａのバンドを有していた。これら後者のバンドは、α鎖特異的ブロットに対する活性を示さなかった。ＩｇＡのα鎖を認識する子宮内膜症患者のＩｇＧ抗体の存在を、高度に精製した血清ＩｇＡ１を使用したウェスタンブロットによって確認した（図７を参照のこと）。それに対して、これらの画分以外のＩｇＡα鎖を含む子宮内膜抗原に対するＩｇＡ自己抗体は認められなかった。

【００４９】

同一の患者（Ｗ１３４５）由来の卵巣異所性子宮内膜サンプルは、分子量５４、４７、４３、および３４ｋＤａの自己抗原タンパク質を有していた（示さず）。５４ｋＤａのタンパク質は、ヒツジ抗α$_2$－ＨＳＧ特異的抗体で発色させたウェスタンブロットにより、α$_2$－ＨＳＧであると判定された。α$_2$－ＨＳＧはまた、ウェスタンブロットによって異なる患者（Ｗ１５１７）由来の腹腔洗浄液中で検出された。５４ｋＤａの抗原に加えて、１８６、１２６、６８、４３、３７、および３４ｋＤａの抗原が、腹腔内貯留液中に存在していた（図２）。

【００５０】

プールした患者および各血清を用いて、２つの分子量マーカーとの反応性が認められた（図１ｃおよび図３を参照のこと）。これらのマーカーは、２０５ｋＤのミオシンおよび４２ｋＤの炭酸脱水酵素ＩＩであった。プールした男性対照およびいくつかの各男性血清の両方を用いたミオシンバンドとの反応性もまた認められた。それに対して、脱水素酵素ＩＩとの反応性は子宮内膜症血清に特異的であり、このタンパク質は他の研究者によって子

宮内膜症における自己抗原[7]、[8]と報告されている。

【００５１】

要約すれば、正常および異所性子宮内膜ならびに腹腔内貯留液由来の調製物中の自己抗原を同定した。以前に公開された報告と一致して、α$_2$－ＨＳＧ、トランスフェリンおよび炭酸脱水酵素ＩＩは、子宮内膜症患者由来の血清によって認識される自己抗原である。さらに、本明細書中に示した結果は、ＩｇＡのα鎖はまた、同一の血清によって認識される自己抗原であることを示す。これらのタンパク質がタンパク質レベルで有意な相同性を示さないので、４つ全てのタンパク質は共通のペプチド・エピトープを共有する可能性は少ない。しかし、４つのタンパク質のうち３つ（α$_2$－ＨＳＧ、ＩｇＡｌのα鎖、およびおそらく炭酸脱水酵素ＩＩ）が共通の炭水化物エピトープを共有する。このＯ結合炭水化物構造は、ジャックフルーツ（Ａｒｔｏｃａｒｐｕｓ　ｉｎｔｅｇｒｉｆｏｌｉａ）のレクチン、ジャカリンによって認識されるＧａｌβ１－３ＮＡｃＧａｌエピトープを含む。本明細書中に示した結果は、α$_2$－ＨＳＧおよびカルビニック脱水素酵素が、子宮内膜症血清中に存在する自己抗体によって認識される自己抗原であるという以前の報告を確認するものである。本発明の研究は、これらの結果を拡大して、ＩｇＡおよびヘモペキシンもまた子宮内膜症患者由来の血清によって認識される自己抗原であることを示している。

【００５２】

（実施例３）

ジャカリン予備吸着により、自己抗体結合が排除される。

【００５３】

α$_2$－ヘーレマンス・シュミット糖タンパク質（α$_2$－ＨＳＧ）を、子宮内膜症反応におけるジャカリン反応性炭水化物の潜在的役割を調査するためのモデル抗原として選択した。市販のα$_2$－ＨＳＧを使用した最初の実験は、不満足な証明となった。これは、これらの調製物中にある糖形態が多様なためだった。ほとんどのシアリル化された糖タンパク質と共通して、α$_2$－ＨＳＧは保存中に末端のシアル酸残基を喪失する。正常なヒト血清由来の完全にシアリル化されたα$_2$－ＨＳＧを、新たに調製した。その炭水化物含有量についての調製の偏りを避けるため、レクチンベースの親和性精製プロトコールを避け、代わりに陰イオン交換クロマトグラフィーとゲル濾過クロマトグラフィーとの組み合わせを使用した。ｐＨ８．０でのＭｏｎｏＱカラム上での陰イオン交換、続いてｐＨ５．０での第２のＭｏｎｏＱカラム、そして最後にスーパーロース１２カラムによるゲル濾過段階からなる３段階の処理により、分子量５８ｋＤａの完全にシアリル化された糖形態のα$_2$－ＨＳＧが精製された。

【００５４】

第１の陰イオン交換カラム由来の部分精製α$_2$－ＨＳＧ画分を使用して実験を行った場合、ウェスタンブロットによる子宮内膜症血清との自己反応性はジャカリンアガロースでの予備インキュベーション後に消滅した（図３）。ＳＹＰＲＯオレンジ染色ＳＤＳ－ＰＡＧＥゲルにおいて１４本のタンパク質バンドが認められた（図３、レーンａ）。子宮内膜症血清との反応性は、５つのタンパク質で認められた（図３、レーンｂ）。反応性タンパク質は、約２３０、１８８、１６８、１２０、および５８ｋＤａの分子量を有しており、５８ｋＤａのものはα$_2$－ＨＳＧであった。これら５本のバンド全ての子宮内膜症血清におけるＩｇＧによる結合は、ジャカリンアガロースとのインキュベーション後に消滅し（図３、レーンｄ）、これは、自己抗体の結合は画分中に存在する全てのジャカリン結合タンパク質に対してであって、ただα$_2$－ＨＳＧに対してだけでないことを示す。吸着前および後の、画分１３のゲルのタンパク質染色は、活性の喪失が画分中のタンパク質の非特異的喪失によって引き起こされたのではないことを示す。吸着および非吸着タンパク質の濃度は、ウェスタンブロット法に使用するゲル上に添加する総タンパク質量が均一となるように調整した。

【００５５】

ジャカリンによって認識される炭水化物構造は非常に特異的であり、結合はＴｆの存在に依存する。ジャカリンは、いくつかのＯ結合少糖類の内に存在する二糖類Ｇａｌβ１－３

ＧａｌＮＡｃを認識する。Ｇａｌβ１－３ＧａｌＮＡｃは、多数の血漿タンパク質のうち非常に限られたいくつかによって発現する。これらのタンパク質には、ＩｇＡ１、ＩｇＤ、Ｃ１阻害剤、ヘモペキシン、プラスミノゲン、α１－抗トリプシン、α２－マクログルブリン、８Ｓ－α３糖タンパク質、絨毛性ゴナドトロピン（ｈＣＧ）、およびα$_2$－ＨＳＧが含まれる（１５）。ｈＣＧに関して、ｈＣＧの受容体がＴｆ様抗原も発現すること、随伴性の子宮内膜症を罹患しているか、罹患していないか、いずれもの不妊症患者で見出された自己抗体が受容体に結合すること[5]は興味深い。

【００５６】

（実施例４）

炭水化物の除去による自己抗体結合の停止。

【００５７】

ジャカリンによる自己抗体によって認識されるタンパク質の除去は、それ自体に共通の炭水化物エピトープ（群）が含まれるかどうかは決定できない。炭水化物が子宮内膜症の自己抗体によるα$_2$－ＨＳＧの認識に不可欠であるかどうかを判定するために、脱グリコシル化α$_2$－ＨＳＧとの反応性を調査した。α$_2$－ＨＳＧ炭水化物は、２段階の処理で除去された。第一段階では、ノイラミニダーゼを使用して、末端シアル酸を切断した。第二段階は、ＥｎｄｏＦ／ＰＮＧアーゼ消化工程を更に使用して、炭水化物を完全に除去した。未処理、ノイラミニダーゼ処理、および脱グリコシル化α$_2$－ＨＳＧを、等量のタンパク質をロードするウェスタンブロット分析に供した（図４）。シアル酸および全ての炭水化物の除去を、ヒツジ抗α$_2$－ＨＳＧとの反応性で示される分子量の減少によって確認した（図４、レーンＡ～Ｃ）。

【００５８】

ヒツジ抗α$_2$－ＨＳＧ抗体は、ペプチドに反応性を示し、二重免疫拡散法によるジャカリン結合タンパク質との反応性を示さない（データ示さず）。ノイラミニダーゼでの末端シアル酸の除去により、患者の血清との反応性は減少した（図４、レーンＤおよびＥ）。この画分に残存する反応性は未消化α$_2$－ＨＳＧと同一の分子量においてで、これは自己抗体結合が末端シアル酸の存在に依存することを示している。ノイラミニダーゼ処理とその後に続くＥｎｄｏＦ／ＰＮＧアーゼ消化による炭水化物の完全な除去で、反応性は完全に消滅した（図４、レーンＦ）。類似の部分精製α$_2$－ＨＳＧ処理により、５つ全ての反応性タンパク質への結合が減少するので、ノイラミニダーゼ後の自己抗体結合のこの減少はα$_2$－ＨＳＧに制限されない（図３、レーンｃ）。

【００５９】

【表１】

表１

同定した子宮内膜症抗原とジャカリン結合活性との相関性

抗原	本研究の子宮内膜症抗原／（公開された研究）	本研究のジャカリン反応性／（公開された研究）
α—ＨＳＧ	あり／[9,10]	あり／[63]
炭酸脱水素酵素Ｉ／II	あり／[6-8]	なし／未知
ヘモペキシン	あり／なし	あり／[15,40]
ＩｇＡ	あり／なし	あり／[15]に多数概説
ＣＤ２３	なし／ 可溶性形態の増加[53-55]	なし／Gal-GalNac レクチン様 ジャカリンとして作用[64,65]
ｈＣＧ受容体	なし／[5]	なし／[66]

これらの結果は（トランスフェリンを除いて）、通常の炭水化物エピトープがα$_2$－ＨＳＧ、炭酸脱水酵素、ヘモペキシン、およびＩｇＡ１のａ鎖に認識されることを示している

（本研究、および公開された研究において同定された抗原、ならびにそれらのジャカリン反応性の要約は、表１を参照のこと）。全ての同定した抗原（トランスフェリンおよび炭酸脱水酵素ＩＩを除く）がレクチンジャカリンに結合することが知られていることと、抗原からの炭水化物の除去により子宮内膜症血清のこれらの抗原への結合能力が失われることに基づいてこの結論に至った。

【００６０】

（実施例５）

ウシフェチュインへの自己抗体の結合

ヒトα$_2$－ＨＳＧのホモログであるウシフェチュインは、ヒトα$_2$－ＨＳＧと同一のジャカリン結合炭水化物部分を有する。ヒトＳＧペプチドに対して惹起したヒツジ抗血清は、ウシフェチュインと交差反応を示さず、このことから、かなりの配列相同性にも関わらず２種の間の抗原性が有意に異なると示唆される。ＥＬＩＳＡおよびウシフェチュインでのウェスタンブロットによれば、子宮内膜症患者由来の血清は正の反応性を示す（図５）。フェチュインのノイラミニダーゼ処理は、－ＨＳＧと対照的に、ＥＬＩＳＡでの自己抗体反応性をわずかに増加させた（図５）。しかし、ウシフェチュインのより完全な脱シアリル化または完全な脱グリコシル化により、抗体結合は消滅し、これにより子宮内膜症患者の自己抗体反応は炭水化物依存性であるというさらなる証拠が得られた（示さず）。完全なノイラミニダーゼ処理後のフェチュインへの結合の増加は、ウシフェチュインがそのヒト対応物に比べより強くシアリル化されることに起因しうる。

【００６１】

（実施例６）

自己抗体結合のＤ－ガラクトース依存性。

【００６２】

ジャカリン結合は、Ｇａｌｂ１－３ＮａｃＧａｌ成分の存在に依存する。もし自己抗体反応がこのエピトープを含んでいたら、ジャカリン結合の場合と同様に、結合はＤ－ガラクトースによって競合的に阻害されるはずである。ウェスタンブロットでのａ$_2$－ＨＳＧへの子宮内膜症ＩｇＧの結合は、０．８Ｍ　Ｄ－ガラクトースの存在下で完全に消滅し、これはＤ－ガラクトースが、自己抗体によって認識されるエピトープの一部を形成しうることを示している（示さず）。

【００６３】

（実施例７）

子宮内膜症血清により認識される血清自己抗原は、同じエピトープを共有する。

【００６４】

同一の炭水化物エピトープが上記で同定された異種抗原との自己反応性の原因となるため、ここで自己抗体結合は、過剰な異種の抗原の存在下では遮断されるはずである。このことは以下の例で証明された：１０倍過剰の高度に精製されたａ$_2$－ＨＳＧまたはウシフェチュインの添加は、どちらも自己抗体の炭酸脱水酵素ＩＩおよび７２ｋＤａの抗原への結合を阻害した（図６）。

【００６５】

子宮内膜症の７２ｋＤａ自己抗原は、トランスフェリンよりもむしろヘモペキシンであろうか？　これまでに記載の抗原のうち、トランスフェリンを除く全てが、ジャカリンによって認識された。このような炭水化物構造はトランスフェリン上には発現せず、ジャカリンはトランスフェリンと結合しない[15]。このことから、以下の３つの可能性が残る：ジャカリン炭水化物反応とは別個の抗トランスフェリン反応が子宮内膜症血清に存在するか、またはトランスフェリンで精製する反応性がトランスフェリン調製物中の夾雑物に対するものであるか、またはその両方かである。正常子宮内膜Ｗ１３４５の陰イオン交換画分中には、～７０ｋＤａの２つの反応性タンパク質が見出された。一方は、塩勾配の早い箇所でトランスフェリンと同一の画分中に溶出した。他方のタンパク質はより高い塩濃度で、ＩｇＡと同一の画分中に溶出された。スイス２Ｄデータベースを調査した結果、類似の分子量および等電点のジャカリン結合タンパク質がヘモペキシンを含むことを示した。へ

モペキシンをZn^{2+}金属キレートクロマトグラフィー、および陰イオン交換クロマトグラフィーにより精製した場合に、均質な調整物が得られた。ヘモペキシン特異的抗体を含む画分のウェスタンブロット法により、同一性を確認した。子宮内膜症患者の血清に対して試験したところ、このタンパク質は自己抗原として認識された（図７）。トランスフェリンに対し特異的な抗体での同じタンパク質のウェスタンブロット分析は、精製ヘモペキシンとの反応性を示されなかった。同一のカラム由来の画分をトランスフェリン反応性について調査したところ、陰イオン交換カラム由来のより早期のピークがトランスフェリンを含むことがわかった。抗ヘモペキシンでのトランスフェリン含有画分のウェスタンブロット分析により、ＳＤＳ－ＰＡＧＥでの移動度がトランスフェリンと類似のヘモペキシンの存在が明らかになった。二重免疫拡散法で抗ヘモペキシンおよび抗トランスフェリンがトランスフェリンのピーク画分に対して沈降反応させた場合に一致線は示されず、これは、抗血清が抗原特異的であることを表している（示さず）。このトランスフェリン調製物中のジャカリン結合活性の存在を、ジャカリンに対する二重免疫拡散法での沈降線の存在によって確認した（示さず）。トランスフェリンで共精製される抗原の性質を決定するため、さらなる実験が現在進行中であるが、しかし７２ｋＤａ結合活性が純粋なα_2－ＨＳＧ調製物の添加により阻害されることは興味深いことである（図６）。

【００６６】

（引例文献）

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２０．Ａｗａｄａｌｌａ Ｓ．Ｇ．，Ｆｒｉｅｄｍａｎ Ｃ．Ｉ．，Ｈａｑ Ａ．Ｕ．，Ｒｏｈ Ｓ．Ｉ．，Ｃｈｉｎ Ｎ．Ｗ．およびＫｉｍ Ｍ．Ｈ．（１９８７）「局所腹腔因子：子宮内膜症に関連する不妊症への役割」Ａｍ Ｊ Ｏｂｓｔｅｔ Ｇｙｎｅｃｏｌ，１５７，１２０７．
２１．Ｍｏｒｉ Ｈ．，Ｓａｗａｉｒｉ Ｍ．，Ｎａｋａｇａｗａ Ｍ．，Ｉｔｏｈ Ｎ．，Ｗａｄａ Ｋ．およびＴａｍａｙａ Ｔ．（１９９２）「子宮内膜症患者由来の腹腔マクロファージ中のインターロイキン１（ＩＬ－１）β伝令リボ核酸（ｍＲＮＡ）およびＩＬ－１受容体アンタゴニストｍＲＮＡの発現」Ｆｅｒｔｉｌ Ｓｔｅｒｉｌ，５７，５３５．
２２．Ｔａｋｅｔａｎｉ Ｙ．，Ｋｕｏ Ｔ．Ｍ．およびＭｉｚｕｎｏ Ｍ．（１９９２）「未処置または処置された子宮内膜症の女性の不妊症における腹腔内貯留液中のサイトカインレベルと胚毒性との比較」Ａｍ Ｊ Ｏｂｓｔｅｔ Ｇｙｎｅｃｏｌ，１６７，２６５．
２３．Ｆａｋｉｈ Ｈ．，Ｂａｇｇｅｔｔ Ｂ．，Ｈｏｌｔｚ Ｇ．，Ｔｓａｎｇ Ｋ．Ｙ．，Ｌｅｅ Ｊ．Ｃ．およびＷｉｌｌｉａｍｓｏｎ Ｈ．Ｏ．（１９８７）「インターロイキン１：子宮内膜症に関連する不妊症における可能性のある役割」Ｆｅｒｔｉｌ Ｓｔｅｒｉｌ，４７，２１３．
２４．Ｈｉｌｌ Ｊ．Ａ．およびＡｎｄｅｒｓｏｎ Ｄ．Ｊ．（１９８９）「子宮内膜

症を持つおよび持たない、妊娠可能および不妊症の女性由来の、腹腔内貯留液の存在下でのリンパ球活性」Ａｍ Ｊ Ｏｂｓｔｅｔ Ｇｙｎｅｃｏｌ，１６１，８６１．
２５．Ｋｏｙａｍａ Ｎ．，Ｍａｔｓｕｕｒａ Ｋ．およびＯｋａｍｕｒａ Ｈ．（１９９３）「子宮内膜症患者の腹腔内貯留液中のサイトカイン」Ｉｎｔ Ｊ Ｇｙｎａｅｃｏｌ Ｏｂｓｔｅｔ，４３，４５．
２６．Ｏｖｅｒｔｏｎ Ｃ．，Ｆｅｒｎａｎｄｅｚ－Ｓｈａｗ Ｓ．，Ｈｉｃｋｓ Ｂ．，Ｂａｒｌｏｗ Ｄ．およびＳｔａｒｋｅｙ Ｐ．（１９９６）「腹腔内貯留液サイトカインおよび子宮内膜症と痛みとの関連」Ｈｕｍ Ｒｅｐｒｏｄ，１１，３８０．
２７．Ｈａｒａｄａ Ｔ．，Ｙｏｓｈｉｏｋａ Ｈ．，Ｙｏｓｈｉｄａ Ｓ．，Ｉｗａｂｅ Ｔ．，Ｏｎｏｈａｒａ Ｙ．，Ｔａｎｉｋａｗａ Ｍ．およびＴｅｒａｋａｗａ Ｎ．（１９９７）「活動性子宮内膜症を伴う不妊症の腹腔内貯留液中のインターロイキン６レベルの増加」Ａｍ Ｊ Ｏｂｓｔｅｔ Ｇｙｎｅｃｏｌ，１７６，５９３．
２８．Ｒｉｅｒ Ｓ．Ｅ．，Ｚａｒｍａｋｏｕｐｉｓ Ｐ．Ｎ．，Ｈｕ Ｘ．およびＢｅｃｋｅｒ Ｊ．Ｌ．（１９９５）「異所的子宮内膜間質細胞におけるインターロイキン６反応の制御不全：子宮内膜症の女性の腹腔内貯留液中の可溶性受容体の減少との相関関係」Ｊ Ｃｌｉｎ Ｅｎｄｏｃｒｉｎｏｌ Ｍｅｔａｂ，８０，１４３１．
２９．Ｒｉｅｒ Ｓ．Ｅ．，Ｐａｒｓｏｎｓ Ａ．Ｋ．およびＢｅｃｋｅｒ Ｊ．Ｌ．（１９９４）「子宮内膜症患者由来の腹腔白血球によるインターロイキン６産生の変化」Ｆｅｒｔｉｌ Ｓｔｅｒｉｌ，６１，２９４．
３０．Ｂｕｙａｌｏｓ Ｒ．Ｐ．，Ｆｕｎａｒｉ Ｖ．Ａ，Ａｚｚｉｚ Ｒ．，Ｗａｔｓｏｎ Ｊ．Ｍ．およびＭａｒｔｉｎｅｚ－Ｍａｚａ Ｏ．（１９９２）「骨盤疾患患者の腹腔内貯留液中のインターロイキン６レベルの上昇」Ｆｅｒｔｉｌ Ｓｔｅｒｉｌ，５８，３０２．
３１．Ｒａｎａ Ｎ．，Ｂｒａｕｎ Ｄ．Ｐ．，Ｈｏｕｓｅ Ｒ．，Ｇｅｂｅｌ Ｈ．，Ｒｏｔｍａｎ Ｃ．およびＤｍｏｗｓｋｉ Ｗ．Ｐ．（１９９６）「子宮内膜症の女性における腹腔マクロファージによるサイトカインの基本および刺激分泌」Ｆｅｒｔｉｌ Ｓｔｅｒｉｌ，６５，９２５．
３２．Ｅｉｓｅｒｍａｎｎ Ｊ．，Ｇａｓｔ Ｍ．Ｊ．，Ｐｉｎｅｄａ Ｊ．，Ｏｄｅｍ Ｒ．Ｒ．およびＣｏｌｌｉｎｓ Ｊ．Ｌ．（１９８８）「腹腔鏡下手術を受けた女性の腹腔内貯留液中の腫瘍開始因子」Ｆｅｒｔｉｌ Ｓｔｅｒｉｌ，５０，５７３．
３３．Ｈａｌｍｅ Ｊ．（１９８９）「ｉｎ ｖｉｖｏおよびインビトロでのヒト腹腔マクロファージによる腫瘍開始因子αの放出」Ａｍ Ｊ Ｏｂｓｔｅｔ Ｇｙｎｅｃｏｌ，１６１，１７１８．
３４．Ｋａｂｉｒ Ｓ．およびＷｉｇｚｅｌｌ Ｈ．（１９８９）「インターロイキン１の阻害剤としての新規の尿シアロ糖タンパク質」Ｃｌｉｎ Ｅｘｐ Ｉｍｍｕｎｏｌ，７７，８９．
３５．Ｌｅｂｒｅｔｏｎ Ｊ．Ｐ．，Ｊｏｉｓｅｌ Ｆ．，Ｒａｏｕｌｔ Ｊ．Ｐ．，Ｌａｎｎｕｚｅｌ Ｂ．，Ｒｏｇｅｚ Ｊ．Ｐ．およびＨｕｍｂｅｒｔ Ｇ．（１９７９）「炎症過程におけるヒトα２ＨＳ糖タンパク質の血清濃度：α２ＨＳ糖タンパク質は陰性急性期反応物質であることの証明」Ｊ Ｃｌｉｎ Ｉｎｖｅｓｔ，６４，１１１８．
３６．Ａｋｈｏｕｎｄｉ Ｃ．，Ａｍｉｏｔ Ｍ．，Ａｕｂｅｒｇｅｒ Ｐ．，Ｌｅ Ｃａｍ Ａ．およびＲｏｓｓｉ Ｂ．（１９９４）「インスリンおよびインターロイキン１はｐｐ６３（肝臓癌株における急性期リンタンパク質）を個別に制御する」Ｊ Ｂｉｏｌ Ｃｈｅｍ，２６９，１５９２５．
３７．Ｄｅｍｅｔｒｉｏｕ Ｍ．，Ｂｉｎｋｅｒｔ Ｃ．，Ｓｕｋｈｕ Ｂ．，Ｔｅｎｅｎｂａｕｍ Ｈ．Ｃ．およびＤｅｎｎｉｓ Ｊ．Ｗ．（１９９６）「フェチュイン／α２－ＨＳ糖タンパク質は、形質転換成長因子－βＩＩ型受容体模倣物およびサイトカインアンタゴニストである」Ｊ Ｂｉｏｌ Ｃｈｅｍ，２７１，１２７５５．
３８．Ｂｒｕｎｅｒ Ｋ．Ｌ．，Ｅｉｓｅｎｂｅｒｇ Ｅ．，Ｇｏｒｓｔｅｉｎ Ｆ．およびＯｓｔｅｅｎ Ｋ．Ｇ．（１９９９）「プロゲステロンおよび形質転換成長因子β

は、子宮内膜症実験モデルにおいて子宮内膜基質メタロプロテイナーゼ抑制を対等に制御する（現在進行中の引用）」Ｓｔｅｒｏｉｄｓ，６４，６４８．
３９．Ｋｕｐｋｅｒ Ｗ．，Ｓｃｈｕｌｔｚｅ－Ｍｏｓｇａｕ Ａ．およびＤｉｅｄｒｉｃｈ Ｋ．（１９９８）「子宮内膜症の女性の腹腔環境のパラクリン変換」Ｈｕｍ Ｒｅｐｒｏｄ Ｕｐｄａｔｅ，４，７１９．
４０．Ｐｉｌａｔｔｅ Ｙ．，Ｒｏｓｔｏｋｅｒ Ｇ．，Ｖｉｏｌｌｅａｕ Ｋ．，Ｂａｓｔａ Ｍ．およびＨａｍｍｅｒ Ｃ．Ｈ．（１９９５）「ガラクトース特異的レクチンジャカリンと相互作用するヒト血漿糖タンパク質のさらなる同定」Ｊ Ｃｈｏｒｏｍａｔｏｇｒ Ｂ Ｂｉｏｍｅｄ Ａｐｐｌ，６６８，１．
４１．Ｍａｓｓｏｖａ Ｉ．，Ｋｏｔｒａ Ｌ．Ｐ．，Ｆｒｉｄｍａｎ Ｒ．およびＭｏｂａｓｈｅｒｙ Ｓ．（１９９８）「基質メタロプロテイナーゼ：構造、進化、および多様化」Ｆａｓａｂ Ｊ，１２，１０７５．
４２．Ｓｏｌｏｍｏｎ Ｊ．Ｃ．，Ｓｔｏｌｌ Ｍ．Ｓ．，Ｐｅｎｆｏｌｄ Ｐ．，Ａｂｂｏｔｔ Ｗ．Ｍ．，Ｃｈｉｌｄｓ Ｒ．Ａ．，Ｈａｎｆｌａｎｄ Ｐ．およびＦｅｉｚｉ Ｔ．（１９９１）「固定化糖脂質および新糖脂質を使用した可溶性で１４，０００ダルトンのウシ心筋レクチンの結合特異性についての研究」Ｃａｒｂｏｈｙｄｒ Ｒｅｓ，２１３，２９３．
４３．Ａｈｍｅｄ Ｈ．，Ａｌｌｅｎ Ｈ．Ｊ．，Ｓｈａｒｍａ Ａ．およびＭａｔｔａ Ｋ．Ｌ．（１９９０）「ヒト脾臓ガラプチン：炭水化物結合特異性および結合部位の特徴づけ」Ｂｉｏｃｈｅｍｉｓｔｒｙ，２９，５３１５．
４４．Ｂａｒｏｎｄｅｓ Ｓ．Ｈ．，Ｃｏｏｐｅｒ Ｄ．Ｎ．，Ｇｉｔｔ Ｍ．Ａ．およびＬｅｆｆｌｅｒ Ｈ．（１９９４）「ガレクチン 動物レクチンの一大ファミリーの構造および機能」Ｊｏｕｒｎａｌ ｏｆ Ｂｉｏｌｏｇｉｃａｌ Ｃｈｅｍｉｓｔｒｙ，２６９，２０８０７．
４５．Ｋｏｐｉｔｚ Ｊ．，ｖｏｎ Ｒｅｉｔｚｅｎｓｔｅｉｎ Ｃ．，Ｂｕｒｃｈｅｒｔ Ｍ．，Ｃａｎｔｚ Ｍ．およびＧａｂｉｉｕｓ Ｈ．Ｊ．（１９９８）「ガレクチン１はガングリオシドＧＭ１の主要な受容体である（培養物中のヒト神経芽細胞腫に対する細胞表面ガングリオシドシアリダーゼの成長調節活性の生成物）」Ｊｏｕｒｎａｌ ｏｆ Ｂｉｏｌｏｇｉｃａｌ Ｃｈｅｍｉｓｔｒｙ，２７３，１１２０５．
４６．Ｏｃｈｉｅｎｇ Ｊ．，Ｇｒｅｅｎ Ｂ．，Ｅｖａｎｓ Ｓ．，Ｊａｍｅｓ Ｏ．およびＷａｒｆｉｅｌｄ Ｐ．（１９９８）「基質メタロプロテイナーゼによるガレクチン３の生物学的機能の調整」Ｂｉｏｃｈｉｍｉｃａ ｅｔ Ｂｉｏｐｈｙｓｉｃａ Ａｃｔａ，１３７９，９７．
４７．Ｏｃｈｉｅｎｇ Ｊ．，Ｆｒｉｄｍａｎ Ｒ．，Ｎａｎｇｉａ－Ｍａｋｋｅｒ Ｐ．，Ｋｌｅｉｎｅｒ Ｄ．Ｅ．，Ｌｉｏｔｔａ Ｌ．Ａ．，Ｓｔｅｔｌｅｒ－Ｓｔｅｖｅｎｓｏｎ Ｗ．Ｇ．およびＲａｚ Ａ．（１９９４）「ガレクチン３は、ヒト基質メタロプロテイナーゼ２および９の新規の基質である」Ｂｉｏｃｈｅｍｉｓｔｒｙ，３３，１４１０９．
４８．Ｂｒｕｎｅｒ Ｋ．Ｌ．，Ｍａｔｒｉｓｉａｎ Ｌ．Ｍ．，Ｒｏｄｇｅｒｓ Ｗ．Ｈ．，Ｇｏｒｓｔｅｉｎ Ｆ．およびＯｓｔｅｅｎ Ｋ．Ｇ．（１９９７）「基質メタロプロテイナーゼの抑制は、ヌードマウスにおいてヒト子宮内膜症による異所性損傷の確立を阻害する」Ｊ Ｃｌｉｎ Ｉｎｖｅｓｔ，９９，２８５１．
４９．Ｃｈｏｅ Ｙ．Ｓ．，Ｓｈｉｍ Ｃ．，Ｃｈｏｉ Ｄ．，Ｌｅｅ Ｃ．Ｓ．，Ｌｅｅ Ｋ．Ｋ．およびＫｉｍ Ｋ．（１９９７）「マウス尿中のガレクチン１ ｍＲＮＡの発現は、胚盤胞移植中は卵巣ステロイドの調節下にある」Ｍｏｌｅｃｕｌａｒ Ｒｅｐｒｏｄｕｃｔｉｏｎ ＆ Ｄｅｖｅｌｏｐｍｅｎｔ，４８，２６１．
５０．Ｖａｎ ｄｅｎ Ｂｒｕｌｅ Ｆ．Ａ．，Ｆｅｒｎａｎｄｅｚ Ｐ．Ｌ．，Ｂｕｉｃｕ Ｃ．，Ｌｉｕ Ｆ．Ｔ．，Ｊａｃｋｅｒｓ Ｐ．，Ｌａｍｂｏｔｔｅ Ｒ．およびＣａｓｔｒｏｎｏｖｏ Ｖ．（１９９７）「妊娠第１期のヒト胚形成間のガレクチン１およびガレクチン３発現の相違」Ｄｅｖｅｌｏｐｍｅｎｔａｌ Ｄｙｎａｍｉｃｓ，２０

９，３９９．
５１．Ｐｈｉｌｌｉｐｓ Ｂ．，Ｋｎｉｓｌｅｙ Ｋ．，Ｗｅｉｔｌａｕｆ Ｋ．Ｄ．，Ｄｏｒｓｅｔｔ Ｊ．，Ｌｅｅ Ｖ．およびＷｅｉｔｌａｕｆ Ｈ．（１９９６）「妊娠マウスの生殖管中の２つのβガラクトシド結合レクチン発現の相違」Ｂｉｏｌｏｇｙ ｏｆ Ｒｅｐｒｏｄｕｃｔｉｏｎ，５５，５４８．
５２．Ｂｙｒｊａｌｓｅｎ Ｉ．，Ｌａｒｓｅｎ Ｐ．Ｍ．，Ｆｅｙ Ｓ．Ｊ．およびＣｈｒｉｓｔｉａｎｓｅｎ Ｃ．（１９９５）「正常な月経周期中の発現が周期的に変化したヒト子宮内膜タンパク質：タンパク質配列解析による特性決定」Ｈｕｍ Ｒｅｐｒｏｄ，１０，２７６０．
５３．Ｏｄｕｋｏｙａ Ｏ．Ａ．，Ｂａｎｓａｌ Ａ．，Ｗｉｌｓｏｎ Ａ．Ｐ．，Ｗｅｅｔｍａｎ Ａ．Ｐ．およびＣｏｏｋｅ Ｉ．Ｄ．（１９９５）「子宮内膜症患者の血清－可溶性ＣＤ２３およびダナゾールおよび酢酸ロイプロリド蓄積注射による治療効果」Ｈｕｍ Ｒｅｐｒｏｄ，１０，９４２．
５４．Ｏｄｕｋｏｙａ Ｏ．，Ｂａｎｓａｌ Ａ．およびＣｏｏｋｅ Ｉ．（１９９６）「子宮内膜症患者の血清子宮内膜ＩｇＧ抗体および可溶性ＣＤ２３濃度」Ａｃｔａ Ｏｂｓｔｅｔ Ｇｙｎｅｃｏｌ Ｓｃａｎｄ，７５，９２７．
５５．Ｏｄｕｋｏｙａ Ｏ．Ａ．，Ｂａｎｓａｌ Ａ．，Ｗｉｌｓｏｎ Ｐ．，Ｌｉｍ Ｋ．，Ｗｅｅｔｍａｎ Ａ．Ｐ．およびＣｏｏｋｅ Ｉ．Ｄ．（１９９６）「子宮内膜症患者の腹腔内貯留液中の可溶性ＣＤ２３タンパク質」Ｈｕｍ Ｒｅｐｒｏｄ，１１，２０１８．
５６．Ｇａｎｇｕｌｙ Ｓ．，Ｓａｒｋａｒ Ｄ．およびＧｈｏｓｈ Ｊ．Ｊ．（１９７６）「異なる子宮不全条件下でのヒト子宮内膜組織、子宮液、および血漿中のシアル酸およびシアリダーゼ活性」Ａｃｔａ Ｅｎｄｏｃｒｉｎｏｌ（Ｃｏｐｅｎｈ），８１，５７４．
５７．Ｎｅｌｓｏｎ Ｊ．Ｄ．，Ｊａｔｏ－Ｒｏｄｒｉｇｕｅｚ Ｊ．Ｊ．，Ｌａｂｒｉｅ Ｆ．およびＭｏｏｋｅｒｊｅａ Ｓ．（１９７７）「ラットの発情周期中の子宮内膜におけるグリコシル転移酵素およびＵＤＰ－ガラクトースピロホスファターゼ活性」Ｊ Ｅｎｄｏｃｒｉｎｏｌ，７３，５３．
５８．Ｚｈｕ Ｚ．，Ｄｅｎｇ Ｈ．，Ｆｅｎｄｅｒｓｏｎ Ｂ．Ａ．，Ｎｕｄｅｌｍａｎ Ｅ．Ｄ．およびＴｓｕｉ Ｚ．（１９９０）「月経周期、妊娠、および加齢におけるヒト子宮筋層および子宮内膜のスフィンゴ糖脂質ならびにその変化」Ｊ Ｒｅｐｒｏｄ Ｆｅｒｔｉｌ，８８，７１．
５９．Ｔａｋａｍａｔｓｕ Ｋ．，Ｋａｍｅｉ Ｋ．，Ｋｕｂｏｓｈｉｒｏ Ｋ．，Ｋｉｇｕｃｈｉ Ｋ．，Ｎｏｚａｗａ Ｓ．およびＩｗａｍｏｒｉ Ｍ．（１９９３）「ヒト子宮頸上皮および子宮内皮における黄体期に特徴的なＩ３ＳＯ３－ＧａｌＣｅｒの誘導およびファローピウス管の異なる領域における卵胞期に特徴的なガングリオシド由来の負電荷勾配の形成」Ｂｉｏｃｈｉｍ Ｂｉｏｐｈｙｓ Ａｃｔａ，１１７０，２３２．
６０．Ｒａｖｎ Ｖ．およびＪｅｎｓｅｎ Ｈ．（１９８８）「ヒト子宮内膜におけるトムゼン－フリーデンライヒ関連抗原：モノクローナル抗体４９Ｈ．８を使用した免疫組織化学研究、予備試験」Ａｐｍｉｓ，９６，５５２．
６１．Ｒａｖｎ Ｖ．，Ｔｅｇｌｂｊａｅｒｇ Ｃ．Ｓ．，Ｍａｎｄｅｌ Ｕ．およびＤａｂｅｌｓｔｅｅｎ Ｅ．（１９９２）「正常なヒト子宮内膜周期における２型鎖組織－血液群抗原の分布」Ｃｅｌｌ Ｔｉｓｓｕｅ Ｒｅｓ，２７０，４２５．
６２．Ｃｒａｍｅｒ Ｄ．Ｗ．，Ｈｏｒｎｓｔｅｉｎ Ｍ．Ｄ．，Ｎｇ Ｗ．Ｇ．およびＢａｒｂｉｅｒｉ Ｒ．Ｌ．（１９９６）「ガラクトース－１リン酸ウリジル転移酵素（ＧＡＬＴ）のＮ３１４Ｄ変異に関連する子宮内膜症」Ｍｏｌ Ｈｕｍ Ｒｅｐｒｏｄ，２，１４９．
６３．Ｔｏ Ｗ．Ｙ．，Ｌｅｕｎｇ Ｊ．Ｃ．およびＬａｉ Ｋ．Ｎ．（１９９５）「ジャカリン結合タンパク質としてのヒト血清α２－ＨＳ糖タンパク質の同定および性質決定」Ｂｉｏｃｈｉｍ Ｂｉｏｐｈｙｓ Ａｃｔａ，１２４９，５８．

６４．Ｋｉｊｉｍｏｔｏ－Ｏｃｈｉａｉ Ｓ．およびＵｅｄｅ Ｔ．（１９９５）「ＣＤ２３分子は、ＥＢＶ形質転換ヒトＢ細胞株の細胞凝集におけるガラクトース結合レクチンとして作用する」Ｇｌｙｃｏｂｉｏｌｏｇｙ，５，４４３．
６５．Ｋｉｊｉｍｏｔｏ－Ｏｃｈｉａｉ Ｓ．，Ｈｏｒｉｍｏｔｏ Ｅ．およびＵｅｄｅ Ｔ．（１９９４）「ＣＤ２３分子と糖タンパク質のガラクトース残基との間の相互作用の証明」Ｉｍｍｕｎｏｌｏｇｙ Ｌｅｔｔｅｒｓ，４０，４９．
６６．Ｓａｋａｉ Ｈ．，Ｙａｍａｇｉｓｈｉ Ｆ．，Ｍｉｕｒａ Ｍ．，Ｈａｔａ Ｋ．，Ｋｏｙａｍａ Ｉ．，Ｓａｋａｇｉｓｈｉ Ｙ．およびＫｏｍｏｄａ Ｔ．（１９９４）「レクチン連続アフィニティークロマトグラフィーによって同定されたヒト尿絨毛性ゴナドトロピン糖鎖の不均一性：良性疾患と悪性疾患との間の相違」Ｔｕｍｏｕｒ Ｂｉｏｌ，１５，２３０．

【図面の簡単な説明】

【図１】

図１ａは子宮内膜症患者由来のホモジナイズした正常子宮内膜のＭｏｎｏＱ陰イオン交換クロマトグラフィーを示す溶出プロフィールである。ホモジネートをプロテインＧカラムを通過させてＩｇＧを除去後、ＭｏｎｏＱカラムに添加した。塩勾配開始前の最初のピークは、複数の１ｍｌのホモジネートのロードを示す。溶出条件：５０ｍＭ Ｔｒｉｓ／ＨＣｌ（ｐＨ８．０）。同一の緩衝液中で、０～０．５ＮａＣｌの線形勾配（１Ｍ ＮａＣｌを終点とする）で溶出を行い、これは破線で示した。ゲルローディングに対応する画分（図１ｂ～ｄ）をグラフの上部に示す。

図１ｂは、図１ａに示される選択されたカラム画分の５～１５％勾配ゲルを還元条件下で泳動しＳＹＰＲＯオレンジで染色した結果を示す図である。

図１ｃはプールした子宮内膜症の血清中に存在するＩｇＧによって認識されるホモジネート画分中のタンパク質を示すオートラジオグラフィーを示す図である。プールした正常な男性血清を使用した同一のブロットの剥離および再探索によっても、同一のバンドとの反応性を示さなかった。ＩｇＧ結合活性は対照血清を使用した画分２４中で検出されたが、子宮内膜症の血清を使用した場合は類似の結合は認められなかった。４５．２ｋＤａの分子量マーカーである炭酸脱水酵素ＩＩは反応性を示す。この同一の反応性は、対照血清では認められなかった。

図１ｄは子宮内膜症の血清のＩｇＡ結合活性を示すオートラジオグラフィーを示す図である。一次血清不在での抗ＩｇＡ ａ鎖特異的抗体でのブロットの探索により、これらの画分中にＩｇＡが存在することが示された。

【図２】

図２は、還元およびアルキル化条件下、５～１５％ＳＤＳ－ＰＡＧＥゲル上で流した腹水（Ｗ１５１７）のウェスタンブロットを示す図である。次いで、ウェスタンブロットを、ヒツジ抗ヒトα$_2$－ＨＳＧ、これに続いてＨＲＰ－ヤギ抗ヒツジ（レーンａ）で、または子宮内膜症の血清を１：１００希釈したもので、これに続いてＨＲＰ－ヤギＩｇＧ ｇ鎖特異的抗体（１：１０００希釈）で、のいずれかでインキュベートした。レーンａのα$_2$－ＨＳＧ、およびレーンｂの同分子量のバンドがはっきりと認められる。

【図３】

図３ａはα$_2$－ＨＳＧの部分精製画分（ＭｏｎｏＱカラム、ｐＨ８．０）を示すＳＤＳ－ＰＡＧＥゲルである。ＳＹＰＲＯオレンジでの染色後に１４本のタンパク質バンドが認められる。

図３ｂはα$_2$－ＨＳＧの部分精製画分を泳動し、子宮内膜症血清ＨＲＰヤギ抗ヒトＩｇＧ抗体で探索した、ゲルのウェスタンブロットである。５本のタンパク質バンドが検出された。

図３ｃは、タンパク質画分を電気泳動前にノイラミニダーゼで処理したこと以外は図３ｂに記載通りのウェスタンブロットである。タンパク質全体に結合する抗体が、明白に減少している。

図３ｄは、α$_2$－ＨＳＧ画分を電気泳動前にジャカリンアガロースで予備吸着させること

以外は図３ｂに記載通りのウェスタンブロットである。抗体結合が全部除去されている。図３ｅは、分子量マーカーの存在下での、ミオシンおよび炭酸脱水酵素ＩＩとの自己反応性を示す。

【図４】

図４はα$_2$－ＨＳＧの部分精製画分を泳動し、子宮内膜症血清ＨＲＰヤギ抗ヒトＩｇＧ抗体で探索したゲルのウェスタンブロットを示し、これはα$_2$－ＨＳＧの脱グリコシル化が自己抗体結合を消失させることを表している。シアル酸の末端の切断にはノイラミニダーゼを使用した（レーンＢ、Ｅ、Ｈ）。次いで、ＥｎｄｏＦ／ＰＮＧアーゼ消化を使用して、炭水化物を完全に除去した（レーンＣ、Ｆ、Ｈ）。シアル酸および全炭水化物の除去は、ヤギ抗α$_2$－ＨＳＧとの反応性によって示される分子量の減少によって確認した（レーンＡ～Ｃ）。レーンＡ、Ｄ、およびＧは未処理であった。ノイラミニダーゼでの末端シアル酸の除去により、患者の血清との反応性が減少した（レーンＤ対Ｅ）。この画分の残存する反応性は、未消化α$_2$－ＨＳＧと同一の分子量であり、これは、自己抗体結合が末端シアル酸の存在に依存することを示す。ノイラミニダーゼ処理後のＥｎｄｏＦ／ＰＮＧアーゼ消化による炭水化物の完全な除去により、反応性が完全に消失した（レーンＦ）。プールした男性対照血清の使用では、同等の反応性は認められなかった（レーンＧ～Ｉ）。

【図５】

図５ａは、ウシフェチュインの脱シアリル化が子宮内膜症の血清との反応性を増加させることを表している。ＥＬＩＳＡで測定したところ、ノイラミニダーゼでの処理後に、わずかだが有意な結合の増加が認められる。

図５ｂはノイラミニダーゼ処理後のフェチュインでの分子量減少を示すＳＹＰＲＯオレンジ染色ゲルを示す。レーンＡは未処理である；レーンＢはノイラミニダーゼ処理である。

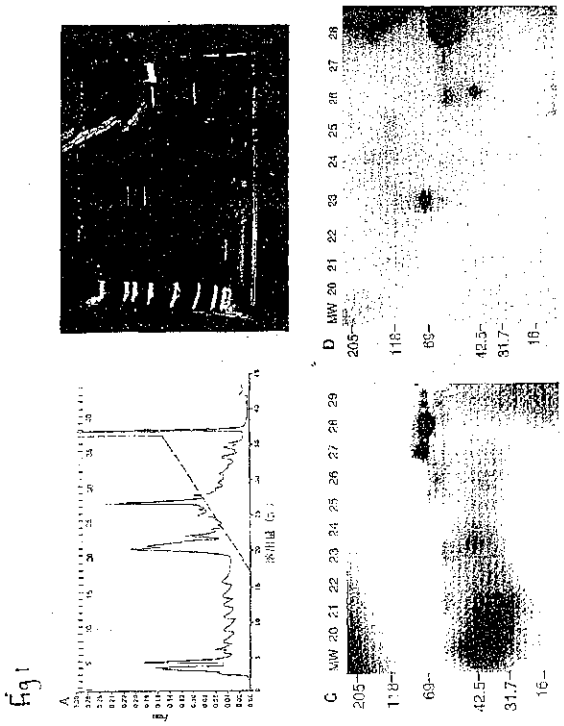
【図６】

図６はどちらも、７２ｋＤａ抗原および炭酸脱水酵素ＩＩへの結合がα^2－ＨＳＧの存在下で阻害されたことを表す、グラフおよびラジオオートグラフィーである。

【図７】

図７は還元およびアルキル化泳動した５～１５％勾配ゲルからニトロセルロースに転写した後の、精製タンパク質のウェスタンブロット分析である。精製ヘモペキシン（レーンＡ）、α$_2$－ＨＳＧ（レーンＢ）、および血清ＩｇＡ１（レーンＣ）は、子宮内膜症血清中のＩｇＧ抗体によって認識される自己抗原である。

【図１】



【図３】

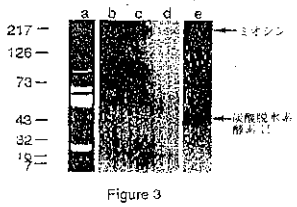
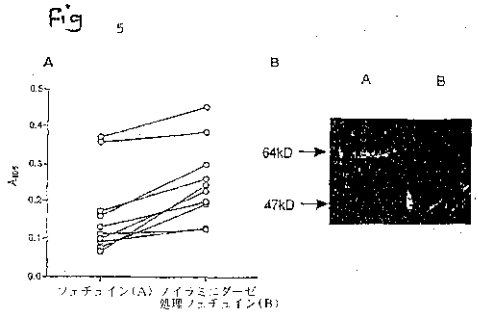
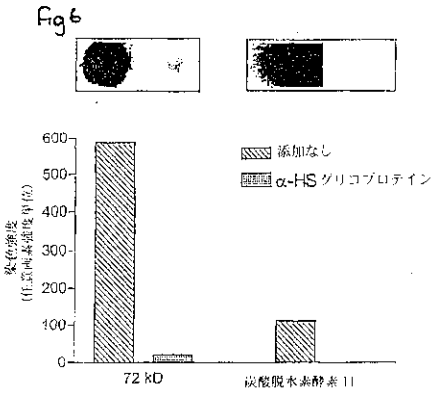


Figure 3

【図５】



【図６】



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(54) Title: DIAGNOSTIC ASSAY FOR ENDOMETRIOSIS

(57) Abstract: The present invention provides a method for detecting endometriosis in a patient and is an improvement over invasive and expensive surgical procedures. The method employs immunoassays which detect autoantibodies in a serum sample which react with Thomsen Friedenreich antigen (TF). Increased levels of autoantibodies in a serum sample from the patient which bind to Tf like antigen is indicative of endometriosis in the patient.

DIAGNOSTIC ASSAY FOR ENDOMETRIOSIS

BACKGROUND OF THE INVENTION

Endometriosis is a common disorder characterized by the growth of endometrial cells at extrauterine (ectopic) sites. It is a common disease which may affect up to 10% of reproductive age women (1). Although the etiology of endometriosis remains enigmatic, altered cellular and humoral immune function is clearly a feature of established disease (2-4).

Autoantibodies to endometrial antigens and deposition of complement components have been described in a number of studies (reviewed in 2) and a number of serum, peritoneal fluid and endometrial antigens have been described. Perhaps the best characterized tissue antigens described, thus far, are the human chorionic gonadotropin receptor (5) and isoforms I and II of the enzyme carbonic anhydrase 6-8. Antibodies to transferrin and α_2-Heremans Schmidt glycoprotein (α_2-HSG) have also been described and proposed as diagnostic markers 9, 10. While considerable work has been carried out in terms of measuring the incidence of these antibodies in endometriosis, reproductive diseases, and other autoimmune diseases, the nature of the epitopes involved has received scant attention. The identified antigens are all glycoproteins. With only one apparent exception (5), carbohydrate antigens on these proteins have not been evaluated.

In accordance with the present invention, it has been surprisingly found that a common carbohydrate

moiety is present on the different aforementioned endometrial antigens. The common carbohydrate moiety is the Thomsen-Friedenreich related antigen, Galβ1-3GalNAc, also referred to as Tf antigen or Tf-like antigen. As used herein, Tf-like antigen also encompasses Tf antigen. Tf antigen is a cryptic disaccharide structure masked by sialic acid. The sialic acid moieties may be removed by sialidases such as neuraminidase. Tf antigen is present on human erythrocytes and is a tumor-associated antigen in epithelial tissues.

The present invention provides diagnostic methods based on autoantibody reactivity with Tf-like antigen. The diagnostic methods are helpful in determining the presence of endometriosis in a patient and are an improvement over the current invasive methods of diagnosis.

SUMMARY OF THE INVENTION

The present invention provides methods for diagnosing endometriosis in a patient. In one embodiment of the invention, a method for diagnosing endometriosis in a patient comprises the steps of

(a) obtaining a serum sample from said patient, (b) incubating Thomsen-Friedenreich (Tf)-like antigen with said serum sample,

(c) detecting autoantibody reactivity with the Tf-like antigen in said sample, and;

(d) correlating an increased level of autoantibody reactivity to the Tf-like antigen in the serum sample with a diagnosis of endometriosis in said patient.

Antibody reactivity may be determined by immunoassays such as immunometric or competitive assays.

In one embodiment of the invention, an immunometric assay comprises the steps of:

(a) immobilizing Tf-like antigen on a solid support,

(b) adding an aliquot of serum sample from a patient to the Tf-like antigen bound on the solid support and incubating,

(c) adding a labeled anti-human immunoglobulin to the solid support wherein said anti-human immunoglobulin is part of a signal producing system,

(d) separating free labeled antibody from bound antibody,

(e) measuring the signal generated by the solution containing the solid support and;

(f) correlating an increase in signal strength with a diagnosis of endometriosis in the patient.

In another embodiment of the invention, a competitive immunoassay comprises the steps of:

(a) preparing a reaction mixture by incubating a constant amount of a labeled antibody which is bound to Tf-like antigen with different concentrations of a serum sample from a patient wherein said labeled antibody is part of a signal producing system,

(b) separating bound labeled antibodies from free autoantibodies,

(c) measuring the signal generated by the labeled antibody in the reaction mixture and;

(d) correlating a decrease in signal strength after addition of the serum sample from a patient with a diagnosis of endometriosis in the patient.

In another embodiment of the invention, a competitive immunoassay comprises the steps of:

(a) preparing a reaction mixture by incubating a first antibody which is bound to Tf-like antigen with

different concentrations of a serum sample from a patient,

(b) adding a constant amount of a second antibody to the reaction mixture wherein said second antibody recognizes a constant region of the heavy chain of the first antibody and wherein the second antibody is labeled and part of a signal producing system,

(c) separating free labeled second antibody from bound antibody,

(d) measuring the signal generated by the second antibody in the reaction mixture; and

(e) correlating a decreased level of signal strength with a diagnosis of endometriosis in the patient.

In those cases where the antibody is enzyme labeled, the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled antibody followed by incubation are performed prior to measuring the generated signal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is an elution profile showing MonoQ anion exchange chromatography of homogenized eutopic endometrium from an endometriosis patient. The homogenate was passed through a protein G column to remove IgG prior to loading on the MonoQ column. Initial peaks prior to the start of the salt gradient represent multiple 1ml homogenate loads. Elution conditions: 50mM Tris/HCl pH8.0. Elution was in the same buffer with a linear 0-0.5 NaCl gradient (stepped to 1M NaCl) and is denoted by the dashed line. Fractions corresponding to the gel loading (Figures 1b-d) are indicated along the top of the graph.

Figure 1b shows a 5-15% gradient gel of selected column fractions indicated in Figure 1a run under reducing conditions and stained with SYPRO Orange.

Figure 1c is an autoradiograph displaying proteins in the homogenate fractions recognized by IgG present in pooled endometriosis serum. Stripping and reprobing of the same blot using pooled normal male serum showed no reactivity with the same bands. IgG binding activity was detected in fraction 24 using control sera but similar binding was not seen with endometriosis sera. The 45.2kDa molecular weight marker carbonic anhydrase II shows reactivity. This same reactivity was not observed with the control sera.

Figure 1d is an autoradiograph showing IgA binding activity of the endometriosis sera. Probing of the blot with anti-IgA a-chain specific antibody in the absence of a primary serum showed that IgA was present in these fractions.

Figure 2 is a western blot of peritoneal fluid (W1517) run out on a 5-15% SDS-PAGE gel under reduced and alkylated conditions. The western blot was then

incubated with either sheep anti-human α_2-HSG followed by HRP-goat anti-sheep (lane a) or with a 1:100 dilution of endometriosis sera followed by HRP-goat IgG g-chain specific antibody (1:1000 dilution). Clearly visible are α_2-HSG in lane a and bands of equivalent molecular weight in lane b.

Figure 3a is an SDS-PAGE gel showing a partially purified fraction of α_2-HSG (MonoQ column, pH 8.0). Fourteen protein bands are visible after staining with SYPRO Orange.

Figure 3b is a western blot of a gel run with partially purified fraction of α_2-HSG and probed with endometriosis sera HRP goat anti-human IgG antibody. Five protein bands were detected.

Figure 3c is a western blot as described in Figure 3b except that the protein fraction was treated with neuraminidase prior to electrophoresis. Antibody binding to all of the proteins is clearly reduced.

Figure 3d is a western blot as described for Figure 3b except that the α_2-HSG fraction was subjected to preadsorption with jacalin agarose prior to electrophoresis. All antibody binding is removed.

Figure 3e shows autoreactivity with myosin and carbonic anhydrase II in the molecular weight markers.

Figure 4 is a western blot of a gel run with partially purified fraction of α_2-HSG and probed with endometriosis sera HRP goat anti-human IgG antibody which demonstrates that deglycosylation of α_2-HSG abolishes autoantibody binding. Neuraminidase was used to cleave terminal sialic acid (lanes B, E, H). Complete removal of carbohydrate was then achieved using EndoF/PNG'ase digestion (lanes C, F, H). Removal of sialic acid and all carbohydrate was confirmed by

reduction in molecular weight as shown by reactivity with goat anti-α_2-HSG (lane A-C). Lanes A, D, and G were untreated. Removal of terminal sialic acid with neuraminidase reduced reactivity with patient serum (lane D versus E). The remaining reactivity in this fraction was at the same molecular weight as the undigested α_2-HSG, indicating that the autoantibody binding is dependent on the presence of terminal sialic acid. Complete removal of carbohydrate by neuraminidase treatment followed by EndoF/PNG'ase digestion completely abolished reactivity (lane F). No equivalent reactivity was observed using pooled male control sera (lanes G-I).

Figure 5a demonstrates that desialylation of bovine fetuin increases reactivity with endometriosis sera. As measured by ELISA, a slight but significant increase in binding is seen following treatment with neuraminidase. Figure 5b is a SYPRO Orange stained gel showing the molecular weight reduction in fetuin following neuraminidase treatment. Lane A is untreated; lane B is neuraminidase treated.

Figure 6 both graphically and by radioautography depicts that binding to the 72kDa antigen and carbonic anhydrase II is inhibited in the presence of α_2-HSG.

Figure 7 is a western blot analysis of purified proteins following transfer to nitrocellulose from 5-15% gradient gels run reduced and alkylated. Purified hemopexin (lane A), α_2-HSG (lane B), and serum IgA1 (lane C) are autoantigens recognized by IgG antibodies in endometriosis sera.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides diagnostic methods for detecting endometriosis in a patient. The diagnostic methods are based on immunoassays which detect the presence of autoantibodies in a patient's serum reacting with Thomsen-Friedenreich (Tf) antigen, including Tf-like antigen. As used herein, Tf-like antigen also encompasses Tf antigen. Increased levels of autoantibodies in a serum sample which bind Tf-like antigen, when compared to normal serum levels of autoantibodies which bind Tf-like antigen, correlate with a diagnosis of endometriosis in the patient.

A blood sample may be conveniently drawn from a patient by venipuncture or other suitable means. A serum sample may be prepared from the blood sample using well known methods.

There are many different types of immunoassays which may be used in the methods of the present invention. Any of the well known immunoassays may be adapted to detect the level of autoantibodies in a serum sample which react with the Tf-like antigen, such as e.g., enzyme linked immunoabsorbent assay (ELISA), fluorescent immunosorbent assay (FIA), chemical linked immunosorbent assay (CLIA), radioimmuno assay (RIA), and immunoblotting. For a review of the different immunoassays which may be used, see: *The Immunoassay Handbook*, David Wild, ed., Stockton Press, New York, 1994. Preferably, a competitive immunoassay with solid phase separation or an immunometric assay for antibody testing is used. See, The Immunoassay Handbook, chapter 2.

In a typical assay, the reagents include a serum sample from a patient, the autoantibodies to be detected (contained in the serum sample), Tf-like antigen, and means for producing a detectable signal.

Thus, in one embodiment of the invention, the method for diagnosing endometriosis in a patient employs an immunometric assay for antibody testing. In this embodiment, the Tf-like antigen is immobilized on a solid support or surface such as a bead, plate, slide or microtiter dish. An aliquot of serum sample from a patient is added to the solid support and allowed to incubate with the Tf-like antigen in a liquid phase. An antibody that recognizes a constant region in human autoantibodies present in the serum which have reacted with the Tf-like antigen is added. This antibody is an anti-human immunoglobulin and is also part of a signal producing system. Anti-human immunoglobulin which is specific for IgA, IgG, or IgM heavy chain constant regions may be employed. After separating the solid support from the liquid phase, the support phase is examined for a detectable signal. The presence of the signal on the solid support indicates that autoantibodies to Tf-like antigen present in the serum sample have bound to the Tf-like antigen on the solid support.

The signal producing system is made up of one or more components, at least one of which is a label, which generate a detectable signal that relates to the amount of bound and/or unbound label i.e., the amount of label bound or unbound to the Tf-like antigen. The label is a molecule that produces or which may be induced to produce a signal. Examples of labels include fluorescers, enzymes, chemiluminescers, photosensitizers or suspendable particles. The signal is detected and may be measured by detecting enzyme activity, luminescence or light absorbance. Radiolabels may also be used and levels of radioactivity detected and measured using a scintillation counter.

Examples of enzymes which may be used to label the anti-human immunoglobulin include β-D-galactosidase, horseradish peroxidase, alkaline phosphatase, and glucose-6-phosphate dehydrogenase ("G6PDH"). Examples of fluorescers which may be used to label the anti-human immunoglobulin include fluorescein, isothiocyanate, rhodamine compounds, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. Chemiluminescers include e.g., isoluminol.

Free labeled antibody is separated from bound antibody and if necessary, an appropriate substrate with which the label, e.g., enzyme, reacts is added and allowed to incubate.

In a preferred embodiment, the anti-human immunoglobulin is enzyme labeled with either horseradish peroxidase or alkaline phosphatase.

The amount of color, fluorescence, luminescence, or radioactivity present in the reaction (depending on the signal producing system used) is proportionate to the amount of autoantibodies in a patient's serum which react with the Tf-like antigen. Quantification of optical density may be performed using spectrophotometric methods. Quantification of radiolabel signal may be performed using scintillation counting.

Increased levels of autoantibodies reacting with Tf-like antigen over normal serum levels correlate with a diagnosis of endometriosis in the patient.

In another embodiment of the invention, the method for diagnosing endometriosis in a patient employs a competitive immunoassay where a known antibody and a patient's autoantibodies compete for binding to Tf. In this embodiment, a constant amount of a labeled antibody which is known to bind to Tf-like antigen is incubated

with different concentrations of a serum sample from a patient. For example, the mouse monoclonal antibody 49H.8, (Rahman and Longenecker, 1982, J. Immun. 129(5): 2021-4) known to bind to Tf, may be used. Other monoclonal antibodies which bind to Tf-like antigen and which may be used as antibody in the competitive immunoassay include 155H7 and 170H82 (Longenecker et al. 1987, J. Nat. Cancer Inst., 78(3): 489-96, A78-G/A7 (Karsten et al. 1995, Hybridoma 14(1): 37-44), HB-T1 (DAKO Co.), RS1-114 and AHB-25B (Stein et al. 1989, Cancer Res. 49(1): 32-7), HT8 (Metcalfe et al., 1984, Br. J. Cancer 49(3): 337-42), 161H4 (Longenecker et al., 1987), HH8 (Clausen et al. 1988), and BW835 (Hanish et al, 1995, Cancer Res. 55(18): 4036-40). As described above, the antibody may be labeled with a fluorescer, enzyme, chemiluminescer, photosensitizer, suspendable particles, or radioisotope. Preferably, the known antibody is enzyme labeled. After incubation, bound labeled antibodies are separated from free autoantibodies. Depending on the signal producing system used and if necessary, an appropriate substrate with which the labeled antibody reacts is added and allowed to incubate. The signal generated by the sample is then measured. A decrease in optical density or radioactivity from before and after addition of the serum sample or between experimental and control samples, is indicative that autoantibodies in the serum sample have bound to Tf. Decreased optical density or radiolabeled signal when compared to experimental serum samples from normal patients, correlates with a diagnosis of endometriosis in a patient.

In a preferred embodiment of the competitive immunoassay, an indirect method using two antibodies is used. The first antibody is a Tf-like antigen specific antibody as described in the preceding paragraph with

the exception that it is not labeled. The first antibody is incubated with different concentrations of a serum sample from a patient. A constant amount of a second antibody is then added to the sample. The second antibody recognizes constant regions of the heavy chains of the first antibody. For example, the second antibody may be an antibody which recognizes constant regions of the heavy chains of mouse immunoglobulin which has reacted with the Tf-like antigen (anti-mouse immunoglobulin). The second antibody is labeled with a fluorophore, chemilophore or radioisotope, as described above. Free labeled second antibody is separated from bound antibody. If an enzyme-labeled antibody is used, an appropriate substrate with which the enzyme label reacts is added and allowed to incubate. A decrease in optical density or radioactivity from before and after addition of the serum sample or between experimental and control samples is indicative that autoantibodies in the serum sample have bound to Tf. Decreased optical density or radioactivity when compared to experimental serum samples from normal patients correlates with a diagnosis of endometriosis in a patient.

Enzymes may be covalently linked to Tf-like antigen reactive antibodies for use in the methods of the invention using well known methods. There are many well known conjugation methods. For example, alkaline phosphatase and horseradish peroxidase may be conjugated to antibodies using glutaraldehyde. Horseradish peroxidase may also be conjugated using the periodate method. Commercial kits for enzyme conjugating antibodies are widely available. Enzyme conjugated anti-human and anti-mouse immunoglobulin specific antibodies are available from multiple commercial sources.

Biotin labeled antibodies may be used as an alternative to enzyme linked antibodies. In such cases, bound antibody would be detected using commercially available streptavidin-horseradish peroxidase detection systems.

Enzyme labeled antibodies produce different signal sources, depending on the substrate. Signal generation involves the addition of substrate to the reaction mixture. Common peroxidase substrates include ABTS®(2,2'-azinobis(ethylbenzothiazoline-6-sulfonate)), OPD (*O*-phenylenediamine) and TMB (3,3', 5,5'-tetramethylbenzidine). These substrates require the presence of hydrogen peroxide. *p*-nitrophenyl phospate is a commonly used alkaline phosphatase substrate. During an incubation period, the enzyme gradually converts a proportion of the substrate to its end product. At the end of the incubation period, a stopping reagent is added which stops enzyme activity. Signal strength is determined by measuring optical density, usually via spectrophotometer.

Alkaline phosphatase labeled antibodies may also be measured by fluorometry. Thus in the immunoassays of the present invention, the substrate 4-methylumbelliferyl phosphate (4-UMP) may be used. Alkaline phosphatase dephosphorylated 4-UMP to form 4-methylumbelliferone (4-MU), the fluorophore. Incident light is at 365 nm and emitted light is at 448 nm.

For use in the methods of the present invention, Tf-like antigen may be obtained from various sources. For example, Tf-like antigen may be purified from conditioned culture medium used to cultivate tumor cell lines such as the adenocarcinoma cell line LS174T, obtainable through the American Type Culture Collection (ATCC). Transitional cell carcinoma lines may also

serve as source of Tf-like antigen. Tf-like antigen may be purified from such conditioned culture medium by affinity chromatograpy using an MAb 49H.8-CnBr activated Sepharose column. Gel filtration may also be performed for additional purification. Still further purification of Tf-like antigen may be achieved by utilizing lectin affinity chromatography with insolubilized peanut agglutinin (PNA) or other lectin. PNA may be obtained from E-Y Laboratories, San Mateo, California.

Purified serum proteins bearing Tf-like antigens such as IgA, hemopexin, and alpha-2-Heremans Schmidt may also be used as antigens. A preferred source of Tf-like antigen is commercially synthesized Tf-like antigens covalently linked to bovine serum albumin. Tf-like antigen and its glycoforms are available from commercial vendors such as BioCarb as recently described by Dahlenborg et al. (1997) *In. J. Cancer 70*:63-71. Alternatively, Tf-like antigens may be obtained by custom synthesis from commercial vendors. Monoclonal antibodies which react with Tf-like antigen are readily available.

MAb49.H (isotype IgM) may be prepared and purified as reported by Rahman et al., (1982) *J. Immunol. 129*:2021-2024 and Longenecker et al. (1984) *Int. J. Cancer* 33:123-129. The disclosure of these articles and other articles cited in this application are incorporated herein as if fully set forth.

In order to practice the methods of the present invention, the relevant immunoassay must be standardized. Since Tf-like antigen is usually associated with a glycoprotein, it is naturally heterogenous. The source of such heterogeneity may include sialylation. To this end, an ampoule of an international standard (IS) or international reference

preparation (IRP) should be obtained. The National Institute for Biological Standards and Control (NIBSC, Blanche Lane, South Mimms, Potters Bar, Herts EN63QG) may prepare such a sample of Tf-like antigen and assign an international unit (IU). Multiple sets of secondary standards, from which future lots of calibrators may be assigned values, should also be prepared.

The immunoassays of the present invention require the use of calibrators in order to assign values or concentrations to unknown samples. Typically, a set of about six calibrators is run prior to the unknown samples from which a calibration curve is plotted. The concentrations of the unknown samples are determined by interpolation. Interpolation is best carried out by a computer program. For a discussion on calibration, see *The Immunoassay Handbook*, chapter 2.

The present invention also provides therapies for reducing, preventing and/or ameliorating endometriosis in a patient. In this embodiment of the invention Tf-like antigen or an analog thereof, is injected into a patient. The injected Tf-like antigen or analog thereof competes with Tf-like antigen present in endometrium of a patient for autoantibody binding.

The following examples further illustrate the invention.

EXAMPLE 1

Materials and methods

Preparation of tissue homogenate

Fresh ectopic and eutopic endometrium, peritoneal fluid, and serum were obtained from hysterectomy patients with the informed consent of the patients. Fresh tissue (1-2 grams) was added to 10 ml ice-cold PBS containing Complete Protease Inhibitor Cocktailä (Boehringer Mannheim, Indianapolis, IN) and maintained at 4°C throughout the preparation. The tissue was homogenized for 1 minute with a Polytron homogenizer (Brinkman, Lucerne, Switzerland) followed by centrifugation at 13000 g for 10 minutes. The supernatant was collected and sonicated using a Branson 250 sonifier (Danbury, CT). The tissue homogenate was 0.22 mm filtered and stored at 20°C.

Preparation of serum

Peripheral venous blood (60 ml) was drawn from healthy volunteers into glass vacutainer tubes (no additive) and allowed to clot for 4 hours at room temperature. Serum was then removed to sterile tubes and delipidated by centrifugation at 12000 g for 30 minutes at 4°C.

Purification of tissue autoantigens

Following homogenization, tissue antigens were passed through a Protein G FPLC column (Amersham Pharmacia, Piscataway, NJ) to remove IgG. Proteins not retained by the protein G column were then applied to an anion exchange column (MonoQ FPLC, Amersham Pharmacia, Piscataway, NJ) following equilibration in 50mM sodium phosphate pH 8.0. Bound proteins were eluted from the

column using a linear 0-0.5 M NaCl gradient in the same buffer, followed by a step gradient to 1 M NaCl also in the same buffer. All proteins of interest eluted prior to the 1 M NaCl gradient.

Purification of Serum Autoantigens

α_2-HSG was purified from normal whole human serum by anion exchange chromatography as described for the tissue antigens above using a HiPrep 16/10 Q XL column. This partially purified α_2-HSG was used in some experiments as indicated in the test. Further purification was carried out on a second anion exchange column (Mono Q) equilibrated with 20 mM piperazine buffer pH 5.0 and eluted in the same buffer using a NaCl gradient as described above. A final homogeneous preparation of α_2-HSG was obtained following gel filtration on an FPLC Superose 12 column (Amersham Pharmacia, Piscataway, NJ). α_2-HSG concentrations were monitored by ELISA.

IgA1 was purified from whole serum by anion exchange chromatography and jacalin agarose affinity chromatography as previously described (11).

Transferrin and Hemopexin were purified from whole serum by metal chelate chromatography on a column packed with Chelating Sepharose Fast Flow (Amersham Pharmacia, Piscataway, NJ) charged with Zn^{2+} ions. The column and serum were pre-equilibrated in 30 mM sodium phosphate buffer containing 150 mM NaCl. Zinc binding proteins were eluted using a linear 0-0.5 M imidazole gradient in the same buffer. The resulting transferrin and hemopexin-containing fractions (as determined by western blot analysis) were separately pooled and further purified on a mono Q anion exchange column at pH 8.0 as already described. This protocol resulted in an electrophoretically pure hemopexin preparation.

Transferrin was not however resolved from hemopexin in the transferrin preparation.

Modification of carbohydrate-epitopes on glycoproteins

Terminal sialic acid moieties were removed from glycoproteins by treatment with agarose-conjugated neuraminidase. One milligram of protein in 0.5 ml was incubated with 5 Units of neuraminidase (Sigma, St Louis, MO) in 50 mM sodium acetate, 150 mM sodium chloride, 4 mM calcium chloride, pH 5.5 overnight at 37°C. Agarose beads were removed by centrifugation at 4000 g for 5 minutes. Supernatants were removed and stored at 4°C.

Core carbohydrate groups were removed from glycoproteins by treatment with endoglycosidase F (Endo F) and peptide-N-glycosidase F (PNG'ase F). This was obtained as a commercial kit from Glyko (Novato, CA). Forty micrograms of neuraminidase-treated protein were denatured by heating to 100°C for 2 minutes in the presence of 20 mM sodium phosphate pH 7.5, 50 mM EDTA, 0.1 % v/v SDS, 0.5 % b-mercaptoethanol. To avoid inhibition of PNG'ase F by SDS, 0.1% Tween-20 was added to the denatured sample after cooling. The denatured protein was then incubated with 667 deglycosylation Units (DGU) of the Endo F / PNG'ase F mix at 37°C for 18 hours.

Removal of jacalin-reactive glycoproteins from samples was achieved by pre-adsorption with an excess of jacalin-conjugated agarose beads (Vector, Burlingame, CA). Samples were incubated with jacalin-conjugated agarose for 30 minutes at room temperature before centrifugation at 4000g for 5 minutes. Supernatants were removed and stored at 4°C.

Antibodies and Glycoproteins

Immunoglobulin-fractions of sheep anti-human α_2-HSG and sheep anti-human transferrin were obtained from Biodesign International (Kennebunk, ME). Goat anti-human hemopexin antiserum was obtained from Kent Laboratories (Redmond,WA). HRP-conjugated rabbit anti-sheep IgG and HRP-conjugated rabbit anti-goat IgG were both from Jackson Immunoresearch Laboratories (West Grove, PA). HRP-conjugated goat-anti human IgA (a-chain specific) and HRP-conjugated goat-anti human IgG (g-chain specific) were purchased from (Sigma). Commercial α_2-HSG was obtained from Calbiochem-Novabiochem (La Jolla, CA).

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (12). Briefly, equal volumes of 2x gel loading buffer were added to protein samples and boiled for 5 minutes. Where appropriate, proteins were resolved under reducing conditions (5 % v/v b-mercaptoethanol in gel loading buffer). Re-association of reduced proteins was prevented by adding iodoacetamide (final concentration 60 mM) to samples after boiling. Protein samples were resolved using 4 % -15 % gradient gels (Biorad, Hercules, CA). For direct visualization of proteins, gels were placed in 0.02 % v/v SYPRO-Orange (Biorad, Hercules, CA) in 7.5 % v/v acetic acid for 30 minutes. Gels were then rinsed in 7.5 % acetic acid and viewed on a gel imager (Alpha Innotech Corporation, San Leandro, CA).

Immunoblotting

Proteins were transferred to nitrocellulose membranes from SDS-gels using a Biorad Transblot SD semi-

dry blotter. Ponceau Red staining confirmed transfer of proteins to nitrocellulose membranes. Membranes were blocked overnight at 4°C in 5 % w/v fat-free powdered milk, 0.5 % Tween-20 in PBS. Primary antibody or serum was added to blocking solution then incubated for 2 hours at room temperature. Membranes were then washed 6 times for 5 minutes per wash in PBS. The HRP-conjugated secondary antibodies were diluted in 3 % w/v fat-free milk powder with 0.05 % v/v Tween-20 in PBS. Membranes were incubated for 2 hours at room temperature before washing 6 times for 5 minutes as described. Proteins were then detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia, Piscataway, NJ). Where appropriate, nitrocellulose membranes were stripped and re-probed by washing twice for 5 minutes per wash in 0.9 % w/v NaCl before agitation in 100 mM Glycine-HCl pH 1.5 for 30 minutes at room temperature. Membranes were then washed 3 times for 5 minutes per wash with PBS pH 7.4. The membranes were blocked and probed with antibodies as described.

Enzyme Linked Immunosorbent Assay (ELISA)

Wells of Falcon Microtest III microtitre plates (Becton Dickinson, Oxnard, CA) were coated overnight at 4°C with 10 µl protein diluted to 100 µl in sodium carbonate buffer pH9.2. Plates were then washed 3 times in PBS containing 0.05 % v/v Tween-20. Wells were blocked with 3 % w/v BSA in PBS / Tween-20 (blocking buffer) for 1 hour at 37°C before washing 3 times. Primary antibody or test serum was diluted in blocking buffer as appropriate and 100 µl added to each well. Plates were incubated for 2 hours at 37°C and washed 3 times in PBS / Tween-20. HRP-conjugated secondary antibody was diluted in blocking buffer and 100 µl added to each well. Plates were then washed 3 times. 100 ml

of the colorimetric HRP-substrate, ABTS was added to each well and incubated for 30 minutes at room temperature. Plates were read at 405nm with a Dynatech plate reader (Chantilly, VA) Data was analyzed using Graphpad Prism software.

EXAMPLE 2

Identification of autoantigens in ectopic endometrium, eutopic endometrium, peritoneal fluid and sera.

Soluble endometrial protein preparations were prepared from hysterectomy tissue as described in the materials and methods, and subjected to protein G chromatography to remove IgG. The resulting IgG-free preparations were then subjected to anion exchange chromatography (figure 1a). Fractions from this column were then analyzed by SDS-PAGE under reducing conditions (figure 1b). Protein bands on identical gels were transferred to nitrocellulose for western blot analysis. Development of the western blots was carried out using sera from both pooled endometriosis patients and pooled normal male donors as primary antibody sources. Figure 1c shows a typical example of the antigens identified in a proliferative phase eutopic endometrium from a patient (W1345). Autoreactive IgG was restricted to the endometriosis patient sera (Figure 1c) as compared to the same blots probed with a control serum from a normal male donor (not shown).

IgG g-chain binding was detected against fraction 24 of the mono Q column with the control serum but not in other fractions (not shown). Interestingly, a similar reactivity against fraction 24 was not present in blots developed using the endometriosis serum. The molecular weight of the reactive proteins detected using pooled endometriosis sera correlate well with those described by other workers 6, 10. A total of 10 autoreactive bands were detected on a western blot probed with pooled endometriosis sera. A 72 kDa band was detected in fraction 23 with both IgA α-chain specific and IgG γ-chain specific second antibodies (figures 1c and 1d). This band was initially identified as transferrin on the

basis of its molecular weight and the NaCl concentration at which it eluted from the MonoQ column (the elution of transferrin at this position was confirmed by western blot analysis using a sheep anti-transferrin antibody, not shown). A 54kDa protein was also detected in this fraction with the IgG γ-chain specific antibody but not the anti-IgA α-chain specific antibody. This protein elutes in a position identical to partially desialylated α_2-HSG (data not shown). α_2-HSG has previously been identified as an autoantigen in endometriosis patients (10). The IgG reactive band of 59kDa observed in fractions 26 and 29 is the α-chain of IgA and the elution positions correspond to the known elution positions, under these run conditions, of monomeric and dimeric IgA respectively. The identity of this protein as IgA α-chain was confirmed by stripping and reprobing the blot with an anti α-chain specific antibody (not shown). This blot also showed the presence of anti-α-chain reactive bands in fraction 23 (the transferrin containing fraction). The presence of IgA antigens in this fraction most likely arises from IgA covalently complexed with other serum proteins [13, 14]. Fraction 26 also contained a reactive band at 72 kDa and fraction 28 and 29 had bands at 69 kDa. These later bands showed no activity on the α-chain specific blot. The presence of an IgG antibody in endometriosis patients which recognizes the α-chain of IgA was confirmed by western blot using highly purified serum IgA1 (see Figure 7). In contrast, no IgA autoantibodies against endometrial antigens other than in those fractions, which contain IgA α-chain, were observed.

An ovarian ectopic endometrial sample from the same patient (w1345) had autoantigenic proteins of molecular

weights 54, 47, 43, and 34 KDa (not shown). The 54 kDa protein was α_2-HSG as determined by a western blot developed with a sheep anti-α_2-HSG specific antibody. α_2-HSG was also detected in peritoneal lavage fluid from a different patient (W1517) by western blot. In addition to the 54 kDa antigen, antigens of 186, 126, 68, 43, 37, and 34 kDa were present in the peritoneal fluid (figure 2).

Reactivity with two molecular weight markers was observed with both pooled patients and individual sera (see figures 1c and figure 3). These markers were myosin at 205kD and carbonic anhydrase II at 42kD. Reactivity with the myosin band was also seen with both the pooled male control sera and some individual male sera. In contrast, reactivity with anhydrase II was specific to the endometriosis sera and this protein has been reported by other workers as an autoantigen in endometriosis [7, 8].

In summary, autoantigens in preparations from eutopic and ectopic endometrium and peritoneal lavage have been identified. In agreement with previously published reports, α_2-HSG, transferrin and carbonic anhydrase II are autoantigens recognized by sera from endometriosis patients. In addition, results presented here also demonstrate that the α-chain of IgA is also an autoantigen recognized by the same sera. Since these proteins show no significant homology at the protein level, it is unlikely that a common peptide epitope is shared by all four proteins. Three of the four proteins, α_2-HSG, α-chain of IgA1 and possibly carbonic anhydrase II, do however, share a common carbohydrate epitope. This O-linked carbohydrate structure contains a Galβ1-3NAcGal epitope which is recognized by the jackfruit (Artocarpus integrifolia) lectin jacalin. The results

presented here confirm previous reports that α_2-HSG and carbonic anhydrase are among the autoantigens recognized by autoantibodies present in endometriosis sera. The present study extends these results to show that IgA1 and hemopexin are also autoantigens recognized by sera from endometriosis patients.

EXAMPLE 3

Jacalin preadsorption removes autoantibody binding.

α_2-Heremans Schmidt glycoprotein (α_2-HSG) was chosen as a model antigen to investigate a potential role for jacalin reactive carbohydrates in the endometriosis autoantibody response. Initial experiments using α_2-HSG purchased from commercial sources proved to be unsatisfactory. This was because of the variability of glycoforms present in these preparations. In common with most sialated glycoproteins, α_2-HSG loses terminal sialic acid residues during storage. Fully sialated α_2-HSG from normal human serum was freshly prepared. In order to avoid biasing the preparation with respect to its carbohydrate content, lectin based affinity purification protocols were avoided and instead a combination of anion exchange and gel filtration chromatography was used. A three step procedure of anion exchange on a MonoQ column at pH 8.0, followed by a second MonoQ column at pH 5.0, and a final gel filtration step on a superose 12 column, resulted in the purification of the fully sialated glycoform of α_2-HSG with a molecular weight of 58kDa.

When experiments were carried out using a partially purified α_2-HSG fraction from the first anion exchange column, autoreactivity with endometriosis serum on western blot was abolished following pre-incubation with jacalin agarose (figure 3). Fourteen protein bands were observed on SYPRO orange stained SDS-PAGE gels (figure 3 lane a). Reactivity with endometriosis sera was observed with 5 proteins (figure 3 lane b). The reactive proteins had approximate molecular weights of 230, 188, 168, 120, and 58kDa; the 58kDa being α_2-HSG. Binding by IgG in endometriosis sera of all of these 5 bands was abolished following incubation with jacalin agarose (figure 3 lane

d), indicating that the binding of the autoantibodies was to all of the jacalin binding proteins present in the fraction and not just to α_2-HSG. Protein staining of gels of pre- and post-absorbed fraction 13 show that the removal of activity was not caused by a non-specific removal of proteins in the fraction. The protein concentrations of adsorbed and unadsorbed were adjusted such that total protein loading on the gels used for western blotting was uniform.

The carbohydrate structure recognized by jacalin is very specific and binding is dependent on the presence of the Tf. Jacalin recognizes the disaccharide Galβ1-3GalNAc, which is present within some O-linked oligosaccharides. Galβ1-3GalNAc is expressed by a very limited number of the many plasma proteins. These proteins include IgA1, IgD, C1-inhibitor, hemopexin, plasminogen, α1-antitrypsin, α2-macroglubulin, 8S-α3 glycoprotein, chorionic gonadotropin (hCG) and α_2-HSG (15). With regard to hCG, it is of interest that the receptor for hCG expresses also expresses Tf-like antigen and that autoantibodies found in infertile patients both with and without concomitant endometriosis bind to the receptor [5].

EXAMPLE 4

Removal of Carbohydrate Abolishes Autoantibody Binding.

Removal of proteins recognized by the autoantibodies by jacalin does not in itself determine whether a common carbohydrate epitope(s) is involved. To determine whether the carbohydrate is essential for the recognition of α_2-HSG by the endometriosis autoantibodies, reactivity with deglycosylated α_2-HSG was investigated. α_2-HSG carbohydrate was removed in a two step procedure. First, neuraminidase was used to cleave terminal sialic acid. Second, complete removal of carbohydrate was then achieved using a further EndoF/PNG'ase digestion step. Untreated, neuraminidase treated, and deglycosylated α_2-HSG were then subjected to western blot analysis at equal protein loads (Figure 4). Removal of sialic acid and all carbohydrate was confirmed by reduction in molecular weight as shown by reactivity with sheep anti-α_2-HSG (Figure 4 lanes A-C).

The sheep anti-α_2-HSG antibody is reactive with the peptide and shows no reactivity with other jacalin binding proteins on double immunodiffusion (data not shown). Removal of terminal sialic acid with neuraminidase reduced reactivity with patient serum (Figure 4 lanes D and E). The remaining reactivity in this fraction was at the same molecular weight as the undigested α_2-HSG, indicating that the autoantibody binding is dependent on the presence of terminal sialic acid. Complete removal of carbohydrate by neuraminidase treatment followed by EndoF/PNG'ase digestion completely abolished reactivity (Figure 4 lane F). This reduction in autoantibody binding following neuraminidase was not restricted to α_2-HSG since similar treatment of the

partially purified α_2-HSG fraction resulted in reduced binding to all 5 reactive proteins (Figure 3 lane c).

Table 1

Correlation of Identified Endometriosis Antigens and Jacalin Binding Activity

Antigen	Endometriosis Antigen In this Study/ (published study)	Jacalin Reactivity In this Study/ (published reactivity)
α-HSG	Yes/[9,10]	Yes/[63]
Carbonic anhydrase I/II	Yes/[6-8]	no/unknown
Hemopexin	Yes/no	Yes/[15,43]
IgA	Yes/no reviewed in [16]	Yes/multiple
CD23	No/soluble form elevated [53-55]	No/acts as a Gal-GalNac lectin like jacalin [64,65]
hCG Receptor	No/[5]	No/[66]

These results show that (with the exception of transferrin) a common carbohydrate epitope is recognized on a_2-HSG, carbonic anhydrase, hemopexin and the a-chain of IgA1 (see table 1 for a summary of antigens identified in this, and published studies, and their jacalin reactivity). This conclusion was reached on the basis that all of the identified antigens (with the exception of transferrin and carbonic anhydrase II) are known to bind the lectin jacalin, and that the removal of carbohydrate from the antigens removed the ability of endometriosis sera to bind these antigens.

EXAMPLE 5

Binding of Autoantibody to Bovine Fetuin

Bovine fetuin, the homologue of human a_2-HSG, bears the same jacalin binding carbohydrate moiety as human a_2-HSG. Sheep antiserum raised against human SG peptide does not show cross-reactivity with bovine fetuin, suggesting significant differences in antigenicity between the two species despite considerable sequence homology. Sera from endometriosis patients show positive reactivity by ELISA and western blot with bovine fetuin (Figure 5). Neuraminidase treatment of fetuin, in contrast to -HSG, slightly increased autoantibody reactivity on ELISA (Figure 5). However, more complete desialylation, or complete deglycosylation of bovine fetuin abolished antibody binding, giving additional evidence that the autoantibody response in endometriosis patients is carbohydrate dependent (not shown). The increase in binding to fetuin following incomplete neuraminidase treatment may result from bovine fetuin being more heavily sialated than its human counterpart.

EXAMPLE 6

D-Galactose dependency of autoantibody binding.

Jacalin binding is dependent on the presence of a Galb1-3NacGal moiety. If the autoantibody response involves this epitope, binding should be competitively inhibited by D-galactose, as is the case for jacalin binding. Binding of endometriosis IgG to a_2-HSG on western blots was completely abolished in the presence of 0.8M D-Galactose, indicating that D-Galactose may form a part of the epitope recognized by the autoantibodies (not shown).

EXAMPLE 7

Serum autoantigens recognized by endometriosis serum share a common epitope.

Since a common carbohydrate epitope accounts for the autoreactivity with the different antigens identified above, then autoantibody binding should be blocked in the presence of an excess of a different antigen. This proved to be the case: addition of a 10 fold excess of highly purified a_1-HSG or bovine fetuin both inhibited binding of autoantibody to carbonic anhydrase II and the 72kDa antigen (Figure 6).

Is hemopexin, rather than transferrin, the 72kDa autoantigen in endometriosis? Of the antigens described thus far, all, with the exception of transferrin, express the O-linked carbohydrates recognized by jacalin. No such carbohydrate structures are expressed on transferrin, and jacalin does not bind to transferrin [15]. This leaves three possibilities: either an anti-transferrin response distinct from the jacalin carbohydrate response is present in endometriosis sera, or the reactivity which purifies with transferrin is against a contaminant in the transferrin preparations, or both. Two reactive proteins of ~70kDa were found in the anion exchange fractions of eutopic endometrium W1345. One eluted early in the salt gradient in the same fraction as transferrin. The other protein eluted at higher salt concentration in the same fractions as IgA. Examination of the Swiss 2D database indicates that jacalin-binding proteins of similar molecular weight and isoelectric point include hemopexin. When hemopexin was purified by Zn^{2+} metal chelate chromatography and anion exchange chromatography, a homogeneous preparation was obtained. Identity was confirmed by western blotting of fractions with a hemopexin specific antibody. When

tested against endometriosis patient serum this protein was recognized as an autoantigen (Figure 7). Western blot analysis of the same protein with an antibody specific for transferrin showed no reactivity with the purified hemopexin. When fractions from the same column were investigated for the transferrin reactivity, an earlier peak from the anion exchange column was found to contain transferrin. Western blot analysis of the transferrin-containing fractions with anti-hemopexin revealed the presence of hemopexin with similar mobility to transferrin on SDS-PAGE. Double immunodiffusion showed no lines of identity when anti-hemopexin and anti-transferrin were precipitated against the peak transferrin fraction, indicating that the antisera were antigen specific (not shown). The presence of jacalin binding activity in this transferrin preparation was confirmed by the presence of a precipitin line in double immunodiffusion against jacalin (not shown). Further experiments are currently underway to determine the nature of the antigen which co-purifies with transferrin, but it is of interest that the 72kDa binding activity was inhibited by the addition of a pure a_2-HSG preparation (figure 6).

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CLAIMS

What is claimed is:

1. A method for diagnosing endometriosis in a patient, said method comprising:

(a) obtaining a serum sample from said patient,

(b) incubating Thomsen-Friedenreich (Tf)-like antigen with said serum sample,

(c) detecting autoantibody reactivity with the Tf-like antigen in the sample, and;

(d) correlating an increased level of autoantibody reactivity to the Tf-like antigen in the serum sample with a diagnosis of endometriosis in said patient.

2. The method of claim 1 wherein the autoantibody reactivity is determined by immunoassay.

3. The method of claim 2 wherein the immunoassay is an immunometric assay.

4. The method of claim 2 wherein the immunoassay is a competitive immunoassay.

5. The method of claim 3 comprising:

(a) immobilizing Tf-like antigen on a solid support,

(b) adding an aliquot of serum sample from a patient to the Tf-like antigen bound on the solid support and incubating,

(c) adding a labeled anti-human immunoglobulin to the solid support wherein said anti-human immunoglobulin is part of a signal producing system,

(d) separating free labeled antibody from bound antibody,

(e) measuring the signal generated by the solution containing the solid support; and

(f) correlating an increase in signal strength with a diagnosis of endometriosis in the patient.

6. The competitive immunoassay of claim 4 comprising:

(a) preparing a reaction mixture by incubating a constant amount of a labeled antibody bound to Tf-like antigen with different concentrations of a serum sample from a patient wherein said labeled antibody is part of a signal producing system,

(b) separating bound labeled antibodies from free autoantibodies,

(c) measuring the signal generated by the labeled antibody in the reaction mixture, and;

(d) correlating a decrease in signal strength after addition of the serum sample from a patient with a diagnosis of endometriosis in the patient.

7. The competitive immunoassay of claim 4 comprising:

(a) preparing a reaction mixture by incubating a first antibody which is bound to Tf-like antigen with different concentrations of a serum sample from a patient,

(b) adding a constant amount of a second antibody to the reaction mixture wherein said second

antibody recognizes a constant region of the heavy chain of the first antibody and wherein the second antibody is labeled and part of a signal producing system,

(c) separating free labeled second antibody from bound antibody,

(d) measuring the signal generated by the second antibody in the reaction mixture, and;

(e) correlating a decreased level of signal strength with a diagnosis of endometriosis in the patient.

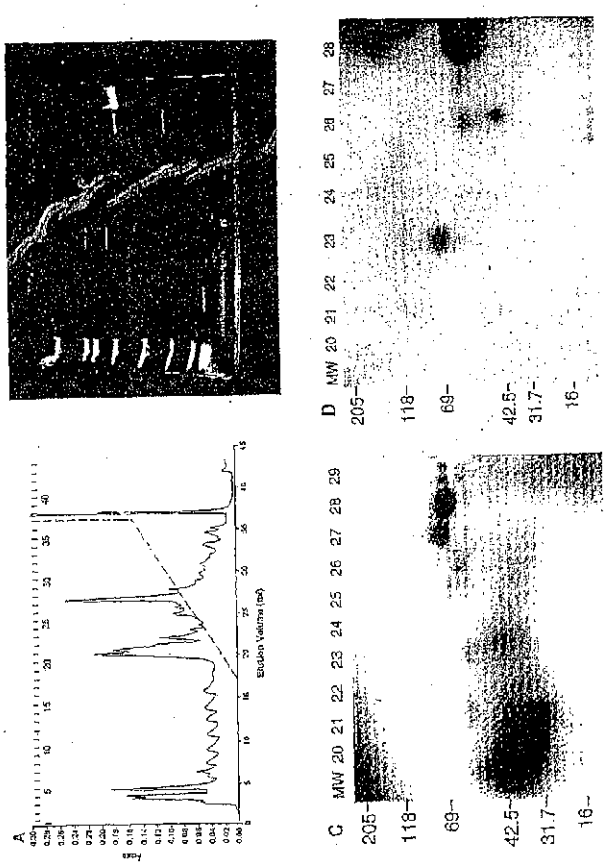
8. The method of claim 5 wherein the antibody is enzyme-labeled and the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled anti-human immunoglobulin followed by incubating are performed between steps (d) and (e).

9. The method of claim 6 wherein the antibody is enzyme-labeled and the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled anti-human immunoglobulin followed by incubating are performed between steps (b) and (c).

10. The method of claim 7 wherein the second antibody is enzyme-labeled and the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled anti-human immunoglobulin followed by incubating are performed between steps (c) and (d).

11. The method of any of claims 5, 6, or 7 wherein the labeled antibody is radiolabeled.

12. The method of any of claims 5, 6, or 7 wherein the labeled antibody is biotin labeled.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Elution Volume (ml)
B
C
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69
42.5
31.7
16
D
MW
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31.7
16

Fig 1

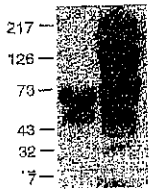


Figure 2

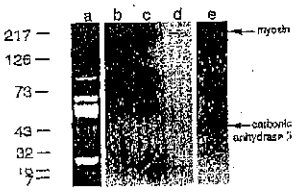


Figure 3

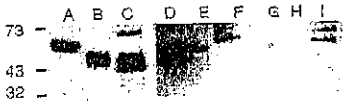
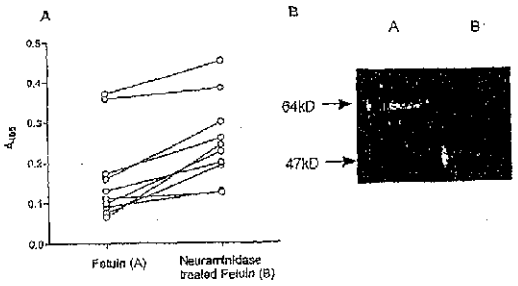


Figure 4

Fig 5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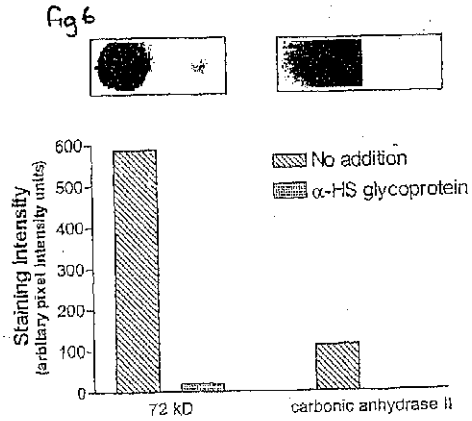
47kD
Fetuin (A)
Neuraminidase treated Fetuin (B)

Fig 6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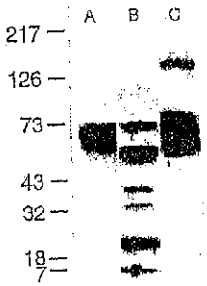

Figure 7

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(54) Title: DIAGNOSTIC ASSAY FOR ENDOMETRIOSIS

(57) Abstract: The present invention provides a method for detecting endometriosis in a patient and is an improvement over invasive and expensive surgical procedures. The method employs immunoassays which detect autoantibodies in a serum sample which react with Thomsen-Friedenreich antigen (TF). Increased levels of autoantibodies in a serum sample from the patient which bind to TF-Ice antigen is indicative of endometriosis in the patient.

【国際公開パンフレット（コレクトバージョン）】

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: DIAGNOSTIC ASSAY FOR ENDOMETRIOSIS

(57) Abstract: The present invention provides a method for detecting endometriosis in a patient and is an improvement over invasive and expensive surgical procedures. The method employs immunoassays which detect autoantibodies in a serum sample which react with Thomsen-Friedenreich antigen (TF). Increased levels of autoantibodies in a serum sample from the patient which bind to TF-like antigen is indicative of endometriosis in the patient.

DIAGNOSTIC ASSAY FOR ENDOMETRIOSIS

BACKGROUND OF THE INVENTION

Endometriosis is a common disorder characterized by the growth of endometrial cells at extrauterine (ectopic) sites. It is a common disease which may affect up to 10% of reproductive age women (1). Although the etiology of endometriosis remains enigmatic, altered cellular and humoral immune function is clearly a feature of established disease (2-4).

Autoantibodies to endometrial antigens and deposition of complement components have been described in a number of studies (reviewed in 2) and a number of serum, peritoneal fluid and endometrial antigens have been described. Perhaps the best characterized tissue antigens described, thus far, are the human chorionic gonadotropin receptor (5) and isoforms I and II of the enzyme carbonic anhydrase 6-8. Antibodies to transferrin and α_2-Heremans Schmidt glycoprotein (α_2-HSG) have also been described and proposed as diagnostic markers 9, 10. While considerable work has been carried out in terms of measuring the incidence of these antibodies in endometriosis, reproductive diseases, and other autoimmune diseases, the nature of the epitopes involved has received scant attention. The identified antigens are all glycoproteins. With only one apparent exception (5), carbohydrate antigens on these proteins have not been evaluated.

In accordance with the present invention, it has been surprisingly found that a common carbohydrate

moiety is present on the different aforementioned endometrial antigens. The common carbohydrate moiety is the Thomsen-Friedenreich related antigen, Galβ1-3GalNAc, also referred to as Tf antigen or Tf-like antigen. As used herein, Tf like antigen also encompasses Tf antigen. Tf antigen is a cryptic disaccharide structure masked by sialic acid. The sialic acid moieties may be removed by sialidases such as neuraminidase. Tf antigen is present on human erythrocytes and is a tumor-associated antigen in epithelial tissues.

The present invention provides diagnostic methods based on autoantibody reactivity with Tf-like antigen. The diagnostic methods are helpful in determining the presence of endometriosis in a patient and are an improvement over the current invasive methods of diagnosis.

SUMMARY OF THE INVENTION

The present invention provides methods for diagnosing endometriosis in a patient. In one embodiment of the invention, a method for diagnosing endometriosis in a patient comprises the steps of

(a) obtaining a serum sample from said patient, (b) incubating Thomsen-Friedenreich (Tf)-like antigen with said serum sample,

(c) detecting autoantibody reactivity with the Tf-like antigen in said sample, and;

(d) correlating an increased level of autoantibody reactivity to the Tf-like antigen in the serum sample with a diagnosis of endometriosis in said patient.

Antibody reactivity may be determined by immunoassays such as immunometric or competitive assays.

In one embodiment of the invention, an immunometric assay comprises the steps of:

(a) immobilizing Tf-like antigen on a solid support,

(b) adding an aliquot of serum sample from a patient to the Tf-like antigen bound on the solid support and incubating,

(c) adding a labeled anti-human immunoglobulin to the solid support wherein said anti-human immunoglobulin is part of a signal producing system,

(d) separating free labeled antibody from bound antibody,

(e) measuring the signal generated by the solution containing the solid support and;

(f) correlating an increase in signal strength with a diagnosis of endometriosis in the patient.

In another embodiment of the invention, a competitive immunoassay comprises the steps of:

(a) preparing a reaction mixture by incubating a constant amount of a labeled antibody which is bound to Tf-like antigen with different concentrations of a serum sample from a patient wherein said labeled antibody is part of a signal producing system,

(b) separating bound labeled antibodies from free autoantibodies,

(c) measuring the signal generated by the labeled antibody in the reaction mixture and;

(d) correlating a decrease in signal strength after addition of the serum sample from a patient with a diagnosis of endometriosis in the patient.

In another embodiment of the invention, a competitive immunoassay comprises the steps of:

(a) preparing a reaction mixture by incubating a first antibody which is bound to Tf-like antigen with

different concentrations of a serum sample from a patient,

(b) adding a constant amount of a second antibody to the reaction mixture wherein said second antibody recognizes a constant region of the heavy chain of the first antibody and wherein the second antibody is labeled and part of a signal producing system,

(c) separating free labeled second antibody from bound antibody,

(d) measuring the signal generated by the second antibody in the reaction mixture; and

(e) correlating a decreased level of signal strength with a diagnosis of endometriosis in the patient.

In those cases where the antibody is enzyme labeled, the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled antibody followed by incubation are performed prior to measuring the generated signal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is an elution profile showing MonoQ anion exchange chromatography of homogenized eutopic endometrium from an endometriosis patient. The homogenate was passed through a protein G column to remove IgG prior to loading on the MonoQ column. Initial peaks prior to the start of the salt gradient represent multiple 1ml homogenate loads. Elution conditions: 50mM Tris/HCl pH8.0. Elution was in the same buffer with a linear 0-0.5 NaCl gradient (stepped to 1M NaCl) and is denoted by the dashed line. Fractions corresponding to the gel loading (Figures 1b-d) are indicated along the top of the graph.

Figure 1b shows a 5-15% gradient gel of selected column fractions indicated in Figure 1a run under reducing conditions and stained with SYPRO Orange.

Figure 1c is an autoradiograph displaying proteins in the homogenate fractions recognized by IgG present in pooled endometriosis serum. Stripping and reprobing of the same blot using pooled normal male serum showed no reactivity with the same bands. IgG binding activity was detected in fraction 24 using control sera but similar binding was not seen with endometriosis sera. The 45.2kDa molecular weight marker carbonic anhydrase II shows reactivity. This same reactivity was not observed with the control sera.

Figure 1d is an autoradiograph showing IgA binding activity of the endometriosis sera. Probing of the blot with anti-IgA a-chain specific antibody in the absence of a primary serum showed that IgA was present in these fractions.

Figure 2 is a western blot of peritoneal fluid (W1517) run out on a 5-15% SDS-PAGE gel under reduced and alkylated conditions. The western blot was then

incubated with either sheep anti-human α_2-HSG followed by HRP-goat anti-sheep (lane a) or with a 1:100 dilution of endometriosis sera followed by HRP-goat IgG g-chain specific antibody (1:1000 dilution). Clearly visible are α_2-HSG in lane a and bands of equivalent molecular weight in lane b.

Figure 3a is an SDS-PAGE gel showing a partially purified fraction of α_2-HSG (MonoQ column, pH 8.0). Fourteen protein bands are visible after staining with SYPRO Orange.

Figure 3b is a western blot of a gel run with partially purified fraction of α_2-HSG and probed with endometriosis sera HRP goat anti-human IgG antibody. Five protein bands were detected.

Figure 3c is a western blot as described in Figure 3b except that the protein fraction was treated with neuraminidase prior to electrophoresis. Antibody binding to all of the proteins is clearly reduced.

Figure 3d is a western blot as described for Figure 3b except that the α_2-HSG fraction was subjected to preadsorption with jacalin agarose prior to electrophoresis. All antibody binding is removed.

Figure 3e shows autoreactivity with myosin and carbonic anhydrase II in the molecular weight markers.

Figure 4 is a western blot of a gel run with partially purified fraction of α_2-HSG and probed with endometriosis sera HRP goat anti-human IgG antibody which demonstrates that deglycosylation of α_2-HSG abolishes autoantibody binding. Neuraminidase was used to cleave terminal sialic acid (lanes B, E, H). Complete removal of carbohydrate was then achieved using EndoF/PNG'ase digestion (lanes C, F, H). Removal of sialic acid and all carbohydrate was confirmed by

reduction in molecular weight as shown by reactivity with goat anti-α_2-HSG (lane A-C). Lanes A, D, and G were untreated. Removal of terminal sialic acid with neuraminidase reduced reactivity with patient serum (lane D versus E). The remaining reactivity in this fraction was at the same molecular weight as the undigested α_2-HSG, indicating that the autoantibody binding is dependent on the presence of terminal sialic acid. Complete removal of carbohydrate by neuraminidase treatment followed by EndoF/PNG'ase digestion completely abolished reactivity (lane F). No equivalent reactivity was observed using pooled male control sera (lanes G-I).

Figure 5a demonstrates that desialylation of bovine fetuin increases reactivity with endometriosis sera. As measured by ELISA, a slight but significant increase in binding is seen following treatment with neuraminidase. Figure 5b is a SYPRO Orange stained gel showing the molecular weight reduction in fetuin following neuraminidase treatment. Lane A is untreated; lane B is neuraminidase treated.

Figure 6 both graphically and by radioautography depicts that binding to the 72kDa antigen and carbonic anhydrase II is inhibited in the presence of α_2-HSG.

Figure 7 is a western blot analysis of purified proteins following transfer to nitrocellulose from 5-15% gradient gels run reduced and alkylated. Purified hemopexin (lane A), α_2-HSG (lane B), and serum IgA1 (lane C) are autoantigens recognized by IgG antibodies in endometriosis sera.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides diagnostic methods for detecting endometriosis in a patient. The diagnostic methods are based on immunoassays which detect the presence of autoantibodies in a patient's serum reacting with Thomsen-Friedenreich (Tf) antigen, including Tf-like antigen. As used herein, Tf-like antigen also encompasses Tf antigen. Increased levels of autoantibodies in a serum sample which bind Tf-like antigen, when compared to normal serum levels of autoantibodies which bind Tf-like antigen, correlate with a diagnosis of endometriosis in the patient.

A blood sample may be conveniently drawn from a patient by venipuncture or other suitable means. A serum sample may be prepared from the blood sample using well known methods.

There are many different types of immunoassays which may be used in the methods of the present invention. Any of the well known immunoassays may be adapted to detect the level of autoantibodies in a serum sample which react with the Tf-like antigen, such as e.g., enzyme linked immunoabsorbent assay (ELISA), fluorescent immunosorbent assay (FIA), chemical linked immunosorbent assay (CLIA), radioimmuno assay (RIA), and immunoblotting. For a review of the different immunoassays which may be used, see: *The Immunoassay Handbook*, David Wild, ed., Stockton Press, New York, 1994. Preferably, a competitive immunoassay with solid phase separation or an immunometric assay for antibody testing is used. See, The Immunoassay Handbook, chapter 2.

In a typical assay, the reagents include a serum sample from a patient, the autoantibodies to be detected (contained in the serum sample), Tf-like antigen, and means for producing a detectable signal.

Thus, in one embodiment of the invention, the method for diagnosing endometriosis in a patient employs an immunometric assay for antibody testing. In this embodiment, the Tf-like antigen is immobilized on a solid support or surface such as a bead, plate, slide or microtiter dish. An aliquot of serum sample from a patient is added to the solid support and allowed to incubate with the Tf-like antigen in a liquid phase. An antibody that recognizes a constant region in human autoantibodies present in the serum which have reacted with the Tf-like antigen is added. This antibody is an anti-human immunoglobulin and is also part of a signal producing system. Anti-human immunoglobulin which is specific for IgA, IgG, or IgM heavy chain constant regions may be employed. After separating the solid support from the liquid phase, the support phase is examined for a detectable signal. The presence of the signal on the solid support indicates that autoantibodies to Tf-like antigen present in the serum sample have bound to the Tf-like antigen on the solid support.

The signal producing system is made up of one or more components, at least one of which is a label, which generate a detectable signal that relates to the amount of bound and/or unbound label i.e., the amount of label bound or unbound to the Tf-like antigen. The label is a molecule that produces or which may be induced to produce a signal. Examples of labels include fluorescers, enzymes, chemiluminescers, photosensitizers or suspendable particles. The signal is detected and may be measured by detecting enzyme activity, luminescence or light absorbance. Radiolabels may also be used and levels of radioactivity detected and measured using a scintillation counter.

Examples of enzymes which may be used to label the anti-human immunoglobulin include β-D-galactosidase, horseradish peroxidase, alkaline phosphatase, and glucose-6-phosphate dehydrogenase ("G6PDH"). Examples of fluorescers which may be used to label the anti-human immunoglobulin include fluorescein, isothiocyanate, rhodamine compounds, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. Chemiluminescers include e.g., isoluminol.

Free labeled antibody is separated from bound antibody and if necessary, an appropriate substrate with which the label, e.g., enzyme, reacts is added and allowed to incubate.

In a preferred embodiment, the anti-human immunoglobulin is enzyme labeled with either horseradish peroxidase or alkaline phosphatase.

The amount of color, fluorescence, luminescence, or radioactivity present in the reaction (depending on the signal producing system used) is proportionate to the amount of autoantibodies in a patient's serum which react with the Tf-like antigen. Quantification of optical density may be performed using spectrophotometric methods. Quantification of radiolabel signal may be performed using scintillation counting.

Increased levels of autoantibodies reacting with Tf-like antigen over normal serum levels correlate with a diagnosis of endometriosis in the patient.

In another embodiment of the invention, the method for diagnosing endometriosis in a patient employs a competitive immunoassay where a known antibody and a patient's autoantibodies compete for binding to Tf. In this embodiment, a constant amount of a labeled antibody which is known to bind to Tf-like antigen is incubated

with different concentrations of a serum sample from a patient. For example, the mouse monoclonal antibody 49H.8, (Rahman and Longenecker, 1982, J. Immun. 129(5): 2021-4) known to bind to Tf, may be used. Other monoclonal antibodies which bind to Tf-like antigen and which may be used as antibody in the competitive immunoassay include 155H7 and 170H82 (Longenecker et al. 1987, J. Nat. Cancer Inst., 78(3): 489-96, A78-G/A7 (Karsten et al. 1995, Hybridoma 14(1): 37-44), HB-T1 (DAKO Co.), RS1-114 and AHB-25B (Stein et al. 1989, Cancer Res. 49(1): 32-7), HT8 (Metcalfe et al., 1984, Br. J. Cancer 49(3): 337-43), 161H4 (Longenecker et al., 1987), HH8 (Clauson et al. 1988), and BW835 (Hanish et al, 1995, Cancer Res. 55(18): 4036-40). As described above, the antibody may be labeled with a fluorescer, enzyme, chemiluminescer, photosensitizer, suspendable particles, or radioisotope. Preferably, the known antibody is enzyme labeled. After incubation, bound labeled antibodies are separated from free autoantibodies. Depending on the signal producing system used and if necessary, an appropriate substrate with which the labeled antibody reacts is added and allowed to incubate. The signal generated by the sample is then measured. A decrease in optical density or radioactivity from before and after addition of the serum sample or between experimental and control samples, is indicative that autoantibodies in the serum sample have bound to Tf. Decreased optical density or radiolabeled signal when compared to experimental serum samples from normal patients, correlates with a diagnosis of endometriosis in a patient.

In a preferred embodiment of the competitive immunoassay, an indirect method using two antibodies is used. The first antibody is a Tf-like antigen specific antibody as described in the preceding paragraph with

the exception that it is not labeled. The first antibody is incubated with different concentrations of a serum sample from a patient. A constant amount of a second antibody is then added to the sample. The second antibody recognizes constant regions of the heavy chains of the first antibody. For example, the second antibody may be an antibody which recognizes constant regions of the heavy chains of mouse immunoglobulin which has reacted with the Tf-like antigen (anti-mouse immunoglobulin). The second antibody is labeled with a fluorophore, chemilophore or radioisotope, as described above. Free labeled second antibody is separated from bound antibody. If an enzyme-labeled antibody is used, an appropriate substrate with which the enzyme label reacts is added and allowed to incubate. A decrease in optical density or radioactivity from before and after addition of the serum sample or between experimental and control samples is indicative that autoantibodies in the serum sample have bound to Tf. Decreased optical density or radioactivity when compared to experimental serum samples from normal patients correlates with a diagnosis of endometriosis in a patient.

Enzymes may be covalently linked to Tf-like antigen reactive antibodies for use in the methods of the invention using well known methods. There are many well known conjugation methods. For example, alkaline phosphatase and horseradish peroxidase may be conjugated to antibodies using glutaraldehyde. Horseradish peroxidase may also be conjugated using the periodate method. Commercial kits for enzyme conjugating antibodies are widely available. Enzyme conjugated anti-human and anti-mouse immunoglobulin specific antibodies are available from multiple commercial sources.

Biotin labeled antibodies may be used as an alternative to enzyme linked antibodies. In such cases, bound antibody would be detected using commercially available streptavidin-horseradish peroxidase detection systems.

Enzyme labeled antibodies produce different signal sources, depending on the substrate. Signal generation involves the addition of substrate to the reaction mixture. Common peroxidase substrates include ABTS®(2,2'-azinobis(ethylbenzothiazoline-6-sulfonate)), OPD (O-phenylenediamine) and TMB (3,3', 5,5'-tetramethylbenzidine). These substrates require the presence of hydrogen peroxide. *p*-nitrophenyl phospate is a commonly used alkaline phosphatase substrate. During an incubation period, the enzyme gradually converts a proportion of the substrate to its end product. At the end of the incubation period, a stopping reagent is added which stops enzyme activity. Signal strength is determined by measuring optical density, usually via spectrophotometer.

Alkaline phosphatase labeled antibodies may also be measured by fluorometry. Thus in the immunoassays of the present invention, the substrate 4-methylumbelliferyl phosphate (4-UMP) may be used. Alkaline phosphatase dephosphorylated 4-UMP to form 4-methylumbelliferone (4-MU), the fluorophore. Incident light is at 365 nm and emitted light is at 448 nm.

For use in the methods of the present invention, Tf-like antigen may be obtained from various sources. For example, Tf-like antigen may be purified from conditioned culture medium used to cultivate tumor cell lines such as the adenocarcinoma cell line LS174T, obtainable through the American Type Culture Collection (ATCC). Transitional cell carcinoma lines may also

serve as source of Tf-like antigen. Tf-like antigen may be purified from such conditioned culture medium by affinity chromatography using an MAb 49H.8-CnBr activated Sepharose column. Gel filtration may also be performed for additional purification. Still further purification of Tf-like antigen may be achieved by utilizing lectin affinity chromatography with insolubilized peanut agglutinin (PNA) or other lectin. PNA may be obtained from E-Y Laboratories, San Mateo, California.

Purified serum proteins bearing Tf-like antigens such as IgA, hemopexin, and alpha-2-Heremans Schmidt may also be used as antigens. A preferred source of Tf-like antigen is commercially synthesized Tf-like antigens covalently linked to bovine serum albumin. Tf-like antigen and its glycoforms are available from commercial vendors such as BioCarb as recently described by Dahlenborg et al. (1997) *In. J. Cancer* 70:63-71. Alternatively, Tf-like antigens may be obtained by custom synthesis from commercial vendors. Monoclonal antibodies which react with Tf-like antigen are readily available.

MAb49.H (isotype IgM) may be prepared and purified as reported by Rahman et al., (1982) *J. Immunol.* *129*:2021-2024 and Longenecker et al. (1984) *Int. J. Cancer* *33*:123-129. The disclosure of these articles and other articles cited in this application are incorporated herein as if fully set forth.

In order to practice the methods of the present invention, the relevant immunoassay must be standardized. Since Tf-like antigen is usually associated with a glycoprotein, it is naturally heterogenous. The source of such heterogeneity may include sialylation. To this end, an ampoule of an international standard (IS) or international reference

preparation (IRP) should be obtained. The National Institute for Biological Standards and Control (NIBSC, Blanche Lane, South Mimms, Potters Bar, Herts EN63QG) may prepare such a sample of Tf-like antigen and assign an international unit (IU). Multiple sets of secondary standards, from which future lots of calibrators may be assigned values, should also be prepared.

The immunoassays of the present invention require the use of calibrators in order to assign values or concentrations to unknown samples. Typically, a set of about six calibrators is run prior to the unknown samples from which a calibration curve is plotted. The concentrations of the unknown samples are determined by interpolation. Interpolation is best carried out by a computer program. For a discussion on calibration, *see The Immunoassay Handbook*, chapter 2.

The present invention also provides therapies for reducing, preventing and/or ameliorating endometriosis in a patient. In this embodiment of the invention Tf-like antigen or an analog thereof, is injected into a patient. The injected Tf-like antigen or analog thereof competes with Tf-like antigen present in endometrium of a patient for autoantibody binding.

The following examples further illustrate the invention.

EXAMPLE 1

Materials and methods

Preparation of tissue homogenate

Fresh ectopic and eutopic endometrium, peritoneal fluid, and serum were obtained from hysterectomy patients with the informed consent of the patients. Fresh tissue (1-2 grams) was added to 10 ml ice-cold PBS containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN) and maintained at 4°C throughout the preparation. The tissue was homogenized for 1 minute with a Polytron homogenizer (Brinkman, Lucerne, Switzerland) followed by centrifugation at 13000 g for 10 minutes. The supernatant was collected and sonicated using a Branson 250 sonifier (Danbury, CT). The tissue homogenate was 0.22 mm filtered and stored at 20°C.

Preparation of serum

Peripheral venous blood (60 ml) was drawn from healthy volunteers into glass vacutainer tubes (no additive) and allowed to clot for 4 hours at room temperature. Serum was then removed to sterile tubes and delipidated by centrifugation at 12000 g for 30 minutes at 4°C.

Purification of tissue autoantigens

Following homogenization, tissue antigens were passed through a Protein G FPLC column (Amersham Pharmacia, Piscataway, NJ) to remove IgG. Proteins not retained by the protein G column were then applied to an anion exchange column (MonoQ FPLC, Amersham Pharmacia, Piscataway, NJ) following equilibration in 50mM sodium phosphate pH 8.0. Bound proteins were eluted from the

column using a linear 0-0.5 M NaCl gradient in the same buffer, followed by a step gradient to 1 M NaCl also in the same buffer. All proteins of interest eluted prior to the 1 M NaCl gradient.

Purification of Serum Autoantigens

α_2-HSG was purified from normal whole human serum by anion exchange chromatography as described for the tissue antigens above using a HiPrep 16/10 Q XL column. This partially purified α_2-HSG was used in some experiments as indicated in the test. Further purification was carried out on a second anion exchange column (Mono Q) equilibrated with 20 mM piperazine buffer pH 5.0 and eluted in the same buffer using a NaCl gradient as described above. A final homogeneous preparation of α_2-HSG was obtained following gel filtration on an FPLC Superose 12 column (Amersham Pharmacia, Piscataway, NJ). α_2-HSG concentrations were monitored by ELISA.

IgA1 was purified from whole serum by anion exchange chromatography and jacalin agarose affinity chromatography as previously described (11).

Transferrin and Hemopexin were purified from whole serum by metal chelate chromatography on a column packed with Chelating Sepharose Fast Flow (Amersham Pharmacia, Piscataway, NJ) charged with Zn^{2+} ions. The column and serum were pre-equilibrated in 20 mM sodium phosphate buffer containing 150 mM NaCl. Zinc binding proteins were eluted using a linear 0-0.5 M imidazole gradient in the same buffer. The resulting transferrin and hemopexin-containing fractions (as determined by western blot analysis) were separately pooled and further purified on a mono Q anion exchange column at pH 8.0 as already described. This protocol resulted in an electrophoretically pure hemopexin preparation.

Transferrin was not however resolved from hemopexin in the transferrin preparation.

Modification of carbohydrate-epitopes on glycoproteins

Terminal sialic acid moieties were removed from glycoproteins by treatment with agarose-conjugated neuraminidase. One milligram of protein in 0.5 ml was incubated with 5 Units of neuraminidase (Sigma, St Louis, MO) in 50 mM sodium acetate, 150 mM sodium chloride, 4 mM calcium chloride, pH 5.5 overnight at 37°C. Agarose beads were removed by centrifugation at 4000 g for 5 minutes. Supernatants were removed and stored at 4°C.

Core carbohydrate groups were removed from glycoproteins by treatment with endoglycosidase F (Endo F) and peptide-N-glycosidase F (PNG'ase F). This was obtained as a commercial kit from Glyko (Novato, CA). Forty micrograms of neuraminidase-treated protein were denatured by heating to 100°C for 2 minutes in the presence of 20 mM sodium phosphate pH 7.5, 50 mM EDTA, 0.1 % v/v SDS, 0.5 % b-mercaptoethanol. To avoid inhibition of PNG'ase F by SDS, 0.1% Tween-20 was added to the denatured sample after cooling. The denatured protein was then incubated with 667 deglycosylation Units (DGU) of the Endo F / PNG'ase F mix at 37°C for 18 hours.

Removal of jacalin-reactive glycoproteins from samples was achieved by pre-adsorption with an excess of jacalin-conjugated agarose beads (Vector, Burlingame, CA). Samples were incubated with jacalin-conjugated agarose for 30 minutes at room temperature before centrifugation at 4000g for 5 minutes. Supernatants were removed and stored at 4°C.

Antibodies and Glycoproteins

Immunoglobulin-fractions of sheep anti-human α_2-HSG and sheep anti-human transferrin were obtained from Biodesign International (Kennebunk, ME). Goat anti-human hemopexin antiserum was obtained from Kent Laboratories (Redmond, WA). HRP-conjugated rabbit anti-sheep IgG and HRP-conjugated rabbit anti-goat IgG were both from Jackson Immunoresearch Laboratories (West Grove, PA). HRP-conjugated goat-anti human IgA (a-chain specific) and HRP-conjugated goat-anti human IgG (g-chain specific) were purchased from (Sigma). Commercial α_2-HSG was obtained from Calbiochem-Novabiochem (La Jolla, CA).

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (12). Briefly, equal volumes of 2x gel loading buffer were added to protein samples and boiled for 5 minutes. Where appropriate, proteins were resolved under reducing conditions (5 % v/v b-mercaptoethanol in gel loading buffer). Re-association of reduced proteins was prevented by adding iodoacetamide (final concentration 60 mM) to samples after boiling. Protein samples were resolved using 4 % -15 % gradient gels (Biorad, Hercules, CA). For direct visualization of proteins, gels were placed in 0.02 % v/v SYPRO-Orange (Biorad, Hercules, CA) in 7.5 % v/v acetic acid for 30 minutes. Gels were then rinsed in 7.5 % acetic acid and viewed on a gel imager (Alpha Innotech Corporation, San Leandro, CA).

Immunoblotting

Proteins were transferred to nitrocellulose membranes from SDS-gels using a Biorad Transblot SD semi-

dry blotter. Ponceau Red staining confirmed transfer of proteins to nitrocellulose membranes. Membranes were blocked overnight at 4°C in 5 % w/v fat-free powdered milk, 0.5 % Tween-20 in PBS. Primary antibody or serum was added to blocking solution then incubated for 2 hours at room temperature. Membranes were then washed 6 times for 5 minutes per wash in PBS. The HRP-conjugated secondary antibodies were diluted in 3 % w/v fat-free milk powder with 0.05 % v/v Tween-20 in PBS. Membranes were incubated for 2 hours at room temperature before washing 6 times for 5 minutes as described. Proteins were then detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia, Piscataway, NJ). Where appropriate, nitrocellulose membranes were stripped and re-probed by washing twice for 5 minutes per wash in 0.9 % w/v NaCl before agitation in 100 mM Glycine-HCl pH 1.5 for 30 minutes at room temperature. Membranes were then washed 3 times for 5 minutes per wash with PBS pH 7.4. The membranes were blocked and probed with antibodies as described.

Enzyme Linked Immunosorbent Assay (ELISA)

Wells of Falcon Microtest III microtitre plates (Becton Dickinson, Oxnard, CA) were coated overnight at 4°C with 10 µl protein diluted to 100 µl in sodium carbonate buffer pH9.2. Plates were then washed 3 times in PBS containing 0.05 % v/v Tween-20. Wells were blocked with 3 % w/v BSA in PBS / Tween-20 (blocking buffer) for 1 hour at 37°C before washing 3 times. Primary antibody or test serum was diluted in blocking buffer as appropriate and 100 µl added to each well. Plates were incubated for 2 hours at 37°C and washed 3 times in PBS / Tween-20. HRP-conjugated secondary antibody was diluted in blocking buffer and 100 µl added to each well. Plates were then washed 3 times. 100 ml

of the colorimetric HRP-substrate, ABTS was added to each well and incubated for 30 minutes at room temperature. Plates were read at 405nm with a Dynatech plate reader (Chantilly, VA) Data was analyzed using Graphpad Prism software.

EXAMPLE 2

Identification of autoantigens in ectopic endometrium, eutopic endometrium, peritoneal fluid and sera.

Soluble endometrial protein preparations were prepared from hysterectomy tissue as described in the materials and methods, and subjected to protein G chromatography to remove IgG. The resulting IgG-free preparations were then subjected to anion exchange chromatography (figure 1a). Fractions from this column were then analyzed by SDS-PAGE under reducing conditions (figure 1b). Protein bands on identical gels were transferred to nitrocellulose for western blot analysis. Development of the western blots was carried out using sera from both pooled endometriosis patients and pooled normal male donors as primary antibody sources. Figure 1c shows a typical example of the antigens identified in a proliferative phase eutopic endometrium from a patient (W1345). Autoreactive IgG was restricted to the endometriosis patient sera (Figure 1c) as compared to the same blots probed with a control serum from a normal male donor (not shown).

IgG g-chain binding was detected against fraction 24 of the mono Q column with the control serum but not in other fractions (not shown). Interestingly, a similar reactivity against fraction 24 was not present in blots developed using the endometriosis serum. The molecular weight of the reactive proteins detected using pooled endometriosis sera correlate well with those described by other workers 6, 10. A total of 10 autoreactive bands were detected on a western blot probed with pooled endometriosis sera. A 72 kDa band was detected in fraction 23 with both IgA α-chain specific and IgG γ-chain specific second antibodies (figures 1c and 1d). This band was initially identified as transferrin on the

basis of its molecular weight and the NaCl concentration at which it eluted from the MonoQ column (the elution of transferrin at this position was confirmed by western blot analysis using a sheep anti-transferrin antibody, not shown). A 54kDa protein was also detected in this fraction with the IgG γ-chain specific antibody but not the anti-IgA α-chain specific antibody. This protein elutes in a position identical to partially desialylated α_2-HSG (data not shown). α_2-HSG has previously been identified as an autoantigen in endometriosis patients (10). The IgG reactive band of 59kDa observed in fractions 26 and 29 is the α-chain of IgA and the elution positions correspond to the known elution positions, under these run conditions, of monomeric and dimeric IgA respectively. The identity of this protein as IgA α-chain was confirmed by stripping and reprobing the blot with an anti α-chain specific antibody (not shown). This blot also showed the presence of anti-α-chain reactive bands in fraction 23 (the transferrin containing fraction). The presence of IgA antigens in this fraction most likely arises from IgA covalently complexed with other serum proteins [13, 14]. Fraction 26 also contained a reactive band at 72 kDa and fraction 28 and 29 had bands at 69 kDa. These later bands showed no activity on the α-chain specific blot. The presence of an IgG antibody in endometriosis patients which recognizes the α-chain of IgA was confirmed by western blot using highly purified serum IgA1 (see Figure 7). In contrast, no IgA autoantibodies against endometrial antigens other than in those fractions, which contain IgA α-chain, were observed.

An ovarian ectopic endometrial sample from the same patient (w1345) had autoantigenic proteins of molecular

weights 54, 47, 43, and 34 KDa (not shown). The 54 kDa protein was α_2-HSG as determined by a western blot developed with a sheep anti-α_2-HSG specific antibody. α_2-HSG was also detected in peritoneal lavage fluid from a different patient (W1517) by western blot. In addition to the 54 kDa antigen, antigens of 186, 126, 68, 43, 37, and 34 kDa were present in the peritoneal fluid (Figure 2).

Reactivity with two molecular weight markers was observed with both pooled patients and individual sera (see figures 1c and figure 3). These markers were myosin at 205kD and carbonic anhydrase II at 42kD. Reactivity with the myosin band was also seen with both the pooled male control sera and some individual male sera. In contrast, reactivity with anhydrase II was specific to the endometriosis sera and this protein has been reported by other workers as an autoantigen in endometriosis [7, 8].

In summary, autoantigens in preparations from eutopic and ectopic endometrium and peritoneal lavage have been identified. In agreement with previously published reports, α_2-HSG, transferrin and carbonic anhydrase II are autoantigens recognized by sera from endometriosis patients. In addition, results presented here also demonstrate that the α-chain of IgA is also an autoantigen recognized by the same sera. Since these proteins show no significant homology at the protein level, it is unlikely that a common peptide epitope is shared by all four proteins. Three of the four proteins, α_2-HSG, α-chain of IgA1 and possibly carbonic anhydrase II, do however, share a common carbohydrate epitope. This O-linked carbohydrate structure contains a Galβ1-3NAcGal epitope which is recognized by the jackfruit (Artocarpus integrifolia) lectin jacalin. The results

presented here confirm previous reports that α_1-HSG and carbonic anhydrase are among the autoantigens recognized by autoantibodies present in endometriosis sera. The present study extends these results to show that IgA1 and hemopexin are also autoantigens recognized by sera from endometriosis patients.

EXAMPLE 3

Jacalin preadsorption removes autoantibody binding.

α_2-Heremans Schmidt glycoprotein (α_2-HSG) was chosen as a model antigen to investigate a potential role for jacalin reactive carbohydrates in the endometriosis autoantibody response. Initial experiments using α_2-HSG purchased from commercial sources proved to be unsatisfactory. This was because of the variability of glycoforms present in these preparations. In common with most sialated glycoproteins, α_2-HSG loses terminal sialic acid residues during storage. Fully sialated α_2-HSG from normal human serum was freshly prepared. In order to avoid biasing the preparation with respect to its carbohydrate content, lectin based affinity purification protocols were avoided and instead a combination of anion exchange and gel filtration chromatography was used. A three step procedure of anion exchange on a MonoQ column at pH 8.0, followed by a second MonoQ column at pH 5.0, and a final gel filtration step on a superose 12 column, resulted in the purification of the fully sialated glycoform of α_2-HSG with a molecular weight of 58kDa.

When experiments were carried out using a partially purified α_2-HSG fraction from the first anion exchange column, autoreactivity with endometriosis serum on western blot was abolished following pre-incubation with jacalin agarose (figure 3). Fourteen protein bands were observed on SYPRO orange stained SDS-PAGE gels (figure 3 lane a). Reactivity with endometriosis sera was observed with 5 proteins (figure 3 lane b). The reactive proteins had approximate molecular weights of 230, 188, 168, 120, and 58kDa; the 58kDa being α_2-HSG. Binding by IgG in endometriosis sera of all of these 5 bands was abolished following incubation with jacalin agarose (figure 3 lane

d), indicating that the binding of the autoantibodies was to all of the jacalin binding proteins present in the fraction and not just to α_2-HSG. Protein staining of gels of pre- and post-absorbed fraction 13 show that the removal of activity was not caused by a non-specific removal of proteins in the fraction. The protein concentrations of adsorbed and unadsorbed were adjusted such that total protein loading on the gels used for western blotting was uniform.

The carbohydrate structure recognized by jacalin is very specific and binding is dependent on the presence of the Tf. Jacalin recognizes the disaccharide Galβ1-3GalNAc, which is present within some O-linked oligosaccharides. Galβ1-3GalNAc is expressed by a very limited number of the many plasma proteins. These proteins include IgA1, IgD, C1-inhibitor, hemopexin, plasminogen, α1-antitrypsin, α2-macroglubulin, 8S-α3 glycoprotein, chorionic gonadotropin (hCG) and α_2-HSG (15). With regard to hCG, it is of interest that the receptor for hCG expresses also expresses Tf-like antigen and that autoantibodies found in infertile patients both with and without concomitant endometriosis bind to the receptor [5].

EXAMPLE 4

Removal of Carbohydrate Abolishes Autoantibody Binding.

Removal of proteins recognized by the autoantibodies by jacalin does not in itself determine whether a common carbohydrate epitope(s) is involved. To determine whether the carbohydrate is essential for the recognition of α_2-HSG by the endometriosis autoantibodies, reactivity with deglycosylated α_2-HSG was investigated. α_2-HSG carbohydrate was removed in a two step procedure. First, neuraminidase was used to cleave terminal sialic acid. Second, complete removal of carbohydrate was then achieved using a further EndoF/PNG'ase digestion step. Untreated, neuraminidase treated, and deglycosylated α_2-HSG were then subjected to western blot analysis at equal protein loads (Figure 4). Removal of sialic acid and all carbohydrate was confirmed by reduction in molecular weight as shown by reactivity with sheep anti-α_2-HSG (Figure 4 lanes A-C).

The sheep anti-α_2-HSG antibody is reactive with the peptide and shows no reactivity with other jacalin binding proteins on double immunodiffusion (data not shown). Removal of terminal sialic acid with neuraminidase reduced reactivity with patient serum (Figure 4 lanes D and E). The remaining reactivity in this fraction was at the same molecular weight as the undigested α_2-HSG, indicating that the autoantibody binding is dependent on the presence of terminal sialic acid. Complete removal of carbohydrate by neuraminidase treatment followed by EndoF/PNG'ase digestion completely abolished reactivity (Figure 4 lane F). This reduction in autoantibody binding following neuraminidase was not restricted to α_2-HSG since similar treatment of the

partially purified α_2-HSG fraction resulted in reduced binding to all 5 reactive proteins (Figure 3 lane c).

Table 1

Correlation of Identified Endometriosis Antigens and Jacalin Binding Activity

Antigen	Endometriosis Antigen In this Study/ (published study)	Jacalin Reactivity In this study/ (published reactivity)
α-HSG	Yes/[5,10]	Yes/[63]
Carbonic anhydrase I/II	Yes/[5-8]	no/unknown
Hemopexin	Yes/no	Yes/[15,60]
IgA	Yes/no reviewed in [15]	Yes/multiple
CD23	No/soluble form elevated [52-55]	No/acts as a Gal-GalNac lectin like jacalin [64,65]
hCG Receptor	No/[5]	No/[66]

These results show that (with the exception of transferrin) a common carbohydrate epitope is recognized on a_2-HSG, carbonic anhydrase, hemopexin and the a-chain of IgA1 (see table 1 for a summary of antigens identified in this, and published studies, and their jacalin reactivity). This conclusion was reached on the basis that all of the identified antigens (with the exception of transferrin and carbonic anhydrase II) are known to bind the lectin jacalin, and that the removal of carbohydrate from the antigens removed the ability of endometriosis sera to bind these antigens.

EXAMPLE 5

Binding of Autoantibody to Bovine Fetuin

Bovine fetuin, the homologue of human a_2-HSG, bears the same jacalin binding carbohydrate moiety as human a_2-HSG. Sheep antiserum raised against human SG peptide does not show cross-reactivity with bovine fetuin, suggesting significant differences in antigenicity between the two species despite considerable sequence homology. Sera from endometriosis patients show positive reactivity by ELISA and western blot with bovine fetuin (Figure 5). Neuraminidase treatment of fetuin, in contrast to -HSG, slightly increased autoantibody reactivity on ELISA (Figure 5). However, more complete desialylation, or complete deglycosylation of bovine fetuin abolished antibody binding, giving additional evidence that the autoantibody response in endometriosis patients is carbohydrate dependent (not shown). The increase in binding to fetuin following incomplete neuraminidase treatment may result from bovine fetuin being more heavily sialated than its human counterpart.

EXAMPLE 6

D-Galactose dependency of autoantibody binding.

Jacalin binding is dependent on the presence of a Galb1-3NacGal moiety. If the autoantibody response involves this epitope, binding should be competitively inhibited by D-galactose, as is the case for jacalin binding. Binding of endometriosis IgG to a_2-HSG on western blots was completely abolished in the presence of 0.8M D-Galactose, indicating that D-Galactose may form a part of the epitope recognized by the autoantibodies (not shown).

EXAMPLE 7

Serum autoantigens recognized by endometriosis serum share a common epitope.

Since a common carbohydrate epitope accounts for the autoreactivity with the different antigens identified above, then autoantibody binding should be blocked in the presence of an excess of a different antigen. This proved to be the case: addition of a 10 fold excess of highly purified α-HSG or bovine fetuin both inhibited binding of autoantibody to carbonic anhydrase II and the 72kDa antigen (Figure 6).

Is hemopexin, rather than transferrin, the 72kDa autoantigen in endometriosis? Of the antigens described thus far, all, with the exception of transferrin, express the O-linked carbohydrates recognized by jacalin. No such carbohydrate structures are expressed on transferrin, and jacalin does not bind to transferrin [15]. This leaves three possibilities: either an anti-transferrin response distinct from the jacalin carbohydrate response is present in endometriosis sera, or the reactivity which purifies with transferrin is against a contaminant in the transferrin preparations, or both. Two reactive proteins of ~70kDa were found in the anion exchange fractions of eutopic endometrium W1345. One eluted early in the salt gradient in the same fraction as transferrin. The other protein eluted at higher salt concentration in the same fractions as IgA. Examination of the Swiss 2D database indicates that jacalin-binding proteins of similar molecular weight and isoelectric point include hemopexin. When hemopexin was purified by Zn^{2+} metal chelate chromatography and anion exchange chromatography, a homogeneous preparation was obtained. Identity was confirmed by western blotting of fractions with a hemopexin specific antibody. When

tested against endometriosis patient serum this protein was recognized as an autoantigen (Figure 7). Western blot analysis of the same protein with an antibody specific for transferrin showed no reactivity with the purified hemopexin. When fractions from the same column were investigated for the transferrin reactivity, an earlier peak from the anion exchange column was found to contain transferrin. Western blot analysis of the transferrin-containing fractions with anti-hemopexin revealed the presence of hemopexin with similar mobility to transferrin on SDS-PAGE. Double immunodiffusion showed no lines of identity when anti-hemopexin and anti-transferrin were precipitated against the peak transferrin fraction, indicating that the antisera were antigen specific (not shown). The presence of jacalin binding activity in this transferrin preparation was confirmed by the presence of a precipitin line in double immunodiffusion against jacalin (not shown). Further experiments are currently underway to determine the nature of the antigen which co-purifies with transferrin, but it is of interest that the 72kDa binding activity was inhibited by the addition of a pure a_2-HSG preparation (figure 6).

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CLAIMS

What is claimed is:

1. A method for diagnosing endometriosis in a patient, said method comprising:

(a) obtaining a serum sample from said patient,

(b) incubating Thomsen Friedenreich (Tf)-like antigen with said serum sample,

(c) detecting autoantibody reactivity with the Tf-like antigen in the sample, and;

(d) correlating an increased level of autoantibody reactivity to the Tf-like antigen in the serum sample with a diagnosis of endometriosis in said patient.

2. The method of claim 1 wherein the autoantibody reactivity is determined by immunoassay.

3. The method of claim 2 wherein the immunoassay is an immunometric assay.

4. The method of claim 2 wherein the immunoassay is a competitive immunoassay.

5. The method of claim 3 comprising:

(a) immobilizing Tf-like antigen on a solid support,

(b) adding an aliquot of serum sample from a patient to the Tf-like antigen bound on the solid support and incubating,

(c) adding a labeled anti-human immunoglobulin to the solid support wherein said anti-human immunoglobulin is part of a signal producing system,

(d) separating free labeled antibody from bound antibody,

(e) measuring the signal generated by the solution containing the solid support; and

(f) correlating an increase in signal strength with a diagnosis of endometriosis in the patient.

6. The competitive immunoassay of claim 4 comprising:

(a) preparing a reaction mixture by incubating a constant amount of a labeled antibody bound to Tf-like antigen with different concentrations of a serum sample from a patient wherein said labeled antibody is part of a signal producing system,

(b) separating bound labeled antibodies from free autoantibodies,

(c) measuring the signal generated by the labeled antibody in the reaction mixture, and;

(d) correlating a decrease in signal strength after addition of the serum sample from a patient with a diagnosis of endometriosis in the patient.

7. The competitive immunoassay of claim 4 comprising:

(a) preparing a reaction mixture by incubating a first antibody which is bound to Tf-like antigen with different concentrations of a serum sample from a patient,

(b) adding a constant amount of a second antibody to the reaction mixture wherein said second

antibody recognizes a constant region of the heavy chain of the first antibody and wherein the second antibody is labeled and part of a signal producing system,

(c) separating free labeled second antibody from bound antibody,

(d) measuring the signal generated by the second antibody in the reaction mixture, and;

(e) correlating a decreased level of signal strength with a diagnosis of endometriosis in the patient.

8. The method of claim 5 wherein the antibody is enzyme-labeled and the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled anti-human immunoglobulin followed by incubating are performed between steps (d) and (e).

9. The method of claim 6 wherein the antibody is enzyme-labeled and the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled anti-human immunoglobulin followed by incubating are performed between steps (b) and (c).

10. The method of claim 7 wherein the second antibody is enzyme-labeled and the additional steps of adding a substrate to the solid support which reacts with the enzyme-labeled anti-human immunoglobulin followed by incubating are performed between steps (c) and (d).

11. The method of any of claims 5, 6, or 7 wherein the labeled antibody is radiolabeled.

12. The method of any of claims 5, 6, or 7 wherein the labeled antibody is biotin labeled.

FIG. 1

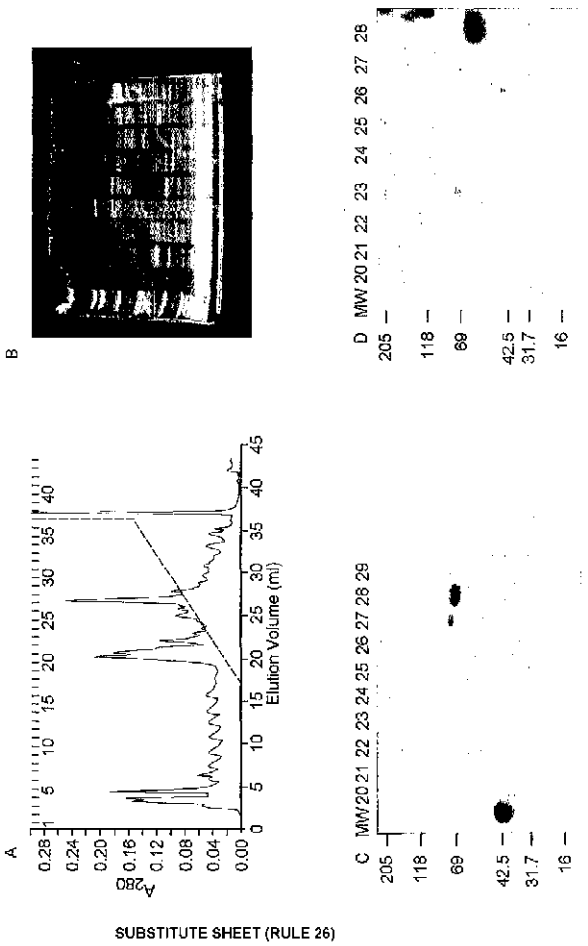

A
A280
Elution Volume (ml)
B
C MW 20 21 22 23 24 25 26 27 28 29
205
118
69
42.5
31.7
16
D MW 20 21 22 23 24 25 26 27 28
205
118
69
42.5
31.7
16

FIG. 2

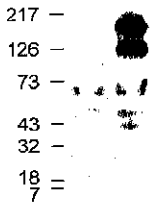


FIG. 3

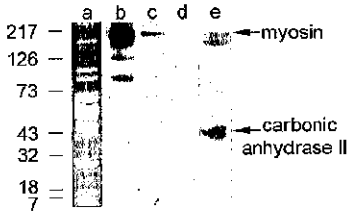


FIG. 4

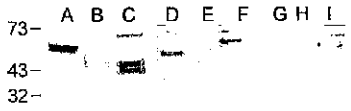


FIG. 5

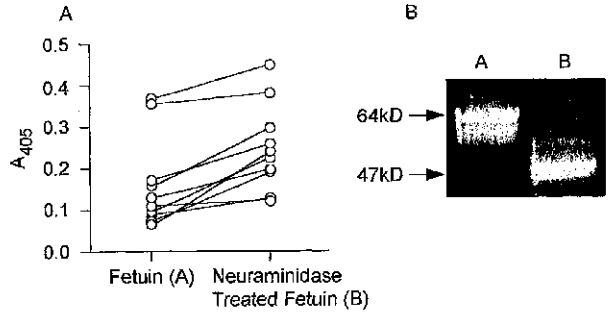

A
B
0.5
0.4
0.3
0.2
0.1
0.0
A_{405}
Fetuin (A)
Neuraminidase Treated Fetuin (B)
A
B
64kD
47kD

FIG. 6

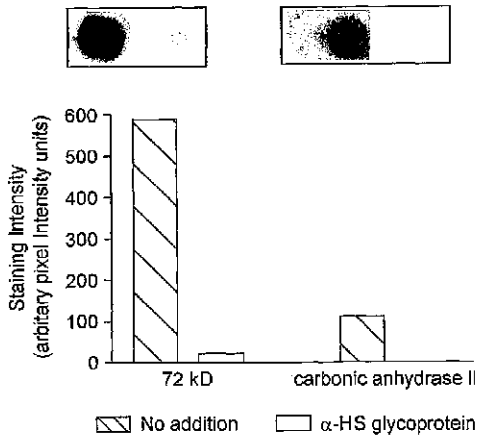
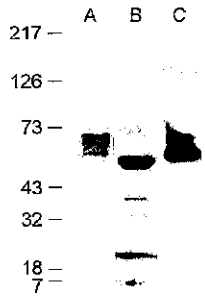

Staining Intensity (arbitary pixel Intensity units)
600
500
400
300
200
100
0
72 kD
carbonic anhydrase II
No addition
α-HS glycoprotein

FIG. 7



【国際調査報告】

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/12802

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/564

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LANG GILLIAN A ET AL: "Autoantibodies in endometriosis sera recognize a Thomsen-Friedenreich-like carbohydrate antigen" JOURNAL OF AUTOIMMUNITY, LONDON, GB, vol. 16, no. 2, March 2001 (2001-03), pages 151-161, XP002196573 ISSN: 0896-8411 the whole document	1-12

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in 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 but 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
3 June 2002	11/06/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Pellegrini, P

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/12802

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
A	SUBBI PILLAI ET AL: "Antibodies to encometrial transferrin and alpha 2-Heremans Schmidt (HS) glycoprotein in patients with endometriosis" AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY, MUNKSGAARD INTERNATIONAL PUBLISHERS, COPENHAGEN, DK, vol. 35, no. 5, 1996, pages 483-494, XP000910506 ISSN: 1046-7408 cited in the application abstract	1-12
A	D'CRUZ O J ET AL: "ANTIBODIES TO CARBONIC ANHYDRASE IN ENDOMETRIOSIS: PREVALENCE, SPECIFICITY AND RELATIONSHIP TO CLINICAL AND LABORATORY PARAMETERS" FERTILITY AND STERILITY, ELSEVIER SCIENCE INC, NEW YORK, NY, US, vol. 4, no. 66, October 1996 (1996-10), pages 547-556, XP001073608 ISSN: 0015-0282 cited in the application abstract	1-12
A	CONFINO E ET AL: "PERITONEAL FLUID AND SERUM AUTOANTIBODY LEVELS IN PATIENTS WITH ENDOMETRIOSIS" FERTILITY AND STERILITY, ELSEVIER SCIENCE INC, NEW YORK, NY, US, vol. 2, no. 58, February 1990 (1990-02), pages 242-245, XP001073574 ISSN: 0015-0282 abstract	1-12
A	BURNS W N ET AL: "PATHOPHYSIOLOGY OF ENDOMETRIOSIS- ASSOCIATED INFERTILITY" CLINICAL OBSTETRICS AND GYNECOLOGY, HARPER AND ROW, HAGERSTOWN, MD, US, vol. 3, no. 42, September 1999 (1999-09), pages 586-610, XP001073561 ISSN: 0009-9201 page 94, column 1, paragraph 2 -page 95, column 1, paragraph 3	1-12

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专利名称(译)	子宫内膜异位症的诊断分析		
公开(公告)号	JP2004501349A	公开(公告)日	2004-01-15
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申请(专利权)人(译)	研究公司技术公司		
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IPC分类号	G01N33/53 G01N33/543 G01N33/564		
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代理人(译)	三好秀 三好康夫		
优先权	60/198882 2000-04-19 US		
其他公开文献	JP2004501349A5		
外部链接	Espacenet		

摘要(译)

本发明提供了一种检测患者子宫内膜异位症的方法，该方法是对侵入性和昂贵的外科手术的改进。该方法使用免疫测定来检测血清样品中的自身抗体，其与Tomsen-Friedenreich抗原（Tf）反应。来自与Tf样抗原结合的患者的血清样品中自身抗体水平的增加指示患者的子宫内膜异位症。