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(54)【発明の名称】ｐａｎ特異的モノクローナル抗体

(57)【要約】
本発明は、ヒトの血液サンプル中におけるグリケーションされたヘモグロビンの全ての構造バリアントの量を測定するためのpan 特異的モノクローナル抗体を記載する。ある一人の個体の血液中におけるヘモグロビンのグリケーションの割合を測定することによって、糖尿病患者におけるグルコースレベルコントロールの有用な指標が提供される。

【特許請求の範囲】
【請求項１】
式：
グリコシル-(NH)-Val-His-またはグリコシル-(NH)-Val-His-AA、
式中、グリコシル-(NH)-Val は非酵素的にグリケーションされたバリン残基を表し、Hisはヘモグロビンの天然型β－鎖の２番目のアミノ酸を表し、AAは１～３個のアミノ酸残基を意味する
のグリケーションされたペプチド残基と特異的に結合する抗体結合部位を含有してなる、pan 特異的である、すなわちグリコシルヘモグロビンの全ての構造バリアントと反応することができる、モノクローナル抗体またはその断片。
【請求項２】
AAが、ヒトヘモグロビンの全ての構造バリアントのN-末端のβ－鎖の一部に対応する１～３個のアミノ酸配列である請求項１記載のモノクローナル抗体またはその断片。
【請求項３】
AAが、-Leu-Aa 、-Leu-Thr-Aa 、-Leu-Thr-Pro-Aa であり、Aaが、AA基中の残りの数のアミノ酸を意味する請求項２記載のモノクローナル抗体またはその断片。
【請求項４】
前記抗体が、Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH （DSMZ）にアクセッション番号DSM ACC2495 として寄託されているハイブリドーマDAK Hb1c-1により得ることができる前記請求項いずれか１つに記載のモノクローナル抗体またはその断片。
【請求項５】
前記抗体が、pan 特異的、すなわちHbA1c 、HbC1c およびHbS1c を含むヒトヘモグロビンの全ての構造バリアントと反応することができる、前記請求項いずれか１つに記載のモノクローナル抗体またはその断片。
【請求項６】
前記抗体が請求項７～１１いずれか１つに記載の方法により得ることができる、前記請求項いずれか１つに記載のモノクローナル抗体またはその断片。
【請求項７】
抗体が免疫原性キャリア物質に化学的に連結されたグリケーションされたペプチドにより得ることができる抗原を含有する免疫原に対して惹起され、該グリケーションされたペプチドが、ヒトヘモグロビンのN-末端のβ－鎖に対応する２～５個のアミノ酸単位を有する、モノクローナル抗体またはその断片の産生方法。
【請求項８】
免疫原が、式：
［フルクトシル-(NH)-Val-His-AA-R］n-キャリア
式中、フルクトシル-(NH)-Valは非酵素的にグリケーションされたバリン残基を表し、His はヘモグロビンの天然型β－鎖の２番目のアミノ酸を表し、AAは１つ以上のさらなるアミノ酸残基を意味し、R は結合基であり、キャリアは免疫原性キャリア物質であり、n は１～該キャリア上で利用可能なカップリング部位の数の整数である、
である請求項７記載のモノクローナル抗体またはその断片の産生方法。
【請求項９】
AAが、ヒトヘモグロビンの全ての構造バリアントのN-末端のβ－鎖の一部に対応する１～３個のアミノ酸配列である請求項８記載のモノクローナル抗体またはその断片の産生方法。
【請求項１０】
前記キャリアが、ヒトヘモグロビン以外の免疫原性タンパク質またはペプチドである請求項８記載のモノクローナル抗体またはその断片の産生方法。
【請求項１１】
抗体が、ヒトヘモグロビンA1c 、C1c およびS1c のβ－鎖のグリケーションされたN-末端

ペプチド配列への立体構造的なアクセスを提供するのに十分露出された後のヒトヘモグロビンA1c 、C1c およびS1c のβ－鎖のグリケーションされたN-末端ペプチド配列に特異的に結合する前記請求項いずれか１つに記載のモノクローナル抗体またはその断片の使用方法。

【請求項１２】

前記グリケーションされたペプチド配列が、物理的または化学的な変性または消化により抗体結合部位に対して露出されている請求項１１記載のモノクローナル抗体またはその断片の使用方法。

【請求項１３】

前記グリケーションされたペプチド配列が、カオトロピック剤を用いた変性により抗体結合部位に対して露出されている請求項１１または１２記載のモノクローナル抗体またはその断片の使用方法。

【請求項１４】

カオトロピック剤が、グアニジン、尿素、チオシアン酸カリウムまたは界面活性剤からなる群より選ばれる請求項１３記載のモノクローナル抗体またはその断片の使用方法。

【請求項１５】

前記抗体が、固相に吸着されているヒトヘモグロビンの全ての構造バリアントのβ－鎖のグリケーションされたN-末端のペプチド配列に特異的に結合する前記請求項いずれか１つに記載のモノクローナル抗体またはその断片の使用方法。

【請求項１６】

固相が、好ましくはマイクロタイタープレート、微粒子、膜、チューブ、ピン、チップまたはディスクからなる群より選ばれる物質から構成される請求項１５記載のモノクローナル抗体またはその断片の使用方法。

【請求項１７】

ヒト血液中のグリケーションされたヘモグロビンのレベルの決定のための前記請求項いずれか１つに記載のモノクローナル抗体またはその断片の使用。

【請求項１８】

ヒト血液中のグリケーションされたHbS および／またはHbC のレベルの決定のための前記請求項いずれか１つに記載のモノクローナル抗体またはその断片の使用。

【請求項１９】

ヒト血液中のグリケーションされたヘテロ接合型HbA 、HbS および／またはHbC のレベルの決定のための前記請求項いずれか１つに記載のモノクローナル抗体またはその断片の使用。

【請求項２０】

二粒子比濁アッセイにおいて測定されるグリケーションされたヘモグロビンのレベルの決定のための前記請求項いずれか１つに記載のモノクローナル抗体またはその断片の使用。

【発明の詳細な説明】

【０００１】

本発明は、ヒトの血液サンプル中におけるグリケーションされたヘモグロビンの全ての構造バリアントの量の測定方法に関する。ある一人の個体の血液中におけるヘモグロビンのグリケーションの割合を測定することによって、糖尿病患者におけるグルコースレベルコントロールの有用な指標が提供される。

【０００２】

特に、本発明はモノクローナル抗体またはその断片の調製方法に関するものであり、これはヒトのヘモグロビンなどのグリケーションされたN-末端ペプチド残基を特異的に認識する。

【０００３】

糖尿病患者は、グルコースを通常の方法で代謝することができず、その結果、患者の血液中および尿中にグルコースが蓄積する。従来より、このような体液中のグルコースレベルは糖尿病の状況の状態の指標として取り扱われており、言い換えるとこのレベルは、服用

するインスリンまたは他の薬剤の量の指針として、または患者の食生活を変更する必要性の指針として用いられている。

【０００４】

直近の食事の時間および内容、直近のインスリン注射などに依存して、グルコースレベルが広い範囲で変動するという点を除いては、これはそれなりに十分役に立っている。このように、示される値はその瞬間の状況を反映することになり、このことは、糖尿病の状況の長期間の状態と正確には合致しないかもしれない。

【０００５】

糖尿病の状況の別の結果として、糖尿病患者の血液中のグリケーションされたヘモグロビンの量が増加するということが知られている。ヘモグロビン（Hb）は、アミノ酸の四本の鎖（サブユニット）からなる四量体タンパク質であり、鎖のそれぞれは約143 単位であり、合計で約64000g/molの分子量を有する。ヘモグロビン中のβ－サブユニットのN-末端のバリンは、グルコースと反応することが可能である。グルコースとバリンのα- アミノ基とに関する非酵素的な反応によって、ヘモグロビンのグリケーションが生じる。反応物の間にシッフ塩基が形成された後、グルコースはアマドリ転位を受けて1-デオキシフルクト- バリンを形成する。最終的な複合体での1-デオキシフルクトースとバリンとの間の結合は共有結合であり、不可逆性である。グリケーション反応は、反応物、たとえばヘモグロビンとグルコースレベルに支配される。糖尿病ではない個体についての、糖尿病のコントロールと合併症のトライアル（Diabetes Control and Complication Trial ）（DCCT）の基準範囲は、約4-6%のグリケーションされたヘモグロビンである。β－鎖のN-末端上に1-デオキシフルクト- バリンを伴うヘモグロビン四量体は、グリケーションされたヘモグロビン; たとえばHb1cまたはHbA1c 、HbC1c またはHbS1c と認められる。

【０００６】

HbC の特徴は、サハラ以南の地域において30% が患者であり、アフリカ系アメリカ人の2.3%が患者であることである。合衆国におけるHbの最も一般的なバリアントであるHbS の特徴は、アフリカ系アメリカ人の7.8%が患者であることである。ひとまとめにすると、アメリカ黒人の少なくとも10% が、HbC の特徴またはHbS の特徴のいずれかを持っている。世界人口では混血がどんどん増加しつつある。したがって、グリケーションされたヘモグロビンの全ての構造バリアントと同様に反応する抗体を持つことがますます重要となる。もし、利用可能な抗体がグリケーションされたヘモグロビンの全ての構造バリアントとは反応しない場合、HbS および／またはHbC の特徴を備える患者からのHb1cの割合の測定が不正確になるだろう。

【０００７】

糖尿病患者におけるグルコースレベルは、血液中のグルコースレベルに直接依存するグリケーションの割合を高めるのに十分に高く、これは糖尿病の状況の重篤度を反映する。それゆえに、ヘモグロビンA1c のレベルは約6 から12% に上昇する。ヘモグロビンA が循環する寿命は約120 日間であることから、グリケーションされたヘモグロビンの測定には、その期間の平均的なグルコースレベルを反映するという意味があるであろう。特に、グルコースが多い食事によって、グリケーションされたヘモグロビンまたは血清アルブミンのレベルが即座に高く反映されるわけではない。したがって、グリケーションされたヘモグロビンの含有量の測定によって、循環しているグルコースの平均的なレベルのより本当に近い臨床像が与えられ、したがって患者の長期間の状況のより本当に近い臨床像が与えられる。

【０００８】

グリケーションされたヘモグロビンレベルを測定するために用いられる一般的な方法には、溶血サンプルをボロン酸塩アフィニティーカラムに通す工程が含まれる。この方法によって、HbA_{1c} と、リジン残基ならびにヘモグロビンのα- 鎖およびβ－鎖の両者のN-末端のバリン残基上で形成されるケトアミン構造体とを含む、グリケーションされたヘモグロビンの総量を測定する。このカラムを洗浄し、そして分光光度法でグリケーションされたヘモグロビンを測定する。

【０００９】
機器によって自動的に分析するために、微粒子強化免疫比濁法（Microparticle enhanced turbidimetric immunoassay technique）を利用することができる。これらの機器は、ある特定の診断用アッセイのために用意したものではなく、そして極めて長いサンプルのサイクル時間に備えている。現在のところ、グリケーションされたヘモグロビンの測定のためのこの方法の実用性は、ありきたりの臨床分析として高まっている。これらの免疫アッセイのいくつかは、微粒子強化競合免疫比濁阻害法（microparticle enhanced competitive turbidimetric inhibition immunoassay technique ）の原理に基づいている。
【００１０】
米国特許第4,247,533 号には、HbA1c の注射によって、ヒツジの中でHbA1c に対する抗体を報告されるように惹起させたことと、グリケーションされていないヘモグロビンを吸着させることでポリクローナル抗体を供給し、これによってHbA1c とグリケーションされていないヘモグロビンとを区別するといった分析方法が開示されている。次いで、このような抗体は、サンプル中のグリケーションされたヘモグロビンの割合を測定するための試験の基礎を形成する。
【００１１】
従来の技術では、ヘモグロビンA_{1c} 抗体と、その抗体がどのようにしてグリケーションされたヘモグロビンのバリアントA と反応することができるかについてだけが記載されている。グリケーションされたヘモグロビンの他のバリアントの影響力については言及されておらず、そしてグリケーションされたヘモグロビンの全ての構造バリアントに対するモノクローナル抗体についても考慮されていない。この抗体はHbS1c およびHbC1c を認識しないという事実が原因で、HbS1c およびHbC1c を有する被験体において、グリケーションされたヘモグロビンのパーセンテージの免疫アッセイ測定をこの抗体を用いて行うことによって、事実上低いグリケーションされたヘモグロビンのパーセンテージが生じてしまう。
【００１２】
Hb1cの全ての構造バリアントについて特異的であるHb1c抗体は、pan 特異的とみなされる。
【００１３】
したがって、上記で認められた欠点を現さない上記の種類の抗体についての必要性が残っている。
【００１４】
pan 特異的である抗体、すなわちグリケーションされたヘモグロビンの全ての構造バリアントについて特異的な抗体を提供することが、したがって本発明の目的である。
【００１５】
HbA1c 、HbC1c およびHbS1c の免疫アッセイ測定法に基づいて、患者の長期間の血液の糖レベルを測定するための抗体を提供することも目的である。
【００１６】
患者の全血サンプル中のグリケーションされたヘモグロビンであるHbA1c 、HbC1c およびHbS1c の含有量を測定するための、このような抗体を提供することは、本発明の別の目的である。
【００１７】
これらの目的および他の目的は、特許請求の範囲に規定される本発明によって達成される。
【００１８】
本発明は、pan 特異的、すなわちグリケーションされたヘモグロビンの全ての構造バリアントと反応することが可能であるモノクローナル抗体またはその断片に関する。このグリケーションされたヘモグロビンの構造バリアントとしては、HbA1c 、HbC1c およびHbS1c が好ましい。
【００１９】
このモノクローナル抗体が、HbS およびHbC を持つ患者におけるグリケーションされたヘ

モグロビンレベルの測定に関する現行の免疫アッセイの改良をもたらすことが好ましい。

【００２０】

本発明のモノクローナル抗体は、その抗体がヒトのヘモグロビンA 、C およびS のβ－鎖のグリケーションされたN-末端のペプチド配列と特異的に結合する抗体結合部位を含有するという特徴を有する。

【００２１】

本発明のモノクローナル抗体試薬は、グリケーションされたヘモグロビン中の線状エピトープについてのその特異的な結合親和性に特徴を有する。したがって、本明細書で用いられるような「抗体試薬」または「抗体結合部位」という用語は、このようなペプチドのエピトープについて特異的なモノクローナル抗体結合部位を含有するあらゆる物質を示すことになり、そしてこの物質はどのようにして得られたものであってもよい。したがって、このような用語には、全ての抗体および適切な断片または多官能性形態が包含される。全ての抗体の形態である場合、たとえばIgG 、IgM などの公知の免疫グロブリンのいずれかのクラスおよびサブクラスに属することが可能である。ペプチドのエピトープについての特異的な結合親和性を有する、いずれかのそのような免疫グロブリンのあらゆる断片を用いることが可能であり、たとえば、IgG の断片は従来よりFab 、Fab'およびF $(ab')_2$ として知られている。さらに、免疫グロブリンまたはその断片の凝集体、ポリマー、誘導体、複合体(conjugates)およびハイブリッドを、適切である場合には用いることが可能である。さらに、単鎖抗体または組み換え手段によって得られるあらゆる他の断片を、適切である場合には用いることが可能である。

【００２２】

グリケーションされた残基は、HbA1c 、HbS1c およびHbC1c の顕著な構造上の特徴である。本発明の抗体には、グルコースと末端のアミンとの間の反応生成物のアマドリ転位で形成される1-デオキシフルクトシル糖質単位を最低限含有するエピトープまたは決定部位と、天然型HbA1c 、HbS1c およびHbC1c の配列に対応する位置にあるHbA1c 、HbS1c およびHbC1c のN-末端の配列の少なくとも１つのアミノ酸単位を含有する、そこから伸びているペプチド配列とが必要である。エピトープを特徴付けるペプチド配列中の他のアミノ酸単位は、天然型HbA1c 、HbS1c およびHbC1c 配列中に見られるアミノ酸単位と同一であってもよく、異なっていてもよい。このように、このエピトープには、抗体に対する少なくとも二つの接触部位または結合部位という特徴があり、このような部位はグリケーションされたN-末端のHbA1c 、HbS1c およびHbC1c 配列に対して特有である。

【００２３】

この抗体は、次の式：

グリコシル-(NH)-Val-His-またはグリコシル-(NH)-Val-His-AA、

式中、グリコシル-(NH)-Val は非酵素的にグリケーションされたバリン残基を表し、Hisはヘモグロビンの天然型β－鎖の2 番目のアミノ酸を表し、AAは１つ以上のさらなるアミノ酸残基である、

のグリケーションされたペプチド残基と特異的に結合することが好ましい。

【００２４】

１つの好ましい態様において、AAとは、ヒトのヘモグロビンの全ての構造バリアントのN-末端のβ－鎖の一部に対応する1 ～3 個のアミノ酸の配列である。AAとしては、-Leu-Aa 、-Leu-Thr-Aa 、-Leu-Thr-Pro-Aa であることが好ましく、そしてAaとは、AA基中のアミノ酸の残りの数を意味する。

【００２５】

本発明のモノクローナル抗体またはその断片としては、ハイブリドーマDAK Hb1c-1を用いて得ることができるものが好ましく、このハイブリドーマは、Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH （DSMZ）にアクセッション番号DSM ACC2495 として寄託されている。

【００２６】

さらに本発明は、モノクローナル抗体またはその断片を産生する方法に関し、ここで、こ

の抗体はpan 特異的、すなわちグリケーションされたヘモグロビンの全ての構造バリアントと反応することが可能である。

【００２７】

抗体またはその断片は、特許請求の範囲に規定された方法によって得られ得るものであることが好ましい。

【００２８】

本発明のモノクローナル抗体またはその断片を産生する方法は、免疫原性キャリア物質に化学的に連結させたグリケーションされたペプチドを含有する免疫原に対する抗体を惹起させる、ここで該グリケーションされたペプチドがヒトのヘモグロビンのβ－鎖のN-末端に対応する2 ～5 個のアミノ酸単位を有する、工程を含む。

この免疫原は次の式：

［フルクトシル-(NH)-Val-His-AA-R］$_n$ - キャリア

式中、フルクトシル- (NH)-Valは非酵素的にグリケーションされたバリン残基を表し、His はヘモグロビンの天然型β－鎖の2 番目のアミノ酸を表し、AAは１つ以上のさらなるアミノ酸残基を意味し、R は結合基であり、キャリアは免疫原性キャリア物質であり、n は１～当該キャリア上で利用可能なカップリング部位の数までの整数である、

であることが好ましい。

【００２９】

１つの好ましい態様において、AAとは１つ以上のさらなるアミノ酸残基である。別の好ましい態様において、AAとは、ヒトのヘモグロビンの全ての構造バリアントのβ－鎖のN-末端部分に対応する1 ～3 個のアミノ酸の配列である。

【００３０】

結合基であるR は、本質的には、都合が良くかつ安定したあらゆる構造であり得る。このような結合基であるR は、通常は、水素を除いて1 ～約20の間の原子を含有し、そして窒素、酸素および硫黄などのヘテロ原子を含む脂肪族鎖の形態である。様々な基を介してこのグリケーションされた残基を結合させて、メチレン、エーテル、チオエーテル、イミノなどを含む結合鎖のR を形成させることが可能である。当業者であれば、そこから免疫原を調製するために選択するところの幅広い種類の結合基を分かっているだろう。

【００３１】

好ましい態様において、キャリアはヒトのヘモグロビン以外の免疫原性タンパク質またはペプチドである。

【００３２】

最も一般的な意味において適切な免疫グロブリンの産生を刺激するために用いられる免疫原は、免疫原性キャリア物質に化学的に連結させられた１つ以上のグリケーションされたペプチド残基を含有する。グリケーションされたペプチド残基にカップリングすることが可能な官能基を有することが通常知られているあらゆるものから、この免疫原性キャリア物質を選択することが可能である。十分なサイズと免疫原性とを持つ、たとえば炭水化物、多糖類、リポ多糖、核酸などの他の物質を同様に用いることも可能ではあるが、多くの場合、キャリアはタンパク質またはポリペプチドである。主として、免疫原性タンパク質および免疫原性ポリペプチドの分子量は4000g/mol ～10000000g/mol の間であり、15000g/molを超える量であることが好ましく、そして50000g/molを超える量であることがより普通である。別の種の血流内に導入する場合、一般的に、１つの動物種から得られるタンパク質が免疫原性となる。特に有用なタンパク質は、アルブミン、グロブリン、酵素、ヘモシアニン、グルテリン、明らかに非タンパク質性の組成を有するタンパク質などである。

【００３３】

好ましい態様において、測定されるサンプル中でタンパク質を適切に変性または消化することによって、本発明のモノクローナル抗体またはその断片に接触可能な天然型HbA1c 、HbS1c およびHbC1c 分子のグリケーションされたN-末端のペプチド残基を作製する。

【００３４】

別の好ましい態様において、天然由来のグリケーションされたヘモグロビン、たとえばHb

A1c 、HbS1c およびHbC1c を化学的にまたは酵素的に消化することによって、グリケーションされた断片を産生することが可能である。伝統的なカップリング手段、たとえばグルタルアルデヒドまたはカルボジイミドを用いて、キャリアにこの断片をカップリングさせることが可能であり、そしてこの複合体を免疫原として用いる。

【００３５】

本発明の抗体またはその断片を、次の工程によって産生することも好ましい：

【００３６】

A.ペプチド免疫原の調製

HbA 、S およびC のβ－鎖上にあるN-末端のバリン残基のグリケーション部位に対する抗体を得るために、免疫原として短いペプチド（五量体）を用いることが好ましい。この抗原としては、Hb1cの四量体にシステインを付加したもの、すなわちフルクトシル-V-H-L-T-Cが好ましい場合がある。

【００３７】

B.ヒトHb1cペプチドによるマウスの免疫化

モノクローナル抗体を分泌するハイブリドーマを生育するために用いる一般的な方法は、当業者に十分知られている。

【００３８】

実際に利用した方法の実例は、ジャーナル・オブ・イムノロジカル・メソッズ（Journal of Immunological Methods）、1980, 39:285に記載されている。

【００３９】

CF1 マウスとBALB/cマウスとの交配によるメスのF1ハイブリッドを用いることが好ましいが、他の系統のマウスを用いても構わない。この免疫化計画およびペプチド- キャリア複合体のレベルを、十分な量の抗原的に刺激されたリンパ球が形成されるように選択することができる。好ましい態様においては、Hb1cペプチドとの複合体の状態の50μg の精製タンパク質誘導体（PPD 、結核菌（M. tuberculosis ）由来の変性タンパク質の、一部が未知の混合物）によって、二週間の間隔を空けて四回、腹腔経由でマウスを免疫化する。最終免疫の3 ～5 日後、融合のために、動物から脾臓細胞を抽出する。

【００４０】

他の種からのハイブリドーマによって産生されるモノクローナル抗体も好ましく用いることができる。

【００４１】

C.細胞融合

免疫化マウスから脾臓を無菌的に取り出し、そして脾臓細胞の懸濁液を調製する。適切な融合プロモーターの存在下で、この脾臓細胞を適切な細胞株由来のマウスミエローマ細胞と融合させる。好ましい態様において、ミエローマ細胞に対する脾臓細胞の好ましい割合は、約20:1～約2:1 であり、そして約10^8 個の脾臓細胞あたり0.5 ～1.5ml の量で、適切に融合培地を用いる。

【００４２】

細胞融合のために用いられるマウスミエローマ細胞は十分に知られている。好ましいマウスミエローマ細胞として、P3-X63-Ag 8.653 を用いてもよい。

【００４３】

融合プロモーターとしては、平均分子量が1000～4000g/mol のポリエチレングリコールが好ましい。技術的に公知の他の融合プロモーターを用いることも可能である。好ましい態様において、平均分子量が1500のポリエチレングリコールを用いることができる。

【００４４】

D.融合した細胞のスクリーニング

個々に分かれている容器（たとえばマイクロタイタープレート）中で、融合していない脾臓細胞、融合していないマウスミエローマ細胞および融合化ハイブリドーマ細胞から構成される混合物を、融合していないマウスミエローマ細胞を生育させない選択培地で好ましくは希釈し、そして融合していないマウスミエローマ細胞を死滅させるのに十分な期間（

約1 週間）培養する。薬物耐性（たとえば、8-アザグアニンに対する耐性）を有し、かつ融合していないマウスミエローマ細胞を生育させない培養培地、たとえばHAT 培地を用いる。選択培地中で、融合していないミエローマ細胞は死滅する。融合していない脾臓細胞は形質転換されなかった細胞であることから、ある特定の期間（1 週間）が経過すると、これらは徐々に死滅する。他方、融合した細胞は、親のミエローマ細胞の腫瘍としての性質と、親の脾臓細胞の性質との両方を有することから、この選択培地中でこれらが生存することができる。

【００４５】

E.ELISA によるグリケーションされたヘモグロビンの測定

特異的な免疫性認識の原理、およびモノクローナル抗体と、抗体がそれに対して特有かつ特異的に結合する抗原性エピトープとの間の反応に基づくこの測定は好ましいものである。たとえば、ELISA 試験によって認識および結合を検出してもよく、この場合、固相支持体、たとえばプラスチック製のウェルの底部に血液サンプルまたはキャリブレーターを固定する。ヒトのヘモグロビンを固定化することによって、抗体の結合のために抗原性エピトープを露出させてもよい。抗体、酵素標識化二次抗体および酵素の基質を、固定化抗原（ヒトのヘモグロビン）と相互作用させてもよく、それによって、抗体- 抗原複合体を形成させることが好ましい。何回かの希釈、インキュベーション工程および洗浄工程、その後に結合した試薬と遊離している試薬とを分離させる。抗体の抗原との結合と、その結果として生じる酵素の基質との相互作用反応の結果として、発色反応が生じる。色素の形成によって血液サンプル中にHb1cが存在することが示され、そして発色の強度によって、そのサンプル中のグリケーションされたエピトープの定量測定を提供する。技術的に知られている他の免疫アッセイ形式によってグリケーションされたヘモグロビンを測定するために、本発明の抗体を用いてもよい。

【００４６】

F.所望の抗体を産生するハイブリドーマ細胞のクローニングとその抗体の産生

限界希釈法などの適切な方法によって、所望の抗体を産生することができるハイブリドーマ細胞をクローニングし、そして所望の抗体を次の方法によって産生することが可能である。適切な培地中で、ある特定の時間ハイブリドーマ細胞を培養し、そしてその培養物の上清からこのハイブリドーマ細胞によって産生されるモノクローナル抗体を単離することが可能である。

【００４７】

本発明はさらに、モノクローナル抗体またはその断片を用いる方法にも関する。

【００４８】

未知の量のグリケーションされたヘモグロビンを含有する血液サンプルと反応させるという通常の方法で、本発明の抗体またはその断片を好ましく用いることが可能であり、そしてグリケーションの程度を測定するために較正された標準と、反応の程度を比較することが可能である。蛍光、免疫アッセイなどによって、HbA1c 、HbS1c およびHbC1c におけるグリケーションされたエピトープについてのそれらの結合力を失うことなく公知の方法にてモノクローナル抗体に読み取り可能な基を適切に結合させることによって、データを出力することが可能である。

【００４９】

本発明のモノクローナル抗体は、グリケーションされたN-末端のペプチド残基の全ての構造バリアントに、好ましくはHbA 、HbS およびHbC で見られるものに特異的に結合する。エピトープが都合良く露出している場合、この抗体は天然型HbA1c 、HbS1c およびHbC1c 分子中のエピトープに結合することが可能である。エピトープに立体構造的にアクセスすることは、任意の効果的な方法で達成することが可能である。無処理タンパク質におけるエピトープの露出は、少なくともエピトープの領域中で物理的または化学的変性または消化を行うことによって達成されると理解されている。このような変性または消化をエピトープの領域に限定することが可能であり、またはこのような変性または消化には、より全体的なすなわちさらに実質的に完全な三次構造の変性、およびタンパク質の二次構造の変

性、あるいはタンパク質の部分消化または完全消化を包含することができる。

【００５０】

好ましい態様において、この抗体は、ヒトヘモグロビンA1c 、C1c およびS1c のβ－鎖のグリケーションされたN-末端ペプチド配列への立体構造的なアクセスを提供するに十分な程度に露出させた後、ヒトヘモグロビンA1c 、C1c およびS1c のβ－鎖のグリケーションされたN-末端ペプチド配列に特異的に結合する。

【００５１】

加熱、超音波処理、高pHまたは低pHへの暴露などの物理的手段によるタンパク質の通常の処理方法を含む様々な方法によって、変性を成し遂げることが可能であり、溶液中での薬剤またはカオトロープを用いての相互作用による化学的変性が好ましい。

【００５２】

たとえば、グアニジンと加熱、グアニジンとSDS 、またはグアニジンとジチオスレイトールのように、化学的手段および／または化学的かつ物理的手段を組み合わせて用いるのであれば、タンパク質の変性を最も効率的に成し遂げることが可能である。グアニジンのような特に強力なカオトロープが好ましい。当然のことながら、抗体の結合のための溶液に接触しやすい露出されるエピトープが含まれているわずかな量のタンパク質の実質的な不溶化、凝集または沈殿生成という結果を招く変性条件は、避けることになる。有用な免疫結合をもたらすために、溶液中または懸濁液中に十分な量の変性タンパク質が残っていなければならない。必要な可溶化の程度は、意図されるまたは所望される結合の状況に依存する。

【００５３】

好ましい態様においては、物理的または化学的変性または消化によって、抗体結合部位に対してグリケーションされたペプチド配列を露出させる。

【００５４】

別の好ましい態様においては、カオトロピック剤を用いての変性によって、抗体結合部位に対してグリケーションされたペプチド配列を露出させる。このカオトロピック剤としては、限定されないが、グアニジン、尿素および種々の界面活性剤（例えば、ドデシル硫酸ナトリウム（SDS ）など）が挙げられ、他には、限定されないが、デオキシコール酸塩および特定の胆汁酸塩、3-（3-コラミドプロピル(cholamidopropyl) ）- ジメチル- アンモニオ-1- プロパンスルホン酸塩、メタノール、プロパノール、アセトニトリルなどの有機溶媒、ならびにチオシアン酸カリウムなどの特定の塩が挙げられる。たとえばTriton X（登録商標）、Tween （登録商標）、nonidet NP-40 （登録商標）およびオクチルグルコシドなどの非イオン性界面活性剤もタンパク質の変性剤として機能することが可能である。

【００５５】

得られる水性混合物中であらゆるHbA1c 、HbS1c およびHbC1c を変性させるのに十分なレベルのカオトロープが存在する水溶液を用いて、特定の血液サンプルの有意な量のHbA1c 、HbS1c およびHbC1c を変性させて、サンプル（たとえば、全血または赤血球の溶血物）との混合による抗体結合のためにグリケーションされたエピトープを露出させる。全血がサンプルである場合では、カオトロープは赤血球を溶血させることにも役立ち、その結果、ヘモグロビンが放出され、そしてプロテアーゼが不活化される。

【００５６】

混合物を短時間加熱することによって、変性プロセスを顕著に促進させてもよい。37℃以下の温度では、カオトロープによる変性には1 時間～数時間かかる可能性があり、それに対して50℃よりも高い温度では、1 分間かそれよりも短時間で十分な変性に到達することができることが分かった。抗体および他のタンパク質性試薬の著しい変性を防止して、その後に混合物へ添加するために、別々の工程として、または、カオトロープがこのような試薬の変性を実質的に無効にし、さらに有意な程度のHbA1c 、HbS1c およびHbC1c が再び天然型になるのを防ぐことによってエピトープの露出を防止するであろうレベルに試薬溶液を添加することによって、サンプル- カオトロープ混合物を希釈することが普通である。

【００５７】
好ましい態様においては、抗体は、固相に吸着されているグリケーションされたヒトヘモグロビンのβ－鎖中のグリケーションされたN-末端のペプチド配列と特異的に結合する。ここで、この固相としては、ポリスチレン、ジビニルベンゼン、ブタジエン、ポリカーボネート、ポリアクリルアミド、ポリアクリル酸、ポリアクリルアミド、ポリエチレン、ポリプロピレン、フッ素化ポリマー、ポリアミドまたはそのブロックコポリマー、金、炭素、セルロース、誘導体化セルロースまたはガラスからなる群より選択されるものが好ましい。
【００５８】
好ましい態様において、ヒトの血液中のグリケーションされたヘモグロビンレベルを測定するために、モノクローナル抗体またはその断片を用いてもよい。さらに、ヒトの血液中のグリケーションされたHbS および／またはHbC レベルを測定するために、モノクローナル抗体またはその断片を用いることもまた好ましい。
【００５９】
さらに好ましい態様において、ヒトの血液中のグリケーションされたヘテロ接合型HbA 、HbS および／またはHbC レベルを測定するために、モノクローナル抗体またはその断片を用いてもよい。
【００６０】
さらに好ましい態様においては、グリケーションされたヘモグロビンレベルを測定するために、二つのミクロスフェアによる比濁法（turbidimetric two-microsphere method）が提供される。この方法は、サンプルと、モノクローナル抗体またはその断片が結合しているミクロスフェアとを混合する、ここで後者は免疫粒子と称される、工程、または、サンプルと、ミクロスフェアとを混合し、次いで免疫ミクロスフェアと混合する工程を含む。得られる免疫凝集物の濁度を測定する。
【００６１】
ミクロスフェアは、好ましくは、ポリスチレン、ジビニルベンゼン、ブタジエン、ポリカーボネート、ポリアクリルアミド、ポリアクリル酸、ポリアクリルアミド、ポリエチレン、ポリプロピレン、フッ素化ポリマー、ポリアミドまたはそのブロックコポリマー(co-block polymer)、金またはシリカからなる群より選択される。
【００６２】
この例の方法において用いられるミクロスフェアは0.01～5 μｍ の粒子サイズを有し、これは、通常用いられる緩衝液中で分散させることによって用いられる。
【００６３】
次の非限定的な実施例は、本発明の特定の特徴および態様を明らかにすることを目的とするものである。
【００６４】
実施例１：
国際特許出願WO86/03494号に記載されているとおりの、シェパードおよびアサトン（Sheppard and Atherton ）の固相法を用いて、1-デオキシフルクトシル-Val-His-AA ペプチドを合成した。フルオレニルメトキシカルボニル（Fmoc）アミノ酸を用いて、カップリング試薬としてPyBOP （ベンゾトリアゾール-1- イル- オキシ- トリス- ピロリジノ- ホスホリウムヘキサフルオロホスフェート）およびHOBt（N-ヒドロキシ- ベンゾトリアゾール）を使用して、このペプチドを合成した。
【００６５】
トリチル基（trt ）で側鎖を保護したシステインおよびヒスチジン、ならびに第三ブチル基（tBu ）で側鎖を保護したスレオニンを用いた。ロイシンは側鎖を保護せずに用いた。
【００６６】
公知の方法を修正することによって、フルクトースアミノ酸複合体であるN-（デオキシ-D- フルクトース-1- イル）-L- バリンを合成した。要約すれば、ジ- イソプロピリデンで保護されたグルコースをトリフレートとして活性化し、そしてバリンのベンジルエステル

と結合させた。得られた2,3:4,5-ジ-O- イソプロピリデン-N- （デオキシ-D- フルクトース-1- イル）-L- バリン- ベンジルエステルを、エタノール中のPd/C上で水素化し、凍結乾燥し、そして最後に、Fmoc保護を伴わないペプチド合成に用いた。TLC 分析および^{1}Hまたは^{13}C のNMR スペクトルによって、生成物の純度を確認した。

【００６７】

自動化固相合成（カルバイオケム- ノババイオケム社（Calbiochem-Novabiochem Ltd）の結晶化装置）によってこのペプチドを合成し、TFA （トリフルオロ酢酸）-TES（トリエチルシラン）- グリセロール-H_2O（90%:4%:3%:3%）によって樹脂から切断し、沈殿させ、そしてメチル- 第三ブチル- エーテルで洗浄し、遠心分離に付し、そして凍結乾燥した。

【００６８】

HPLC（C_{18} 逆相カラム、0.1%のTFA 中の典型的な5-25% のMeCNの段階的勾配を使用）によって精製を行った。MALDI-TOF 質量分析法によってこのペプチドの実態を確認し、そして分析用HPLCによる分析によって、その純度が90% よりも高いことを最終的に保証した。

【００６９】

実施例２：

ギバ財団（Giba Foundation ）のシンポジウム1986, 119:25に記載されたとおりに、最初の免疫化の四週間前に、カルメット- ゲラン杆菌（BCG ）ワクチンを注射して、マウスにおける免疫応答の準備を行った。

【００７０】

ペプチド免疫原を作製するために、キャリア混合物のツベルクリンPPD に複合化させた。グルタルジアルデヒド、すなわちCHO-（CH2 ）$_3$-CHO を用いて、ペプチドとキャリア（PPD ）とを複合化させた。グルタルジアルデヒドは、ペプチドおよびキャリア上のアミノ基と反応する。

【００７１】

したがって、これらの免疫原は次の式を有する：

フルクトシル-Val-His-Leu-Thr-Cys-CH-（CH2 ）$_3$-CH-PPD

【００７２】

実施例３：

CF1 マウスとBALB/cマウスとの交配によるメスのF1ハイブリッド（12週齢）をBCG-ワクチンで準備し、そして上記のペプチド-PPD複合体によって免疫化した。リン酸塩緩衝液（PBS ）中に溶解したペプチド-PPD複合体と水酸化アルミニウム（1mg/用量）との50μｇ の混合物によって、マウスの腹腔内に四回免疫化した。グリケーションされたヘモグロビンに対する高力価の抗体を有するマウスを、融合のために選択した。最終免疫化の三日後に、この免疫化マウス由来の脾臓細胞を、細胞融合のために用いた。

【００７３】

選択した免疫化マウス由来の脾臓細胞およびBALB/c起源のミエローマ細胞（P3-X63-Ag 8.653 ）を約3:1 の割合で混合し、そして50% のポリエチレングリコール1500（ロッシュ・ダイアグノスティクス（Roche Diagnostics GmbH）株式会社の製品）の存在下で融合させた（ジェイ．ダブリュ．ゴッディング（J. W. Goding）：モノクローナル抗体：原理と実践、アカデミック・プレス社（Academic Press）、サンディエゴ、1996）。この融合した細胞を、10% のFCS 、ヒポキサンチン、アミノプテリンおよびチミジンを含有するRPMI-1640 培地（HAT 培地）中に懸濁して、1 ×10^6 細胞/mL のレベルとした。この懸濁液を、96- 穴のマイクロプレート（コスター（Costar）社）に、ウェル当たり200 μL の量で分配した。

【００７４】

この細胞を、CO2 インキュベーター（5%のCO2 、37℃）中でインキュベーションを行った。14日後、ハイブリドーマ細胞のスクリーニングを行った。

【００７５】

抗原として4.7%のHbA1c および16.7% のHbA1c を含有するヘモグロビンキャリブレーターでコーティングしたマイクロタイタープレートを用いるELISA 法によって、ハイブリドー

マ細胞の培養物から産生された抗体を含有する上清を検出した。120 μg/mLのキャリブレーターを、炭酸塩／重炭酸塩緩衝液（pH9.6 ）を用いて、37℃で15分間かけて、プラスチック製のマイクロタイターのウェル上に固定した。結合しなかった抗原を取り除く洗浄工程の後に、0.1%のTween20 を含有する50mMのトリス-HCl緩衝液に希釈したハイブリドーマの培養物の上清をそれぞれのウェルに添加し、そして室温で一時間反応させた。結合しなかった抗体を取り除く別の洗浄工程の後に、ヤギの抗マウス抗体と酵素である西洋ワサビペルオキシダーゼとの複合体を添加した。インキュベーション工程および三度目の洗浄工程の後に、基質である3,3,5,5-テトラメチルベンジジン（TMB ）との反応によって、結合している複合体を検出した。硫酸を用いてこの反応を停止させ、そして450nm における吸光度を測定した。グリケーションされたヘモグロビンキャリブレーターである4.7%のHbA1c および16.7% のHbA1c と反応する、強度が高くなっている陽性のウェルからの細胞だけを選択した。

【００７６】

これらの陽性細胞を、限界希釈法によって3 回クローニングした。得られたクローンであるDAK Hb1c-1を90% のFCS 、10% のDMSO中に懸濁し、そして-150℃で貯蔵した。

【００７７】

実施例４：

線状抗体エピトープマッピングとは、ポリペプチド配列またはタンパク質配列に由来する、可能性のある全てのペプチドの体系的なスクリーニングである。エピトープマッピングによって、所定の抗体に対する相互作用部位を形成するアミノ酸および誘導体の直線状の広がりに関する情報が得られる。

【００７８】

ヘモグロビンのβ－鎖のグリケーションされたエピトープについてのDAK Hb1c-1の特異性は確定されている。

【００７９】

1 ～5 アミノ酸長のペプチドを含有するペプチドライブラリーを、アミノ酸のN-末端の保護基としてFmocを用いて、固相（アビメド（Abimed）社の膜）上でC-末端からN-末端まで手動で合成した。ペプチド合成の詳細な説明については、実施例１に記載する。次の四つのペプチドを合成した：

【００８０】

フルクトシル-Hb のβ－鎖

N-末端1-デオキシフルクトシル- ヘモグロビンのβ－鎖の1 ～5 アミノ酸長のペプチドアナログ。

【００８１】

Hbのβ－鎖

N-末端ヘモグロビンのβ－鎖の1 ～5 アミノ酸長のペプチドアナログ（1-デオキシフルクトシルβ－鎖を引いたもの；基幹コントロール）。

【００８２】

Hbのα- 鎖

N-末端ヘモグロビンのα- 鎖の5 アミノ酸長のペプチドアナログ（陰性の配列コントロール）。

【００８３】

フルクトシル-Hb のα- 鎖

N 末端のバリン残基における1-デオキシフルクトシルを含むヘモグロビンのα- 鎖の5 アミノ酸長のペプチドアナログ（陰性の1-デオキシフルクトシル配列コントロール）。

【００８４】

ペプチドのスポットを伴った膜を、DAK Hb1c-1および無関係な抗体（陰性の抗体コントロール、抗トロポニンＩ、ダコ（DAKO）社、製品番号0 9528）とともにインキュベートした。その後、膜をHRP 複合化ヤギの抗マウス抗体（ダコ社、製品番号P 0447）とともにインキュベートして、3-アミノ-9- エチルカルバゾールを基質として用いて、目に見えるよう

にした。

【００８５】

【表１】

アミノ酸の数 ペプチドの名称	5	4	3	2	1
フルクトシル－Hb β－鎖	++	++	++	+	-
Hb β－鎖	-	-	-	-	-
Hb α－鎖	-	N.D.	N.D.	N.D.	N.D.
フルクトシル－Hb α－鎖	-	N.D.	N.D.	N.D.	N.D.

【００８６】

表１：抗Hb_{1c}抗体のクローンDAK Hb1c-1のエピトープマッピングのためのスポットペプチドライブラリー。抗Hb_{1c}抗体のクローンDAK Hb1c-1とのインキュベーションと、それに続く視覚化の後の、ペプチドのスポットの強度を示す。高い強度（++）、中程度の強度（＋）、陰性（－）、測定せず（N.D.）。

【００８７】

DAK Hb1c-1は、1-デオキシフルクトシル・ヘモグロビンβ－鎖の二量体とは穏やかに反応し、そして1-デオキシフルクトシル・ヘモグロビンβ－鎖三量体～五量体とは強く反応する（表１を参照すること）。DAK Hb1c-1は、1-デオキシフルクトシル- バリンそのものとは反応しない。DAK Hb1c-1は、ヘモグロビンβ－鎖のフルクトシル化されていないペプチド、1-デオキシフルクトシル・ヘモグロビンα- 鎖五量体およびヘモグロビンα- 鎖五量体とは反応しない。DAK Hb1c-1とは対照的に、陰性の抗体コントロールは、いずれのペプチドとも反応しなかった。

【００８８】

要約すれば、ヘモグロビンβ－鎖の1-デオキシフルクトシル化N-末端部分についての、抗Hb1c抗体のクローンDAK Hb1c-1の特異性が実証された。

【００８９】

実施例５：

全血サンプル、赤血球の溶血物またはキャリブレーター由来のペプチド、タンパク質を、96- 穴のマイクロタイタープレートの表面に結合させた。結合しなかったタンパク質を取り除く洗浄工程に続いて、モノクローナル抗体であるDAK Hb1c-1を添加した。インキュベーションの後に、結合しなかった抗体を洗い落とし、そしてヤギの抗マウス抗体と酵素である西洋ワサビペルオキシダーゼとの複合体を添加した。別の洗浄工程の後に、基質である3,3,5,5-テトラメチルベンジジン（TMB ）との反応によって、結合している複合体を検出した。酸を添加してこの反応を停止させ、分光測光的に読み取られる比色定量の終点を得た。

【００９０】

実施例６：

1-デオキシフルクトシルをヘモグロビンβ－鎖のアミノ末端に結合させなかった点を除いて、HbA_0はHbA_{1c} と同じである。

【００９１】

DAK Hb1c-1の、HbA_0ならびにHbC_0の17マーのペプチドおよびHbS_0の17マーのペプチドとの反応を、上記のELISA によって分析した。

【００９２】

HbA_0またはHbC_0の17マーのペプチドもしくはHbS_0の17マーのペプチドのいずれかでコーティングしたマイクロタイタープレートにDAK Hb1c-1を添加した場合、測定される値はバックグラウンドの値よりも高くはなかった。

【００９３】

結論としては、DAK Hb1c-1は、HbA_0、HbC_0またはHbS_0との反応性を示さなかった。

【００９４】

実施例７：

DAK Hb1c-1と天然型HbA_{1c}との溶液中での反応を、競合的ELISA法で分析した。溶液中で、DAK Hb1c-1抗体と一緒にHbA_{1c}キャリブレーターのプレインキュベーションを行い、何らかの抗原-抗体複合体が形成されるかどうかを試験した。次いで、抗原-抗体の溶液をマイクロタイターのウェルに移した。ここには同一の抗原を固相上に吸着させた（固定化抗原）。溶液中の抗原は過剰に存在した。溶液中で抗原と複合体を形成していない抗体は、この固定化抗原と結合することが可能であった。上記の二次抗体によって、結合しているDAK Hb1c-1抗体を検出した。コントロールとして、溶液中でHbA1cキャリブレーターとのプレインキュベーションを行っていない同一量の抗体を、マイクロタイタープレートの１つのレーンに添加し、続いて上記のプロトコールに付した。

【００９５】

溶液中でHbA_{1c}と一緒にDAK Hb1c-1抗体のプレインキュベーションを行っても、何らの効果も見られなかったことから、DAK Hb1c-1は、溶液中で天然型HbA_{1c}とは結合しないとの結論を出した。

【００９６】

実施例８：

DAK Hb1c-1およびHEM13の、ヘモグロビンの構造バリアントを含有するヒトの血液との反応性を、等電点電気泳動（IEF）とそれに続くイムノブロットによって分析した。

【００９７】

ヘモグロビンの構造バリアントを分離する目的で、IEFポリアクリルアミドゲル（ファストゲル（PhastGel）IEF-5-8、ファルマシア社）を用いるファルマシア社のファストシステム（Pharmacia's PhastSystem）によって、HbCA、HbSSおよびHbAAを含有する赤血球の溶血物を電気泳動的に分離した。次いで、このゲルを押し付けて、PVDF膜上へのタンパク質のブロッティングを行い、そして、ブロッキング剤を用いて非特異的染色をブロッキングした。次いで、DAK Hb1c-1（図１Ａを参照すること）およびHEM13（図１Ｂを参照すること）のそれぞれと一緒に、この膜のインキュベーションを行った。HRP-複合化ヤギの抗マウス免疫グロブリン（ダコA/S社、製品番号P 0447）を二次抗体として用いた。最後に、ダコ社の液体DAB+基質-色素原システム（liquid DAB+ Substrate-Chromogen System）（ダコA/S社、製品番号K 3468）を用いて、HRP活性を目に見えるようにした。

【００９８】

ホモ接合型HbSSを持つ、糖尿病ではない患者からの赤血球の溶血物を、モノクローナル抗体であるクローンDAK Hb1c-1を用いて分析して、強度が高く明確なHbS_{1c}のバンドを同定した（図１Ａを参照すること）。対照的に、ホモ接合型HbSSを持つ患者からの赤血球の溶血物をHEM13を用いて分析すると、HbS_{1c}がわずかに染色されることが分かった（図１Ｂを参照すること）。

【００９９】

ヘテロ接合型ヘモグロビンCAを持つ、糖尿病ではない個体からの赤血球の溶血物を、DAK Hb1cおよびHEM 13を用いて分析すると、HbC_{1c}のそれぞれ高強度のバンドおよび中程度の強度のバンドが目に見えるように現れた。DAK Hb1cおよびHEM13の両者については、ほぼ同じ強度の強いHbA_{1c}のバンドが観察された。

【０１００】

コントロールとして、ヘモグロビンAを持つ糖尿病患者からの赤血球の溶血物のプールについても試験を行った。CおよびSとは関係のない、ヘモグロビンの他のバリアントがこの患者のプールの一部であってもよい。DAK Hb1c-1およびHEM 13の両者について、ほぼ同一の強さのHbA1cのバンドが現れるまで、基質と一緒にそれぞれの膜のインキュベーションを行った。

【０１０１】

最終的に、我々は、我々のpan特異的モノクローナルグリケーションされたヘモグロビン

抗体であるクローンDAK Hb1c-1が、ヒトの血液サンプル由来のHbA_{1c}、HbS_{1c}およびHbC_{1c}と反応することを実証した。

【０１０２】

実施例９：

成熟ヘモグロビンは四量体タンパク質であり、二つのα-サブユニットおよび二つのβ－サブユニット、$\alpha_2\ \beta_2$からなる。Hbの構造バリアントは、ペプチドの一次構造中に、遺伝的に決められた変異を有する。A、SおよびCは、β－鎖のN-末端から６番目のアミノ酸残基だけが異なっている：

【０１０３】

【化１】

HbA_{1c} fructosyl-V-H-L-T-P-E-E-K-T-A-V-N-A-L-W-G-K.....

HbC_{1c} fructosyl-V-H-L-T-P-K-E-K-T-A-V-N-A-L-W-G-K.....

HbS_{1c} fructosyl-V-H-L-T-P-V-E-K-T-A-V-N-A-L-W-G-K.....

【０１０４】

抗体がpan 特異的であるか、すなわち抗体がグリケーションされたヘモグロビンの全ての構造バリアントと反応するかどうかを試験するために、我々は、DAK Hb1c-1抗体の、グリケーションされたヘモグロビンA、CおよびSのβ－サブユニットのN-末端に対応する三種類の17マーのグリケーションされたペプチドとの反応について、ELISA 法によって試験した（図２）。これらのペプチド配列を上記に示す。コントロールとして、ヘモグロビンA を持つ糖尿病患者からの赤血球の溶血物のプールについても、DAK Hb1c-1のELISA 法により試験をした。

【０１０５】

精製したヘモグロビンA1c を用いてマウスを免疫化することによって、モノクローナルHbA_{1c}抗体のクローンHEM13 を惹起した。我々は、DAK Hb1c-1およびHbA_{1c}抗体クローンHEM13 の反応と、グリケーションされたヘモグロビンペプチドおよび赤血球抽出物であるヘモグロビンA とを比較した。

【０１０６】

DAK Hb1c-1のグリケーションされたヘモグロビンペプチドに対する親和性は、滴定曲線を比較することによって判断されるように、同じペプチドに対するHEM13 の親和性よりも明らかに強い（図２および図３を参照すること）。

【０１０７】

DAK Hb1c-1は三種類のペプチドの全てについて同じように反応する（図１）のに対して、HEM13 はA1c、S1cおよびC1c ペプチドについて異なる反応性を示す（図３）。これらの結果は、DAK Hb1c-1抗体がpan 特異的であることを実証する。

【０１０８】

この実施例では、グリケーションされたヘモグロビンA、CおよびSの構造バリアントのβ－鎖の全ての最初の4 個のアミノ酸に１つの無関係なアミノ酸（ここではシステイン）を加えたものに対応するところの5 マーのグリケーションされたペプチドを注射することによって、グリケーションされたヘモグロビンの全ての構造バリアントについて同じように反応する抗体を惹起することが可能であることを実証する。

【０１０９】

実施例１０：

「ERL 教育プログラム（ERL Educational Programme ）」からの血液サンプルを試験した。陽イオン交換クロマトグラフィーによって、それぞれのサンプルにおけるHbA_{1c}のパーセンテージを測定した（表２中のIFCC/DCCT-値；IFCC：国際臨床化学連合（International Federation of Clinical Chemistry）；DCCT：糖尿病のコントロールと合併症のトライアル）。ヘテロ接合型のサンプルは二つの異なるヘモグロビンβ－サブユニット、たとえばASまたはACから構成されるのに対して、ホモ接合型の血液サンプルは二つの同一のβ－サブユニット、たとえばAAまたはSSから構成される。

【０１１０】
【表２】

	IFCC	DCCT	DAK-Hb1c-1
HbAA	5.0	6.0	5.4
HbAA	5.7	6.5	6.2
HbAA	6.3	7.0	6.5
HbAA	7.0	7.6	7.9
HbAA	7.5	8.0	8.5
HbAA	8.1	8.4	8.7
HbAA	8.8	8.9	9.4
HbAA	9.4	9.4	9.7
HbAA	10.0	9.8	10.0
HbAS	4.3	5.5	4.7
HbAC	4.3	5.5	4.0

表２： 血液サンプル中のHb1cのパーセンテージ。血液サンプルACおよびASは、ヘテロ接合型ヘモグロビンを含む。

【０１１１】
上記のELISA 法の形式にて、DAK Hb1c-1を用いて、全血サンプル中のHbA1c のパーセンテージを4-10% の範囲内で測定した。IFCCおよびDCCTによって推奨されている標準的な方法によって測定された値と同じレベルでのHbA1c のパーセンテージを得た。
【０１１２】
要約すれば、当該抗体は、HbA1c 、 HbC1cおよび HbS1cに対して正確な特異性を有することが実証される。
【０１１３】
実施例１１：
二つのミクロスフェアによる比濁法によって、グリケーションされたヘモグロビンレベルを測定した。この準備において、グリケーションされたヘモグロビンを非共有結合的にミクロスフェアに吸着させ、次いで、ミクロスフェアに共有結合したグリケーションされたヘモグロビン抗体を伴う抗原を検出した。免疫ミクロスフェアと複合化した抗原ミクロスフェアを構成する、得られるネットワークの濁度を測定した。
【０１１４】

出願人又は代理人の書類記号 P062 WO 01	国際出願番号 RO/DK 25 APR 2002

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寄託機関のあて名（郵便番号及び国名も記載）
Mascheroder Weg 1b
D-38124 Braunschweig

寄託の日付	アクセッション番号
6 March 2001	DSM ACC2495

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【図面の簡単な説明】
【０１１５】
【図１】
【図２】
【図３】

【図１】

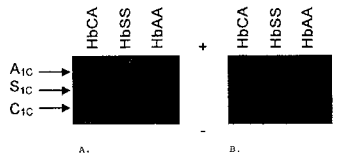

Figure 1

【図２】

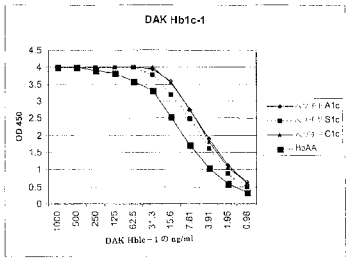

Figure2：ELISA試験により測定される、DAK Hb1c-1と、ヘモグロビンAA（HbAA）を持つ患者からの赤血球の溶血物ならびにヘモグロビンA1c、C1cおよびS1cに対応するIT-マー修飾ヘプチドとの反応性

【図３】

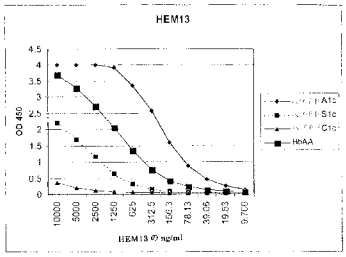

Figure3：ELISA試験により測定される、HEM13と、ヘモグロビンAA（HbAA）を持つ患者からの赤血球の溶血物ならびにヘモグロビンA1c、C1cおよびS1cに対応するIT-マー修飾ヘプチドとの反応性

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(54) **Title:** PAN-SPECIFIC MONOCLONAL ANTIBODY

(57) **Abstract:** The invention describes pan-specific monoclonal antibodies for the determination of the amount of all structural variants of glycated hemoglobin in human blood samples. The determination of the rate of glycation of hemoglobin in an individual's blood provides a useful index of glucose level control in diabetics.

Pan-specific monoclonal antibody

This invention relates to the determination of the amount of all structural variants of glycated hemoglobin in human blood samples. The determination of the rate of glycation of hemoglobin in an individual's blood provides a useful index of glucose level control in diabetics.

In particular, the present invention concerns the preparation of monoclonal antibodies or a fragment thereof, which recognize specifically the glycated N-terminal peptide residue in such human hemoglobin.

Patients afflicted with diabetes are incapable of metabolizing glucose in a conventional manner resulting in a build-up of glucose in their blood and urine. Conventionally, the glucose level in such body fluids is taken as a measure of the state of the diabetic condition, which in turn is used as a guide for the amount of insulin or other agent to be taken or of the need to change the patient's diet.

This works moderately well except that the glucose level may fluctuate widely in dependence on time and content of the last meal, the last insulin injection, and the like. Thus, the reading will reflect an instantaneous condition, which may not truly identify the longer-term state of the diabetic condition.

It is known that another effect of the diabetic condition is an increase in the amount of glycated hemoglobin in the blood of the diabetic. Hemoglobin (Hb) is a protein tetramer consisting of four chains (subunits) of amino acids, each of about 143 units and having a total

molecular weight of approximately 64,000 g/mol. The N-terminal valine of the β-subunit in hemoglobin can react with glucose. The glycation of hemoglobin occurs by a non-enzymatic reaction involving glucose and the alpha-amino group of valine. Following a Schiff base formation between the reactants, the glucose undergoes an Amadori rearrangement forming 1-deoxyfructo-valine. The final complex binding between 1-deoxyfructose and valine is covalent and irreversible. The glycation reaction is governed by the concentration of the reactants, e.g., hemoglobin and glucose. The Diabetes Control and Complication Trial (DCCT) reference interval for non-diabetic individuals is approximately 4-6% glycated hemoglobin. Hemoglobin tetramers with a 1-deoxyfructo-valine on the N-terminus of a β-chain are identified as being glycated hemoglobin; e.g. Hb1c or HbA1c, HbC1c or HbS1c.

HbC trait has a prevalence of 30% in parts of sub-Saharan and 2.3% among African Americans. HbS trait, the most commonly variant of Hb in US, has a prevalence of 7.8% of African Americans. All together, at least 10% of black Americans have either HbC or HbS trait. The world population is getting more and more mixed with respect to race. Therefore, it is increasingly important to have an antibody that reacts with all structural variants of glycated hemoglobin in the same manner. If the available antibody do not react with all structural variants of glycated hemoglobin, Hb1c percent measurements from patients with traits of HbS and/or HbC will be incorrect.

Glucose levels in diabetics are sufficiently high to increase the rate of glycation depending directly on the glucose level in the blood, which reflects the severity

of the diabetic condition. Therefore, the hemoglobin A1c level is raised to about 6 to 12%. Since the circulating life span of hemoglobin A is about 120 days, a glycated hemoglobin measurement will give a value, which reflects an average glucose level for that period. Notably a meal high in glucose will not be reflected in instantly high levels of glycated hemoglobin or serum albumin. Thus, measurement of the glycated-hemoglobin content gives a truer picture of the average circulating glucose levels and thus a truer picture of the long-term condition of the patient.

A common method used to determine the level of glycated hemoglobin involves passing a lysed blood sample through a boronate affinity column. The method determines the total glycated hemoglobin, including HbA_{1c} and ketoamine structures formed on lysine and N-terminal valine residues of both the α- and β-chains of hemoglobin. The column is washed and the glycated hemoglobin determined spectrophotometrically.

Microparticle enhanced turbidimetric immunoassay techniques are available for automatic analyses by instruments. These instruments are not dedicated to a certain diagnostic assay and have a very high sample cycle time. Currently, the utility of this technique for determination of glycated hemoglobin is growing as rutine clinical analysis. Some of these immunoassays are based on the principle of microparticle enhanced competitive turbidimetric inhibition immunoassay techniques.

US Patent no. 4,247,533 discloses an analytical technique wherein antibodies to HbAlc were reportedly raised in a sheep by injection of HbAlc and absorbed with nonglycated

hemoglobin to provide polyclonal antibodies, which distinguished between HbAlc and nonglycated hemoglobin. Such antibodies then form the basis for a test to determine the proportion of glycated hemoglobin in a sample.

In the prior art only the hemoglobin A_{1c} antibody and how the antibody is able to react with the A variant of glycated hemoglobin is described. The influence of the other variants of glycated hemoglobin is not mentioned and a monoclonal antibody against all structural variants of glycated-hemoglobin has neither been considered. Due to the fact that the antibody does not recognise HbS1c and HbC1c, immunoassay measurement of glycated-hemoglobin percentages in subjects with HbS1c and HbC1c using that antibody will lead to truly decreased percentages of glycated hemoglobin.

An Hb1c antibody, which is specific for all structural variants of Hb1c, is considered as pan specific.

Accordingly, there remains a need for an antibody of the above-mentioned kind, which does not exhibit the above-identified drawbacks.

It is accordingly an objective of the present invention to provide an antibody, which is pan-specific, i.e. specific for all structural variants of glycated hemoglobin.

It is also the objective to provide an antibody for determining the long-term blood sugar level of a patient based on an immunoassay determination of HbAlc, HbC1c and HbS1c.

It is another objective of the invention to provide such an antibody for determining the content of glycated hemoglobin, HbAlc, HbC1c and HbS1c, in a patient's whole blood sample.

These and the other objects are achieved by the invention as defined in the claims.

The invention relates to a monoclonal antibody or a fragment thereof, which is pan-specific, i.e. able to react with all structural variants of glycated-hemoglobin. The structural variants of glycated-hemoglobin are preferably HbAlc, HbC1c and HbS1c.

The monoclonal antibody preferably leads to improvement of existing immunoassay with respect to measurements of the level of glycated hemoglobin in patients with HbS and HbC.

The monoclonal antibody of the present invention is characterized in that the antibody comprises an antibody combining site, which binds specifically to the glycated N-terminal peptide sequence of human hemoglobin A, C and S ß-chains.

The monoclonal antibody reagent of the present invention is characterized by its specific binding affinity for the linear epitope in glycated hemoglobin. Therefore, as used herein the term "antibody reagent" or "antibody combining site" will refer to any material however obtained which comprises a monoclonal antibody combining site specific for such peptide epitope. Such term therefore includes whole antibodies as well as appropriate fragments or

polyfunctionalized forms thereof. When in the form of whole antibody, it can belong to any of the classes and subclasses of known immunoglobulins, e.g. IgG, IgM and so forth. Any fragment of any such immunoglobulins which retains specific binding affinity for the peptide epitope can also be employed, for instance, the fragments of IgG conventionally known as Fab, Fab' and $F(ab')_2$. In addition, aggregates, polymers, derivates, conjugates, and hybrids of immunoglobulins, or their fragments can be used where appropriate. Furthermore, single chain antibodies or any other fragment obtained by recombinant means can be used where appropriate.

The glycated residue is the distinguishing structural feature of HbAlc, HbSlc and HbClc. An antibody of the present invention requires an epitope or determinant site comprising minimally the 1-deoxyfructosyl carbohydrate unit, formed upon Amadori rearrangement of the reaction product between glucose and the terminal amine, and a peptide sequence extending therefrom comprising at least one of the amino acid units of the HbAlc, HbSlc and HbClc N-terminal sequence in the position corresponding to the native HbAlc, HbSlc and HbClc sequence. The other amino acid units in the peptide sequence characterizing the epitope may be the same or different as those appearing in the native HbAlc, HbSlc and HbClc sequence. In this way, the epitope is characterized by at least two contact or binding sites with the antibody, which sites are unique to the glycated N-terminal HbAlc, HbSlc and HbClc sequence.

The antibody will preferably specifically bind a glycated peptide residue of the formula:

Glycosyl-(NH)-Val-His- or Glycosyl-(NH)-Val-His-AA,

wherein Glycosyl-(NH)-Val represents a non-enzymatically glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin and AA means one or more additional amino acid residues.

In a preferred embodiment AA is a sequence of from 1 to 3 amino acids corresponding to a part of the N-terminal β-chain of all structural variants of human hemoglobin. AA is preferably -Leu-Aa; -Leu-Thr-Aa; -Leu-Thr-Pro-Aa, and Aa designates the remaining number of amino acids in the AA group.

A preferred monoclonal antibody or a fragment thereof according to the invention is obtainable using the hybridoma DAK Hb1c-1, which is deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) as accession no. DSM ACC2495.

The invention also relates to a method of producing a monoclonal antibody or a fragment thereof, wherein the antibody is pan-specific, i.e. able to react with all structural variants of glycated hemoglobin.

The antibody or a fragment thereof may preferably be obtainable by the method as defined in claims.

The method of producing a monoclonal antibody or a fragment thereof according to the invention comprising the step of raising an antibody against an immunogen comprising a glycated peptide chemically linked to an immunogenic carrier material, the glycated peptide having

from 2 to 5 amino acid units corresponding to the N-terminal in the β-chain of human hemoglobin.
The immunogen is preferably of the formula:

[Fructosyl-(NH)-Val-His-AA-R]$_n$-Carrier

wherein Fructosyl-(NH)-Val represents a nonenzymatic glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin, AA means one or more additional amino acid residues, R is a linking group, Carrier is an immunogenic carrier material, and n is an integer from 1 to the number of available coupling sites on said carrier.

In a preferred embodiment AA is one or more additional amino acid residues. In another preferred embodiment AA is a sequence of from 1 to 3 amino acids corresponding to the N-terminal part of the β-chain of all structural variants of human hemoglobin.

Linking group R can be essentially any convenient and stable structure. Such linking group R will usually be in the form of an aliphatic chain comprising between 1 and approximately 20 atoms, excluding hydrogen, and including heteroatoms such as nitrogen, oxygen, and sulfur. The glycated residue can be joined through a variety of groups to form linking chain R, including methylene, ether, thioether, imino, and the like. One skilled in the art will have a wide variety of linking groups from which to choose to prepare the immunogen.

In a preferred embodiment the carrier is an immunogenic protein or peptide other than human hemoglobin.

The immunogen used to stimulate production of appropriate immunoglobulins in the most general sense will comprise one or more of the glycated peptide residues chemically linked to an immunogenic carrier material. The immunogenic carrier material can be selected from any of those conventionally known having functional groups available for coupling to the glycated peptide residue. In most cases, the carrier is a protein or polypeptide, although other materials such as carbohydrates, polysaccharides, lipopolysaccharides, nucleic acids, and the like of sufficient size and immunogenicity can likewise be used. For the most part, immunogenic proteins and polypeptides will have molecular weights between 4,000 g/mol and 10,000,000 g/mol, preferably greater than 15,000 g/mol, and more usually greater than 50,000 g/mol. Generally, proteins taken from one animal species will be immunogenic when introduced into the blood stream of another species. Particularly useful proteins are albumins, globulins, enzymes, hemocyanins, glutelins, proteins having significant nonproteinaceous constituents, and the like.

In a preferred embodiment the glycated N-terminal peptide residue of the native HbAlc, HbSlc and HbClc molecule is made accessible to the monoclonal antibody or a fragment thereof of the present invention by appropriate denaturation or digestion of the protein in the sample to be assayed.

In another preferred embodiment the glycated fragment can be produced by chemical or enzymatic digestion of naturally occurring glycated hemoglobin, e.g. HbAlc, HbSlc and HbClc. This fragment can be coupled to a carrier using classical coupling procedures, e.g.,

glutaraldehyde or carbodiimide, and the conjugate used as an immunogen.

The antibody or a fragment thereof according to the invention may preferably be produced by the following step:

A. Preparation of peptide immunogen

To obtain antibodies against the glycation site of N-terminal valine residue on the β-chain of Hb A, S and C a short peptide (pentamer) is preferably used as an immunogen. The antigen may preferably be Hb1c 4-mer plus cysteine, i.e. fructosyl-V-H-L-T-C.

B. Immunization of mize with human Hb1c peptides

The general method used to develop hybridomas secreting monoclonal antibodies is well known to a person skilled in the art.

An illustration of the techniques utilized in the practice is described in Journal of Immunological Methods, 1980, 39:285.

Female F1 hybrids of CF1 x BALB/c mice may preferably be used, but mice of other strains may also be used. The immunization plan and the concentration of peptide-carrier complex may be selected to form a sufficient amount of antigenically stimulated lymphocytes. In a preferred embodiment mice are immunized intraperitoneally with 50 micrograms of purified protein derivates (PPD, a

partly unknown mixture of denatured proteins from *M. tuberculosis*) in complex with the Hb1c peptide four times at 2-week intervals. 3-5 days after the final immunization, the spleen cells are extracted from the animal for fusion.

Monoclonal antibodies produced by hybridomas from other species may also preferably be used.

C. Cell fusion

The spleen is aseptically taken out from the immunized mouse, and a suspension of the spleen cells is prepared. The spleen cells are fused with mouse myeloma cells from a suitable cell line in the presence of a suitable fusion promotor. In a preferred embodiment the preferred ratio of the spleen cells to the myeloma cells is from about 20:1 to about 2:1, and a fusion medium is suitably used in an amount of 0.5 to 1.5 ml per about 10^8 spleen cells.

The mouse myeloma cells used for cell fusion are well known. P3-X63-Ag 8.653 may preferably be used as a mouse myeloma cell.

The fusion promoter is preferably polyethylene glycol having an average molecular weight of 1000 to 4000 g/mol. Other fusion promoters known in the art can also be used. In a preferred embodiment polyethylene glycol having an average molecular weight of 1500 may be used.

D. Screening of the fused cells

In a separate container (such as a microtiter plate), a mixture composed of the unfused spleen cells, unfused mouse myeloma cells and the fused hybridoma cells is preferably diluted with a selective medium not supporting the unfused mouse myeloma cells and cultured for a period sufficient to kill the unfused mouse myeloma cells (about 1 week). A culture medium having drug resistance (for example, resistance to 8-azaguanine) and not supporting the unfused mouse myeloma cells, such as HAT medium, is used. In the selective medium, the unfused myeloma cells die. Since the unfused spleen cells are untransformed cells, they die away after a certain period of time (1 week). The fused cells, on the other hand, can survive in the selective medium since they have both the tumoral nature of the parent myeloma cells and the nature of the parent spleen cells.

E. Determination of glycated hemoglobin by ELISA

The determination is preferably based on the principle of specific immunologic recognition and reaction between a monoclonal antibody and the antigenic epitope to which the antibody uniquely and specifically binds. The recognition and binding may be detected, for example, by an ELISA test, wherein blood samples or calibrators are immobilized on a solid phase support, such as the bottom of a plastic well. By immobilizing human hemoglobin the antigenic epitope may be exposed for antibody binding. The antibody, secondary enzyme-labeled antibody and enzyme substrate may interact with the immobilized antigen (human hemoglobin) thereby antibody-antigen

complexes are preferably formed. A number of dilution, incubation and washing steps, then allow separation of bound and free reagents. A colour forming reaction takes place as a result of binding of the antibody to antigen and the consequent reaction of the enzyme upon its substrate interaction. The formation of colour indicates the presence of Hb1c in the blood sample, and the intensity of the colour provides a quantitative measure of the amount of glycated epitope in the sample. The antibody of the present invention may be used to measure glycated hemoglobin in other immunological assay formats known in the art.

F. Cloning of the hybridoma cells producing the desired antibody and the production of the antibody

Hybridoma cells capable of producing the desired antibody are cloned by a suitable method such as limiting dilution method, and the desired antibody can be produced by the following method. Hybridoma cells are cultured in a suitable medium for a certain period of time, and the monoclonal antibody produced by the hybridoma cells can be isolated from the supernatant of the culture.

The invention also relates to a method of using a monoclonal antibody or a fragment thereof.

The antibodies or a fragment thereof according to the invention can preferably be used in conventional manner to react with blood samples containing unknown quantities of glycated hemoglobin and the extent of reaction can be compared with calibrated standards to determine the

extent of glycation. The read-out can be by fluorescence, by immunoassay, or the like, by joining suitably readable groups to the monoclonal antibodies in known manner without loss of their binding power for the glycated epitope in HbAlc, HbSlc and HbClc.

The monoclonal antibodies of the present invention are specific for binding to all structural variants of glycated N-terminal peptide residue preferable found in HbA, HbS and HbC. The antibodies are able to bind to the epitope in the native HbAlc, HbSlc and HbClc molecule when the epitope is appropriately exposed. Steric access to the epitope can be obtained in any effective manner. Exposure of the epitope in the intact protein is understood to be accomplished by a physical or chemical denaturation or digestion at least in the region of the epitope. Such denaturation or digestion can be localized to the region of the epitope or can involve a more general, or even substantially complete denaturation of the tertiary, and additionally the secondary structure of the protein, or partial or complete digestion of the protein.

In a preferred embodiment the antibody binds specifically to the glycated N-terminal peptide sequence in the β-chain of human hemoglobin Alc, Clc and Slc after having been exposed sufficiently to provide steric access thereto.

Denaturation can be accomplished in a variety of ways including conventional treatment of the protein by physical means such as heat, sonication, high or low pH and, as is preferable, chemical denaturation by interaction with an agent or chaotrope in solution.

Protein denaturation can be most effectively accomplished if combinations of chemical and/or chemical and physical means are used, e.g. guanidine and heat, guanidine and SDS, or guanidine and dithiothreitol. Particularly strong chaotropes such as guanidine are preferred. Of course, denaturing conditions which result in substantial insolubilization, aggregation, or precipitation of the protein implying an insignificant amount of the exposed epitope is accessible to the solution for antibody binding will be avoided. A sufficient amount of the denatured protein must remain in solution or suspension in order to obtain useful immunobinding. The extent of solubilization necessary will depend upon the circumstances of the intended or desired binding.

In a preferred embodiment the glycated peptide sequence is exposed to the antibodybinding site by physical or chemical denaturation or digestion.

In another preferred embodiment the glycated peptide sequence is exposed to the antibody binding site by denaturation with a chaotropic agent. The chaotropic agents include, without limitation, guanidine, urea, and various detergents such as sodium dodecylsulfate (SDS) and others, without limitation, including deoxycholoate and certain bile salts, 3-(3-cholamidopropyl)-dimethyl-ammonio-1-propanesulfonate, organic solvents such as methanol, propanol, acetonitrile and certain salts such as potassium thiocyanate. Non-ionic detergents such as Triton X®, Tween®, nonidet NP-40®, and octyl-glucosides can also function as protein denaturants.

A significant amount of HbAlc, HbSlc and HbClc in a particular blood sample can be denatured to expose the

glycated epitope for antibody binding by combining the sample, e.g., whole blood or red cell hemolysate, with an aqueous solution of the chaotrope present at sufficient concentration to denature any HbAlc, HbSlc and HbClc in the resulting aqueous mixture. Where whole blood is the sample, the chaotrope also serves to lyse red blood cells to release hemoglobin and to inactivate proteases.

The denaturation process may be significantly accelerated by heating the mixture for a short period of time. It has been found that at temperatures below 37°C , denaturation by the chaotrope can take from one to several hours, whereas at temperatures above 50°C sufficient denaturation can be attained in a minute or less. In order to prevent significant denaturation of the antibody and other proteinaceous reagents to be subsequently added to the mixture, the sample-chaotrope mixture will normally be diluted as a separate step or by addition of reagent solutions to a level that the chaotrope is substantially ineffective to denature such reagents, yet will preserve the exposure of the epitope by preventing significant renaturation of HbAlc, HbSlc and HbClc.

In a preferred embodiment the antibody binds specifically to a glycated N-terminal peptide sequence in the β-chain of glycated human hemoglobin, which has been adsorbed to a solid phase, wherein the solid phase preferably is selected from the group consisting of polystyrene, divinylbenzene, butadiene, polycarbonate, polyacrylamide, polyacrylic acid, polyacryl amide, polyethylene, polypropylene, fluorinated polymers, poly amides, or co-block polymers hereof, gold, carbon, cellulose, derivatized cellulose, or glass.

In a preferred embodiment the monoclonal antibody or a fragment thereof may be used for the determination of the level of glycated hemoglobin in human blood. The monoclonal antibody or a fragment thereof may also preferably be used for the determination of the level of glycated HbS and/or HbC in human blood.

In a further preferred embodiment the monoclonal antibody or a fragment thereof may be used for the determination of the level of glycated heterozygous HbA, HbS and/or HbC in human blood.

In a further preferred embodiment there is provided a turbidimetric two-microsphere method for determination of the level of glycated hemoglobin. This method comprises mixing a sample with microspheres and microspheres on which the monoclonal antibody or a fragment thereof is bound; the latter is referred to as immunoparticles, or with microspheres and subsequently with immunomicrospheres. The resulting immunoagglutinates is measured turbidimetrically.

The microspheres are preferably selected from the group consisting of polystyrene, divinylbenzene, butadiene, polycarbonate, polyacrylamide, polyacrylic acid, polyacryl amide, polyethylene, polypropylene, fluorinated polymers, poly amides, or co-block polymers hereof, gold, or silica.

Microspheres to be used in the method of the present example have a particle size of from 0.01 to 5 micro meters, which are used by dispersing in a usually used buffer solution.

The following experimental non-limiting examples are intended to illustrate certain features and embodiments of the invention.

Example 1:

The 1-deoxyfructosyl-Val-His-AA peptide was synthesized using the solid-phase method of Sheppard and Atherton as described in international patent application no. WO 86/03494. The peptide was synthesized with fluorenylmethoxycarbonyl (Fmoc) amino acids, using PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphorium hexafluorophosphate) and HOBt (N-hydroxy-benzotriazole) as coupling reagents.

Cysteine and histidin were used with trityl (trt), and threonin with tertiary butyl (tBu) side-chain protection. Leucin was used without side-chain protection.

The fructose amino acid conjugate, N-(deoxy-D-fructos-1-yl)-L-valine, was synthesized by modifications of known methods. In short, Di-isopropyliden protected glucose was activated as the trifluate and conjugated to the benzyl ester of valine. The resulting 2,3:4,5-Di-*O*-isopropyliden-N-(deoxy-D-fructos-1-yl)-L-valine-benzyl ester was hydrogenated over Pd/C in ethanol, lyophilized, and finally used in the peptide synthesis without Fmoc protection. The purity of the product was verified by TLC analysis and ^{1}H or ^{13}C NMR spectra.

The peptide was synthesized by automated solid phase synthesis (Crystal instrument from Calbiochem-Novabiochem Ltd) cleaved from the resin with TFA (trifluoroacetic acid) – TES (triethylsilan) – Glycerol – H_2O (90% : 4% :

3% : 3%), precipitated and washed with methyl-tertiary-butyl-ether, centrifuged and lyophilised.

Purification was performed by HPLC (C_{18} reversed-phase column, using a step-wise gradient of typically 5-25% MeCN in 0.1% TFA). The identity of the peptide was verified by MALDI-TOF mass spectroscopy and the purity was finally assured by an analytical HPLC analysis to be better than 90%.

Example 2:

Four weeks before first immunization Bacille Calmette Guérin (BCG) vaccine was injected to prime the immune response in the mice as described in Giba Foundation Symposium, 1986, 119: 25.

To make the peptide immunogen it was conjugated to a carrier mixture, Tuberculin PPD. Peptide and carrier (PPD) were conjugated with glutardialdehyde, i.e. CHO-$(CH2)_3$-CHO. Glutardialdehyde reacts with amino-groups on the peptide and the carrier.

The immunogens thus have the following formula:

Fructosyl-Val-His-Leu-Thr-Cys-CH-$(CH2)_3$-CH-PPD

Example 3:

Female F1 hybrids of CF1 x BALB/c mice (12 weeks old) primed with BCG-vaccine were immunized with the peptide-PPD complex described above. Mice were immunized four times intraperitoneally with a mixture of 50 micrograms

of peptide-PPD complex dissolved in phosphate buffered saline (PBS) and aluminium hydroxide (1 mg/dose). A mouse with high titer of antibodies against glycated hemoglobin was selected for fusion. Three days after the final immunization, spleen cells from the immunized mouse was used for cell fusion.

Spleen cells from the selected immunized mouse and myeloma cells of BALB/c origin (P3-X63-Ag 8.653) were mixed in a ratio of about 3:1 and fused in the presence of 50% polyethylene glycol 1500 (a product of Roche Diagnostics GmbH)(J. W. Goding: Monoclonal Antibodies: Principle and Practice, Academic Press, San Diego, 1996). The fused cells were suspended in RPMI-1640 medium, containing 10% FCS, hypoxanthine, aminopterin and thymidine (HAT media) to give a concentration of 1×10^6 cells/mL. The suspension was distributed to 96-well microplates (Costar) in an amount of 200 μL per well.

The cells were incubated in a CO2 incubator (5% CO2, 37°C). After 14 days hybridoma cells were screened.

Supernatants containing antibodies produced by the hybridoma cell cultures were detected by an ELISA technique using microtiter plates coated with hemoglobin calibrator containing 4.7% HbA1c and 16.7% HbA1c as antigens. 120 μg/mL of the calibrators were immobilized onto plastic microtiter wells using a carbonate/bicarbonate buffer (pH 9.6) for 15 minutes at 37°C. After a washing step that removes unbound antigen the hybridoma culture supernatants diluted in 50 mM Tris-HCl buffer containing 0.1% Tween 20 were added to each well and allowed to react for one hour at room temperature. After another washing step to remove unbound

antibody a conjugate of a goat anti-mouse antibody and the enzyme horseradish peroxidase was added. After incubation and a third washing step the bound conjugate was detected by reaction with the substrate 3,3,5,5-tetramethylbenzidine (TMB). The reaction was stopped with sulphuric acid and the absorbance at 450 nm was measured. Only cells from positive wells reacting with glycated hemoglobin calibrators, 4.7% HbA1c and 16.7% HbA1c, with increasing intensity were selected.

These positive cells were cloned three times by limiting dilution method. The resulting clone, DAK Hb1c-1, was suspended in 90% FCS; 10% DMSO and stored at -150°C.

Example 4:

A linear antibody epitope mapping is the systematic screening of all possible peptides derived from a polypeptide or protein sequence. Epitope mapping yields information on the linear stretch of amino acids and derivatives that form an interaction site to a given antibody.

The specificity of DAK Hb1c-1 for the glycated epitope of hemoglobin β-chain has been determined.

A peptide library containing 1-5 amino acid long peptides manually synthesized from the C- to the N-terminal end on a solid phase (Abimed membrane) using Fmoc as protecting group of the N-terminal end of amino acids. Detailed description of peptide synthesis is described in Example 1. The following four peptides were synthesized:

Fructosyl-Hb β-chain

1-5 amino acid long peptide analogs of N-terminal 1-deoxyfructosyl-hemoglobin β-chain.

Hb β-chain
1-5 amino acid long peptide analog of N-terminal hemoglobin β-chain (minus 1-deoxyfructosyl β-chain; backbone control).

Hb α-chain
5 amino acid long peptide analog of N-terminal hemogobin α-chain (negative sequence control).

Fructosyl-Hb α-chain
5 amino acid long peptide analogs of hemoglobin α-chain incl. 1-deoxyfructosyl at the N-terminal valine residue (negative 1-deoxyfructosyl sequence control).

The membranes with peptide spots were incubated with DAK Hb1c-1 and an irrelevant antibody (a negative antibody control, anti-troponin I, DAKO, product no. O 9528). Thereafter, the membranes were incubated with HRP conjugated goat-anti-mouse antibodies (DAKO, product no. P 0447) and visualized using 3-amino-9-ethylcarbazole as substrate.

Number of Amino Acids / ***Peptide name***	5	4	3	2	1
Fructosyl-Hb β-chain	++	++	++	+	-
Hb β-chain	-	-	-	-	-
Hb α-chain	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Fructosyl-Hb α-chain	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*

Table 1: Spot peptide library for epitope mapping of anti-Hb_{1c} antibody clone DAK Hb1c-1. Intensity of peptide spots after incubation with anti-Hb1c antibody clone DAK Hb1c-1 followed by visualization are shown; strong intensity (++), medium intensity (+), negative (-), not determined (N.D.).

DAK Hb1c-1 reacts moderately with 1-deoxyfructosyl 2-mer of hemoglobin β-chain, strongly with 1-deoxyfructosyl 3-mer to 5-mer hemoglobin β-chain (see Table 1). DAK Hb1c-1 does not react with 1-deoxyfructosyl-valine alone. DAK Hb1c-1 does not react with non-fructosylated peptides of hemoglobin β-chain, 1-deoxyfructosyl 5-mer hemoglobin α-chain and 5-mer hemoglobin α-chain. In contrast to DAK Hb1c-1, the negative antibody control did not react with any of the peptides.

In summary, the specificity of anti-Hb1c antibody clone DAK Hb1c-1 for the 1-deoxyfructosylated N-terminal part of hemoglobin β-chain has been demonstrated.

Example 5:

Peptides, proteins from a whole blood sample, red cellhemolysate or calibrators were allowed to bind to the surface of a 96-well microtiter plate. Following a washing step to remove unbound proteins, the monoclonal antibody, DAK Hb1c-1, was added. After incubation, the unbound antibody was washed away, and a conjugate of a goat-anti-mouse antibody and the enzyme horseradish peroxidase was added. After another washing step the bound conjugate was detected by reaction with 3,3,5,5-tetramethylbenzidine (TMB) substrate. The reaction was

stopped by adding acid to give a colorimetric end point that was read spectrophotometrically.

Example 6:

HbA_0 is identical to HbA_{1c} except that there is no 1-deoxyfructosyl linked to the amino-terminus of the hemoglobin β-chain.

The reaction of DAK Hb1c-1 with HbA_0 and 17-mer peptides of HbC_0 and HbS_0 was analysed by the ELISA described above.

When DAK Hb1c-1 was added to microtiter plates coated with either HbA_0 or 17-mers peptides of HbC_0 or HbS_0 the values measured were no higher than the background values.

In conclusion, no reactivity of DAK Hb1c-1 with HbA_0, HbC_0 or HbS_0 was demonstrated.

Example 7:

The reaction of DAK Hb1c-1 with native HbA_{1c} in solution was analyzed in a competitive ELISA. HbA_{1c} calibrator in solution was pre-incubated with DAK Hb1c-1 antibody to test if any antigen-antibody complexes were formed. The solution of antigen-antibody was then transferred to microtiter-wells, where the same antigen was adsorbed on the solid phase (immobilized antigen). The antigen in solution was in excess. The antibody not in complex with the antigen in solution was able to bind to the immobilized antigen. A secondary antibody as described above detected the bound DAK Hb1c-1 antibody. As a

control the same amount of antibody not pre-incubated with HbA1c calibrator in solution was added to one lane of the microtiter plate and followingly subjected to the protocol described above.

No effect of pre-incubation of DAK Hb1c-1 antibody with HbA_{1c} in solution was observed, concluding that DAK Hb1c-1 does not bind to native HbA_{1c} in solution.

Example 8:

The reactivity of DAK Hb1c-1 and HEM13 with human blood containing structural variants of hemoglobin have been analysed by isoelectric focusing (IEF) followed by immunoblotting.

Red cell hemolysate containing HbCA, HbSS and HbAA were electrophoretically separated by Pharmacia's PhastSystem using IEF polyacrylamide gel (PhastGel IEF - 5-8, Pharmacia) in order to separate structural variants of hemoglobin. Following, the gels were subjected to press blotting of proteins onto a PVDF membrane and blocked for non-specific staining with a blocking agent. The membranes were then incubated with DAK Hb1c-1 (see figure 1 A) and HEM13 (see figure 1 B), respectively. HRP-conjugated goat anti-mouse immunoglobulin (DAKO A/S, product no. P 0447) was used as secondary antibody. Finally, HRP activity were visualized using DAKO liquid DAB+ Substrate-Chromogen System (DAKO A/S, product no. K 3468).

A distinct HbS_{1c} band with strong intensity was identified analysing red cell hemolysate from a non-diabetic patient with homozygous HbSS with the monoclonal

antibody, clone DAK Hb1c-1 (see Figure 1, A). In contrast, weak HbS_{1c} staining was revealed with HEM13 analyzing red cell hemolysate from a patient with homozygous HbSS (see Figure 1 B).

Analyzing red cell hemolysate from a non-diabetic individual with heterozygous hemoglobin CA, HbC_{1c} bands with strong and moderate intensity were visualized with DAK Hb1c and HEM 13, respectively. A strong HbA_{1c} band with approximately the same intensity was observed for both DAK Hb1c and HEM13.

As a control a pool of red cell hemolysate from diabetes patients with hemoglobin A was also tested. Other variants of hemoglobin not related to C and S may be a part of this patient pool. Each membrane was incubated with substrate until HbA1c bands with approximately the same intensity were revealed on both DAK Hb1c-1 and HEM 13.

Conclusively, we have demonstrated that our pan-specific monoclonal glycated hemoglobin antibody, clone DAK Hb1c-1, reacts with HbA_{1c}, HbS_{1c} and HbC_{1c} from human blood samples.

Example 9:

Adult hemoglobin is a tetrameric protein, consisting of two α-subunits and two β-subunits, $\alpha_2\beta_2$. Structural variants of Hb contain genetically determined changes in the primary structure of the peptides. A, S and C differ only in the sixth amino acid residue from the N-terminal of the β-chain:

HbA_{1c} fructosyl-V-H-L-T-P-E-E-K-T-A-V-N-A-L-W-G-K.....
HbC_{1c} fructosyl-V-H-L-T-P-K-E-K-T-A-V-N-A-L-W-G-K.....
HbS_{1c} fructosyl-V-H-L-T-P-V-E-K-T-A-V-N-A-L-W-G-K.....

To test if the antibody is pan-specific, i.e. reacting with all structural variants of glycated hemoglobin, we have tested the reaction of DAK Hb1c-1 antibody with three 17-mer glycated peptides corresponding to the N-terminal end of glycated hemoglobin A, C and S β-subunits by ELISA (Figure 2). The peptide sequences are shown above. As a control a pool of red cell hemolysate from diabetes patients with hemoglobin A was also tested by DAK Hb1c-1 ELISA.

Monoclonal HbA_{1c} antibody clone HEM13 has been raised by immunization of mice with purified hemoglobin A1c. We have compared the reaction of DAK Hb1c-1 and HbA_{1c} antibody clone HEM13 with the glycated hemoglobin peptides and erythrocyte extract of hemoglobin A.

The affinity of DAK Hb1c-1 for the glycated hemoglobin peptides is significantly stronger than the affinity of HEM13 for the same peptides as judged by comparing the titration curves (see Figure 2 and Figure 3).

HEM13 shows different reactions with the A1c, S1c and C1c peptides (Figure 3) whereas DAK Hb1c-1 reacts in the same manner with all three peptides (Figure 1). These results demonstrates that the DAK Hb1c-1 antibody is pan-specific.

This example verifies that an antibody that reacts in the same manner with all structural variants of glycated hemoglobin can be raised by injection of a glycated 5-mer

peptide corresponding to the first 4 amino acids of all structural variants of glycated hemoglobin A, C and S β-chain plus one irrelevant amino acid (here cysteine).

Example 10:

Blood samples from the "ERL Educational Programme" have been tested. The HbA1c-percentage of each sample has been determined by cation-exchange chromatography (IFCC/DCCT-values in table 2; IFCC: International Federation of Clinical Chemistry; DCCT: Diabetes Control and Complication Trial). A homozygotic blood sample consists of two identical β-subunits, e.g. AA or SS, whereas heterozygotic samples consists of two different hemoglobin β-subunits, e.g. AS or AC.

	IFCC	DCCT	DAK-Hb1c-1
HbAA	5.0	6.0	5.4
HbAA	5.7	6.5	6.2
HbAA	6.3	7.0	6.5
HbAA	7.0	7.6	7.9
HbAA	7.5	8.0	8.5
HbAA	8.1	8.4	8.7
HbAA	8.8	8.9	9.4
HbAA	9.4	9.4	9.7
HbAA	10.0	9.8	10.0
HbAS	4.3	5.5	4.7
HbAC	4.3	5.5	4.0

Table 2: Percentage of Hb1c in blood samples. The blood sample AC and AS contain heterozygotic hemoglobin.

At a range from 4-10% the HbA1c percentage was measured in whole blood samples using DAK Hb1c-1 in the above-mentioned ELISA format. HbA1c percentages at the same

levels as the values determined by reference methods recommended by IFCC and DCCT were obtained.

In summary, it is demonstrated that the antibody in question has the correct specificity against HbA1c, HbC1c and HbS1c.

Example 11:

The level of glycated hemoglobin was measured in a two-microsphere turbidimetry assay. In this set-up glycated hemoglobin was non-covalently adsorbed to microspheres followed by detection of the antigen with glycated hemoglobin antibodies covalently bound to microspheres. The resulting network constituting antigen microspheres complexed with immunomicrospheres were measured turbidimetrically.

Applicant's or agent's file reference	International application No.
P062 WO 01	RO/DK 25 APR 2002

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 7, line 19

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b
D-38124 Braunschweig

Date of deposit	Accession Number
6 March 2001	DSM ACC2495

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on: 16 MAY 2002 (16.05.02)
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Form PCT/RO/134 (July1998)

Claims:

1. A monoclonal antibody or a fragment thereof, wherein said antibody is pan-specific, i.e. able to react with all structural variants of glycosyl-hemoglobin, said antibody or a fragment thereof comprises an antibody combining site, which binds specifically to a glycated peptide residue of the formula:

Glycosyl-(NH)-Val-His- or Glycosyl-(NH)-Val-His-AA,

wherein Glycosyl-(NH)-Val represents a non-enzymatically glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin and AA means 1 to 3 amino acid residues.

2. A monoclonal antibody or a fragment thereof according to claim 1, wherein AA is a sequence of from 1 to 3 amino acids corresponding to a part of the N-terminal β-chain of all structural variants of human hemoglobin.

3. A monoclonal antibody or a fragment thereof according to claim 2, wherein AA is -Leu-Aa; -Leu-Thr-Aa; -Leu-Thr-Pro-Aa, and Aa designates the remaining number of amino acids in the AA group.

4. A monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody is obtainable by hybridoma DAK Hb1c-1 deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) as accession no. DSM ACC2495.

5. A monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody

is pan-specific, i.e. able to react with all structural variants of human hemoglobin including HbA1c, HbC1c and HbS1c.

6. A monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody being obtainable by a method as defined in any one of the claims 7-11.

7. A method of producing a monoclonal antibody or a fragment thereof, wherein said antibody has been raised against an immunogen comprising an antigen obtainable by a glycated peptide chemically linked to an immunogenic carrier material, the glycated peptide having from 2 to 5 amino acid units corresponding to the N-terminal β-chain of human hemoglobin.

8. A method of producing a monoclonal antibody or a fragment thereof according to claim 7, wherein the immunogen is of the formula:

[Fructosyl-(NH)-Val-His-AA-R]n-Carrier

wherein Fructosyl-(NH)-Val represents a non-enzymatic glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin, AA means one or more additional amino acid residues, R is a linking group, Carrier is an immunogenic carrier material, and n is an integer from 1 to the number of available coupling sites on said carrier.

9. A method of producing a monoclonal antibody or a fragment thereof according to claim 8, wherein AA is a sequence of from 1 to 3 amino acids corresponding to a

part of the N-terminal β-chain of all structural variants of human hemoglobin.

10. A method of producing a monoclonal antibody or a fragment thereof according to claim 8, wherein said Carrier is immunogenic proteins or peptides other than human hemoglobin.

11. A method of using a monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody binds specifically to said glycated N-terminal peptide sequence of human hemoglobin A1c, C1c and S1c ß-chains after having been exposed sufficiently to provide steric access thereto.

12. A method of using a monoclonal antibody or a fragment thereof according to claim 11, wherein said glycated peptide sequence is exposed to the antibody combining site by physical or chemical denaturation or digestion.

13. A method of using a monoclonal antibody or a fragment thereof according to claims 11-12, wherein said glycated peptide sequence is exposed to the antibody combining site by denaturation with a chaotropic agent.

14. A method of using a monoclonal antibody or a fragment thereof according to claim 13, wherein the chaotropic agent is selected from the group consisting of gluanidine, urea, potassium-thiocyanate, or detergent.

15. A method of using a monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody binds specifically to a glycated N-terminal peptide sequence of the β-chain of all

structural variants of human hemoglobin, which has been adsorbed to a solid phase.

16. A method of using a monoclonal antibody or a fragment thereof according to claim 15, wherein the solid phase preferably is constituted by a material selected from the group consisting of microtiter plates, micro particles, membranes, tubes, pins, chips or discs.

17. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated hemoglobin in human blood.

18. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated HbS and/or HbC in human blood.

19. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated heterozygous HbA, HbS and/or HbC in human blood.

20. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated hemoglobin measured in a two-particle turbidimetric assay.

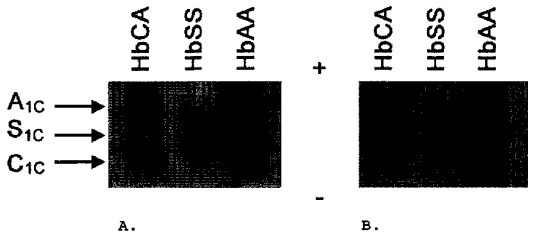

Figure 1

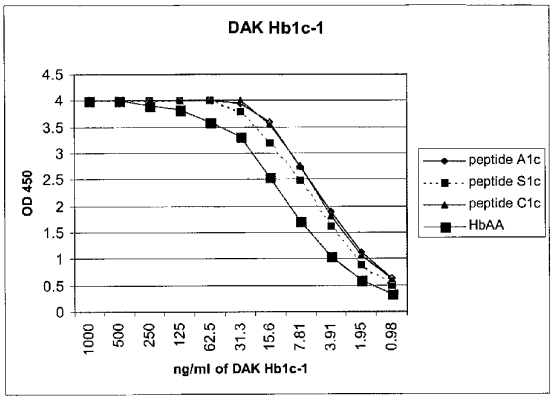

Figure 2: Reactivity of DAK Hb1c-1 with red cell hemolysate from patients with hemoglobin AA (HbAA) and 17-mer glycated peptides corresponding to hemoglobin A1c, C1c and S1c as determined by an ELISA test.

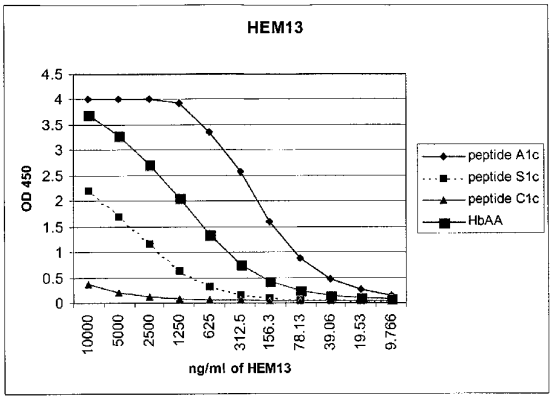

Figure 3. Reactivity of HEM13 with red cell hemolysate from patients with hemoglobin AA (HbAA) and 17-mer glycated peptides corresponding to hemoglobin A1c, C1c and S1c as determined by an ELISA test.

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(54) Title: PAN-SPECIFIC MONOCLONAL ANTIBODY

(57) Abstract: The invention describes pan-specific monoclonal antibodies for the determination of the amount of all structural variants of glycated hemoglobin in human blood samples. The determination of the rate of glycation of hemoglobin in an individual's blood provides a useful index of glucose level control in diabetics.

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(54) Title: PAN-SPECIFIC MONOCLONAL ANTIBODY

(57) Abstract: The invention describes pan-specific monoclonal antibodies for the determination of the amount of all structural variants of glycated hemoglobin in human blood samples. The determination of the rate of glycation of hemoglobin in an individual's blood provides a useful index of glucose level control in diabetics.

Pan-specific monoclonal antibody

This invention relates to the determination of the amount of all structural variants of glycated hemoglobin in human blood samples. The determination of the rate of glycation of hemoglobin in an individual's blood provides a useful index of glucose level control in diabetics.

In particular, the present invention concerns the preparation of monoclonal antibodies or a fragment thereof, which recognize specifically the glycated N-terminal peptide residue in such human hemoglobin.

Patients afflicted with diabetes are incapable of metabolizing glucose in a conventional manner resulting in a build-up of glucose in their blood and urine. Conventionally, the glucose level in such body fluids is taken as a measure of the state of the diabetic condition, which in turn is used as a guide for the amount of insulin or other agent to be taken or of the need to change the patient's diet.

This works moderately well except that the glucose level may fluctuate widely in dependence on time and content of the last meal, the last insulin injection, and the like. Thus, the reading will reflect an instantaneous condition, which may not truly identify the longer-term state of the diabetic condition.

It is known that another effect of the diabetic condition is an increase in the amount of glycated hemoglobin in the blood of the diabetic. Hemoglobin (Hb) is a protein tetramer consisting of four chains (subunits) of amino acids, each of about 143 units and having a total

molecular weight of approximately 64,000 g/mol. The N-terminal valine of the β-subunit in hemoglobin can react with glucose. The glycation of hemoglobin occurs by a non-enzymatic reaction involving glucose and the alpha-amino group of valine. Following a Schiff base formation between the reactants, the glucose undergoes an Amadori rearrangement forming 1-deoxyfructo-valine. The final complex binding between 1-deoxyfructose and valine is covalent and irreversible. The glycation reaction is governed by the concentration of the reactants, e.g., hemoglobin and glucose. The Diabetes Control and Complication Trial (DCCT) reference interval for non-diabetic individuals is approximately 4-6% glycated hemoglobin. Hemoglobin tetramers with a 1-deoxyfructo-valine on the N-terminus of a β-chain are identified as being glycated hemoglobin; e.g. Hb1c or HbA1c, HbC1c or HbS1c.

HbC trait has a prevalence of 30% in parts of sub-Saharan and 2.3% among African Americans. HbS trait, the most commonly variant of Hb in US, has a prevalence of 7.8% of African Americans. All together, at least 10% of black Americans have either HbC or HbS trait. The world population is getting more and more mixed with respect to race. Therefore, it is increasingly important to have an antibody that reacts with all structural variants of glycated hemoglobin in the same manner. If the available antibody do not react with all structural variants of glycated hemoglobin, Hb1c percent measurements from patients with traits of HbS and/or HbC will be incorrect.

Glucose levels in diabetics are sufficiently high to increase the rate of glycation depending directly on the glucose level in the blood, which reflects the severity

of the diabetic condition. Therefore, the hemoglobin A1c level is raised to about 6 to 12%. Since the circulating life span of hemoglobin A is about 120 days, a glycated hemoglobin measurement will give a value, which reflects an average glucose level for that period. Notably a meal high in glucose will not be reflected in instantly high levels of glycated hemoglobin or serum albumin. Thus, measurement of the glycated-hemoglobin content gives a truer picture of the average circulating glucose levels and thus a truer picture of the long-term condition of the patient.

A common method used to determine the level of glycated hemoglobin involves passing a lysed blood sample through a boronate affinity column. The method determines the total glycated hemoglobin, including HbA_{1c} and ketoamine structures formed on lysine and N-terminal valine residues of both the α- and β-chains of hemoglobin. The column is washed and the glycated hemoglobin determined spectrophotometrically.

Microparticle enhanced turbidimetric immunoassay techniques are available for automatic analyses by instruments. These instruments are not dedicated to a certain diagnostic assay and have a very high sample cycle time. Currently, the utility of this technique for determination of glycated hemoglobin is growing as rutine clinical analysis. Some of these immunoassays are based on the principle of microparticle enhanced competitive turbidimetric inhibition immunoassay techniques.

US Patent no. 4,247,533 discloses an analytical technique wherein antibodies to HbAlc were reportedly raised in a sheep by injection of HbAlc and absorbed with nonglycated

hemoglobin to provide polyclonal antibodies, which distinguished between HbAlc and nonglycated hemoglobin. Such antibodies then form the basis for a test to determine the proportion of glycated hemoglobin in a sample.

In the prior art only the hemoglobin A_{1c} antibody and how the antibody is able to react with the A variant of glycated hemoglobin is described. The influence of the other variants of glycated hemoglobin is not mentioned and a monoclonal antibody against all structural variants of glycated-hemoglobin has neither been considered. Due to the fact that the antibody does not recognise HbS1c and HbC1c, immunoassay measurement of glycated-hemoglobin percentages in subjects with HbS1c and HbC1c using that antibody will lead to truly decreased percentages of glycated hemoglobin.

An Hb1c antibody, which is specific for all structural variants of Hb1c, is considered as pan specific.

Accordingly, there remains a need for an antibody of the above-mentioned kind, which does not exhibit the above-identified drawbacks.

It is accordingly an objective of the present invention to provide an antibody, which is pan-specific, i.e. specific for all structural variants of glycated hemoglobin.

It is also the objective to provide an antibody for determining the long-term blood sugar level of a patient based on an immunoassay determination of HbAlc, HbC1c and HbS1c.

It is another objective of the invention to provide such an antibody for determining the content of glycated hemoglobin, HbAlc, HbClc and HbSlc, in a patient's whole blood sample.

These and the other objects are achieved by the invention as defined in the claims.

The invention relates to a monoclonal antibody or a fragment thereof, which is pan-specific, i.e. able to react with all structural variants of glycated-hemoglobin. The structural variants of glycated-hemoglobin are preferably HbAlc, HbClc and HbSlc.

The monoclonal antibody preferably leads to improvement of existing immunoassay with respect to measurements of the level of glycated hemoglobin in patients with HbS and HbC.

The monoclonal antibody of the present invention is characterized in that the antibody comprises an antibody combining site, which binds specifically to the glycated N-terminal peptide sequence of human hemoglobin A, C and S ß-chains.

The monoclonal antibody reagent of the present invention is characterized by its specific binding affinity for the linear epitope in glycated hemoglobin. Therefore, as used herein the term "antibody reagent" or "antibody combining site" will refer to any material however obtained which comprises a monoclonal antibody combining site specific for such peptide epitope. Such term therefore includes whole antibodies as well as appropriate fragments or

polyfunctionalized forms thereof. When in the form of whole antibody, it can belong to any of the classes and subclasses of known immunoglobulins, e.g. IgG, IgM and so forth. Any fragment of any such immunoglobulins which retains specific binding affinity for the peptide epitope can also be employed, for instance, the fragments of IgG conventionally known as Fab, Fab' and $F(ab')_2$. In addition, aggregates, polymers, derivates, conjugates, and hybrids of immunoglobulins, or their fragments can be used where appropriate. Furthermore, single chain antibodies or any other fragment obtained by recombinant means can be used where appropriate.

The glycated residue is the distinguishing structural feature of HbAlc, HbSlc and HbClc. An antibody of the present invention requires an epitope or determinant site comprising minimally the 1-deoxyfructosyl carbohydrate unit, formed upon Amadori rearrangement of the reaction product between glucose and the terminal amine, and a peptide sequence extending therefrom comprising at least one of the amino acid units of the HbAlc, HbSlc and HbClc N-terminal sequence in the position corresponding to the native HbAlc, HbSlc and HbClc sequence. The other amino acid units in the peptide sequence characterizing the epitope may be the same or different as those appearing in the native HbAlc, HbSlc and HbClc sequence. In this way, the epitope is characterized by at least two contact or binding sites with the antibody, which sites are unique to the glycated N-terminal HbAlc, HbSlc and HbClc sequence.

The antibody will preferably specifically bind a glycated peptide residue of the formula:

Glycosyl-(NH)-Val-His- or Glycosyl-(NH)-Val-His-AA,

wherein Glycosyl-(NH)-Val represents a non-enzymatically glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin and AA means one or more additional amino acid residues.

In a preferred embodiment AA is a sequence of from 1 to 3 amino acids corresponding to a part of the N-terminal β-chain of all structural variants of human hemoglobin. AA is preferably -Leu-Aa; -Leu-Thr-Aa; -Leu-Thr-Pro-Aa, and Aa designates the remaining number of amino acids in the AA group.

A preferred monoclonal antibody or a fragment thereof according to the invention is obtainable using the hybridoma DAK Hb1c-1, which is deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) as accession no. DSM ACC2495.

The invention also relates to a method of producing a monoclonal antibody or a fragment thereof, wherein the antibody is pan-specific, i.e. able to react with all structural variants of glycated hemoglobin.

The antibody or a fragment thereof may preferably be obtainable by the method as defined in claims.

The method of producing a monoclonal antibody or a fragment thereof according to the invention comprising the step of raising an antibody against an immunogen comprising a glycated peptide chemically linked to an immunogenic carrier material, the glycated peptide having

from 2 to 5 amino acid units corresponding to the N-terminal in the β-chain of human hemoglobin.
The immunogen is preferably of the formula:

[Fructosyl-(NH)-Val-His-AA-R]$_n$-Carrier

wherein Fructosyl-(NH)-Val represents a nonenzymatic glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin, AA means one or more additional amino acid residues, R is a linking group, Carrier is an immunogenic carrier material, and n is an integer from 1 to the number of available coupling sites on said carrier.

In a preferred embodiment AA is one or more additional amino acid residues. In another preferred embodiment AA is a sequence of from 1 to 3 amino acids corresponding to the N-terminal part of the β-chain of all structural variants of human hemoglobin.

Linking group R can be essentially any convenient and stable structure. Such linking group R will usually be in the form of an aliphatic chain comprising between 1 and approximately 20 atoms, excluding hydrogen, and including heteroatoms such as nitrogen, oxygen, and sulfur. The glycated residue can be joined through a variety of groups to form linking chain R, including methylene, ether, thioether, imino, and the like. One skilled in the art will have a wide variety of linking groups from which to choose to prepare the immunogen.

In a preferred embodiment the carrier is an immunogenic protein or peptide other than human hemoglobin.

The immunogen used to stimulate production of appropriate immunoglobulins in the most general sense will comprise one or more of the glycated peptide residues chemically linked to an immunogenic carrier material. The immunogenic carrier material can be selected from any of those conventionally known having functional groups available for coupling to the glycated peptide residue. In most cases, the carrier is a protein or polypeptide, although other materials such as carbohydrates, polysaccharides, lipopolysaccharides, nucleic acids, and the like of sufficient size and immunogenicity can likewise be used. For the most part, immunogenic proteins and polypeptides will have molecular weights between 4,000 g/mol and 10,000,000 g/mol, preferably greater than 15,000 g/mol, and more usually greater than 50,000 g/mol. Generally, proteins taken from one animal species will be immunogenic when introduced into the blood stream of another species. Particularly useful proteins are albumins, globulins, enzymes, hemocyanins, glutelins, proteins having significant nonproteinaceous constituents, and the like.

In a preferred embodiment the glycated N-terminal peptide residue of the native HbAlc, HbSlc and HbClc molecule is made accessible to the monoclonal antibody or a fragment thereof of the present invention by appropriate denaturation or digestion of the protein in the sample to be assayed.

In another preferred embodiment the glycated fragment can be produced by chemical or enzymatic digestion of naturally occurring glycated hemoglobin, e.g. HbAlc, HbSlc and HbClc. This fragment can be coupled to a carrier using classical coupling procedures, e.g.,

glutaraldehyde or carbodiimide, and the conjugate used as an immunogen.

The antibody or a fragment thereof according to the invention may preferably be produced by the following step:

A. Preparation of peptide immunogen

To obtain antibodies against the glycation site of N-terminal valine residue on the β-chain of Hb A, S and C a short peptide (pentamer) is preferably used as an immunogen. The antigen may preferably be Hb1c 4-mer plus cysteine, i.e. fructosyl-V-H-L-T-C.

B. Immunization of mize with human Hb1c peptides

The general method used to develop hybridomas secreting monoclonal antibodies is well known to a person skilled in the art.

An illustration of the techniques utilized in the practice is described in Journal of Immunological Methods, 1980, 39:285.

Female F1 hybrids of CF1 x BALB/c mice may preferably be used, but mice of other strains may also be used. The immunization plan and the concentration of peptide-carrier complex may be selected to form a sufficient amount of antigenically stimulated lymphocytes. In a preferred embodiment mice are immunized intraperitoneally with 50 micrograms of purified protein derivates (PPD, a

partly unknown mixture of denatured proteins from *M. tuberculosis*) in complex with the Hb1c peptide four times at 2-week intervals. 3-5 days after the final immunization, the spleen cells are extracted from the animal for fusion.

Monoclonal antibodies produced by hybridomas from other species may also preferably be used.

C. Cell fusion

The spleen is aseptically taken out from the immunized mouse, and a suspension of the spleen cells is prepared. The spleen cells are fused with mouse myeloma cells from a suitable cell line in the presence of a suitable fusion promotor. In a preferred embodiment the preferred ratio of the spleen cells to the myeloma cells is from about 20:1 to about 2:1, and a fusion medium is suitably used in an amount of 0.5 to 1.5 ml per about 10^8 spleen cells.

The mouse myeloma cells used for cell fusion are well known. P3-X63-Ag 8.653 may preferably be used as a mouse myeloma cell.

The fusion promoter is preferably polyethylene glycol having an average molecular weight of 1000 to 4000 g/mol. Other fusion promoters known in the art can also be used. In a preferred embodiment polyethylene glycol having an average molecular weight of 1500 may be used.

D. Screening of the fused cells

In a separate container (such as a microtiter plate), a mixture composed of the unfused spleen cells, unfused mouse myeloma cells and the fused hybridoma cells is preferably diluted with a selective medium not supporting the unfused mouse myeloma cells and cultured for a period sufficient to kill the unfused mouse myeloma cells (about 1 week). A culture medium having drug resistance (for example, resistance to 8-azaguanine) and not supporting the unfused mouse myeloma cells, such as HAT medium, is used. In the selective medium, the unfused myeloma cells die. Since the unfused spleen cells are untransformed cells, they die away after a certain period of time (1 week). The fused cells, on the other hand, can survive in the selective medium since they have both the tumoral nature of the parent myeloma cells and the nature of the parent spleen cells.

E. Determination of glycated hemoglobin by ELISA

The determination is preferably based on the principle of specific immunologic recognition and reaction between a monoclonal antibody and the antigenic epitope to which the antibody uniquely and specifically binds. The recognition and binding may be detected, for example, by an ELISA test, wherein blood samples or calibrators are immobilized on a solid phase support, such as the bottom of a plastic well. By immobilizing human hemoglobin the antigenic epitope may be exposed for antibody binding. The antibody, secondary enzyme-labeled antibody and enzyme substrate may interact with the immobilized antigen (human hemoglobin) thereby antibody-antigen

complexes are preferably formed. A number of dilution, incubation and washing steps, then allow separation of bound and free reagents. A colour forming reaction takes place as a result of binding of the antibody to antigen and the consequent reaction of the enzyme upon its substrate interaction. The formation of colour indicates the presence of Hb1c in the blood sample, and the intensity of the colour provides a quantitative measure of the amount of glycated epitope in the sample. The antibody of the present invention may be used to measure glycated hemoglobin in other immunological assay formats known in the art.

F. Cloning of the hybridoma cells producing the desired antibody and the production of the antibody

Hybridoma cells capable of producing the desired antibody are cloned by a suitable method such as limiting dilution method, and the desired antibody can be produced by the following method. Hybridoma cells are cultured in a suitable medium for a certain period of time, and the monoclonal antibody produced by the hybridoma cells can be isolated from the supernatant of the culture.

The invention also relates to a method of using a monoclonal antibody or a fragment thereof.

The antibodies or a fragment thereof according to the invention can preferably be used in conventional manner to react with blood samples containing unknown quantities of glycated hemoglobin and the extent of reaction can be compared with calibrated standards to determine the

extent of glycation. The read-out can be by fluorescence, by immunoassay, or the like, by joining suitably readable groups to the monoclonal antibodies in known manner without loss of their binding power for the glycated epitope in HbAlc, HbSlc and HbClc.

The monoclonal antibodies of the present invention are specific for binding to all structural variants of glycated N-terminal peptide residue preferable found in HbA, HbS and HbC. The antibodies are able to bind to the epitope in the native HbAlc, HbSlc and HbClc molecule when the epitope is appropriately exposed. Steric access to the epitope can be obtained in any effective manner. Exposure of the epitope in the intact protein is understood to be accomplished by a physical or chemical denaturation or digestion at least in the region of the epitope. Such denaturation or digestion can be localized to the region of the epitope or can involve a more general, or even substantially complete denaturation of the tertiary, and additionally the secondary structure of the protein, or partial or complete digestion of the protein.

In a preferred embodiment the antibody binds specifically to the glycated N-terminal peptide sequence in the β-chain of human hemoglobin Alc, Clc and Slc after having been exposed sufficiently to provide steric access thereto.

Denaturation can be accomplished in a variety of ways including conventional treatment of the protein by physical means such as heat, sonication, high or low pH and, as is preferable, chemical denaturation by interaction with an agent or chaotrope in solution.

Protein denaturation can be most effectively accomplished if combinations of chemical and/or chemical and physical means are used, e.g. guanidine and heat, guanidine and SDS, or guanidine and dithiothreitol. Particularly strong chaotropes such as guanidine are preferred. Of course, denaturing conditions which result in substantial insolubilization, aggregation, or precipitation of the protein implying an insignificant amount of the exposed epitope is accessible to the solution for antibody binding will be avoided. A sufficient amount of the denatured protein must remain in solution or suspension in order to obtain useful immunobinding. The extent of solubilization necessary will depend upon the circumstances of the intended or desired binding.

In a preferred embodiment the glycated peptide sequence is exposed to the antibodybinding site by physical or chemical denaturation or digestion.

In another preferred embodiment the glycated peptide sequence is exposed to the antibody binding site by denaturation with a chaotropic agent. The chaotropic agents include, without limitation, guanidine, urea, and various detergents such as sodium dodecylsulfate (SDS) and others, without limitation, including deoxycholoate and certain bile salts, 3-(3-cholamidopropyl)-dimethyl-ammonio-1-propanesulfonate, organic solvents such as methanol, propanol, acetonitrile and certain salts such as potassium thiocyanate. Non-ionic detergents such as Triton X®, Tween®, nonidet NP-40®, and octyl-glucosides can also function as protein denaturants.

A significant amount of HbAlc, HbSlc and HbClc in a particular blood sample can be denatured to expose the

glycated epitope for antibody binding by combining the sample, e.g., whole blood or red cell hemolysate, with an aqueous solution of the chaotrope present at sufficient concentration to denature any HbAlc, HbSlc and HbClc in the resulting aqueous mixture. Where whole blood is the sample, the chaotrope also serves to lyse red blood cells to release hemoglobin and to inactivate proteases.

The denaturation process may be significantly accelerated by heating the mixture for a short period of time. It has been found that at temperatures below 37°C , denaturation by the chaotrope can take from one to several hours, whereas at temperatures above 50°C sufficient denaturation can be attained in a minute or less. In order to prevent significant denaturation of the antibody and other proteinaceous reagents to be subsequently added to the mixture, the sample-chaotrope mixture will normally be diluted as a separate step or by addition of reagent solutions to a level that the chaotrope is substantially ineffective to denature such reagents, yet will preserve the exposure of the epitope by preventing significant renaturation of HbAlc, HbSlc and HbClc.

In a preferred embodiment the antibody binds specifically to a glycated N-terminal peptide sequence in the β-chain of glycated human hemoglobin, which has been adsorbed to a solid phase, wherein the solid phase preferably is selected from the group consisting of polystyrene, divinylbenzene, butadiene, polycarbonate, polyacrylamide, polyacrylic acid, polyacryl amide, polyethylene, polypropylene, fluorinated polymers, poly amides, or co-block polymers hereof, gold, carbon, cellulose, derivatized cellulose, or glass.

In a preferred embodiment the monoclonal antibody or a fragment thereof may be used for the determination of the level of glycated hemoglobin in human blood. The monoclonal antibody or a fragment thereof may also preferably be used for the determination of the level of glycated HbS and/or HbC in human blood.

In a further preferred embodiment the monoclonal antibody or a fragment thereof may be used for the determination of the level of glycated heterozygous HbA, HbS and/or HbC in human blood.

In a further preferred embodiment there is provided a turbidimetric two-microsphere method for determination of the level of glycated hemoglobin. This method comprises mixing a sample with microspheres and microspheres on which the monoclonal antibody or a fragment thereof is bound; the latter is referred to as immunoparticles, or with microspheres and subsequently with immunomicrospheres. The resulting immunoagglutinates is measured turbidimetrically.

The microspheres are preferably selected from the group consisting of polystyrene, divinylbenzene, butadiene, polycarbonate, polyacrylamide, polyacrylic acid, polyacryl amide, polyethylene, polypropylene, fluorinated polymers, poly amides, or co-block polymers hereof, gold, or silica.

Microspheres to be used in the method of the present example have a particle size of from 0.01 to 5 micro meters, which are used by dispersing in a usually used buffer solution.

The following experimental non-limiting examples are intended to illustrate certain features and embodiments of the invention.

Example 1:

The 1-deoxyfructosyl-Val-His-AA peptide was synthesized using the solid-phase method of Sheppard and Atherton as described in international patent application no. WO 86/03494. The peptide was synthesized with fluorenylmethoxycarbonyl (Fmoc) amino acids, using PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphorium hexafluorophosphate) and HOBt (N-hydroxy-benzotriazole) as coupling reagents.

Cysteine and histidin were used with trityl (trt), and threonin with tertiary butyl (tBu) side-chain protection. Leucin was used without side-chain protection.

The fructose amino acid conjugate, N-(deoxy-D-fructos-1-yl)-L-valine, was synthesized by modifications of known methods. In short, Di-isopropyliden protected glucose was activated as the trifluate and conjugated to the benzyl ester of valine. The resulting 2,3:4,5-Di-O-isopropyliden-N-(deoxy-D-fructos-1-yl)-L-valine-benzyl ester was hydrogenated over Pd/C in ethanol, lyophilized, and finally used in the peptide synthesis without Fmoc protection. The purity of the product was verified by TLC analysis and ^{1}H or ^{13}C NMR spectra.

The peptide was synthesized by automated solid phase synthesis (Crystal instrument from Calbiochem-Novabiochem Ltd) cleaved from the resin with TFA (trifluoroacetic acid) - TES (triethylsilan) - Glycerol - H_2O (90% : 4% :

3% : 3%), precipitated and washed with methyl-tertiary-butyl-ether, centrifuged and lyophilised.

Purification was performed by HPLC (C_{18} reversed-phase column, using a step-wise gradient of typically 5-25% MeCN in 0.1% TFA). The identity of the peptide was verified by MALDI-TOF mass spectroscopy and the purity was finally assured by an analytical HPLC analysis to be better than 90%.

Example 2:

Four weeks before first immunization Bacille Calmette Guérin (BCG) vaccine was injected to prime the immune response in the mice as described in Giba Foundation Symposium, 1986, 119: 25.

To make the peptide immunogen it was conjugated to a carrier mixture, Tuberculin PPD. Peptide and carrier (PPD) were conjugated with glutardialdehyde, i.e. CHO-$(CH2)_3$-CHO. Glutardialdehyde reacts with amino-groups on the peptide and the carrier.

The immunogens thus have the following formula:

Fructosyl-Val-His-Leu-Thr-Cys-CH-$(CH2)_3$-CH-PPD

Example 3:

Female F1 hybrids of CF1 x BALB/c mice (12 weeks old) primed with BCG-vaccine were immunized with the peptide-PPD complex described above. Mice were immunized four times intraperitoneally with a mixture of 50 micrograms

of peptide-PPD complex dissolved in phosphate buffered saline (PBS) and aluminium hydroxide (1 mg/dose). A mouse with high titer of antibodies against glycated hemoglobin was selected for fusion. Three days after the final immunization, spleen cells from the immunized mouse was used for cell fusion.

Spleen cells from the selected immunized mouse and myeloma cells of BALB/c origin (P3-X63-Ag 8.653) were mixed in a ratio of about 3:1 and fused in the presence of 50% polyethylene glycol 1500 (a product of Roche Diagnostics GmbH)(J. W. Goding: Monoclonal Antibodies: Principle and Practice, Academic Press, San Diego, 1996). The fused cells were suspended in RPMI-1640 medium, containing 10% FCS, hypoxanthine, aminopterin and thymidine (HAT media) to give a concentration of 1×10^6 cells/mL. The suspension was distributed to 96-well microplates (Costar) in an amount of 200 μL per well.

The cells were incubated in a CO2 incubator (5% CO2, 37°C). After 14 days hybridoma cells were screened.

Supernatants containing antibodies produced by the hybridoma cell cultures were detected by an ELISA technique using microtiter plates coated with hemoglobin calibrator containing 4.7% HbA1c and 16.7% HbA1c as antigens. 120 μg/mL of the calibrators were immobilized onto plastic microtiter wells using a carbonate/bicarbonate buffer (pH 9.6) for 15 minutes at 37°C. After a washing step that removes unbound antigen the hybridoma culture supernatants diluted in 50 mM Tris-HCl buffer containing 0.1% Tween 20 were added to each well and allowed to react for one hour at room temperature. After another washing step to remove unbound

antibody a conjugate of a goat anti-mouse antibody and the enzyme horseradish peroxidase was added. After incubation and a third washing step the bound conjugate was detected by reaction with the substrate 3,3,5,5-tetramethylbenzidine (TMB). The reaction was stopped with sulphuric acid and the absorbance at 450 nm was measured. Only cells from positive wells reacting with glycated hemoglobin calibrators, 4.7% HbA1c and 16.7% HbA1c, with increasing intensity were selected.

These positive cells were cloned three times by limiting dilution method. The resulting clone, DAK Hb1c-1, was suspended in 90% FCS; 10% DMSO and stored at -150°C.

Example 4:

A linear antibody epitope mapping is the systematic screening of all possible peptides derived from a polypeptide or protein sequence. Epitope mapping yields information on the linear stretch of amino acids and derivatives that form an interaction site to a given antibody.

The specificity of DAK Hb1c-1 for the glycated epitope of hemoglobin β-chain has been determined.

A peptide library containing 1-5 amino acid long peptides manually synthesized from the C- to the N-terminal end on a solid phase (Abimed membrane) using Fmoc as protecting group of the N-terminal end of amino acids. Detailed description of peptide synthesis is described in Example 1. The following four peptides were synthesized:

Fructosyl-Hb β-chain

1-5 amino acid long peptide analogs of N-terminal 1-deoxyfructosyl-hemoglobin β-chain.

Hb β-chain
1-5 amino acid long peptide analog of N-terminal hemoglobin β-chain (minus 1-deoxyfructosyl β-chain; backbone control).

Hb α-chain
5 amino acid long peptide analog of N-terminal hemogobin α-chain (negative sequence control).

Fructosyl-Hb α-chain
5 amino acid long peptide analogs of hemoglobin α-chain incl. 1-deoxyfructosyl at the N-terminal valine residue (negative 1-deoxyfructosyl sequence control).

The membranes with peptide spots were incubated with DAK Hb1c-1 and an irrelevant antibody (a negative antibody control, anti-troponin I, DAKO, product no. O 9528). Thereafter, the membranes were incubated with HRP conjugated goat-anti-mouse antibodies (DAKO, product no. P 0447) and visualized using 3-amino-9-ethylcarbazole as substrate.

Number of Amino Acids / *Peptide name*	5	4	3	2	1
Fructosyl-Hb β-chain	++	++	++	+	-
Hb β-chain	-	-	-	-	-
Hb α-chain	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Fructosyl-Hb α-chain	-	*N.D.*	*N.D.*	*N.D.*	*N.D.*

Table 1: Spot peptide library for epitope mapping of anti-Hb_{1c} antibody clone DAK Hb1c-1. Intensity of peptide spots after incubation with anti-Hb1c antibody clone DAK Hb1c-1 followed by visualization are shown; strong intensity (++), medium intensity (+), negative (-), not determined (N.D.).

DAK Hb1c-1 reacts moderately with 1-deoxyfructosyl 2-mer of hemoglobin β-chain, strongly with 1-deoxyfructosyl 3-mer to 5-mer hemoglobin β-chain (see Table 1). DAK Hb1c-1 does not react with 1-deoxyfructosyl-valine alone. DAK Hb1c-1 does not react with non-fructosylated peptides of hemoglobin β-chain, 1-deoxyfructosyl 5-mer hemoglobin α-chain and 5-mer hemoglobin α-chain. In contrast to DAK Hb1c-1, the negative antibody control did not react with any of the peptides.

In summary, the specificity of anti-Hb1c antibody clone DAK Hb1c-1 for the 1-deoxyfructosylated N-terminal part of hemoglobin β-chain has been demonstrated.

Example 5:

Peptides, proteins from a whole blood sample, red cellhemolysate or calibrators were allowed to bind to the surface of a 96-well microtiter plate. Following a washing step to remove unbound proteins, the monoclonal antibody, DAK Hb1c-1, was added. After incubation, the unbound antibody was washed away, and a conjugate of a goat-anti-mouse antibody and the enzyme horseradish peroxidase was added. After another washing step the bound conjugate was detected by reaction with 3,3,5,5-tetramethylbenzidine (TMB) substrate. The reaction was

stopped by adding acid to give a colorimetric end point that was read spectrophotometrically.

Example 6:

HbA_0 is identical to HbA_{1c} except that there is no 1-deoxyfructosyl linked to the amino-terminus of the hemoglobin β-chain.

The reaction of DAK Hb1c-1 with HbA_0 and 17-mer peptides of HbC_0 and HbS_0 was analysed by the ELISA described above.

When DAK Hb1c-1 was added to microtiter plates coated with either HbA_0 or 17-mers peptides of HbC_0 or HbS_0 the values measured were no higher than the background values.

In conclusion, no reactivity of DAK Hb1c-1 with HbA_0, HbC_0 or HbS_0 was demonstrated.

Example 7:

The reaction of DAK Hb1c-1 with native HbA_{1c} in solution was analyzed in a competitive ELISA. HbA_{1c} calibrator in solution was pre-incubated with DAK Hb1c-1 antibody to test if any antigen-antibody complexes were formed. The solution of antigen-antibody was then transferred to microtiter-wells, where the same antigen was adsorbed on the solid phase (immobilized antigen). The antigen in solution was in excess. The antibody not in complex with the antigen in solution was able to bind to the immobilized antigen. A secondary antibody as described above detected the bound DAK Hb1c-1 antibody. As a

control the same amount of antibody not pre-incubated with HbA1c calibrator in solution was added to one lane of the microtiter plate and followingly subjected to the protocol described above.

No effect of pre-incubation of DAK Hb1c-1 antibody with HbA_{1c} in solution was observed, concluding that DAK Hb1c-1 does not bind to native HbA_{1c} in solution.

Example 8:

The reactivity of DAK Hb1c-1 and HEM13 with human blood containing structural variants of hemoglobin have been analysed by isoelectric focusing (IEF) followed by immunoblotting.

Red cell hemolysate containing HbCA, HbSS and HbAA were electrophoretically separated by Pharmacia's PhastSystem using IEF polyacrylamide gel (PhastGel IEF - 5-8, Pharmacia) in order to separate structural variants of hemoglobin. Following, the gels were subjected to press blotting of proteins onto a PVDF membrane and blocked for non-specific staining with a blocking agent. The membranes were then incubated with DAK Hb1c-1 (see figure 1 A) and HEM13 (see figure 1 B), respectively. HRP-conjugated goat anti-mouse immunoglobulin (DAKO A/S, product no. P 0447) was used as secondary antibody. Finally, HRP activity were visualized using DAKO liquid DAB+ Substrate-Chromogen System (DAKO A/S, product no. K 3468).

A distinct HbS_{1c} band with strong intensity was identified analysing red cell hemolysate from a non-diabetic patient with homozygous HbSS with the monoclonal

antibody, clone DAK Hb1c-1 (see Figure 1, A). In contrast, weak HbS_{1c} staining was revealed with HEM13 analyzing red cell hemolysate from a patient with homozygous HbSS (see Figure 1 B).

Analyzing red cell hemolysate from a non-diabetic individual with heterozygous hemoglobin CA, HbC_{1c} bands with strong and moderate intensity were visualized with DAK Hb1c and HEM 13, respectively. A strong HbA_{1c} band with approximately the same intensity was observed for both DAK Hb1c and HEM13.

As a control a pool of red cell hemolysate from diabetes patients with hemoglobin A was also tested. Other variants of hemoglobin not related to C and S may be a part of this patient pool. Each membrane was incubated with substrate until HbA1c bands with approximately the same intensity were revealed on both DAK Hb1c-1 and HEM 13.

Conclusively, we have demonstrated that our pan-specific monoclonal glycated hemoglobin antibody, clone DAK Hb1c-1, reacts with HbA_{1c}, HbS_{1c} and HbC_{1c} from human blood samples.

Example 9:

Adult hemoglobin is a tetrameric protein, consisting of two α-subunits and two β-subunits, $\alpha_2\beta_2$. Structural variants of Hb contain genetically determined changes in the primary structure of the peptides. A, S and C differ only in the sixth amino acid residue from the N-terminal of the β-chain:

HbA_{1c} fructosyl-V-H-L-T-P-E-E-K-T-A-V-N-A-L-W-G-K.....
HbC_{1c} fructosyl-V-H-L-T-P-K-E-K-T-A-V-N-A-L-W-G-K.....
HbS_{1c} fructosyl-V-H-L-T-P-V-E-K-T-A-V-N-A-L-W-G-K.....

To test if the antibody is pan-specific, i.e. reacting with all structural variants of glycated hemoglobin, we have tested the reaction of DAK Hb1c-1 antibody with three 17-mer glycated peptides corresponding to the N-terminal end of glycated hemoglobin A, C and S β-subunits by ELISA (Figure 2). The peptide sequences are shown above. As a control a pool of red cell hemolysate from diabetes patients with hemoglobin A was also tested by DAK Hb1c-1 ELISA.

Monoclonal HbA_{1c} antibody clone HEM13 has been raised by immunization of mice with purified hemoglobin A1c. We have compared the reaction of DAK Hb1c-1 and HbA_{1c} antibody clone HEM13 with the glycated hemoglobin peptides and erythrocyte extract of hemoglobin A.

The affinity of DAK Hb1c-1 for the glycated hemoglobin peptides is significantly stronger than the affinity of HEM13 for the same peptides as judged by comparing the titration curves (see Figure 2 and Figure 3).

HEM13 shows different reactions with the A1c, S1c and C1c peptides (Figure 3) whereas DAK Hb1c-1 reacts in the same manner with all three peptides (Figure 1). These results demonstrates that the DAK Hb1c-1 antibody is pan-specific.

This example verifies that an antibody that reacts in the same manner with all structural variants of glycated hemoglobin can be raised by injection of a glycated 5-mer

peptide corresponding to the first 4 amino acids of all structural variants of glycated hemoglobin A, C and S β-chain plus one irrelevant amino acid (here cysteine).

Example 10:

Blood samples from the "ERL Educational Programme" have been tested. The HbA_{1c}-percentage of each sample has been determined by cation-exchange chromatography (IFCC/DCCT-values in table 2; IFCC: International Federation of Clinical Chemistry; DCCT: Diabetes Control and Complication Trial). A homozygotic blood sample consists of two identical β-subunits, e.g. AA or SS, whereas heterozygotic samples consists of two different hemoglobin β-subunits, e.g. AS or AC.

	IFCC	DCCT	DAK-Hb1c-1
HbAA	5.0	6.0	5.4
HbAA	5.7	6.5	6.2
HbAA	6.3	7.0	6.5
HbAA	7.0	7.6	7.9
HbAA	7.5	8.0	8.5
HbAA	8.1	8.4	8.7
HbAA	8.8	8.9	9.4
HbAA	9.4	9.4	9.7
HbAA	10.0	9.8	10.0
HbAS	4.3	5.5	4.7
HbAC	4.3	5.5	4.0

Table 2: Percentage of Hb1c in blood samples. The blood sample AC and AS contain heterozygotic hemoglobin.

At a range from 4-10% the HbA1c percentage was measured in whole blood samples using DAK Hb1c-1 in the above-mentioned ELISA format. HbA1c percentages at the same

levels as the values determined by reference methods recommended by IFCC and DCCT were obtained.

In summary, it is demonstrated that the antibody in question has the correct specificity against HbA1c, HbC1c and HbS1c.

Example 11:

The level of glycated hemoglobin was measured in a two-microsphere turbidimetry assay. In this set-up glycated hemoglobin was non-covalently adsorbed to microspheres followed by detection of the antigen with glycated hemoglobin antibodies covalently bound to microspheres. The resulting network constituting antigen microspheres complexed with immunomicrospheres were measured turbidimetrically.

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Applicant's or agent's file reference	International application No.
P062 WO 01	RO/DK 25 APR 2002

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 7, line 19.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b
D-38124 Braunschweig

Date of deposit	Accession Number
6 March 2001	DSM ACC2495

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on: 16 MAY 2002 (16.05.02)
Authorized officer	Authorized officer Jean-Luc MARTIN

Form PCT/RO/134 (July1998)

Claims:

1. A monoclonal antibody or a fragment thereof, wherein said antibody is pan-specific, i.e. able to react with all structural variants of glycosyl-hemoglobin, said antibody or a fragment thereof comprises an antibody combining site, which binds specifically to a glycated peptide residue of the formula:

Glycosyl-(NH)-Val-His- or Glycosyl-(NH)-Val-His-AA,

wherein Glycosyl-(NH)-Val represents a non-enzymatically glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin and AA means 1 to 3 amino acid residues.

2. A monoclonal antibody or a fragment thereof according to claim 1, wherein AA is a sequence of from 1 to 3 amino acids corresponding to a part of the N-terminal β-chain of all structural variants of human hemoglobin.

3. A monoclonal antibody or a fragment thereof according to claim 2, wherein AA is -Leu-Aa; -Leu-Thr-Aa; -Leu-Thr-Pro-Aa, and Aa designates the remaining number of amino acids in the AA group.

4. A monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody is obtainable by hybridoma DAK Hb1c-1 deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) as accession no. DSM ACC2495.

5. A monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody

is pan-specific, i.e. able to react with all structural variants of human hemoglobin including HbA1c, HbC1c and HbS1c.

6. A monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody being obtainable by a method as defined in any one of the claims 7-11.

7. A method of producing a monoclonal antibody or a fragment thereof, wherein said antibody has been raised against an immunogen comprising an antigen obtainable by a glycated peptide chemically linked to an immunogenic carrier material, the glycated peptide having from 2 to 5 amino acid units corresponding to the N-terminal β-chain of human hemoglobin.

8. A method of producing a monoclonal antibody or a fragment thereof according to claim 7, wherein the immunogen is of the formula:

[Fructosyl-(NH)-Val-His-AA-R]n-Carrier

wherein Fructosyl-(NH)-Val represents a non-enzymatic glycated valine residue, His represents the second amino acid in the native β-chain of hemoglobin, AA means one or more additional amino acid residues, R is a linking group, Carrier is an immunogenic carrier material, and n is an integer from 1 to the number of available coupling sites on said carrier.

9. A method of producing a monoclonal antibody or a fragment thereof according to claim 8, wherein AA is a sequence of from 1 to 3 amino acids corresponding to a

part of the N-terminal β-chain of all structural variants of human hemoglobin.

10. A method of producing a monoclonal antibody or a fragment thereof according to claim 8, wherein said Carrier is immunogenic proteins or peptides other than human hemoglobin.

11. A method of using a monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody binds specifically to said glycated N-terminal peptide sequence of human hemoglobin A1c, C1c and S1c ß-chains after having been exposed sufficiently to provide steric access thereto.

12. A method of using a monoclonal antibody or a fragment thereof according to claim 11, wherein said glycated peptide sequence is exposed to the antibody combining site by physical or chemical denaturation or digestion.

13. A method of using a monoclonal antibody or a fragment thereof according to claims 11-12, wherein said glycated peptide sequence is exposed to the antibody combining site by denaturation with a chaotropic agent.

14. A method of using a monoclonal antibody or a fragment thereof according to claim 13, wherein the chaotropic agent is selected from the group consisting of gluanidine, urea, potassium-thiocyanate, or detergent.

15. A method of using a monoclonal antibody or a fragment thereof according to any one of the preceding claims, wherein said antibody binds specifically to a glycated N-terminal peptide sequence of the β-chain of all

structural variants of human hemoglobin, which has been adsorbed to a solid phase.

16. A method of using a monoclonal antibody or a fragment thereof according to claim 15, wherein the solid phase preferably is constituted by a material selected from the group consisting of microtiter plates, micro particles, membranes, tubes, pins, chips or discs.

17. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated hemoglobin in human blood.

18. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated HbS and/or HbC in human blood.

19. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated heterozygous HbA, HbS and/or HbC in human blood.

20. Use of a monoclonal antibody or a fragment thereof according to anyone of the preceding claims for the determination of the level of glycated hemoglobin measured in a two-particle turbidimetric assay.

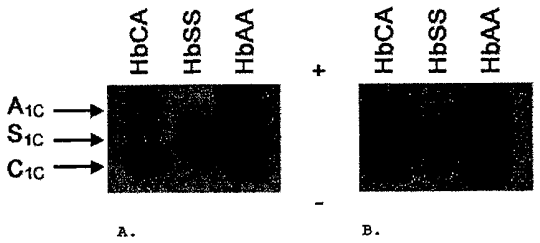

Figure 1

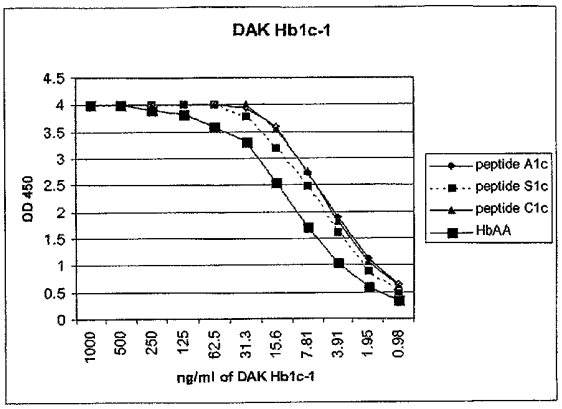

Figure 2: Reactivity of DAK Hb1c-1 with red cell hemolysate from patients with hemoglobin AA (HbAA) and 17-mer glycated peptides corresponding to hemoglobin A1c, C1c and S1c as determined by an ELISA test.

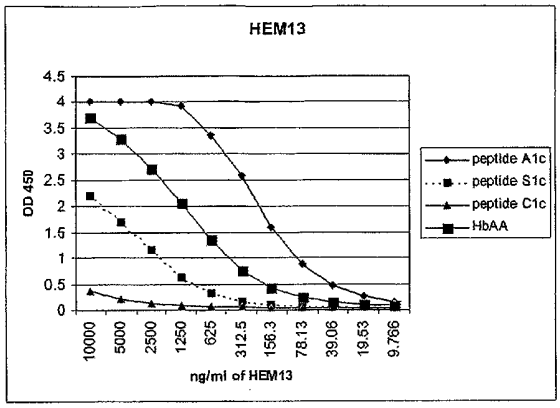

Figure 3. Reactivity of HEM13 with red cell hemolysate from patients with hemoglobin AA (HbAA) and 17-mer glycated peptides corresponding to hemoglobin A1c, C1c and S1c as determined by an ELISA test.

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK 02/00267

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/28 G01N33/577 G01N33/68 G01N33/72

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 C07K 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)
BIOSIS, WPI Data, EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 34 39 610 A (BOEHRINGER MANNHEIM GMBH) 30 April 1986 (1986-04-30) page 5, column 26-29 page 6, line 10-21 page 7, column 14-20 examples 4,6	1-20
X	US 4 727 036 A (KNOWLES WILLIAM J ET AL) 23 February 1988 (1988-02-23) column 1, line 47 -column 2, line 15 column 3, line 60 -column 4, line 10 column 10, line 13 -column 11, line 27 claims	1-20
A	EP 0 315 864 A (MILES INC) 17 May 1989 (1989-05-17) page 2, line 10-20 page 7, line 55 -page 11, line 39	20

☐ 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
12 May 2003	19/05/2003

Name and mailing address of the ISA	Authorized officer
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	COVONE-VAN HEES, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 02/00267

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 7-19 are directed to a method of treatment and/or diagnosis of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 02/00267

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摘要(译)

本发明描述了泛特异性单克隆抗体，用于测定人血液样品中糖化血红蛋白的所有结构变体的量。确定个体血液中血红蛋白的糖化速率提供了糖尿病患者中葡萄糖水平控制的有用指标。