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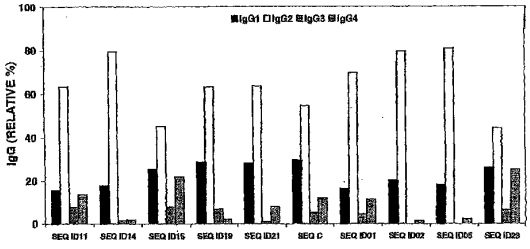
(54)【発明の名称】第ＶＩＩＩ因子の抗原エピトープ、当該エピトープに対して向けられたインヒビターおよびそれらの使用

(57)【要約】
本発明は、抗原性ポリペプチド配列に向けられたインヒビターに対する抗原性ポリペプチド配列（第ＶＩＩＩ因子のエピトープ）および前記インヒビターに向けられた抗インヒビターに関する。本発明はまた、少なくとも一つの上記分子を含有する医薬組成物及び診断装置に関する。
【選択図】図３



【特許請求の範囲】
【請求項１】
次のものから成る群より選択される第ＶＩＩＩ因子ポリペプチド配列の抗原エピトープ：
－　次の配列によって規定される、セリン２０１８からヒスチジン２０３１までを含有してなるエピトープ：
配列番号：１０：

Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
1 5 10

－　次の配列によって規定される、チロシン５５５からグルタミン５６５までのエピトープ：
配列番号：１７：

Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
1 5 10

－　次の配列によって規定される、ロイシン７３０からセリン７４１までのエピトープ：
配列番号：２０：

Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
1 5 10

末端のアミノ酸セリンおよび／または最初のアミノ酸ロイシンが欠失しているかもしれない
－　次の配列によって規定される、セリン８１７からセリン８３０までのエピトープ：
配列番号：２１：

Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
1 5 10

－　次の配列によって規定される、アスパラギン酸２１２８からアスパラギン酸２１３８までのエピトープ：
配列番号：２４：

Asn Val Asp Ser Ser Gly Ile Lys His Asn
1 5 10

－　次の配列によって規定される、セリン２２０４からグルタミン２２２２までのエピトープ：
配列番号：２７：

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

－　次の配列によって規定される、イソロイシン２２６２からグルタミン２２７０までのエピトープ：
配列番号：３０：

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

－　次の配列によって規定される、ロイシン２２７３からセリン２２８９までのエピトープ：
配列番号：３１：

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
1 5 10 15
Ser

－　次の配列によって規定される、プロリン２２９２からチロシン２３０５までのエピトープ：
配列番号：３２：

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

末端のトリペプチドＴｈｒ－Ａｒｇ－Ｔｙｒの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、グルタミン酸２３２２からチロシン２３３２までのエピトープ：
配列番号：３３：

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

【請求項２】
請求項１に記載の一つ以上の第ＶＩＩＩ因子の抗原配列エピトープ、および所望により次のものから成る群より選択される第ＶＩＩＩ因子の抗原配列エピトープを含有する第ＶＩＩＩ因子ポリペプチド配列の抗原エピトープのプール：
－　次の配列によって規定される、アルギニン１６４８からチロシン１６６４までのエピトープ：
配列番号：１：

Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1 5 10 15
Tyr

テトラペプチドＡｒｇ－Ａｓｐ－Ｉｌｅ－Ｔｈｒの一つ以上のアミノ酸が、またはペプチドの最後のアミノ酸Ａｓｐ－Ｔｙｒの一つまたは二つのアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸１６８１からアルギニン１６９６までのエピトープ：
配列番号：２：

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
1 5 10 15

エピトープのＡｓｐ－Ｇｌｕ－Ａｓｐ－Ｇｌｕの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、スレオニン１７３９からチロシン１７４８までのエピトープ：
配列番号：３：

Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
1 5 10

－　次の配列によって規定される、アスパラギン１７７７からフェニルアラニン１７８５までのエピトープ：
配列番号：４：

Asn Gln Ala Ser Arg Pro Tyr Ser Phe
1 5

末端のジペプチドＳｅｒ－ＰｈｅまたはテトラペプチドＰｒｏ－Ｔｙｒ－Ｓｅｒ－Ｐｈｅの一つまたは二つのアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、グルタミン酸１７９４からチロシン１８１５までのエピトープ：
配列番号：５：

Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
1 5 10 15
Asn Glu Thr Lys Thr Tyr
20

最初のトリペプチドＧｌｕ－Ａｓｐ－Ｇｌｎまたは最初のノナペプチドＧｌｕ－Ａｓｐ－Ｇｌｎ－Ａｒｇ－Ｇｌｎ－Ｇｌｙ－Ａｌａ－Ｇｌｕ－Ｐｒｏの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、メチオニン１８２３からアスパラギン酸１８３１までのエピトープ：
配列番号：６：

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

－　次の配列によって規定される、グルタミン酸１８８５からフェニルアラニン１８９１までのエピトープ：
配列番号：７：

Glu Thr Lys Ser Trp Tyr Phe
1 5

－　次の配列によって規定される、グルタミン酸１８８５からアラニン１９０１までのエピトープ：
配列番号：８：

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

ヘプタペプチドＧｌｕ－Ｔｈｒ－Ｌｙｓ－Ｓｅｒ－Ｔｒｐ－Ｐｈｅ－ＴｈｒのまたはトリペプチドＣｙｓ－Ａｒｇ－Ａｌａの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸１９０９からアルギニン１９１７までのエピトープ：
配列番号：９：

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

－　次の配列によって規定される、アラニン１０８からバリン１２８までのエピトープ：
配列番号：１１：

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

末端のアミノ酸アラニンおよび／またはバリンが欠失しているかもしれない

－　次の配列によって規定される、グルタミン酸１８１からロイシン１９２までのエピトープ：
配列番号：１２：

Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
1 5

末端のジペプチドＴｈｒ－Ｌｅｕの一つまたは二つのアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸２０３からアラニン２２７までのエピトープ：
配列番号：１３：

Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
1 5 10 15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
20

ノナペプチドＡｓｐ－Ａｒｇ－Ａｓｐ－Ａｌａ－Ａｌａ－Ｓｅｒ－Ａｌａ－Ａｒｇ－Ａｌａの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸３２７からメチオニン３５５までのエピトープ：
配列番号：１４：

Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
1 5 10 15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
20 25

ジペプチドＡｓｐ－Ｓｅｒの一つ以上のアミノ酸がまたはオクタペプチドＡｓｐ－Ａｓｐ－Ｌｅｕ－Ｔｈｒ－Ａｓｐ－Ｓｅｒ－Ｇｌｕ－Ｍｅｔの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸４０３からリジン４２５までのエピトープ：
配列番号：１５：

Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
1 5 10 15
Ile Gly Arg Lys Tyr Lys Lys
20

テトラペプチドＡｓｐ－Ａｓｐ－Ａｒｇ－Ｓｅｒの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、バリン５１７からアルギニン５２７までのエピトープ：
配列番号：１６：

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

ジペプチドＰｒｏ－Ａｒｇの一つまたは二つのアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、ヒスチジン６９３からグリシン７０１までのエピトープ：
配列番号：１８：

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

－　次の配列によって規定される、セリン７１０からアスパラギン酸７２５までのエピトープ：
配列番号：１９：
Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

－　次の配列によって規定される、イソロイシン２０８１からセリン２０９５までのエピトープ：
配列番号：２２：
Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

テトラペプチドＩｌｅ－Ｈｉｓ－Ｇｌｙ－Ｉｌｅの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、チロシン２１０５からグリシン２１２１までのエピトープ：
配列番号：２３：
Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15
Gly

トリペプチドＴｙｒ－Ｓｅｒ－Ｌｅｕの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、ヒスチジン２１５２からアルギニン２１６３までのエピトープ：
配列番号：２５：
His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg
1 5 10

－　次の配列によって規定される、セリン２１８１からアスパラギン２１９８までのエピトープ：
配列番号：２６：
Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr
1 5 10 15
Asn

末端のトリペプチドＰｈｅ－Ｔｈｒ－Ａｓｎ（Ｐ１１）の一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、グルタミン２２３５からロイシン２２５１までのエピトープ：
配列番号：２８：
Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

末端のジペプチドＳｅｒ－Ｌｅｕの一つまたは二つのアミノ酸がまたはテトラペプチドＶａｌ－Ｌｙｓ－Ｓｅｒ－Ｌｅｕの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、グリシン２２４２からロイシン２２５１までのエピト

ープ：
配列番号：２９：

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

末端のジペプチドＳｅｒ－Ｌｅｕの一つまたは二つのアミノ酸が欠失しているかもしれない。
【請求項３】
次のもののいずれかを含む立体配置的なエピトープ：
－　請求項１に記載の少なくとも二つの異なるエピトープ、または
－　請求項１に記載の少なくとも一つのエピトープ配列および請求項２に記載のエピトープ配列のプールから選択するエピトープ。
【請求項４】
請求項１に記載の抗原エピトープ配列の一つから選択されたアミノ酸配列または請求項２に記載のエピトープのプールから選択される一つ以上のエピトープ配列を有する組み換え第ＶＩＩＩ因子。
【請求項５】
請求項１または３に記載のエピトープ配列に結合されるキャリアタンパク質またはキャリアペプチドからなる複合体。
【請求項６】
請求項１から５のいずれか一つに記載のエピトープ配列との、エピトープ配列のプールとのおよび／または複合体との免疫親和性を示す第ＶＩＩＩ因子ポリペプチド配列のインヒビター。
【請求項７】
抗第ＶＩＩＩ因子抗体または抗体フラグメントである請求項６に記載のインヒビター。
【請求項８】
請求項６または７に記載の第ＶＩＩＩ因子インヒビターに向けられた抗インヒビター。
【請求項９】
抗－抗第ＶＩＩＩ因子のイディオタイプ抗体または抗体フラグメントである請求項８に記載の抗インヒビター。
【請求項１０】
適切な製薬学上の担体および、請求項１～９のいずれか一つに記載のエピトープ配列、エピトープ配列のプール、複合体、組み換え第ＶＩＩＩ因子、インヒビターおよび／または抗インヒビターから成る群より選択される少なくとも一つの成分を含む医薬組成物。
【請求項１１】
請求項１～９のいずれか一つに記載のエピトープ配列、エピトープ配列のプール、複合体、インヒビターおよび／または抗インヒビターから成る群より選択される少なくとも一つの成分を含む、診断用装置および／または精製用装置。
【請求項１２】
診断キットである請求項１１に記載の装置。
【請求項１３】
クロマトグラフィーカラムまたはフィルターである請求項１１に記載の装置。
【請求項１４】
第ＶＩＩＩ因子ポリペプチド配列のインヒビターによって引き起こされる哺乳動物の免疫障害を予防するおよび／または治療するための薬剤を調製するための請求項１０に記載の医薬組成物の使用。
【請求項１５】
哺乳動物（ヒトを含む）の患者から得られた体液（血清）の処理方法であって、前記体液中に存在する第ＶＩＩＩ因子ポリペプチド配列のインヒビターを前記クロマトグラフィーカラム中に含まれるエピトープ、プールまたは複合体に結合させるために、前記体液を請

求項１３に記載のクロマトグラフィーカラム中に添加し、カラムの溶出後、第ＶＩＩＩ因子ポリペプチド配列のインヒビターを有さない体液が哺乳動物の患者に再注入するために回収される方法。

【請求項１６】

請求項６～９のいずれか一つに記載のインヒビターおよび／または抗インヒビターを検出して入手するためのプロセスであって、次のステップを含むプロセス：

－　請求項１～５のいずれか一つに記載のエピトープ配列、エピトープ配列のプールおよび／または複合体からなる群から、固体担体（好ましくはクロマトグラフィーカラムの固体担体）に付着される一つの成分を選択するステップ、

－　患者から得られた第ＶＩＩＩ因子ポリペプチド配列のインヒビターを含有する体液（血清）を、前記クロマトグラフィーカラムに通過させるステップ、

－　前記カラムを溶出するステップ、

－　前記成分と免疫親和性を示した第ＶＩＩＩ因子ポリペプチド配列のインヒビターを含む画分を集めるステップ。

【請求項１７】

請求項１６に記載のプロセスであって、さらに次のステップを含むプロセス：

－　集められた第ＶＩＩＩ因子のインヒビターを固体担体（好ましくはクロマトグラフィーカラムの固体担体）上に付着させるステップと、

－　前記固体担体上に第ＶＩＩＩ因子の抗インヒビターを含む哺乳動物の患者由来の体液を通過させるステップと、

－　担体を好ましくは前記カラムを溶出することによって洗浄するステップ、および

－　前記第ＶＩＩＩ因子のインヒビターと免疫親和性を示した第ＶＩＩＩ因子の抗インヒビター含む画分を集めるステップ。

【発明の詳細な説明】

【０００１】

発明の主題

本発明は、抗第ＶＩＩＩ因子インヒビターに対する第ＶＩＩＩ因子の抗原性ポリペプチド配列（エピトープ）に関し、この抗第ＶＩＩＩ因子インヒビターはこれらのポリペプチド配列に向けられたものである。そして本発明は、当該抗第ＶＩＩＩ因子インヒビターに向けられた抗インヒビターに関する。

【０００２】

本発明はまた、少なくとも一つの上記分子を含有する医薬組成物および診断用デバイスに関する。

【０００３】

発明の技術的背景

第ＶＩＩＩ因子は、三つの構造ドメインＡ、ＢおよびＣから形成され、２，３３２のアミノ酸から成る巨大な複数ドメインタンパク質であり、これらのドメインはＡ１：ａ１：Ａ２：ａ２：Ｂ：ａ３：Ａ３：Ｃ１：Ｃ２の順番に配置されている。Ａドメインには４０％を超えるホモロジーがあり、そしてセルロプラスミンともホモロジーを有する（最近の総説であるプラット（２０００）およびサエンコ（１９９９）を参照すること）。第Ｖ因子のＡドメインおよび第ＶＩＩＩ因子のＡドメインとの間にも３０％のホモロジーが存在する。Ｃドメインは二回現れ、そして複合糖質および最終的に負電荷を帯びたリン脂質と結合することが可能であると報告されている。Ｃドメインには、負電荷を帯びたリン脂質と結合することが可能なレクチンとのホモロジーも見られる。血小板付着部位もこの領域（Ｃ２ドメイン）内に位置する（フォスターら（１９９０））。

【０００４】

これらの抗原決定基は、フラグメント３５１－３６５（Ａ１ドメイン重鎖）、フラグメント７１３－７４０（Ａ２ドメイン）、フラグメント１６７０－１６８４（Ａ３ドメイン軽鎖）（軽鎖のNH_2末端）から成るか、そうでなければ、フラグメント２３０３－２３３２（Ｃ２ドメイン軽鎖）（フォスター　シー、（１９９０））、フラグメント７０１－７

５０、フラグメント１６６３－１６８９、フラグメント３３０－４７２、フラグメント１６９４－１７８２（ＥＰ－０　２０２　８５３）、フラグメント３２２－７４０およびフラグメント２１７０－２３２２から成る。

【０００５】

米国特許第５，７４４，４４６号には、ブタの第ＶＩＩＩ因子またはネズミの第ＶＩＩＩ因子の対応する配列を伴った、Ａ２ドメインのフラグメント３７３－５４０、フラグメント３７３－５０８、フラグメント４４５－５０８、フラグメント４８４－５０８、フラグメント４０４－５０８、フラグメント４８９－５０８およびフラグメント４８４－４８９から成る群より選択されるアミノ酸配列を有するヒト／動物のハイブリッド第ＶＩＩＩ因子が記載されており、当該ハイブリッドは第ＶＩＩＩ因子欠乏症の治療に用いられる。

【０００６】

これらの種々の部位を認識する抗体は、第ＶＩＩＩ因子の活性化や、ｖＷｆ、第ＩＸａ因子、第Ｘａ因子、ＡＰＣまたはリン脂質の結合を妨害する。第ＶＩＩＩ因子に応答する特異的な抗体は、個体間でかなりのばらつきがあり、そしてインヒビター抗体についてのエピトープを、第ＶＩＩＩ因子のすべてのドメインについて確定する必要がある（最近の総説であるスキャンデラ、２０００；ローラー、２０００を参照すること）。

【０００７】

その他の抗体－インビトロでの標準的な活性試験を阻害しないもの－は、分子中の活性部位から実質的に少し離れた位置の部位にそれらの抗体が接触している間に、血液凝固カスケードのその他の構成成分と共に、第ＶＩＩＩ因子の挙動に影響を与えることが可能である。これらの抗体は、第ＶＩＩＩ因子の自然の状態のフォールディングを、いくつかの特性を変化させることによって、妨害することが可能である。

【０００８】

注入された第ＶＩＩＩ因子活性を中和する同種異系抗体（インヒビター）が出現することによって、第ＶＩＩＩ因子の代償療法が著しく困難になるかもしれない。報告されているインヒビターの血友病患者における発生率には、相当程度のばらつきがある。発生率は６－３５％前後の範囲に分布している（バーミレンら、１９９８）。たとえば大規模な欠失およびイントロン２２の逆位などのような遺伝的素因を有していそうな者は、インヒビターならびにＭＨＣクラスＩ遺伝子およびクラスＩＩ遺伝子として免疫応答に関与する遺伝子が合同して高頻度で見られる（タッデンハムおよびマックベイ、１９９８）。ある第ＶＩＩＩ因子の産物から別の産物に繰り返し切り替わること、およびいくつかの第ＶＩＩＩ因子濃縮物の免疫原性がより強いという可能性があることも、インヒビターが出現することの説明となるかもしれない（バーミレンら、１９９８）。第ＶＩＩＩ因子を調製するための方法が異なることが、その構造、物理化学的特性または本来の微小環境に影響を与えるかもしれない；ラウブら、（１９９９）；ラウトら、（１９９８））。臨床上の関連性を有する抗第ＶＩＩＩ因子の自己抗体は、非血友病患者の間ではまれである（集団における一年間の頻度：１－５／10^6）（モリソンおよびルドラム）（１９９５）。これらは多数の自己免疫疾患と関連性を有し、そして多くの場合、生命に関わる出血を特徴とする。他方、抗第ＶＩＩＩ因子抗体は健康な被験者中でも記載されており（エイルジマン（Ａｌｇｉｍａｎ）ら、１９９２；モロー（Ｍｏｒｅａｕ）ら、２０００）、ここでは、被験者における循環する第ＶＩＩＩ因子のレベルに何らの明白な影響も見られない。

【０００９】

炎症部位において、プロフェッショナル抗原提示細胞によってＣＤ４　Ｔ細胞に提示される場合、自己タンパク質または誘導されたペプチドは免疫応答を導き出すかもしれない。個々の第ＶＩＩＩ因子ドメインの配列をまたぐ、重なり合った合成ペプチドのプールを用いて、レディングら（２０００）は、健康な被験者および血友病患者において、第ＶＩＩＩ因子反応性$CD4^+$を示した。いくつかの第ＶＩＩＩ因子ドメインが認められた：Ａ３ドメインがより強くかつ高頻度で認められ、そして各ドメインはいくつかのエピトープを形成する。

【００１０】

第ＶＩＩＩ因子の明確なタンパク質分解フラグメント、多数の組み換えペプチドライブラリー、または合成ペプチドアレイを用いての、たとえばウエスタンブロッティング、免疫沈降、および酵素結合抗体免疫吸着アッセイ（ＥＬＩＳＡ）などの技術を利用して、主にＡ２ドメインおよびＣ２ドメイン中に位置する、異なる第ＶＩＩＩ因子－インヒビター結合部位のマッピングを行った。しかしながら、これらの技術はどれもインヒビターエピトープおよび非インヒビターエピトープを特定するためのモデルを構築することができなかった。わずかに数個のエピトープが別個の配列（＜２０アミノ酸残基）に対してマッピングされている。この問題を解決するために、パルマーら（１９９７）は、第ＶＩＩＩ因子の完全な配列の残基の８０％を表す９６種のウンデカマーペプチド（１１アミノ酸残基）を合成した。彼らは、９人の患者の阻害性抗体のエピトープ特異性を決定することに成功した。他の有用な技術は、第ＶＩＩＩ因子の遺伝子突然変異を分析することであり、そしてそのような突然変異による第ＶＩＩＩ因子分子ならびにファージディスプレイ技術（ファンデンブリンクら、２０００）への影響を分析することである。しかしながら、これらのすべての方法論は時間を要し、費用が高くつく傾向があり、そして患者を確保できるかどうかに大きく依存する。第ＶＩＩＩ因子分子のある特定の領域は、一般的に認識されているインヒビターエピトープのかたまり、たとえばＡ２ドメイン、Ａ３ドメイン、およびＣ２ドメイン内の領域などを含有する「ホットスポット」かもしれない。インヒビターの反応を引き起こす際のこれらの「ホットスポット」の理由には、まだ完全に理解されていない点が残っている（ライスナーら、１９９５）。

【００１１】

現在のところ、血友病患者、臨床医および「分離屋」の間で有力な考えは、すべての病原性の血漿汚染物質および二次作用が除かれた、利用可能な精製第ＶＩＩＩ因子を持つことである。

【００１２】

血友病の犬、重篤複合免疫不全マウス、血友病のマウスなどの異なる動物モデルを用いることは可能であった。しかし、現在までのところ、第ＶＩＩＩ因子調製品の免疫原性もしくは免疫調節効果を、または臨床的に宿主に投与する前にその宿主の感受性を予測することが可能な、満足できる実験モデルは存在しない。

【００１３】

抗第ＶＩＩＩ因子免疫応答が現れる患者は、危険で、攻撃的でかつ極めて高価な手段を講じることが必要な、深刻な状態にあることが分かる。

【００１４】

高い頻度で行われる治療の一つに、プロトロンビン複合体濃縮物と共同してまたは共同せずに、第ＶＩＩＩ因子を非常に多い投与量で投与すること（１５０ＩＵ／ｋｇ、一日に二回）によって免疫トレランスを誘導することがあり、「ボンプロトコール」とされる。治療の選択の余地としては、ＰＣＣ（好ましくは活性化ＰＣＣ〔ＡＰＣＣ〕）または第ＶＩＩａ因子を用いて、第ＶＩＩＩ因子インヒビター活性を回避することもある。これらの代替的な作用物質を注入した結果としての特異的抗体が産生されたが、治療を弱めるものであった。治療前または治療中に、ブタの第ＶＩＩＩ因子と実質的に交差反応を起こさない抗体を有する患者の止血を、代替的な作用物質としてブタの第ＶＩＩＩ因子を用いて実施できるかもしれない（ローラー、２０００）。

【００１５】

インヒビターの産生を阻害するための見込みのある代替的な研究手法としては、レセプター・リガンド結合シグナル事象（エベンスタインら、２０００）を経由して媒介されるＴ細胞／Ｂ細胞の協力を妨害することである。ヒトＴ細胞ＣＤ４０リガンド（ＣＤ　１５４）に対するヒト化マウスモノクローナル抗体を用いて、予備的な臨床試験を実施した。

【００１６】

インヒビターのレベルを下げるための有利な戦略は、総ＩｇＧの固相への吸着が可能となる体外循環に患者をさらすことにある。

【００１７】

免疫吸着は、総ヒト免疫グロブリンに対するセファロース結合スタフィロコッカスのプロテインＡまたはセファロース結合ポリクローナルヒツジ抗体となるかもしれない（クノッフおよびデルフラー、１９９９）。外来タンパク質（プロテインＡ、ヒツジ抗ヒトＩｇ）はカラムから漏出することもあり、そして臓器移植者の免疫機構を引き起こした；その上、衛生面（ＩＣＨトピックＱ５Ａ、指示書９２／７９／ＥＣ）での問題が生じるかもしれない。

【００１８】

多価の静注用免疫グロブリン（ＩＶＩＧ）の注入－ここでは免疫抑制療法と適切に組み合わせている－は、比較的有効であることが分かった。しかしながら、この有効性の理由はまだ完全には明らかにはなっていない。ＩｇＧ合成のフィードバック阻害、ＩｇＧクリアランスの刺激またはサプレッサーＴ細胞の活性化などの種々の仮説が提唱されてきた。一つの興味深い解釈は、これらの市販の静注用免疫グロブリンは、抗第ＶＩＩＩ因子抗体の種々の部分（イディオタイプ）と反応することができ、そしてこれらの抗体を中和することができる抗体を含んでいるのかもしれない、というものである（ディートリッヒら（１９９２））。

【００１９】

残念なことに、これらの研究手法のいずれも安全性、有効性、能力およびコストの点で満足できるものではないことが分かっている。

【００２０】

エピトープの構造の予測についての最新技術は、不連続なアミノ酸残基が最も重要なエピトープを構成しているらしいこと、および多くの場合、エピトープの構造予測を複雑な四次元の難問にせしめるエピトープの予測式に、結合の動力学を組み入れることができないこと（バンレゲンモーテル、方法：メソッズインエンザイモロジーの手引書、９、第４６５－４７２頁、１９９６）といった事実に制限されていた。

【００２１】

著者によれば、未処理のタンパク質に対して産生された抗体のほとんどは、親タンパク質に由来するあらゆるペプチドフラグメントとも反応せず、そしてこのことは、このような抗体が不連続のエピトープ（立体配置的なエピトープ）に対して向けられていることを示唆しているという。

【００２２】

この著者はさらに、抗原性の予測の成功率が低いのは、予測は連続的なエピトープだけを対象としているという事実が原因であり、そして立体的な特徴を常に一次元の直鎖ペプチドモデルに帰着させてエピトープの複雑さを緩和することは現実的ではないと述べている。

【００２３】

同様に、合成ペプチドアレイを用いて新規第ＶＩＩＩ因子インヒビターエピトープを特定しているパルマーら（１９９７）は、それぞれの患者の抗第ＶＩＩＩ因子抗体反応性のパターンがポリクローナルのように思われ、タンパク質のアミノ末端およびカルボキシル末端の範囲に位置する複数の部位に対して向けられており、そして調査されたそれぞれの血漿についてユニークであるらしいことを言及している（上記も参照すること）。さらに、この著者は、合成ペプチドアッセイだけでの結果に基づく第ＶＩＩＩ因子凝固活性に関して、あらゆる所定の抗体：エピトープ相互作用の持つ重要性を予測することは困難であることを言及している。（異なる第ＶＩＩＩ因子ドメインの構造と機能との間の関係について不完全な理解と、インヒビターおよび非阻害性抗体の両者が患者の血漿内に存在するかもしれないという可能性が原因である。）

【００２４】

したがって、最新技術の文献には、巨大分子（たとえば第ＶＩＩＩ因子など）上で抗原性直鎖ペプチドを特定することが示されておらず、第ＶＩＩＩ因子に対して向けられたインヒビターによって引き起こされる免疫障害の診断および／または治療に線状エピトープを用いることができるかもしれない。

【００２５】
国際特許出願第ＷＯ９６／０２５７２号には、第ＶＩＩＩ因子の抗原性フラグメントおよびエピトープ配列ならびにこれらの配列のいくつかに対して向けられたインヒビターが記載されている。
【００２６】
発明の目的
本発明は、免疫障害（特に第ＶＩＩＩ因子のインヒビター、とりわけ第ＶＩＩＩ因子の、フォンウィルブランド因子（ｖＷｆ）、第ＩＸ因子および／または膜のリン脂質（ＰＬ）との結合のインヒビターによって引き起こされる障害）の診断および／または（予防を含む）治療を改良するために、第ＶＩＩＩ因子の新規抗原エピトープを得ることを目的とし、当該エピトープによって、非阻害性のおよび阻害性の抗第ＶＩＩＩ因子同種異系抗体または自己抗体（同種異系免疫グロブリンまたは自己免疫グロブリン）の間でのスクリーニングが可能となる。
【００２７】
本発明の別の目的は、これらの抗原エピトープとの免疫親和性を示すインヒビターを得ること、および抗インヒビター、特に抗体または（Ｔ）細胞レセプターを得ることであり、この抗インヒビターは上記の当該インヒビターに対して向けられたものであり、その目的は、免疫障害の診断および／または治療（または予防）を改良することである。
【００２８】
本発明のさらなる目的は、治療および診断の分野でのＧＭＰ基準（ＩＣＨトピックＱＳＡ、指示書９２／７９／ＥＣなど）にしたがって、汚染物質（ウイルス、プリオンなど）がない高純度の当該分子を工業レベルで得ることである。
【００２９】
発明の概要
本発明は第ＶＩＩＩ因子の抗原性ポリペプチド配列（エピトープ）に関する。この因子の完全な配列はベルハーら（１９８４）によって記載され、そしてこの因子は、完全な第ＶＩＩＩ因子配列のアミノ酸番号の付与の基準を提供する。
【００３０】
「第ＶＩＩＩ因子の完全なポリペプチド配列」とは、自然なままのヒトの配列または動物の配列と理解され、これらのものはグリコシル化されていてもよく、そして血漿のプールから、特に寒冷沈降物内からの精製によって、合成によっておよび／または第ＶＩＩＩ因子の遺伝子操作によって得られたものである（血液凝固メカニズムには関与しない部分の配列は削除されていてもよい）。
【００３１】
本発明は特に－　次のものから成る群より選択される第ＶＩＩＩ因子の抗原エピトープ配列に関する：
－　次の配列によって規定される、セリン２０１８からヒスチジン２０３１までを含有してなるエピトープ：
配列番号：１０：

```
Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
1               5                  10
```

－　次の配列によって規定される、チロシン５５５からグルタミン５６５までのエピトープ：
配列番号：１７：

```
Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
1               5                  10
```

－　次の配列（Ｐ４）によって規定される、ロイシン７３０からセリン７４１までのエピトープ：

配列番号：２０：

Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
1 5 10

末端のアミノ酸セリン（Ｐ４）がおよび／または最初のアミノ酸ロイシンが欠失しているかもしれない

－　次の配列（Ｐ５）によって規定される、セリン８１７からセリン８３０までのエピトープ：

配列番号：２１：

Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
1 5 10

－　次の配列によって規定される、アスパラギン２１２８からアスパラギン２１３８までのエピトープ：

配列番号：２４：

Asn Val Asp Ser Ser Gly Ile Lys His Asn
1 5 10

－　次の配列（Ｐ１２）によって規定される、セリン２２０４からグルタミン２２２２までのエピトープ：

配列番号：２７：

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

－　次の配列によって規定される、イソロイシン２２６２からグルタミン２２７０までのエピトープ：

配列番号：３０：

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

－　次の配列（Ｐ１４）によって規定される、ロイシン２２７３からセリン２２８９までのエピトープ：

配列番号：３１：

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
1 5 10 15
Ser

－　次の配列（Ｐ１５）によって規定される、プロリン２２９２からチロシン２３０５までのエピトープ：

配列番号：３２：

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

リン脂質のフォンウィルブランド因子結合部位に関わる末端のトリペプチドＴｈｒ－Ａｒｇ－Ｔｙｒの一つ以上のアミノ酸が欠失しているかもしれない

－　次の配列（Ｐ１６）によって規定される、グルタミン酸２３２２からチロシン２３３２までのエピトープ：

配列番号：３３：

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

【００３２】
本発明はさらに、当該エピトープの主要な部分に関する。当該エピトープからは一つ以上の末端のアミノ酸、好ましくは一つ、二つまたは三つのアミノ酸を削除することが可能であり、または、親水性、柔軟性および接近可能性といった特性と同じ特性を示す一つ以上のアミノ酸によって、当該エピトープを置換することが可能である。
【００３３】
本発明のエピトープのいくつかが、ヒトインヒビターエピトープまたはいくつかの因子結合部位もしくは既知のモノクローナル抗体の結合部位、特にモノクローナル抗体Ｍａｓ５３１Ｐの結合部位として知られているＣ２部位もしくは結合部位ＥＳＨ８、ならびにリン脂質、第Ｘａ因子もしくはフォンウィルブランド因子結合部位の主な決定基内に含まれていることも知られている。しかしながら、本発明の特異的エピトープまたはそれらの主要な部分が、当該結合部位の好ましい部分として選択されるか、または当該結合部位との潜在的な重なり合いを含んでいてもよい。
【００３４】
これらのエピトープ配列は、パーカーおよびホッジズ（１９８６）によって規定される高い親水性、カープラスおよびシュルツ（１９８５）によって規定される高い柔軟性、およびジャニン（１９７９）によって規定される高い接近可能性という特に有利な特徴を有する。
【００３５】
これらのエピトープは、特に第ＶＩＩＩ因子タンパク質の表面上に露出しており、そして明白な抗原性および免疫原性の特性を示す。
【００３６】
本発明の別の側面は修飾（組み換えまたはトランスジェニック）第ＶＩＩＩ因子に関し、遺伝子工学によって得られ、そして上記に確認されたエピトープまたは当該エピトープの主要部分の一つ以上のものを削除されるかもしれない。
【００３７】
都合が良いことに、当該第ＶＩＩＩ因子はなお単数（または複数）の凝固因子の結合を許容するものの、免疫原性はより小さくなり、当該修飾第ＶＩＩＩ因子または天然第ＶＩＩＩ因子に対して向けられたインヒビターの形成を誘導しないかまたは誘導の程度がより小さくなるだろう。
【００３８】
当該エピトープはさらに独立して免疫原性を有し（すなわち、たとえばＢＳＡ、ＫＬＨ、ヘモシアニンなどのサイズが大きなタンパク質と複合化しなくても、これらのエピトープは免疫原性を有する）、そして好ましくは、たとえば抗第ＶＩＩＩ因子抗体などの第ＶＩＩＩ因子のインヒビターの内部での免疫親和性を示し、および／またはＴリンパ球およびおそらくＢリンパ球のレセプターについての免疫親和性を示す、という点も好都合である。
【００３９】
これらのエピトープおよび／または当該エピトープの主要部分は、これらがウサギの中に注入された場合、免疫反応（抗体の合成）を誘導する。
【００４０】
当該配列が、モノクローナル抗体およびポリクローナル抗体に対して実質的に免疫原性を有するという特徴は予想外であるものの、合成によって容易にかつ都合良く得るには、この配列は十分短い。
【００４１】
本発明はさらに、本発明の上記に特定された少なくとも二つの異なる配列のエピトープおよび／または少なくとも二つの当該エピトープの主要部分を含有する立体配置的なエピト

ープに関する。
【００４２】
この立体配置的なエピトープは二つ以上の異なるポリペプチド配列の部分から構成され、この部分は、タンパク質がその三次構造または四次構造にてフォールディングする場合、互いの近傍に位置する。
【００４３】
これらのエピトープは、第ＶＩＩＩ因子のインヒビターによって、特に（主要組織適合遺伝子座（ＭＨＣ　Ｉおよび／またはＭＨＣ　ＩＩ）を経由して）Ｂリンパ球およびＴリンパ球および／または抗第ＶＩＩＩ因子抗体（スキャンデラら（２０００）；レディングら、（２０００））によって、好ましくは同時に「認識される」（すなわち、免疫親和性を示す）という能力を有する。
【００４４】
より強力な免疫原性を示す複合体を形成するように、当該エピトープおよび／または当該エピトープの主要部分が、キャリアタンパク質またはキャリアペプチド、たとえばＢＳＡ、またはＫＬＨ、ヘモシアニンなどと複合体化することが好ましい。
【００４５】
本発明はさらに、上記の線状エピトープ（配列番号：１０、配列番号：１７、配列番号：２０、配列番号：２１、配列番号：２４、配列番号：２７、配列番号：３０、配列番号：３１、配列番号：３２、配列番号：３３）のエピトープ混合物を含有する第ＶＩＩＩ因子の抗原エピトープのプールに、または当該エピトープもしくは最新技術として既に記載されている少なくとも一つの当該エピトープおよび一つ以上のさらなる第ＶＩＩＩ因子の抗原エピトープを含有してもよいプールから作られる立体配置的なエピトープを含有する第ＶＩＩＩ因子の抗原エピトープのプールに関し、そして好ましくは、以下のものから成る群から選択されるものに関する：
－　次の配列によって規定される、アルギニン１６４８からチロシン１６６４までのエピトープ：
配列番号：１：

Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1 5 10 15
Tyr

テトラペプチドＡｒｇ－Ａｓｐ－Ｉｌｅ－Ｔｈｒ（Ｐ７）の一つ以上のアミノ酸が、またはジペプチドＡｓｐ－Ｔｙｒの最後の一つまたは二つのアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸１６８１からアルギニン１６９６（Ｐ８）までのエピトープ：
配列番号：２：

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
1 5 10 15

エピトープのＡｓｐ－Ｇｌｕ－Ａｓｐ－Ｇｌｕの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、スレオニン１７３９からチロシン１７４８までのエピトープ：
配列番号：３：

Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
1 5 10

－　次の配列によって規定される、アスパラギン１７７７からフェニルアラニン１７８５までのエピトープ：

配列番号：４：

Asn Gln Ala Ser Arg Pro Tyr Ser Phe
1 5

末端のジペプチドＳｅｒ－ＰｈｅまたはテトラペプチドＰｒｏ－Ｔｙｒ－Ｓｅｒ－Ｐｈｅの一つ以上のアミノ酸が欠失しているかもしれない

－　次の配列によって規定される、グルタミン酸１７９４からチロシン１８１５までのエピトープ：

配列番号：５：

Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
1 5 10 15
Asn Glu Thr Lys Thr Tyr

最初のトリペプチドＧｌｕ－Ａｓｐ－Ｇｌｎ（Ｐ９）または最初のノナペプチドＧｌｕ－Ａｓｐ－Ｇｌｎ－Ａｒｇ－Ｇｌｎ－Ｇｌｙ－Ａｌａ－Ｇｌｕ－Ｐｒｏの一つ以上のアミノ酸が欠失しているかもしれない

－　次の配列によって規定される、メチオニン１８２３からアスパラギン酸１８３１までのエピトープ：

配列番号：６：

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

－　次の配列によって規定される、グルタミン酸１８８５からフェニルアラニン１８９１までのエピトープ：

配列番号：７：

Glu Thr Lys Ser Trp Tyr Phe
1 5

－　次の配列によって規定される、グルタミン酸１８８５からアラニン１９０１までのエピトープ：

配列番号：８：

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

ヘプタペプチドＧｌｕ－Ｔｈｒ－Ｌｙｓ－Ｓｅｒ－Ｔｒｐ－Ｐｈｅ－ＴｈｒのまたはトリペプチドＣｙｓ－Ａｒｇ－Ａｌａの一つ以上のアミノ酸が欠失しているかもしれない

－　次の配列によって規定される、アスパラギン酸１９０９からアルギニン１９１７までのエピトープ：

配列番号：９：

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

－　次の配列によって規定される、アラニン１０８からバリン１２８までのエピトープ：

配列番号：１１：

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

末端のアミノ酸アラニンおよびバリン（Ｐ１）が欠失しているかもしれない
－　次の配列によって規定される、グルタミン酸１８１からロイシン１９２までのエピトープ：
配列番号：１２：

Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
1 5

末端のジペプチドＴｈｒ－Ｌｅｕの一つまたは二つのアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸２０３からアラニン２２７までのエピトープ：
配列番号：１３：

Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
1 5 10 15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
20 25

ノナペプチドＡｓｐ－Ａｒｇ－Ａｓｐ－Ａｌａ－Ａｌａ－Ｓｅｒ－Ａｌａ－Ａｒｇ－Ａｌａの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸３２７からメチオニン３５５までのエピトープ：
配列番号：１４：

Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
1 5 10 15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
20 25

末端のジペプチドＡｓｐ－Ｓｅｒの一つ以上のアミノ酸がまたはオクタペプチドＡｓｐ－Ａｓｐ－Ｌｅｕ－Ｔｈｒ－Ａｓｐ－Ｓｅｒ－Ｇｌｕ－Ｍｅｔ（Ｐ２）の一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、アスパラギン酸４０３からリジン４２５までのエピトープ：
配列番号：１５：

Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
1 5 10 15
Ile Gly Arg Lys Tyr Lys Lys
20

テトラペプチドＡｓｐ－Ａｓｐ－Ａｒｇ－Ｓｅｒ（Ｐ３）の一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、バリン５１７からアルギニン５２７までのエピトープ：
配列番号：１６：

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

ジペプチドＰｒｏ－Ａｒｇの一つまたは二つのアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、ヒスチジン６９３からグリシン７０１までのエピトープ：
配列番号：１８：

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

－　次の配列（Ｐ４）によって規定される、セリン７１０からアスパラギン酸７２５までのエピトープ：
配列番号：１９：

Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

－　次の配列によって規定される、イソロイシン２０８１からセリン２０９５までのエピトープ：
配列番号：２２：

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

テトラペプチドＩｌｅ－Ｈｉｓ－Ｇｌｙ－Ｉｌｅの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、チロシン２１０５からグリシン２１２１までのエピトープ：
配列番号：２３：

Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15
Gly

トリペプチドＴｙｒ－Ｓｅｒ－Ｌｅｕ（Ｐ１０）の一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列（Ｐ１３）によって規定される、グルタミン２２３５からロイシン２２５１までのエピトープ：
配列番号：２８：

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

末端のジペプチドＳｅｒ－Ｌｅｕの一つまたは二つのアミノ酸がまたはテトラペプチドＶａｌ－Ｌｙｓ－Ｓｅｒ－Ｌｅｕの一つ以上のアミノ酸が欠失しているかもしれない
－　次の配列によって規定される、グリシン２２４２からロイシン２２５１までのエピトープ：
配列番号：２９：

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

末端のジペプチドＳｅｒ－Ｌｅｕの一つまたは二つのアミノ酸が欠失しているかもしれず

、当該エピトープは既知のモノクローナル抗体結合部位ＥＳＨ８　２２４８－２２８５と部分的に重なり合っている可能性を示している。

【００４６】

本発明の別の側面は、抗原エピトープとの、当該エピトープの主要部分とのおよび／または本発明の複合体との免疫親和性を示す第ＶＩＩＩ因子のインヒビターに関する。

【００４７】

インヒビターとは、当該第ＶＩＩＩ因子に結合し、そして（当該第ＶＩＩＩ因子に対する体液性免疫応答および／または細胞性免疫応答を特徴とする）免疫障害を引き起こす能力を有する、あらゆる生体分子または細胞（たとえばＴリンパ球など）を意味すると理解される。

【００４８】

特に、このようなインヒビターは、当該第ＶＩＩＩ因子を不活性化する、および／またはフォンウィルブランド因子へのおよび／または膜のリン脂質への第ＶＩＩＩ因子の結合を阻害する抗第ＶＩＩＩ因子モノクローナル抗体またはポリクローナル抗体もしくは抗体フラグメント（たとえば当該抗体の超可変性Ｆａｂ部分）である可能性がある。

【００４９】

ヒトの免疫機構を含む「キメラ」動物－たとえば、ヒト抗体を産生するｈｕ－ＳＣＩＤマウスまたはトランスジェニックマウスなど－によって、またはファージディスプレイ技術または特にＥＰＶによる不死化Ｂ細胞のようなその他の抗体産生技術によって当該インヒビターが合成されることは好都合である。

【００５０】

本発明の別の側面は、既に記載されている当該第ＶＩＩＩ因子インヒビターに対して向けられた抗インヒビターに関する。

【００５１】

第ＶＩＩＩ因子インヒビターに対して向けられた抗インヒビターは、確実に不活性化するか、または第ＶＩＩＩ因子への結合を防止するもしくは弱めるというような方法によって当該インヒビターを妨害する能力を有する、あらゆる化学物質または生体分子、細胞および／または細胞フラグメント（レセプター）を意味すると理解される。

【００５２】

このような抗インヒビターは、天然のまたは遺伝子工学によって得られる、抗－抗第ＶＩＩＩ因子のイディオタイプ（モノクローナルまたはポリクローナル）抗体または抗体フラグメントであることが好ましい。

【００５３】

本発明の別の側面は、適切な製薬学上の担体または希釈剤および当該エピトープまたはそのプール、それらエピトープに対して向けられた第ＶＩＩＩ因子のインヒビター、当該インヒビターに対して向けられた抗インヒビター、および／またはこれらの混合物から成る群より選択される成分を含む医薬組成物に関する。

【００５４】

当該医薬組成物中に存在する適切な製薬学上の担体または希釈剤（そしてもしかすると補助剤または賦形剤）のタイプおよび量は投与方法にしたがって変化してもよく、そして本発明の医薬組成物の治療上の特性を改良するために、または起こり得る副作用を減少させるために、補助剤を組み合わせることもあり得る。本発明の医薬組成物において用いられる適切な製薬学的に許容される担体は、当業者によって周知のものであり、薬剤師によって一般的に用いられる方法にしたがって選択され、そして固体、液体または気体の無害の製薬学的に許容される担体が挙げられる。活性成分／製薬学的に許容される担体のパーセンテージについては、極めて大きな範囲内で変更してもよいが、（ヒトを含む）患者の耐性および患者への考えられる副作用、ならびに投与の頻度および／または投与様式によってのみ制限される。

【００５５】

これらのエピトープおよび／または当該エピトープの主要部分、本発明の複合体またはそ

れらのプール、それらに対して向けられたインヒビター、当該インヒビターに対して向けられた抗インヒビター、および／またはそれらの混合物から成る群より選択される成分を含む、診断用装置および／または精製用装置、たとえば診断キット、アフィニティーフィルター、またはクロマトグラフィーカラムに関する。当該装置は、患者のスクリーニングを可能とし、当該患者において存在する最も重要なインヒビターを検出でき、そして十分な特異性および感度を伴う陽性試験を可能とする当該エピトープのプールを含む点で都合が良い。

【００５６】

したがって、精製用装置は、これらのエピトープおよび／またはエピトープの主要部分を含み、クロマトグラフィーカラムの固相に付着しているクロマトグラフィーカラムで構成されることも可能である。

【００５７】

患者に由来し、そして第ＶＩＩＩ因子のインヒビターを含有する体液（たとえば血清など）が、当該インヒビター（たとえば抗体など）が当該エピトープもしくは当該主要部分またはそのプールに特異的に付着しながら固体担体（クロマトグラフィーカラム）を通過する。溶出の後に、抗インヒビター（抗－抗第ＶＩＩＩ因子のイディオタイプ抗体）と反応させることによって、当該インヒビターを集めることが可能である。

【００５８】

第ＶＩＩＩ因子のインヒビターがその固相上に付着している固体担体（クロマトグラフィーカラム）を通過したこれらの抗インヒビターによって、血清中に存在する抗－抗第ＶＩＩＩ因子のイディオタイプ抗体をキャラクタライズすることも可能である。

【００５９】

当該エピトープまたはそのプールと結合させることによって第ＶＩＩＩ因子のインヒビターを除去した後で、当該患者に体液（血液または血清もしくは得られる分画）を再注入すること（生体外での治療）も可能である；当該インヒビターは、ヒト患者に適用される透析法に推奨されているのと同様に、体液（血液または血清）から取り除かれる。

【００６０】

本発明はさらに、第ＶＩＩＩ因子に対するインヒビターによって引き起こされる病状を示す患者を治療する方法（生体外での治療）であって、患者から当該体液（血液または血清）を抽出する工程、本発明のエピトープまたはそのプールが結合している固体担体上で反応を行う工程、そして当該エピトープ、主要部分またはそのプールが固定されたインヒビターを除去した後で当該体液を患者に再注入する工程を含む方法に関する。

【００６１】

本発明の最後の側面は、免疫障害、特に第ＶＩＩＩ因子のインヒビターによって、第ＶＩＩＩ因子の第ＩＸ因子および／または第Ｘ因子および／またはフォンウィルブランド因子（ｖＷＦ）への結合のインヒビターによって、および／または第ＶＩＩＩ因子の膜のリン脂質への結合のインヒビターによって引き起こされる免疫障害を予防するおよび／または治療するために用いられる薬剤を調製するための本発明の医薬組成物の使用に関する。

【００６２】

添付された図を参照しつつ次の非限定的な実施例において、本発明を具体的に説明する。

【００６３】

図面の簡単な説明

図１は、第ＶＩＩＩ因子のＡ３配列の１から３７１に番号を付け替えられたアミノ酸の親水性、柔軟性および接近可能性の（それぞれのアミノ酸についての表面値の）グラフを表す。

【００６４】

図２ａは、ペプチド－セファロースカラム上でのアフィニティークロマトグラフィーによる、ヒト抗－配列番号：３２抗体の精製に関する溶出プロファイルを表す。コーン画分ＩＩ＋ＩＩＩ溶液（５０ｍｌ）をカラム（１ｍｌのゲル）上に添加し流速を１ｍｌ／分とした。実施例に記載されたようにして、特異的抗体の分離を実施した。矢印は、特異的ヒト

抗－配列番号：３２抗体の位置を示す。

【００６５】

図２ｂは、コーン画分ＩＩ＋ＩＩＩから精製された抗－（配列番号：３２）ＩｇＧの存在下での第ＶＩＩＩ因子凝固活性を表す。実施例に記載されたようにして、その量が増加する抗－配列番号：３２の存在下で、第ＶＩＩＩ因子の凝固活性を測定した。第ＶＩＩＩ因子活性の％＝（抗体存在下での第ＶＩＩＩ因子活性／抗体非存在下での第ＶＩＩＩ因子活性）×１００。

【００６６】

図３は、ウエスタンブロッティング後の、第ＶＩＩＩ因子ポリペプチドに対するヒト抗ペプチド抗体の免疫反応を表す（パネルＡの左側から右側への方向：ＨＡＰ１からＨＡＰ４までのヒト抗体であり、第ＶＩＩＩ因子ＨＣ内で見られた異なる第ＶＩＩＩ因子エピトープ配列について特異的である－表２も参照すること、そしてパネルＢ：Ｐ５ペプチドおよび第ＶＩＩＩ因子ＬＣ配列、Ｐ７、Ｐ８およびＰ９について特異的なヒト抗体－表２も参照すること）。ＲＡＰ９のレーンは、ペプチド配列Arg^{1797}－Tyr^{1815}について特異的な精製ウサギ抗体に対する第ＶＩＩＩ因子ポリペプチドの反応性を示す（表２も参照すること）。

【００６７】

図４は、４種のインヒビター血漿の、異なるペプチド配列とのＥＬＩＳＡでの反応性を表す。４人の患者の血漿中に存在するインヒビターを、選択された異なる第ＶＩＩＩ因子エピトープ合成ペプチドを被覆抗原として用いるＥＬＩＳＡ試験によって、縦軸で表されているように分析した。

【００６８】

実施例

材料と方法

試薬

ＭＡＳ５３０ｐ（ハーラン－セララブ社、インディアナポリス、インディアナ州）は、第ＶＩＩＩ因子重鎖の４４ｋＤａのＡ２ドメインに対して特異的なマウスモノクローナル抗体である。ビオチン標識化ウサギＩｇＧ－抗マウスＩｇＧをダコパッツ社（コペンハーゲン、デンマーク）から購入した。ビオチン標識化ヤギＩｇＧ－抗ヒトＩｇＧおよびビオチン標識化マウスＩｇＧ－抗ウサギＩｇＧをシグマケミカルズ社（セントルイス、ミズーリ州）から入手し、精製α－トロンビン（３０００ＩＵ／ｍｇ）、ストレプトアビジン－ペルオキシダーゼ複合体、オボアルブミン（ＯＶＡ）、ウシ血清アルブミン（ＢＳＡ）、スカシ貝ヘモシアニン（ＫＬＨ）、およびｏ－フェニレンジアミン（ＯＰＤ）をシグマケミカルズ社（セントルイス、ミズーリ州）から購入した。カゼインをメルク社（ダルムシュタット、ドイツ）から入手した。４－クロロ－１－ナフトールおよびビオチン化分子量マーカーをバイオ－ラッドラボラトリーズ社（ハーキュリーズ、カリフォルニア州）から入手した。フロイントのアジュバントはディフコ社（デトロイト、ミシガン州）からのものであった。

【００６９】

第ＶＩＩＩ因子濃縮物

血漿第ＶＩＩＩ因子（ｐ－ＦＶＩＩＩ）は、イオン交換クロマトグラフィーで精製した溶媒／界面活性剤処理済み第ＶＩＩＩ因子濃縮物（タンパク質１ｍｇあたり１００ＩＵ）（ＦＶＩＩＩ　Ｃｏｎｃ．　ＳＤ、シーエーエフ－ディーシーエフ赤十字社、ブリュッセル、ベルギー）であった。アルブミンを含まない組み換え第ＶＩＩＩ因子（ｒＦＶＩＩＩ）を、ハイランド社（グレンデール、カリフォルニア州）から入手した。

【００７０】

血漿分画免疫グロブリン

４，８００人の無給の提供者由来の多量の血漿プールを、徐々にエタノール濃度を高めて沈殿させた後のものから、コーン（Ｃｏｈｎ）分画ＩＩ＋ＩＩＩを得た。この画分は、すべてのＩｇクラスおよびサブクラスを含有する。比濁法によってＩｇＧの組成を測定した

。各サブクラスの相対的なパーセントは、ＩｇＧ１、ＩｇＧ２、ＩｇＧ３およびＩｇＧ４についてそれぞれ６３．７；３０．１；３．４および２．８であった（分画ＩＩ＋ＩＩＩの三つの異なるバッチについての平均値）。

【００７１】

第ＶＩＩＩ因子濃縮物、第ＶＩＩＩ因子の活性および活性の阻害

コアグロメーターＫＣ４Ａ（シグマダイアグノスティックス社）上で用いるように適合化させたワンステージ凝固アッセイにて、第ＶＩＩＩ因子の活性を測定した。このアッセイでは、危険な血友病Ａ血漿（オラガノンテクニカ社、ケンブリッジ、イギリス）とインスツルメンテイションラボラトリー社（ウォリントン、イギリス）のＡＰＰＴ試薬とを用いる。第５国際標準第ＶＩＩＩ因子濃縮物８８／６４０（５．４ＩＵ／ｍｌ）（エヌアイビーエスシー（ＮＩＢＳＣ）社、ポッターズバー、イギリス）と相対化することによって、有効性を算出した。ベセズダアッセイの変法にしたがって、精製ウサギＩｇＧ調製品および精製ヒトＩｇＧ調製品にて第ＶＩＩＩ因子阻害活性を測定した。簡単に言えば、アフィニティー精製されたＩｇＧを連続的に希釈し、そして第ＶＩＩＩ因子濃縮物８８／６４０（１ＩＵ／ｍｌ）の存在下、３７℃にて１時間インキュベートした。残りの第ＶＩＩＩ因子の活性を、上記のようにして測定した。

【００７２】

α－トロンビンによる第ＶＩＩＩ因子の活性化およびイムノブロッティングは、他の箇所に記載した（ピールリンクら、１９９７）。

【００７３】

ペプチドの合成、ペプチドのキャリアタンパク質への複合化およびウサギ抗ペプチド抗血清の作製は、ネオシステム社（ストラスブール、フランス）によって行われた。

【００７４】

アフィニティークロマトグラフィーによるウサギ抗体およびヒト抗体の精製

ウサギ抗体およびヒト抗体を精製するために、製造メーカーの取扱説明書にしたがって、５ｍｇの異なるペプチドをそれぞれ１ｍｌのプレパックのＮＨＳ－活性化セファロース（ファルマシアバイオテック社、ウプサラ、スウェーデン）に結合させた。自動液体クロマトグラフィーシステム（ＡＥＫＴＡｅｘｐｌｏｒｅｒ　１００Ａ、ファルマシア社、ウプサラ、スウェーデン）を用いて、５０ｍｌのウサギ抗血清、またはコーン分画（分画ＩＩ＋ＩＩＩ；１３ｍｇタンパク質／ｍｌ）の後に得られた１００ｍｌのヒト血漿分画のいずれか一方から、特異的な抗ペプチド抗体を精製した。簡単に言えば、５倍の体積のＴＥ緩衝液（２０ｍＭのトリス－塩酸、ｐＨ７．２、１５０ｍＭのＮａＣｌおよび０．０２％のNaN_3）に対してサンプルを３回透析し、そして１ｍｌ／分の流速にてカラム上に添加した。２ｍｌ／分にて、５０ｍｌのＴＥ緩衝液および１ＭのＮａＣｌを含有する３０ｍｌのＴＥを用いて、このカラムを順次洗浄した。吸着後に、５ｍｌの０．１Ｍのクエン酸、ｐＨ２．５によって物質を（１ｍｌ／分で）溶出し、そして５ｍｌの１Ｍのトリス－塩酸、ｐＨ９．０中に直接回収した。最後に、サンプルを１０倍の体積の平衡化緩衝液に対して透析し、そしてＣｅｎｔｒｉｐｒｅｐ－３０（アミコン社、ビバリー、マサチューセッツ州）で濃縮した。バイオラッドタンパク質アッセイによって、Ｉｇの回収を測定した。

【００７５】

結果

第ＶＩＩＩ因子の線状エピトープの可能性があるものの選択

８から２５残基の範囲の、３０を超える表面領域（線状エピトープ）－高い親水性、高い柔軟性および高い接近可能性に特徴を有する－が第ＶＩＩＩ因子分子上で特定された。これらが外側の位置にある可能性が高いことに基づいて（Ａ３について図１を参照すること）、特定された、１３個またはそれを超える鎖長のアミノ酸残基と適合する１６種の直鎖ペプチド（Ｐ１からＰ１６）を選択した。これらのペプチドを合成し、そして特異的抗血清を作製するためにオボアルブミンと結合させた（以下では表１）。Ｐ８は、シマら（１９８８）に記載されたエピトープを含み、外部コントロールとして用いた。

【００７６】

ウサギモデルを用いて当該合成線状エピトープから得られた実験結果

ウサギ抗第ＶＩＩＩ因子－ペプチド抗血清および回収されたアフィニティー精製免疫グロブリンの特性に関する結果を、表１に要約している。

【００７７】

Ａドメイン、Ｂドメイン、Ｃ１ドメインおよびＣ２ドメインの中から、１６種の合成ペプチド（１０から２０アミノ酸）を選択した。オボアルブミンとの複合化の後に、ＯＶＡ－ペプチド複合体をウサギの中に注入し、そして第ＶＩＩＩ因子抗ペプチド抗血清のＲＡＰ１からＲＡＰ１６について調査した。

【００７８】

より正確に言えば、二羽のウサギをそれぞれ第ＶＩＩＩ因子－ペプチド－オボアルブミン調製品を用いて免疫化した。特異的抗血清のＲＡＰ１からＲＡＰ１６（カラムｂ、表１）を調製し、そしてｒＦＶＩＩＩまたは第ＶＩＩＩ因子－ペプチド－ＫＬＨを抗原として用いるＥＬＩＳＡ（カラムｃ、表１）にてアッセイを行った。５０％が結合する血清の希釈率の逆数の対数に負号をつけたものとして、ＥＬＩＳＡの力価を表現する。次いで、ペプチド結合セファロースでのクロマトグラフィーによって、免疫グロブリンを精製した。イムノブロッティングの後に抗第ＶＩＩＩ因子ペプチドＩｇによって認識された第ＶＩＩＩ因子のドメインを（カラムｄ、表１に）示し、そしてＩｇタンパク質の回収の程度を、免疫グロブリンを標準として用いて測定した（カラムｅ、表１）。ＢＵ／ｍｇタンパク質で表示される阻害活性を、第ＶＩＩＩ因子中和活性アッセイにて測定した（カラムｆ、表１）。

【００７９】

第ＶＩＩＩ因子ペプチドの免疫原性およびウサギ抗第ＶＩＩＩ因子ペプチド抗血清のキャラクタライゼイション

ＫＬＨタンパク質に結合する第ＶＩＩＩ因子－ペプチドであって、対応する異なる第ＶＩＩＩ因子－ペプチド、または精製ｒＦＶＩＩＩのいずれか一方を抗原として用いるＥＬＩＳＡによって、第ＶＩＩＩ因子抗ペプチド抗血清の反応性を測定した。それぞれの抗第ＶＩＩＩ因子－ペプチド抗血清の結合反応は、ウサギでの免疫応答を導くために用いられる第ＶＩＩＩ因子ペプチド、およびｒＦＶＩＩＩの両者について特異的であった（表１を参照すること）。

【００８０】

ウサギ抗ペプチド抗体の第ＶＩＩＩ因子エピトープの特異性を明らかにするために、ｒＦＶＩＩＩおよびトロンビンにて処理した後に得られるｒＦＶＩＩＩのフラグメントをＳＤＳ－ＰＡＧＥによって分離し、そしてウサギＩｇＧの異なる調製品を用いるウエスタンブロッティングによって分析した。予想された通り、選択された線状エピトープを含有する、対応する第ＶＩＩＩ因子フラグメントに対して、ほとんどの抗血清（１４／１６、８７％）が強く反応することが示された（表１を参照すること）。

【００８１】

ウサギ抗第ＶＩＩＩ因子ペプチド抗体の精製

下記の方法に記載の通りに、ペプチド－セファロースにてのアフィニティークロマトグラフィーによって特異的ウサギＩｇＧを精製した。ワンステージ凝固アッセイにて第ＶＩＩＩ因子中和活性を測定した場合、二種のウサギＩｇＧ精製調製品：配列番号：１４について特異的なＩｇＧに対応するＲＡＰ２および配列番号：０１について特異的なＩｇＧに対応するＲＡＰ７による顕著な阻害が見られた。

【００８２】

ヒトｒＦＶＩＩＩを用いるイムノブロッティングによる、ウサギ抗第ＶＩＩＩ因子ペプチド抗体のエピトープマッピング

ウサギ抗ペプチド抗体の第ＶＩＩＩ因子エピトープの特異性を明らかにするために、ｒＦＶＩＩＩおよびトロンビンにて処理した後に得られるｒＦＶＩＩＩのフラグメントをＳＤＳ－ＰＡＧＥによって分離し、そしてウサギＩｇＧの異なる調製品（ＲＡＰ１からＲＡＰ１７のＩｇ）を用いるウエスタンブロッティングによって分析した。

【００８３】
それぞれ重鎖および軽鎖について特異的な二種のモノクローナル抗体であるＭｏＡｂ５３０ｐおよびＭｏＡｂｌ８の混合物を用いて、それぞれのレーンにおいて、ｒＦＶＩＩＩの重鎖（ＨＣ）および軽鎖（ＬＣ）ならびにそれらのトロンビンによるタンパク質分解産物（４４ｋＤａおよび７２ｋＤａ）を特定した。ＭｏＡｂ１８は、トロンビンによる活性化後に得られる、第ＶＩＩＩ因子の軽鎖フラグメントのNH_2末端を認識し、このフラグメントはあまりにも小さいため、電気泳動後のゲルに残っていることを検証することができなかった。１７種のウサギ免疫グロブリン調製品のうちの１４種が、ｒＦＶＩＩＩおよびｐＦＶＩＩＩの両者と強く反応した。抗血清のＲＡＰ１、ＲＡＰ２、ＲＡＰ３、ＲＡＰ４は、（２００ｋＤａから９２ｋＤａの）重鎖を独占的に認識した。抗血清のＲＡＰ１およびＲＡＰ２は５０－ｋＤａのＡ１ドメインフラグメントと反応し；ＲＡＰ３およびＲＡＰ４は４４－ｋＤａのフラグメント（ドメインＡ２）と結合し；（Ｂドメインについて特異的な）ＲＡＰ５は高分子量の第ＶＩＩＩ因子重鎖（約２００－ｋＤａ）と結合した。

【００８４】
ＲＡＰ７、ＲＡＰ８、およびＲＡＰ９は、８０－ｋＤａの軽鎖のダブレットと反応した。ＲＡＰ９およびＲＡＰ１２からＲＡＰ１７の抗体は、７２－ｋＤａの第ＶＩＩＩ因子軽鎖フラグメントも検出した。予想された通り、それぞれの反応性抗血清は、対応する、選択された線状エピトープを含有する第ＶＩＩＩ因子フラグメントと強く反応することが示された。ＲＡＰ６またはＲＡＰ１０と、ＨＣ第ＶＩＩＩ因子フラグメントまたはＬＣの第ＶＩＩＩ因子フラグメントとの間の反応は、ゲル中では検出できなかった。

【００８５】
ヒト自己抗体を精製し、キャラクタライズするための当該合成線状エピトープから得られた実験結果
表２は、健康な被験者のコーン画分ＩＩ＋ＩＩＩに由来するヒト抗第ＶＩＩＩ因子抗体のキャラクタライゼイションに関する。

【００８６】
今までのところ、１３種の異なる第ＶＩＩＩ因子ペプチドに結合したセファロースにて、ヒト抗ペプチドＩｇＧ調製品（ＨＡＰ１からＨＡＰ１７まで）を精製した（カラムａ、表２）。これらのＩｇ（カラムｂ、表２）をイムノブロッティングによって分析した。ｒＦＶＩＩＩのＨＣ鎖またはＬＣ鎖への結合、そしてｒＦＶＩＩＩのトロンビンフラグメントへの結合を、それぞれ表２のカラムｃおよびｄに示す。第ＶＩＩＩ因子－ドメイン反応性を表２のカラムｅに示す。矢印は、８０－ｋＤａのバンドの強度が低下したことを意味する。アフィニティー精製後のＩｇの回収の程度（カラムｆ、表２）を、μｇ／１０ｍｇの添加された画分ＩＩ＋ＩＩＩで表現する（材料と方法を参照すること）。１３種のＩｇ調製品のそれぞれの存在下でのインキュベートの後で、凝固アッセイの阻害をベセズダアッセイにて測定した（カラムｇ、表２）。

【００８７】
健康な提供者におけるヒト抗第ＶＩＩＩ因子抗体を免疫精製するための第ＶＩＩＩ因子ペプチドの使用
健康な被験者中に存在するヒト抗第ＶＩＩＩ因子抗体の調製およびキャラクタライゼイションを行うために、我々は、選択された特異的抗ペプチド抗体の存在を示すための免疫グロブリンに富むコーン画分ＩＩ＋ＩＩＩを分析した。適切なペプチド（表２を参照すること）に結合したセファロースにてのアフィニティークロマトグラフィーによって、ヒト抗第ＶＩＩＩ因子－ペプチド抗体（ＨＡＰ１からＨＡＰ１１、ＨＡＰ１６およびＨＡＰ１７）を精製した。典型的な例として、図２に、Ｃ２ドメインで見られる配列である配列番号：３２によって得られたクロマトグラフを示す。表２には、それぞれの第ＶＩＩＩ因子ドメイン（Ａ１、Ａ２、Ａ３、Ｂ、Ｃ１およびＣ２）において選択された１７種のエピトープ配列によって得られた結果が要約されている。用いられた１３種の第ＶＩＩＩ因子ペプチドのそれぞれについて特異的な免疫グロブリンのかなりの量が、出発血漿分画ＩＩ＋ＩＩＩから得られた。得られた精製ヒト抗体の特異性について、血漿第ＶＩＩＩ因子、組み

換え第ＶＩＩＩ因子、およびトロンビンによるタンパク質分解後に得られたフラグメントを用いてのイムノブロッティングによって直接テストした（表２を参照すること）。

【００８８】

ヒト精製抗体調製品におけるＩｇＧのアイソタイプの分布は、極めて不均一であることが分かった。興味深い点としては、回収されたＩｇＧのうちの４０から７９％のものが、ＩｇＧ２サブクラスに属していたことであった。ほとんどの調製品において、ＩｇＧ４は過剰に（最大で２５％まで）表示されているように思われた。

【００８９】

ヒト抗第ＶＩＩＩ因子－ペプチド抗体調製品のすべてについての第ＶＩＩＩ因子活性を阻害する能力を、ワンステージ凝固アッセイによってテストした。表２には、テストを行った１３種の調製品のうちの７種（５４％）、それぞれ配列番号：１４、配列番号：１９、配列番号：２、配列番号：５、配列番号：２２、配列番号：３２および配列番号：３３が阻害活性を示したことが記載されている。典型的な例として、抗－配列番号：３２のＩｇ濃度の第ＶＩＩＩ因子活性の阻害を関数の形として図２に示す。

【００９０】

第ＶＩＩＩ因子に対するヒト抗第ＶＩＩＩ因子ペプチドＩｇ免疫特異性

得られた精製ヒト抗体の特異性について、血漿第ＶＩＩＩ因子、組み換え第ＶＩＩＩ因子、およびトロンビンによるタンパク質分解後に得られたフラグメントを用いてのイムノブロッティングによってテストした。第ＶＩＩＩ因子－ＨＣ－もしくは第ＶＩＩＩ因子－ＬＣ－特異的マウスモノクローナル抗体または第ＶＩＩＩ因子－ペプチド－特異的ウサギポリクローナル抗体のいずれか一方を用いて、第ＶＩＩＩ因子フラグメントを再度特定した。ビオチン化ヤギ抗ヒトＩｇＧの結合後に、ヒト抗体を特定した。図３には、第ＶＩＩＩ因子ペプチド配列番号：１１（Ser^{109}－Lys^{127}）、配列番号：１４（Cys^{329}－Asp^{348}）、配列番号：１５（Tyr^{407}－Lys^{425}）または配列番号：１９（Cys^{711}－Asp^{725}）に結合したセファロースにて精製された４種のヒト抗体調製品との、高分子量の第ＶＩＩＩ因子（≧９２－ｋＤａ）の免疫反応が示されている。Ser^{109}－Lys^{127}－セファロースまたはCys^{329}－Asp^{348}－セファロースにて精製されたヒト抗体によって、５０－ｋＤａの第ＶＩＩＩ因子フラグメント（ドメインＡ１）が認められ、そしてTyr^{407}－Lys^{425}－セファロースおよびCys^{711}－Asp^{725}－セファロースにて精製された免疫グロブリンによって、４４－ｋＤａの第ＶＩＩＩ因子フラグメント（Ａ２）が認められた。抗－（Ser^{817}－Ser^{830}）免疫グロブリン調製品（ＨＡＰ５）が第ＶＩＩＩ因子フラグメントに対して反応しないことから、このエピトープがドメインＢのアミノ末端に位置することが確認される（図３）。配列番号：１（Arg^{1652}－Tyr^{1664}）のペプチドまたは配列番号：２（Asp^{1681}－Arg^{1696}）のペプチドに結合したセファロースにて精製されたヒト抗体は、８０－ｋＤａの第ＶＩＩＩ因子軽鎖（図３）と強く反応した。両調製品について、８０－ｋＤａのものと反応したバンドがトロンビンによるタンパク質分解後に消失したことは、エピトープが、予想された通りに、第ＶＩＩＩ因子Ａ３ドメインのNH_2末端の部分にあるａ３酸性ペプチド内に位置していることを示す。配列番号：５（Ａ３ドメイン内にあるArg^{1797}－Tyr^{1815}）のペプチドについて特異的なヒト抗体をイムノブロッティングによって分析したところ、ｒＦＶＩＩＩについてのそれらの抗体の特異性は、８０－ｋＤａの第ＶＩＩＩ因子軽鎖およびその７２－ｋＤａのトロンビンフラグメントに制限されているように思われた。

【００９１】

ｒＦＶＩＩＩを用いてのＥＬＩＳＡにて陽性反応が得られたのにも関わらず、第ＶＩＩＩ因子ペプチドの配列Ｃおよび配列番号：２３について特異的な抗体調製品を用いても、ｒＦＶＩＩＩ鎖またはフラグメントとの免疫反応は検出されなかった。このことは、これらの免疫グロブリン調製品は立体配置的なエピトープを認識することを意味するのかもしれない。

【００９２】

血友病Ａ患者の血漿におけるヒト抗第ＶＩＩＩ因子抗体をキャラクタライズするための第ＶＩＩＩ因子合成ペプチドの使用

ＥＬＩＳＡ実験にて選択されたペプチドを使用し、血友病Ａの血漿において抗第ＶＩＩＩ因子抗体の特異性が存在するかどうかを測定した。これらのペプチドをマイクロプレート上で被覆した（ＰＢＳ緩衝液１ｍｌあたり２５μｇ、４℃で１６時間）。患者血漿をトリス－カゼイン緩衝液で１／１０から１／１０００に希釈したものを、被覆されたペプチドと３７℃で２時間反応させた。結合したヒトＩｇＧを、方法の欄に記載されたようにして測定した。コントロールのサンプルを健康な提供者の血漿プールとした。図４は、４人の血友病Ａ患者の血漿を用いて得られた結果を示している。光学濃度は、二つの独立した実験の平均値（患者のＯＤ－健常者の血漿プールのＯＤ）を補正したものである。

【００９３】

分子モデルでのエピトープの予測

ペンバートンら（１９９７）は第ＶＩＩＩ因子のＡドメインの分子モデルを構築した。この三次元モデルから、第ＶＩＩＩ因子の活性の重要な領域について予測し検討することが可能となる。このモデルを用いて、パーカーおよびホッジのアルゴリズムによって特性された第ＶＩＩＩ因子ペプチドエピトープの位置を決定した。これらのアルゴリズムによって予測された通り、Ａドメイン内に位置するすべてのペプチドは第ＶＩＩＩ因子表面上で見出され、そして細部に至るまで完全に利用可能なものであった。

【００９４】

エピトープと第ＩＸａ因子結合ループとの間の重なり合い（Glu^{1811}－Tyr^{1815}にまたがった共通する５残基）から、フィブリンクロット形成時の、対応する抗－（Arg^{1797}－Tyr^{1815}）抗体の阻害作用を明らかにすることができるかもしれない。

【００９５】

結果の分析

凝固試験において、ウサギの抗－（Cys^{329}－Asp^{348}）抗体および抗－（Arg^{1653}－Tyr^{1664}）抗体の存在下では第ＶＩＩＩ因子活性の顕著な阻害が記録された。しかし、異なる阻害様式が観察された。抗－（Arg^{1653}－Tyr^{1664}）による阻害は、第ＶＩＩＩ因子活性の激しい低下を伴う二次反応速度式に従う。抗－（Cys^{329}－Asp^{348}）Ｉｇの有効性はより小さく、そして、抗体の濃度に非線形的な依存性を伴ったさらに複雑なタイプの反応を示す。エピトープのArg^{1652}－Tyr^{1664}およびそれに近接する主な結合部位のｖＷＦ（Glu^{1675}－Glu^{1684}の残基）は、酸性軽鎖ペプチドａ３内に位置する。ウエスタンブロッティングによって示されるとおり、トロンビン処理の後にＡ３ドメインからａ３が遊離し、抗－（Arg^{1652}－Tyr^{1664}）Ｉｇが活性化第ＶＩＩＩ因子にさらに結合することを妨害する。同様の結果がシマら（１９９１）によって報告されており、彼らは、第ＶＩＩＩ因子活性を中和するウサギポリクローナル抗体の結合部位として、第ＶＩＩＩ因子の配列Asp^{1663}－Ser^{1669}を記載していた。エピトープのCys^{329}－Asp^{348}は、活性化されたプロテインＣの切断部位（Arg^{336}）およびトロンビンの切断部位（Arg^{372}）に近接するものとして記載されている（ａ１中の）酸性のAsp^{348}－Lys^{362}配列と重なり合っていた。これはヒト血友病インヒビターのターゲットである。抗－（Asp^{348}－Lys^{362}）抗体は、タンパク質分解または第Ｘ因子相互作用部位（Met^{337}－Arg^{372}）（サエンコら、１９９９およびスキャンデラら、２０００）を妨害するかもしれない。

【００９６】

１３種のＩｇ調製品のすべてについて、第ＶＩＩＩ因子－中和活性を測定した。７種のヒトＩｇ調製品は、凝血促進活性を阻害することを示した。これらはそれぞれアミノ酸残基Cys^{711}－Asp^{725}、Tyr^{1681}－Arg^{1696}、およびArg^{1797}－Tyr^{1815}について特異的であった。Cys^{711}－Asp^{725}配列は、Tyr^{718}、Tyr^{719}、およびTyr^{723}において硫酸化チロシンを含有し、そして活

性化とＨＣのタンパク質分解との両方を促進するものとして記載された第ＶＩＩＩ因子ＨＣ領域のＬｙｓ^{713}－Ａｒｇ^{740}と重なり合う。添加された硫酸化群は、トロンビンまたは第Ｘ因子－活性化複合体において見られるような他の成分と適切に相互作用を行うために必要なのかもしれない。この配列もさらに領域Ｇｌｙ^{701}－Ｓｅｒ^{750}と重なり合っており、阻害性マウスモノクローナル抗体によってかすかに認められる。ペプチドＰ８（Ｔｙｒ^{1681}－Ａｒｇ^{1696}）（第ＶＩＩＩ因子ＬＣ）は、シマら、１９９１によって既に記載されている配列Ｇｌｕ^{1684}－Ａｒｇ^{1689}を含有する。これはトロンビン活性化部位のＡｒｇ^{1689}－Ｓｅｒ^{1690}を含む。Ｐ４（Ｃｙｓ^{711}－Ａｓｐ^{725}）はさらに、患者抗体のレパートリーをジーンファージディスプレイ技術にて分析することによって検出されたＡｓｐ^{712}－Ａｌａ^{736}配列に含有される。これは、患者における潜在的な補助的インヒビターとして提案されている（ファンデンブリンクら、２０００）。ペプチドＰ９（Ａｒｇ^{1797}－Ｔｙｒ^{1815}）は第Ｘａ因子結合部位を含有する（下記を参照すること）。

【００９７】

ヒトから精製されたかまたはウサギ内で産生された１６種の抗第ＶＩＩＩ因子－ペプチド免疫グロブリンのうち、７種は試験の条件下で第ＶＩＩＩ因子活性を中和した。小さなペプチドの配列と免疫結合アッセイを用いて、我々はさらなる新規エピトープの証拠を提供した。我々は、新規エピトープがＡ１ドメイン（Ｓｅｒ^{109}－Ｃｙｓ^{127}残基）内、Ａ２ドメイン（Ｃｙｓ^{407}－Ｌｙｓ^{425}）内、およびＢドメイン（Ｓｅｒ^{817}－Ｓｅｒ^{830}およびＧｌｕ^{1078}－Ｐｒｏ^{1092}）内に位置することを見出した。

【００９８】

変性第ＶＩＩＩ因子を用いて免疫精製された自己抗体が、健康な被験者内および正常ヒト免疫グロブリンのプール（分画ＩＩを処理したもの、上記を参照すること）内で報告されている（エイルジマンら、１９９２およびモローら、２０００）。血流からの変性第ＶＩＩＩ因子またはそのフラグメントのクリアランスにおけるおよび／または免疫トレランスにおける、考えられる役割が示唆された。

【００９９】

第ＶＩＩＩ因子の治療および治療用第ＶＩＩＩ因子濃縮物の質を改善するために、第ＶＩＩＩ因子エピトープを特定することは達成されるべき主な課題である。第ＶＩＩＩ因子エピトープ配列は、患者ポリクローナル抗第ＶＩＩＩ因子Ｉｇが、全体的な阻害活性および活性の調節に寄与しているかどうかを決定することの助けになる。これらエピトープ配列はさらに、治療中の患者における抗第ＶＩＩＩ因子の特異性がいつものように転換することを監視することにも用いることが可能かもしれない。第ＶＩＩＩ因子エピトープのこのようなキャラクタライゼイションと、フォールディングしている分子上でのこれらエピトープの位置のモデルによって、血友病患者および非血友病患者の両者におけるインヒビターの取り扱い（検出、フォローアップおよび第ＶＩＩＩ因子エピトープペプチドの治療目的の使用など）が改善される。

【表１】

表1　ウサギ抗第ＶＩＩＩ因子ペプチド抗体のキャラクタライゼイション

配列番号(a)	ウサギ抗血清(b)	ＥＬＩＳＡ力価(c)		第ＶＩＩＩ因子ドメインの認識(d)	RAP-IgGの回収の程度(e) μg/ 血清 mL	インヒビター力価(f) BU/mg
		P-KLH	r-FVIII			
SEQ ID 11	RAP1	2.5	2.2	A1	27	-
SEQ ID 14	RAP2	3.6	2.5	A1/a1	55	1,5
SEQ ID 15	RAP3	2.5	3.2	A2	268	-
SEQ ID 19	RAP4	2.5	1.3	A2/a2	12	-
SEQ ID 21	RAP5	4.6	3.9	B	106	-
SEQ C	RAP6	3.8	2.9	-	14	-
SEQ ID 01	RAP7	3.9	3.9	a3 ↓	35	0,5
SEQ ID 02	RAP8	1.9	0.9	a3/A3 ↓	3	-
SEQ ID 05	RAP9	3.8	2.6	A3	42	-
SEQ ID 23	RAP10	3.9	0.8	-	65	-
SEQ ID 22	RAP11	ND	ND	ND	ND	ND
SEQ ID 26	RAP12	4,1	1,1	C2	ND	ND
SEQ ID 27	RAP13	3,7	1,1	C2	ND	ND
SEQ ID 28	RAP14	3,8	0,9	C2	ND	ND
SEQ ID 31	RAP15	3,2	0,7	C2	ND	ND
SEQ ID 32	RAP16	3,5	1,8	C2	ND	ND
SEQ ID 33	RAP17	4,8	1,2	C2	ND	ND

【表２】

表２　ヒト抗第ＶＩＩＩ因子ペプチド自己抗体のキャラクタライゼイション

配列番号(a)	ヒト抗ペプチド Ig(b)	イムノブロッティングにおける第ＶＩＩＩ因子の反応性（トロンビンなし）(c)	イムノブロッティングにおける第ＶＩＩＩ因子の反応性（トロンビンあり）(c) (トロンビンあり) (d)	第ＶＩＩＩ因子ドメイン (e)	HAP-IgG の回収の程度 (f) μg/10mgのIgG	第ＶＩＩＩ因子阻害活性 (g) BU/mg
SEQ ID 11	HAP1	>92kDa	50kDa	A1	0,27	-
SEQ ID 14	HAP2	>92kDa	50kDa	A1/a1	1,07	3,4
SEQ ID 15	HAP3	>92kDa	44kDa	A2	0,06	-
SEQ ID 19	HAP4	92kDa	44kDa	A2/a2	0,12	+
SEQ ID 21	HAP5	>100kDa	-	B	0,26	-
SEQ C	HAP6	-	-	-	0,03	-
SEQ ID 01	HAP7	80kDa	80kDa	a3 ↓	0,20	-
SEQ ID 02	HAP8	80kDa	80kDa	a3/A3 ↓	0,01	+
SEQ ID 05	HAP9	80kDa	72kDa	A3	0,08	+
SEQ ID 23	HAP10	-	-	-	0,11	-
SEQ ID 22	HAP11	ND	ND	ND	0,98	4,3
SEQ ID 26	HAP12	ND	ND	ND	ND	ND
SEQ ID 27	HAP13	ND	ND	ND	ND	ND
SEQ ID 28	HAP14	ND	ND	ND	ND	ND
SEQ ID 31	HAP15	ND	ND	ND	ND	ND
SEQ ID 32	HAP16	80kDa	72 kDa	A3C1C2	2,40	6,3
SEQ ID 33	HAP17	ND	ND	ND	1,06	2,4

+：１００μｇ／ｍｌにおいて２５％を超える阻害性

【０１００】

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【図面の簡単な説明】

【０１０１】

【図１】図１は、第ＶＩＩＩ因子のＡ３配列の１から３７１に番号を付け替えられたアミノ酸の親水性、柔軟性および接近可能性の（それぞれのアミノ酸についての表面値の）グラフを表す。

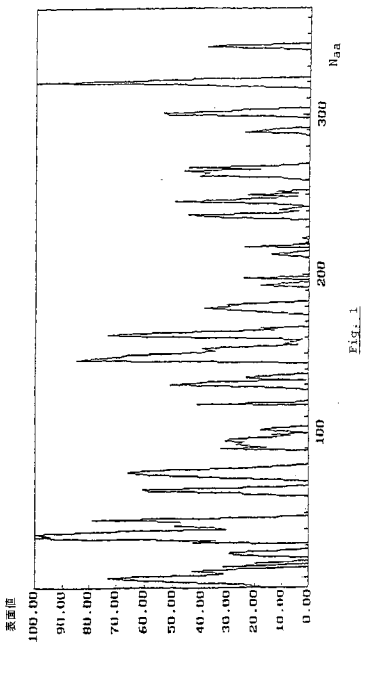
【図２ａ】図２ａは、ペプチド－セファロースカラム上でのアフィニティークロマトグラフィーによる、ヒト抗－配列番号：３２抗体の精製に関する溶出プロファイルを表す。

【図２ｂ】図２ｂは、コーン画分ＩＩ＋ＩＩＩから精製された抗－（配列番号：３２）ＩｇＧの存在下での第ＶＩＩＩ因子凝固活性を表す。

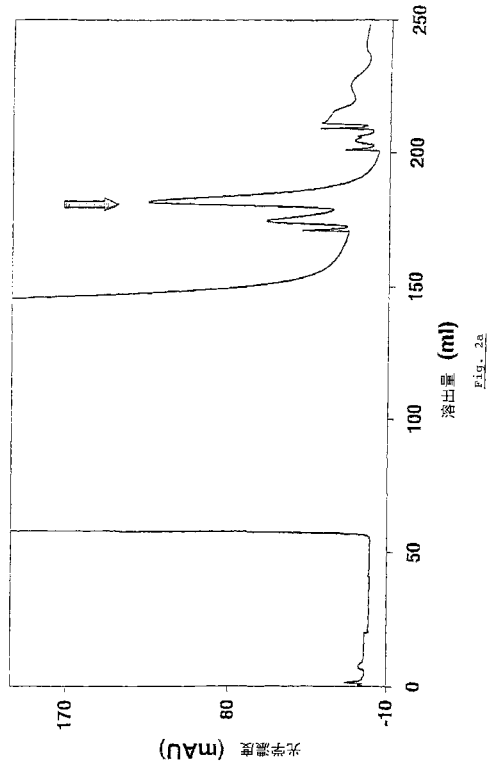
【図３】図３は、ウエスタンブロッティング後の、第ＶＩＩＩ因子ポリペプチドに対するヒト抗ペプチド抗体の免疫反応を表す。

【図４】図４は、４種のインヒビター血漿の、異なるペプチド配列とのＥＬＩＳＡでの反応性を表す。

【図１】



【図２ａ】



【図２ｂ】

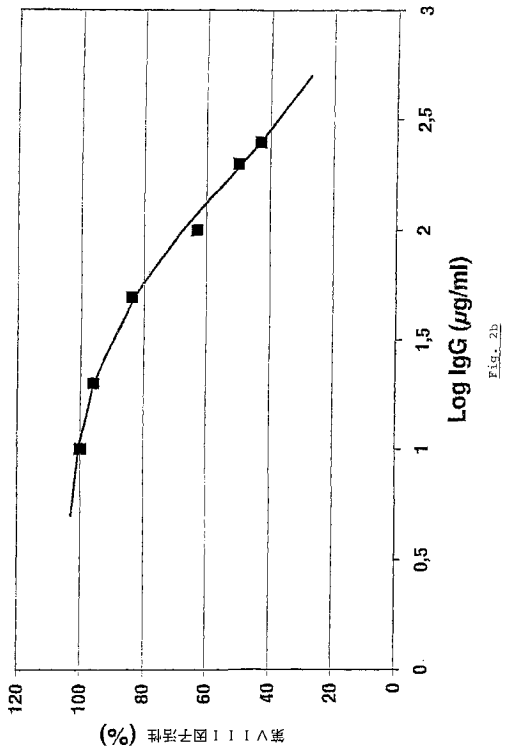


Fig. 2b

【図３】

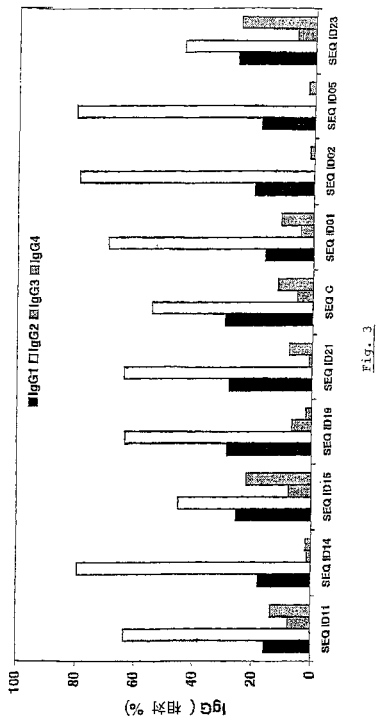


Fig. 3

【図４】

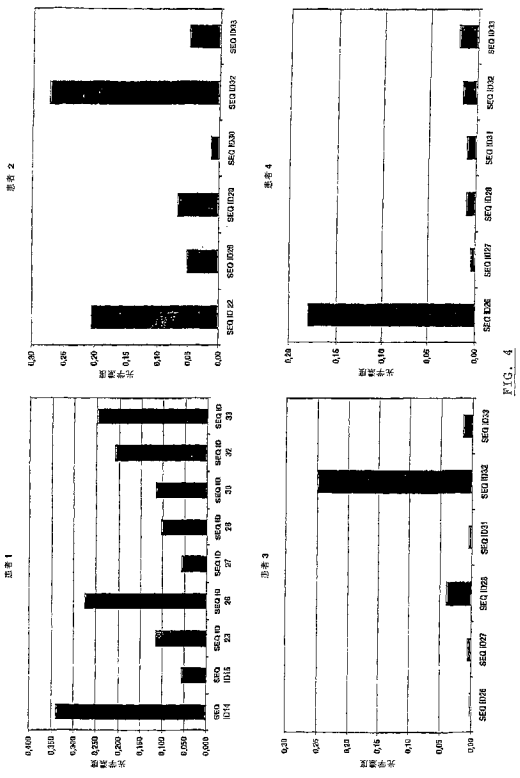


FIG. 4

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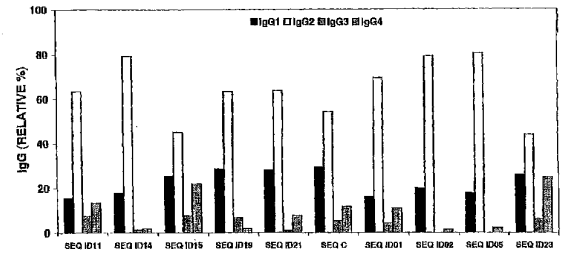
(54) Title: ANTIGENIC EPITOPES OF FACTOR VIII, INHIBITORS DIRECTED AGAINST SAID EPITOPES AND USE THEREOF



(57) Abstract: The present invention is related to antigenic polypeptide sequence (epitope of factor VIII) to the inhibitors which are directed against these sequence and to anti-inhibitors which are directed against said inhibitors. The present invention is also related to pharmaceutical composition and to a diagnostic device comprising at least one of the above mentioned molecules.

WO 02/090542 A2

ANTIGENIC EPITOPES OF FACTOR VIII, INHIBITORS DIRECTED AGAINST SAID EPITOPES AND USE THEREOF

Subject of the invention

[0001] The present invention relates to the antigenic polypeptide sequences (epitopes) of factor VIII to the anti-FVIII inhibitors which are directed against these sequences and to anti-inhibitors which are directed against said anti-FVIII inhibitors.

[0002] The present invention also relates to a pharmaceutical composition and to a diagnostic device comprising at least one of the above mentioned molecules.

Technical background underlying the invention

[0003] FVIII is a large multi-domain protein of 2,332 amino acids made up of three structural domains, A, B and C which are arranged in the order A1:a1:A2:a2:B:a3:A3:C1:C2. The A domains possess more than 40% homology and are also homologous to ceruloplasmin (for recent review, see Pratt (2000) and Saenko (1999)). 30% homology also exists between the A domains of factor V and FVIII. The C domain occurs twice and is reported to be able to bind glyco-conjugates and phospholipids having a net negative charge. It exhibits homology with lectins which are able to bind to negatively charged phospholipids. The platelet attachment site has been located in this region (C2 domain) (Foster et al., (1990)).

[0004] These antigenic determinants consist of fragments 351 - 365 (A1 domain - heavy chain), 713 - 740 (A2 domain), 1670 - 1684 (A3 domain - light chain) (NH_2 end of the light chain) or else 2303 - 2332 (C2 domain - light chain) (Foster C, (1990)), fragments 701 - 750, 1663 - 1689, 330 - 472, 1694 - 1782 (EP-0 202 853), 322 - 740 and 2170 - 2322.

[0005] The U.S patent 5,744,446 describes an hybrid human/animal Factor VIII having a sequence of amino acids selected from the group of the A2 domain fragments 373-540, 373-508, 445-508, 484-508, 404-508, 489-508 and 484-489, with corresponding sequences of porcine or murine Factor VIII, said hybrid being used for the treatment of Factor VIII deficiencies.

[0006] The antibodies which recognize these various sites interfere, with the activation of FVIII, the binding of vWf, FIXa, FXa, APC or phospholipids. The specific antibody response to FVIII vary considerably among individuals, and epitopes for inhibitor antibodies have to be determined for all FVIII domains (see for recent review Scandella, 2000; Lollar, 2000).

[0007] Other antibodies, which do not inhibit standard activity tests in vitro, can exert an influence on the behavior of FVIII with the other constituents of the coagulation cascade while attaching themselves to sites in the molecule which are at a substantial distance from the active sites. These antibodies, can interfere with the natural state of folding of FVIII by altering some of its properties.

[0008] Emergence of alloantibodies (inhibitors) that neutralize infused FVIII activity may seriously complicate FVIII replacement therapy. Reported inhibitor incidence rates in hemophiliacs vary considerably. They range around 6-35% (Vermylen et al, 1998). Candidates for genetic

predispositions such as large deletions and intron 22 inversion have been found associated with a high incidence of inhibitors and genes that are involved in the immune response as genes MHC class I and class II (Tuddenham and McVey, 1998). Repeat switching from one FVIII product to another and the possibility that some FVIII concentrates are more immunogenic may also explain the appearance of inhibitors (Vermylen et al, 1998). Different methods of preparing FVIII could exert an influence on its structure, its physicochemical properties or its natural micro-environment; Laub et al. (1999); Raut et al. (1998)). Clinically relevant anti-FVIII autoantibodies are rare in non-hemophilic patients (annual frequency in the population: 1-5/10^6) (Morrisson and Ludlam) (1995). They are associated with a number of autoimmune diseases and are often characterized by life-threatening hemorrhage. On the other hand, anti-FVIII antibodies have also been described in healthy subjects (Algiman et al, 1992; Moreau et al, 2000), without any apparent effect on the subjects' levels of circulating FVIII.

[0009] Self proteins or derived peptides may elicit an immune response if presented to CD4 T cells at inflammatory sites by professional antigen presenting cells. Using pools of overlapping synthetic peptides spanning the sequences of individual FVIII domains, Reding et al. (2000) showed reactive $CD4^+$ to FVIII in healthy subjects and hemophilia patients. Several FVIII domains were recognized: A3 domain was recognized more strongly and frequently and each domain forms several epitopes.

[0010] Techniques such as western blotting, immunoprecipitation, and enzyme-linked immunosorbent assays (ELISAs), using well-defined FVIII proteolytic fragments, a large recombinant peptide library, or synthetic peptide arrays, have been used to map different FVIII-inhibitor

binding sites located mainly in the A2 and C2 domains. However, none of these techniques has made it possible to build a model for identification of inhibitor and non-inhibitor epitopes. Only a few epitopes have been mapped to discrete sequences (<20 amino-acid residues). To solve this problem, Palmer et al (1997) synthesized 96 undecamer peptides (11 amino-acid residues) representing 80% of the complete residue sequence of FVIII. They succeeded in determining the epitope specificity of 9 patients' inhibitory antibodies. Other useful techniques are analysis of FVIII gene mutations and their effects on the FVIII molecule as well as phage display technology (van den Brink et al, 2000). All these methodologies, however, are time consuming, rather costly, and largely dependent on patient availability. Certain areas of the FVIII molecule may be "hot spots" containing commonly recognized clusters of inhibitor epitopes, e.g., regions in the A2 domain, A3 domain, and C2 domain. The reason for these "hot spots" in generating an inhibitor response remains poorly understood (Reisner et al, 1995).

[0011] Currently, a predominant notion among hemophilic patients, clinicians and "fractionators" is that of having available a purified FVIII which is devoid of all pathogenic plasma contaminants and secondary effects.

[0012] Different animal models could be used as hemophilia dogs, scid mice, hemophilia mice ... but until now, no satisfactory experimental model exists which makes it possible to forecast the immunogenicity or the immuno-modulatory effect of the FVIII preparations, or the susceptibility of the host, before they have been administered clinically.

[0013] Patients who develop an anti-FVIII immune response find themselves in a serious situation which

necessitates the use of severe, aggressive and excessively expensive measures.

[0014] One of the frequently treatment, is the induction of immune tolerance by administration of very high doses of FVIII (150 IU/kg twice a day) in association or not with prothrombin complex concentrates and is assigned as "Bonn Protocol". Treatment options are also to by-pass the FVIII inhibitor activity by use of PCC (preferably an activated PCC [APCC]) or FVIIa. Specific antibodies as consequence of the infusion of these alternative agents could be produced, impairing the treatment. As an alternative agent porcine FVIII may be used to achieve haemostasis in patients with antibodies that do not substantially crossreact with porcine FVIII before or during the treatment (Lollar, 2000).

[0015] A potential alternative approach to inhibit the production of inhibitors is blockade of the T cell/B cell collaboration mediated by through receptor ligand binding signal events (Ewenstein et al, 2000). Preliminary clinical trials were performed using a humanized mouse monoclonal antibody to human T cell CD40 ligand (CD 154).

[0016] A profitable strategy for reducing the level of inhibitors has consisted in subjecting patients to an extracorporeal circulation to enable solid-phase absorption of the total IgG.

[0017] The immunoabsorbant could be sepharose-bound staphylococcal protein A or sepharose-bound polyclonal sheep antibodies to total human immunoglobulin (Knobf and Derfler, 1999). The foreign proteins (protein A, sheep anti-human Ig) could leak from the column and triggered the immune system of the recipient; moreover problems could raised as sanitisation (ICH Topic Q5A, Directive 92/79/EC).

[0018] The infusion of polyvalent intravenous immunoglobulins (IVIG), where appropriate combined with an immunosuppressive treatment, has been found to be relatively effective, although the reason for this effectiveness is still not fully established. Various hypotheses involving feed-back inhibition of IgG synthesis, stimulation of IgG clearance or activation of T suppressor cells have been advanced. An interesting explanation is that these commercial intravenous immunoglobulins might contain antibodies which are able to react with the variable parts (idiotypes) of the anti-FVIII antibodies and neutralize these antibodies (Dietrich et al. (1992)).

[0019] Unfortunately, none of these approaches has been found to be satisfactory in terms of safety, efficacy, efficiency and cost.

[0020] The state of the art in epitope structure prediction was limited given to the fact that non-continuous amino acid residues seem to constitute most important epitope and that the dynamics of binding is often not integrated into the epitope prediction equation making epitope structure prediction a complex four-dimensional problem (Van Regenmortel, Methods: A companion to Methods in Enzymology, 9, page 465-472, 1996).

[0021] According to the author, most of the antibodies raised against intact proteins do not react with any peptide fragment derived from the parent protein indicating that such antibodies are directed to discontinuous epitopes (conformational epitopes).

[0022] This author states also that low success rate of antigenic prediction is due to the fact that predictions concerns only continuous epitopes and it is unrealistic to reduce the complexity of epitopes that always possess conformational features to one dimensional linear peptide model.

[0023] Similarly, Palmer et al. (1997) using synthetic peptide arrays to identify novel Factor VIII inhibitor epitopes note that each patient pattern of anti-factor VIII antibody reactivity appears to be polyclonal, directed against multiple sites located within the amino and carboxyl terminus of the protein and seems to be unique for each plasma investigated (see also above).
Moreover, this author notes that it is difficult to predict the importance that any given antibody: epitope interaction may have on Factor VIII coagulation activity based on the results of synthetic peptide assays alone (due to the uncomplete understanding of the relationship between structure and function of different factor VIII domains and the possibility that both inhibitor and non-inhibitory antibodies may be present in a patient's plasma.

[0024] Therefore, the documents of the state of the art do not suggest to identify antigenic linear peptides upon a macro-molecule (such as Factor VIII) and that linear epitopes could be used for the diagnostic and/or the therapy of immune disorders induced by inhibitors directed against Factor VIII.

[0025] The international patent application WO96/02572 describes antigenic fragment and epitope sequences of factor VIII and inhibitors directed against some of these sequences.

Aims of the invention

[0026] The present invention aims to obtain new antigenic epitopes of factor VIII in order to improve the diagnosis and/or the therapy (including the prevention) of immune disorders (in particular those induced by inhibitors of FVIII, especially inhibitors of the binding of FVIII to the von Willebrand factor (vWf), to the FIX and/or to membrane phospholipids (PL)), said epitopes allowing a

screening between non-inhibitory and inhibitory anti-FVIII allo- or auto-antibodies (allo- or auto-immunoglobulins).

[0027] Another aim of the invention is to obtain inhibitors which exhibit an immunoaffinity with these antigenic epitopes, as well as to obtain anti-inhibitors, in particular antibodies or (T)cell receptors, which are directed against the abovementioned said inhibitors and whose purpose is to improve the diagnosis and/or therapy (or prevention) of immune disorders.

[0028] A further aim of the invention is to obtain said molecules at high purity, in industrial level, without contaminants (viruses, prions,...) and according to the GMP practices in the field of therapy and diagnostics (ICH topic QSA, Directive 92/79/EC, etc.).

Summary of the invention

[0029] The present invention relates to the antigenic polypeptide sequences (epitopes) of factor VIII whose complete sequence is described by Verhar et al. (1984) and which provides the reference for the amino-acids numerotation of the complete factor VIII sequence.

[0030] The "complete polypeptide sequence of factor VIII" is understood to be the natural human or animal sequence, which may be glycosylated and which has been obtained by purification from pools of plasma, in particular cryoprecipitate, by synthesis and/or by genetic manipulation (sequence from which portions which are not involved in the mechanism of blood coagulation may have been deleted) of factor VIII.

[0031] The present invention relates, in particular, to an antigenic epitope sequence of factor VIII which is selected from the group consisting of :

- the epitope comprised between serine 2018 and histidine 2031 inclusive, defined by the following sequence:

SEQ ID No:10:

Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
1 5 10

- the epitope tyrosine 555 to glutamine 565 inclusive, defined by the following sequence:

SEQ ID No:17:

Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
1 5 10

- the epitope leucine 730 to serine 741 inclusive, defined by the following sequence (P4):

SEQ ID No:20:

Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
1 5 10

possibly deleted from the terminal amino acid serine (P4) and/or the first amino acid leucine

- the epitope serine 817 to serine 830 inclusive, defined by the following sequence (P5):

SEQ ID No:21:

Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
1 5 10

- the epitope asparagine 2128 to asparagine 2138 inclusive, defined by the following sequence:

SEQ ID No:24:

Asn Val Asp Ser Ser Gly Ile Lys His Asn
1 5 10

- the epitope serine 2204 to glutamine 2222 inclusive, defined by the following sequence (P12):

SEQ ID No:27:

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

- the epitope isoleucine 2262 to glutamine 2270 inclusive, defined by the following sequence:

SEQ ID No:30:

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

- the epitope leucine 2273 to serine 2289 inclusive, defined by the following sequence (P14):

SEQ ID No:31:

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
1 5 10 15
Ser

- the epitope proline 2292 to tyrosine 2305 inclusive, defined by the following sequence (P15):

SEQ ID No:32:

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

possibly deleted from one or more amino acids of the terminal tripeptide Thr-Arg-Tyr involved in the phospholipid von Willebrand factor binding site

- the epitope glutamic acid 2322 to tyrosine 2332 inclusive, defined by the following sequence (P16):

SEQ ID No:33:

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

[0032] The invention also relates to the major parts of the said epitopes. Said epitopes can be deleted from one or more terminal amino acids, preferably from one, two or three amino acids, or can be replaced by one or more amino acids that present the same characteristic of hydrophilicity, flexibility and accessibility.

[0033] It is also known that some of the epitopes according to the invention are comprised in major determinants of human inhibitors epitopes or several factors binding sites or binding sites of known monoclonal antibodies, especially the portion C2 that is known to be the binding site of the monoclonal antibody Mas531P or the binding site ESH8 as well as phospholipids, Factor Xa or the von Willebrand factor binding site. However, the specific epitopes according to the invention or their major

parts are preferred selected portions of said binding sites or may include a possible overlapping with said binding sites.

[0034] These epitopes sequences are particularly advantageously characterized by high hydrophilicity, which has been defined by Parker and Hodges (1986), considerable flexibility, which has been defined by Karplus and Schultz (1985) and considerable accessibility, which has been defined by Janin (1979).

[0035] These epitopes are, in particular, exposed on the surface of the factor VIII protein and exhibit pronounced antigenic and immunogenic characteristics.

[0036] Another aspect of the present invention is related to a modified (recombinant or transgenic) FVIII, possibly obtained by genetic engineering, and deleted from one or more of the above-identified epitopes or major parts of said epitopes.

[0037] Advantageously, said FVIII still allows the binding of coagulation factor(s), but will be less immunogenic and will not induce or induce less the formation of inhibitors directed against said modified FVIII or natural FVIII.

[0038] Advantageously, said epitopes are also independently immunogenic (that is to say they are immunogenic even without being complexed with a protein of large size such as BSA, KLH, haemocyanin, etc.), and preferably exhibit an immunoaffinity within inhibitors of factor VIII, such as anti-factor VIII antibodies, and/or exhibit an immunoaffinity for the receptors of the T lymphocytes and possibly B lymphocytes.

[0039] These epitopes and/or major parts of said epitopes induce an immune reaction (antibody synthesis) when they are injected into a rabbit.

[0040] Said sequences are unexpectedly characterized

by substantial immunogenicity towards monoclonal and polyclonal antibodies, but are sufficiently short to be readily and advantageously obtained by synthesis.

[0041] The present invention also relates to the conformational epitopes which comprise at least two different sequence epitopes and/or at least two major parts of said epitopes according to the invention and above identified.

[0042] The conformational epitopes are made up of two or more different portions of a polypeptide sequence, which portions are located in proximity to each other when the protein is folded in its tertiary or quaternary structure.

[0043] These epitopes are capable of being "recognized" (that is to say of exhibiting an immunoaffinity), preferably simultaneously, with inhibitors of factor VIII, in particular B and T lymphocytes (by way of the major histocompatibility locus (MHC I and/or II)) and/or anti-factor VIII antibodies (Scandella et al. (2000); Reding et al. (2000)).

[0044] Preferably, the said epitopes and/or the major parts of said epitopes are complexed with a carrier protein or a carrier peptide, such as BSA, or KLH haemocyanin, as to form a complex exhibiting a more powerful immunogenicity.

[0045] The present invention is also related to a pool of antigenic epitopes of factor VIII which comprises a mixture of the epitopes above mentioned linear epitopes (SEQ ID 10, SEQ ID 17, SEQ ID 20, SEQ ID 21, SEQ ID 24, SEQ ID 27, SEQ ID 30, SEQ ID 31, SEQ ID 32, SEQ ID 33) or conformational epitopes made of said epitopes or a pool which may comprise at least one of said epitopes and one or more additional antigenic epitopes of factor VIII already described in the state of the art and preferably selecting

from the group consisting of :

- the epitope arginine 1648 to tyrosine 1664 inclusive, defined by the following sequence:

SEQ ID No:1:

Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1 5 10 15
Tyr

and possibly deleted from one or more amino acids of the tetrapeptide Arg-Asp-Ile-Thr (P7), or one or two of the last amino acids of the dipeptide Asp-Tyr

- the epitope aspartic acid 1681 to arginine 1696 (P8) inclusive, defined by the following sequence:

SEQ ID No:2:

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
1 5 10 15

possibly deleted from one or more amino acids of the epitope Asp-Glu-Asp-Glu,

- the epitope threonine 1739 to tyrosine 1748 inclusive, defined by the following sequence:

SEQ ID No:3:

Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
1 5 10

- the epitope asparagine 1777 to phenylalanine 1785 inclusive, defined by the following sequence:

SEQ ID No:4:

Asn Gln Ala Ser Arg Pro Tyr Ser Phe
1 5

possibly deleted from one or more amino acids of the terminal dipeptide Ser-Phe or tetrapeptide Pro-Tyr-Ser-Phe

- the epitope glutamic acid 1794 to tyrosine 1815 inclusive, defined by the following sequence:

SEQ ID No:5:

Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
1 5 10 15
Asn Glu Thr Lys Thr Tyr

20

possibly deleted from one or more amino acids of the first tripeptide Glu-Asp-Gln (P9) or the first nonapeptide Glu-Asp-Gln-Arg-Gln-Gly-Ala-Glu-Pro

- the epitope methionine 1823 to aspartic acid 1831 inclusive, defined by the following sequence:

SEQ ID No:6:

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

- the epitope glutamic acid 1885 to phenylalanine 1891 inclusive, defined by the following sequence:

SEQ ID No:7:

Glu Thr Lys Ser Trp Tyr Phe
1 5

- the epitope glutamic acid 1885 to alanine 1901 inclusive, defined by the following sequence:

SEQ ID No:8:

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

possibly deleted from one or more amino acids from the heptapeptide Glu-Thr-Lys-Ser-Trp-Phe-Thr or from the tripeptide Cys-Arg-Ala.

- the epitope aspartic acid 1909 to arginine 1917 inclusive, defined by the following sequence:

SEQ ID No:9:

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

- the epitope alanine 108 to valine 128 inclusive, defined by the following sequence:

SEQ ID No:11:

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

possibly deleted from the terminal amino acids alanine and valine (P1)

- the epitope glutamic acid 181 to leucine 192 inclusive, defined by the following sequence:

SEQ ID No:12:

Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
1 5

possibly deleted from one or two amino acids of the terminal dipeptide Thr-Leu

- the epitope aspartic acid 203 to alanine 227 inclusive, defined by the following sequence:

SEQ ID No:13:

Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
1 5 10 15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
20 25

possibly deleted from one or more amino acids of the nonapeptide Asp-Arg-Asp-Ala-Ala-Ser-Ala-Arg-Ala

- the epitope aspartic acid 327 to methionine 355 inclusive, defined by the following sequence:

SEQ ID No:14:

Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
1 5 10 15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
20 25

possibly deleted from one or more amino acids from the terminal dipeptide Asp-Ser or the octapeptide Asp-Asp-Leu-Thr-Asp-Ser-Glu-Met (P2).

- the epitope aspartic acid 403 to lysine 425 inclusive, defined by the following sequence:

SEQ ID No:15:

Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
1 5 10 15
Ile Gly Arg Lys Tyr Lys Lys
20

possibly deleted from one or more amino acids of the tetrapeptide Asp-Asp-Arg-Ser (P3),

- the epitope valine 517 to arginine 527 inclusive, defined by the following sequence:

SEQ ID No:16:

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

possibly deleted from one or the two amino acids of the dipeptide Pro-Arg,

- the epitope histidine 693 to glycine 701 inclusive, defined by the following sequence:

SEQ ID No:18:

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

- the epitope serine 710 to aspartic acid 725 inclusive, defined by the following sequence (P4):

SEQ ID No:19:

Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

- the epitope isoleucine 2081 to serine 2095 inclusive, defined by the following sequence:

SEQ ID No:22:

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

possibly deleted from one or more amino acids from the tetrapeptide Ile-His-Gly-Ile

- the epitope tyrosine 2105 to glycine 2121 inclusive, defined by the following sequence:

SEQ ID No:23:

Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15
Gly

possibly deleted from one or more amino acids of the tripeptide Tyr-Ser-Leu (P10)

- the epitope glutamine 2235 to leucine 2251 inclusive, defined by the following sequence (P13):

SEQ ID No:28:

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu or one or more amino acids of the tetrapeptide Val-Lys-Ser-Leu

- the epitope glycine 2242 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:29:

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu, said epitope presenting a possible partial overlapping with a known monoclonal antibody binding site ESH8 2248-2285

[0046] Another aspect of the present invention relates to an inhibitor of factor VIII which exhibits an immunoaffinity with antigenic epitopes, with the major parts of said epitopes and/or with the complex according to the invention.

[0047] An inhibitor is understood to mean any biological molecule or cell (such as a T-lymphocyte) binding to said FVIII and capable of giving rise to immune disorders (characterized by humoral immune response and/or cellular immune response against said FVIII).

[0048] In particular, such an inhibitor can be an anti-factor VIII monoclonal or polyclonal antibody or antibody fragment (such as the hypervariable Fab portion of the said antibody) which inactivates the said factor VIII and/or which inhibits the binding of factor VIII to the von Willebrand factor and/or to membrane phospholipids.

[0049] Advantageously, the said inhibitors are synthesized by a "chimaeric" animal which comprises a human immune system, such as an hu-SCID mouse or transgenic mouse

producing human antibodies or other antibodies production technologies as phage display technology or immortalized B-cells, by EPV in particular.

[0050] Another aspect of the invention relates to an anti-inhibitor which is directed against the said previously described factor VIII inhibitor.

[0051] An anti-inhibitor which is directed against the factor VIII inhibitor is understood to mean any chemical or biological molecule, a cell and/or a cell fragment (receptor) which is capable of interfering with the said inhibitor in such a way as to ensure its inactivation or avoid or reduce its binding to the factor VIII.

[0052] Preferably, such an anti-inhibitor is an anti-anti-factor VIII idiotype (monoclonal or polyclonal) antibody or antibody fragment, natural or obtained by genetic engineering.

[0053] Another aspect of the invention relates to a pharmaceutical composition which comprises an adequate pharmaceutical carrier or a diluant and an element selected from the group consisting of said epitopes or a pool thereof, an inhibitor of factor VIII which is directed against them, an anti-inhibitor which is directed against the said inhibitor, and/or a mixture of these.

[0054] The type and amount of adequate pharmaceutical carrier or diluant (and possibly adjuvant or excipient) present in said pharmaceutical composition, may vary according to the method of administration and is possibly combined an adjuvant in order to improve therapeutical properties of the pharmaceutical composition according to the invention or to reduce its possible side effects. Suitable pharmaceutical acceptable carriers used in the pharmaceutical composition according to the invention are well known by the person skilled in the art

and are selected according to the methods generally applied by pharmacists and may include solid, liquid or gaseous non-toxic pharmaceutically acceptable carriers. The percentage of active product / pharmaceutical acceptable carrier may vary within very large ranges only limited by the tolerance and the possible side effects on patients (including humans), and by frequency and/or mode of administration.

[0055] Another aspect of the invention relates to a diagnostic and/or purification device, such as a diagnostic kit, an affinity filter, or a chromatography column which comprises an element which is selected from the group consisting of these epitopes and/or major parts of said epitopes, the complex according to the invention or a pool thereof, an inhibitor which is directed against them, an anti-inhibitor which is directed against said inhibitor, and/or a mixture of these. Advantageously, said device comprises the pool of said epitopes which allow a screening of patients and may detect the most important inhibitors present in said patients and which allow a positive test with enough specificity and sensibility.

[0056] The purification device can therefore consist of a chromatography column which comprises these epitopes and/or major parts of epitopes, attached to the solid phase of the chromatography column.

[0057] A physiological liquid (such as serum), which is derived from a patient and which comprises inhibitors of factor VIII pass through a solid support (chromatography column), with said inhibitors (for example antibodies) becoming attached specifically to said epitopes or said major parts or a pool thereof. Following elution, it is possible to collect said inhibitors by causing them to react with anti-inhibitors (anti-anti-factor VIII idiotype antibodies).

[0058] It is also possible to characterize the anti-anti-factor VIII idiotype antibodies which are present in a serum by these anti-inhibitors passed through a solid support (chromatography column) on which inhibitors of factor VIII have been attached to the solid phase.

[0059] It is also possible to reinject (*ex vivo* treatment) the physiological liquid (blood or serum or a derived fraction) to said patient after its inhibitors of factor VIII have been removed by binding with said epitopes or a pool thereof; said inhibitors being removed from the physiological fluid (blood or serum) similarly as proposed for dialysis method applied to human patients.

[0060] The present invention is also related to a method of treatment (*ex vivo* treatment) of a patient suffering from a pathology induced by inhibitors to the factor VIII which comprises the steps of extracting said physiological liquid (blood or serum) from the patient, obtaining its reaction upon a solid support binding the epitopes or a pool thereof according to the invention and reinjecting said physiological liquid to the patient after the removing of the inhibitors having fixed said epitopes, majors parts or a pool thereof.

[0061] A final aspect of the invention relates to the use of the pharmaceutical composition according to the invention for preparing a medicament used for preventing and/or treating immune disorders, in particular those induced by inhibitors of factor VIII, inhibitors of the binding of factor VIII to the factor IX and/or the factor X and/or the von Willebrand factor (vWF) and/or inhibitors of the binding of factor VIII to membrane phospholipids.

[0062] The present invention will be described in details in the following non-limiting examples in reference to the enclosed figures.

Brief description of the figures

[0063] Figure 1 depicts the hydrophilicity, flexibility and accessibility graph of the A3 sequence of Factor VIII renumbered 1 to 371 amino acids (surface value for each amino acid).

[0064] Figure 2a represents the elution profile related to the purification of human anti-SEQ ID 32 antibodies by affinity chromatography on peptide-Sepharose column. Cohn fraction II+III solution (50 ml) was loaded onto the column (1 ml gel) at a flow rate of 1 ml/min. The separation of specific antibodies was performed as described hereafter. The arrow indicates the position of specific human anti-SEQ ID 32 antibodies.

[0065] Figure 2b represents FVIII clotting activity in the presence of anti-(SEQ ID 32) IgG purified from Cohn fraction II+III. The clotting activity of FVIII was measured as described hereafter in the presence of increasing amount of anti-SEQ ID 32. The % of FVIII activity =(FVIII activity in the presence of antibody/FVIII activity in absence of antibody)*100.

[0066] Figure 3 represents the human anti-peptide antibody immunoreactions with FVIII polypeptides after western blotting (panel A from left to right : human antibodies HAP1 through HAP4, specific for different FVIII epitope sequences found in the FVIII HC - see also table 2 and panel B : human antibodies specific for the P5 peptide and the FVIII LC sequences, P7, P8 and P9 - see also table 2). The RAP9 lane shows the reactivity of FVIII polypeptides towards purified rabbit antibodies specific for the peptide sequence Arg^{1797}-Tyr^{1815} (see also table 2).

[0067] Figure 4 represents ELISA reactivity of 4 inhibitor plasmas with different peptide sequences. Inhibitors present in 4 patients plasmas were analyzed by

ELISA test using as coated antigens the different selected FVIII epitopes synthetic peptides as indicated in ordinate.

Examples

Materials and Methods

Reagents

[0068] MAS530p (Harlan-Seralab, Indianapolis, IN) is a mouse monoclonal antibody specific for the 44-kDa A2 domain of the factor VIII heavy chain. Biotin-labeled rabbit IgG anti-mouse IgG was purchased from Dakopatts (Copenhagen, Denmark). Biotin-labeled goat IgG anti-human IgG and biotin-labeled mouse IgG anti-rabbit IgG were obtained from Sigma Chemicals (St Louis, MI), purified α-thrombin (3000 IU/mg), streptavidin-peroxidase conjugate, ovalbumin (OVA), bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), and o-phenylenediamine (OPD) were purchased from Sigma Chemicals (St. Louis, MI). Casein was obtained from Merck (Darmstadt, Germany). 4-chloro-1-naphtol and biotinylated molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA). Freund's adjuvant was from Difco (Detroit, Michigan).

FVIII concentrates

[0069] Plasma FVIII (p-FVIII) was a solvent/detergent-treated FVIII concentrate (100 IU/mg protein) purified by ion exchange chromatography (FVIII Conc. SD, CAF-DCF- Red Cross , Brussels, Belgium). Albumin-free recombinant FVIII (rFVIII) was obtained from Hyland (Glendale, CA).

Plasma fraction immunoglobulins

[0070] Cohn Fraction II+III was obtained from large plasma pool from 4,800 unpaid donors, after precipitation in the presence of increasing ethanol concentration. This fraction contains all Ig classes and subclasses. IgG composition was determined by nephelometry. The relative percentage of each subclass was 63,7; 30,1; 3,4 and 2,8 for IgG1, IgG2, IgG3 and IgG4 respectively (average values for 3 different batches of FII+III).

Factor VIII concentrates, Factor VIII activity and activity inhibition

[0071] Factor VIII activity was determined in a one-stage clotting assay adapted for use on the Coagulometer KC4A (Sigma Diagnostics). The assay uses severe hemophilia A plasma (Organon Teknika, Cambridge, UK) and APPT reagent from Instrumentation Laboratory (Warrington, UK). Potencies were calculated relative to the 5th International Standard FVIII concentrate 88/640 (5.4 IU/ml) (NIBSC, Potters Bar, UK). FVIII-inhibitory activity was measured in purified rabbit and human IgG preparations according to the modified Bethesda assay. Briefly, affinity-purified IgGs were serially diluted and incubated for 1 h in the presence of FVIII concentrate 88/640 (1 IU/ml) at 37°C. The residual FVIII activity was measured as described above.

[0072] Activation of factor VIII by α-thrombin and immunoblotting has been described elsewhere (Peerlinck et al, 1997).

[0073] Synthesis of peptides, conjugation of peptides to carrier proteins and production of rabbit anti-peptide antisera were performed by Neosystem (Strasbourg, France).

Purification of rabbit and human antibodies by affinity chromatography

[0074] For purification of rabbit and human antibodies, 5 mg of each different peptide was coupled to 1 ml pre-packed NHS-activated Sepharose (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Specific anti-peptide antibodies were purified with an automated liquid chromatography system (ÄKTAexplorer 100A, Pharmacia Uppsala, Sweden) either from 50 ml rabbit antiserum or from 100 ml of a human plasma fraction, obtained after Cohn fractionation (fraction II+III; 13 mg protein/ml). Briefly, samples were dialyzed 3 times against 5 volumes of TE buffer (20 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.02% NaN_3) and loaded onto the column at a flow rate of 1 ml/min. The column was sequentially washed at 2 ml/min with 50 ml TE buffer and 30 ml TE containing 1 M NaCl. After absorption, the material was eluted (1 ml/min) with 5 ml of 0.1 M citric acid pH 2.5 and directly recovered in 5 ml of 1M Tris-HCl, pH 9.0. Samples were finally dialyzed versus 10 volumes of equilibration buffer and concentrated on Centriprep-30 (Amicon, Beverly, MA). Ig recovery was determined by the Bio-Rad protein assay.

Results

Selection of potential factor-VIII linear epitopes

[0075] More than 30 surface regions (linear epitopes) spanning 8 to 25 residues, characterized by a high hydrophilicity, flexibility and accessibility were identified on the FVIII molecule. On the basis of their high probability of an outer location (see Fig. 1 for A3), 16 linear peptides (P1 to P16) were selected, matching identified stretches of 13 or more amino-acid residues. These peptides were synthesized and coupled to ovalbumin

for production of specific antiserum (Table 1, hereafter). P8 includes the epitope described by Shima et al (1988) and was used as an external control.

Experimental results obtained from said synthesized linear epitopes using the rabbit model

[0076] Results are summarized in Table 1 which concerns the characterization of rabbit anti-FVIII-peptide antisera and recovered affinity-purified of immunoglobulins.

[0077] Sixteen synthetic peptides (from 10 to 20 amino acids) were selected in the A, B, C1 and C2 domains. After conjugation with ovalbumin, the OVA-peptide conjugates were injected into rabbits and FVIII anti-peptide antisera RAP1 to RAP16 were studied.

[0078] More precisely, two rabbits were immunized with each FVIII-peptide-ovalbumin preparation. Specific antisera RAP1 to RAP16 (column b, Table 1) were prepared and assayed in an ELISA (column c, Table 1) using rFVIII or FVIII-peptide-KLH as the antigen. ELISA titre is expressed as the negative log of the reciprocal of the serum dilution giving 50% binding. The immunoglobulins were then purified by chromatography on peptide-bound Sepharose. The FVIII domain recognized by the anti-FVIII peptide Ig after immunoblotting is shown in (column d, Table 1) and Ig protein recoveries (column e, Table 1) were measured using immunoglobulins as the standard. The inhibitory activity, expressed in BU/mg protein, was determined in a FVIII neutralizing activity assay (column f, Table 1).

Immunogenicity of FVIII peptides and characterization of rabbit anti-FVIII peptide antisera

[0079] The reactivity of FVIII anti-peptide antisera was measured by an ELISA using, as antigen, either the

different corresponding FVIII-peptide coupled to KLH protein or purified rFVIII. The binding reaction of each anti-FVIII-peptide antiserum was specific both for the FVIII peptide used to elicit the immune response in rabbit and for rFVIII (see Table 1).

[0080] To demonstrate the FVIII epitope specificity of the rabbit anti-peptide antibodies, rFVIII and the rFVIII fragments obtained after treatment with thrombin were resolved by SDS-PAGE and analyzed by western blotting with the different preparations of rabbit IgGs. As expected, most antisera (14/16, 87%), showed a strong reaction with the corresponding FVIII fragment containing the selected linear epitope (see Table 1).

Purification of rabbit-anti-FVIII peptide antibodies

[0081] The specific rabbit IgG were purified by affinity chromatography on peptide-Sepharose as described under Methods. When FVIII-neutralizing activity was measured in a one-stage clotting assay, significant inhibition was found with two rabbit IgG purified preparations: RAP2, corresponding to IgG specific for SEQ ID No. 14 and RAP7 specific for SEQ ID No: 01.

Epitope mapping of rabbit anti-FVIII peptide antibodies by immunoblotting with human rFVIII

[0082] To demonstrate the FVIII epitope specificity of the rabbit anti-peptide antibodies, rFVIII and the rFVIII fragments obtained after treatment with thrombin were resolved by SDS-PAGE and analyzed by western blotting with different preparations of rabbit IgGs (RAP1 to RAP17 Igs).

[0083] In each run, the rFVIII heavy chain (HC) and light chain (LC) and their thrombin proteolysis products (44 kDa and 72 kDa) were identified with a mixture of two

monoclonal antibodies, MoAb 530p and MoAb18, respectively specific for the heavy and light chain. MoAb18 recognizes the NH_2-terminal light-chain FVIII fragment obtained after thrombin activation, which proved too small to remain in the gel after electrophoresis. Fourteen of the 17 rabbit immunoglobulin preparations reacted strongly with both rFVIII and pFVIII. Antisera RAP1, RAP2, RAP3, RAP4 recognized exclusively the heavy chains (200 kDa to 92 kDa). Antisera RAP1 and RAP2 reacted with the 50-kDa A1-domain fragment; RAP3 and RAP4 bound to the 44-kDa fragment (domain A2); RAP5 (specific for the B domain) bound to the high-molecular-weight FVIII heavy chain (about 200-kDa).

[0084] RAP7, RAP8, and RAP9 reacted with the 80-kDa light-chain doublet. RAP9 and RAP12 to RAP17 antibodies also detected the 72-kDa FVIII light-chain fragment. As expected, each reactive antiserum showed a strong reaction with the corresponding FVIII fragment containing the selected linear epitope. No reaction was detectable in the gels between RAP6 or RAP10 and the HC or LC FVIII fragments.

Experimental results obtained from said synthesized linear epitopes to purify and characterize human autoantibodies

[0085] Table 2 concerns the characterization of human anti-FVIII antibodies from Cohn fraction II+III of healthy individuals.

[0086] Human anti-peptide IgG preparations (HAP1 through HAP17) were so far purified on Sepharose coupled to 13 different FVIII peptides (column a, Table 2). The Igs (column b, Table 2) were analyzed by immunoblotting. Binding to the rFVIII HC or LC chains and to the rFVIII thrombin fragment is shown respectively in columns c and d, Table 2. FVIII-domain reactivity is shown in column e,

Table 2. Arrows indicate a decrease in 80-kDa band intensity. Ig recovery (column f, Table 2) after affinity purification is expressed in µg/10 mg loaded FII+III (see Materials and Methods). Inhibition of the clotting assay was determined after incubation in the presence of each of the 13 Ig preparations in the Bethesda assay (column g, Table 2).

Use of FVIII peptides for the immunopurification of human anti-FVIII antibodies in healthy donors

[0087] To prepare and characterize human anti-FVIII antibodies present in healthy individuals, we analyzed Cohn fraction II+III, rich in immunoglobulins, for the presence of selected specific anti-peptide antibodies. Human anti-FVIII-peptide antibodies (HAP1 to HAP11, HAP16 and HAP17) were purified by affinity chromatography on Sepharose coupled to the appropriate peptide (see Table 2). As a typical example, figure 2 shows the chromatographic profile obtained with SEQ ID 32, a sequence found in C2 domain. Table 2 summarizes the results obtained with 17 epitopic sequences selected in each FVIII domain (A1, A2, A3, B, C1 and C2). Significant amounts of immunoglobulins, specific for each of the 13 FVIII peptides used, were obtained from the starting plasma fraction II+III. The specificity of the resulting purified human antibodies was directly tested by immunoblotting with plasma FVIII, recombinant FVIII, and the fragments obtained after thrombin proteolysis (see Table 2).

[0088] The IgG isotype distribution in the human purified antibody preparations was found to be quite heterogeneous. Interestingly, 40 to 79% of the recovered IgGs belonged to the IgG2 subclass. In most preparations, IgG4 appeared to be over-represented (up to 25%).

[0089] All the human anti-FVIII-peptide antibody preparations were tested for the capacity to inhibit FVIII activity in a one-stage clotting assay. Table 2 shows that seven out of 13 preparations tested (54%) displayed inhibitory activity, SEQ ID No: 14, SEQ ID No: 19, SEQ ID No: 2, SEQ ID No: 5, SEQ ID No: 22, SEQ ID No: 32 and SEQ ID No: 33, respectively. As a typical example, the inhibition of FVIII activity in function of anti-SEQ ID 32 Ig concentration is shown in figure 2.

Human anti-FVIII-peptide Ig immunospecificity towards FVIII

[0090] The specificity of the resulting purified human antibodies was tested by immunoblotting with plasma FVIII, recombinant FVIII, and the fragments obtained after thrombin proteolysis. Again, the FVIII fragments were identified with either FVIII-HC- or FVIII-LC-specific mouse monoclonal antibodies or FVIII-peptide-specific rabbit polyclonal antibodies. The human antibodies were identified after binding of biotinylated goat anti-human IgG. Figure 3 shows the immunoreaction of high-molecular-weight FVIII (≥ 92-kDa) with four human antibody preparations, purified on Sepharose coupled to FVIII peptide SEQ ID No: 11 (Ser^{109}-Lys^{127}), SEQ ID No: 14 (Cys^{329}- Asp^{348}), SEQ ID No: 15 (Tyr^{407}-Lys^{425}) or SEQ ID No: 19 (Cys^{711}-Asp^{725}). The 50-kDa FVIII fragment (domain A1) was recognized by human antibodies purified on Ser^{109}- Lys^{127} or Cys^{329}-Asp^{348}-Sepharose and the 44-kDa FVIII fragment (A2) by immunoglobulins purified on Tyr^{407}-Lys^{425} and Cys^{711}-Asp^{725}-Sepharose. The lack of reactivity of the anti-(Ser^{817}-Ser^{830}) immunoglobulin preparation (HAP5) with the FVIII fragments confirms that this epitope is located in the amino-terminal end of domain B (Figure 3). Human antibodies purified on Sepharose coupled to peptide SEQ ID No: 1 (Arg^{1652}-Tyr^{1664}) or

SEQ ID No: 2 (Asp^{1681}-Arg^{1696}) reacted strongly with the 80-kDa FVIII light chain (Figure 3). For both preparations, the reaction with the 80-kDa band disappeared after thrombin proteolysis, indicating that the epitopes, as expected, are located in the *a3* acidic peptide at the NH_2-terminal part of the FVIII A3 domain. When human antibodies specific for peptide SEQ ID No: 5 (Arg^{1797}-Tyr^{1815} in A3 domain) were analyzed by immunoblotting, their specificity for rFVIII appeared restricted to the 80-kDa FVIII light chain and its 72-kDa thrombin fragment.

[0091] No immunoreaction with the rFVIII chains or fragments was detected with antibody preparations specific for FVIII peptides SEQ C and SEQ ID No: 23, although a positive reaction was obtained in the ELISA using rFVIII. This could mean that these immunoglobulin preparations recognize a conformational epitope.

Use of FVIII synthetic peptides to characterize human anti-FVIII antibodies in hemophilia A patient plasmas

[0092] The selected peptides were used in ELISA experiment to determine the anti-FVIII antibody specificity's present in hemophilia A plasmas. The peptides were coated on microplate (25 μg/ml in PBS buffer during 16h at 4°C). A 1/10 to 1/1000 dilution of plasma patient in Tris-casein buffer was reacted with the coated peptide for 2h at 37°C. The bound human IgG was measured as described in Methods. Control samples were plasma pools of healthy donors. Figure 4 shows the results obtained with the plasma of 4 hemophilia A patients. The optical densities are corrected average values (OD patient-OD normal plasma pool) of two independent experiments.

Molecular model epitope prediction

[0093] Pemberton et al (1997) have built a molecular model of the A domains of FVIII. This 3-D model makes it possible to explore predictions for important regions of FVIII activity. The model was used to locate the FVIII-peptide epitopes identified by the Parker and Hodge algorithms. As predicted by these algorithms, all peptides located in the A domains were found on the FVIII surface and were fully accessible to specific.

[0094] The overlap between the epitope and the FIXa-binding loop (5 common residues spanning Glu^{1811}-Tyr^{1815}) may explain the inhibitory action of the corresponding anti-(Arg^{1797}-Tyr^{1815}) antibodies on formation of the fibrin clot.

Analysis of the results

[0095] In the clotting test, significant inhibition of FVIII activity was recorded in the presence of rabbit anti-(Cys^{329}-Asp^{348}) and anti-(Arg^{1653}-Tyr^{1664}) antibodies, but different inhibition patterns were observed. Inhibition by anti-(Arg^{1653}-Tyr^{1664}) follows second-order kinetics with a drastic reduction in FVIII activity. Anti-(Cys^{329}-Asp^{348}) Ig is less efficient and shows a more complex type of reaction, with a non-linear dependence on the antibody concentration. Epitope Arg^{1652}-Tyr^{1664} and the adjacent major binding site vWF (residues Glu^{1675}-Glu^{1684}) are located in the acidic light-chain peptide *a3*. As shown by western blotting, *a3* is released from the A3 domain after thrombin treatment, preventing further binding of anti-(Arg^{1652}-Tyr^{1664}) Ig to activated FVIII. Similar results have been reported by Shima et al (1991), who described the FVIII sequence Asp^{1663}-Ser^{1669} as a binding site of rabbit polyclonal antibodies neutralizing FVIII activity. Epitope Cys^{329}-Asp^{348} overlapped the acidic Asp^{348}-Lys^{362} sequence (in *a1*) described as adjacent to the activated protein C

(Arg^{336}) and thrombin (Arg^{372}) cleavage sites. It is the target of human hemophilic inhibitors. Anti-(Asp^{348}-Lys^{362}) antibodies may interfere with proteolysis or with the FX interaction site (Met^{337}-Arg^{372}) (Saenko et al., 1999 and Scandella et al., 2000).

[0096] FVIII-neutralizing activity was measured in all 13 Ig preparations. Seven human Ig preparations displayed inhibition of procoagulant activity, these being specific for amino-acid residues Cys^{711}-Asp^{725}, Tyr^{1681}-Arg^{1696}, and Arg^{1797}-Tyr^{1815} respectively. The Cys^{711}-Asp^{725} sequence contains sulfated tyrosines at Tyr^{718}, Tyr^{719}, and Tyr^{723}, and overlaps with the FVIII HC region Lys^{713}-Arg^{740} described as promoting both activation and HC proteolysis. The additional sulfated groups may be required for proper interaction with thrombin or another component as in the FX-activating complex. The sequence also overlaps with region Gly^{701}-Ser^{750}, recognized by a weakly inhibitory mouse monoclonal antibody. Peptide P8 (Tyr^{1681}-Arg^{1696}) (FVIII LC) includes the sequence Glu^{1684}-Arg^{1689} already described by Shima et al, 1991. It contains the thrombin activation site Arg^{1689}-Ser^{1690}. P4 (Cys^{711}-Asp^{725}) is also included in the Asp^{712}-Ala^{736} sequence detected by analysis of the patient antibody repertoire by gene phage display technology. It is proposed as a possible additional inhibitor in patients (van den Brink et al, 2000). Peptide P9 (Arg^{1797}-Tyr^{1815}) contains the FXa binding site (see below).

[0097] Of the 16 anti-FVIII-peptide immunoglobulins purified from humans or produced in rabbits, 7 did neutralize FVIII activity under the tested conditions. Using small peptide sequences and immunobinding assays, we have provided evidence for additional new epitopes. We have located new epitopes in the A1 domain (residues Ser^{109}-Cys^{127}), the A2 domain (Cys^{407}-Lys^{425}), and the B domain (Ser^{817}-Ser^{830} and Glu^{1078}-Pro^{1092}).

[0098] Autoantibodies immunopurified with denatured FVIII have been reported in healthy subjects and in pools of normal human immunoglobulins (processed fraction II, see above) (Algiman et al., 1992 and Moreau et al., 2000). A possible role in clearance of denatured FVIII or its fragments from the bloodstream and/or in the immunotolerance was suggested.

[0099] Identification of the FVIII epitopes is a major challenge to be met in order to improve FVIII treatment and the quality of therapeutic FVIII concentrates. FVIII epitope sequences help to determine the contribution of patient polyclonal anti-FVIII Igs to overall inhibitory and regulatory activity. They could also be used to monitor the usual switch in anti-FVIII specificity in a patient during treatment. Said characterization of FVIII epitopes and a model of their locations on the folded molecule improves the treatment of inhibitors in both hemophilic and non- hemophilic patients (detection, follow-up, therapeutic use of FVIII epitope peptides...).

Table 1 Characterization of rabbit anti-FVIII peptides antibodies

SEQ ID(a)	Rabbit Antiserum(b)	ELISA Titre(c)		FVIII domain recognize(d)	RAP-IgG Recovery(e) µg/ml serum	Inhibitor Titre(f) BU/mg
		P-KLH	r-FVIII			
SEQ ID 11	RAP1	2.5	2.2	A1	27	-
SEQ ID 14	RAP2	3.6	2.5	A1/a1	55	1,5
SEQ ID 15	RAP3	2.5	3.2	A2	268	-
SEQ ID 19	RAP4	2.5	1.3	A2/a2	12	-
SEQ ID 21	RAP5	4.6	3.9	B	106	-
SEQ C	RAP6	3.8	2.9	-	14	-
SEQ ID 01	RAP7	3.9	3.9	a3 ↓	35	0,5
SEQ ID 02	RAP8	1.9	0.9	a3/A3 ↓	3	-
SEQ ID 05	RAP9	3.8	2.6	A3	42	-
SEQ ID 23	RAP10	3.9	0.8	-	65	-
SEQ ID 22	RAP11	ND	ND	ND	ND	ND
SEQ ID 26	RAP12	4,1	1,1	C2	ND	ND
SEQ ID 27	RAP13	3,7	1,1	C2	ND	ND
SEQ ID 28	RAP14	3,8	0,9	C2	ND	ND
SEQ ID 31	RAP15	3,2	0,7	C2	ND	ND
SEQ ID 32	RAP16	3,5	1,8	C2	ND	ND
SEQ ID 33	RAP17	4,8	1,2	C2	ND	ND

Table 2 Characterization of human anti-FVIII peptides autoantibodies

SEQ ID(a)	Human Anti-peptide Ig(b)	FVIII reactivity on immunoblot (-thrombin)(c)	FVIII reactivity on immunoblot (+thrombin)(d)	FVIII domain(e)	HAP-IgG Recovery(f) µg/10 mgIgG	FVIII inhibitory Activity(g) BU/mg
SEQ ID 11	HAP1	>92kDa	50kDa	A1	0,27	-
SEQ ID 14	HAP2	>92kDa	50kDa	A1/a1	1,07	3,4
SEQ ID 15	HAP3	>92kDa	44kDa	A2	0,06	-
SEQ ID 19	HAP4	92kDa	44kDa	A2/a2	0,12	+
SEQ ID 21	HAP5	>100kDa	-	B	0,26	-
SEQ C	HAP6	-	-	-	0,03	-
SEQ ID 01	HAP7	80kDa	80kDa	a3 ↓	0,20	-
SEQ ID 02	HAP8	80kDa	80kDa	a3/A3 ↓	0,01	+
SEQ ID 05	HAP9	80kDa	72kDa	A3	0,08	+
SEQ ID 23	HAP10	-	-	-	0,11	-
SEQ ID 22	HAP11	ND	ND	ND	0,98	4,3
SEQ ID 26	HAP12	ND	ND	ND	ND	ND
SEQ ID 27	HAP13	ND	ND	ND	ND	ND
SEQ ID 28	HAP14	ND	ND	ND	ND	ND
SEQ ID 31	HAP15	ND	ND	ND	ND	ND
SEQ ID 32	HAP16	80kDa	72 kDa	A3C1C2	2,40	6,3
SEQ ID 33	HAP17	ND	ND	ND	1,06	2,4

+: Inhibition >25% at 100µg/ml

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CLAIMS

1. An antigenic epitope of FVIII polypeptide sequence, which is selected from the group consisting of :

- the epitope comprised between serine 2018 and histidine 2031 inclusive, defined by the following sequence:
  SEQ ID No:10:
  Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
  1 5 10
- the epitope tyrosine 555 to glutamine 565 inclusive defined by the following sequence:
  SEQ ID No:17:
  Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
  1 5 10
- the epitope leucine 730 to serine 741 inclusive, defined by the following sequence:
  SEQ ID No:20:
  Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
  1 5 10
  possibly deleted from the terminal amino acid serine and/or the first amino acid leucine
- the epitope serine 817 to serine 830 inclusive, defined by the following sequence:
  SEQ ID No:21:
  Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
  1 5 10
- the epitope asparagine acid 2128 to asparagine acid 2138 inclusive, defined by the following sequence:
  SEQ ID No:24:
  Asn Val Asp Ser Ser Gly Ile Lys His Asn
  1 5 10
- the epitope serine 2204 to glutamine 2222 inclusive, defined by the following sequence:

SEQ ID No:27:

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

- the epitope isoleucine 2262 to glutamine 2270 inclusive, defined by the following sequence:

SEQ ID No:30:

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

- the epitope leucine 2273 to serine 2289 inclusive, defined by the following sequence:

SEQ ID No:31:

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
1 5 10 15
Ser

- the epitope proline 2292 to tyrosine 2305 inclusive, defined by the following sequence:

SEQ ID No:32:

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

possibly deleted from one or more amino acids of the terminal tripeptide Thr-Arg-Tyr

- the epitope glutamic acid 2322 to tyrosine 2332 inclusive, defined by the following sequence:

SEQ ID No:33:

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

2.A pool of antigenic epitopes of factor VIII polypeptide sequence which comprise one or more antigenic sequence epitope of factor VIII according to claim 1 and possibly an antigenic sequence epitope of factor VIII which is selected from the group consisting of :

- the epitope arginine 1648 to tyrosine 1664 inclusive, defined by the following sequence:

SEQ ID No:1:

```
Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1               5                  10                  15
Tyr
```

possibly deleted from one or more amino acids of the tetrapeptide Arg-Asp-Ile-Thr or one or two of the last amino acids of the peptide Asp-Tyr.

- the epitope aspartic acid 1681 to arginine 1696 inclusive, defined by the following sequence:

SEQ ID No:2:

```
Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
 1               5                  10                  15
```

possibly deleted from one or more amino acids of the epitope Asp-Glu-Asp-Glu.

- the epitope threonine 1739 to tyrosine 1748 inclusive, defined by the following sequence:

SEQ ID No:3:

```
Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
 1               5                  10
```

- the epitope asparagine 1777 to phenylalanine 1785 inclusive, defined by the following sequence:

SEQ ID No:4:

```
Asn Gln Ala Ser Arg Pro Tyr Ser Phe
 1               5
```

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Phe or the tetrapeptide Pro-Tyr-Ser-Phe.

- the epitope glutamic acid 1794 to tyrosine 1815 inclusive, defined by the following sequence:

SEQ ID No:5:

```
Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
 1               5                  10                  15
Asn Glu Thr Lys Thr Tyr
            20
```

possibly deleted from one or more amino acids from the first tripeptide Glu-Asp-Gln or the first nonapeptide Glu-Asp-Gln-Arg-Gln-Gly-Ala-Glu-Pro.

- the epitope methionine 1823 to aspartic acid 1831, defined by the following sequence:

SEQ ID No:6:

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

- the epitope glutamic acid 1885 to phenylalanine 1891 inclusive, defined by the following sequence:

SEQ ID No:7:

Glu Thr Lys Ser Trp Tyr Phe
1 5

- the epitope glutamic acid 1885 to alanine 1901 inclusive, defined by the following sequence:

SEQ ID No:8:

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

possibly deleted from one or more amino acids from the heptapeptide Gly-Thr-Lys-Ser-Trp-Phe-Thr or from the tripeptide Cys-Arg-Ala.

- the epitope aspartic acid 1909 to arginine 1917 inclusive, defined by the following sequence:

SEQ ID No:9:

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

- the epitope alanine 108 to valine 128 inclusive, defined by the following sequence:

SEQ ID No:11:

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

possibly deleted from the terminal amino acids alanine and/or valine

- the epitope glutamic acid 181 to leucine 192 inclusive, defined by the following sequence:

SEQ ID No:12:

```
Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
 1               5
```

possibly deleted from one or two amino acids of the terminal dipeptide Thr-Leu

- the epitope aspartic acid 203 to alanine 227 inclusive, defined by the following sequence:

SEQ ID No:13:

```
Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
 1               5                  10                  15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
            20
```

possibly deleted from one or more amino acids of the nonapeptide Asp-Arg-Asp-Ala-Ala-Ser-Ala-Arg-Ala

- the epitope aspartic acid 327 to methionine 355 inclusive, defined by the following sequence:

SEQ ID No:14:

```
Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
 1               5                  10                  15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
            20                  25
```

possibly deleted from one or more amino acids of the dipeptide Asp-Ser or the octapeptide Asp-Asp-Leu-Thr-Asp-Ser-Glu-Met.

- the epitope aspartic acid 403 to lysine 425 inclusive, defined by the following sequence:

SEQ ID No:15:

```
Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
 1               5                  10                  15
Ile Gly Arg Lys Tyr Lys Lys
            20
```

possibly deleted from one or more amino acids of the tetrapeptide Asp-Asp-Arg-Ser.

- the epitope valine 517 to arginine 527 inclusive, defined by the following sequence:

SEQ ID No:16:

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

possibly deleted from one or the two amino acids of the dipeptide Pro-Arg

- the epitope histidine 693 to glycine 701 inclusive, defined by the following sequence:

SEQ ID No:18

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

- the epitope serine 710 to aspartic acid 725 inclusive, defined by the following sequence:

SEQ ID No:19

Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

- the epitope isoleucine 2081 to serine 2095 inclusive, defined by the following sequence

SEQ ID No:22:

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

possibly deleted from one or more amino acids of the tetrapeptide Ile-His-Gly-Ile

- the epitope tyrosine 2105 to glycine 2121 inclusive, defined by the following sequence:

SEQ ID No:23:

Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15

Gly

possibly deleted from one or more amino acids of the tripeptide Tyr-Ser-Leu

- the epitope histidine 2152 to arginine 2163 inclusive, defined by the following sequence:

SEQ ID No:25:

His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg
1 5 10

- the epitope serine 2181 to asparagine 2198 inclusive, defined by the following sequence:

SEQ ID No:26:

Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr
1 5 10 15
Asn

possibly deleted from one or more amino acids from the terminal tripeptide Phe-Thr-Asn (P11)

- the epitope glutamine 2235 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:28:

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu or one or more amino acids of the tetrapeptide Val-Lys-Ser-Leu.

- the epitope glycine 2242 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:29:

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu

3. A conformational epitope which contains:

- either at least two different epitopes according to the claim 1 or,
- at least one epitope sequence according to claim 1 and an epitope selecting from the pool of epitopes sequences of claim 2.

4. A recombinant factor VIII having an amino acid sequence deleted from one of the antigenic epitopes sequences according to claim 1 or one or more epitopes sequences selected from the pool of epitopes of claim 2.

5. A complex comprising a carrier protein or a carrier peptide linked to an epitope sequence according to the claim 1 or 3.

6. An inhibitor of factor VIII polypeptide sequence which exhibits an immunoaffinity with the epitope sequence, the pool of epitopes sequences and/or the complex according to any one of the preceding claims 1 to 5.

7. The inhibitor according to Claim 6, which is an anti-factor VIII antibody or antibody fragment.

8. An anti-inhibitor, which is directed against the inhibitor of factor VIII according to Claim 6 or 7.

9. The anti-inhibitor according to Claim 8, which is an anti-anti-factor VIII idiotype antibody or antibody fragment.

10. A pharmaceutical composition, which comprises an adequate pharmaceutical carrier and at least one element selected from the group consisting of the epitope sequence, the pool of epitopes sequences, the complex, the recombinant factor VIII, the inhibitor and/or the anti-inhibitor according to any one of the preceding claims 1 to 9.

11. A diagnostic and/or purification device, which comprises at least one element which is selected from the group consisting of the epitope sequence, the pool of epitopes sequences, the complex, the inhibitor and/or the anti-inhibitor according to any one of the preceding Claims 1 to 9.

12. The device according to Claim 11, which is a diagnostic kit.

13. The device according to Claim 11, which is a chromatography column or filter.

14. Use of the pharmaceutical composition according to Claim 10 for the manufacture of a medicament in the treatment and/or the prevention of an immune disorder in a mammal induced by inhibitors of factor VIII polypeptide sequence.

15. A method of treatment of a physiological fluid (serum), obtained from a mammal patient (including a human) wherein said physiological fluid is put into the chromatography column according to the claim 13 in order to allow a binding of the inhibitors of factor VIII polypeptide sequence present in said physiological fluid with epitope, pool or complex comprised in said chromatography column wherein after elution of the column a physiological fluid without inhibitors of factor VIII polypeptide sequence is recovered for its reinjection to the mammal patient.

16. A process for identifying and obtaining inhibitors and/or anti-inhibitors according to any of the preceeding Claims 6 to 9, comprising the steps of:

- selecting an element from the group consisting of the epitope sequence, the pool of epitopes sequences and/or the complex according to any one of the preceding claims 1 to 5, attached to a solid support (preferably a solid support of a chromatography column),
- passing a physiological fluid (serum) obtained from a patient containing inhibitors of factor VIII polypeptide sequence through said chromatography column,
- eluting said column, and
- collecting the fractions containing inhibitors of factor VIII polypeptide sequence which have exhibited an immunoaffinity with said element.

17. The process according to Claim 16, further comprising the steps of:

- attaching the collected inhibitors of factor VIII upon a solid support (preferably a solid support of a chromatography column),
- passing a physiological fluid from a mammal patient containing anti-inhibitors of factor VIII upon said solid support,
- washing the support, preferably by eluting said column, and
- collecting the fractions containing anti-inhibitors of factor VIII which have exhibited an immunoaffinity with said inhibitors of factor VIII.

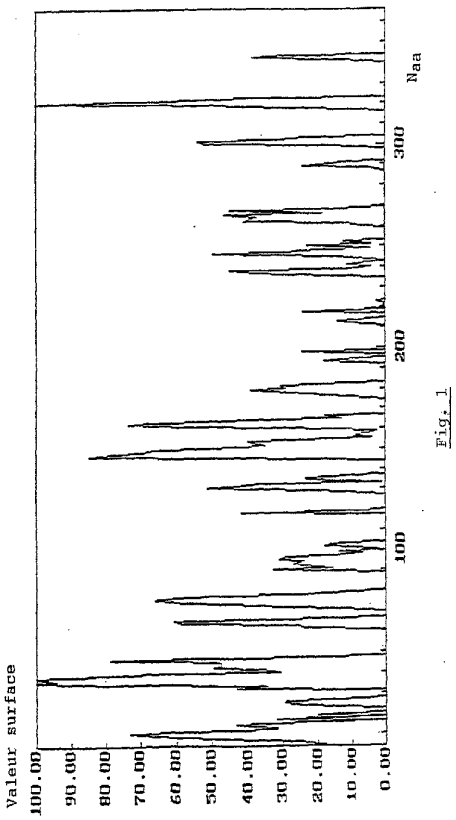
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Fig. 1

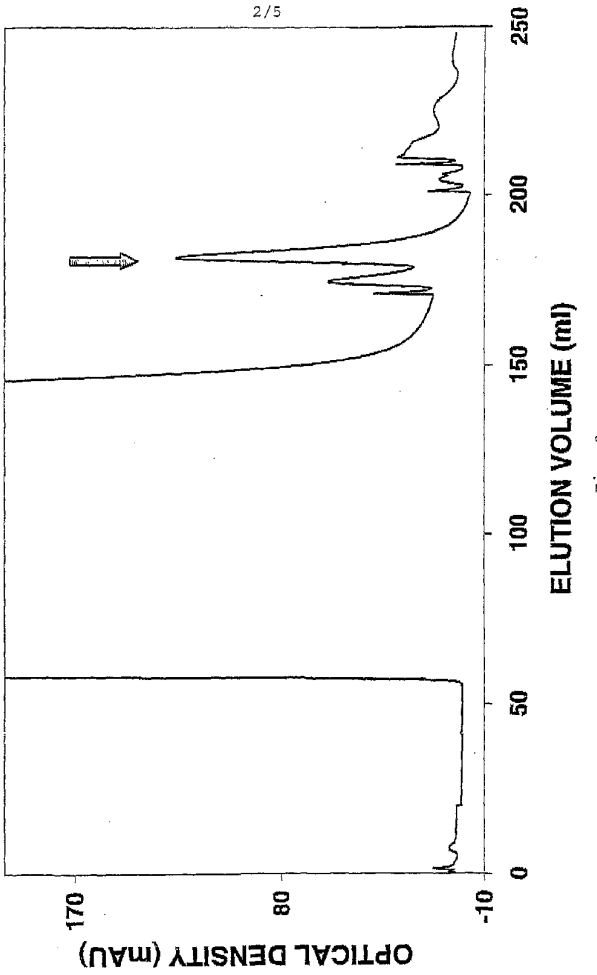
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Fig. 2a

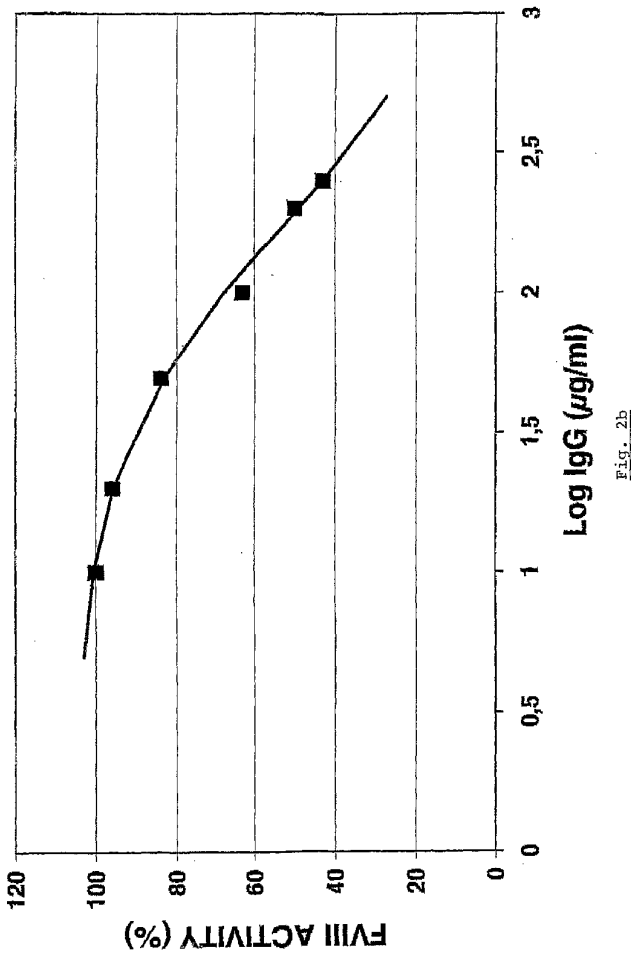
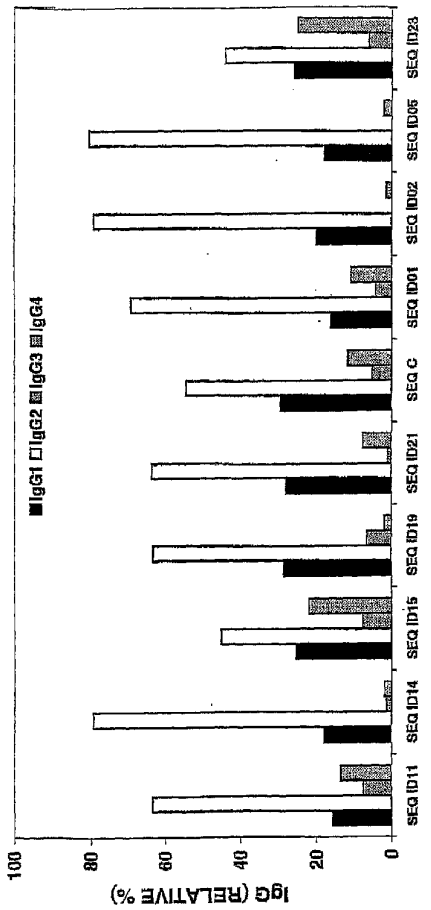


Fig. 2b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SEQ C
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Fig. 3

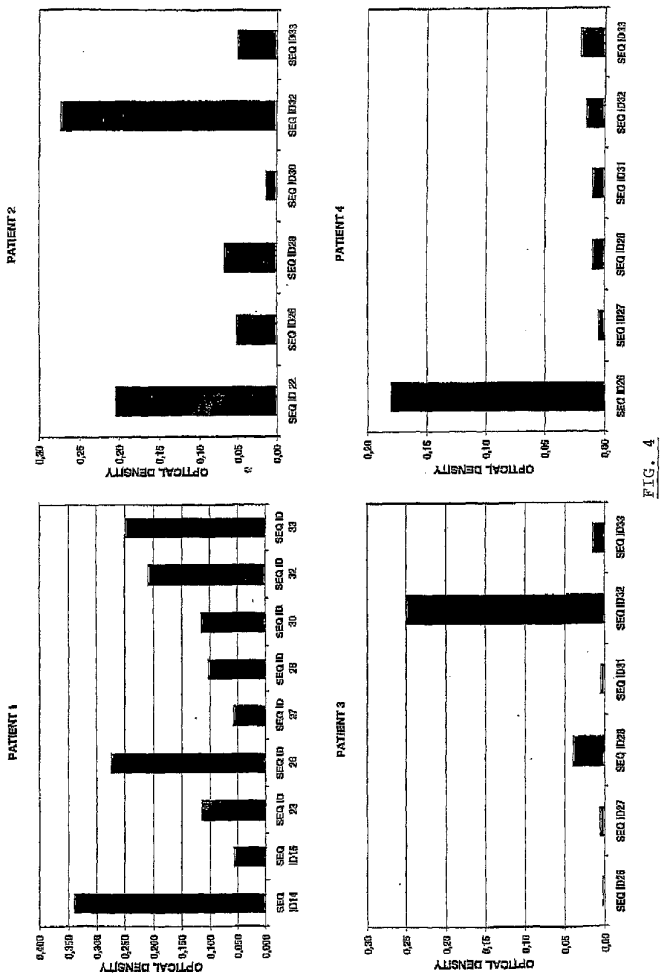

FIG. 4

SEQUENCE LISTING

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Di Gianbattista, Mario

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1 5 10 15

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<210> 31
<211> 17
<212> PRT
<213> Homo sapiens

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1 5 10 15

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1 5 10

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<400> 33

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1 5 10

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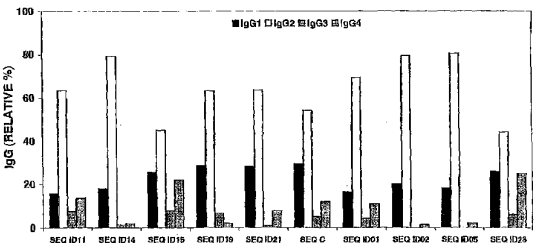

(57) Abstract: The present invention is related to antigenic polypeptide sequence (epitope of factor VIII) to the inhibitors which are directed against these sequence and to anti-inhibitors which are directed against said inhibitors. The present invention is also related to pharmaceutical composition and to a diagnostic device comprising at least one of the above mentioned molecules.

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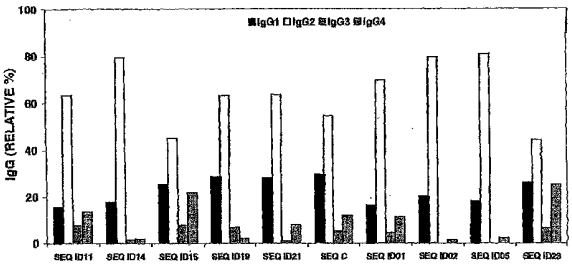

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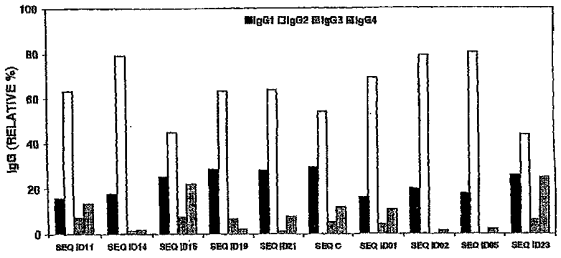

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WO 02/090542 A2

ANTIGENIC EPITOPES OF FACTOR VIII, INHIBITORS DIRECTED AGAINST SAID EPITOPES AND USE THEREOF

Subject of the invention

[0001] The present invention relates to the antigenic polypeptide sequences (epitopes) of factor VIII to the anti-FVIII inhibitors which are directed against these sequences and to anti-inhibitors which are directed against said anti-FVIII inhibitors.

[0002] The present invention also relates to a pharmaceutical composition and to a diagnostic device comprising at least one of the above mentioned molecules.

Technical background underlying the invention

[0003] FVIII is a large multi-domain protein of 2,332 amino acids made up of three structural domains, A, B and C which are arranged in the order A1:a1:A2:a2:B:a3:A3:C1:C2. The A domains possess more than 40% homology and are also homologous to ceruloplasmin (for recent review, see Pratt (2000) and Saenko (1999)). 30% homology also exists between the A domains of factor V and FVIII. The C domain occurs twice and is reported to be able to bind glyco-conjugates and phospholipids having a net negative charge. It exhibits homology with lectins which are able to bind to negatively charged phospholipids. The platelet attachment site has been located in this region (C2 domain) (Foster et al., (1990)).

[0004] These antigenic determinants consist of fragments 351 - 365 (A1 domain - heavy chain), 713 - 740 (A2 domain), 1670 - 1684 (A3 domain - light chain) (NH_2 end of the light chain) or else 2303 - 2332 (C2 domain - light chain) (Foster C, (1990)), fragments 701 - 750, 1663 - 1689, 330 - 472, 1694 - 1782 (EP-0 202 853), 322 - 740 and 2170 - 2322.

[0005] The U.S patent 5,744,446 describes an hybrid human/animal Factor VIII having a sequence of amino acids selected from the group of the A2 domain fragments 373-540, 373-508, 445-508, 484-508, 404-508, 489-508 and 484-489, with corresponding sequences of porcine or murine Factor VIII, said hybrid being used for the treatment of Factor VIII deficiencies.

[0006] The antibodies which recognize these various sites interfere, with the activation of FVIII, the binding of vWf, FIXa, FXa, APC or phospholipids. The specific antibody response to FVIII vary considerably among individuals, and epitopes for inhibitor antibodies have to be determined for all FVIII domains (see for recent review Scandella, 2000; Lollar, 2000).

[0007] Other antibodies, which do not inhibit standard activity tests in vitro, can exert an influence on the behavior of FVIII with the other constituents of the coagulation cascade while attaching themselves to sites in the molecule which are at a substantial distance from the active sites. These antibodies, can interfere with the natural state of folding of FVIII by altering some of its properties.

[0008] Emergence of alloantibodies (inhibitors) that neutralize infused FVIII activity may seriously complicate FVIII replacement therapy. Reported inhibitor incidence rates in hemophiliacs vary considerably. They range around 6-35% (Vermylen et al, 1998). Candidates for genetic

predispositions such as large deletions and intron 22 inversion have been found associated with a high incidence of inhibitors and genes that are involved in the immune response as genes MHC class I and class II (Tuddenham and McVey, 1998). Repeat switching from one FVIII product to another and the possibility that some FVIII concentrates are more immunogenic may also explain the appearance of inhibitors (Vermylen et al, 1998). Different methods of preparing FVIII could exert an influence on its structure, its physicochemical properties or its natural micro-environment; Laub et al. (1999); Raut et al. (1998)). Clinically relevant anti-FVIII autoantibodies are rare in non-hemophilic patients (annual frequency in the population: 1-5/10^6) (Morrisson and Ludlam) (1995). They are associated with a number of autoimmune diseases and are often characterized by life-threatening hemorrhage. On the other hand, anti-FVIII antibodies have also been described in healthy subjects (Algiman et al, 1992; Moreau et al, 2000), without any apparent effect on the subjects' levels of circulating FVIII.

[0009] Self proteins or derived peptides may elicit an immune response if presented to CD4 T cells at inflammatory sites by professional antigen presenting cells. Using pools of overlapping synthetic peptides spanning the sequences of individual FVIII domains, Reding et al. (2000) showed reactive $CD4^+$ to FVIII in healthy subjects and hemophilia patients. Several FVIII domains were recognized: A3 domain was recognized more strongly and frequently and each domain forms several epitopes.

[0010] Techniques such as western blotting, immunoprecipitation, and enzyme-linked immunosorbent assays (ELISAs), using well-defined FVIII proteolytic fragments, a large recombinant peptide library, or synthetic peptide arrays, have been used to map different FVIII-inhibitor

binding sites located mainly in the A2 and C2 domains. However, none of these techniques has made it possible to build a model for identification of inhibitor and non-inhibitor epitopes. Only a few epitopes have been mapped to discrete sequences (<20 amino-acid residues). To solve this problem, Palmer et al (1997) synthesized 96 undecamer peptides (11 amino-acid residues) representing 80% of the complete residue sequence of FVIII. They succeeded in determining the epitope specificity of 9 patients' inhibitory antibodies. Other useful techniques are analysis of FVIII gene mutations and their effects on the FVIII molecule as well as phage display technology (van den Brink et al, 2000). All these methodologies, however, are time consuming, rather costly, and largely dependent on patient availability. Certain areas of the FVIII molecule may be "hot spots" containing commonly recognized clusters of inhibitor epitopes, e.g., regions in the A2 domain, A3 domain, and C2 domain. The reason for these "hot spots" in generating an inhibitor response remains poorly understood (Reisner et al, 1995).

[0011] Currently, a predominant notion among hemophilic patients, clinicians and "fractionators" is that of having available a purified FVIII which is devoid of all pathogenic plasma contaminants and secondary effects.

[0012] Different animal models could be used as hemophilia dogs, scid mice, hemophilia mice ... but until now, no satisfactory experimental model exists which makes it possible to forecast the immunogenicity or the immuno-modulatory effect of the FVIII preparations, or the susceptibility of the host, before they have been administered clinically.

[0013] Patients who develop an anti-FVIII immune response find themselves in a serious situation which

necessitates the use of severe, aggressive and excessively expensive measures.

[0014] One of the frequently treatment, is the induction of immune tolerance by administration of very high doses of FVIII (150 IU/kg twice a day) in association or not with prothrombin complex concentrates and is assigned as "Bonn Protocol". Treatment options are also to by-pass the FVIII inhibitor activity by use of PCC (preferably an activated PCC [APCC]) or FVIIa. Specific antibodies as consequence of the infusion of these alternative agents could be produced, impairing the treatment. As an alternative agent porcine FVIII may be used to achieve haemostasis in patients with antibodies that do not substantially crossreact with porcine FVIII before or during the treatment (Lollar, 2000).

[0015] A potential alternative approach to inhibit the production of inhibitors is blockade of the T cell/B cell collaboration mediated by through receptor ligand binding signal events (Ewenstein et al, 2000). Preliminary clinical trials were performed using a humanized mouse monoclonal antibody to human T cell CD40 ligand (CD 154).

[0016] A profitable strategy for reducing the level of inhibitors has consisted in subjecting patients to an extracorporeal circulation to enable solid-phase absorption of the total IgG.

[0017] The immunoabsorbant could be sepharose-bound staphylococcal protein A or sepharose-bound polyclonal sheep antibodies to total human immunoglobulin (Knobf and Derfler, 1999). The foreign proteins (protein A, sheep anti-human Ig) could leak from the column and triggered the immune system of the recipient; moreover problems could raised as sanitisation (ICH Topic Q5A, Directive 92/79/EC).

[0018] The infusion of polyvalent intravenous immunoglobulins (IVIG), where appropriate combined with an immunosuppressive treatment, has been found to be relatively effective, although the reason for this effectiveness is still not fully established. Various hypotheses involving feed-back inhibition of IgG synthesis, stimulation of IgG clearance or activation of T suppressor cells have been advanced. An interesting explanation is that these commercial intravenous immunoglobulins might contain antibodies which are able to react with the variable parts (idiotypes) of the anti-FVIII antibodies and neutralize these antibodies (Dietrich et al. (1992)).

[0019] Unfortunately, none of these approaches has been found to be satisfactory in terms of safety, efficacy, efficiency and cost.

[0020] The state of the art in epitope structure prediction was limited given to the fact that non-continuous amino acid residues seem to constitute most important epitope and that the dynamics of binding is often not integrated into the epitope prediction equation making epitope structure prediction a complex four-dimensional problem (Van Regenmortel, Methods: A companion to Methods in Enzymology, 9, page 465-472, 1996).

[0021] According to the author, most of the antibodies raised against intact proteins do not react with any peptide fragment derived from the parent protein indicating that such antibodies are directed to discontinuous epitopes (conformational epitopes).

[0022] This author states also that low success rate of antigenic prediction is due to the fact that predictions concerns only continuous epitopes and it is unrealistic to reduce the complexity of epitopes that always possess conformational features to one dimensional linear peptide model.

[0023] Similarly, Palmer et al. (1997) using synthetic peptide arrays to identify novel Factor VIII inhibitor epitopes note that each patient pattern of anti-factor VIII antibody reactivity appears to be polyclonal, directed against multiple sites located within the amino and carboxyl terminus of the protein and seems to be unique for each plasma investigated (see also above).
Moreover, this author notes that it is difficult to predict the importance that any given antibody: epitope interaction may have on Factor VIII coagulation activity based on the results of synthetic peptide assays alone (due to the uncomplete understanding of the relationship between structure and function of different factor VIII domains and the possibility that both inhibitor and non-inhibitory antibodies may be present in a patient's plasma.

[0024] Therefore, the documents of the state of the art do not suggest to identify antigenic linear peptides upon a macro-molecule (such as Factor VIII) and that linear epitopes could be used for the diagnostic and/or the therapy of immune disorders induced by inhibitors directed against Factor VIII.

[0025] The international patent application WO96/02572 describes antigenic fragment and epitope sequences of factor VIII and inhibitors directed against some of these sequences.

Aims of the invention

[0026] The present invention aims to obtain new antigenic epitopes of factor VIII in order to improve the diagnosis and/or the therapy (including the prevention) of immune disorders (in particular those induced by inhibitors of FVIII, especially inhibitors of the binding of FVIII to the von Willebrand factor (vWf), to the FIX and/or to membrane phospholipids (PL)), said epitopes allowing a

screening between non-inhibitory and inhibitory anti-FVIII allo- or auto-antibodies (allo- or auto-immunoglobulins).

[0027] Another aim of the invention is to obtain inhibitors which exhibit an immunoaffinity with these antigenic epitopes, as well as to obtain anti-inhibitors, in particular antibodies or (T)cell receptors, which are directed against the abovementioned said inhibitors and whose purpose is to improve the diagnosis and/or therapy (or prevention) of immune disorders.

[0028] A further aim of the invention is to obtain said molecules at high purity, in industrial level, without contaminants (viruses, prions,...) and according to the GMP practices in the field of therapy and diagnostics (ICH topic QSA, Directive 92/79/EC, etc.).

Summary of the invention

[0029] The present invention relates to the antigenic polypeptide sequences (epitopes) of factor VIII whose complete sequence is described by Verhar et al. (1984) and which provides the reference for the amino-acids numerotation of the complete factor VIII sequence.

[0030] The "complete polypeptide sequence of factor VIII" is understood to be the natural human or animal sequence, which may be glycosylated and which has been obtained by purification from pools of plasma, in particular cryoprecipitate, by synthesis and/or by genetic manipulation (sequence from which portions which are not involved in the mechanism of blood coagulation may have been deleted) of factor VIII.

[0031] The present invention relates, in particular, to an antigenic epitope sequence of factor VIII which is selected from the group consisting of :

- the epitope comprised between serine 2018 and histidine 2031 inclusive, defined by the following sequence:

SEQ ID No:10:

Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
1 5 10

- the epitope tyrosine 555 to glutamine 565 inclusive, defined by the following sequence:

SEQ ID No:17:

Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
1 5 10

- the epitope leucine 730 to serine 741 inclusive, defined by the following sequence (P4):

SEQ ID No:20:

Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
1 5 10

possibly deleted from the terminal amino acid serine (P4) and/or the first amino acid leucine

- the epitope serine 817 to serine 830 inclusive, defined by the following sequence (P5):

SEQ ID No:21:

Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
1 5 10

- the epitope asparagine 2128 to asparagine 2138 inclusive, defined by the following sequence:

SEQ ID No:24:

Asn Val Asp Ser Ser Gly Ile Lys His Asn
1 5 10

- the epitope serine 2204 to glutamine 2222 inclusive, defined by the following sequence (P12):

SEQ ID No:27:

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

- the epitope isoleucine 2262 to glutamine 2270 inclusive, defined by the following sequence:

SEQ ID No:30:

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

- the epitope leucine 2273 to serine 2289 inclusive, defined by the following sequence (P14):

SEQ ID No:31:

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Glu Asp
1 5 10 15
Ser

- the epitope proline 2292 to tyrosine 2305 inclusive, defined by the following sequence (P15):

SEQ ID No:32:

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

possibly deleted from one or more amino acids of the terminal tripeptide Thr-Arg-Tyr involved in the phospholipid von Willebrand factor binding site

- the epitope glutamic acid 2322 to tyrosine 2332 inclusive, defined by the following sequence (P16):

SEQ ID No:33:

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

[0032] The invention also relates to the major parts of the said epitopes. Said epitopes can be deleted from one or more terminal amino acids, preferably from one, two or three amino acids, or can be replaced by one or more amino acids that present the same characteristic of hydrophilicity, flexibility and accessibility.

[0033] It is also known that some of the epitopes according to the invention are comprised in major determinants of human inhibitors epitopes or several factors binding sites or binding sites of known monoclonal antibodies, especially the portion C2 that is known to be the binding site of the monoclonal antibody Mas531P or the binding site ESH8 as well as phospholipids, Factor Xa or the von Willebrand factor binding site. However, the specific epitopes according to the invention or their major

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parts are preferred selected portions of said binding sites or may include a possible overlapping with said binding sites.

[0034] These epitopes sequences are particularly advantageously characterized by high hydrophilicity, which has been defined by Parker and Hodges (1986), considerable flexibility, which has been defined by Karplus and Schultz (1985) and considerable accessibility, which has been defined by Janin (1979).

[0035] These epitopes are, in particular, exposed on the surface of the factor VIII protein and exhibit pronounced antigenic and immunogenic characteristics.

[0036] Another aspect of the present invention is related to a modified (recombinant or transgenic) FVIII, possibly obtained by genetic engineering, and deleted from one or more of the above-identified epitopes or major parts of said epitopes.

[0037] Advantageously, said FVIII still allows the binding of coagulation factor(s), but will be less immunogenic and will not induce or induce less the formation of inhibitors directed against said modified FVIII or natural FVIII.

[0038] Advantageously, said epitopes are also independently immunogenic (that is to say they are immunogenic even without being complexed with a protein of large size such as BSA, KLH, haemocyanin, etc.), and preferably exhibit an immunoaffinity within inhibitors of factor VIII, such as anti-factor VIII antibodies, and/or exhibit an immunoaffinity for the receptors of the T lymphocytes and possibly B lymphocytes.

[0039] These epitopes and/or major parts of said epitopes induce an immune reaction (antibody synthesis) when they are injected into a rabbit.

[0040] Said sequences are unexpectedly characterized

by substantial immunogenicity towards monoclonal and polyclonal antibodies, but are sufficiently short to be readily and advantageously obtained by synthesis.

[0041] The present invention also relates to the conformational epitopes which comprise at least two different sequence epitopes and/or at least two major parts of said epitopes according to the invention and above identified.

[0042] The conformational epitopes are made up of two or more different portions of a polypeptide sequence, which portions are located in proximity to each other when the protein is folded in its tertiary or quaternary structure.

[0043] These epitopes are capable of being "recognized" (that is to say of exhibiting an immunoaffinity), preferably simultaneously, with inhibitors of factor VIII, in particular B and T lymphocytes (by way of the major histocompatibility locus (MHC I and/or II)) and/or anti-factor VIII antibodies (Scandella et al. (2000); Reding et al. (2000)).

[0044] Preferably, the said epitopes and/or the major parts of said epitopes are complexed with a carrier protein or a carrier peptide, such as BSA, or KLH haemocyanin, as to form a complex exhibiting a more powerful immunogenicity.

[0045] The present invention is also related to a pool of antigenic epitopes of factor VIII which comprises a mixture of the epitopes above mentioned linear epitopes (SEQ ID 10, SEQ ID 17, SEQ ID 20, SEQ ID 21, SEQ ID 24, SEQ ID 27, SEQ ID 30, SEQ ID 31, SEQ ID 32, SEQ ID 33) or conformational epitopes made of said epitopes or a pool which may comprise at least one of said epitopes and one or more additional antigenic epitopes of factor VIII already described in the state of the art and preferably selecting

from the group consisting of :

- the epitope arginine 1648 to tyrosine 1664 inclusive, defined by the following sequence:

SEQ ID No:1:

Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1 5 10 15
Tyr

and possibly deleted from one or more amino acids of the tetrapeptide Arg-Asp-Ile-Thr (P7), or one or two of the last amino acids of the dipeptide Asp-Tyr

- the epitope aspartic acid 1681 to arginine 1696 (P8) inclusive, defined by the following sequence:

SEQ ID No:2:

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
1 5 10 15

possibly deleted from one or more amino acids of the epitope Asp-Glu-Asp-Glu,

- the epitope threonine 1739 to tyrosine 1748 inclusive, defined by the following sequence:

SEQ ID No:3:

Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
1 5 10

- the epitope asparagine 1777 to phenylalanine 1785 inclusive, defined by the following sequence:

SEQ ID No:4:

Asn Gln Ala Ser Arg Pro Tyr Ser Phe
1 5

possibly deleted from one or more amino acids of the terminal dipeptide Ser-Phe or tetrapeptide Pro-Tyr-Ser-Phe

- the epitope glutamic acid 1794 to tyrosine 1815 inclusive, defined by the following sequence:

SEQ ID No:5:

Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
1 5 10 15
Asn Glu Thr Lys Thr Tyr

20

possibly deleted from one or more amino acids of the first tripeptide Glu-Asp-Gln (P9) or the first nonapeptide Glu-Asp-Gln-Arg-Gln-Gly-Ala-Glu-Pro

- the epitope methionine 1823 to aspartic acid 1831 inclusive, defined by the following sequence:

SEQ ID No:6:

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

- the epitope glutamic acid 1885 to phenylalanine 1891 inclusive, defined by the following sequence:

SEQ ID No:7:

Glu Thr Lys Ser Trp Tyr Phe
1 5

- the epitope glutamic acid 1885 to alanine 1901 inclusive, defined by the following sequence:

SEQ ID No:8:

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

possibly deleted from one or more amino acids from the heptapeptide Glu-Thr-Lys-Ser-Trp-Phe-Thr or from the tripeptide Cys-Arg-Ala.

- the epitope aspartic acid 1909 to arginine 1917 inclusive, defined by the following sequence:

SEQ ID No:9:

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

- the epitope alanine 108 to valine 128 inclusive, defined by the following sequence:

SEQ ID No:11:

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

possibly deleted from the terminal amino acids alanine and valine (P1)

- the epitope glutamic acid 181 to leucine 192 inclusive, defined by the following sequence:

SEQ ID No:12:

```
Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
 1               5
```

possibly deleted from one or two amino acids of the terminal dipeptide Thr-Leu

- the epitope aspartic acid 203 to alanine 227 inclusive, defined by the following sequence:

SEQ ID No:13:

```
Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
 1               5                  10                  15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
            20                  25
```

possibly deleted from one or more amino acids of the nonapeptide Asp-Arg-Asp-Ala-Ala-Ser-Ala-Arg-Ala

- the epitope aspartic acid 327 to methionine 355 inclusive, defined by the following sequence:

SEQ ID No:14:

```
Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
 1               5                  10                  15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
            20                  25
```

possibly deleted from one or more amino acids from the terminal dipeptide Asp-Ser or the octapeptide Asp-Asp-Leu-Thr-Asp-Ser-Glu-Met (P2).

- the epitope aspartic acid 403 to lysine 425 inclusive, defined by the following sequence:

SEQ ID No:15:

```
Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
 1               5                  10                  15
Ile Gly Arg Lys Tyr Lys Lys
            20
```

possibly deleted from one or more amino acids of the tetrapeptide Asp-Asp-Arg-Ser (P3),

- the epitope valine 517 to arginine 527 inclusive, defined by the following sequence:

SEQ ID No:16:

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

possibly deleted from one or the two amino acids of the dipeptide Pro-Arg,

- the epitope histidine 693 to glycine 701 inclusive, defined by the following sequence:

SEQ ID No:18:

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

- the epitope serine 710 to aspartic acid 725 inclusive, defined by the following sequence (P4):

SEQ ID No:19:

Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

- the epitope isoleucine 2081 to serine 2095 inclusive, defined by the following sequence:

SEQ ID No:22:

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

possibly deleted from one or more amino acids from the tetrapeptide Ile-His-Gly-Ile

- the epitope tyrosine 2105 to glycine 2121 inclusive, defined by the following sequence:

SEQ ID No:23:

Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15
Gly

possibly deleted from one or more amino acids of the tripeptide Tyr-Ser-Leu (P10)

- the epitope glutamine 2235 to leucine 2251 inclusive, defined by the following sequence (P13):

SEQ ID No:28:

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu or one or more amino acids of the tetrapeptide Val-Lys-Ser-Leu

- the epitope glycine 2242 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:29:

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu, said epitope presenting a possible partial overlapping with a known monoclonal antibody binding site ESH8 2248-2285

[0046] Another aspect of the present invention relates to an inhibitor of factor VIII which exhibits an immunoaffinity with antigenic epitopes, with the major parts of said epitopes and/or with the complex according to the invention.

[0047] An inhibitor is understood to mean any biological molecule or cell (such as a T-lymphocyte) binding to said FVIII and capable of giving rise to immune disorders (characterized by humoral immune response and/or cellular immune response against said FVIII).

[0048] In particular, such an inhibitor can be an anti-factor VIII monoclonal or polyclonal antibody or antibody fragment (such as the hypervariable Fab portion of the said antibody) which inactivates the said factor VIII and/or which inhibits the binding of factor VIII to the von Willebrand factor and/or to membrane phospholipids.

[0049] Advantageously, the said inhibitors are synthesized by a "chimaeric" animal which comprises a human immune system, such as an hu-SCID mouse or transgenic mouse

producing human antibodies or other antibodies production technologies as phage display technology or immortalized B-cells, by EPV in particular.

[0050] Another aspect of the invention relates to an anti-inhibitor which is directed against the said previously described factor VIII inhibitor.

[0051] An anti-inhibitor which is directed against the factor VIII inhibitor is understood to mean any chemical or biological molecule, a cell and/or a cell fragment (receptor) which is capable of interfering with the said inhibitor in such a way as to ensure its inactivation or avoid or reduce its binding to the factor VIII.

[0052] Preferably, such an anti-inhibitor is an anti-anti-factor VIII idiotype (monoclonal or polyclonal) antibody or antibody fragment, natural or obtained by genetic engineering.

[0053] Another aspect of the invention relates to a pharmaceutical composition which comprises an adequate pharmaceutical carrier or a diluant and an element selected from the group consisting of said epitopes or a pool thereof, an inhibitor of factor VIII which is directed against them, an anti-inhibitor which is directed against the said inhibitor, and/or a mixture of these.

[0054] The type and amount of adequate pharmaceutical carrier or diluant (and possibly adjuvant or excipient) present in said pharmaceutical composition, may vary according to the method of administration and is possibly combined an adjuvant in order to improve therapeutical properties of the pharmaceutical composition according to the invention or to reduce its possible side effects. Suitable pharmaceutical acceptable carriers used in the pharmaceutical composition according to the invention are well known by the person skilled in the art

and are selected according to the methods generally applied by pharmacists and may include solid, liquid or gaseous non-toxic pharmaceutically acceptable carriers. The percentage of active product / pharmaceutical acceptable carrier may vary within very large ranges only limited by the tolerance and the possible side effects on patients (including humans), and by frequency and/or mode of administration.

[0055] Another aspect of the invention relates to a diagnostic and/or purification device, such as a diagnostic kit, an affinity filter, or a chromatography column which comprises an element which is selected from the group consisting of these epitopes and/or major parts of said epitopes, the complex according to the invention or a pool thereof, an inhibitor which is directed against them, an anti-inhibitor which is directed against said inhibitor, and/or a mixture of these. Advantageously, said device comprises the pool of said epitopes which allow a screening of patients and may detect the most important inhibitors present in said patients and which allow a positive test with enough specificity and sensibility.

[0056] The purification device can therefore consist of a chromatography column which comprises these epitopes and/or major parts of epitopes, attached to the solid phase of the chromatography column.

[0057] A physiological liquid (such as serum), which is derived from a patient and which comprises inhibitors of factor VIII pass through a solid support (chromatography column), with said inhibitors (for example antibodies) becoming attached specifically to said epitopes or said major parts or a pool thereof. Following elution, it is possible to collect said inhibitors by causing them to react with anti-inhibitors (anti-anti-factor VIII idiotype antibodies).

[0058] It is also possible to characterize the anti-anti-factor VIII idiotype antibodies which are present in a serum by these anti-inhibitors passed through a solid support (chromatography column) on which inhibitors of factor VIII have been attached to the solid phase.

[0059] It is also possible to reinject (*ex vivo* treatment) the physiological liquid (blood or serum or a derived fraction) to said patient after its inhibitors of factor VIII have been removed by binding with said epitopes or a pool thereof; said inhibitors being removed from the physiological fluid (blood or serum) similarly as proposed for dialysis method applied to human patients.

[0060] The present invention is also related to a method of treatment (*ex vivo* treatment) of a patient suffering from a pathology induced by inhibitors to the factor VIII which comprises the steps of extracting said physiological liquid (blood or serum) from the patient, obtaining its reaction upon a solid support binding the epitopes or a pool thereof according to the invention and reinjecting said physiological liquid to the patient after the removing of the inhibitors having fixed said epitopes, majors parts or a pool thereof.

[0061] A final aspect of the invention relates to the use of the pharmaceutical composition according to the invention for preparing a medicament used for preventing and/or treating immune disorders, in particular those induced by inhibitors of factor VIII, inhibitors of the binding of factor VIII to the factor IX and/or the factor X and/or the von Willebrand factor (vWF) and/or inhibitors of the binding of factor VIII to membrane phospholipids.

[0062] The present invention will be described in details in the following non-limiting examples in reference to the enclosed figures.

Brief description of the figures

[0063] Figure 1 depicts the hydrophilicity, flexibility and accessibility graph of the A3 sequence of Factor VIII renumbered 1 to 371 amino acids (surface value for each amino acid).

[0064] Figure 2a represents the elution profile related to the purification of human anti-SEQ ID 32 antibodies by affinity chromatography on peptide-Sepharose column. Cohn fraction II+III solution (50 ml) was loaded onto the column (1 ml gel) at a flow rate of 1 ml/min. The separation of specific antibodies was performed as described hereafter. The arrow indicates the position of specific human anti-SEQ ID 32 antibodies.

[0065] Figure 2b represents FVIII clotting activity in the presence of anti-(SEQ ID 32) IgG purified from Cohn fraction II+III. The clotting activity of FVIII was measured as described hereafter in the presence of increasing amount of anti-SEQ ID 32. The % of FVIII activity =(FVIII activity in the presence of antibody/FVIII activity in absence of antibody)*100.

[0066] Figure 3 represents the human anti-peptide antibody immunoreactions with FVIII polypeptides after western blotting (panel A from left to right : human antibodies HAP1 through HAP4, specific for different FVIII epitope sequences found in the FVIII HC - see also table 2 and panel B : human antibodies specific for the P5 peptide and the FVIII LC sequences, P7, P8 and P9 - see also table 2). The RAP9 lane shows the reactivity of FVIII polypeptides towards purified rabbit antibodies specific for the peptide sequence Arg^{1797}-Tyr^{1815} (see also table 2).

[0067] Figure 4 represents ELISA reactivity of 4 inhibitor plasmas with different peptide sequences. Inhibitors present in 4 patients plasmas were analyzed by

ELISA test using as coated antigens the different selected FVIII epitopes synthetic peptides as indicated in ordinate.

Examples

Materials and Methods

Reagents

[0068] MAS530p (Harlan-Seralab, Indianapolis, IN) is a mouse monoclonal antibody specific for the 44-kDa A2 domain of the factor VIII heavy chain. Biotin-labeled rabbit IgG anti-mouse IgG was purchased from Dakopatts (Copenhagen, Denmark). Biotin-labeled goat IgG anti-human IgG and biotin-labeled mouse IgG anti-rabbit IgG were obtained from Sigma Chemicals (St Louis, MI), purified α-thrombin (3000 IU/mg), streptavidin-peroxidase conjugate, ovalbumin (OVA), bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), and o-phenylenediamine (OPD) were purchased from Sigma Chemicals (St. Louis, MI). Casein was obtained from Merck (Darmstadt, Germany). 4-chloro-1-naphtol and biotinylated molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA). Freund's adjuvant was from Difco (Detroit, Michigan).

FVIII concentrates

[0069] Plasma FVIII (p-FVIII) was a solvent/detergent-treated FVIII concentrate (100 IU/mg protein) purified by ion exchange chromatography (FVIII Conc. SD, CAF-DCF- Red Cross , Brussels, Belgium). Albumin-free recombinant FVIII (rFVIII) was obtained from Hyland (Glendale, CA).

Plasma fraction immunoglobulins

[0070] Cohn Fraction II+III was obtained from large plasma pool from 4,800 unpaid donors, after precipitation in the presence of increasing ethanol concentration. This fraction contains all Ig classes and subclasses. IgG composition was determined by nephelometry. The relative percentage of each subclass was 63,7; 30,1; 3,4 and 2,8 for IgG1, IgG2, IgG3 and IgG4 respectively (average values for 3 different batches of FII+III).

Factor VIII concentrates, Factor VIII activity and activity inhibition

[0071] Factor VIII activity was determined in a one-stage clotting assay adapted for use on the Coagulometer KC4A (Sigma Diagnostics). The assay uses severe hemophilia A plasma (Organon Teknika, Cambridge, UK) and APPT reagent from Instrumentation Laboratory (Warrington, UK). Potencies were calculated relative to the 5th International Standard FVIII concentrate 88/640 (5.4 IU/ml) (NIBSC, Potters Bar, UK). FVIII-inhibitory activity was measured in purified rabbit and human IgG preparations according to the modified Bethesda assay. Briefly, affinity-purified IgGs were serially diluted and incubated for 1 h in the presence of FVIII concentrate 88/640 (1 IU/ml) at 37°C. The residual FVIII activity was measured as described above.

[0072] Activation of factor VIII by α-thrombin and immunoblotting has been described elsewhere (Peerlinck et al, 1997).

[0073] Synthesis of peptides, conjugation of peptides to carrier proteins and production of rabbit anti-peptide antisera were performed by Neosystem (Strasbourg, France).

Purification of rabbit and human antibodies by affinity chromatography

[0074] For purification of rabbit and human antibodies, 5 mg of each different peptide was coupled to 1 ml pre-packed NHS-activated Sepharose (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Specific anti-peptide antibodies were purified with an automated liquid chromatography system (ÄKTAexplorer 100A, Pharmacia Uppsala, Sweden) either from 50 ml rabbit antiserum or from 100 ml of a human plasma fraction, obtained after Cohn fractionation (fraction II+III; 13 mg protein/ml). Briefly, samples were dialyzed 3 times against 5 volumes of TE buffer (20 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.02% NaN_3) and loaded onto the column at a flow rate of 1 ml/min. The column was sequentially washed at 2 ml/min with 50 ml TE buffer and 30 ml TE containing 1 M NaCl. After absorption, the material was eluted (1 ml/min) with 5 ml of 0.1 M citric acid pH 2.5 and directly recovered in 5 ml of 1M Tris-HCl, pH 9.0. Samples were finally dialyzed versus 10 volumes of equilibration buffer and concentrated on Centriprep-30 (Amicon, Beverly, MA). Ig recovery was determined by the Bio-Rad protein assay.

Results

Selection of potential factor-VIII linear epitopes

[0075] More than 30 surface regions (linear epitopes) spanning 8 to 25 residues, characterized by a high hydrophilicity, flexibility and accessibility were identified on the FVIII molecule. On the basis of their high probability of an outer location (see Fig. 1 for A3), 16 linear peptides (P1 to P16) were selected, matching identified stretches of 13 or more amino-acid residues. These peptides were synthesized and coupled to ovalbumin

for production of specific antiserum (Table 1, hereafter). P8 includes the epitope described by Shima et al (1988) and was used as an external control.

Experimental results obtained from said synthesized linear epitopes using the rabbit model

[0076] Results are summarized in Table 1 which concerns the characterization of rabbit anti-FVIII-peptide antisera and recovered affinity-purified of immunoglobulins.

[0077] Sixteen synthetic peptides (from 10 to 20 amino acids) were selected in the A, B, C1 and C2 domains. After conjugation with ovalbumin, the OVA-peptide conjugates were injected into rabbits and FVIII anti-peptide antisera RAP1 to RAP16 were studied.

[0078] More precisely, two rabbits were immunized with each FVIII-peptide-ovalbumin preparation. Specific antisera RAP1 to RAP16 (column b, Table 1) were prepared and assayed in an ELISA (column c, Table 1) using rFVIII or FVIII-peptide-KLH as the antigen. ELISA titre is expressed as the negative log of the reciprocal of the serum dilution giving 50% binding. The immunoglobulins were then purified by chromatography on peptide-bound Sepharose. The FVIII domain recognized by the anti-FVIII peptide Ig after immunoblotting is shown in (column d, Table 1) and Ig protein recoveries (column e, Table 1) were measured using immunoglobulins as the standard. The inhibitory activity, expressed in BU/mg protein, was determined in a FVIII neutralizing activity assay (column f, Table 1).

Immunogenicity of FVIII peptides and characterization of rabbit anti-FVIII peptide antisera

[0079] The reactivity of FVIII anti-peptide antisera was measured by an ELISA using, as antigen, either the

different corresponding FVIII-peptide coupled to KLH protein or purified rFVIII. The binding reaction of each anti-FVIII-peptide antiserum was specific both for the FVIII peptide used to elicit the immune response in rabbit and for rFVIII (see Table 1).

[0080] To demonstrate the FVIII epitope specificity of the rabbit anti-peptide antibodies, rFVIII and the rFVIII fragments obtained after treatment with thrombin were resolved by SDS-PAGE and analyzed by western blotting with the different preparations of rabbit IgGs. As expected, most antisera (14/16, 87%), showed a strong reaction with the corresponding FVIII fragment containing the selected linear epitope (see Table 1).

Purification of rabbit-anti-FVIII peptide antibodies

[0081] The specific rabbit IgG were purified by affinity chromatography on peptide-Sepharose as described under Methods. When FVIII-neutralizing activity was measured in a one-stage clotting assay, significant inhibition was found with two rabbit IgG purified preparations: RAP2, corresponding to IgG specific for SEQ ID No. 14 and RAP7 specific for SEQ ID No: 01.

Epitope mapping of rabbit anti-FVIII peptide antibodies by immunoblotting with human rFVIII

[0082] To demonstrate the FVIII epitope specificity of the rabbit anti-peptide antibodies, rFVIII and the rFVIII fragments obtained after treatment with thrombin were resolved by SDS-PAGE and analyzed by western blotting with different preparations of rabbit IgGs (RAP1 to RAP17 Igs).

[0083] In each run, the rFVIII heavy chain (HC) and light chain (LC) and their thrombin proteolysis products (44 kDa and 72 kDa) were identified with a mixture of two

monoclonal antibodies, MoAb 530p and MoAb18, respectively specific for the heavy and light chain. MoAb18 recognizes the NH_2-terminal light-chain FVIII fragment obtained after thrombin activation, which proved too small to remain in the gel after electrophoresis. Fourteen of the 17 rabbit immunoglobulin preparations reacted strongly with both rFVIII and pFVIII. Antisera RAP1, RAP2, RAP3, RAP4 recognized exclusively the heavy chains (200 kDa to 92 kDa). Antisera RAP1 and RAP2 reacted with the 50-kDa A1-domain fragment; RAP3 and RAP4 bound to the 44-kDa fragment (domain A2); RAP5 (specific for the B domain) bound to the high-molecular-weight FVIII heavy chain (about 200-kDa).

[0084] RAP7, RAP8, and RAP9 reacted with the 80-kDa light-chain doublet. RAP9 and RAP12 to RAP17 antibodies also detected the 72-kDa FVIII light-chain fragment. As expected, each reactive antiserum showed a strong reaction with the corresponding FVIII fragment containing the selected linear epitope. No reaction was detectable in the gels between RAP6 or RAP10 and the HC or LC FVIII fragments.

Experimental results obtained from said synthesized linear epitopes to purify and characterize human autoantibodies

[0085] Table 2 concerns the characterization of human anti-FVIII antibodies from Cohn fraction II+III of healthy individuals.

[0086] Human anti-peptide IgG preparations (HAP1 through HAP17) were so far purified on Sepharose coupled to 13 different FVIII peptides (column a, Table 2). The Igs (column b, Table 2) were analyzed by immunoblotting. Binding to the rFVIII HC or LC chains and to the rFVIII thrombin fragment is shown respectively in columns c and d, Table 2. FVIII-domain reactivity is shown in column e,

Table 2. Arrows indicate a decrease in 80-kDa band intensity. Ig recovery (column f, Table 2) after affinity purification is expressed in µg/10 mg loaded FII+III (see Materials and Methods). Inhibition of the clotting assay was determined after incubation in the presence of each of the 13 Ig preparations in the Bethesda assay (column g, Table 2).

Use of FVIII peptides for the immunopurification of human anti-FVIII antibodies in healthy donors

[0087] To prepare and characterize human anti-FVIII antibodies present in healthy individuals, we analyzed Cohn fraction II+III, rich in immunoglobulins, for the presence of selected specific anti-peptide antibodies. Human anti-FVIII-peptide antibodies (HAP1 to HAP11, HAP16 and HAP17) were purified by affinity chromatography on Sepharose coupled to the appropriate peptide (see Table 2). As a typical example, figure 2 shows the chromatographic profile obtained with SEQ ID 32, a sequence found in C2 domain. Table 2 summarizes the results obtained with 17 epitopic sequences selected in each FVIII domain (A1, A2, A3, B, C1 and C2). Significant amounts of immunoglobulins, specific for each of the 13 FVIII peptides used, were obtained from the starting plasma fraction II+III. The specificity of the resulting purified human antibodies was directly tested by immunoblotting with plasma FVIII, recombinant FVIII, and the fragments obtained after thrombin proteolysis (see Table 2).

[0088] The IgG isotype distribution in the human purified antibody preparations was found to be quite heterogeneous. Interestingly, 40 to 79% of the recovered IgGs belonged to the IgG2 subclass. In most preparations, IgG4 appeared to be over-represented (up to 25%).

[0089] All the human anti-FVIII-peptide antibody preparations were tested for the capacity to inhibit FVIII activity in a one-stage clotting assay. Table 2 shows that seven out of 13 preparations tested (54%) displayed inhibitory activity, SEQ ID No: 14, SEQ ID No: 19, SEQ ID No: 2, SEQ ID No: 5, SEQ ID No: 22, SEQ ID No: 32 and SEQ ID No: 33, respectively. As a typical example, the inhibition of FVIII activity in function of anti-SEQ ID 32 Ig concentration is shown in figure 2.

Human anti-FVIII-peptide Ig immunospecificity towards FVIII

[0090] The specificity of the resulting purified human antibodies was tested by immunoblotting with plasma FVIII, recombinant FVIII, and the fragments obtained after thrombin proteolysis. Again, the FVIII fragments were identified with either FVIII-HC- or FVIII-LC-specific mouse monoclonal antibodies or FVIII-peptide-specific rabbit polyclonal antibodies. The human antibodies were identified after binding of biotinylated goat anti-human IgG. Figure 3 shows the immunoreaction of high-molecular-weight FVIII (≥ 92-kDa) with four human antibody preparations, purified on Sepharose coupled to FVIII peptide SEQ ID No: 11 (Ser^{109}-Lys^{127}), SEQ ID No: 14 (Cys^{329}- Asp^{348}), SEQ ID No: 15 (Tyr^{407}-Lys^{425}) or SEQ ID No: 19 (Cys^{711}-Asp^{725}). The 50-kDa FVIII fragment (domain A1) was recognized by human antibodies purified on Ser^{109}- Lys^{127} or Cys^{329}-Asp^{348}-Sepharose and the 44-kDa FVIII fragment (A2) by immunoglobulins purified on Tyr^{407}-Lys^{425} and Cys^{711}-Asp^{725}-Sepharose. The lack of reactivity of the anti-(Ser^{817}-Ser^{830}) immunoglobulin preparation (HAP5) with the FVIII fragments confirms that this epitope is located in the amino-terminal end of domain B (Figure 3). Human antibodies purified on Sepharose coupled to peptide SEQ ID No: 1 (Arg^{1652}-Tyr^{1664}) or

SEQ ID No: 2 (Asp^{1681}-Arg^{1696}) reacted strongly with the 80-kDa FVIII light chain (Figure 3). For both preparations, the reaction with the 80-kDa band disappeared after thrombin proteolysis, indicating that the epitopes, as expected, are located in the *a3* acidic peptide at the NH_2-terminal part of the FVIII A3 domain. When human antibodies specific for peptide SEQ ID No: 5 (Arg^{1797}-Tyr^{1815} in A3 domain) were analyzed by immunoblotting, their specificity for rFVIII appeared restricted to the 80-kDa FVIII light chain and its 72-kDa thrombin fragment.

[0091] No immunoreaction with the rFVIII chains or fragments was detected with antibody preparations specific for FVIII peptides SEQ C and SEQ ID No: 23, although a positive reaction was obtained in the ELISA using rFVIII. This could mean that these immunoglobulin preparations recognize a conformational epitope.

Use of FVIII synthetic peptides to characterize human anti-FVIII antibodies in hemophilia A patient plasmas

[0092] The selected peptides were used in ELISA experiment to determine the anti-FVIII antibody specificity's present in hemophilia A plasmas. The peptides were coated on microplate (25 µg/ml in PBS buffer during 16h at 4°C). A 1/10 to 1/1000 dilution of plasma patient in Tris-casein buffer was reacted with the coated peptide for 2h at 37°C. The bound human IgG was measured as described in Methods. Control samples were plasma pools of healthy donors. Figure 4 shows the results obtained with the plasma of 4 hemophilia A patients. The optical densities are corrected average values (OD patient-OD normal plasma pool) of two independent experiments.

Molecular model epitope prediction

[0093] Pemberton et al (1997) have built a molecular model of the A domains of FVIII. This 3-D model makes it possible to explore predictions for important regions of FVIII activity. The model was used to locate the FVIII-peptide epitopes identified by the Parker and Hodge algorithms. As predicted by these algorithms, all peptides located in the A domains were found on the FVIII surface and were fully accessible to specific.

[0094] The overlap between the epitope and the FIXa-binding loop (5 common residues spanning Glu^{1811}-Tyr^{1815}) may explain the inhibitory action of the corresponding anti-(Arg^{1797}-Tyr^{1815}) antibodies on formation of the fibrin clot.

Analysis of the results

[0095] In the clotting test, significant inhibition of FVIII activity was recorded in the presence of rabbit anti-(Cys^{329}-Asp^{348}) and anti-(Arg^{1653}-Tyr^{1664}) antibodies, but different inhibition patterns were observed. Inhibition by anti-(Arg^{1653}-Tyr^{1664}) follows second-order kinetics with a drastic reduction in FVIII activity. Anti-(Cys^{329}-Asp^{348}) Ig is less efficient and shows a more complex type of reaction, with a non-linear dependence on the antibody concentration. Epitope Arg^{1652}-Tyr^{1664} and the adjacent major binding site vWF (residues Glu^{1675}-Glu^{1684}) are located in the acidic light-chain peptide *a3*. As shown by western blotting, *a3* is released from the A3 domain after thrombin treatment, preventing further binding of anti-(Arg^{1652}-Tyr^{1664}) Ig to activated FVIII. Similar results have been reported by Shima et al (1991), who described the FVIII sequence Asp^{1663}-Ser^{1669} as a binding site of rabbit polyclonal antibodies neutralizing FVIII activity. Epitope Cys^{329}-Asp^{348} overlapped the acidic Asp^{348}-Lys^{362} sequence (in *a1*) described as adjacent to the activated protein C

(Arg^{336}) and thrombin (Arg^{372}) cleavage sites. It is the target of human hemophilic inhibitors. Anti-(Asp^{348}-Lys^{362}) antibodies may interfere with proteolysis or with the FX interaction site (Met^{337}-Arg^{372}) (Saenko et al., 1999 and Scandella et al., 2000).

[0096] FVIII-neutralizing activity was measured in all 13 Ig preparations. Seven human Ig preparations displayed inhibition of procoagulant activity, these being specific for amino-acid residues Cys^{711}-Asp^{725}, Tyr^{1681}-Arg^{1696}, and Arg^{1797}-Tyr^{1815} respectively. The Cys^{711}-Asp^{725} sequence contains sulfated tyrosines at Tyr^{718}, Tyr^{719}, and Tyr^{723}, and overlaps with the FVIII HC region Lys^{713}-Arg^{740} described as promoting both activation and HC proteolysis. The additional sulfated groups may be required for proper interaction with thrombin or another component as in the FX-activating complex. The sequence also overlaps with region Gly^{701}-Ser^{750}, recognized by a weakly inhibitory mouse monoclonal antibody. Peptide P8 (Tyr^{1681}-Arg^{1696}) (FVIII LC) includes the sequence Glu^{1684}-Arg^{1689} already described by Shima et al, 1991. It contains the thrombin activation site Arg^{1689}-Ser^{1690}. P4 (Cys^{711}-Asp^{725}) is also included in the Asp^{712}-Ala^{736} sequence detected by analysis of the patient antibody repertoire by gene phage display technology. It is proposed as a possible additional inhibitor in patients (van den Brink et al, 2000). Peptide P9 (Arg^{1797}-Tyr^{1815}) contains the FXa binding site (see below).

[0097] Of the 16 anti-FVIII-peptide immunoglobulins purified from humans or produced in rabbits, 7 did neutralize FVIII activity under the tested conditions. Using small peptide sequences and immunobinding assays, we have provided evidence for additional new epitopes. We have located new epitopes in the A1 domain (residues Ser^{109}-Cys^{127}), the A2 domain (Cys^{407}-Lys^{425}), and the B domain (Ser^{817}-Ser^{830} and Glu^{1078}-Pro^{1092}).

[0098] Autoantibodies immunopurified with denatured FVIII have been reported in healthy subjects and in pools of normal human immunoglobulins (processed fraction II, see above) (Algiman et al., 1992 and Moreau et al., 2000). A possible role in clearance of denatured FVIII or its fragments from the bloodstream and/or in the immunotolerance was suggested.

[0099] Identification of the FVIII epitopes is a major challenge to be met in order to improve FVIII treatment and the quality of therapeutic FVIII concentrates. FVIII epitope sequences help to determine the contribution of patient polyclonal anti-FVIII Igs to overall inhibitory and regulatory activity. They could also be used to monitor the usual switch in anti-FVIII specificity in a patient during treatment. Said characterization of FVIII epitopes and a model of their locations on the folded molecule improves the treatment of inhibitors in both hemophilic and non- hemophilic patients (detection, follow-up, therapeutic use of FVIII epitope peptides...).

Table 1 Characterization of rabbit anti-FVIII peptides antibodies

SEQ ID(a)	Rabbit Antiserum(b)	ELISA Titre(c)		FVIII domain recognize(d)	RAP-IgG Recovery(e) µg/ml serum	Inhibitor Titre(f) BU/mg
		P-KLH	r-FVIII			
SEQ ID 11	RAP1	2.5	2.2	A1	27	-
SEQ ID 14	RAP2	3.6	2.5	A1/a1	55	1,5
SEQ ID 15	RAP3	2.5	3.2	A2	268	-
SEQ ID 19	RAP4	2.5	1.3	A2/a2	13	-
SEQ ID 21	RAP5	4.6	3.9	B	106	-
SEQ C	RAP6	3.8	2.9	-	14	-
SEQ ID 01	RAP7	3.9	3.9	a3 ↓	35	0,5
SEQ ID 02	RAP8	1.9	0.9	a3/A3 ↓	3	-
SEQ ID 05	RAP9	3.8	2.6	A3	42	-
SEQ ID 23	RAP10	3.9	0.8	-	65	-
SEQ ID 22	RAP11	ND	ND	ND	ND	ND
SEQ ID 26	RAP12	4,1	1,1	C2	ND	ND
SEQ ID 27	RAP13	3,7	1,1	C2	ND	ND
SEQ ID 28	RAP14	3,8	0,9	C2	ND	ND
SEQ ID 31	RAP15	3,2	0,7	C2	ND	ND
SEQ ID 32	RAP16	3,5	1,8	C2	ND	ND
SEQ ID 33	RAP17	4,8	1,2	C2	ND	ND

Table 2 Characterization of human anti-FVIII peptides autoantibodies

SEQ ID(a)	Human Anti-peptide Ig(b)	FVIII reactivity on immunoblot (-thrombin)(c)	FVIII reactivity on immunoblot (+thrombin)(d)	FVIII domain(e)	HAP-IgG Recovery(f) µg/10 mgIgG	FVIII inhibitory Activity(g) BU/mg
SEQ ID 11	HAP1	>92kDa	50kDa	A1	0,27	-
SEQ ID 14	HAP2	>92kDa	50kDa	A1/a1	1,07	3,4
SEQ ID 15	HAP3	>92kDa	44kDa	A2	0,06	-
SEQ ID 19	HAP4	92kDa	44kDa	A2/a2	0,12	+
SEQ ID 21	HAP5	>100kDa	-	B	0,26	-
SEQ C	HAP6	-	-	-	0,03	-
SEQ ID 01	HAP7	80kDa	80kDa	a3 ↓	0,20	-
SEQ ID 02	HAP8	80kDa	80kDa	a3/A3 ↓	0,01	+
SEQ ID 05	HAP9	80kDa	72kDa	A3	0,08	+
SEQ ID 23	HAP10	-	-	-	0,11	-
SEQ ID 22	HAP11	ND	ND	ND	0,98	4,3
SEQ ID 26	HAP12	ND	ND	ND	ND	ND
SEQ ID 27	HAP13	ND	ND	ND	ND	ND
SEQ ID 28	HAP14	ND	ND	ND	ND	ND
SEQ ID 31	HAP15	ND	ND	ND	ND	ND
SEQ ID 32	HAP16	80kDa	72 kDa	A3C1C2	2,40	6,3
SEQ ID 33	HAP17	ND	ND	ND	1,06	2,4

+: Inhibition >25% at 100µg/ml

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CLAIMS

1. An antigenic epitope of FVIII polypeptide sequence, which is selected from the group consisting of :

- the epitope comprised between serine 2018 and histidine 2031 inclusive, defined by the following sequence:
  SEQ ID No:10:
  Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
  1 5 10
- the epitope tyrosine 555 to glutamine 565 inclusive defined by the following sequence:
  SEQ ID No:17:
  Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
  1 5 10
- the epitope leucine 730 to serine 741 inclusive, defined by the following sequence:
  SEQ ID No:20:
  Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
  1 5 10
  possibly deleted from the terminal amino acid serine and/or the first amino acid leucine
- the epitope serine 817 to serine 830 inclusive, defined by the following sequence:
  SEQ ID No:21:
  Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
  1 5 10
- the epitope asparagine acid 2128 to asparagine acid 2138 inclusive, defined by the following sequence:
  SEQ ID No:24:
  Asn Val Asp Ser Ser Gly Ile Lys His Asn
  1 5 10
- the epitope serine 2204 to glutamine 2222 inclusive, defined by the following sequence:

SEQ ID No:27:

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

- the epitope isoleucine 2262 to glutamine 2270 inclusive, defined by the following sequence:

SEQ ID No:30:

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

- the epitope leucine 2273 to serine 2289 inclusive, defined by the following sequence:

SEQ ID No:31:

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
1 5 10 15
Ser

- the epitope proline 2292 to tyrosine 2305 inclusive, defined by the following sequence:

SEQ ID No:32:

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

possibly deleted from one or more amino acids of the terminal tripeptide Thr-Arg-Tyr

- the epitope glutamic acid 2322 to tyrosine 2332 inclusive, defined by the following sequence:

SEQ ID No:33:

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

2.A pool of antigenic epitopes of factor VIII polypeptide sequence which comprise one or more antigenic sequence epitope of factor VIII according to claim 1 and possibly an antigenic sequence epitope of factor VIII which is selected from the group consisting of :

- the epitope arginine 1648 to tyrosine 1664 inclusive, defined by the following sequence:

SEQ ID No:1:

Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1 5 10 15
Tyr

possibly deleted from one or more amino acids of the tetrapeptide Arg-Asp-Ile-Thr or one or two of the last amino acids of the peptide Asp-Tyr.

- the epitope aspartic acid 1681 to arginine 1696 inclusive, defined by the following sequence:

SEQ ID No:2:

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
1 5 10 15

possibly deleted from one or more amino acids of the epitope Asp-Glu-Asp-Glu.

- the epitope threonine 1739 to tyrosine 1748 inclusive, defined by the following sequence:

SEQ ID No:3:

Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
1 5 10

- the epitope asparagine 1777 to phenylalanine 1785 inclusive, defined by the following sequence:

SEQ ID No:4:

Asn Gln Ala Ser Arg Pro Tyr Ser Phe
1 5

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Phe or the tetrapeptide Pro-Tyr-Ser-Phe.

- the epitope glutamic acid 1794 to tyrosine 1815 inclusive, defined by the following sequence:

SEQ ID No:5:

Glu Asp Gln Arg Glu Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
1 5 10 15
Asn Glu Thr Lys Thr Tyr
20

42

possibly deleted from one or more amino acids from the first tripeptide Glu-Asp-Gln or the first nonapeptide Glu-Asp-Gln-Arg-Gln-Gly-Ala-Glu-Pro.

- the epitope methionine 1823 to aspartic acid 1831, defined by the following sequence:

SEQ ID No:6:

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

- the epitope glutamic acid 1885 to phenylalanine 1891 inclusive, defined by the following sequence:

SEQ ID No:7:

Glu Thr Lys Ser Trp Tyr Phe
1 5

- the epitope glutamic acid 1885 to alanine 1901 inclusive, defined by the following sequence:

SEQ ID No:8:

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

possibly deleted from one or more amino acids from the heptapeptide Gly-Thr-Lys-Ser-Trp-Phe-Thr or from the tripeptide Cys-Arg-Ala.

- the epitope aspartic acid 1909 to arginine 1917 inclusive, defined by the following sequence:

SEQ ID No:9:

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

- the epitope alanine 108 to valine 128 inclusive, defined by the following sequence:

SEQ ID No:11:

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

possibly deleted from the terminal amino acids alanine and/or valine

43

- the epitope glutamic acid 181 to leucine 192 inclusive, defined by the following sequence:

SEQ ID No:12:

Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
1 5

possibly deleted from one or two amino acids of the terminal dipeptide Thr-Leu

- the epitope aspartic acid 203 to alanine 227 inclusive, defined by the following sequence:

SEQ ID No:13:

Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
1 5 10 15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
20

possibly deleted from one or more amino acids of the nonapeptide Asp-Arg-Asp-Ala-Ala-Ser-Ala-Arg-Ala

- the epitope aspartic acid 327 to methionine 355 inclusive, defined by the following sequence:

SEQ ID No:14:

Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
1 5 10 15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
20 25

possibly deleted from one or more amino acids of the dipeptide Asp-Ser or the octapeptide Asp-Asp-Leu-Thr-Asp-Ser-Glu-Met.

- the epitope aspartic acid 403 to lysine 425 inclusive, defined by the following sequence:

SEQ ID No:15:

Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
1 5 10 15
Ile Gly Arg Lys Tyr Lys Lys
20

possibly deleted from one or more amino acids of the tetrapeptide Asp-Asp-Arg-Ser.

- the epitope valine 517 to arginine 527 inclusive, defined by the following sequence:

SEQ ID No:16:

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

possibly deleted from one or the two amino acids of the dipeptide Pro-Arg

- the epitope histidine 693 to glycine 701 inclusive, defined by the following sequence:

SEQ ID No:18

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

- the epitope serine 710 to aspartic acid 725 inclusive, defined by the following sequence:

SEQ ID No:19

Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

- the epitope isoleucine 2081 to serine 2095 inclusive, defined by the following sequence

SEQ ID No:22:

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

possibly deleted from one or more amino acids of the tetrapeptide Ile-His-Gly-Ile

- the epitope tyrosine 2105 to glycine 2121 inclusive, defined by the following sequence:

SEQ ID No:23:

Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15
Gly

possibly deleted from one or more amino acids of the tripeptide Tyr-Ser-Leu

- the epitope histidine 2152 to arginine 2163 inclusive, defined by the following sequence:

SEQ ID No:25:

His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg
1 5 10

- the epitope serine 2181 to asparagine 2198 inclusive, defined by the following sequence:

SEQ ID No:26:

Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr
1 5 10 15
Asn

possibly deleted from one or more amino acids from the terminal tripeptide Phe-Thr-Asn (P11)

- the epitope glutamine 2235 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:28:

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu or one or more amino acids of the tetrapeptide Val-Lys-Ser-Leu.

- the epitope glycine 2242 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:29:

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu

3. A conformational epitope which contains:

- either at least two different epitopes according to the claim 1 or,
- at least one epitope sequence according to claim 1 and an epitope selecting from the pool of epitopes sequences of claim 2.

4. A recombinant factor VIII having an amino acid sequence deleted from one of the antigenic epitopes sequences according to claim 1 or one or more epitopes sequences selected from the pool of epitopes of claim 2.

5. A complex comprising a carrier protein or a carrier peptide linked to an epitope sequence according to the claim 1 or 3.

6. An inhibitor of factor VIII polypeptide sequence which exhibits an immunoaffinity with the epitope sequence, the pool of epitopes sequences and/or the complex according to any one of the preceding claims 1 to 5.

7. The inhibitor according to Claim 6, which is an anti-factor VIII antibody or antibody fragment.

8. An anti-inhibitor, which is directed against the inhibitor of factor VIII according to Claim 6 or 7.

9. The anti-inhibitor according to Claim 8, which is an anti-anti-factor VIII idiotype antibody or antibody fragment.

10. A pharmaceutical composition, which comprises an adequate pharmaceutical carrier and at least one element selected from the group consisting of the epitope sequence, the pool of epitopes sequences, the complex, the recombinant factor VIII, the inhibitor and/or the anti-inhibitor according to any one of the preceding claims 1 to 9.

11. A diagnostic and/or purification device, which comprises at least one element which is selected from the group consisting of the epitope sequence, the pool of epitopes sequences, the complex, the inhibitor and/or the anti-inhibitor according to any one of the preceding Claims 1 to 9.

12. The device according to Claim 11, which is a diagnostic kit.

13. The device according to Claim 11, which is a chromatography column or filter.

14. Use of the pharmaceutical composition according to Claim 10 for the manufacture of a medicament in the treatment and/or the prevention of an immune disorder in a mammal induced by inhibitors of factor VIII polypeptide sequence.

15. A method of treatment of a physiological fluid (serum), obtained from a mammal patient (including a human) wherein said physiological fluid is put into the chromatography column according to the claim 13 in order to allow a binding of the inhibitors of factor VIII polypeptide sequence present in said physiological fluid with epitope, pool or complex comprised in said chromatography column wherein after elution of the column a physiological fluid without inhibitors of factor VIII polypeptide sequence is recovered for its reinjection to the mammal patient.

16. A process for identifying and obtaining inhibitors and/or anti-inhibitors according to any of the preceeding Claims 6 to 9, comprising the steps of:

- selecting an element from the group consisting of the epitope sequence, the pool of epitopes sequences and/or the complex according to any one of the preceding claims 1 to 5, attached to a solid support (preferably a solid support of a chromatography column),
- passing a physiological fluid (serum) obtained from a patient containing inhibitors of factor VIII polypeptide sequence through said chromatography column,
- eluting said column, and
- collecting the fractions containing inhibitors of factor VIII polypeptide sequence which have exhibited an immunoaffinity with said element.

17. The process according to Claim 16, further comprising the steps of:

- attaching the collected inhibitors of factor VIII upon a solid support (preferably a solid support of a chromatography column),
- passing a physiological fluid from a mammal patient containing anti-inhibitors of factor VIII upon said solid support,
- washing the support, preferably by eluting said column, and
- collecting the fractions containing anti-inhibitors of factor VIII which have exhibited an immunoaffinity with said inhibitors of factor VIII.

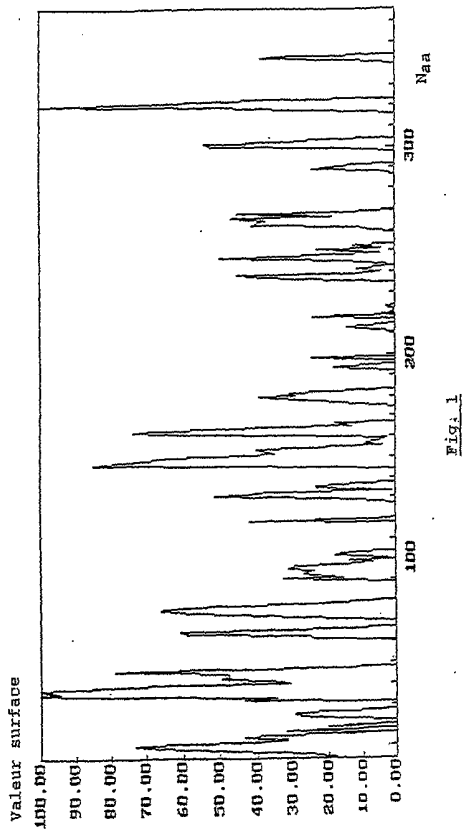
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Fig. 1

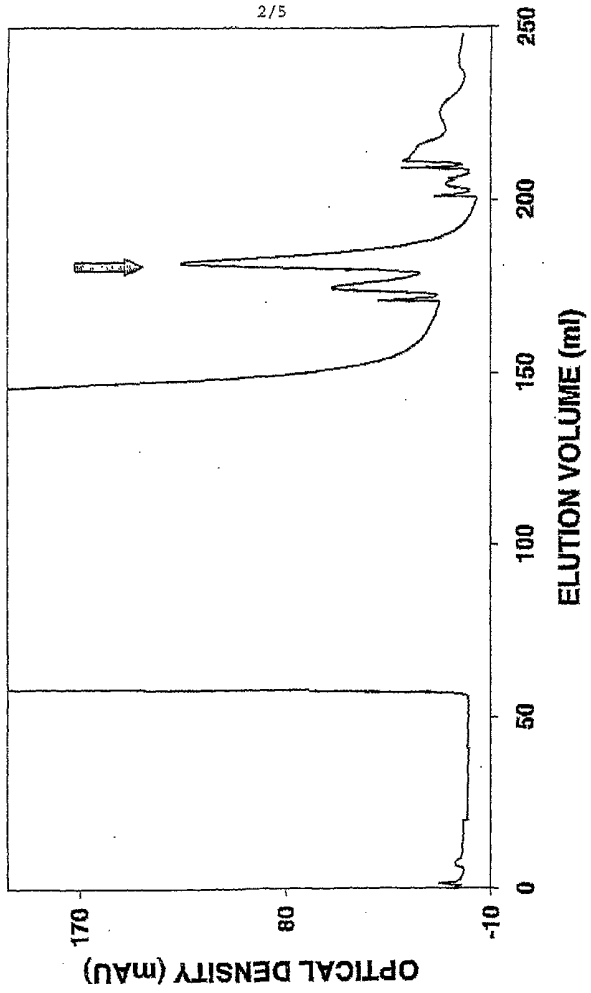
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Fig. 2a

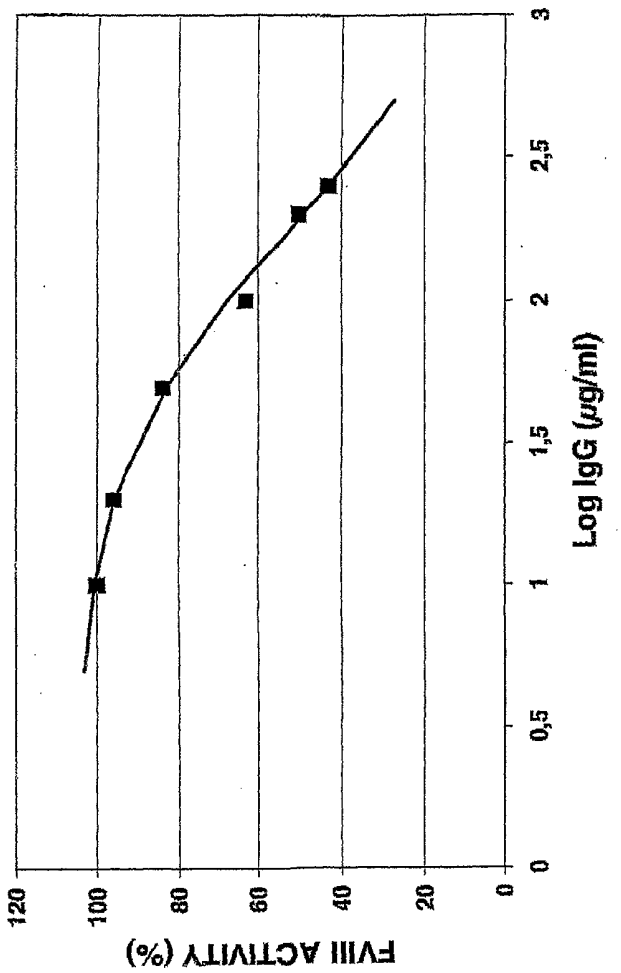
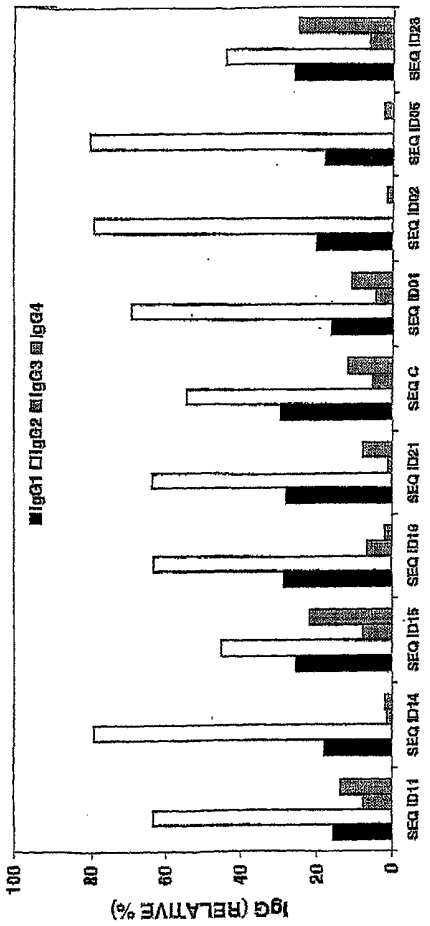


Fig. 2b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SEQ C
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SEQ ID02
SEQ ID05
SEQ ID28

Fig. 3

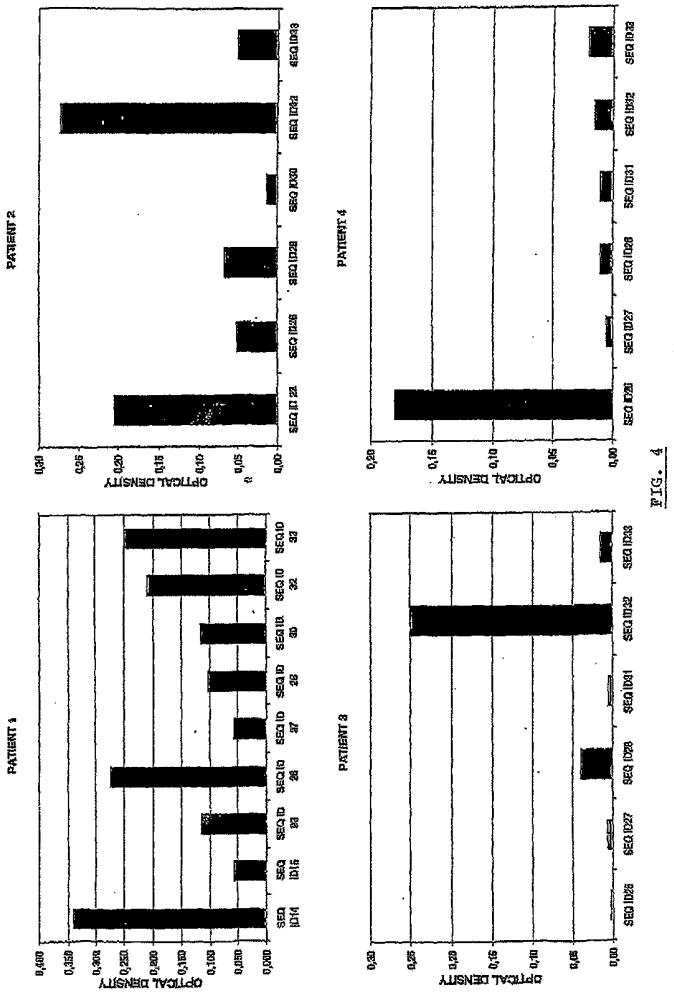

FIG. 4

54

SEQUENCE LISTING

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Di Gianbattista, Mario

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【国際調査報告】

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 02/00070

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/755 C07K16/36 C07K16/42 A61K38/37
A61K39/395 G01N33/53

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 C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, SEQUENCE SEARCH, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUGIHARA T ET AL: "Identification of plasma antibody epitopes and gene abnormalities in Japanese hemophilia A patients with factor VIII inhibitor." NAGOYA JOURNAL OF MEDICAL SCIENCE. JAPAN MAY 2000, vol. 63, no. 1-2, May 2000 (2000-05), pages 25-39, XP008014431 ISSN: 0027-7622 abstract; table 2 ---	1-14,16, 17
X	WO 96 02572 A (CROIX ROUGE DE BELGIQUE ;GIAMBATTISTA MARIO DI (BE); LAUB RUTH (BE) 1 February 1996 (1996-02-01) claims 1-30 --- -/--	1-14,16, 17

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

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"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
30 May 2003	07. 06. 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	Gurdjian, D

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

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 02/00070

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	VEHAR G ET AL: "STRUCTURE OF HUMAN FACTOR VIII" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 312, no. 5992, 1 November 1984 (1984-11-01), pages 337-342, XP002017256 ISSN: 0028-0836 abstract; figure 3 ---	1-14,16, 17
A	EP 0 965 597 A (MOCHIDA PHARM CO LTD) 22 December 1999 (1999-12-22) claim 6; figures SEQ.ID.8,10 ---	1-14,16, 17
A	BOWEN D J ET AL: "Analysis of the BglI restriction fragment length polymorphism in the human factor VIII gene using "virtual PCR"--a novel approach employing the polymerase chain reaction in the absence of sequence information for the locus." HUMAN GENETICS. GERMANY AUG 1996, vol. 98, no. 2, August 1996 (1996-08), pages 219-222, XP001151994 ISSN: 0340-6717 abstract; figure 1 -----	1-14,16, 17

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BE 02/00070

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 claim 15 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 6,8
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:

see FURTHER INFORMATION sheet PCT/ISA/210

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:

see additional sheet

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. [X] 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.:

1-17

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.

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

International Application No. PCT/BE 02 00070

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 6,8

Present claims 6,8 relate to a product defined by reference to a desirable characteristic or property, namely being an inhibitor of factor VIII polypeptide sequence which exhibits an immunoaffinity with the epitope sequence of claims 1 to 5 and an anti-inhibitor, which is directed against the inhibitor of factor VIII according to Claim 6 or 7.

The claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the products by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the anti FVIII antoibodies products , have been searched e.g those prepared in the example page 28-30 and tables 1,2

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

International Application No. PCT/BE 02 /00070

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.10 ,corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

2. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.17 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

3. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.20 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

4. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.21 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

5. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.24 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for

International Application No. PCT/BE 02 /00070

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

identifying inhibitors or anti-inhibitors .

6. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.27 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

7. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.30 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

8. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.31 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

9. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.32 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for identifying inhibitors or anti-inhibitors .

10. Claims: 1-17 partially

Antigenic epitope of polypeptides of Factor VIII with amino acid sequence with seq.id.33 corresponding epitopes with one or more antigenic sequence epitope of Factor VIII , recombinant deletion Factor VIII variant ,complex, inhibitor , anti-inhibitor antibody , pharmaceutical composition , diagnostic and/or purification device , process for

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

identifying inhibitors or anti-inhibitors .

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/BE 02/00070

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9602572	A	01-02-1996	BE	1008491 A3	07-05-1996
			WO	9602572 A2	01-02-1996
			CA	2195126 A1	01-02-1996
			EP	0804484 A1	05-11-1997
			JP	10506610 T	30-06-1998
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			WO	9829453 A1	09-07-1998

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Ｇ０１Ｎ 30/48	Ｇ０１Ｎ 30/88	Ｊ	
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Ｇ０１Ｎ 33/15	Ｇ０１Ｎ 33/50	Ｚ	
Ｇ０１Ｎ 33/50	Ｇ０１Ｎ 33/53	Ｄ	
Ｇ０１Ｎ 33/53	Ｇ０１Ｎ 33/531	Ａ	
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ベルギー, ベー７０９０ ブライン－ルーコンテ, ルー プロウシャート ２４

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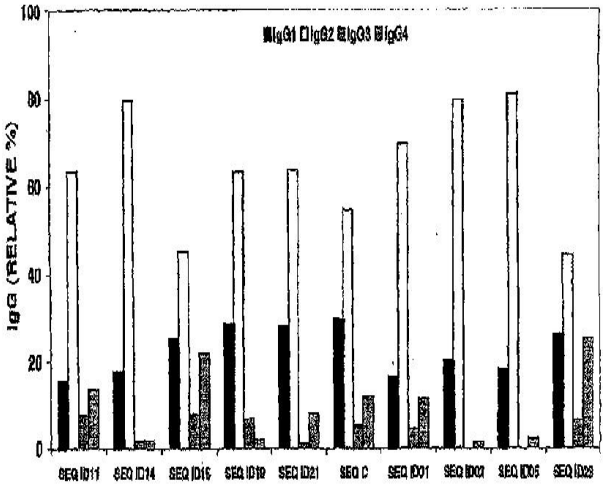
4C085 AA13 BB11 EE01 GG01
4H045 AA11 AA30 AA40 BA14 BA15 BA16 BA17 BA18 BA40 CA42
DA66 DA75 EA20 EA22 FA74 FA82 GA21

专利名称(译)	因子VIII的抗原表位，针对所述表位的抑制剂及其用途		
公开(公告)号	JP2004535393A	公开(公告)日	2004-11-25
申请号	JP2002587603	申请日	2002-05-06
[标]申请(专利权)人(译)	中央部门去分馏空蒙特德·拉克鲁瓦胭脂ES硒麦芽酒萨尔瓦多		
申请(专利权)人(译)	中央部门去分馏空蒙特德拉十字-...日ES本身强麦萨尔瓦多.		
[标]发明人	ローブルス ディジャムバチスタマリオ		
发明人	ローブ, ルス ディ ジャムバチスタ, マリオ		
IPC分类号	G01N33/50 A61K38/00 A61K39/00 A61K39/395 A61P7/00 A61P37/00 B01J20/281 C07K1/16 C07K14/755 C07K16/36 C07K16/42 C07K19/00 C12N15/12 G01N30/08 G01N30/88 G01N33/15 G01N33/53 G01N33/531 G01N33/566 G01N33/68 G01N30/48		
CPC分类号	G01N33/6878 A61K38/00 A61K39/00 A61K39/0008 A61K2039/505 C07K14/755 C07K16/36 C07K2317/21 C07K2317/76 G01N2333/755 Y10S530/806		
FI分类号	C07K14/755 A61K39/395.D A61P7/00 A61P37/00 C07K1/16 C07K16/36 C07K16/42 C07K19/00 G01N30/08.L G01N30/48.R G01N30/88.J G01N33/15.Z G01N33/50.Z G01N33/53.D G01N33/531.A G01N33/566 A61K37/02		
F-TERM分类号	2G045/AA40 2G045/BB03 2G045/BB20 2G045/CB01 2G045/CB17 2G045/DA36 2G045/DA37 2G045/FB03 2G045/FB06 4C084/AA02 4C084/AA06 4C084/AA07 4C084/DC15 4C084/MA66 4C084/NA05 4C084/NA06 4C084/NA14 4C084/ZA511 4C084/ZB071 4C084/ZC781 4C085/AA13 4C085/BB11 4C085/EE01 4C085/GG01 4H045/AA11 4H045/AA30 4H045/AA40 4H045/BA14 4H045/BA15 4H045/BA16 4H045/BA17 4H045/BA18 4H045/BA40 4H045/CA42 4H045/DA66 4H045/DA75 4H045/EA20 4H045/EA22 4H045/FA74 4H045/FA82 4H045/GA21		
代理人(译)	Kazehaya信明 浅野纪子		
优先权	09/853080 2001-05-09 US		
其他公开文献	JP2004535393A5 JP4500494B2		
外部链接	Espacenet		

摘要(译)

本发明涉及针对针对抗原多肽序列的抑制剂的抗原多肽序列（因子VIII的表位）和针对所述抑制剂的抗抑制剂。本发明还涉及含有至少一种上述分子的药物组合物和诊断装置。 点域


IgG1 IgG2 IgG3 IgG4
IgG (RELATIVE %)
100
80
60
40
20
0
SEQ ID11
SEQ ID14
SEQ ID16
SEQ ID19
SEQ ID21
SEQ C
SEQ ID01
SEQ ID03
SEQ ID05
SEQ ID23