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(54)【発明の名称】扁平上皮癌に対する特異的モノクローナル抗体の同定と開発

(57)【要約】
本発明は、肺癌、食道癌および子宮頸癌などの扁平上皮癌抗原に選択性の高いモノクローナル抗体、抗体フラグメントおよび抗体コンジュゲートに関する。本発明は、本発明の新規抗体を使用して、患者の生物学的サンプル中の扁平上皮癌抗原の発現を検出することによる診断または予後のｉｎ　ｖｉｖｏおよびｉｎ　ｖｉｔｒｏ両方の臨床的スクリーニング法に関する。本発明はさらに、上記のスクリーニング方法を実施するためのキットを提供する。さらに、抗体コンジュゲートは、抗腫瘍作用を有する種々の薬剤を効果的に送達するのに用いることができる。本発明の抗体を、腫瘍細胞へのＡＤＣＣをターゲットとして、結合していない形態で患者に投与することもできる。

【特許請求の範囲】
【請求項１】
以下の特徴を有する、ヒト癌関連タンパク質抗原に特異的なモノクローナル抗体：
ａ．　該抗原は、癌ではない正常なヒト組織で検出されないこと、
ｂ．　該抗原は、ヒトにおいて特異的な免疫原であること、
ｃ．　該抗原が、結腸癌を有するヒトにおいて、細胞性免疫として発現される免疫応答を誘導すること。
【請求項２】
マウスモノクローナル抗体ＡＤ６（ＡＴＣＣ　ＰＴＡ－２４６０）またはモノクローナル抗体ＡＤ６に特異的に結合する癌関連エピトープに特異的に結合する抗体である、請求項１記載の抗体。
【請求項３】
マウスモノクローナル抗体ＡＤ７（ＡＴＣＣ　ＰＴＡ－２４５９）またはモノクローナル抗体ＡＤ７に特異的に結合する癌関連エピトープに特異的に結合する抗体である、請求項１記載の抗体。
【請求項４】
マウスモノクローナル抗体５Ｃ６（ＡＴＣＣ　ＰＴＡ－２４５８）またはモノクローナル抗体５Ｃ６に特異的に結合する癌関連エピトープに特異的に結合する抗体である、請求項１記載の抗体。
【請求項５】
マウスモノクローナル抗体ＡＨ１（ＡＴＣＣ　ＰＴＡ－２４５７）またはモノクローナル抗体ＡＨ１に特異的に結合する癌関連エピトープに特異的に結合する抗体である、請求項１記載の抗体。
【請求項６】
固相支持体上に固定化された、請求項１記載の抗体。
【請求項７】
検出可能な標識が付された、請求項１記載の抗体。
【請求項８】
検出可能な標識が放射性標識である、請求項７記載の抗体。
【請求項９】
細胞傷害性放射性核種にコンジュゲートされた、請求項１記載の抗体。
【請求項１０】
細胞傷害性薬剤にコンジュゲートされた、請求項１記載の抗体。
【請求項１１】
細胞傷害性タンパク質にコンジュゲートされた、請求項１記載の抗体。
【請求項１２】
請求項１０記載の抗体を含む、製薬上許容され得る担体に組み合わされた組成物。
【請求項１３】
請求項１１記載の抗体を含む、製薬上許容され得る担体に組み合わされた組成物。
【請求項１４】
請求項１記載のモノクローナル抗体に対するモノクローナル抗体。
【請求項１５】
サンプル中のマウスモノクローナル抗体ＡＤ６（ＡＴＣＣ　ＰＴＡ－２４６０）に結合する癌関連抗原を検出するためのイムノアッセイ法であって、
ａ．　結合に効果的な量の抗体をサンプルと接触させること；および
ｂ．　精製癌関連タンパク質抗原への抗体の結合を検出することにより抗原を検出すること；
を含む上記方法。
【請求項１６】
サンプル中のマウスモノクローナル抗体ＡＤ７（ＡＴＣＣ　ＰＴＡ－２４５９）に結合す

る癌関連抗原を検出するためのイムノアッセイ法であって、
ａ．　結合に効果的な量の抗体をサンプルと接触させること；および
ｂ．　精製癌関連タンパク質抗原への抗体の結合を検出することにより抗原を検出すること；
を含む上記方法。
【請求項１７】
サンプル中のマウスモノクローナル抗体ＡＨ１（ＡＴＣＣ ＰＴＡ－２４５７）に結合する癌関連抗原を検出するためのイムノアッセイ法であって、
ａ．　結合に効果的な量の抗体をサンプルと接触させること；および
ｂ．　精製癌関連タンパク質抗原への抗体の結合を検出することにより抗原を検出すること；
を含む上記方法。
【請求項１８】
サンプル中のマウスモノクローナル抗体５Ｃ６（ＡＴＣＣ ＰＴＡ－２４５８）に結合する癌関連抗原を検出するためのイムノアッセイ法であって、
ａ．　結合に効果的な量の抗体をサンプルと接触させること；および
ｂ．　精製癌関連タンパク質抗原への抗体の結合を検出することにより抗原を検出すること；
を含む上記方法。
【請求項１９】
患者の癌を診断するための方法であって、以下の：
ａ．　癌を患っていると疑われる患者から組織標本を切除すること；
ｂ．　モノクローナル抗体ＡＤ６（ＡＴＣＣ ＰＴＡ－２４６０）と該標本を接触させること；
ｃ．　免疫組織学的染色により標本を染色すること；および
ｄ．　上記染色により抗原抗体複合体の存在を検出すること；
を含む上記方法。
【請求項２０】
患者の癌を診断するための方法であって、以下の：
ａ．　癌を患っていると疑われる患者から組織標本を切除すること；
ｂ．　モノクローナル抗体ＡＤ７（ＡＴＣＣ ＰＴＡ－２４５９）と該標本を接触させること；
ｃ．　免疫組織学的染色により標本を染色すること；および
ｄ．　上記染色により抗原抗体複合体の存在を検出すること；
を含む上記方法。
【請求項２１】
患者の癌を診断するための方法であって、以下の：
ａ．　癌を患っていると疑われる患者から組織標本を切除すること；
ｂ．　モノクローナル抗体５Ｃ６（ＡＴＣＣ ＰＴＡ－２４５８）と該標本を接触させること；
ｃ．　免疫組織学的染色により標本を染色すること；および
ｄ．　上記染色により抗原抗体複合体の存在を検出すること；
を含む上記方法。
【請求項２２】
患者の癌を診断するための方法であって、以下の：
ａ．　癌を患っていると疑われる患者から組織標本を切除すること；
ｂ．　モノクローナル抗体ＡＨ１（ＡＴＣＣ ＰＴＡ－２４５７）と該標本を接触させること；
ｃ．　免疫組織学的染色により標本を染色すること；および
ｄ．　上記染色により抗原抗体複合体の存在を検出すること；

を含む上記方法。
【請求項２３】
モノクローナル抗体ＡＤ６を含む組成物を投与することを含む、癌患者のＡＤＣＣ反応を誘導する方法。
【請求項２４】
モノクローナル抗体ＡＤ７を含む組成物を投与することを含む、癌患者のＡＤＣＣ反応を誘導する方法。
【請求項２５】
モノクローナル抗体５Ｃ６を含む組成物を投与することを含む、癌患者のＡＤＣＣ反応を誘導する方法。
【請求項２６】
モノクローナル抗体ＡＨ１を含む組成物を投与することを含む、癌患者のＡＤＣＣ反応を誘導する方法。
【発明の詳細な説明】
【０００１】
１．緒言
本発明は、肺癌、食道癌および子宮頸癌を含む扁平上皮癌抗原に高度の選択性を示す新規抗体、抗体フラグメントおよび抗体コンジュゲートに関する。本発明は、本発明の新規抗体を使用して、患者の生物学的サンプル中の扁平上皮癌抗原の発現を検出することによる診断または予後のｉｎ ｖｉｖｏおよびｉｎ ｖｉｔｒｏ両方の臨床的スクリーニング法に関する。本発明はさらに、上記のスクリーニング方法を実施するためのキットを提供する。かかるキットは、診断、予見または予後の癌の指標として扁平上皮癌抗原の発現について患者をスクリーニングするために使用することができる。さらに、抗体コンジュゲートは、抗腫瘍作用を有する種々の薬剤、非限定的な例としては抗癌剤、毒物、免疫応答調節剤、放射性同位元素などを効果的に送達するのに用いることができる。免疫応答調節剤はまた、融合タンパク質の形で提供され得、放射性同位元素はまた、単鎖のＦｖ鎖構築物に結合されて送達され得る。腫瘍細胞へのＡＤＣＣをターゲットとして、本発明の抗体を結合していない形態で患者に投与することもできる。
【０００２】
２．発明の背景
肺癌は多くの人が患う一般的な悪性腫瘍のうちの１つである。腫瘍と診断されれば、全身的な予後は悪く、５年生存率はわずか１３％である。しかし、肺癌の早期の検出および治療は５年の生存率を著しく改善することができる。疾患が早期に検出され、外科的切除が可能であれば、５年の生存率は４０％まで増加する。肺癌の発生危険率の高い者については、集中的なモニタリングは疾患の発症の減少には効果がなく、気管支の正常細胞においてトランスフォーメーション最初に生じる時を疾患の早期段階と定義することはある意味不可能であることが分かってきた。
【０００３】
腫瘍を関連する抗原へのモノクローナル抗体は、癌の診断、および腫瘍部位に、放射性同位元素、化学抗癌剤および毒素などの様々な抗腫瘍剤をターゲティングすることに有用な試薬を提供する。癌関連抗原に反応する多くのモノクローナル抗体が知られている。既知の抗体は、糖タンパク質（炭水化物部分に結合する場合が大部分である）を含む種々の癌関連抗原に結合する例えば、特定のタイプの癌の糖タンパク質抗原に結合するモノクローナル抗体は、米国特許第４，７３７，５７９号、米国特許第４，７５３，８９４号、４，５７９，８２７号、および米国特許第４，７１３，３５２号に記載されている。
【０００４】
扁平上皮癌の場合、血清などの循環系の体液または気管支分泌物中に、多くの腫瘍細胞の糖タンパク質が循環されるので、モノクローナル抗体を使用してＥＬＩＳＡで循環する抗原を検出できることは早期検出への可能なアプローチである。ＰＳＡおよびＣＥＡなどの腫瘍マーカーの検出は、このアプローチである。例として、の気管支洗浄液の上清を、循

環する抗原が上清液中に存在する気管支上皮の形質転換細胞の早期検出に使用し得る。現在のところ、痰サンプル中の所定の腫瘍細胞を調べるために設計されたアッセイは、正常扁平上皮細胞と異常な扁平上皮細胞とを区別するモノクローナル抗体は未だないため、効果的ではなく、悪性度の表現型の変化の前に遺伝的に変化した細胞が検出されることがわかった。

【０００５】

多くの研究は、非特異的で、全てではないがほとんどの上皮細胞と反応するサイトケラチン（ｃｙｔｏｋｅｒａｔｉｎ）抗体などの上皮細胞マーカーを使用してきた。したがって、より優れたモノクローナル抗体に基づく診断および予後のマーカー、および臨床的に肺癌を特定するために用いるためのより感度の高い試験が必要である。理想的なモノクローナル抗体は、疾患が臨床的には明らかでない早期段階で、特異的腫瘍抗原の発現を検出するものである。モノクローナル抗体によって特定される腫瘍抗原は、細胞の表面に発現される特異的な腫瘍抗原に対する免疫治療の標的としても用いることができる。かかる抗体はまた、予後の値に基づいて、疾患の異なる段階において発現されるか、または機能するマーカー、すなわち、ｅ－カフェリン（ｅ－ｃａｐｈｅｒｉｎ）、増殖因子または受容体などを同定することができる。

【０００６】

３．発明の概要

本発明は、扁平上皮癌に選択性の高いモノクローナル抗体、抗体フラグメントおよび抗体コンジュゲートに関する。さらに詳細には、本発明の新規抗体、抗体フラグメントおよび抗体コンジュゲートは、扁平上皮癌上に認められる細胞膜抗原に結合するが、正常細胞または他の型の癌とは反応しないか、するとしてもわずかである。本発明は、扁平上皮癌に選択性の高いモノクローナル抗体を産生するハイブリドーマ細胞株ハイブリドーマにも関する。

【０００７】

本発明の他の実施形態においては、本発明の抗体は、扁平上皮癌を検出するために設計されたｉｎ ｖｉｖｏもしくはｉｎ ｖｉｔｒｏの診断または予後の診断方法に用いることができる。例えば、抗体は、ヒトの肺、子宮頸部または他の組織における悪性症状の存在を検出するように設計された方法に用いることができる。サンプル中に存在し得る他の細胞型から扁平上皮癌細胞を識別できる本発明の抗体と、組織を接触させればよい。抗体が細胞に結合できる条件下で接触させると、標本の細胞への抗体の結合の存否が検出できる。さらなる診断方法は、検出可能なシグナルを有する薬剤で標識した本発明の精製抗体または抗体フラグメントを被験者に投与して生体内での腫瘍の位置を診断する方法である。腫瘍の位置は、体外シントグラフィー（ｅｘｔｅｒｎａｌ ｓｃｉｎｔｏｇｒａｐｈｙ）、放射断層撮影法あるいは放射核スキャニングを使用して検出される。

【０００８】

本発明はさらに、例えば、腫瘍細胞と反応する本発明の抗体の治療用途にも関する。例えば、抗癌剤、毒素、免疫応答調節剤、酵素および放射性同位元素を含む種々の抗腫瘍作用を有する薬剤の標的選択的キャリアとして働く新規抗体コンジュゲートを使用することができる。

【０００９】

あるいはまた、モノクローナル抗体は改変されずに、すなわち、コンジュゲートした形態ではなくても、扁平上皮癌の患者の治療に用いることができる。本発明の抗体は、特にヒトのリンパ細胞、マクロファージおよび補体の存在下での癌細胞をターゲティングして溶解させることができる抗体依存的細胞傷害性（ＡＤＣＣ）をメディエートするのに特に適している。

【００１０】

本発明は、本発明の抗体によって特定される抗原も含む。さらに、扁平上皮癌に対して免疫接種されるワクチンとして、抗体によって特定される、精製されまたはクローン化された抗原を使用する方法も包含する。

【００１１】
４．図面の簡単な説明
図面の簡単な説明については、後述する。
【００１２】
５．発明の説明
本発明は、扁平上皮癌に特異性の高い新規モノクローナル抗体に関する。より具体的には、正常組織とは反応しないかまたはしてもわずかにしか反応しないが、肺および子宮頸部の扁平上皮癌と特異的に反応する抗体に関する。係る抗体には、設計されたＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１１が含まれる。ＥＬＩＳＡと免疫蛍光分析を使用して分析すると、４種の抗体はすべて肺癌抗原およびヒト肺癌細胞株に対する反応性を有している。さらに、該抗体は強いＡＤＣＣおよび免疫組織化学反応性を示す。
【００１３】
５．１．　扁平上皮癌特異的抗体
本発明は、正常なヒトの組織と全くまたはほとんど反応性を示さない肺および子宮頸部の扁平上皮癌に特異性の高い新規抗体に関する。本発明の新規抗体は、ＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１である。設計された抗体を用いて、それに結合する抗原を単離および解析することができる。したがって、抗体は、これが反応する細胞表面糖タンパク質の同定、単離および／または解析に用いることができる。
【００１４】
本明細書中に使用する用語「ＡＤ６抗体」は、完全な欠失部分のないポリクローナル抗体、およびハイブリドーマＡＴＣＣＰＴＡ－２４６０によって産生されるマウスＡＤ６モノクローナル抗体のような、完全な欠失部分のないモノクローナル抗体、およびＡＤ６抗体と同じ抗原決定基に結合し得るキメラ抗体分子を含む。上記ＡＤ６抗体は、Ｆａｂ、Ｆ（ａｂ’）、Ｆｖフラグメントなどの、抗体の抗原結合領域を含有する抗体フラグメントを含む。ＡＤ６抗体はさらに融合タンパク質も含む。
【００１５】
本明細書中に使用する用語「５Ｃ６抗体」は、完全な欠失部分のないポリクローナル抗体、およびハイブリドーマＡＴＣＣＰＴＡ－２４５８によって産生されるマウス５Ｃ６モノクローナル抗体のような、完全な欠失部分のないモノクローナル抗体、および５Ｃ６抗体と同じ抗原決定基に結合し得るキメラ抗体分子を含む。上記５Ｃ６抗体は、Ｆａｂ、Ｆ（ａｂ’）、Ｆｖフラグメントなどの、抗体の抗原結合領域を含有する抗体フラグメントを含む。５Ｃ６抗体はさらに融合タンパク質も含む。
【００１６】
本明細書中に使用する用語「ＡＤ７抗体」は、完全な、欠失部分のないポリクローナル抗体、およびハイブリドーマＡＴＣＣＰＴＡ－２４５９によって産生されるマウスＡＤ７モノクローナル抗体のような、完全な欠失部分のないモノクローナル抗体、およびＡＤ７抗体と同じ抗原決定基に結合し得るキメラ抗体分子を含む。上記ＡＤ７抗体は、Ｆａｂ、Ｆ（ａｂ’）、Ｆｖフラグメントなどの、抗体の抗原結合領域を含有する抗体フラグメントを含む。ＡＤ７抗体はさらに融合タンパク質も含む。
【００１７】
本明細書中に使用する用語「ＡＨ１抗体」は、完全な欠失部分のないポリクローナル抗体、およびハイブリドーマＡＴＣＣＰＴＡ－２４５７によって産生されるマウスＡＨ１モノクローナル抗体のような、完全な欠失部分のないモノクローナル抗体、およびＡＨ１抗体と同じ抗原決定基に結合し得るキメラ抗体分子を含む。上記ＡＨ１抗体は、Ｆａｂ、Ｆ（ａｂ’）、Ｆｖフラグメントなどの、抗体の抗原結合領域を含有する抗体フラグメントを含む。ＡＨ１抗体はさらに融合タンパク質も含む。
【００１８】
本発明はさらに、扁平上皮癌細胞表面抗原に特異的な抗体を産生し得るハイブリドーマ細胞株を含む。かかるハイブリドーマ細胞株は、限定はされないが、ハイブリドーマＡＴＣＣ ＰＴＡ－２４５７、ハイブリドーマ ＡＴＣＣ ＰＴＡ－２４５９、ハイブリドーマＡ

ＴＣＣ ＰＴＡ－２４Ｓ８、およびハイブリドーマＡＴＣＣＰＴＡ－２４６０などである。

【００１９】

本発明のＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１などの扁平上皮癌細胞表面抗原に特異的な抗体を産生するマウスハイブリドーマは、マウスの融合パートナーの細胞（ＳＰ２／０など）と扁平上皮癌細胞表面抗原で免疫したマウスから単離した脾臓細胞とを融合することにより作製する。マウスの免疫は、対照とする抗原を非精製または粗精製な状態で含む調製物で行ってもよい。マウスの免疫は、種々の慣用技術にしたがって行えばよい。例えば、マウスに、抗原調調製物を、初回免疫と追加免疫をする。

【００２０】

本発明のモノクローナル抗体は、培養中の継代細胞株により抗体分子を産生させる任意の技法によって得ることができる。これらは、限定はしないが、例えば、Ｋｏｈｌｅｒおよびｍｉｌｓｔｅｉｎ（１９７５．Ｎａｔｕｒｅ ２５６：４９５－４９７；および米国特許第４，３７６，１１０号）に記載のハイブリドーマ技術、ヒトＢ細胞ハイブリドーマ技術（Ｋｏｓｂｏｒら、１９８３，Ｉｍｍｕｎｏｌｏｇｙ Ｔｏｄａｙ ４：７２；Ｃｏｌｅら、１９８３，Ｐ，ｒｏｃ．Ｎａｔｌ Ａｃａｄ．Ｓｃｉ，ＵＳＡ ８０：２０２６－２０３０），ＥＢＶ－ハイブリドーマ技術（Ｃｏｌｅら、１９８５．Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ Ａｃａｄ Ｃａｎｃｅｒ Ｔｈｅｒａｐｙ．Ａｌａｎ Ｒ．Ｌｉｓｓ，Ｌｎｃ．，ｐｐ．７７－９６）などである。かかる抗体は、ＩｇＧ、ＩｇＭ、ＩｇＥ、ＩｇＡ、ＩｇＤおよびこれらのあらゆるサブクラスを含む、いかなる免疫グロブリンクラスであってもよい。モノクローナル抗体を産生するハイブリドーマは、ｉｎ ｖｉｔｒｏまたはｉｎ ｖｉｖｏで培養することができる。ｉｎ ｖｉｖｏで高力価のモノクローナル抗体を産生することが好ましい。

【００２１】

さらに、適切な抗原特異性を有するマウス抗体分子から得られた遺伝子と適切な生物学的活性を有するヒト抗体分子から得られた遺伝子と一緒にしてスプライシングさせることによる、「キメラ抗体」の生産のために開発された技術を用いることができる（Ｍｏｒｒｉｓｏｎら、１９８４，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．，８１：６８５１－６８５５；Ｎｅｕｂｅｒｇｅｒら、１９８４，Ｎａｔｕｒｅ．３１２：６０４－６０８；Ｔａｋｅｄａら、１９８５，Ｎａｔｕｒｅ ３１４：４５２－４５４）。Ｆｅｌｌら（１９８９，３０ Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．，８６：８５０７－８５１１）に記載の２段階相同組換え法を用いてキメラ抗体を作製することもできる。あるいは、ヒト化抗体作製のために開発された技術（米国特許第．５，５８５，０８９号）または一本鎖抗体（米国特許第４，９４６，７７８号、Ｂｉｒｄ，１９８８，Ｓｃｉｅｎｃｅ ２４２：４２３－４２６；Ｈｕｓｔｏｎら、１９８８，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ ＵＳＡ，８５：５８７９－５８８３；およびＷａｒｄら、１９８９，Ｎａｔｕｒｅ ３３４：５４４－５４６）を用いて、扁平上皮癌を特異的に認識する抗体を作製することができる。

【００２２】

本発明のモノクローナル抗体は、マウスの腹腔に、抗体を分泌するハイブリドーマ細胞を接種し、適切な日数を経た後、高力価の抗体を含んでいる腹水を回収し、抗体をそこから単離することにより、大量に産生できる。モノクローナル抗体は、ｉｎ ｖｉｔｒｏのハイブリドーマ細胞を培養し、該細胞培養培地から分泌されたｍＡＢを分離することことにより生産され得る（参照、Ｃｏｌｅら、１９８５年、「モノクローナル抗体および癌治療」Ａｌａｎ Ｒ．Ｌｉｓｓ社）。あるいは、抗体遺伝子を、ウイルスのベクターに挿入して遺伝子治療の形態で使用することができ、その場合、抗体遺伝子を受け取った患者が自分の免疫系によって治療抗体を生産することができる。

【００２３】

さらに、当技術分野で既に確立された方法で、活性のある抗原結合活性部位を含んでいる抗体のフラグメントなどの抗体フラグメントを提供することもできる（例えばＭｅｔｈｏ

ｄｓ ｉｎ Ｅｎｚｙｍｏｌｏｇｙ １９８６、（Ａｅａｄｅｍｉｃ Ｐｒｅｓｓ）．１２１：６６３－６９など参照）。

【００２４】

本発明のＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１抗体の抗イディオタイプ抗体もまた、本発明の範囲内に含まれる。ＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１抗体および／またはそのフラグメントを免疫原として用いることにより、抗イディオタイプ抗体生産することができる。係る抗イディオタイプ抗体は腫瘍に対する体液性応答を検出するための診断薬として有用である。また、ワクチンのような治療適用にも有用であり、扁平上皮癌細胞を有している患者の抗腫瘍応答を誘導する。

【００２５】

ＡＤ６、５Ｃ６、ＡＤ７および／またはＡＨ１抗体と同様の結合特異性を有し、かつ細胞傷害性薬剤と組合わせたキメラ抗体は、本発明に包含される。このような免疫毒素は、例えばジフテリア毒素のような細胞傷害性薬剤と融合したＡＤ６、５Ｃ６、ＡＤ７あるいはＡＨ１モノクローナル抗体の抗原結合領域を含む融合タンパク質をコードし得る組換えＤＮＡ分子を生産するために当技術分野で公知の遺伝子工学技術を使用して作製することができる。さらに、ヒト化抗体または完全なヒト抗体は、組換え遺伝子技術を使用して作製でき、こうして得られる抗体は、それが引き起こす抗マウス応答が低減される。

【００２６】

本発明は種々の薬剤の標的選択性キャリアとして働く新規抗体コンジュゲートを包含し、かかる薬剤には、抗癌剤、毒素、免疫応答調節剤、酵素およびアイソトープが含まれる。係る薬剤は、扁平上皮癌細胞の表面に薬剤がターゲティングするのに利用するために、本発明のモノクローナル抗体にコンジュゲートさせることができる。係る細胞傷害性薬剤は、少数挙げるとすれば、例えば、ビンカ・アルカロイド（ｖｉｎｃａ ａｌｋａｌｏｉｄｓ）、リシン、タキソール、ドクソルブシン、メトトレキセート、マイトマイシンＣおよびサイトカラシンＢがある。免疫応答調節剤も、融合タンパク質の形態で提供され得る。また、アイソトープを単鎖Ｆｖ鎖構築物にコンジュゲートさせて送達することも可能である。

【００２７】

本発明の特異的抗体を産生するために、肺の扁平上皮癌由来の糖タンパク質を免疫原として、単離した。抗原は、手術の際に同種の材料を集め、これをＳｅｐｈａｄｅｘＧ－２００クロマトグラフィーによって分画して調製した。Ｂａｌｂ／ｃマウスを、ヒトの肺癌細胞の部分的に精製された可溶性膜抗原で免疫し、十分な時間経過後、マウスを殺して、体液性抗体産生リンパ細胞（例えば脾臓細胞）を得、マウスミエローマ細胞株ＳＰ２／０－Ａｇ１４と融合させた。

【００２８】

融合後、得られた細胞は、ＨＡＴ培地等の選択的な培地中で増殖し得るので、生存した細胞を、限界希釈法を用いて該培地中で増殖させて、その上清を所望の特異性を有するモノクローナル抗体についてスクリーニングした。他のタンパク質および他の混入物から単離するモノクローナル抗体の単離および精製のための様々な従来技術が存在する。

【００２９】

４つのハイブリッドクローンが、所望の特異性を有する抗体を産生することを見出した。これらの抗体はＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１と命名した。４つのモノクローナル抗体はすべて、およびＥＬＩＳＡおよび免疫蛍光検査法の分析により、肺癌抗原およびヒト肺癌細胞株に反応性であることがわかった。抗体は、正常組織、骨髄あるいは組織学的にタイプの異なる他の腫瘍細胞株とは反応しなかった。さらに、ウェスタンブロット分析により、肺癌細胞株Ｐ３およびＰ６に由来するタンパク質抽出物および部分的に精製した肺癌細胞膜抗原を使用して実施した。４種の異なる抗体により様々な分子量の抗原が検出され、各々のモノクローナル抗体が、細胞膜上に発現した別個の抗原を認識することがわかった。

【００３０】

これらの抗体は、肺、食道および子宮頸癌を含む多くの扁平上皮癌にバイオマーカーを特定することがわかった。本発明者らは、抗体、すなわち５Ｃ６とＡＤ７は、市販の抗体より子宮頸部扁平上皮悪性腫瘍においてはるかに感度が高いということを明らかにした。さらに、パップスメア（ｐａｐ ｓｍｅａｒ）試験を細胞が固定化されるように薄層調製技術を用いて実施する場合は、腫瘍抗原を測定および特定するための材料としてサイトスピンを用いることができる。

【００３１】

５２．扁平上皮癌特異的抗原の検出の診断アッセイ

本発明によると、本発明の扁平上皮癌特異的モノクローナル抗体は、肺、食道、子宮頸癌などの疾患の早期診断に有用であり得る。さらに、抗原レベルをモニターおよび定量して、疾患の進行段階を予知するため、および患者の治療に用いる有効な薬剤の評価に用いることができる。

【００３２】

患者から得たサンプル中の扁平上皮癌特異的抗原の検出は、多くの方法で達成され得る。患者の生物学的サンプル中の扁平上皮癌特異的抗原の好ましい診断法には、例えば、本発明の特異的抗体と抗原を反応させて検出する方法などがある。本発明に有用な抗体を用いて、扁平上皮癌特異的抗原またはそのフラグメントの定量的または定性的な検出が可能である。例えば、本発明のモノクローナル抗体を用いて、例えば、組織学的および細胞学的標本中の癌細胞を検出することができる。

【００３３】

例えば、免疫ペルオキシダーゼ染色技術を用いて、組織標本を陽性染色にて分析できる（Ｇａｒｒｉｇｕｅｓ ｅｔ ａｌ．，１９８２，Ｉｎｔ．Ｊ．Ｃａｎｃｅｒ ２９：５１１）。本発明の実施において有用なイムノアッセイとしては、限定される訳ではないが、少数例示するとすれば、ウェスタンブロット、ラジオイムノアッセイ、ＥＬＩＳＡ（酵素連結免疫吸着アッセイ）、「サンドイッチ」イムノアッセイ、免疫沈降アッセイ、沈降反応、ゲル拡散沈殿反応（ｇｅｌ ｄｉｆｆｕｓｉｏｎ ｐｒｅｃｉｐｉｔｉｎ ｒｅａｃｔｉｏｎｓ）、免疫拡散アッセイ、凝集アッセイ、補体結合アッセイ、イムノラジオメトリックアッセイ、蛍光イムノアッセイ、プロテインＡイムノアッセイ、などの技法を用いたアッセイ系が挙げられる。

【００３４】

扁平上皮癌特異的抗原タンパク質を含み得る生物学的サンプル、例えば、肺組織または他の生物学的組織は、特定の癌のリスクを有すると疑われる患者から採取する。全組織の一部または細胞を当技術分野で公知の種々の可溶化混液のうちのいずれかを用いて可溶化する。例えば、１リットル中８Ｍの尿素、２０ｍｌの界面活性剤ＮｏｎｉｄｅｔＰ－４０、２０ｍｌのアンホライト（ｐＨ３．５～１０）、２０ｍｌの２－メルカプトエタノール、および０．２ｍＭのフェニルメチルスルホニルフッ素（ＰＭＳＦ）（脱イオン蒸留水で１リットルにする）を含む溶解バッファーの添加により、組織を可溶化することができる。

【００３５】

扁平上皮癌特異的抗原の発現を検出するためのイムノアッセイは、典型的には、免疫特異的抗原抗体反応の起こる条件下で、組織サンプルなどの患者から得た生物学的サンプルを、本発明のモノクローナル抗体（すなわち、ＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１）に接触させて、抗体の免疫特異的結合量を検出または測定することを含む。特定の態様では、例えば、係る抗体の結合を用いて産生の存在および／または増加を検出することができる。

【００３６】

扁平上皮癌特異的抗原タンパク質を含み得る生物学的サンプル、例えば、肺組織または他の生物学的組織は、特定の癌のリスクを有すると疑われる患者から採取する。全組織の一部または細胞を当技術分野で公知の種々の可溶化混液のうちのいずれかを用いて可溶化する。例えば、１リットル中８Ｍの尿素、２０ｍｌの界面活性剤ＮｏｎｉｄｅｔＰ－４０、２０ｍｌのアンホライト（ｐＨ３．５～１０）、２０ｍｌの２－メルカプトエタノール、

および０．２ｍＭのフェニルメチルスルホニルフッ素（ＰＭＳＦ）（脱イオン蒸留水で１リットルにする）を含む溶解バッファーの添加により、組織を可溶化することができる。

【００３７】

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【００３８】

本発明のある実施態様では、組織抽出液などの生物学的サンプルは、サンプル中に存在するタンパク質全てを固定化する目的で固相支持体またはキャリア（ニトロセルロースなど）に接着させる。そして、支持体を適切なバッファーで洗浄して、ＡＤ６、５Ｃ６、ＡＤ７および／またはＡＨ１などの検出可能な標識モノクローナル抗体で処理する。その後、固相支持体を結合していない抗体を除去するためにバッファーで２回洗浄する。固相支持体に結合した抗体の量を、公知の方法で測定する。抗体または抗体フラグメントを用いて、ラジオイムノアッセイ（ＲＩＡ）の使用により、扁平上皮癌特異的抗原を検出することができる（例えば、Ｗｅｉｎｔｒａｕｂ，Ｂ．，Ｐｒｉｎｃｉｐｌｅｓ ｏｆ Ｒａｄｉｏｉｍｕｎｏａｓｓａｙｓ，Ｓｅｖｅｎｔｈ Ｔｒａｉｎｉｎｇ Ｃｏｕｒｓｅ ｏｎ Ｒａｄｉｏｌｉｇａｎｄ Ａｓｓａｙ Ｔｅｃｈｎｉｑｕｅｓ．Ｔｈｅ Ｅｎｄｏｃｒｉｎｅ Ｓｏｃｉｅｔｙ，Ｍａｒｃｈ １９８６参照）。放射活性同位元素を、ガンマカウンターまたはシンチレーションカウンターを使用する方法により、またはオートラジオグラフィーにより検出することができる。

【００３９】

モノクローナル抗体は、蛍光化合物で標識することもできる。最も汎用される蛍光標識化合物は、フルオロセインイソチオシアネート、ローダミン、フィコエリスリンおよびフルオレサミンである。同様に、生物発光化合物を用いてモノクローナル抗体を標識することができる。生物発光タンパク質の存在は、発光の存在を検出することにより検出される。標識の目的のために生物発光化合物の重要な物は、ルシフェリン、ルシフェラーゼおよびアエクオリンである。

【００４０】

本発明の抗体を用いて、ｉｎ ｖｉｖｏでの診断適用に用いることもできる。例えば、抗体または抗体から調製されるフラグメントを用いて、ヒト患者体内の転移腫瘍などの腫瘍を予測することができる。精製抗体またはそのフラグメントは、検出可能なシグナルを付与することができる試薬で標識され、適切な担体と共に、患者に例えば静脈注射により投与する。腫瘍に結合した抗体の局在は、エクスターナルシントグラフィー（ｅｘｔｅｒｎａｌ ｓｃｉｎｔｏｇｒａｐｈｙ）、エミッション・トモグラフィー（ｅｍｉｓｓｉｏｎ ｔｏｍｏｇｒａｐｈｙ）または放射核スキャニングによって、例えばγカメラを用いて検出することができる。

【００４１】

５．３ キット

本発明はさらに、上述のアッセイを実施するためのキットを提供する。本明細書記載のアッセイは、例えば、抗体試薬（扁平上皮癌特異的抗原検出用）を含むプレパックされた診断キットを用いることにより実施できる。例えば、癌などの症状を診断するために臨床で使用することができる。抗体試薬は、ＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１モノクローナル抗体を含む。

【００４２】

非限定的な例としては、本発明のキットは、患者の生物学的サンプルでの扁平上皮細胞特異的抗原を検出および／または測定する構成要素を含み得る。例えば、扁平上皮癌特異的抗原を酵素連結免疫吸着アッセイ（ＥＬＩＳＡ）で検出および／または測定する場合には、かかる構成成分は、生物学的サンプル中の扁平上皮癌特異的抗原のレベルを検出および／または定量するのに用いることができる、扁平上皮癌特異的抗原のエピトープを認識する抗体を含み得る。抗体自体が、放射活性、蛍光、色素または酵素で標識されていてもよい。このような抗体は、ＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１モノクローナル抗体を含む。あるいは、該キットは、標識二次抗体を含んでいてもよい。

【００４３】

５．４　モノクローナル抗体の治療的適用

本発明の抗体は、さまざまな治療方法に用いることができる。モノクローナル抗体は、扁平上皮癌を有する患者の治療に、修飾されないままで、すなわちコンジュゲートされてない形態で用いることもできる。例えば、抗体は補体（ＣＤＣ）またはエフェクター細胞（ＡＤＣＣ）介在性細胞傷害をターゲットとしてもよい。あるいは、抗体を抗癌剤、毒素、放射性各種にコンジュゲートしてもよい。コンジュゲートした抗体を患者に投与し、抗体部分の結合親和性に基づいて腫瘍へ送達された抗癌剤の細胞傷害作用を介して、殺腫瘍効果が向上する。

【００４４】

本発明のキメラ抗体分子は、ヒト定常領域ドメインと抗原結合ドメインを含むように調製するとよく（Ｍｏｒｒｉｓｏｎら、１９８４，Ｐｒｏｃ．Ｎａｔｌ Ａｃａｄ．Ｓｃｉ．Ｕ．Ｓ．Ａ．８１：６８５１；Ｔａｋｅｄａら、１９８５，２５Ｎａｔｕｒｅ，３１４：４５２）、この手法を用いるとヒトの補体の活性化およびＡＣＤＤの介在する能力などの所望のエフェクター機能を有する新規抗体分子を作製できる。

【００４５】

本発明は、本発明の抗体を含む医薬組成物に関する。抗体、抗体フラグメントまたは誘導体の投与量とその処方は、癌治療の臨床分野の当業者が容易に決定できる。例えば、非経口、皮下、筋内、腹腔、経皮または舌下投与などでよい。投与用量は、投与される者の年齢、健康状態、体重、治療頻度、および所望する効果の性質によっても異なる。

【００４６】

本発明の範囲内の組成物は、抗体、フラグメントまたは誘導体が意図する目的を達成するために効果的な量で含まれていれば、あらゆる組成物でよい。個々の必要性は様々であり、各組成物の最適範囲の効果的な量の決定は当業者には公知技術である。効果的な用量は、それぞれのキメラ抗体、モノクローナル抗体コンジュゲートした治療剤、患者およびその臨床的状態の存在および性質に依存するが、体重１ｋｇ当たり約１０ｎｇ～１００ｍｇの範囲内である。好ましい用量は、体重１ｋｇ当たり０．１～１０ｍｇ／ｋｇである。非経口投与のための本発明の抗体、フラグメントまたは誘導体の調剤（イメージング用に検出可能な標識を付した形態、または治療用の遊離もしくは結合した形態など）は、滅菌した凍結乾燥タンパク質、水性または非水性液、懸濁剤および乳化剤を含む。非水性溶媒の例としては、プロピレングリコール、ポリエチレングリコール、オリーブ油などの植物油、およびエチルオレイン酸などの注射可能な有機エステルがある。水性担体は、水、アルコール／水溶液、乳化剤、懸濁剤などがあり、生理的食塩水、緩衝剤、塩化ナトリウム溶液、リンガーデキストロース液、デキストロースと塩化ナトリウム、乳酸加リンガー溶液または不揮発性油などの非経口ビヒクルが挙げられる。静脈投与ビヒクルは、液体およびリンガーデキストロース液などに基づいた栄養補充剤を含み得る。例えば、抗菌剤、抗酸化剤、キレート剤、不活性気体などの保存剤および他の添加物を含んでもよい。概説としては、Ｒｅｍｉｎｇｔｏｎ’ｓ Ｐｈａｎｎａｃｅｕｔｉｃａｌ Ｓｃｉｅｎｃｅ，１６ｔｈ ｅｄ．，Ｍａｃｋ Ｐｕｂｌｉｓｈｉｎｇ Ｃｏ．，Ｅａｓｔｏｎ，ＰＡ，１９８０などを参照のこと。

【００４７】

特に、抗体、そのフラグメントおよび誘導体は、扁平上皮癌を有する患者の治療に有用で

ある。かかる治療は、抗体、そのフラグメントもしくは誘導体、またはそのコンジュゲートの単回または複数回の非経口投与を含み得る。

【００４８】

ＡＤ６、５Ｃ６、ＡＤ７およびＡＨ１抗体が結合する本発明の新規抗原は、治療適用に用いることもできる。抗原を腫瘍から精製して、免疫原として単独で、または適切な免疫アジュバントと一緒に投与する。あるいは、抗原を免疫原として利用するために遺伝子組換えにより作製してもよい。

【００４９】

６．実施例

扁平上皮癌特異的モノクローナル抗体

抗体

下記に、扁平上皮癌の表面に発現される特異的糖タンパク質に対して反応するモノクローナル抗体の産生について記載する。免疫学的アッセイにより、該モノクローナル抗体が癌細胞と反応するが、正常組織とは全くもしくはほとんど反応しないことが示された。さらに、ＡＨ１およびＡＤ７も同様である。

【００５０】

６．１　モノクローナル抗体の調製

ヒト肺扁平上皮癌関連抗原（ＳＬＡＡ）に対するモノクローナル抗体は、Ｈｅｒｚｅｎｂｅｒｇらによって記載された方法の変法を用いて作製した。４週齢のＢａｌｂ／ｃマウスに、腹腔内投与（ｉｐ）にて、外科手術時に患者から摘出したヒト肺扁平上皮癌を回収して、その膜を抽出したものから得た粗精製済みＳＬＡＡ　１００μｇを、完全フロイントアジュバント２００μｌで乳化して免疫した。２週間感覚で、不完全フロイントアジュバント２００μｌで乳化したものを２回腹腔注射した。その後免疫原２０μｇで静脈内投与により最終免疫して、３日後に脾臓細胞を採取した。細胞ハイブリッドを、Ｍｕｒａｒｏらの方法によりマウス非分泌性ミエローマ細胞株ＳＰ２／０－Ａｇ１４を用いて調製した。ハイブリドーマの上清を、ヒト肺扁平上皮癌の数種の細胞株（Ｐ３、Ｐ６およびＨ５９５）および粗精製免疫原を用いてＥＬＩＳＡで特異的抗体産生についてアッセイした。全てのハイブリドーマ株を限界希釈法により２回クローニングした。

【００５１】

腹水での調製のために、４週齢のＢａｌｂ／ｃマウスに、プリスタン投与後、約５×10^6個のハイブリドーマ細胞を接種した。モノクローナル抗体を該マウスの腹水から精製して、プロテインＧアフィニティカラムクロマトグラフィで精製した。精製抗体調製物のタンパク質含量は、１２％ＳＤＳポリアクリルアミドＴｒｉｓ－グリシンゲル上で解析した。

【００５２】

６．２　モノクローナル抗体の調製

６．２．１．　ウェスタンブロット

５Ｃ６、ＡＤ６、ＡＤ７およびＡＨ１抗体を用いて、ウェスタンブロットを行った。タンパク質サンプルは、肺洗浄液標本から得た。サンプル（３μｇ）を１２％ＳＤＳ－ＰＡＧＥ上にロードした。タンパク質をニトロセルロース膜に転写して、西洋ワサビペルオキシダーゼで標識した抗体を用いて染色した。図１に示すように、本発明のモノクローナル抗体はいずれも、肺洗浄液標本から得た扁平上皮癌細胞に発現された異なる抗体に対して反応した。

【００５３】

６．２．２．　扁平上皮癌特異的抗体によるＡＤＣＣ

４時間^{51}Ｃｒ放出アッセイを用いてＡＤＣＣ活性を測定した。標的細胞を肺扁平上皮癌細胞株Ｈ５９５とした。標的細胞を、２００μｌのウシ胎児血清中で１時間２００μＣｉの［^{51}Ｃｒ］クロム酸ナトリウム（２００～５００ｍＣｉ／ｍｇ、Ａｍｅｒｓｈａｍ、Ａｒｌｉｇｔｏｎ，　ＩＬ）で標識した。標的細胞（１×10^4）を、モノクローナル抗体（１．０μｇ／ウェル）の存在下で、エフェクター細胞と共に（比率は、エフェクター

細胞対標的細胞が８０：１になるようにして）Ｕ底９６ウェルプレートでインキュベートした。プレートを５％CO_2存在、湿度条件下で、３７℃にて４時間インキュベートした。上清を回収して、Ｓｋａｔｒｏｎ Ｈａｒｖｅｓｔｅｒフレームを使用してγカウントに供した。溶解率を下記式を用いて計算した：
溶解率（％）＝｛［観察された放出量（ｃｐｍ）－自発的放出量（ｃｐｍ）］／［総放出量（ｃｐｍ）－自発的放出量（ｃｐｍ）］｝×１００
自発的放出量は、培地のみの中でインキュベートした標的細胞から放出された放射活性を測定することにより検出した。総放出量放射活性は、２．５％のＴｒｉｔｏｎ Ｘ－１００で処理した後に測定した。放射性標識クロムの自発的放出は、２．５％のＴｒｉｔｏｎ Ｘ－１００で処理した後に測定した。

【００５４】
図２および図３に示すように、ＡＨ－１、Ｄ６および５Ｃ６モノクローナル抗体は、ヒト肺扁平上皮癌細胞に対して試験した場合のＡＤＣＣ活性を示す。図３Ｂに示すように、ＡＤＣＣ活性は、ヒト結腸癌細胞に対して活性は示さないことから、肺扁平上皮癌細胞に特異的であった。

【００５５】
６．２．３．　扁平上皮癌特異的抗体を用いた免疫組織化学
染色方法は下記の通りである。

【００５６】
Ｍａｙｏ凍結組織法
スライドグラス標本を、１０分間１％パラフォルムアルデヒドで固定化して３回ＰＢＳで洗浄した。内因性ペルオキシダーゼ（１％アジ化ナトリウム＋３％H_2O_2）でブロッキングして１分間水で洗浄した。５％ＮＧＳを加えて１０分後に、１％ＮＧＳで希釈した一次抗体（２μｇ／ｍｌ）を加えて、室温で２時間インキュベートした。スライドグラスをＰＢＳで２～３回洗浄し、ビオチンＲＡＭ（ＤＡＫＯ）と共に室温にて１５分間インキュベートした。スライドグラスをＰＢＳで２～３回洗浄した。ストレプトアビジン（ＨＲＰ）（ＤＡＫＯ）をスライドグラスに添加して室温で１５分間インキュベートした。スライドグラスをＰＢＳで２回洗浄し、水で１回洗浄した。ＤＡＢまたはＡＥＣを反応させた。

【００５７】
パラフィン切片法
スライドグラス標本を、２０分間６０℃で静置した。脱パラフィン化はセーフティキャビネット内で行った、（ｉ）キシレン５分；（ｉｉ）キシレンと１％ヨード５分；キシレン１０秒；（ｉｉｉ）１００％アルコール；（ｉｖ）９５％アルコール、（ｖ）５０％アルコール。スライドグラスを５０％メタノール３％H_2O_2中に１０分間静置し、１分間水で洗浄した。５％ＮＧＳを１０分間添加して、１％ＮＧＳで希釈した一次抗体（１μｇ／ｍｌ）を加えて、室温で一晩インキュベートした。サンプルを水で洗浄し、ビオチンＧＡＭ（ＤＡＫＯ）と共に３０分間室温にてインキュベートした。スライドグラスを水で洗浄し、ストレプトアビジン（ＨＲＰ）（ＤＡＫＯ）をスライドグラスに添加して３０分間インキュベートした。サンプルを水で洗浄し、ＤＡＢ／ＡＥＣを反応させた。

【００５８】
本発明の４種のモノクローナル抗体は、肺の扁平上皮癌の表面に特異的に発現されるが、正常細胞の表面には発現しない抗原に対して反応することがわかった。さらに、抗体は、子宮頸癌細胞の表面に発現された抗原に対しても免疫反応性を有していることが分かった（図７Ａ～Ｂ）。

【００５９】
さらに、ＥＬＩＳＡアッセイを以下の方法を用いて実施した。肺洗浄液を、１：２、１：４、１：８、１：１６、１：３２、１：６４、１：１２８の希釈割合で正確に希釈して１００μ／ウェルで４℃で一晩コーティングした。ウェルをＴＢＳＴで３回洗浄した。ブロッキングをＴＢＳＴ／２％ジェラチンで行った（２００～１００μｌ／ウェル、３７℃、

１時間）。その後、ＴＢＳＴで５回洗浄した。腹水抗体１００μｌ／ｍｌを加えて３０℃で１時間インキュベートした。抗マウスＩｇＧ ＡＰ抗体１：１０００を添加して、３７℃で１時間インキュベートした。ウェルをＴＢＳＴで５回洗浄した。基質Ｐ－ＮＰＰを各ウェルに添加して４０５ｎｍでのエンドポイント吸光度測定を行った。図５および図６に示すように、５Ｃ６およびＡＤ６モノクローナル抗体は、肺洗浄液組織サンプルと反応した。

【００６０】

さらに、細胞フローサイトメトリーを実施して抗体の特異性を調べた。細胞（ＬＳ１７４Ｔ、Ｈ５９６、Ｈ４４１およびＣａＬｕ３）を、フェニルレッド不含培地中で対数増殖期まで増殖させて、０．０２５％トリプシンＥＤＴＡ（ＢｉｏＷｈｉｔｔａｋｅｒ）でフラスコから剥離した。そして、細胞をＰＢＳ（ｐＨ７．４）で洗浄して３０分間培地中に懸濁させてカウントした。全てのその後の手順は、４℃で実施した。

【００６１】

細胞をＰＢＳで３回洗浄した。5×10^{5} 個の細胞を含むサンプルをＰＢＳ２００μｌ中に懸濁して、それぞれの反応試験管に入れた。ビオチン化抗体溶液２００μｌをサンプルに加えて、４℃で３０分間インキュベートした。細胞をＰＢＳで３回洗浄して、５００μｌのＰＢＳに懸濁させた。その後、細胞をフローサイトメトリーで解析した。図８に示すように、肺癌細胞（Ｈ５９６、Ｈ４４１およびＣａＬｕ３）は抗体で染色されたが、対照の結腸癌細胞（ＬＳ１７４Ｔ）はほとんど染色されなかった。

【００６２】

６．３．　ワクチンの研究

同種肺癌細胞（すなわち、扁平上皮癌）から膜調製物を得た。膜抽出物を生細胞から得て、低頻度超音波処理に供した。可溶性物質をＳｅｐｈａｄｅｘ Ｇ－２００で分離して、さらに非連続的ポリアクリルアミドゲル電気泳動に供した。個々のバンドをゲルから単離し、ＤＨＲ（遅延型皮膚過敏症）について調べ、回収してワクチンとして用いた。ＤＨＲについての皮膚試験には、３０μｇの抗原（ＴＡＡ）を用いた。免疫応答を抑制する阻害物質を含んでいたためＳｅｐｈａｄｅｘ画分Ｉをワクチンから除去した。例外なく、自己免疫肺製応答の徴候が現れた。各患者で、細胞性および体液性免疫の増強が観察された。ＤＨＲは５年以上増大したままであった。外科手術とワクチン接種のにより調べた患者（２３７例）の８０％が生存したが、外科手術だけでは４０％しか生存しなかった。

【００６３】

６．４．　ＡＤ７および５Ｃ６重鎖ならびに軽鎖のクローニングと決定

ＡＤ７および５Ｃ６重鎖Ｖ領域のクローニングと増幅に用いたプライマーは以下の通りである：

（ｉ）　ｃＤＮＡ：ＭＨＣＧＳＰ１Ａ：

５’－ＣＡＴ ＧＧＡ ＧＴＴ ＡＧＴ ＴＴＧ ＧＧＣ ＡＧＣ ＡＧＡ－３’

ブリッジ化アンカープライマー；

５’－ＧＧＣ ＣＡＣ ＧＣＧ ＴＣＧ ＡＣＴ ＡＧＴ ＡＣＧ ＧＧＩＩＧＧ ＧＩＩ ＧＧＧ ＩＩＧ－３’

（ｉｉ）増幅：ＭＨＣＧＳＰ２Ａ：

５’－ＣＡＧ ＧＧＧ ＣＣＡ ＧＴＧ ＧＡＴ ＡＧＡ ＣＡＧ ＡＴＧ－３’

ブリッジ化アンカープライマー；

５’－ＧＧＣ ＣＡＣ ＧＣＧ ＴＣＧ ＡＣＴ ＡＧＴ ＡＣＧ ＧＧＩＩＧＧ ＧＩＩ ＧＧＧ ＩＩＧ－３’

以下のプライマーを、ＡＤ７と５Ｃ６の軽鎖Ｖ領域のクローニングと増幅に用いた：

（ｉ）　ｃＤＮＡ：ＭＬＣＧＳＰ１：

５’－ＣＣＴ ＧＴＴ ＧＡＡ ＧＣＴ ＣＴＴ ＧＡＣ ＡＡＴ ＧＧＧ－３’

ブリッジ化アンカープライマー；

５’－ＧＧＣ ＣＡＣ ＧＣＧ ＴＣＧ ＡＣＴ ＡＧＴ ＡＣＧ ＧＧＩＩＧＧ ＧＩＩ ＧＧＧ ＩＩＧ－３’

（ｉｉ） 増幅：ＭＯＳＫＡＰＰＡ：
５’－ＡＣＴ ＧＧＡ ＴＧＧ ＴＧＧ ＧＡＡ ＧＡＴ ＧＧＡ Ｔ－３’
ブリッジ化アンカープライマー；
５’－ＧＧＣ ＣＡＣ ＧＣＧ ＴＣＧ ＡＣＴ ＡＧＴ ＡＣＧ ＧＧＩＩＧＧ ＧＩＩ ＧＧ Ｇ ＩＩＧ－３’。

【００６４】

６．５．ヒト－マウスキメラ抗体遺伝子の構築と発現

レトロウイルスベクターｐＬＨＣＸＩＩとｐＬＮＣＸＩｌを用いて、キメラ重鎖および軽鎖のｃＤＮＡをそれぞれ発現させた。ｐＬＨＣＸＩＩは、ネオ遺伝子の５’側から４５塩基の位置にあるＥｃｏＲＩ部位はあるがベクター骨格中のＥｃｏＲＩ部位が破壊されていることを除いてはベクターＬＮＣＸと同じである。これはＤ．Ｍｉｌｌｅｒ博士（Ｆｒｅｄ Ｈｕｔｃｈｉｎｓｏｎ Ｃａｎｃｅｒ Ｒｅｓｓｅａｒｃｈ Ｃｅｎｔｅｒ，Ｓｅａｔｔｌｅ ＷＡ）より供与いただいた。ベクターはＮ２ベクターからＭ－ＭｕＬＶの５’末端反復配列（ＬＴＲ）をＭ－ＭＳＶＬＲＴに置換して、かつヒトサイトメガロウイルス（ＨＣＶＭ）の最初期遺伝子プロモーター３’からｎｅｏ遺伝子までを含むＢａｌＩ／ＸｍａＩＩＩ断片を挿入することにより得た。標的遺伝子のクローニングのために、ＨｉｎｄＩＩＩ、ＨｐａＩおよびＣｌａＩ部位を含むポリリンカーは、３’からＨＣＭＶプロモーターまでに挿入された。ベクターｐＬＨＣＸＩＩを、ネオマイシン耐性遺伝子を含む１．２ＫｂのＥｃｏＲＩ／ＢａｍＨＩフラグメントをハイグロマイシン耐性を付与する遺伝子を有する１．４ＫｂのＥｃｏＲＩ／ＢａｍＨＩフラグメントと置換して作製した。ｐＬＮＣＸＩＩＨｕＫのレトロウイルス発現構築物を作製するために、キメラκ鎖（ｍＡｂ）をコードするｃＤＮＡを含むＤＮＡ断片をｐＢｌｕｅｓｃｒｉｐｔ構築物をＳｍａＩ／ＣｌａＩで切断することにより得た。得られたフラグメントを、ＨｐａＩ／ＣｌａＩで直鎖状にしたｐＬＮＣＸＩＩベクターにクローニングした。キメラ重鎖をコードするＤＮＡ断片配列をｐＢｌｕｅｓｃｒｉｐｔ構築物から、プラスミドをＸｂａＩで切断して、突出部分をＫｌｅｎｏｗで埋める以外は同様にして得、ＨｉｎｄＩＩＩで切断することにより断片を切り出した。できた断片を、ＨｉｎｄＩＩＩ／ＨｐａＩで直鎖状にしたｐＬＨＣＸＩＩにクローニングして、キメラｍＡｂ重鎖の発現構築物であるｐＬＨＸＩＩＨｕＧ１を作製した。

【００６５】

キメラｍＡｂの開発のために続いて、ＨＣおよびＬＣ発現構築物をＳＰ２／Ｏ細胞に、セルポレーターシステムを用いてエレクトロポレーションによって導入した。エレクトロポレーションは以下の通り行った。簡単に説明すると、ＳＰ２／Ｏ細胞を４．５ｇ／Ｌグルコース含有血清不含ＤＭＥＭ（ＪＲＨ Ｎｉｏｓｃｉｅｎｃｅ，Ｌｅｎｅｘａ，ＫＳ）で洗浄し、5×10^{6}細胞／ｍｌの濃度で同じ培地に懸濁した。プラスミド１００μｇを４℃に保ったエレクトロポレーションチャンバー中の細胞懸濁液１ｍｌに加えた。細胞とＤＮＡの混合物を６５０Ｖ／ｃｍで１３ｍｓにて刺激した（キャパシタンスセッティングは１６００μＦ）。細胞を４℃で１０分間保って、１５％ＦＣＳ含有ＲＰＭＩ１６４０で希釈した。その後、細胞を5×10^{5}細胞／ウェルの濃度で２４ウェルプレートの播種した。３７℃で１５％CO_2条件下で、２４時間インキュベートした後、ハイグロマイシンまたは活性Ｇ４１８含有選択培地を５００～８００μｌ／ｍｌの濃度でそれぞれ添加した。

【００６６】

ヒトマウスキメラｍＡｂ（Ｈｕ ｍＡｂ）産生クローンは、タンパク質不含ＰＦＨＭ－ＩＩハイブリドーマ培地（ＧＩＢＣＯ，ＢＲＬ）で増殖させ、プロテインＧアフィニティクロマトグラフィで精製した。タンパク質濃度は、ＢｉｏＲａｄマイクロアッセイ法またはＬｏｗｒｙ法により測定する。充填済みの４～２０％のＳＤＳ－ポリアクリルアミドＴｒｉｓ－グリシンゲル（Ｎｏｖｅｘ Ｓｙｓｔｅｍ，Ｓａｎ Ｄｉｅｇｏ，ＣＡ）を用いて、２－メルカプトエタノールでの変性後もしくは未変性でタンパク質を解析した。タンパク質ゲルを、Ｌａｅｍｍｌｉの方法でクマシーブリリアントブルーＲ２５０で可視化

した。

【００６７】

本発明は、ここに記載した特定の実施態様に限定されるものではない。記載の実施態様は、本発明のこの態様の単なる例示として示したものであり、機能的に同等な方法および構成要素は本発明の範囲に含まれる。無論、本明細書の記載および例示において、本発明に関する様々な改変が可能であることは、当業者には明らかであろう。かかる改変もまた、特許請求の範囲に含まれる者である。本明細書中に引用する種々の刊行物は、全体を参照として本明細書中に組込むものとする。

【図面の簡単な説明】

【図１】

モノクローナル抗体５Ｃ６、ＡＤ６、ＡＤ７およびＡＨ１による肺洗浄液のウェスタンブロットの結果を示す。

【図２】

図２は、扁平上皮癌特異的抗体ＡＨ１およびＡＤ７ならびにコントロール抗体ＵＰＣ－１０によるＡＤＣＣの結果である。

【図３】

図３Ａは、ヒト扁平上皮癌細胞株Ｈ５９６に対する、扁平上皮癌特異的抗体ＡＨ１、ＡＤ６、ＡＤ７、５Ｃ６およびコントロール抗体ＵＰＣ－ｌＯによるＡＤＣＣの結果である。図３Ｂは、ヒトの結腸癌細胞株ＬＳ１７４Ｔに対する、扁平上皮癌特異的抗体ＡＨ１、ＡＤ６、ＡＤ７、５Ｃ６およびコントロール抗体ＵＰＣ－ｌＯによるＡＤＣＣの結果である。

【図４】

図４は、扁平上皮癌特異的抗体ＡＨ６、ＡＤ７および５Ｃ６を用いた免疫組織染色の結果である。図４Ａは、正常な肺組織をＡＤ６でプロービングした結果である。図４Ｂは正常な肺組織をＡＤ７でプロービングした結果である。図４Ｃは、扁平上皮癌肺洗浄液をＡＤ６でプロービングした結果である。図４Ｄは、扁平上皮癌肺洗浄液を５Ｃ６でプロービングした結果である。図４Ｅは、扁平上皮癌を５Ｃ６でプロービングした結果である。図４Ｆは、扁平上皮癌をＡＤ６でプロービングした結果である。

【図５】

図５は、肺洗浄液を用いた５Ｃ６およびＡＤ６モノクローナル抗体による直接法ＥＬＩＳＡ分析の結果である。

【図６】

図６は、肺洗浄液を用いた５Ｃ６、ＡＤ６、ＡＨ１およびＡＤ７モノクローナル抗体による直接法ＥＬＩＳＡ分析の結果である

【図７】

図７Ａは、ＳＣ６およびＡＤ７モノクローナル抗体を使用するパップスメア試験（Ｐａｐ Ｓｍｅａｒ）の結果である。スライドは形成障害を表わすが、癌特異的抗原を発現する細胞の存在を示す。図７Ｂは、Ｃ５６（上）およびＡＤ７（下）を使用して、子宮頸癌細胞をペルオキシダーゼでｉｎ ｓｉｔｕで染色した結果である。

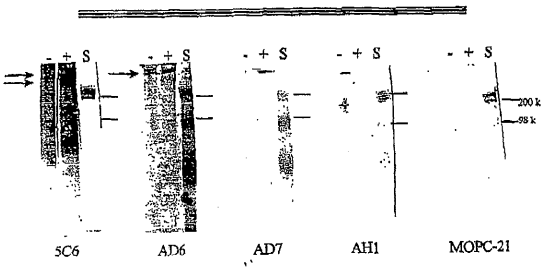
【図８】

図８は、細胞のフローサイトメトリーの結果である。ＬＳ１７４Ｔ結腸癌細胞、Ｈ５９６、Ｈ４４１およびＣａＬｕ３ 肺癌細胞を、ＡＪ６またはＡＤ７抗体で染色した。

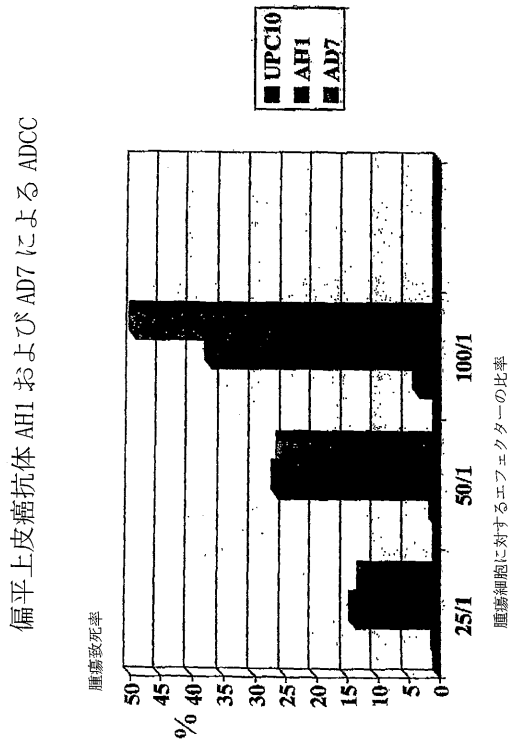
【図１】

肺洗浄液のモノクローナル抗体による
ウェスタンブロッティング

＋ DTT により還元したサンプル
－ 非還元サンプル
S 分子量マーカー

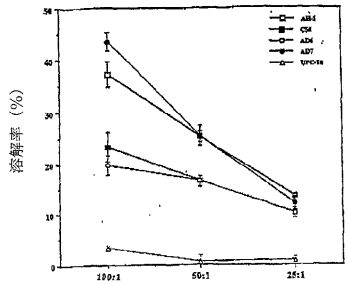


【図２】

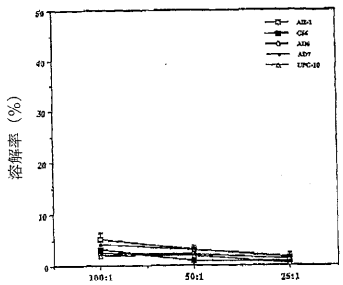


【図３】

ヒト肺偏平上皮癌細胞株 H596 に対する肺偏平上皮癌細胞モノクローナル抗体 AH-1、C56、AD6、AD7 および対照の抗体 UPC-20 の ACDD 活性



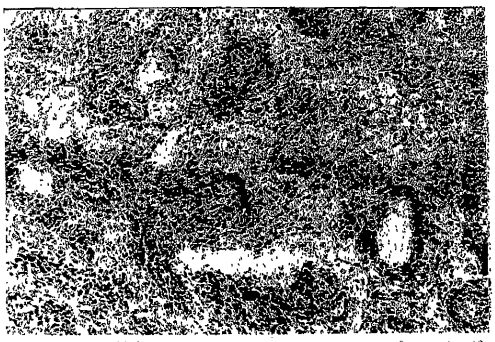
ヒト結腸癌細胞株 L5174T に対する肺偏平上皮癌細胞モノクローナル抗体 AH-1、C56、AD6、AD7 および対照の抗体 UPC-10 の ACDD 活性



【図４】

正常の肺

A.



10 倍拡大　　AD6 でのプロービング

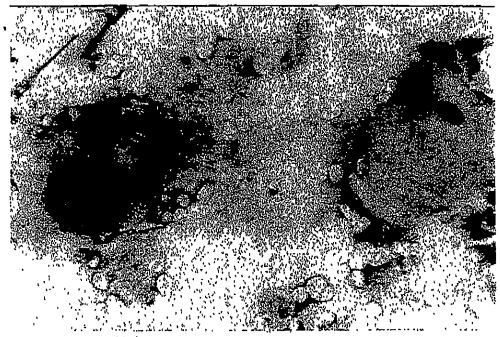
B.

4 倍拡大　　AD7 でのプロービング

4A-B

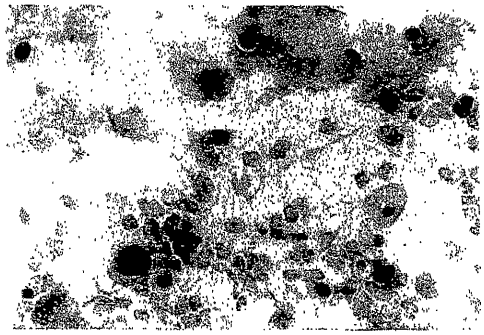
SCC 肺洗浄液

C.



40 倍拡大　　AD6 でのプロービング

D.

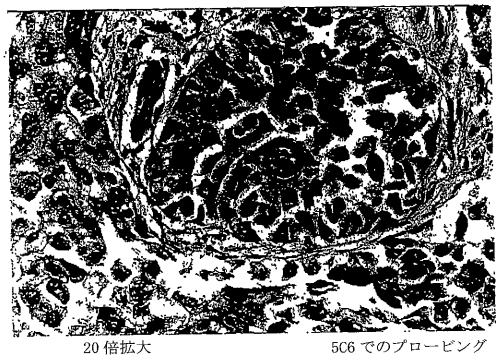


20 倍拡大　　5C6 でのプロービング

4 C-D

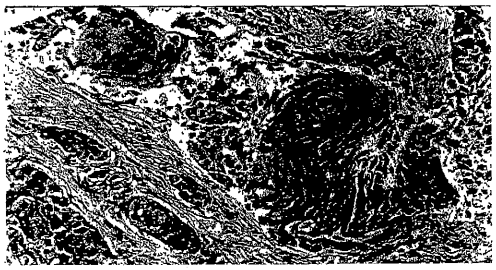
SCC 肺

E.



20 倍拡大　　5C6 でのプロービング

F



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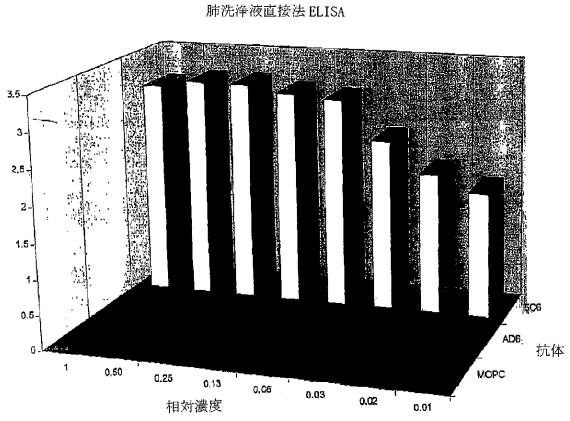
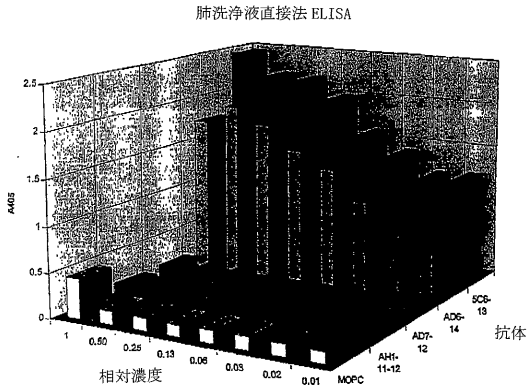
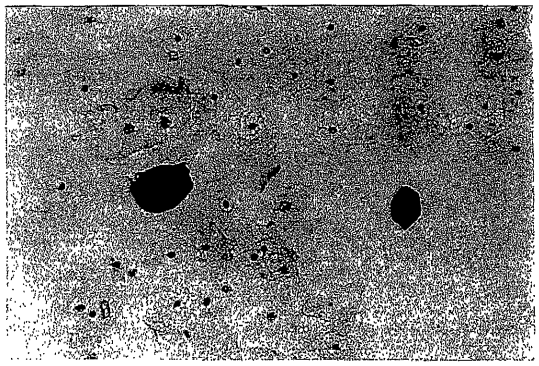


【図６】



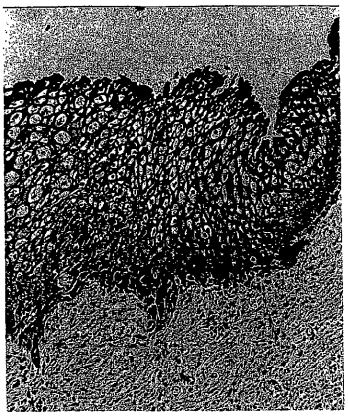
【図７】

偏平上皮癌特異的抗体5C6およびAD7を用いた免疫組織染色による
パップスメア技法



子宮頸部の偏平上皮癌

5C6を用いた
in situ癌細胞
ペルオキシダーゼ
免疫染色 ->

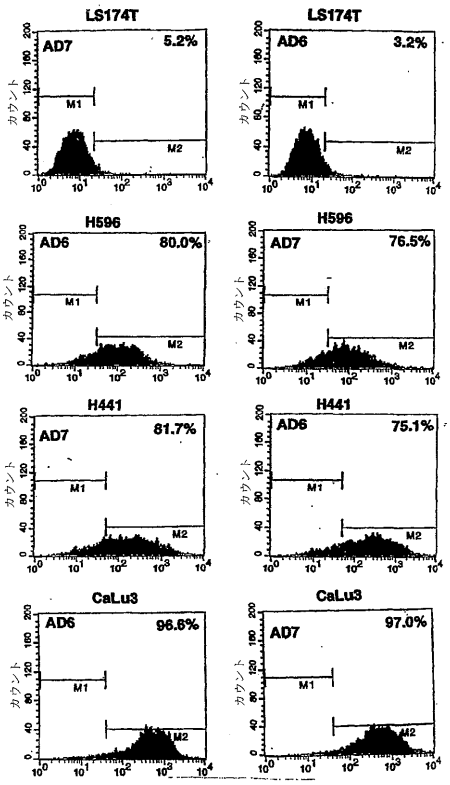


AD7で染色した子宮頸部の
浸潤偏平上皮癌

（下図）

7A

【図８】



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(54) Title: THE IDENTIFICATION AND DEVELOPMENT OF SPECIFIC MONOCLONAL ANTIBODIES TO SQUAMOUS CELL CARCINOMA

(57) Abstract: The present invention relates to novel antibodies, antibody fragments and antibody conjugates which display a high degree of selectivity for squamous cell carcinoma antigens including carcinomas of the lung, esophagus and cervix. The present invention relates to both in vivo and in vitro clinical screening methods for diagnosis or prognosis of carcinomas by means of detecting the expression of squamous cell carcinoma antigens in biological samples of the subject using the novel antibodies of the invention. The invention further provides for kits for carrying out the above described screening methods. Additionally, antibody conjugates may be used to efficiently deliver various agents which have anti-tumor effects to the tumor cell. The antibodies of the invention may also be administered to a patient in non-conjugated form to target ADCC to the tumor cell.

THE IDENTIFICATION AND DEVELOPMENT OF SPECIFIC MONOCLONAL ANTIBODIES TO SQUAMOUS CELL CARCINOMA

1. INTRODUCTION

The present invention relates to novel antibodies, antibody fragments and antibody conjugates which display a high degree of selectivity for squamous cell carcinoma antigens including carcinomas of the lung, esophagus and cervix. The present invention relates to both *in vivo* and *in vitro* clinical screening methods for diagnosis or prognosis of carcinomas by means of detecting the expression of squamous cell carcinoma antigens in biological samples of the subject using the novel antibodies of the invention. The invention further provides for kits for carrying out the above described screening methods. Such kits can be used to screen subjects for expression of squamous cell carcinoma antigens as a diagnostic, predictive or prognostic indicator of cancer. Additionally, antibody conjugates may be used to efficiently deliver various agents which have anti-tumor effects including, but not limited to, chemotherapeutic drugs, toxins, immunological response modifiers, and radioisotopes, immunological response modifiers may also be provided in the form of fusion proteins. Immunological response modifiers may also be provided in the form of fusion proteins and radioisotopes may also be delivered while conjugated to single chain Fv chain constructs. The antibodies of the invention may also be administered to a patient in unconjugated form to target ADCC to the tumor cell.

2. BACKGROUND OF INVENTION

Lung cancer is one of the more common malignancy effecting the population. Once a tumor is diagnosed, the overall prognosis is poor, with an overall five year survival rate of only 13%. However, early detection and treatment of lung cancer can significantly improve 5 year survival rates. In those cases where the disease is detected early and surgical resection is feasible, the five year survival rate increases to 40%. For those at high risk for development of lung cancer intensive

monitoring has not been effective in reducing the incidence of the disease or its outcome due in part to the inability to define early stages of the disease when transformation is first occurring within normal cell populations of the tracheobronchial tree.

Monoclonal antibodies to tumor-associated antigens provide useful reagents for diagnosis of cancer and for targeting of various anti-tumor agents such as radioisotopes, chemotherapeutic drugs and toxins to the site of the tumor. Many monoclonal antibodies reactive against carcinoma -associated antigens are known. These known antibodies bind to a variety of different carcinoma-associated antigens that include glycoproteins but for the most part bind to the carbohydrate moiety. For example, monoclonal antibodies that bind to glycoprotein antigens on specific types of carcinomas include those described in U.S. Patent Nos. 4,737,579; 4,753,894; 4,579,827 and 4,713,352.

Since many tumor cells shed their membrane glycoproteins into surrounding body fluids such as serum, or bronchial secretions in the case of squamous cell carcinomas, the possibility of detecting shed antigen using monoclonal antibodies in an ELISA is a possible approach to early detection. This is the approach taken for detection of tumor markers such as PSA and CEA in serum. As an example, supernatants of the bronchial lavage could be used in immunoassays designed for early detection of transformed cells in bronchial epithelium where shed antigen appears in the supernatant fluid. To date, assays designed to define tumor cells in sputum samples have proven to be ineffective because of the unavailability of monoclonal antibodies capable of differentiating between normal and abnormal squamous cells or to be able to define genotypically altered cells before the phenotypic changes of malignancy are evident.

Most studies have employed epithelial markers such as cytokeratin antibodies which are non-specific and will react with most if not all epithelial cells. Thus, better monoclonal antibody based diagnostic and prognostic markers and more sensitive tests for use in defining clinical lung cancer are needed. Ideally the monoclonal antibodies will detect the expression of a specific tumor antigen at an

early time when clinical disease is not obvious. Such a tumor antigen defined by a monoclonal antibody could be used as a target for immunotherapy directed against the specific tumor antigen expressed on the surface of the cell. Such antibodies may also be of prognostic value based their ability to identify markers expressed at different stages of disease or that function differently, *i.e.*, e-capherin, growth factor or receptor.

3. SUMMARY OF THE INVENTION

The present invention relates to monoclonal antibodies, antibody fragments, and antibody conjugates that are highly selective for squamous cell carcinomas. More specifically, the novel antibodies, antibody fragments and antibody conjugates of the invention are those that bind to a cell membrane antigen found on squamous cell carcinomas but show no or limited reactivity with normal cells or other types of cancers. The invention also relates to hybridoma cell lines that produce monoclonal antibodies that are highly selective for squamous cell carcinomas.

In yet another embodiment of the invention, the antibodies of the invention may be used for *in vitro* or in *vivo* diagnostic and prognostic methods designed to detect squamous cell carcinoma. For instance, the antibody may be used in methods designed to detect the presence of a malignant condition in human lung, cervical or other tissue. The tissue may be contacted with an antibody of the invention which is capable of distinguishing squamous cell carcinoma cells from other cell types which may be present in the sample. Contact is carried out under conditions that allow for binding of the antibody to such cells followed by detecting the presence or absence of binding of the antibody to the cells of the specimen. Additional diagnostic methods include the *in vivo* localization of a tumor by administering to a patient a purified antibody or antibody fragment of the present invention labeled with an agent which gives a detectable signal. The localization of the tumor is then detected using external scintography, emission tomography or radionuclear scanning.

The invention further relates to the use of the antibodies of the invention in therapeutic applications, for example, to react with targeted tumor cells.

For example, novel antibody conjugates that act as target selective carriers of various agents which have antitumor effects including chemotherapeutic drugs, toxins, immunological response modifiers, enzymes and radioisotopes can be used.

Alternatively, the monoclonal antibodies may be used even in unmodified, *i.e.*, not in conjugated form, to treat subjects having squamous cell carcinoma. The antibodies of the present invention are particularly well suited for mediating antibody dependent cellular cytotoxicity (ADCC) which can results in targeted lysis of carcinoma cells in the presence of human lymphocytes, macrophages and complement.

The invention also comprises the antigens identified by the antibodies of the invention. Further encompassed are methods for using the purified or cloned antigens defined by the antibodies as vaccines to immunize against certain squamous cell carcinomas.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Western Blot of Lung Lavage with Monoclonal Antibodies 5C6, AD6, AD7, and AH1.

Figure 2. ADCC With Squamous Cell Carcinoma Specific Antibodies AH1 and AD7 and control antibody UPC-10.

Figure 3A. ADCC With Squamous Cell Carcinoma Specific Antibodies AH1, AD6, AD7 , 5C6 and control antibody UPC-10 against human squamous cell carcinoma cell line H596. Figure 3B. ADCC With Squamous Cell Carcinoma Specific Antibodies AH1, AD6, AD7 , 5C6 and control antibody UPC-10 against human colon carcinoma cell line LS174T.

Figure 4A-F. Immunohistology Using Squamous Cell Carcinoma Specific Antibodies AH6, AD7, and 5C6. Figure 4A. Normal Lung Tissue probed with AD6. Figure 4B. Normal Lung Tissue probed with AD7. Figure 4C. Squamous Cell Carcinoma Lung Lavage probed with AD6. Figure 4D. Squamous Cell Carcinoma Lung Lavage probed with 5C6. Figure 4E. Squamous Cell Carcinoma probed with 5C6. Figure 4F. Squamous Cell Carcinoma probed with AD6.

Figure 5. Direct ELISA assay with 5C6 and AD6 monoclonal antibodies using lung lavage sample.

Figure 6. Direct ELISA assay with 5C6, AD6, AH1 and AD7 monoclonal antibodies using lung lavage sample.

Figure 7A. Pap Smear using 5C6 and AD7 monoclonal antibodies. Slide represents dysplasia but indicates the presence of cell expressing carcinoma specific antigens.

Figure 7B. *In situ* immunoperoxidase staining of cervical carcinoma cells using C56 (top) and AD7 (bottom).

Figure 8. Cell flow cytometry. LS174T colon carcinoma cells, H596, H441 and CaLu3 lung carcinoma cells were stained with AD6 or AD7 antibodies.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel monoclonal antibodies that are highly specific for squamous cell carcinomas. More specifically, the antibodies react with squamous cell carcinoma of the lung and cervix, while showing none or limited reactivity with normal tissue. Such antibodies include those designated AD6, 5C6, AD7, and AH1 . All four antibodies were reactive to lung cancer antigen and to human lung cancer cell lines when assayed using ELIZA and immunofluorescent assays. In addition, the antibodies exhibit strong ADCC and immunohistochemical activity.

5.1 SQUAMOUS CELL CARCINOMA SPECIFIC ANTIBODIES

The present invention relates to novel antibodies that are highly specific for squamous cell carcinoma cells of the lung and cervix while showing none or limited reactivity with normal human tissue. The novel antibodies of the invention are designated AD6, 5C6, AD7, and AH1. The designated antibodies can be used to isolate and characterize the antigen to which they bind. Thus, the antibodies can be used to identify, isolate and/or characterize the immunogenic cell surface glycoproteins to which they react.

The term "AD6 antibody" as used herein includes whole, intact polyclonal and monoclonal antibodies such as the murine AD6 monoclonal antibody produced by hybridoma ATCC No. PTA-2460, and chimeric antibody molecules capable of binding to the same antigenic determinant as the AD6 antibody. The AD6 antibody described above includes fragments thereof containing the active antigen-binding region of the antibody, including Fab, F(ab'), and Fv fragments. The AD6 antibody of the invention also includes fusion proteins.

The term "5C6 antibody" as used herein includes whole, intact polyclonal and monoclonal antibodies such as the murine 5C6 monoclonal antibody produced by hybridoma ATCC No. PTA-2458, and chimeric antibody molecules capable of binding to the same antigenic determinant as the 5C6 antibody. The 5C6 antibody described above includes fragments thereof containing the active antigen-binding region of the antibody, including Fab, F(ab'), and Fv fragments. The 5C6 antibody of the invention also includes fusion proteins.

The term "AD7 antibody" as used herein includes whole, intact polyclonal and monoclonal antibodies such as the murine AD7 monoclonal antibody produced by hybridoma ATCC No. PTA-2459, and chimeric antibody molecules capable of binding to the same antigenic determinant as the AD7 antibody. The AD7 antibody described above includes fragments thereof containing the active antigen-binding region of the antibody, including Fab, F(ab'), and Fv fragments. The AD7 antibody of the invention also includes fusion proteins.

The term "AH1 antibody" as used herein includes whole, intact polyclonal and monoclonal antibodies such as the murine AH1 monoclonal antibody produced by hybridoma ATCC No. PTA-2457, and chimeric antibody molecules capable of binding to the same antigenic determinant as the AH1 antibody. The AH1 antibody described above includes fragments thereof containing the active antigen-binding region of the antibody, including Fab, F(ab'), and Fv fragments. The AH1 antibody of the invention also includes fusion proteins.

The present invention further encompasses the hybridoma cell lines capable of producing antibodies specific for squamous cell carcinoma cell surface

antigens. Such hybridoma cell lines include but are not limited to hybridoma ATCC No. PTA-2457, hybridoma ATCC No. PTA-2459, hybridoma ATCC No. PTA-2458, and hybridoma ATCC No. PTA-2460.

Murine hybridomas which produce mAB specific for squamous cell carcinoma cell surface antigens, such as the AD6, 5C6, AD7 and AH1 antibodies of the present invention, are formed by the fusion of a mouse fusion partner cell, such as SP2/0 and spleen cells isolated from mice immunized with squamous cell carcinoma cell surface antigens. Mice may be immunized with crude or semi-purified preparations containing the antigens of interest. To immunize mice, a variety of different conventional protocols may be followed. For example, mice may receive primary and boosting immunizations of antigenic preparations.

The monoclonal antibodies of the invention may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* **256**:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* **4**:72; Cole et al., 1983, *Proc. Natl. Acad. Sci.* USA **80**:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclasses thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titres of Mabs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used (Morrison et al., 1984, *Proc. Nat'l. Acad. Sci.*, **81**:6851-6855; Neuberger et al., 1984, *Nature*, **312**: 604-608; Takeda et al. 1985, Nature **314**: 452-454). Chimeric antibodies may be produced using a two-step homologous recombination procedure such as that described in Fell et al., (1989, *Proc. Nat'l. Acad. Sci.*, **86**:8507-8511). Alternatively, techniques developed for the

production of humanized antibodies (U.S. Patent No. 5,585,089) or single chain antibodies (U.S. Patent No. 4,946,778 Bird, 1988, Science **242**: 423-426; Huston et al., 1988, *Proc. Nat'l. Acad. Sci USA*, **85**: 5879-5883; and Ward et al., 1989, *Nature* **334**: 544-546) may be used to produce antibodies that specifically recognize squamous carcinoma.

The monoclonal antibodies of the invention may be produced in large quantities by injecting hybridoma cells secreting the antibody into the peritoneal cavity of mice and, after an appropriate time, harvesting the ascites fluid which contains a high titre of the monoclonal antibody and isolating the antibody therefrom. The monoclonal antibodies may be produced by culturing hybridoma cells *in vitro* and isolating the secreted mAB from the cell culture medium (See, Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc.). Alternatively, the gene for the antibody can be placed into a viral vector and used as a form of gene therapy so that the patient receiving the antibody gene can produce therapeutic antibody through his own immune system.

In addition, antibody fragments including fragments thereof containing the active antigen-binding region of the antibody may be prepared using techniques well established in the art (see, for example, 1986, Methods in Enzymology 121:663-69(Academic Press)).

Also included within the scope of the invention are anti-idiotypic antibodies to the AD6, 5C6, AD7 and AH1 antibodies of the invention. The anti-idiotypic antibodies can be produced using the AD6, 5C6, AD7 and AH1 antibodies and/or fragments thereof as immunogens. Such anti-idiotypic antibodies are useful as diagnostic reagents for detecting a humoral response to tumors and in therapeutic applications such as in a vaccine, to induce an anti-tumor response in subjects with squamous cell carcinomas.

Chimeric antibodies having the same binding specificity as the AD6, 5C6, AD7 and/or AH1 antibodies and combined with a cytotoxic agent are encompassed by the present invention. Such immunotoxins can be generated using genetic engineering techniques known in the art to produce recombinant DNA

molecules capable of encoding a fusion protein containing the antigen binding region of the AD6, 5C6, AD7 or AH1 monoclonal antibody fused to a cytotoxic agent such as, for example, diptheria toxin. In addition, humanized or completely human antibodies can be produced using recombinant engineering techniques resulting in production of antibodies that induce less of an anti-mouse response.

The present invention encompasses novel antibody conjugates that act as target selective carriers of various agents which have anti-tumor effects including chemotherapeutic drugs, toxins, immunological response modifiers, enzymes and radioisotopes can be used. Such agents may be conjugated to the monoclonal antibodies of the invention for use in targeting the agent to the surface of squamous cell carcinoma cells. Such cytotoxic agents include for example vinca alkaloids, ricin, taxol, doxorubicin, methotrexate, mitomycin C, and cytochalasin B to name a few. Immunological response modifiers may also be provided in the form of fusion proteins and radioisotopes may also be delivered while conjugated to single chain Fv chain constructs.

To generate the specific antibodies of the present invention, immunogenic glycoproteins derived from squamous cell carcinomas of the lung were isolated. The antigen was prepared from pooled allogeneic material sampled at the time of surgery and fractionated by Sephadex G-200 chromatography. Balb/c mice were immunized with the partially purified soluble membrane antigens of the human lung carcinoma cells and, after a sufficient time, the mice were sacrificed and somatic antibody producing lymphocytes, *e.g.*, spleen cells, were obtained and fused with the murine myeloma cell line SP2/0-Ag14.

Following fusion, the resulting cells were allowed to grow in selective medium, such as HAT-medium, and the surviving cells are grown in such medium using limiting dilution conditions and the supernatant was screened for monoclonal antibodies having the desired specificity. Various conventional methods exist for isolation and purification of the monoclonal antibodies, so as to free them from other proteins and other contaminants.

Four hybrid clones were found to produce antibodies with the desired specificity. These antibodies were designated as AD6, 5C6, AD7, and AH1. All four monoclonal antibodies were found to reactive to lung cancer antigen and to human lung cancer cell lines as assayed by ELISA and immunofluorescent assays. The antibodies did not react with normal tissue, bone marrow or tumor cell lines of other histological types. In addition, western blot analysis was performed using protein extracts derived from lung carcinoma cell lines P3 and P6 and partially purified lung cancer membrane antigens. Antigens of various molecular weight were detected using the four different antibodies indicating that each of the monoclonal antibodies recognized a distinct antigen expressed on the cell membrane

These antibodies appear to identity biomarkers on an array of squamous cell cancers including lung, esophagus and cervix cancer. We have demonstrated that the antibodies, i.e., 5C6 and AD7 are far more sensitive in cervical squamous malignancies than commercially available antibodies. In addition, if the pap smear is performed using thin prep technology so that cells are placed into fixative, the cytospin can be used as a source for measuring and defining a tumor antigen.

5.2. DIAGNOSTIC ASSAYS FOR DETECTION OF SQUAMOUS CELL CARCINOMA SPECIFIC ANTIGENS

In accordance with the invention, the squamous cell carcinoma specific monoclonal antibodies of the present invention can be used for the early diagnosis of diseases such as lung, esophagus and cervical carcinoma. Moreover, the monitoring and quantitation of antigen levels can be used prognostically to stage the progression of the disease and to evaluate the efficacy of agents used to treat a cancer subject. Antigen levels can be monitored from cytospins by direct ELISA or in serum with a capture assay.

The detection of squamous cell carcinoma specific antigens in a sample from a subject can be accomplished by any of a number of methods. Preferred diagnostic methods for the detection of squamous cell carcinoma specific antigens in

the biological sample of a subject can involve, for example, immunoassays wherein the antigens are detected by their interaction with the specific antibodies of the invention. Antibodies useful in the present invention can be used to quantitatively or qualitatively detect the presence of squamous cell carcinoma specific antigens or fragments thereof. For example, the monoclonal antibodies of the invention can be used, for example, to detect carcinoma cells in histological and cytological specimens.

For instance, using an immunoperoxidase staining technique tissue specimens can be analyzed for positive staining (Garrigues et al., 1982, Int. J.Cancer 29:511). Immunoassays useful in the practice of the invention include but are not limited to assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

A biological sample which may contain squamous cell carcinoma specific antigens proteins, such as lung tissue or other biological tissue, is obtained from a subject suspected of having a particular cancer or risk for cancer. Aliquots of whole tissues, or cells, are solubilized using any one of a variety of solubilization cocktails known to those skilled in the art. For example, tissue can be solubilized by addition of lysis buffer comprising (per liter) 8 M urea, 20 ml of Nonidet P-40 surfactant, 20 ml of ampholytes (pH 3.5-10), 20 ml of 2-mecaptoethanol, and 0.2 mM of phenylmethylsulfonyl fluoride (PMSF) in distilled deionized water.

Immunoassays for detecting expression of squamous cell carcinoma specific antigens typically comprise contacting the biological sample, such as a tissue sample derived from a subject, with the monoclonal antibodies of the invention, i.e., AD6, 5C6, AD7, and AH1, under conditions such that an immunospecific antigen-antibody binding reaction can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, for example, can be used to detect the presence and/or increased production

A biological sample which may contain squamous cell carcinoma specific antigens proteins, such as lung tissue or other biological tissue, is obtained from a subject suspected of having a particular cancer or risk for cancer. Aliquots of whole tissues, or cells, are solubilized using any one of a variety of solubilization cocktails known to those skilled in the art. For example, tissue can be solubilized by addition of lysis buffer comprising (per liter) 8 M urea, 20 ml of Nonidet P-40 surfactant, 20 ml of ampholytes (pH 3.5-10), 20 ml of 2-mecaptoethanol, and 0.2 mM of phenylmethylsulfonyl fluoride (PMSF) in distilled deionized water.

Immunoassays for detecting expression of squamous cell carcinoma specific antigens typically comprise contacting the biological sample, such as a tissue sample derived from a subject, with the monoclonal antibodies of the invention, i.e., AD6, 5C6, AD7, and AH1, under conditions such that an immunospecific antigen-antibody binding reaction can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, for example, can be used to detect the presence and/or increased production of squamous cell carcinoma specific antigens wherein the detection or increased production of squamous cell carcinoma specific antigens is an indication of a diseased condition. The levels of squamous cell carcinoma specific antigens in a biological sample are compared to norms established for age and gender-matched normal individuals and for subjects with a variety of non-cancerous or pre-cancerous disease states.

In an embodiment of the invention, the biological sample, such as a tissue extract is brought in contact with a solid phase support or carrier, such as nitrocellulose, for the purpose of immobilizing any proteins present in the sample. The support is then washed with suitable buffers followed by treatment with detectably labeled monoclonal antibodies such as AD6, 5C6, AD7, and/or AH1. The solid phase support is then washed with the buffer a second time to remove unbound antibody. The amount of bound antibody on the solid support is then determined according to well known methods.

antibodies or antibody fragments, it is possible to detect squamous cell carcinoma specific antigen expression through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., *Principles of Radioimmunoassays*, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March 1986). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

The monoclonal antibodies may also be labeled with a fluorescent compound. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin and fluorescamine. Likewise, a bioluminescent compound may be used to label the monoclonal antibodies. The presence of a bioluminescence protein is determined by detecting the presence of luminescence. Important bioluminescence compounds for purposes of labeling are luciferin, luciferase and aequorin.

The antibodies of the invention can also be employed for *in vivo* diagnostic applications. For example, antibodies or fragments prepared from antibodies, can be used to image tumors, including metastatic tumors in human patients. The purified antibody or fragments thereof are labeled with an agent capable of giving a detectable signal and administered in a suitable carrier, for example, intravenously, to a patient. The localization of the tumor-bound antibody is detected by external scintography, emission tomography or radionuclear scanning, using for example, a gamma camera.

5.3. KITS

The present invention further provides for kits for carrying out the above-described assays. The assays described herein can be performed, for example, by utilizing pre-packaged diagnostic kits, comprising at an antibody reagent (for detection of squamous cell carcinoma specific antigens), which can be conveniently used, *e.g.*, in clinical settings to diagnose disorders such as cancer. Such antibody reagents include the monoclonal antibodies AD6, 5C6, AD7, and AH1.

In a nonlimiting embodiment, a kit according to the invention may comprise components which detect and/or measure squamous cell carcinoma specific antigens in the biological sample of a subject. For example, where squamous cell carcinoma specific antigens are detected and/or measured by enzyme linked immunoabsorbent assay (ELISA), such components may comprise an antibody directed to epitopes of the squamous cell carcinoma specific antigens which can be used to detect and/or quantitate the level of squamous cell carcinoma specific antigens expression in the biological sample. The antibody itself may be detectably labeled with a radioactive, flourescent, colorimetric or enzyme label. Such antibodies include the monoclonal antibodies AD6, 5C6, AD7, and AH1. Alternatively, the kit may contain a labeled secondary antibody.

5.4 THERAPEUTIC USES OF MONOCLONAL ANTIBODIES

The antibodies of the present invention may be used therapeutically in a variety of different ways. The monoclonal antibodies may be used in unmodified, *i.e.*, non-conjugated form, to treat subjects having squamous cell carcinoma. For example, the antibodies may be used to direct complement (CDC) or effector cell (ADCC) mediated cytotoxicity. Alternatively, the antibodies may be conjugated to anti-tumor drugs, toxins or radionuclides. Conjugated antibodies can be administered to patients to achieve enhanced tumoricidal effects through the cytotoxic action of the chemotherapeutic agent delivered to the tumor based on the binding affinity of the antibody moiety.

Chimeric antibody molecules of the present invention may be prepared containing a mouse antigen-binding domain with human constant region domains (Morrison et al., 1984, *Proc. Natl Acad. Sci.* U.S.A. 81:6851; Takeda et al., 1985, *Nature*, 314:452) and this approach may be used to generate novel antibody molecules with desirable effector functions such as the ability to activate human complement and mediate ADCC.

The present invention relates to pharmaceutical compositions comprising the antibodies of the present invention. Amounts and regimens for the

administration of antibodies, their fragments or derivatives can be determined readily by those with ordinary skill in the clinical art of treating cancer. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient , frequency of treatment and the nature of the effect desired.

Compositions within the scope of the invention include all compositions wherein the antibody, fragment or derivative is contained in an amount effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. The effective dose is a function of the individual chimeric or monoclonal antibody, the presence and nature of a conjugated therapeutic agent, the patient and his clinical status and can vary from about 10 ng/kg body weight to about 100 mg/kg body weight. The preferred dosages comprise 0.1 to 10 mg/kg body weight. Preparations of the antibody, fragment or derivative of the present invention for parental administration, such as in detectably labeled form for imaging or in a free or conjugated form for therapy, include sterile lyophylized protein, aqueous or non-aqueous solutions, suspensions and emulsions. Examples of non-aqueous solvents are propyleneglygol, polyethyleneglycol, vegetable oil such as olive oil, and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media, parenteral vehicles including sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, such as those based on Ringer's dextrose and the like. Preservatives and other additives may also be present, such as, for example, anti-microbials, antioxidants, chelating agents, and inert gases and the like. See, generally, *Remington's Pharmaceutical Science*, 16th ed., Mack Publishing Co., Easton, PA, 1980.

In particular, the antibodies, fragments and derivatives thereof are useful for treating a subject having a squamous cell carcinoma. Such treatment comprises parenterally administering a single or multiple doses of the antibody, fragment or derivative, or conjugate thereof.

The novel antigens of the invention to which the antibodies AD6, 5C6, AD7 and AH1 bind may also be used for therapeutic applications. The antigens can be purified from tumors and administered, alone as an immunogen, or together with a proper immunological adjuvant. Alternatively, the antigens may be recombinantly produced for use as an immunogen.

6. EXAMPLE: PRODUCTION OF SQUAMOUS CELL CARCINOMASPECIFIC MONOCLONAL ANTIBODIES

The subsection below describes the generation of monoclonal antibodies reactive against specific glycoproteins expressed on the surface of squamous cell carcinoma. Immunological assays indicate that the monoclonal antibodies react with carcinoma cells, while showing none or limited reactivity with normal tissue. In addition AH1 and AD7.

6.1 PREPARATION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies (mAb) against human squamous cell lung carcinoma associated antigens (SLAA) were produced using a modified method described by Herzenberg et al. Four weeks old Balb/c mice were immunized by intra peritoneal (ip) injection with 100 μg of partially purified SLAA obtained from the membrane extracts from pooled human squamous cell lung carcinomas removed from patients at the time of surgery, emulsified with 200 μl of complete Freund's adjuvant. Two ip injections emulsified in 200 μl of incomplete Freund's adjuvant were given at 2 week intervals. Mice were then boosted intravenously with 20 μg of the immunogen and the splenocytes were removed 3 days later. Somatic hybrids were prepared using the mouse non-secreting myeloma cell line SP2/0-Ag14 following the procedue of Muraro et al. Hybridoma supernatents were assayed for specific antibody

production in ELISA using several human squamous cell lung carcinoma cell lines (P3, P6 and H595) and partially purified immunogen. All hybridoma cell lines were cloned twice by limited dilution.

For ascities fluids production, four week old Balb/c mice were pristane-primed and then inoculated with approximately 5 x 106 hybridoma cells. mAb was purified from murine ascite fluids by protein G affinity chromatography. The protein content of purified antibody preparation was analyzed on a 12 % SDS-polyacrylamide Tris-glycine gel.

6.2 CHARACTERIZATION OF MONOCLONAL ANTIBODIES

6.2.1. WESTERN BLOTS

Western Blots were performed using the 5C6, AD6, AD7 and Ah1 antibodies. The protein samples were derived from lung lavage specimens. Samples (3 μg) were loaded onto 12% SDS PAGE. Proteins were transferred to nitrocellulose membranes and stained using antibodies labeled with horseradish peroxidase. As indicated in Figure 1, the monoclonal antibodies of the invention each reacted against different antigens expressed within squamous cell carcinomas derived from lung lavage specimens.

6.2.2. ADCC WITH SQUAMOUS CELL CARCINOMA SPECIFIC ANTIBODIES

A four hour ^{51}Cr release assay was used to measure ADCC activity. Target cells were the lung squamous CA cell line H596. Target cells were labeled with 200 μCi sodium [^{51}Cr] chromate (250-500mCi/mg, Amersham, Arlington, IL) in 200 μl fetal calf serum for 1 hour. Target cells ($1x10^4$)were incubated in 96 U-bottom wells of assay plates with effector cells in a ratio of effector to target cells of 80:1 in the presence of mAb (1.0μg/well). The plates were incubated fro 4 hrs at 37 °C in a humidified atmosphere containing 5% CO2. Supernatant was harvested for gamma counting with the use of Skatron Harvester frames. Specific lysis was calculated with the use of the following formula:

$$\text{Specific Lysis (\%)} = \frac{\text{Observed release (cpm) - spontaneous release (cpm)}}{\text{Total release (cpm)- spontaneous release (cpm)}} \times 100$$

Spontaneous release was determined by measuring the radioactivity released from target cells incubated in medium alone. Total releasable radioactivity was obtained after treatment with 2.5% Triton X-100. Spontaneous release of radio-labeled chromium was measured after treatment with 2.5 % Triton X-100.

As indicated in Figure 2 and Figure 3 the AH-1, D6 and 5C6 monoclonal antibodies demonstrated ADCC activity when tested against human squamous cell lung carcinoma cells. As presented in Figure 3B, the ADCC activity was specific for human squamous cell lung carcinoma cells as indicated by the lack of activity against human colon carcinoma cells.

6.2.3. IMMUNOHISTOCHEMISTRY USING SQUAMOUS CELL CARCINOMA SPECIFIC ANTIBODIES

Staining protocols were as follows:

Mayo Frozen Tissue Protocol

Slides were fixed in 1% paraformaldehyde for 10 minutes followed by rinsing 3X in PBS. A blocking step was carried out with endogenous peroxidase (1% sodium azide +3%H_2O_2 and rinsed in water for 1 minute. 5% NGS was added for 10 minutes followed by a addition of primary antibody diluted in 1% NGS (2μg/ml) and incubation for two hours at RT. Slides were rinsed 2-3X PBS and incubated for 15 minutes at RT with Biotin RAM (DAKO). The slides were rinsed 2-3X with PBS. Streptavidin(HRP) was added to the slides and incubated for 15 minutes at RT. The slides were rinsed twice with PBS and once with water. DAB or AEC.

Paraffin Section Protocol

Slides were placed at 60 degrees for 20 minutes. Deparaffinizing was done in the hood: (i) Xylene, 5min; (ii) Xylene & 1% Iodine, 5 min: Xylene, 10 dips; (iii) 100% alcohol;(iv) 95% alcohol; and (v) 50% alcohol. Slides were placed in 50% methanol/50% 3%H_2O_2 for 10 min. and rinsed in water for 1 min. 5% NGS was added for 10 minutes followed by addition of primary antibody diluted in 1% NGS (1μg/ml) and incubation overnight at RT. Samples were rinsed in water and incubated with Biotin GAM (DAKO) for 30 min at RT. Slides were rinsed in water and incubated

with Streptavidin (HRP) for 30 minutes (DAKO). Samples were rinsed and water and DAB/AEC.

The four monoclonal antibodies of the invention were found to be reactive against antigens specifically expressed on the surface of squamous cell carcinomas of the lung (Figure 4) but not on the surface of normal cells. In addition, the antibodies were found to be immunoreactive against antigens expressed on the surface of cervical carcinoma cells (Figure 7A-B).

In addition ELISA assays were performed using the following protocol. Lung lavage was coated with dilution neat, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 100µl/well at 4° overnight. The wells were washed 3X with TBST. Blocking was carried out with TBST /2% gelatin (200 100µl/well at 37° for one hour followed by washing 5X with TBST. 100µl/well of ascites antibody was added and incubated for 1 hr at 37°. Anti-mouse IgG AP 1:1000 was added and incubated for 1 hr at 37°. The wells were washed 5X with TBST. Substrate P-NPP was added to each well and the endpoint was read at 405nm. As indicated in Figure 5 and 6 the 5C6, and AD6 monoclonal antibodies reacted with lung lavage tissue samples.

In addition, cell flow cytometry was performed to test the specificity of the antibodies. Cells (LS174T, H596, H441 and CaLu3) grown to log phase in culture medium free of phenyl red and removed from flasks with 0.025% trypsin EDTA (BioWhittaker). The cells were then washed with PBS (pH7.4) and ssuspended in culture medium for 30 minutes and counted. All subsequent procedures were performed at 4°C .

The cells were washed three times with PBS. A sample containing $5x10^5$ cells was suspended in 200µl of PBS and delivered to each reaction tube. A solution of 200µl of biotinylated antibody was added to the samples and the mixture was incubated at 4°C for 30 min. The cells were washed three times with PBS and suspended in 500µl of PBS. The cells are then analyzed by flow cytometry. As indicated in Figure 8, lung carcinoma cells (H596, H441 and CaLu3) stained with the antibodies while little staining was observed for the control colon carcinoma cells (LS174T).

6.3 VACCINE STUDIES

Membrane preparations were obtained from pooled allogeneic lung cancer cells , *i.e.*, squamous cell carcinomas. Membrane extracts were obtained from viable cells which were then subjected to low frequency sonication. The soluble material was separated on Sephadex G-200 and further subjected to discontinuous polyacrylamide gel electrophoresis. Individual bands isolated from the gels and tested for DHR (delayed cutaneous hypersensitivity) as described above were pooled and used as a vaccine. Skin testing for DHR employed 30μg antigen (TAA). Sephadex fraction I was eliminated from the vaccine because this fraction contained inhibitory material which suppressed the immune response. The vaccine for therapy utilized 300μg antigen in 0.2ml complete Freund Adjuvant. In no instance was there evidence of an autoimmune pulmonary response. In each patient an enhancement of cell mediated and humoral immunity was observed. DHR continued to enhance over 5 years . 80% of the patients studied (237 in study) survived after surgery and vaccination versus 40% for surgery alone.

6.4 CLONING AND DETERMINATION OF THE AD7 AND 5C6 HEAVY AND LIGHT CHAINS

The following primers are used for cloning and amplification of the AD7 and 5C6 heavy chain V-region:

(i) cDNA: MHCGSP1A:

5'-CAT GGA GTT AGT TTG GGC AGC AGA-3'

Abridged Anchor Primer;

5'-GGC CAC GCG TCG ACT AGT ACG GGIIGG GII GGG IIG-3'

(ii) amplification: MHCGSP2A:

5'-CAG GGG CCA GTG GAT AGA CAG ATG-3'

Abridged Anchor Primer;

5'-GGC CAC GCG TCG ACT AGT ACG GGIIGG GII GGG IIG-3'

The following primers are used for cloning and amplification of the AD7 and 5C6 light chain V-region:

(i) cDNA: MLCGSP1:

5'-CCT GTT GAA GCT CTT GAC AAT GGG-3'

Abridged Anchor Primer;

5'-GGC CAC GCG TCG ACT AGT ACG GGIIGG GII GGG IIG-3'

(ii) amplification: MOSKAPPA:

5'- ACT GGA TGG TGG GAA GAT GGA T-3'

Abridged Anchor Primer;

5'-GGC CAC GCG TCG ACT AGT ACG GGIIGG GII GGG IIG-3'

6.5. CONSTRUCTION AND EXPRESSION OF HUMAN-MOUSE CHIMERIC ANTIBODY GENES

Retroviral vectors pLHCXII and pLNCXII are used for the cDNA expression of the chimeric heavy chain and light chains respectively. pLNCXII is the vector LNCX except that an EcoRI site in the backbone of the vector is destroyed while another EcoRI site located 45 basepairs 5' to the neo gene is retained. LNCX was obtained from Dr. D. Miller (Fred Hutchinson Cancer Research Center, Seattle WA). The vector is derived from N2 vector by replacing the 5' long terminal repeats (LTR) of M-MuLV with M-MSV LTR and inserting a BalI/Xma III fragment containing the human cytomegalovirus (HCMV) immediate early promoter 3' to the neo gene. A polylinker containing HindIII, Hpa I and Cla I site is inserted 3' to the HCMV promotor for cloning of the target gene. The vector pLHCXII is made by replacing a - 1.2 KbEcoR1/BamH1 fragment carrying the neomycin resistant gene with a 1.4 kb BamH1/BamH1 fragment carrying a gene conferring hygromycin resistance. For generating the retroviral expression construct of pLNCXIIHuK, a DNA fragment carrying the cDNA encoding chimeric k chain (mAb) is obtained from its pBluescript construct by cleavage with Sma I/Cla I. The resulting fragment is cloned in Hpa I/ Cla I linearized pLNCXII vector. Similarly, the DNA fragment sequences encoding the chimeric heavy chain is obtained from the pBluescript construct except that the plasmid is cleaved with Xba I, the sticky ends will be filled in by Klenow, and the fragment is excised by subsequent cleavage with HindIII. The

fragment generated is cloned in HindIII/Hpa I linearized pLHCXII to generate pLHXII HuG1, the expression construct of chimeric mAb heavy chain.

For the development of chimeric mAb, the HC and LC expression constructs are sequentially introduced into SP2/O cells by electroporation, using the cell-porator system. Electroporation is carried out as follows. Briefly, SP2/O cells are washed in serum free DMEM with 4.5g/L glucose (JRH Bioscience, Lenexa, KS) and suspended in the same medium at a concentration of 5 x 10^6 cells/ml. One hundred μg of the plasmid is added to 1 ml of the cell suspension in a electroporating chamber at 4°C. The cells and DNA mixture are pulsed at 650V/cm for 13ms (capacitance setting at 1,600 μF). Cells are kept at 4° C for 10 min and diluted in RPMI1640 medium containing 15% FCS. Cells are then distributed in 24 well plates at a concentration 5 x 10^5 cell per well. After incubation at 37° C in 15% CO^2 incubator for 24 hour, selective medium containing hygromycin or active G418 at concentration of 500μg/ml and 800 μg/ml respectively is added.

Human mouse chimeric mAb (Hu mAb) producing clones are grown in protein free hybridoma medium PFHM-II (GIBCO,BRL) and are purified by protein G affinity chromatography. Protein concentration are determined using BioRad microassay procedure or by method of Lowry. The protein is analyzed on a pre-cast 4-20% SDS-polyacrylamide Tris-glycine gel (Novex System, San Diego, CA) with and without denaturation with 2-mercaptoethanol. The protein gel is visualized by staining with Coomassie Brilliant Blue R250 according to the method of Lamemmli.

The present invention is not to be limited in scope by the specific embodiments described herein which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the claims. Various publications are cited herein, the contents of which are hereby incorporated, by reference, in their entireties.

CLAIMS

1. A monoclonal antibody specific for a purified human carcinoma-associated protein antigen, wherein said antigen has the following characteristics:

a. said antigen is not detectable on a normal cancer free human tissue;

b. said antigen is specifically immunogenic in humans; and

c. said antigen induces an immune response in humans havingcolon carcinoma which is expressed as cell mediated immunity.

2. An antibody according to claim 1 which is mouse monoclonal antibody AD6 (ATCC PTA-2457) or an antibody which binds specifically to a carcinoma-associated epitope that specifically binds to monoclonal antibody AD6.

3. An antibody according to claim 1 which is mouse monoclonal antibody AD7 (ATCC PTA-2459) or an antibody which binds specifically to a carcinoma-associated epitope that specifically binds to monoclonal antibody AD7.

4. An antibody according to claim 1 which is mouse monoclonal antibody 5C6 (ATCC PTA-2458) or an antibody which binds specifically to a carcinoma-associated epitope that specifically binds to monoclonal antibody 5C6.

5. An antibody according to claim 1 which is mouse monoclonal antibody AH1 (ATCC PTA-2457) or an antibody which binds specifically to a carcinoma-associated epitope that specifically binds to monoclonal antibody AH1.

6. An antibody according to claim 1 immobilized on a solid phase.

7. An antibody according to claim 1 which is detectably labeled.

8. An antibody according to claim 7 wherein said detectable label is a radiolabel.

9. An antibody according to claim 1 conjugated to a cytotoxic radionuclide

10. An antibody according to claim 1 conjugated to a cytotoxic drug.

11. An antibody according to claim 1 conjugated to a cytotoxic protein.

12. A composition comprising an antibody according to claim 10 in combination with a pharmaceutical acceptable carrier.

13. A composition comprising an antibody according to claim 11 in combination with a pharmaceutically acceptable carrier.

14. A monoclonal antibody against the monoclonal antibody of claim.

15. An immunnoassay for detecting a carcinoma-associated antigen which binds to mouse monoclonal antibody AD6 (ATCC PTA-2460) in a sample comprising:

a. contacting said sample with an effective binding amount of the antibody; and

b. detecting said antigen by detecting the binding of the antibody to the purified carcinoma associated protein antigen.

16. An immunnoassay for detecting a carcinoma-associated antigen which binds to mouse monoclonal antibody AD7 (ATCC PTA-2459) in a sample comprising:

a. contacting said sample with an effective binding amount of the antibody; and

b. detecting said antigen by detecting the binding of the antibody to the purified carcinoma associated protein antigen.

17. An immunnoassay for detecting a carcinoma-associated antigen which binds to mouse monoclonal antibody AH1 (ATCC PTA-2457) in a sample comprising:

a. contacting said sample with an effective binding amount of the antibody; and

b. detecting said antigen by detecting the binding of the antibody to the purified carcinoma associated protein antigen.

18. An immunnoassay for detecting a carcinoma-associated antigen which binds to mouse monoclonal antibody 5C6 (ATCC PTA-2458) in a sample comprising:

a. contacting said sample with an effective binding amount of the antibody; and

b. detecting said antigen by detecting the binding of the antibody to the purified carcinoma associated protein antigen.

19. A method of diagnosing a carcinoma in a patient comprising:

a. removing a historical specimen from a patient suspected of having a carcinoma;

b. contracting the specimen with monoclonal antibody AD6 (ATCC PTA-2460);

c. staining the specimen with an immunohistochemical stain; and

d. detecting the presence of the antigen-antibody complex by the stain.

20. A method of diagnosing a carcinoma in a patient comprising:
a. removing a historical specimen from a patient suspected of having a carcinoma;
b. contracting the specimen with monoclonal antibody AD7 (ATCC PTA-2459);
c. staining the specimen with an immunohistochemical stain; and
d. detecting the presence of the antigen-antibody complex by the stain.

21. A method of diagnosing a carcinoma in a patient comprising:
a. removing a historical specimen from a patient suspected of having a carcinoma;
b. contracting the specimen with monoclonal antibody 5C6 (ATCC PTA-2458);
c. staining the specimen with an immunohistochemical stain; and
d. detecting the presence of the antigen-antibody complex by the stain.

22. A method of diagnosing a carcinoma in a patient comprising:
a. removing a historical specimen from a patient suspected of having a carcinoma;
b. contracting the specimen with monoclonal antibody AH1 (ATCC PTA-2457);
c. staining the specimen with an immunohistochemical stain; and

d. detecting the presence of the antigen-antibody complex by the stain.

23. A method of inducing an ADCC reaction in a subject having a carcinoma comprising administration of a composition comprising an AD6 monoclonal antibody.

24. A method of inducing an ADCC reaction in a subject having a carcinoma comprising administration of a composition comprising an AD7 monoclonal antibody.

25. A method of inducing an ADCC reaction in a subject having a carcinoma comprising administration of a composition comprising an 5C6 monoclonal antibody.

26. A method of inducing an ADCC reaction in a subject having a carcinoma comprising administration of a composition comprising an AH1 monoclonal antibody.

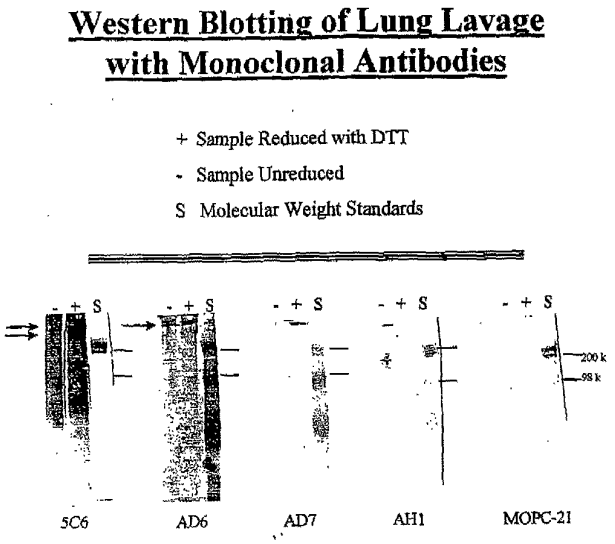

Western Blotting of Lung Lavage with Monoclonal Antibodies
+ Sample Reduced with DTT
- Sample Unreduced
S Molecular Weight Standards
- + S
200 k
98 k
5C6
AD6
AD7
AH1
MOPC-21

Figure 1

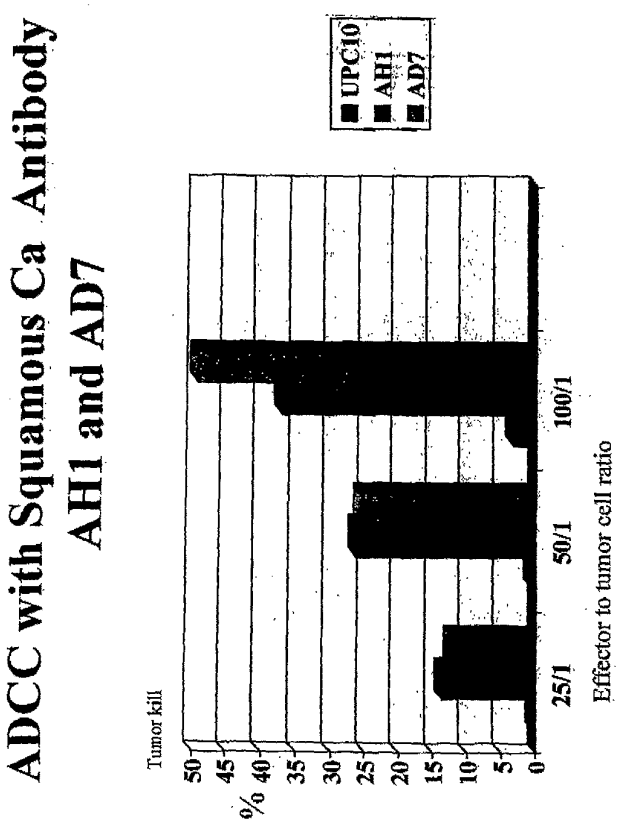
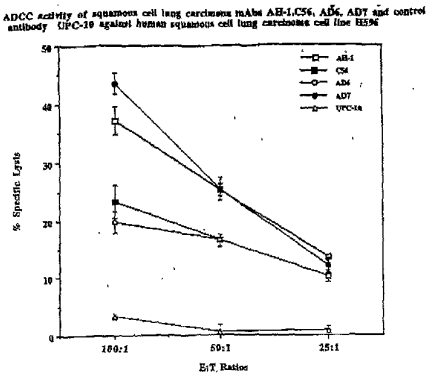

ADCC with Squamous Ca Antibody AH1 and AD7
UPC10
AH1
AD7
Tumor kill
% 50 45 40 35 30 25 20 15 10 5 0
25/1
50/1
100/1
Effector to tumor cell ratio

Figure 2


ADCC activity of squamous cell lung carcinoma mAbs AE-1,C56, AD6, AD7 and control antibody UPC-10 against human squamous cell lung carcinoma cell line H596
% Specific Lysis
0
10
20
30
40
50
100:1
50:1
25:1
E:T Ratios
AE-1
C56
AD6
AD7
UPC-10

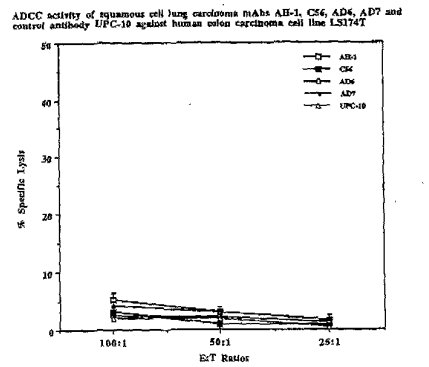
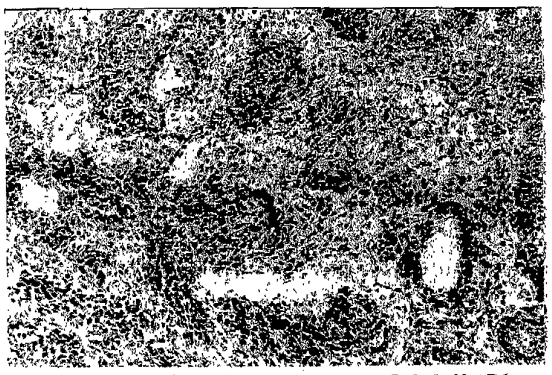

ADCC activity of squamous cell lung carcinoma mAbs AE-1, C56, AD6, AD7 and control antibody UPC-10 against human colon carcinoma cell line LS174T
% Specific Lysis
0
10
20
30
40
50
100:1
50:1
25:1
E:T Ratios
AE-1
C56
AD6
AD7
UPC-10

Figure 3

Normal Lung

A.



10 X Magnification Probed with AD6

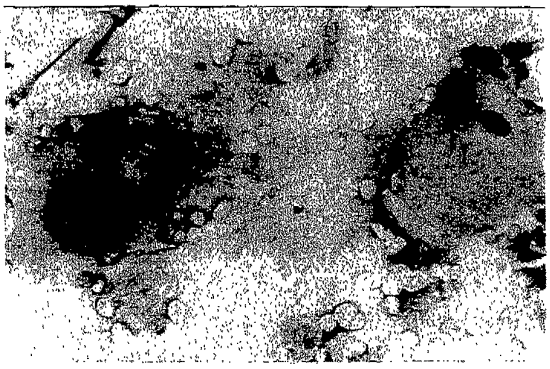
B.

4 X Magnification Probed with AD7

Figure 4A-B

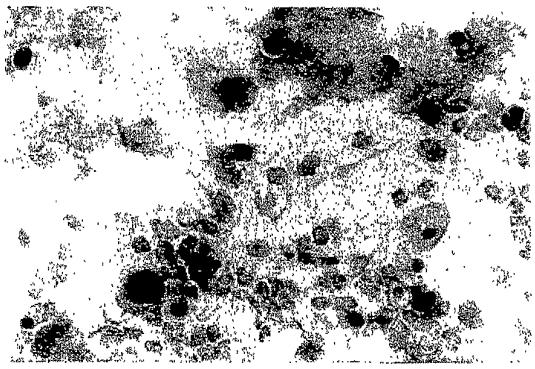
SCC Lung Lavage

C.



40 X Magnification Probed with AD6

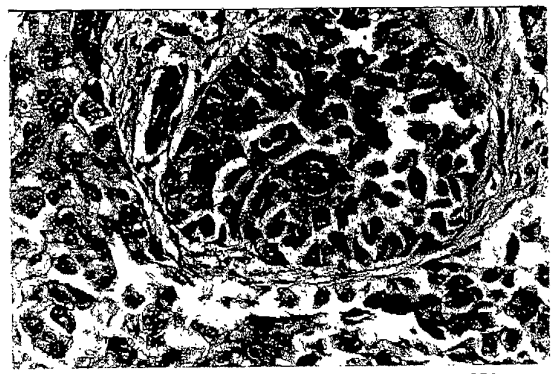
D.



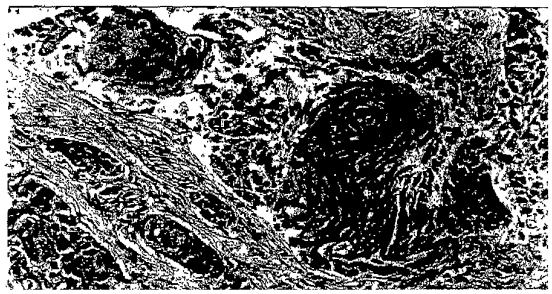
20 X Magnification Probed with 5C6

Figure 4 C-D

SCC Lung



20 X Magnification Probed with 5C6



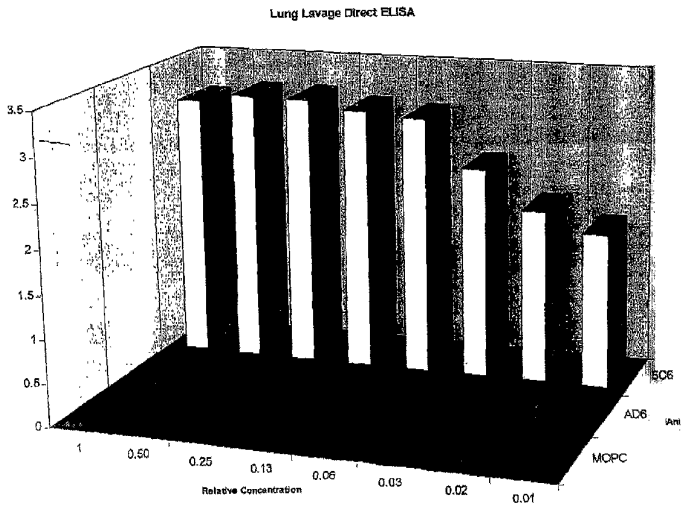

Lung Lavage Direct ELISA
3.5
3
2.5
2
1.5
1
0.5
0
1
0.50
0.25
0.13
0.06
0.03
0.02
0.01
Relative Concentration
SD6
AD6
MOPC

Figure 5

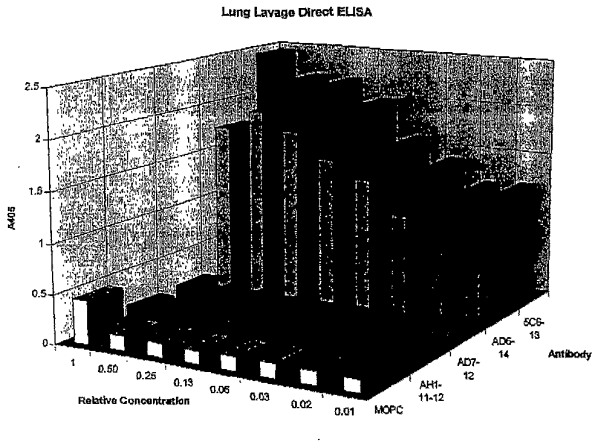

Lung Lavage Direct ELISA
A405
2.5
2
1.5
1
0.5
0
1
0.50
0.25
0.13
0.06
0.03
0.02
0.01
Relative Concentration
MOPC
AH1-11-12
AD7-12
AD6-14
5C6-13
Antibody

Figure 6

Pap Smear Technology utilizing immunohistochemistry with specific Squamous Cancer antibodies, 5C6 and AD7

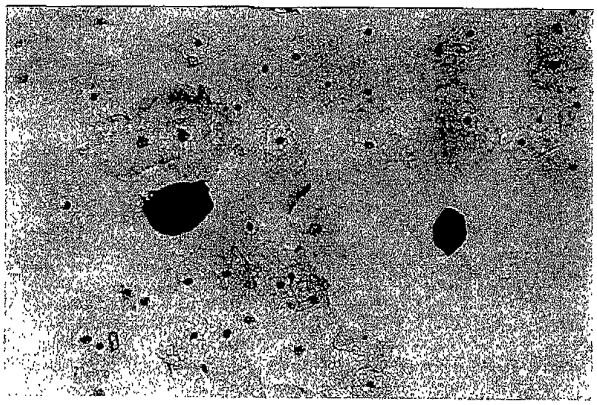
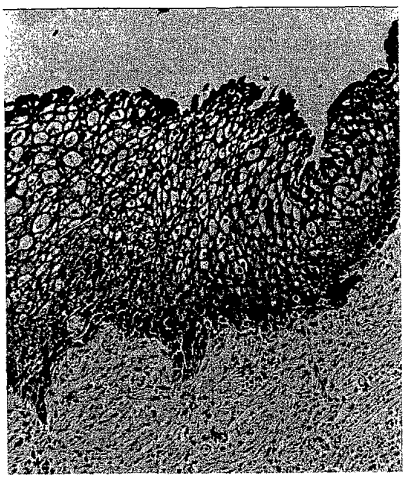


Figure 7A

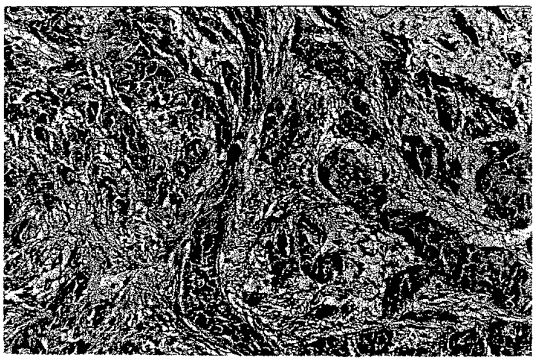
Squamous Carrcinoma of the Cervix

In situ-carcinoma
Immunoperoxidase
Using C56 ->



Invasive squamous carcinoma
of cervix staining with AD7

(bottom)



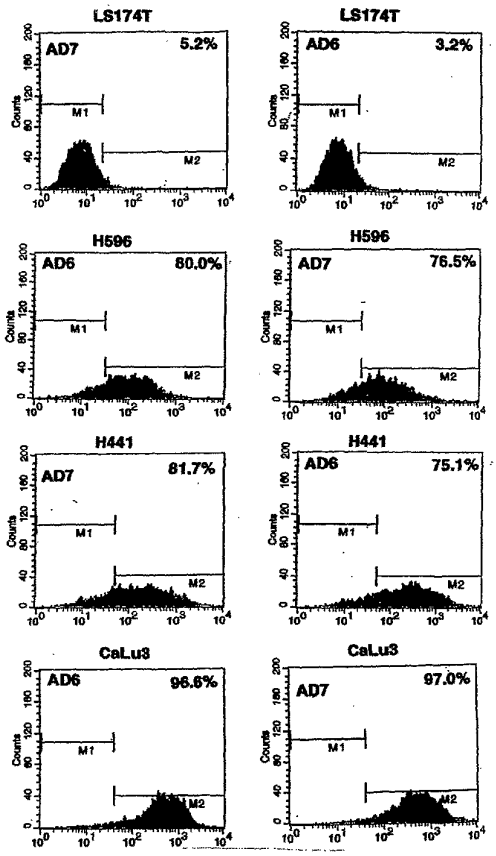

LS174T
AD7
5.2%
LS174T
AD6
3.2%
H596
AD6
80.0%
H596
AD7
76.5%
H441
AD7
81.7%
H441
AD6
75.1%
CaLu3
AD6
96.6%
CaLu3
AD7
97.0%
M1
M2
Counts

Figure 8

INTERNATIONAL SEARCH REPORT

I tional application No.
PCT/US01/26734

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07K 16/00; C12P 21/08
US CL :530/387.1, 387.3, 387.7, 388.2, 388.8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/387.1, 387.3, 387.7, 388.2, 388.8

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
ADISINSIGHT BIOSIS BIOTECHNO CABA CANCERLIT CAPLUS EMBASE ESBIOBASE LIFESCI MEDLINE PASCAL SCISEARCH USPATFULL
search terms:antibody, treat, specific, colon, SEA, SF25

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database USPATFULL, Accession Number 93:39911, (SF-25 Colon adenocarcinoma antigen, and antibodies with recognize this antigen. US 5,212,085 A, TAKAHASHI, 18 May 1993, see entire document.	1, 6-13

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

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 NOVEMBER 2001	27 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SUSAN UNGAR
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26734

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

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

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

2. ☒ Claims Nos.: 14
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:

The claim is drawn to a monoclonal antibody against the monoclonal antibody of claim. Since no claim is recited there is no way to determine which anti-idiotypic antibody should be searched.

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:

Please See Extra 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. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2 and 6–13

Remark on Protest

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

	ational application No.
	PCT/US01/26734

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-13, drawn to a monoclonal antibody specific for purified human carcinoma-associated protein antigen which antigen induces an immune response in humans having colon carcinoma which is expressed as cell mediated immunity.
Group II, claim(s) 15, drawn to an immunoassay with MaB AD6.
Group III, claim(s) 16, drawn to an immunoassay with MaB AD7.
Group IV, claim(s) 17, drawn to an immunoassay with MaB AH1.
Group V, claim(s) 18, drawn to an immunoassay with MaB 5C6.
Group VI, claim(s) 19, drawn to a method of diagnosing a carcinoma with Mab AD6.
Group VII, claim(s) 20, drawn to a method of diagnosing a carcinoma with Mab AD7.
Group VIII, claim(s) 21, drawn to a method of diagnosing a carcinoma with Mab 5C6.
Group IX, claim(s) 22, drawn to a method of diagnosing a carcinoma with Mab AH1.
Group X, claim(s)23, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab AD6.
Group XI, claim(s) 24, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab AD7.
Group XII, claim(s) 25, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab 5C6.
Group XIII, claim 26, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab AH1.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

The claims are deemed to correspond to the species listed above in the following manner:

The following claims are generic:

Claim 1 is generic to a plurality of patentably distinct species comprising monoclonal antibodies with different structures and functions and which bind to different epitopes wherein the species are (a)Mab AD6 (claim 2), (b) Mab AD7 (claim 3), (c) MAb 5C6 (claim 4), (d) Mab AH1 (claim 5).

The inventions listed as Groups I-XIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-XIII appears to be that they all relate to a monoclonal antibody specific for a purified human carcinoma-associated protein antigen wherein said antigen is not detectably on normal cancer free human tissue, is specifically immunogenic in humans and induces an immune response in humans having colon carcinoma which is expressed as cell mediated immunity.

However, US Patent No. 5,213,085 specifically teaches the SF-25 colon adenocarcinoma antigen which is constitutively expressed on colon adenocarcinomas in vivo, which is a cell surface antigen which is isolated(p. 7, Section I) and is not expressed on normal colon tissue (see para 8, p. 9). The reference teaches methods of purifying the antigen (p. 9, Section II), methods of producing the SF-25 antigen by recombinant technology (p. 11, Section IV) and methods of producing monoclonal antibodies to the antigen (para bridging pages 9 and 10)and teaches methods of using both the antibody and the antigen for the diagnosis and treatment of colon cancer (see abstract). The reference further teaches a method of using the antigen, or an immunologically active fragment thereof to elicit the production of antibodies capable of recognizing SF-25 expressing cells for the treatment of colon cancer (p. 19, Section VIII) and Mab SF-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26734

which is specific for and recognizes this antigen (p. 22, last paragraph). Although the reference does not teach that the monoclonal antibody produces a cell-mediated immunity, Sung et al (International J. Cancer, 1995, 61:864-872) specifically teach that SF-25 is capable of stimulating ADCC (see abstract).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to produce a monoclonal antibody, SF-25,against the SF-25 antigen because US Patent No. 5,212,085 specifically teaches that it is constitutively expressed on colon adenocarcinomas *in vivo* but not on normal cells and that the antibody is useful for treatment and diagnosis of colon adenocarcinoma. Further the antigen has been characterized as detectable on colon cancer cells but not on normal tissue. The antigen has been shown to be immunogenic in mice and it would be expected that, since the antigen is not expressed in normal human, that it would be seen as other and that it would be specifically immunogenic in normal humans. Further, given the appropriate carrier protein and adjuvant solutions, it would be expected that the antigen would produce an immune response in humans having colon carcinoma and that the immune response would be a cell mediated immunity, not only because Sung et al have demonstrated the efficacy of Mab SF-25 for ADCC but also because "cell mediated immunity" is not defined in the specification and the production of antibodies by B-cells is a cell mediated immunity. Although the reference does not specifically teach that the antigen produces an immune response in humans with colon cancer, the claimed antigen to which the antibody is specific appears to be the same as the prior art antigen, absent a showing of unobvious differences. The office does not have the facilities and resources to provide the factual evidence needed in order to establish that the product of the prior art does not possess the same material, structural and functional characteristics of the claimed product. In the absence of evidence to the contrary, the burden is on the applicant to prove that the claimed product is different from those taught by the prior art and to establish patentable differences.

Therefore, the technical feature linking the inventions of Groups I-XIII does not constitute a special technical feature as defined by Rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of Group I is considered to be a monoclonal antibody.

The special technical feature of Group II is considered to be an immunoassay.

The special technical feature of Group III is considered to be a different immunoassay.

The special technical feature of Group IV is considered to be a different immunoassay.

The special technical feature of Group V is considered to be a different immunoassay.

The special technical feature of Group VI is considered to be a method of diagnosing carcinoma.

The special technical feature of Group VII is considered to be a different method of diagnosing carcinoma.

The special technical feature of Group VIII is considered to be a different method of diagnosing carcinoma.

The special technical feature of Group IX is considered to be a different method of diagnosing carcinoma.

The special technical feature of Group X is considered to be a method of inducing ADCC.

The special technical feature of Group XI is considered to be a different method of inducing ADCC.

The special technical feature of Group XII is considered to be a different method of inducing ADCC.

The special technical feature of Group XIII is considered to be a different method of inducing ADCC.

Accordingly, Groups I-XIII are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

【国際調査報告】

INTERNATIONAL SEARCH REPORT

	1 ational application No. PCT/US01/28734

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07K 16/00; C12P 21/08
US CL :530/387.1, 387.3, 387.7, 388.8, 388.6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/387.1, 387.3, 387.7, 388.8, 388.6

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
ADISINSIGHT BIOSIS BIOTECHNO CABA CANCERLIT CAPLUS EMBASE ESBIOBASE LIFESCI MEDLINE PASCAL SCISEARCH USPATFULL
search terms:antibody, crent, specific, colon, SEA, SF25

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database USPATFULL, Accession Number 93:39911, (SF-25 Colon adenocarcinoma antigen, and antibodies with recognize this antigen. US 5,212,085 A, TAKAHASHI, 18 May 1993, see entire document.	1, 6-13

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

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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	"&"	document member of the same patent family

Date of the actual completion of the international search 08 NOVEMBER 2001	Date of mailing of the international search report 27 DEC 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUSAN UNGAR Telephone No. (703) 308-0196

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

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/26784

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

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

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

2. ☒ Claims Nos.: 14
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:

The claim is drawn to a monoclonal antibody against the monoclonal antibody of claim. Since no claim is recited there is no way to determine which anti-idiotypic antibody should be searched.

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:

Please See Extra 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. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2 and 6--13

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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

INTERNATIONAL SEARCH REPORT

tional application No.
PCT/US01/40734

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-12, drawn to a monoclonal antibody specific for purified human carcinoma-associated protein antigen which antigen induces an immune response in humans having colon carcinoma which is expressed as cell mediated immunity.
Group II, claim(s) 13, drawn to an immunoassay with MaB AD6.
Group III, claim(s) 14, drawn to an immunoassay with MaB AD7.
Group IV, claim(s) 17, drawn to an immunoassay with MaB AH1.
Group V, claim(s) 18, drawn to an immunoassay with MaB 5C8.
Group VI, claim(s) 19, drawn to a method of diagnosing a carcinoma with Mab AD6.
Group VII, claim(s) 20, drawn to a method of diagnosing a carcinoma with Mab AD7.
Group VIII, claim(s) 21, drawn to a method of diagnosing a carcinoma with Mab 5C8.
Group IX, claim(s) 22, drawn to a method of diagnosing a carcinoma with Mab AH1.
Group X, claim(s)23, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab AD6.
Group XI, claim(s) 24, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab AD7.
Group XII, claim(s) 25, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab 5C8.
Group XIII, claim 26, drawn to a method of inducing an ADCC reaction in a subject comprising administering Mab AH1.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

The claims are deemed to correspond to the species listed above in the following manner:

The following claims are generic:

Claim 1 is generic to a plurality of patentably distinct species comprising monoclonal antibodies with different structures and functions and which bind to different epitopes wherein the species are (a)Mab AD6 (claim 2), (b) Mab AD7 (claim 3), (c) MAb 5C8 (claim 4), (d) Mab AH1 (claim 5).

The inventions listed as Groups I-XIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-XIII appears to be that they all relate to a monoclonal antibody specific for a purified human carcinoma-associated protein antigen wherein said antigen is not detectably on normal cancer free human tissue, is specifically immunogenic in humans and induces an immune response in humans having colon carcinoma which is expressed as cell mediated immunity.

However, US Patent No. 5,212,085 specifically teaches the SF-25 colon adenocarcinoma antigen which is constitutively expressed on colon adenocarcinomas in vivo, which is a cell surface antigen which is isolated(p. 7, Section I) and is not expressed on normal colon tissue (see para 2, p. 9). The reference teaches methods of purifying the antigen (p. 9, Section II), methods of producing the SF-25 antigen by recombinant technology (p. 11, Section IV) and methods of producing monoclonal antibodies to the antigen (para bridging pages 9 and 10)and teaches methods of using both the antibody and the antigen for the diagnosis and treatment of colon cancer (see abstract). The reference further teaches a method of using the antigen, or an immunologically active fragment thereof to elicit the production of antibodies capable of recognizing SF-25 expressing cells for the treatment of colon cancer (p. 19, Section VIII) and Mab SF-25

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/20734

which is specific for and recognizes this antigen (p. 22, last paragraph). Although the reference does not teach that the monoclonal antibody produces a cell-mediated immunity, Sung et al (International J. Cancer, 1995, 61:864-872) specifically teach that SF-25 is capable of stimulating ADCC (see abstract).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to produce a monoclonal antibody, SF-25,against the SF-25 antigen because US Patent No. 5,815,985 specifically teaches that it is constitutively expressed on colon adenocarcinomas *in vivo* but not on normal cells and that the antibody is useful for treatment and diagnosis of colon adenocarcinoma. Further the antigen has been characterized as detectable on colon cancer cells but not on normal tissue. The antigen has been shown to be immunogenic in mice and it would be expected that, since the antigen is not expressed in normal human, that it would be seen as other and that it would be specifically immunogenic in normal humans. Further, given the appropriate carrier protein and adjuvant solutions, it would be expected that the antigen would produce an immune response in humans having colon carcinoma and that the immune response would be a cell mediated immunity, not only because Sung et al have demonstrated the efficacy of Mab SF-25 for ADCC but also because "cell mediated immunity" is not defined in the specification and the production of antibodies by B-cells is a cell mediated immunity. Although the reference does not specifically teach that the antigen produces an immune response in humans with colon cancer, the claimed antigen to which the antibody is specific appears to be the same as the prior art antigen, absent a showing of unobvious differences. The office does not have the facilities and resources to provide the factual evidence needed in order to establish that the product of the prior art does not possess the same material, structural and functional characteristics of the claimed product. In the absence of evidence to the contrary, the burden is on the applicant to prove that the claimed product is different from those taught by the prior art and to establish patentable differences.

Therefore, the technical feature linking the inventions of Groups I-XIII does not constitute a special technical feature as defined by Rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of Group I is considered to be a monoclonal antibody.

The special technical feature of Group II is considered to be an immunoassay.

The special technical feature of Group III is considered to be a different immunoassay.

The special technical feature of Group IV is considered to be a different immunoassay.

The special technical feature of Group V is considered to be a different immunoassay.

The special technical feature of Group VI is considered to be a method of diagnosing carcinoma.

The special technical feature of Group VII is considered to be a different method of diagnosing carcinoma.

The special technical feature of Group VIII is considered to be a different method of diagnosing carcinoma.

The special technical feature of Group IX is considered to be a different method of diagnosing carcinoma.

The special technical feature of Group X is considered to be a method of inducing ADCC.

The special technical feature of Group XI is considered to be a different method of inducing ADCC.

The special technical feature of Group XII is considered to be a different method of inducing ADCC.

The special technical feature of Group XIII is considered to be a different method of inducing ADCC.

Accordingly, Groups I-XIII are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

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

フロントページの続き

(51)Int.Cl.[7]	FI		テーマコード（参考）
Ａ６１Ｐ 1/00	Ａ６１Ｐ 1/00		
Ａ６１Ｐ 35/00	Ａ６１Ｐ 35/00		
Ａ６１Ｐ 37/04	Ａ６１Ｐ 37/04		
Ａ６１Ｐ 43/00	Ａ６１Ｐ 43/00	１２１	
Ｇ０１Ｎ 33/53	Ｇ０１Ｎ 33/53	Ｙ	
Ｇ０１Ｎ 33/574	Ｇ０１Ｎ 33/574	Ａ	
Ｇ０１Ｎ 33/577	Ｇ０１Ｎ 33/574	Ｄ	
// Ｃ０７Ｋ 19/00	Ｇ０１Ｎ 33/577	Ｂ	
Ｃ１２Ｐ 21/08	Ａ６１Ｋ 37/02		
	Ｃ０７Ｋ 19/00		
	Ｃ１２Ｐ 21/08		

(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZW

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摘要(译)

本发明涉及对鳞状细胞癌抗原包括肺癌，食道癌和子宫颈癌具有高度选择性的新型抗体，抗体片段和抗体缀合物。 本发明涉及通过使用本发明的新型抗体检测受试者的生物学样品中鳞状细胞癌抗原的表达来诊断或预后癌症的体内和体外临床筛选方法。 本发明进一步提供了用于进行上述筛选方法的试剂盒。 另外，抗体缀合物可用于将具有抗肿瘤作用的多种试剂有效地递送至肿瘤细胞。 本发明的抗体也可以非缀合形式施用于患者，以将ADCC靶向肿瘤细胞。

【図２】