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(54)【発明の名称】シュードモナスＶ抗原を用いる免疫化のための方法および組成物

(57)【要約】
シュードモナス・アエルギノーザ感染を抑制、調節、または診断する方法を開示する。１つの実施態様において、本法は、患者に有効量のＰｃｒＶ抗原を接種することを含む。

【特許請求の範囲】
【請求項１】
シュードモナス・アエルギノーザ感染を抑制する方法であって、患者に有効量のＰｃｒＶ抗原を接種することを含む方法。
【請求項２】
ＰｃｒＶ抗原がＰｃｒＶタンパク質の断片であり、該断片はＶ抗原に特異的な免疫応答を誘導できる、請求項１の方法。
【請求項３】
患者に、ＰｃｒＶをコード化するＤＮＡを含む遺伝子ワクチンを接種する、請求項１の方法。
【請求項４】
ＤＮＡがＰｃｒＶタンパク質の断片をコード化し、該断片はＰｃｒＶ抗原に特異的な免疫応答を誘導できる、請求項３の方法。
【請求項５】
患者がヒト患者である、請求項１の方法。
【請求項６】
シュードモナス・アエルギノーザ感染を診断する方法であって、患者のサンプルをヌクレオチドプローブにさらす段階を含み、ここで、該プローブはＰｃｒＶコード化核酸に特異的にハイブリッド化し、他の核酸にはハイブリッド化しない方法。
【請求項７】
患者がヒト患者である、請求項６の方法。
【請求項８】
シュードモナス・アエルギノーザ感染を診断する方法であって、
ａ）患者のサンプルを、ｐｃｒＶ遺伝子を増幅するように設計されたヌクレオチドプライマーにさらし、
ｂ）ポリメラーゼ連鎖反応を行い、ここで、ｐｃｒＶ遺伝子が存在すればサンプル中で増幅され、そして
ｃ）シュードモナス・アエルギノーザ感染を増幅産物の存在と相関させる
各段階を含む方法。
【請求項９】
患者がヒト患者である、請求項８の方法。
【請求項１０】
シュードモナス・アエルギノーザ感染を診断する方法であって、
ａ）患者のサンプルをＰｃｒＶ抗原にさらし、そして
ｂ）シュードモナス・アエルギノーザ感染をＰｃｒＶ特異的抗体／抗原複合体の存在と相関させる
各段階を含む方法。
【請求項１１】
シュードモナス・アエルギノーザ感染を抑制する方法であって、患者に有効量の遺伝子ワクチンを接種することを含み、ここで、該遺伝子ワクチンはＰｃｒＶ抗原をコード化する方法。
【請求項１２】
遺伝子ワクチンが全ＰｃｒＶタンパク質をコード化する、請求項１１の方法。
【請求項１３】
遺伝子ワクチンがＰＣＲ　ＰｃｒＶタンパク質の断片をコード化し、該断片はＰｃｒＶ抗原に特異的な免疫応答を誘導できる、請求項１１の方法。
【請求項１４】
患者がヒト患者である、請求項１１の方法。
【請求項１５】
シュードモナス・アエルギノーザ感染を治療または予防する方法であって、ヒト化または

ヒトＰｃｒＶ抗体または抗体断片を得て、そして、該抗体を全身投与する各段階を含み、ここで、該抗体はシュードモナス・アエルギノーザ感染を抑制または予防する方法。
【請求項１６】
シュードモナス・アエルギノーザ感染を治療または予防する方法であって、ヒト化またはヒトＰｃｒＶ抗体または抗体断片を得て、そして、抗体を治療剤として肺に投与する各段階を含む方法。
【請求項１７】
シュードモナス・アエルギノーザ感染を治療する方法であって、シュードモナス・アエルギノーザ感染患者に有効量のＰｃｒＶ抗原を接種する段階を含む方法。
【請求項１８】
ＰｃｒＶ抗原に対して特異的な抗体。
【請求項１９】
抗体がモノクローナルである、請求項１８の抗体。
【請求項２０】
抗体がｍａｂ　１６６である、請求項１９の抗体。
【請求項２１】
ＰｃｒＶポリペプチドアミノ酸配列におけるアミノ酸残基１４４および２５７を含むエピトープを認識する抗ＰｃｒＶモノクローナル抗体またはその断片。
【請求項２２】
ＰｃｒＶポリペプチドアミノ酸配列におけるアミノ酸残基１４４～２５７を含むエピトープを認識する抗ＰｃｒＶモノクローナル抗体またはその断片。
【請求項２３】
図７に示される軽鎖ポリペプチドアミノ酸配列のＣＤＲを含むモノクローナル抗体またはその断片。
【請求項２４】
図６Ｂに示される重鎖ポリペプチドアミノ酸配列のＣＤＲを含むモノクローナル抗体またはその断片。
【請求項２５】
図７に示される軽鎖ポリペプチドアミノ酸配列のＣＤＲと、図６Ｂに示される重鎖ポリペプチドアミノ酸配列のＣＤＲとを含むモノクローナル抗体またはその断片。
【請求項２６】
図７に示される軽鎖ポリペプチドアミノ酸配列のＦＲ領域をさらに含む、請求項２３および２５のいずれかのモノクローナル抗体または断片。
【請求項２７】
図６Ｂに示される重鎖ポリペプチドアミノ酸配列のＦＲ領域をさらに含む、請求項２４および２５のいずれかのモノクローナル抗体または断片。
【請求項２８】
図６Ｂに示される重鎖ポリペプチドアミノ酸配列のＦＲ領域と、図７に示される軽鎖ポリペプチドアミノ酸配列のＦＲ領域とをさらに含む、請求項２５のモノクローナル抗体または断片。
【請求項２９】
ヒト化されている、請求項２１～２８のいずれかの抗体または断片。
【請求項３０】
ヒト型である、請求項２１～２８のいずれかの抗体または断片。
【請求項３１】
請求項２１～３０のいずれかの抗体または断片と、薬学的に受容可能なキャリアとを含む薬学的組成物。
【請求項３２】
請求項２１～３０のいずれかの抗体または断片を、患者におけるシュードモナス感染を処置または防止するために効果的な量で含む薬学的組成物。

【請求項３３】
請求項２１～３０のいずれかの抗体または断片を、患者におけるシュードモナスの病原性を低下させるために効果的な量で含む薬学的組成物。
【請求項３４】
患者におけるシュードモナス感染を処置または防止するための方法であって、請求項３１または３２の組成物の有効量を患者に投与することを含む方法。
【請求項３５】
患者におけるシュードモナスの病原性を低下させるための方法であって、請求項３１または３３の組成物の有効量を患者に投与することを含む方法。
【請求項３６】
ヒト細胞に対するシュードモナスの細胞傷害性を調節するための方法であって、ヒト細胞の存在下で該シュードモナスを請求項２１～３０のいずれかの抗体または断片と接触させることを含む方法。
【請求項３７】
請求項２１～３０のいずれかの抗体または断片をコードする核酸。
【発明の詳細な説明】
【連邦政府後援の研究開発に関する記述】
【０００１】
本発明は、以下の機関により与えられた米国政府支援によりなされた。ＮＩＨ／ＮＩＡＤＡ許可番号Ｒ０１　Ａ１３１６６５－０８、Ｋ０４　ＡＩ０１２８９－０４およびＲ０１　ＨＬ５９２３９－０２。米国は本発明に一定の権利を有する。
【発明の背景】
【０００２】
シュードモナス・アエルギノーザ（Ｐｓｅｕｄｏｍｏｎａｓ　ａｅｒｕｇｉｎｏｓａ）は、重病の個体において致命的な急性肺感染を引き起こすことのできる日和見細菌病原体である（１）。細菌の肺上皮傷害能は、III型媒介分泌および転位機構を介して、真核細胞に直接注入される毒素の発現と関連している（２、３）。
【０００３】
Ｐ．アエルギノーザ（Ｐ．ａｅｒｕｇｉｎｏｓａ）III型分泌および転位装置によりコード化されるタンパク質は、エルシニア（Ｙｅｒｓｉｎｉａ）Ｙｏｐレギュロンのメンバーと高レベルのアミノ酸同一性を示す（４－６）。グラム陰性細菌に発見された全III型系の中で、唯一、Ｐ．アエルギノーザが、エルシニアＶ抗原であるＰｃｒＶに相同性を有する（III型系の概観については６参照）。分泌および転位装置に相同なタンパク質は、植物および動物の両方の病原性細菌によりコード化される。これらの生物は、サルモネラ・チフス菌（Ｓａｌｍｏｎｅｌｌａ　ｔｙｐｈｉｍｕｒｉｕｍ）、シゲラ・フレクスナー（Ｓｈｉｇｅｌｌａ　ｆｌｅｘｎｅｒｉ）、腸病原性大腸菌（Ｅ．ｃｏｌｉ）、クラミジア（Ｃｈｌａｍｙｄｉａ）種などのヒト病原体、およびキサントモナス・カンペストリス（Ｘａｎｔｈａｍｏｎａｓ　ｃａｍｐｅｓｔｒｉｓ）、シュードモナス・シリンゲ（Ｐｓｅｕｄｏｍｏｎａｓ　ｓｙｒｉｎｇａｅ）、エルシニア・アミロボーラ（Ｅｒｗｉｎｉａ　ａｍｙｌｏｖｏｒａ）およびラルストニア・ソラナセアラム（Ｒａｌｓｔｏｎｉａ　ｓｏｌａｎａｃｅａｒｕｍ）などの植物病原体を含む。しかし、唯一、Ｐ．アエルギノーザおよびエルシニアが、Ｖ抗原をコード化する。
【０００４】
ヤール（Ｙａｈｒ）等、１９９７は、ＰｃｒＶをコード化するオペロンの配列を開示し、この配列をＬｃｒＶタンパク質と比較している。それゆえに、ＰｃｒＶのアミノ酸配列は既知であり、ＧｅｎＢａｎｋの寄託番号ＡＦ０１０１４９で入手できる。
【発明の要約】
【０００５】
本発明は、シュードモナスＶ抗原は、致命的な肺感染から動物を守るために使用できるという我々の観察から発展させた方法および組成物を含む。

【０００６】
１つの実施態様において、本発明は、患者（ｐａｔｉｅｎｔ、患者または患蓄）に有効量のＰｃｒＶ抗原を接種することを含む、シュードモナス感染を抑制する方法である。別の実施態様において、ＰｃｒＶをコード化するＤＮＡを、遺伝子ワクチンとして使用する。
【０００７】
１つの好ましい実施態様において、抗原は、組換えタンパク質として発現され、危険にさらされている患者を免疫化するために使用する。
【０００８】
好ましくは、患者は、感染から完全に守られる。
【０００９】
別の実施態様において、ＰｃｒＶをコード化するＤＮＡ（ｐｃｒＶと呼ぶ）またはＤＮＡ断片は、Ｐ．アエルギノーザ感染を検出するために診断的に使用することができる。
【００１０】
別の実施態様において、組換えタンパク質（ｒＰｃｒＶ）は、患者から抗体を検出するために診断的に使用する。ＰｃｒＶに対する患者の抗体応答は、予後に関連し得る。それ故、この実施態様において、組換えタンパク質は、患者の抗体力価を測定することにより、予後の指標として使用する。
【００１１】
本発明はまた、個体を、有効量のＰｃｒＶ抑制剤、特にＰｃｒＶ抗体、抗体誘導体もしくは断片、または抗体擬似体と接触させることにより、個体のシュードモナス感染を抑制する方法を提供する。ＰｃｒＶ抗体、抗体誘導体および抗体断片もまた提供される。
【００１２】
本発明の目的は、シュードモナス感染に対して患者を能動および受動免疫化することである。
【００１３】
本発明の別の目的は、Ｐ．アエルギノーザ感染を診断的に検出することである。
【００１４】
本発明の別の目的は、シュードモナス患者から抗体を診断的に検出することである。
【００１５】
本発明の別の目的、特徴および利点は、明細書、特許請求の範囲および図面を概観した後、当業者には明らかとなろう。
【発明の説明】
【００１６】
我々は、本明細書で、ＰｃｒＶは、III型分泌産物の発現に対して新規な調節効果を有し、III型毒素の転位に関与し、Ｐ．アエルギノーザ感染により誘導される肺損傷から守る最初の抗原であることを開示する。抗ＰｃｒＶ　ＩｇＧを気腔に滴下注入する前に、ＰｃｒＶに対してワクチン接種することにより、攻撃された動物の生存が保証されただけでなく、細菌により引き起こされる肺炎症および損傷も減少した。
【００１７】
ＬｃｒＶ、またはＶ抗原は、Ｙｏｐエフェクタータンパク質の分泌および転位を調節し、エルシニア感染に対するホストサイトカイン応答を変化させることにより、病理発生に細胞外的役割を果たしている、多機能性タンパク質である（７－１１）。この危険な病原因子の唯一の既知の相同体は、ＰｃｒＶと呼ばれる、Ｐ．アエルギノーザによりコード化される細胞外タンパク質である。
【００１８】
本発明の１つの実施態様は、有効量のＰｃｒＶ抗原を用いて患者を免疫化することにより、シュードモナス感染を緩和または抑制する方法である。ここで「有効量」とは、抗原で処置されていない対照被検者または動物と比較して、シュードモナス感染のいくらかの緩和または抑制が示されるに有効な量のＰｃｒＶ抗原を意味する。
【００１９】

ここで「緩和」とは、感染が、非免疫化動物と比較して、少なくとも５０％抑制されることを意味する。好ましくは、感染は完全に予防される。感染の定量評価は、好ましくは、血流または胸水中の細菌量の検査および／または肺損傷パラメーターの検査を含む。例えば、血流または胸水中での細菌の非存在は、感染の予防を示す。肺損傷パラメーターの減少は、感染の緩和を示す。

【００２０】

感染は、痰、血液または胸水中の細菌容量の減少、浸潤物のサイズの減少、酸素付加改善、機械的換気時間の長さの減少、熱降下、および白血球数の減少を含む、いくつかの他の臨床指標により定量的に評価できる。

【００２１】

ここで「ＰｃｒＶ抗原」とは、感染を予防または緩和する免疫応答を引き出すのに必要なＰｃｒＶタンパク質の一部または断片を意味する。我々は、全長ＰｃｒＶタンパク質を、保護を誘導するための抗原として使用している。さらに、我々は、保護的なエピトープを、ＰｃｒＶのアミノ酸１４４～２５７を含む断片に特定している。このエピトープを特定するために、感染および細胞毒性から守るモノクローナル抗体を、段々と小さくした形態の組換えＰｃｒＶに対する結合性について試験した。（ここで「組換えＰｃｒＶ」または「ｒＰｃｒＶ」とは、非天然ホストに配置しておいたＰｃｒＶ遺伝子から産生されたタンパク質を意味する。）この保護により、その領域が突き止められた。

【００２２】

ＰｃｒＶ抗原は、我々が使用した方法、すなわちノバゲン（Ｎｏｖａｇｅｎ）から市販で入手可能な細菌性発現プラスミドｐｅｔ１６ｂにより、最も容易に得られ得る。ｐｃｒＶ遺伝子は、初めて、オペロンの一部として、Ｐ．アエルギノーザ染色体からクローン化された。コード領域を増幅し、２つの異なるベクターに挿入した。１つのベクターは、図１で示すように、Ｐ．アエルギノーザからの発現用である。これは、ＰｃｒＶ発現が、細菌の残りの輸送および中毒装置と協調的に調節されるように、適切なプロモーター配列を含むように我々が修飾した、ハーバート・シュバイツァーからのベクター（参考文献１９）である。第二のプラスミドであるｐＥＴ１６ｂは、大腸菌からの発現および精製目的用である。

【００２３】

この系の利点は、Ｐ．アエルギノーザタンパク質の汚染を心配する必要がなく、タンパク質は非常に大量に産生され、そして１段階精製プロセスであるということである。この状況において、ＰｃｒＶコード領域を増幅し、ｐＥＴ１６ｂベクター上に提供されたヒスチジン標識を有するフレーム内でクローン化する。ＰｃｒＶのアミノ末端に融合した複数のヒスチジン残基により、ニッケル－ＮＴＡカラムを使用したアフィニティークロマトグラフィーが可能となる。それ故、好ましいＰｃｒＶ抗原は、組換えバージョンの天然ＰｃｒＶタンパク質である。

【００２４】

免疫化は、全身的にまたは鼻腔内に実施し得る。これらの個体の免疫化は、好ましくは、他の小児期疾患の普通のワクチン接種法中に開始する。我々は、おそらく５および１０歳頃でのブースター用量により保護の永続を予測する。

【００２５】

別の実施態様において、Ｐ．アエルギノーザ感染を診断的に検出するために、ＰｃｒＶタンパク質をコード化するＤＮＡ、またはこのＤＮＡの相補体を使用する。ＧｅｎＢａｎｋＡＦ０１０１４９でＰｃｒＶ抗原のＤＮＡ配列が得られる。ＰｃｒＶのコード領域は、ヌクレオチド６２６－１５１０である。また、このコード領域の断片またはこの断片の相補体の使用を選択してもよい。成功するプローブは、ＰｃｒＶ　ＤＮＡに特異的にハイブリッド化し、他の領域にはハイブリッド化しないものである。

【００２６】

好ましくは、抗原配列内で少なくとも４０個連続したヌクレオチドのハイブリッド形成プローブ、または配列内で少なくとも２５個連続したヌクレオチドの２つのプライマーを使

用する。当業者は、多くの標準的な型の核酸診断技術が適切であることを理解し、例えば、患者の痰から抽出されたＤＮＡまたはＲＮＡに対する一本鎖４０ヌクレオチドプローブのハイブリッド形成がある。別の例では、患者の痰を、細菌性ＤＮＡまたはＲＮＡ源として使用し、それぞれ、ＰＣＲまたはＲＴ－ＰＣＲ反応の鋳型として使用する。

【００２７】

また、個体から得られたサンプルを、ＰｃｒＶに特異的な抗体と接触させ、対照サンプルと比較して増強した抗体結合を、個体のシュードモナス・アエルギノーザ感染と相関させることにより、個体におけるシュードモナス・アエルギノーザ感染を決定する。

【００２８】

追加の実施態様において、ＰｃｒＶをコード化するＤＮＡを、標準的な分子生物学的方法を使用して、遺伝子ワクチンとして使用する。例えば、当業者には公知の技術について、以下の参考文献を概観し得る。デイビス・エイチ・エル（Ｄａｖｉｓ，Ｈ．Ｌ．）等、「Ｂ型肝炎のＤＮＡワクチン：チンパンジーにおける免疫原性の証拠および他のワクチンとの比較」、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．９３：７２１３－７２１８、１９９６；バリー・エム・エー（Ｂａｒｒｙ，Ｍ．Ａ．）等、「発現－ライブラリー免疫化を使用したマイコプラズマ感染からの保護」、Ｎａｔｕｒｅ ３７７：６３２－６３５、１９９５；キシアング・ゼット・キュー（Ｘｉａｎｇ，Ｚ．Ｑ．）等、「狂犬病ウイルスの核酸ワクチンに対する免疫応答」、Ｖｉｒｏｌｏｇｙ ２０９：５６９－５７９、１９９５。ここで「有効量」の遺伝子ワクチンとは、シュードモナス感染またはシュードモナス感染症状を緩和または排除するに有効な量のワクチンを意味する。

【００２９】

タンパク質または抗原は、患者の抗体を検出するために診断的に使用することもでき、したがって、患者の感染状態を予測できる。好ましくは、シュードモナス感染が疑われる個体から得られたサンプルを、ＰｃｒＶタンパク質またはその断片と接触させ、タンパク質／抗体結合を検出する。その後、（対照サンプルと比較して）増強した抗体結合を、個体のシュードモナス・アエルギノーザ感染と相関させる。

【００３０】

別の実施態様において、本発明は、ＰｃｒＶを阻止することができ、そしてまた長期間にわたって動物において単鎖抗体の産生を生じさせる真核生物ベクターが送達される遺伝子送達実験において配列を利用することできる組換え単鎖抗体を製造するための抗体配列（これは下記および配列番号１～４に報告される）の使用である。この配列はまた、ネズミのモノクローナル抗体をヒト化して、ヒト患者の治療において利用され得る製造物を製造するために利用することができる。

【００３１】

抗体がヒト使用に安全であるとなれば、（ａ）それを全身投与でき、また（ｂ）予防処置または治療法として肺に投与できる。ヒトでＰｃｒＶ抗体を使用するために、抗体は好ましくは「ヒト化」する。一般に、モノクローナル抗体が得られれば、重鎖および軽鎖可変領域をクローン化する。これらのクローン化した断片は、その後、ヒト抗体骨格（定常領域）に挿入する。このようにして、我々は、結合特異性を提供することに加えて、抗体のクラス（ＩｇＧ、ＩｇＡ等）を調節できる。

【００３２】

本発明での使用において、ＰｃｒＶ抗体は、モノクローナル抗体でもポリクローナルでもよい。抗体は、特に治療適用のために、ヒトまたはヒト化され得る。抗体断片または誘導体、例えばＦａｂ、Ｆ（ａｂ'）$_2$またはＦｖも使用してよい。例えば、フストン（Ｈｕｓｔｏｎ）等（Ｉｎｔ．Ｒｅｖ．Ｉｍｍｕｎｏｌ．１０：１９５－２１７、１９９３）に記載のような単鎖抗体は、本明細書に記載の方法にも用途を見出し得る。ここで「有効量」のＰｃｒＶ抗体または抗体断片とは、シュードモナス感染または感染症状を緩和または排除するに十分な量を意味する。

【００３３】

好ましくは、ＰｃｒＶに対するヒトまたはヒト化モノクローナルまたはポリクローナル抗

体を投与し、Ｐ．アエルギノーザによる感染を予防または治療する。Ｐ．アエルギノーザ感染にさらされる危険性の高い患者において、抗体を、感染の予防のために投与できる。さらに、抗体は、感染を治療するために、感染の開始後に、投与してもよい。この場合、抗体は、単独で、または抗生物質と組合せて投与できる。抗生物質と共に抗体を投与することにより、より短いクールまたは低用量の抗生物質の投与が可能となり得、よって、抗生物質耐性生物の出現の危険性が減少する。

【００３４】

我々は、仮定上の少なくとも３つの型の患者を想定する。（１）重度の損傷または熱傷にさらされる危険性のある健康な個体（消防士、軍人、警官）を、永続する保護を与える方法（注射または鼻腔内のいずれか）によりワクチンで免疫化する。ブースターは、損傷後に病院の許可を得て投与する（筋肉内注射）。（２）機械的換気を受けている患者。（３）嚢胞性繊維症と遺伝子診断された患者。

【００３５】

ＰｃｒＶ抗体および抗体断片に加えて、小分子ペプチド擬似体または非ペプチド擬似体を、おそらくＰｃｒＶの作用を妨害することによる、シュードモナス感染の抑制または調節におけるＰｃｒＶ抗体の作用を擬似するように設計できる。かかる小分子擬似体を設計する方法は公知である（例えば、リプカ（Ｒｉｐｋａ）およびリッチ（Ｒｉｃｈ）、Ｃｕｒｒ．Ｏｐｉｎ．Ｃｈｅｍ．Ｂｉｏｌ．２：４４１－４５２、１９９８；フアング（Ｈｕａｎｇ）等、Ｂｉｏｐｏｌｙｍｅｒｓ ４２：３６７－３８２、１９９７；アル－オベイジ（ａｌ－Ｏｂｅｉｄｉ）等、Ｍｏｌ．Ｂｉｏｔｅｃｈｎｏｌ．９：２０５－２２３、１９９８参照）。ＰｃｒＶ抗体に基づき設計した小分子抑制剤を、ＰｃｒＶ－ＰｃｒＶ抗体結合相互作用を妨害する能力についてスクリーニングした。かかるアッセイで活性を示す候補小分子を、例えば、インビトロスクリーニングアッセイを含む、当分野で公知の方法により最適化し得、本明細書に記載の方法のいずれかにより、または当分野で公知のように、シュードモナス感染の抑制または調節についてインビボアッセイでさらに精製し得る。かかる小分子のＰｃｒＶ作用抑制剤は、シュードモナス感染の抑制または調節において本法に有用である。

【００３６】

本発明の別の態様において、ＰｃｒＶタンパク質は、ホスト細胞、特にヒト細胞、より特定すると、ヒト上皮細胞またはマクロファージに存在し得るＰｃｒＶレセプターを同定するために使用し得る。ＰｃｒＶレセプターの同定により、組合せライブラリーなどの小分子ライブラリーを、ＰｃｒＶ結合を妨害する候補についてスクリーニングすることが可能となる。かかる分子は、シュードモナス感染を抑制または調節する方法にも有用である。

【００３７】

レセプター同定における我々の最初の試みは、プルダウン実験でＰｃｒＶを使用することである。ＰｃｒＶを、グルタチオンＳ－トランスフェラーゼ（ＧＳＴ）に融合させ、可溶化細胞抽出物のアフィニティークロマトグラフィーのカラムマトリックスに付着させる。ＰｃｒＶに特異的に結合しているタンパク質を溶出し、同定のためにアミノ末端シークエンスにかけた。平行実験で、ＰｃｒＶを、酵母二重ハイブリッド分析にかける。この場合、ＰｃｒＶは、Ｇａｌ４のＤＮＡ結合ドメインを有するフレーム内に融合させる。クローンが得られれば、適切な酵母ホスト株に形質転換する。Ｇａｌ４ＰｃｒＶ作成物を含む酵母株を、Ｇａｌ４活性化ドメインを有するフレーム内にクローン化したＨｅｌａ細胞ｃＤＮＡバンクを用いて形質転換する。ヒスチジンを利用し、βガラクトシダーゼ（ＰｃｒＶと相互作用するタンパク質）を産生する能力を補完した二重形質転換体を、遺伝学的におよびヌクレオチド配列レベルで分析する。レセプターが細胞糖脂質である場合、我々は、糖脂質を、薄層クロマトグラフィーにより分離し、その後、放射標識細菌でプローブする、オーバーレイ技術を利用する。特異的成分への結合は、オートラジオグラフィーにより監視する。同じように、上皮およびマクロファージタンパク質を、ＳＤＳ－ＰＡＧＥにより分離し、ニトロセルロースにブロットし、放射標識細菌または標識ＰｃｒＶをのせる。ここでも、細菌が結合したタンパク質成分を、その後、オートラジオグラフィーにより同

定する。
【００３８】
シュードモナス種は、動物界およびさらには植物界内の広範囲のホストを感染することが知られている。当業者には明らかなように、本明細書で開示した組成物および方法は、シュードモナス種による感染から生じた疾患または状態の抑制または調節において、広範囲の生物に使用し得る。本発明の組成物および方法は、特にシュードモナス・アエルギノーザへの適用について記載するが、本明細書に教示した方法を他の種に適用することは当業者の能力の十分範囲内である。
【実施例】
【００３９】
１．細胞毒性におけるＰｃｒＶの役割
III型媒介調節／分泌におけるＰｃｒＶの役割を決定するために、我々は、ＰｃｒＶの非極性対立遺伝子を作成し、この作成物を使用して、Ｐ．アエルギノーザＰＡ１０３株（インビトロで細胞毒性が高く（３）、インビボで肺上皮傷害を引き起こす（１２、１３）株である）の野生型対立遺伝子を置換した。細胞毒性および肺損傷は、特異的な細胞毒ＥｘｏＵの産生に起因する（３）。
【００４０】
ＰＡ１０３ΔｐｃｒＶは、ＥｘｏＵ細胞毒（３）、ＰｃｒＶ（５）、および毒素の転位に必要なタンパク質ＰｏｐＤ（１４）を含む、Ｐ．アエルギノーザIII型系により分泌されるいくつかの細胞外産物の発現により特徴づけられた。濃縮培養上清のＳＤＳ－ポリアクリルアミドゲル電気泳動により、親株ＰＡ１０３は、カルシウムキレート剤であるニトリロトリ酢酸（ＮＴＡ）を含む培地中での増殖により、III型タンパク質の産生および分泌が誘導されることが示された（図１）。ＰｃｒＶをコード化する発現クローンが、親株においてトランスで提供される場合、ＮＴＡの存在または非存在に応答する細胞外タンパク質産生は正常である。ＰＡ１０３ΔｐｃｒＶは、カルシウム盲目表現型を示し、細胞外タンパク質産生は、ＮＴＡの存在および非存在の両方で強く誘導される。これらの結果は、分泌系は、完全に機能しているが、調節は解除されていることを示唆する。この調節の解除された表現型は、細胞外Ｙｏｐｓを産生できず、カルシウムの存在または非存在にかかわらず３７℃で増殖し、ほんの一部しかＹｏｐｓの誘導を示さない、ＬｃｒＶ欠損株で報告されたカルシウム非依存性表現型と対照的である（７）。ＰＡ１０３ΔｐｃｒＶを、野生型ＰｃｒＶを発現するクローンで補完することにより、ＮＴＡ誘導に応答した、正常な細胞外タンパク質産生調節が回復した。
【００４１】
Ｐ．アエルギノーザ病理発生に対するＰｃｒＶの寄与を試験するために、２つの感染モデルを使用した。インビトロモデルにおいて、親および数個の変異誘導体株を、ＣＨＯ細胞感染アッセイで、細胞毒性を引き起こす能力について比較した（３）。この実験の陰性対照は、III型病原性決定要因の転位が欠損していることが以前に示されたＰＡ１０３ｐｏｐＤ：：Ω（１４）、およびＥｘｏＵが産生されないために、細胞毒性のないＰＡ１０３ΔｅｘｏＵを含んだ（３、１５）。
【００４２】
３時間感染後、ＣＨＯ細胞は、野生型およびＰｃｒＶを発現するプラスミド作成物で補完したΔｐｃｒＶ株で、トリパンブルーを除外できなかった。ＣＨＯ細胞を、陰性対照株またはＰＡ１０３ΔｐｃｒＶで感染させた場合、染色は起こらなかった（データは示していない）。これらの結果により、ＰｃｒＶ発現は細胞毒性に必要であることが示唆される。精製組換えＰｃｒＶは、外因的に組織培養細胞に加えた場合、細胞毒性ではなかった。転位に必要なIII型タンパク質の分泌は、ｐｃｒＶの欠失により影響を受けなかったので（図１ＡおよびＢ）、ＰＡ１０３ΔｐｃｒＶには、ＥｘｏＵ転位が欠損しているようである。
【００４３】
図１Ａおよび１Ｂは、ＰＡ１０３ΔｐｃｒＶの表現型分析を示す、染色ゲル（図１Ａ）お

よびウエスタンブロット（図１Ｂ）である。トランスのＰｃｒＶを発現するプラスミドを含む、または含まない、親およびΔｐｃｒＶ誘導体を、Ｐ．アエルギノーザのIII型分泌の誘導物質、ニトリロトリ酢酸（ＮＴＡ）の非存在下または存在下で増殖させた。細胞外タンパク質プロフィル（図１Ａ）を、クーマシーブルーで染色したＳＤＳ－ポリアクリルアミドゲル（１０％）上で分析した。Ｐ．アエルギノーザコード化III型タンパク質の移動は左に示し、分子量マーカーの移動は右に示す。図１Ｂは、結合したＩｇＧを検出するために、ＥｘｏＵ、ＰｃｒＶ、およびＰｏｐＤに特異的な抗体および^{125}Ｉ－プロテインＡを使用した重複ゲルのウエスタンブロットである。

【００４４】

野生型および変異Ｐ．アエルギノーザ株は、低および高攻撃用量の細菌を使用して、急性肺感染モデルで試験した。生存測定により、ＰｃｒＶおよびＰｏｐＤが、致命的感染の誘導に必要であることが示された（図２Ａ）。３つの独立した肺損傷測定を利用した実験において（肺の気腔から血流への標識アルブミンの流入、肺の気腔から胸水への標識アルブミンの流入、および肺浮腫を測定する湿潤／乾燥比）、ＰＡ１０３ΔｐｃｒＶ、ベクター対照株（ＰＡ１０３ΔｐｃｒＶｐＵＣＰ１８）、およびＰＡ１０３ｐｏｐＤ：：Ωにより引き起こされる損傷度は、非感染対照動物と全く異ならなかった（図２Ｂ）。ＰＡ１０３ΔｐｃｒＶをトランスのｐｃｒＶで補完することにより、肺損傷レベルは、親株ＰＡ１０３で測定されたものに戻った。合わせると、これらのデータにより、ＰｃｒＶ発現は、急性肺感染モデルにおけるＰ．アエルギノーザの病原性に必要であり、ＰｃｒＶの機能の一部は、III型エフェクタータンパク質を真核細胞に転位する能力に関連があるようである。

【００４５】

図２Ａおよび２Ｂは、Ｐ．アエルギノーザ親および変異株の生存および肺損傷を示す、グラフ（図２Ａ）および棒グラフのセット（図２Ｂ）である。図２Ａに関して、マウスを、５×１０^{5}ｃｆｕの示された各株で攻撃し、生存を１週間監視した。図２Ｂに関して、肺損傷を、肺の気腔から血液への（肺上皮損傷）、胸水への（胸水）標識アルブミンの流入により、または湿潤／乾燥比（肺浮腫）の測定により評価した。２つの細菌感染用量を使用し、黒棒および縞棒により表した。対照ないし試験群での有意差（＊ｐ＜０．００１）を、一方向性分散分析およびダネット（Ｄｕｎｎｅｔ）複数比較試験により決定した。以下の略称を使用した。ＰＡ１０３、親野生型株；ΔｐｃｒＶ、ＰＡ１０３ΔｐｃｒＶ；ΔｐｃｒＶｐＵＣＰｐｃｒＶ、ＰｃｒＶを発現するプラスミドで補完したＰＡ１０３ΔｐｃｒＶ；ΔｐｃｒＶｐＵＣＰ、ベクター対照を有するＰＡ１０３ΔｐｃｒＶ；ｐｏｐＤ：：Ω、転位欠損株であるＰＡ１０３ｐｏｐＤ：：Ω。

２．ＰｃｒＶを用いた免疫化

【００４６】

ＰｃｒＶを用いた免疫化により致命的な肺感染から動物が守られるかを決定するために、組換えＰｃｒＶ（ｒＰｃｒＶ）またはＥｘｏＵ（ｒＥｘｏＵ）を、ヒスチジン標識融合タンパク質として精製し、抗原として使用した。マウスを免疫化し、その後、気腔を介して、致死量のＰＡ１０３株で攻撃した。生存を測定すると、両方のワクチンがマウスを保護した（図３Ａ）。肺損傷を評価すると、ＰｃｒＶワクチン接種を受けた動物のみにおいて、上皮傷害および肺浮腫が有意に低かった（図３Ｂ）。ＰｃｒＶワクチンで免疫化した動物は、その肺における細菌が有意に低く、このことは、シュードモナスＶ抗原の遮断により、肺からの細菌の迅速な除去が促進され、重度の上皮損傷から動物が守られることを示唆する（図３Ｂ）。

【００４７】

図３Ａおよび３Ｂは、生存、肺損傷、および細菌コロニー化に対する免疫化の効果を示す、グラフ（図３Ａ）および棒グラフのセット（図３Ｂ）である。図３Ａに関して、マウスを、示されたように免疫化し（ＰｃｒＶ、ｎ＝１０；ＥｘｏＵ、ｎ＝５；対照、ｎ＝１０）、５×１０^{5}ＣＦＵ／動物のＰＡ１０３株で攻撃した。生存動物の比率を、１週間決定した。Ｍａｎｔｅｌ－Ｃｏｘ　ｌｏｇ　ｒａｎｋ検定によりｐ＜０．０５。図３Ｂに関し

て、ＰＡ１０３の設置４時間後の、ワクチン接種動物の肺損傷評価および細菌コロニー形成。肺上皮損傷、肺浮腫、および細菌負荷；ＰｃｒＶ、ｎ＝９；ＥｘｏＵ、ｎ＝４；および対照、ｎ＝８。肺の細菌の最終数は、Ｙ軸×10^4ＣＦＵの数として示される。ダネット複数比較試験により決定、肺損傷（ｐ＜０．０１）、肺浮腫（ｐ＜０．０５）、および細菌数（ｐ＜０．０５）の有意差（＊）。肺損傷における一方向性分散分析、ｐ＝０．０００５；肺浮腫、ｐ＝０．０４３７；細菌負荷、ｐ＝０．００７５。

【００４８】

治療介入が可能であるかを決定するために、５×10^5ＣＦＵ／マウスの濃度のＰＡ１０３を気腔滴下する１時間前に、マウスを、免疫前のウサギＩｇＧ、またはｒＰｃｒＶ、ｒＥｘｏＵ、またはｒＰｏｐＤに特異的なウサギＩｇＧを用いて受動免疫化した。ｒＰｃｒＶに対する抗体により、致命的感染に対する完全な保護が提供された（図４）。抗ｒＥｘｏＵ　ＩｇＧにより部分的生存が提供され、これは、対照ＩｇＧの投与とは有意に異なっていたが、全ての生存動物が試験中に重病であるようであった。生存は、別のIII型転位タンパク質ＰｏｐＤに対する抗体の受動移動により向上しなかった。これらの結果から、我々は、ＰｃｒＶに対する抗体は、急性肺感染モデルで高度に保護的であり、ＰｃｒＶは、細菌表面上にさらされ得るか、または抗体－抗原相互作用に利用可能な可溶形であり得ると結論する。

【００４９】

図４は、５×10^5ＣＦＵ／マウスのＰＡ１０３株で攻撃して生存した動物数のグラフである。動物を、免疫ＩｇＧ１００μｇまたは非免疫化ウサギ由来の対照ＩｇＧ（ｒＰｃｒＶ、免疫前の血清）で前処置した。各群についてＮ＝１０；Ｍａｎｔｅｌ－Ｃｏｘ　ｌｏｇ　ｒａｎｋ検定により、抗ＰｃｒＶおよび抗ＥｘｏＵ　ＩｇＧ調製物での処理において、＊ｐ＜０．０５対対照群。

【００５０】

ＰｃｒＶが、中和を受け易い場合、抗ｒＰｃｒＶ　ＩｇＧと細菌種菌の併用投与により、肺損傷および致死から完全に守られる。ＩｇＧ調製物を、細菌を肺に滴下注入する前に、種菌（致死種菌よりも１０倍高い用量）と混合し、生存を測定した。唯一、抗ｒＰｃｒＶ　ＩｇＧのみが、この極度な感染から保護した（図５Ａ）。肺損傷は、正常致死量である５×10^5細菌で感染させた動物で測定した。肺の気腔からの標識アルブミンの流出は、抗ｒＰｃｒＶ　ＩｇＧの同時投与後、非感染対照よりも僅か３％高かった（図５Ｂ）。肺から胸水への標識タンパク質の流出減少は、抗ＰｃｒＶを種菌と共に含めた場合、非感染対照と同じであった。珍しいことに、湿潤／乾燥比により測定した肺浮腫は、抗ｒＰｃｒＶまたは抗ｒＰｏｐＤの添加により有意に減少した（図５Ｂ）。従って、細菌と抗ｒＰｃｒＶ　ＩｇＧの併用投与は、ワクチン接種よりも、全ての肺損傷パラメーターを規格化する上で、さらにより効果的であった。これらのデータは、ＰｃｒＶが抗体媒介中和を受け易いことを支持し、肺損傷の臨床関連減少を文書で裏付け；ＰｃｒＶに対する抗体は、シュードモナス・アエルギノーザにより引き起こされる重度の院内肺炎の治療における治療薬として使用し得る。

【００５１】

図５は、ＩｇＧおよび細菌攻撃の併用投与による肺損傷からの生存および保護を示す、グラフ（図５Ａ）および棒グラフのセット（図５Ｂ）である。ＩｇＧ（５μｇ）を、５×10^6（生存アッセイ用、１群あたりｎ＝１０）または５×10^5（肺損傷測定用、１群あたりｎ＝４～６動物）のＰ．アエルギノーザＰＡ１０３株と混合した。この混合物を、肺に滴下注入し、生存（図５Ａ）または肺損傷（図５Ｂ）を評価した。生存では、Ｍａｎｔｅｌ－Ｃｏｘ　ｌｏｇ　ｒａｎｋ検定による抗ＰｃｒＶにおいて＊ｐ＜０．０５対対照ＩｇＧ；肺上皮損傷および肺浮腫では、ダネット複数比較試験により、＊ｐ＜０．０５対対照ＩｇＧ。肺損傷における一方向性分散分析、ｐ＝０．０２６、および肺浮腫、ｐ＜０．０００５。

【００５２】

急性Ｐ．アエルギノーザ感染では、III型媒介中毒の実効効果は、上皮を超えて細菌の播

種を促進し、胸水、脾臓、肝臓、および血流の感染をもたらし得ることである。急性換気装置関連肺炎からの、または熱傷感染からの、Ｐ．アエルギノーザによる血液由来感染の結果、活発な抗生物質処置にもかかわらず、死亡率は４０－８０％であり得る（１６）。ＰｃｒＶは、Ｐ．アエルギノーザのIII型転位複合体の成分であるに違いない。なぜなら、このタンパク質の産生が欠損している変異体は、転位に必要なIII型エフェクターおよびタンパク質を産生および分泌できるにもかかわらず、ＣＨＯ細胞を中毒させることができないか、または肺上皮損傷を引き起こすことができないからである。ＰｏｐＤ（これもまた転位に必要である）と異なり、ＰｃｒＶは、抗体媒介中和を受け易く、これは、抗体は急性感染の有用な治療剤であり得ることを示唆する。

【００５３】

３．実施例１および２の方法

ＰｃｒＶの非極性挿入物の作成および補完。ｐｃｒＧＶＨｐｏｐＢＤおよびフランキング配列をコード化する５．０ｋｂのＥｃｏＲＩ－ＮｓｉＩ制限断片を、対立遺伝子置換ベクターｐＮＯＴ１９にクローン化した（１７）。２つのＮｏｔＩ部位（１つはｐｃｒＧ内で、１つはｐｏｐＢ内）を、スカルプター（Ｓｃｕｌｐｔｏｒ）変異誘発系（アマシャム）を使用して、挿入配列から除去した。内部ＳｓｔＩ制限断片を、ＰｃｒＶから欠失させ、残基１７－２２１をインフレーム欠失させた（ｐＮＯＴΔｐｃｒＶ）。プラスミドの組込みを選択するために、テトラサイクリン耐性（ＴｃΩ）をコード化する遺伝子を、ベクターのＨｉｎｄIII部位にクローン化した（ｐＮＯＴΩΔｐｃｒＶ）。ＭＯＢカセット（１７）を、ＮｏｔＩ断片として加えた。部分二倍体の選択、プラスミド配列の分解能、および対立遺伝子置換の確認を、前記した通り行なった（１８）。シャトルプラスミド（ｐＵＣＰ、１９）を使用して、ｐｃｒＶ欠失を補完したクローンを作成した。ＰｃｒＶのコード配列を増幅し、ＥｘｏＳプロモーター領域の制御下でクローン化した（２０）。ＥｘｏＳの転写は、Ｐ．アエルギノーザにおけるIII型分泌および転位を調節するオペロンと協調的に調節されている（２）。ヌクレオチド配列は、部位特異的変異、ＰＣＲ増幅、またはインフレーム欠失を含む、各ＤＮＡ作成物について確認した。

【００５４】

分泌産物のＳＤＳ－ＰＡＧＥおよびウエスタンブロット分析。Ｐ．アエルギノーザを、誘導（＋ＮＴＡ）または非誘導条件下（－ＮＴＡ）で、III型分泌産物を発現させるために増殖した（１８）。培養物を、５４０ｎｍでの光学密度測定に基づき収集し、上清画分を、硫酸アンモニウムの飽和溶液を最終濃度が５５％となるように添加して濃縮した。ＳＤＳ－ポリアクリルアミドゲル（１１％）の各レーンに、３μｌの２０倍濃縮した上清をのせ、クーマシーブルーで染色した。同一のゲルを、ＥｘｏＵ、ＰｏｐＤ、およびＰｃｒＶを特異的に認識する、ウサギ抗血清のカクテルを使用して、前記の通り（３－５）ウエスタンブロット分析にかけた。^{125}Ｉで標識したプロテインＡを、第二試薬として使用して、結合したＩｇＧを同定した。

【００５５】

感染モデルおよび肺損傷評価。チャイニーズハムスター卵巣細胞（ＣＨＯ）を、細胞毒性およびIII型転位を測定するために設計した、インビトロ感染モデルに使用した（２１）。簡潔には、細菌種菌を、血清を含まない組織培養培地で調製した。血清含有培地中で繁殖させたＣＨＯ細胞を洗浄し、５：１の感染多重度で、様々なＰ．アエルギノーザ株で感染させた。培養物を、組織培養条件下（３７℃、５％CO_2）で3時間インキュベートし、洗浄し、トリパンブルーで染色した。色素への透過性は、位相差写真から決定した。ＥｘｏＵを発現する親株ＰＡ１０３での感染により、インキュベートの3時間後、単層の約８０％がトリパンブルー染色され、インキュベートの４－５時間後、単層は完全に破壊される。マウス感染および肺損傷の評価は、前記した通りに行なった（１６）。簡潔には、雄で８－１２週令の病原体のないＢＡＬＢ／ｃマウスを、シモンセン（Ｓｉｍｏｎｓｅｎ）研究所（ギルロイ、カリフォルニア）から購入し、柵のある状態に飼った。マウスを、簡潔には、吸入メトファン（メトキシフルラン、ピットマン－ムーア（Ｐｉｔｍａｎ－Ｍｏｏｒｅ）、マンデライン（Ｍｕｎｄｅｌｅｉｎ）、イリノイ）で麻酔し、約３０°の角

度で、仰臥位に配置した。１５μｌの細菌性種菌を、中咽頭を介して気管に挿入した修飾２４ゲージ動物栄養補給針（ポッパー・アンド・サンズ・インコーポレイテッド（Ｐｏｐｐｅｒ＆Ｓｏｎｓ，Ｉｎｃ．）、ニューハイドパーク、ニューヨーク）を使用して、左葉にゆっくりと滴下注入した。肺損傷評価を測定する場合、５％マウスアルブミンと共に、リンガーラクテート１ｍｌ中、０．５μＣｉの^{131}Ｉ標識ヒト血清アルブミン（メルク－フロスト（Ｆｒｏｓｓｔ）、ケベック、カナダ）、０．０５μｇの無水エバンスブルーを滴下注入液に加えた。感染の４時間後、マウスを麻酔し、血液を頚動脈穿刺により集め、正中胸骨切開を行なった。肺、胸水、気管、中咽頭、胃、および肝臓を収集し、放射活性を測定した。滴下注入した肺に残り、循環または胸水に侵入した、放射活性アルブミンの比率を、血液量（体重×０．０７）に、終末血液サンプルで測定された計数（１ｍｌあたり）を掛けることにより計算した。肺の湿潤／乾燥比は、１ｍｌの水を肺に加え、混合物をホモジナイズすることにより決定した。ホモジネートは、前以て秤量したアルミニウム皿に置き、８０℃のオーブンで３日間一定重量になるまで乾燥させた。肺ホモジネートは、連続希釈し、細菌を定量評価するためにヒツジ血液寒天上に置いた。

【００５６】

ＰｃｒＶ、ＰｏｐＤ、およびＥｘｏＵに対するウサギ抗血清の産生。ｒＰｃｒＶ、ｒＰｏｐＤ、およびｒＥｘｏＵは、ｐＥＴ１６ｂにおいてヒスチジン標識融合タンパク質として産生し、前記した通りニッケルクロマトグラフィーにより精製した（２２）。ウサギに、フロイント完全アジュバント中で乳化した３００μｇの組換えタンパク質を皮内注射（１０部位）し、フロイント不完全アジュバント中の抗原をブースター投与し、７日間間隔で周期的に採血した。受動免疫化するために、ＩｇＧ画分を、プロテインＡカラムクロマトグラフィー（ピアスケミカルズ、ロックフォード、イリノイ）を使用して単離した。マウスに、５×１０^{5}ＣＦＵのＰＡ１０３株で攻撃する１時間前に、１００μｇのＩｇＧ（腹腔内注射）を注射した。ｒＰｃｒＶおよびｒＥｘｏＵを用いて能動免疫化するために、エンドトキシンを、１％トリトンＸ－１１４での抽出により、タンパク質調製物から除去した（２３）。抽出後、トリトンＸ－１１４を、セファクリルＳ－２００クロマトグラフィーにより除去した。全ワクチン調製物が、リムルスアメーバ様細胞ライセートアッセイ（バイオウィッタカー（ＢｉｏＷｈｉｔｔａｋｅｒ）、ウォーカースヴィレ（Ｗａｌｋｅｒｓｖｉｌｌｅ）、メリーランド）の使用により決定されたように、４０μｇの組換えタンパク質あたり、１ｎｇ以下のエンドトキシンを含んだ。ＢＡＬＢ／ｃマウスに、フロイント完全アジュバント中、１０μｇの組換えタンパク質を皮下注射した。３０日目に、マウスを、フロイントの不完全アジュバント中、追加の１０μｇの抗原をブースター投与した。５１日目に、マウスを、その左肺へのＰ．アエルギノーザの滴下注入により攻撃した。

【００５７】

４．モノクローナル抗体の合成

マウスを、フロイントの完全アジュバント中、１０μｇの精製、ＬＰＳ非含有、組換えＰｃｒＶを用いて免疫化し、２週間後に、フロイントの不完全アジュバント中で乳化した、同量の抗原を用いてブースター投与した。免疫化は皮下で行なった。フロイントの不完全アジュバント中のＰｃｒＶのブースター用量の１週間後に、脾臓をマウスから収集した。

【００５８】

単一の脾臓を、血清を含有しない組織培養培地５ｍｌに置き、片に切断し、穏やかにホモジナイズした。組織の大片を、ホモジネートおよび上清から沈降させ、単一細胞懸濁液を取り出し、１２００ｒｐｍで１０分間遠心分離にかけた。ペレット化した細胞を、１０ｍｌ溶液に再懸濁し、赤血球を５分間溶解し、引き続き、１０ｍｌのウシ胎児血清を敷いた。物質を、１２００ｒｐｍで８分間遠心分離し、上清を廃棄し、細胞を３０ｍｌの培地に懸濁した。脾臓細胞およびミエローマ細胞（Ｐ３×６３Ａｇ８．６５３）を、１２００ｒｐｍで１０分間の遠心分離により収集し、各ペレットを、別々に、１０ｍｌの組織培養培地に懸濁した。１０^{8}脾臓細胞および２×１０^{7}ミエローマ細胞を混合し、１２００ｒｐｍで６分間の遠心分離により一緒にペレット化させた。上清を吸引により取り出し、１ｍｌの３５％ポリエチレングリコール（ＰＥＧ）を加えた。細胞をこの溶液に穏やかに懸濁し

、１０００ｒｐｍで３分間遠心分離した。いくつかの実験では、遠心分離は省いた。
【００５９】
ＰＥＧ添加から正確に８分後、２５ｍｌの培地を加え、細胞を穏やかに再懸濁した。５分間の１２００ｒｐｍでの遠心分離段階の後、細胞ペレットを、３０％ならし培地および７０％完全培地（血清含有）中１ｍｌあたり1×10^{6}の密度で懸濁した。細胞を３７℃で一晩インキュベートした。次の日、細胞を遠心分離により収集し、２００ｍｌの３０％ならし培地およびヒポキサンチン、アミノプテリンおよびチミジン（ＨＡＴ）を含む７０％完全培地中に懸濁した。
【００６０】
１ウェルあたり、約０．２ｍｌのこの細胞懸濁液を、１０個の９６ウェルプレート（９６ウェルプレートあたり１２ｍｌ）に加えた。残りの細胞の密度は、１ｍｌあたり2.5×10^{5}に調整し、細胞を９６ウェル型式で培養した。プレートを、単一のコロニーについて顕微鏡でスクリーニングし、その後、上清を、抗原として組換えＰｃｒＶを使用して、酵素結合免疫吸着検定法により抗体産生について試験した。ＰｃｒＶに反応性の抗体を産生するクローンを、より大きな培養皿に継代培養し、その後、アイソタイプを決定した。
【００６１】
抗体の結合は、ウェルを覆膜する抗原（ヒスチジン標識タンパク質）として組換えＰｃｒＶを使用して、酵素結合免疫吸着検定法で試験した。モノクローナル抗体は、ヒスチジン標識を有さない天然ＰｃｒＶを含むＰ．アエルギノーザ上清を使用して、ウエスタンブロット反応でも試験した。
【００６２】
５．ＰｃｒＶ抗原の同定
我々は、標識されたＰｃｒＶと結合する抗体を産生する約３００個の細胞株を得た。これらの最初の細胞株は保管のために液体窒素で保存された。すべての細胞株は、安定なクローンを単離するために継代培養された。安定なクローンを単離することと同時に、我々は、インビトロアッセイを、動物感染モデルにおける中毒からの保護に対する相関関係を示すものとして開発した。
【００６３】
安定に継代培養され、そしてＥＬＩＳＡにおいてＰｃｒＶに対して反応性の抗体を依然として産生するハイブリドーマ（約８０個の細胞株）を、続いて、下記の技術および仮定を使用して蛍光活性化細胞分取器で試験した。抗体がタイプIII中毒システムを阻止する場合、阻止するモノクローナル抗体が存在するもとでは、より少数の細胞が我々のトキシンによって殺されると推論した。細胞を８０個のモノクローナル抗体のそれぞれにさらし、毒性の細菌を加え、インキュベーションして、死細胞のＤＮＡに対してのみ透過性である色素（ヨウ化プロピジウム）を加えた。過剰な色素を洗い流し、細胞を集め、固定して、ＦＡＣＳによって分析した。死細胞は、色素が漏入し、色素により核内のＤＮＡが染色されるので蛍光を発する。
【００６４】
細胞がウサギポリクローナル抗ＰｃｒＶ、マウスポリクローナル抗ＰｃｒＶまたはｍａｂ１６６および細菌とインキュベーションされた場合、関連しないポリクローナル抗体（抗ＰｏｐＤ）またはそれ以外の７８個のモノクローナル抗体とインキュベーションされたコントロールの場合よりも少数の細胞が死亡したことを我々は見出した。
【００６５】
ｍａｂ１６６は、細菌によってコードされるタイプIII分泌因子（これはＰｃｒＶと名付けられた）に結合することが特異的に見出された。ＰｃｒＶは、細胞死を引き起こす細菌トキシンの輸送を促進する、Ｐ．アエルギノーザと肺細胞との相互作用を媒介する。この反応は、Ｐ．アエルギノーザに対する固有的な免疫応答に関与する肺細胞の除去をもたらすと考えられる。これらの細胞の死は、宿主の上皮細胞をＰ．アエルギノーザのコロニー形成ならびに胸膜液および血流への広がりに対して無防備の状態にする。細菌が血流中に進入すると、Ｐ．アエルギノーザによってコードされる抗生物質抵抗性により、効果的な

処置は有効でなくなる。

【００６６】

Ｐ．アエルギノーザによる急性肺感染の動物モデルにおける細菌の滴下注入前および滴下注入後にｍａｂ１６６によってもたらされる保護は顕著である。ヒトのＰ．アエルギノーザ感染症における介入のための抗体処置様式を設計するためには、ヒトのモノクローナル抗体を製造するか、またはｍａｂ１６６によって規定されるＰｃｒＶの保護的なエピトープで危険性の患者を免疫化するかのいずれかが必要である。下記に記載される研究の目的は、ｍａｂ１６６が結合するＰｃｒＶのアミノ酸配列を明らかにすることである。

【００６７】

結果

我々は、ｍａｂ１６６が結合するアミノ酸残基を明らかにするために分子遺伝学的方法を使用した。ＰｃｒＶは２９４個のアミノ酸を有する。この方法は、ポリメラーゼ連鎖反応を使用して分子の一部をヌクレオチド配列レベルで欠失することからなった。各生成物を、グルタチオンＳトランスフェラーゼタンパク質をコードする遺伝子と読み枠を合わせてタンパク質発現ベクターにクローン化した。この方法では、少数のＰｃｒＶアミノ酸をコードする欠失体が、ウエスタンブロット技術またはドットブロット技術を使用して確実に検出され得る。グルタチオンＳトランスフェラーゼのみをコードするコントロールの細菌溶解物は、我々の抗ＰｃｒＶポリクローナル抗体またはｍａｂ１６６モノクローナル抗体のいずれに対しても反応性を示さなかった。

【００６８】

（１つの全長ＰｃｒＶ発現プラスミドとともに）合計で６６個のクローンを構築し、発現させ、そしてウサギポリクローナル抗ＰｃｒＶ抗血清に対する反応性について調べた。１個のクローンを除くすべてが抗ＰｃｒＶウサギ抗体に結合した。このことにより、発現したタンパク質はＰｃｒＶと読み枠が一致していることが確認された。１個の非反応性クローンは分析から除かれた。Ｃ端欠失体（ｎ＝５個の構築物）はどれもｍａｂ１６６と結合しなかった。このことは、エピトープがタンパク質のＣ末端側の半分に存在することを示唆している。ＰｃｒＶのアミノ酸（ａａ）１３９～２９４をコードするＮ端短縮タンパク質（ｎ＝８個の構築物）のうちの１個だけがｍａｂ１６６に結合した。この実験により、ｍａｂ１６６のエピトープはタンパク質のカルボキシル端側の半分によってコードされているという我々の仮説が確認された。残る５１個の構築物は分子の様々な内部欠失体をコードしていた。下記の表１にまとめられた結合性分析により、ｍａｂ１６６によって認識される最小エピトープがＰｃｒＶのａａ１４４～２５７から構成されることが明らかにされた。

【００６９】

【表１－１】

表１

ＰｃｒＶエピトープマッピング		
すべてのタンパク質はアミノ末端標識ＧＳＴ－ＰｃｒＶ短縮体である。		
アミノ酸	ｐＡｂに対する結合性	ｍＡｂ１６６に対する結合性
1-294（全長）	有	有
（Ｃ端短縮体）		
1-46	有	無
1-76	有	無
1-134	有	無
1-172	有	無
1-75 + 173-294	有	無
（Ｎ端短縮体）		
139-294	有	有
148-294	有	無
159-294	有	無
164-294	有	無
194-294	有	無
261-294	有	無
269-294	有	無
278-294	有	無

【００７０】
【表１－２】

アミノ酸	ｐＡｂに対する結合性	ｍＡｂ１６６に対する結合性
(内部断片)		
139-191	有	無
139-195	有	無
139-234	有	無
139-243	有	無
139-256	有	無
139-257	有	有；弱い
139-258	有	有
139-259	有	有
139-260	有	有
139-261	有	有
139-262	有	有
139-263	有	有
139-264	有	有
139-265	有	有
139-266	有	有
139-274	有	有
139-281	有	有
140-266	有	有
141-266	有	有
142-266	有	有
143-266	有	有
144-266	有	有
145-266	有	無
146-266	有	無
147-266	有	無
148-170	無*	無
148-202	有	無
159-202	有	無
159-209	有	無
159-216	有	無
159-226	有	無
159-234	有	無
164-234	有	無
164-243	有	無
164-256	有	無
164-266	有	無
164-275	有	無
164-281	有	無
194-234	有	無
194-243	有	無
194-256	有	無
194-266	有	無
194-275	有	無
194-281	有	無

【００７１】
【表１－３】

アミノ酸	ｐＡｂに対する結合性	ｍＡｂ１６６に対する結合性
141-258	有	有；弱い
142-258	NT**	有
143-258	有	有
144-258	有	有
141-257	有	有
142-257	有	有
143-257	有	有
144-257	有；弱い	有；弱い

備考
*短縮体１４８～１７０は、ウサギのポリクローナルコントロール抗体によって認識されない唯一の短縮体である。
**ＮＴ；細菌溶解物の量が不十分であるために試験されず。
－予測されるように、ｐＧＥＸ－４Ｔ－２ベクターのコントロール溶解物はいずれの抗体によっても認識されなかった。
－ｍＡｂ１６６によって認識され得るＰｃｒＶの最小エピトープはアミノ酸１４４～２５７から構成されるようである。

【００７２】
６．ＰｃｒＶ特異的抗体の試験
方法
ポリＡ＋ＲＮＡの抽出。ハイブリドーマ細胞株ｍ１６６を、４．５ｇ／ＬのＤ－グルコース、１０ｍＭのＨＥＰＥＳ、５０μＭの２－メルカプトエタノール、３ｍＭのＬ－グルタミンおよび１０％の熱不活性化ウシ胎児血清、１００Ｕ／ｍＬのペニシリンおよび１００μｇ／ｍＬの硫酸ストレプトマイシンを含む完全ダルベッコ最少必須培地で培養した。細胞が７５ｃｍ^{2}フラスコにおいてコンフルエンス状態に達した後、細胞を６００ｒｐｍで５分間の遠心分離によって集めた。細胞のペレットを２ｍＬのＴＲＩｚｏｌ試薬（Ｌｉｆｅ　Ｔｅｃｈｎｏｌｏｇｉｅｓ、Ｇａｉｔｈｅｒｓｂｕｒｇ、ＭＤ）においてホモジネートした。総ＲＮＡ（１００μｇ）が、クロロホルム分画、イソプロパノール沈殿および７０％エタノール洗浄の後で抽出された。ポリＡ＋ＲＮＡ（４μｇ）を、オリゴテックスｍＲＮＡスピンカラム（Ｑｉａｇｅｎ、Ｖａｌｅｎｃｉａ、ＣＡ）を用いて抽出した。
【００７３】
ＲＮＡオリゴのキャッピング。ｍＲＮＡ（２５０ｎｇ）を、非ｍＲＮＡまたは短縮化ｍＲＮＡを脱リン酸化するためにウシ腸ホスファターゼとともに５０℃で１時間インキュベーションした。反応後、フェノール／クロロホルム抽出およびエタノール沈殿を行い、脱リン酸化ＲＮＡをタバコ酸性ピロホスファターゼとともに３７℃で１時間インキュベーションして、５’－キャップ構造を全長ｍＲＮＡから除いた。フェノール／クロロホルム抽出およびエタノール沈殿の後、合成ＲＮＡオリゴ（ＧｅｎｅＲａｃｅｒ　ＲＮＡオリゴ、Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、ＣＡ）を、Ｔ４ＲＮＡリガーゼを用いて３７℃で１時間、脱キャップ化ＲＮＡに連結した。フェノール／クロロホルム抽出およびエタノール沈殿の後、ＲＮＡを１３μＬのジエチルピロカルボネート処理水に懸濁した。
【００７４】
ｍＲＮＡの逆転写。ＲＮＡ－オリゴが連結された全長ｍＲＮＡ（１３μＬ）を、１８ヌクレオチドのｄＴテールを含有する５４塩基対プライマー（ＧｅｎｅＲａｃｅｒオリゴｄＴ、Ｉｎｖｉｔｒｏｇｅｎ）およびトリ骨髄芽球症ウイルス逆転写酵素を用いて、２０μＬの反応において４２℃で１時間逆転写した。反応後、サンプルを滅菌水で４倍に希釈した。
【００７５】
ポリメラーゼ連鎖反応（ＰＣＲ）によるｃＤＮＡ端の増幅。１マイクロリットルのｃＤＮＡをＰＣＲのために使用した。合成ＲＮＡオリゴ配列に由来する５’プライマー（ＧｅｎｅＲａｃｅｒ５’プライマー、Ｉｎｖｉｔｒｏｇｅｎ）およびネズミ免疫グロブリンγ２ｂ鎖ＣＨ１領域特異的プライマーまたはネズミ免疫グロブリンκ鎖ＣＬ領域特異的プライマーを使用した。ＰＣＲ反応のために使用されたサイクル処理パラメーターは下記の通りであった。１）９４℃で２分、１サイクル、２）９４℃で３０秒および７２℃で１分、５

サイクル、３）９４℃で３０秒、７０℃で３０秒および７２℃で１分、５サイクル、４）９４℃で３０秒、６８℃で３０秒および７２℃で１分、２０サイクル、５）７２℃で１０分。

【００７６】

サブクローニングおよびＤＮＡ配列決定。ＰＣＲ産物（ネズミ免疫グロブリンγ２ｂ鎖ＣＨ１領域由来の断片およびネズミ免疫グロブリンκ鎖ＣＬ領域由来の断片）をｐＣＲＩＩベクター（ＴＯＰＯクローニング、Ｉｎｖｉｔｒｏｇｅｎ）にサブクローン化し、そしてＤＮＡ配列を分析するためにＵＣＳＦ　Ｍｏｌｅｃｕｌａｒ　Ｂｉｏｒｅｓｏｕｒｃｅ　Ｃｅｎｔｅｒに付託した。

【００７７】

配列番号１はｍ１６６重鎖ｍＲＮＡのＤＮＡ配列であり、配列番号２はｍ１６６重鎖（ＩｇＧＩＩ$_b$）のアミノ酸配列であり、配列番号３はｍ１６６軽鎖ｍＲＮＡのＤＮＡ配列であり、配列番号４はｍ１６６軽鎖のアミノ酸配列である。図６Ａ、図６Ｂおよび図７により、配列が調べられ、より細部が示される。

【００７８】

商業的意義

本発明の抗体配列は、ＰｃｒＶを阻止することができ、そしてまた長期間にわたって動物において単鎖抗体の産生を生じさせる真核生物ベクターが送達される遺伝子送達実験において配列を利用することができる組換え単鎖抗体を製造するために使用することができる。最後に、本発明の抗体配列は、ネズミのモノクローナル抗体をヒト化して、ヒト患者の治療において利用され得る製造物を製造するために利用することができる。当業者は、ヒト化抗体を作製するために、齧歯類の抗体の可変領域に由来する抗原結合性の相補性決定領域（ＣＤＲ）をヒトの可変ドメインにつなぐことなどの標準的な方法に注目する。

【００７９】

７．ＰｃｒＶに対する単鎖抗体

ａ．単鎖抗体の組み立て

ＶＨ遺伝子およびＶＬ遺伝子が、それぞれの遺伝子について特異的なプライマーを用いたポリメラーゼ連鎖反応（ＰＣＲ）によって増幅された。増幅されたＶＨ断片およびＶＬ断片は、プライマーとともにＰＣＲを使用することによってリンカーとともに組み立てられた。組み立てられた単鎖抗体遺伝子（リンカーを伴うｓｃＦｖ：：ｍ１６６：ＶＨ遺伝子およびＶＬ遺伝子）をクローニングベクターｐＣＲ４Ｔｏｐｏ（Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、ＣＡ）に移した。その後、ｓｃＦｖ：：ｍ１６６のコード配列を、宿主大腸菌としてのＬＭＧ１９４における大腸菌発現ベクターｐＢＡＤ／ｇIII（Ｉｎｖｉｔｒｏｇｅｎ）にサブクローン化した。

【００８０】

ｂ．タンパク質の誘導および精製

タンパク質誘導のために、０．２％グルコースおよび１００μｇのアンピシリンを含有するＲＭ培地において、形質転換大腸菌をオービタル振とう機（２００ｒｐｍ）にて３７℃で一晩培養した。翌日、５ｍＬの培養された大腸菌を５００ｍＬの同じ培地に移して、１００ｒｐｍにおいて室温で３時間インキュベーションした。Ｌ－アラビノースを０．００４％の濃度で加えた後、大腸菌を一晩培養した。３日目に、タンパク質を浸透圧ショック法によって大腸菌の細胞周辺腔から集めた。浸透圧ショック由来のペリプラズムタンパク質を含む溶液を溶解緩衝液に対して一晩透析した。４日目のとき、透析された溶液をニッケル－ＮＴＡカラムに負荷して、ヘキサヒスチジン標識の単鎖抗体を精製した。ニッケルカラムからの溶出液をリン酸塩緩衝化生理的食塩水に対して一晩透析した。５日目に、透析された溶液を遠心分離濃縮器に加えて、より高濃度のｓｃＦｖ：ｍ１６６を作製した。

【００８１】

ｃ．結合試験

精製された単鎖抗体（ｓｃＦｖ：：ｍ１６６）を、組換えＰｃｒＶに対する酵素結合免疫吸着アッセイを使用することによって、そして組換えＰｃｒＶタンパク質およびＰ．アエ

ルギノーザＰＡ１０３の未変性ＰｃｒＶの両方に対する免疫ブロット（ウエスタンブロット）によって調べた。

【００８２】

本発明の単鎖抗体により、我々は、様々なファージディスプレー技術を利用して抗体をヒト化することができ、そしてこれらの技術を使用して抗体の親和性を改善することができる。本発明の単鎖抗体は、（組織学のための）診断ツールとして利用することができるが、治療剤としては利用されない。しかし、単鎖抗体に対する遺伝子は遺伝子治療において利用することができ、その結果、動物は、ある期間にわたって単鎖抗体を産生し、それにより、Ｐ．アエルギノーザ感染からの保護を生じさせることができる。

【００８３】

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【００８４】

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【００８５】

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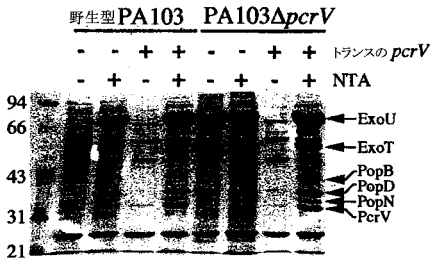
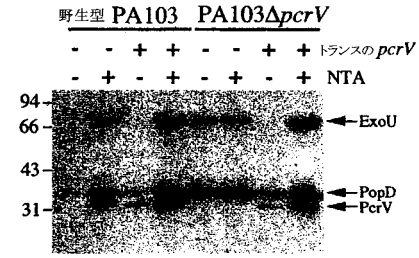
【００９０】
２２．ヤール・ティー・エル、バービエリ・ジェイ・ティーおよびフランク・ディー・ダブリュー、「シュードモナス・アエルギノーザ由来の細胞外酵素Ｓの５３ないし４９ｋＤａ形の遺伝子関係」、Ｊ．Ｂａｃｔｅｒｉｏｌ．１７８：１４１２－１４１９（１９９６）。
２３．アイジ・ワイ（Ａｉｄｉ，Ｙ．）およびパブスト・エム・ジェイ（Ｐａｂｓｔ，Ｍ．Ｊ．）、「トリトンＸ－１１４を使用した相分離によるタンパク質溶液からのエンドトキシンの除去」、Ｊ．Ｉｍｍｕｎｏｌ．Ｍｅｔｈｏｄｓ １３２：１９１－１９５（１９９０）。
【図面の簡単な説明】
【００９１】
【図１】図１Ａおよび図１Ｂは、ＰＡ１０３ΔｐｃｒＶの表現型分析を示す、染色ゲル（図１Ａ）およびウエスタンブロット（図１Ｂ）である。
【図２】図２Ａおよび図２Ｂは、Ｐ．アエルギノーザ親および変異株の生存および肺損傷を示す、グラフ（図２Ａ）および棒グラフのセット（図２Ｂ）である。
【図３】図３Ａおよび図３Ｂは、生存、肺損傷、および細菌コロニー形成に対する免疫化の効果を示す、グラフ（図３Ａ）および棒グラフのセット（図３Ｂ）である。
【図４】図４は、ＰｃｒＶ、ＥｘｏＵ、ＰｏｐＤに特異的なポリクローナル抗体または非免疫化動物由来の対照ＩｇＧを受動投与した後、５×１０^{5}ＣＦＵ／マウスのＰＡ１０３株で攻撃して生存した動物数のグラフである。
【図５】図５は、異なる細菌性抗原に対するＩｇＧおよび細菌攻撃の併用投与による、肺損傷からの生存および保護を示す、グラフ（図５Ａ）および棒グラフのセット（図５Ｂ）である。肺損傷の一方向性分散分析は、ｐ＝０．０２６、および肺浮腫では、ｐ＜０．０００５。
【図６】図６Ａおよび図６Ｂは、さらなる説明情報を伴う配列番号１および配列番号２のプリントアウトである。図６Ａは配列番号１である。図６Ｂは配列番号２である。
【図７】図７は、さらなる説明情報を伴う配列番号３および配列番号４のプリントアウトである。
【図８】図８は、合成された組換え単鎖抗体（ＳＣＦＶ－Ｍ１６６）（配列番号５および配列番号６）である。

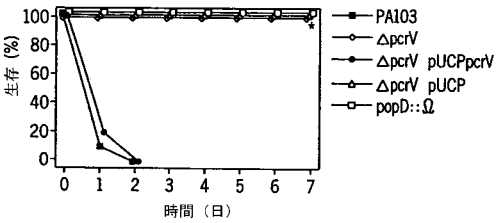
【図１Ａ】



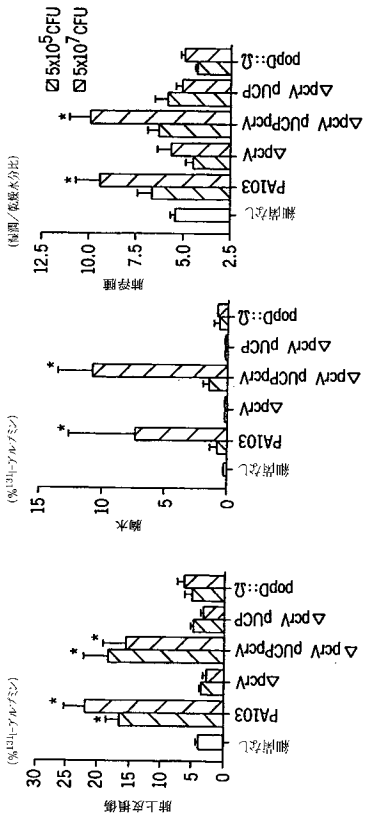
【図１Ｂ】



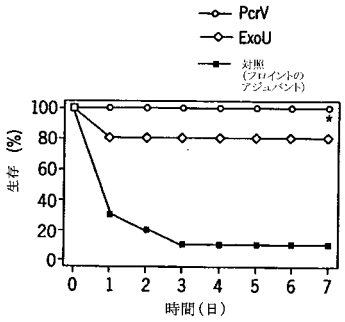
【図２Ａ】



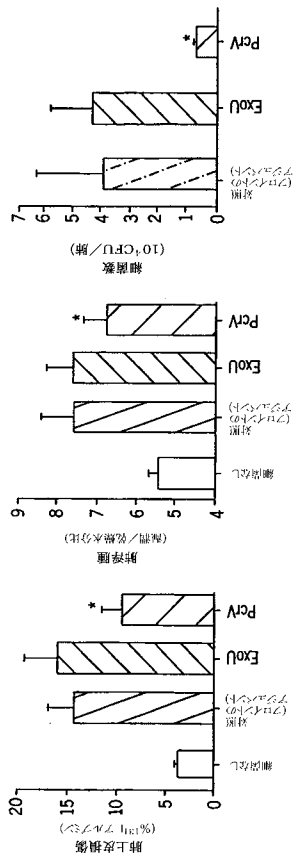
【図２Ｂ】



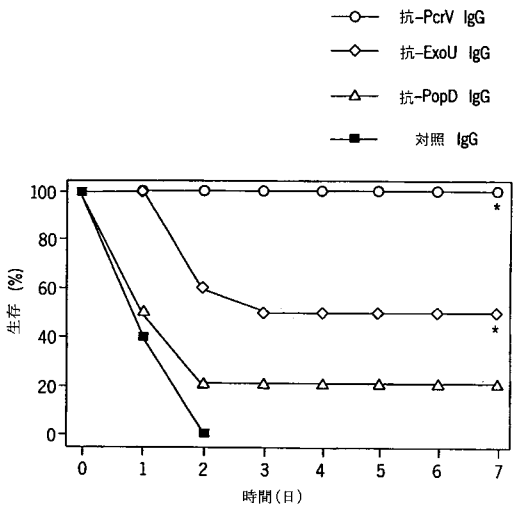
【図３Ａ】



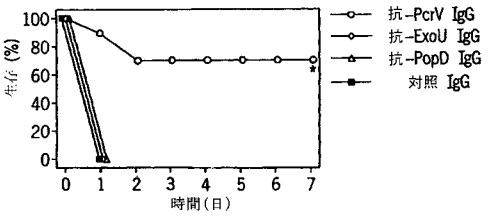
【図３Ｂ】



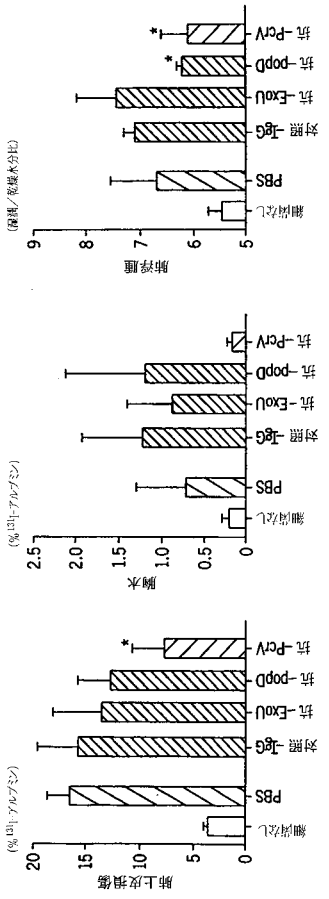
【図４】



【図５Ａ】



【図５Ｂ】



【図６Ａ－１】

m166重鎖
1．m166重鎖（IgG2b）の 完全なmRNA配列：

(転写開始点からポリAテールまで)

CCATCCTCTT CTCATAGAGC CTCCATCAGA GCATGGCTGT CTTGGGGCTG
CTCTTCTGCC TGGTGACATT CCCAAGCTGT GTCCTATCCC AGGTGCAGCT
GAAGCAGTCA GGACCTGGCC TAGTGCAGCC CTCACAGAGC CTGTCCATCA
CCTGCACAGT CTCTGGTTTC TCATTAACTA GCTATGGTGT ACACTGGGTT
CGTCAGTCTC CAGGAAAGGG TCTGGAGTGG CTGGGAGTGA TATGGAGTGG
TGGAGACACA GACTATAATG CAGCTTTCAT ATCCAGACTG AGCATCAGCA
AGGACAATTC CAAGAGCCAA CTCTTCTTTA AAATGAACAG TCTGCGAGCT
ACTGACACAG CCATATATTA CTGTGCCAGA AATAGAGGGG ATATTTACTA
TGATTTCACT TATGGCATGG ACTACTGGGG TCAAGGAACC TCAGTCACCG
TCTCCTCAGC CAAAACAACA CCCCCATCAG TCTATCCACT GGCCCCTGGG
TGTGGAGATA CAACTGGTTC CTCCGTGACT CTGGGATGCC TGGTCAAGGG
CTACTTCCCT GAGTCAGTGA CTGTGACTTG GAACTCTGGA TCCCTGTCCA
GCAGTGTGCA CACCTTCCCA GCTCTCCTGC AGTCTGGACT CTACACTATG
AGCAGCTCAG TGACTGTCCC CTCCAGCACC TGGCCAAGTC AGACCGTCAC
CTGCAGCGTT GCTCACCCAG CCAGCAGCAC CACGGTGGAC AAAAAACTTG
AGCCCAGCGG GCCCATTTCA ACAATCAACC CCTGTCCTCC ATGCAAGGAG

【図６Ａ－２】

TGTCACAAAT GCCCAGCTCC TAACCTCGAG GGTGGACCAT CCGTCTTCAT
CTTCCCTCCA AATATCAAGG ATGTACTCAT GATCTCCCTG ACACCCAAGG
TCACGTGTGT GGTGGTGGAT GTGAGCGAGG ATGACCCAGA CGTCCAGATC
AGCTGGTTTG TGAACAACGT GGAAGTACAC ACAGCTCAGA CACAAACCCA
TAGAGAGGAT TACAACAGTA CTATCCGGGT GGTCAGCACC CTCCCCATCC
AGCACCAGGA CTGGATGAGT GGCAAGGAGT TCAAATGCAA GGTCAACAAC
AAAGACCTCC CATCACCCAT CGAGAGAACC ATCTCAAAAA TTAAAGGGCT
AGTCAGAGCT CCACAAGTAT ACATCTTGCC GCCACCAGCA GAGCAGTTCT
CCAGGAAAGA TGTCAGTCTC ACTTGCCTGG TCGTGGGCTT CAACCCTGGA
GACATCAGTG TGGAGTGGAC CAGCAATGGG CATACAGAGG AGAACTACAA
GGACACCGCA CCAGTCCTGG ACTCTGACGG TTCTTACTTC ATATATAGCA
AGCTCAATAT GAAAACAAGC AAGTGGGAGA AAACAGATTC CTTCTCATGC
AACGTGAGAC ACGAGGGTCT GAAAAATTAC TACCTGAAGA AGACCATCTC
CCGGTCTCCG GGTAAATGAG CTCAGCACCC ACAAAGCTCT CAGGTCCTAA
GAGACACTGG CACCCATATC CATGCATCCC TTGTATAAAT AAAGCATCCA
GCAAAGCCTG GTACCATGTA AAAAAAAAAA AAAAAAAA

【図６Ｂ－２】

[SIG-PEP] MAVLGLLFCLVTFPSCVLS

[VH-REGION]
FR1: QVQLKQSGPGLVQPSQSLSITCTVSGFSLT
CDR1: SYGVH
FR2: WVRQSPGKGLEWLG
CDR2: VIWSGGDTDYNAAFIS
FR3: RLSISKDNSKSQLFFKMNSLRATDTAIYYCAR
CDR3: NRGDIYYDFTYAMDY
FR4: WGQGTSVTVSS

[CH-REGION]
CH:

AKTTPPSVYP LAPGCGDTTG SSVTLGCLVK GYFPESVTVT WNSGSLSSSV
HTFPALLQSG LYTMSSSVTV PSSTWPSQTV TCSVAHPASS TTVDKKLEPS
GPISTINPCP PCKECHKCPA PNLEGGPSVF IFPPNIKDVL MISLTPKVTC
VVVDVSEDDP DVQISWFVNN VEVHTAQTQT HREDYNSTIR VVSTLPIQHQ
DWMSGKEFKC KVNNKDLPSP IERTISKIKG LVRAPQVYIL PPPAEQLSRK
DVSLTCLVVG FNPGDISVEW TSNGHTEENY KDTAPVLDSD GSYFIYSKLN
MKTSKWEKTD SFSCNVRHEG LKNYYLKKTI SRSPGK[STOP]

【図７Ａ】

ｍ１６６軽鎖
１．ｍ１６６軽鎖（κ）の完全なｍＲＮＡ配列：

（転写開始点からポリＡテールまで）

ACACCCTTTG CTGGAGTCAG AATCACACTG ATCACACACA GTCATGAGTG
TGCTCACTCA GGTCCTGGCG TTGCTGCTGC TGTGGCTTAC AGGTGCCAGA
TGTGACATCC AGATGACTCA GTCTCCAGCC TCCCTATCTG CATCTGTGGG
AGAAACTGTC ACCATCACAT GTCGAGCAAG TGGGAATATT CAAAATTATT
TAGCATGGTA TCAGCAGACA CAGGGAAAAT CTCCTCAGCT CCTGGTCTAT
TCTGCAAAAA CCTTAGCAGA TGGTGTGCCA TCAAGGTTCA GTGGCAGTGG
ATCAGGAACA CAATATTCTC TCAAGATCAA CAGCCTGCAG CCTGAAGATT
TTGGGAGTTA TTACTGTCAA CATTTTTGGA GTACTCCGTA CACGTTCGGA
GGGGGGACCA AGCTGGAAAT AAAACGGGCT GATGCTGCAC CAACTGTATC
CATCTTCCCA CCATCCAGTG AGCAGTTAAC ATCTGGAGGT GCCTCAGTCG

【図６Ｂ－１】

２．ｍ１６６重鎖（ＩｇＧ２ｂ）の完全なアミノ酸配列：

（開始コドンから停止コドンまで）

MAVLGLLFCL VTFPSCVLSC VQLKQSGPGL VQPSQSLSIT CTVSGFSLTS
YGVHWVRQSP GKGLEWLGVI WSGGDTDYNA AFISRLSISK DNSKSQLFFK
MNSLRATDTA IYYCARNRGD IYYDFTYAMD YWGQGTSVTV SSAKTTPPSV
YPLAPGCGDT TGSSVTLGCL VKGYFPESVT VTWNSGSLSS SVHTFPALLQ
SGLYTMSSSV TVPSSTWPSQ TVTCSVAHPA SSTTVDKKLE PSGPISTINP
CPPCKECHKC PAPNLEGGPS VFIFPPNIKD VLMISLTPKV TCVVVDVSED
DPDVQISWFV NNVEVHTAQT QTHREDYNST IRVVSTLPIQ HQDWMSGKEF
KCKVNNKDLP SPIERTISKI KGLVRAPQVY ILPPPAEQLS RKDVSLTCLV
VGFNPGDISV EWTSNGHTEE NYKDTAPVLD SDGSYFIYSK LNMKTSDWEK
TDSFSCNVRH EGLKNYYLKK TISRSPGK[STOP]

【図７Ｂ】

TGTGCTTCTT	GAACAACTTC	TACCCCAAAG	ACATCAATGT	CAAGTGGAAG
ATTGATGGCA	GTGAACGACA	AAATGGCGTC	CTGAACAGTT	GGACTGATCA
GGACAGCAAA	GACAGCACCT	ACAGCATGAG	CAGCACCCTC	ACGTTGACCA
AGGACGAGTA	TGAACGACAT	AACAGCTATA	CCTGTGAGGC	CACTCACAAG
ACATCAACTT	CACCCATTGT	CAAGAGCTTC	AACAGGAATG	AGTGTTAGAG
ACAAAGGTCC	TGAGACGCCA	CCACCAGCTC	CCCAGCTCCA	TCCTATCTTC
CCTTCTAAGG	TCTTGGAGGC	TTCCCCACAA	GCGACCTACC	ACTGTTGCGG
TGCTCCAAAC	CTCCTCCCCA	CCTCCTTCTC	CTCCTCCTCC	CTTTCCTTGG
CTTTTATCAT	GCTAATATTT	GCAGAAAATA	TTCAATAAAG	TGAGTCTTTG
CAAAAAAAAA	AAAAAAAAAA	AAAAAAAAA		

【図７Ｃ】

２．ｍ１６６軽鎖（κ）の完全なアミノ酸配列：

(開始コドンから停止コドンまで)

MSVLTQVLAL	LLLWLTGARC	DIQMTQSPAS	LSASVGETVT	ITCRASGNIQ
NYLAWYQQTQ	GKSPQLLVYS	AKTLADGVPS	RFSGSGSGTQ	YSLKINSLQP
EDFGSYYCQH	FWSTPYTFGG	GTKLEIKRAD	AAPTVSIFPP	SSEQLTSGGA
SVVCFLNNFY	PKDINVKWKI	DGSERQNGVL	NSWTDQDSKD	STYSMSSTLT
LTKDEYERHN	SYTCEATHKT	STSPIVKSFN	RNEC[STOP]	

[SIG-PEP] MSVLTQVLALLLLWLTGARC

[VL-REGION]
FR1: DIQMTQSPASLSASVGETVTITC
CDR1: RASGNIQNYLA
FR2: WYQQTQGKSPQLLVY
CDR2: SAKTLAD
FR3: GVSRFSGSGSGTQSLKINSLQPEDFGSYYC
CDR3: QHFWSTPYT
FR4: FGGGTKLEIKR

[CL-REGION]
CL: ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVL
NSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC[STOP]

【図８Ａ】

合成された組換え単鎖抗体（ｓｃＦｖ－ｍ１６６）
１．ＤＮＡ配列：

ATGAAAAAAC	TGCTGTTCGC	GATTCCGCTG	GTGGTGCCGT	TCTATAGCCA
TAGCACCATG	GAGCTCGAGC	GGCAGGTGCA	GCTGAAGCAG	TCAGGACCTG
GCCTAGTGCG	GCCCTCACAG	AGCCTGTCCA	TCACCTGCAC	AGTCTCTGGT
TTCTCATTAA	CTAGCTATGG	TGTACACTGG	GTTCGTCAGT	CTCCAGGAAA
GGGTCTGGAG	TGGCTGGAG	TGATATGGAG	TGGTGGAGAC	ACAGACTATA
ATGCAGCTTT	CATATCCAGA	CTGAGCATCA	GCAAGGACAA	TTCCAAGAGC
CAACTCTTCT	TTAAAATGAA	CAGTCTGCGA	GCTACTGACA	CAGCCATATA
TTACTGTGCC	AGAAATAGAG	GGGATATTTA	CTATGATTTC	ACTTATGCCA
TGGACTACTG	GGGTCAAGGA	ACCTCAGTCA	CCGTCTCCTC	AGGTGGAGGC
GGCTCAGGCG	GAGGTGGCTC	TGGCGGTGGC	GGATCGGACA	TCCAGATGAC
TCAGTCTCCA	GCCTCCCTAT	CTGCATCTGT	GGGAGAAACT	GTCACCATCA
CATGTCGAGC	AAGTGGGAAT	ATTCAAAATT	ATTTAGCATG	GTATCAGCAG
ACACAGGGAA	AATCTCCTCA	GCTCCTGGTC	TATTCTGCAA	AAACCTTAGC
AGATGGTGTG	CCATCAAGGT	TCAGTGGCAG	TGGATCAGGA	ACACAATATT
CTCTCAAGAT	CAACAGCCTG	CAGCCTGAAG	ATTTTGGGAG	TTATTACTGT
CAACATTTTT	GGAGTACTCC	GTACACGTTC	GGAGGGGGGA	CCAAGCTGGA
AATAAAACGG	GCTCTAGAAC	AAAAACTCAT	CTCAGAAGAG	GATCTGAATA
GCGCCGTCGA	CCATCATCAT	CATCATCATT	GA	

【図８Ｂ】

２．アミノ酸配列：

MKKLLFAIPL	VVPFYSHSTM	ELERQVQLKQ	SGPGLVRPSQ	SLSITCTVSG
FSLTSYGVHW	VRQSPGKGLE	WLGVIWSGGD	TDYNAAFISR	LSISKDNSKS
QLFFKMNSLR	ATDTAIYYCA	RNRGDIYYDF	TYAMDYWGQG	TSVTVSSGGG
GSGGGGSGGG	GSDIQMTQSP	ASLSASVGET	VTITCRASGN	IQNYLAWYQQ
TQGKSPQLLV	YSAKTLADGV	PSRFSGSGSG	TQYSLKINSL	QPEDFGSYYC
QHFWSTPYTF	GGGTKLEIKR	ALEQKLISEE	DLNSAVDHHH	HHH[STOP]

【図８Ｃ】

[GENE III SIGNAL SEQUENCE] MKKLLFAIPLVVPFYSHS
[JOINT-1] TMELER
[m166 HEAVY CHAIN]
QVQLKQSGPG LVRPSQSLSI TCTVSGFSLT SYGVHWVRQS PGKGLEWLGV
IWSGGDTDYN AAFISRLSIS KDNSKSQLFF KMNSLRATDT AIYYCARNRG
DIYYDFTYAM DYWGQGTSVT VSS
[scFv-LINKER] GGGGSGGGGSGGGGS
[m166 LIGHT CHAIN]
DIQMTQSPAS LSASVGETVT ITCRASGNIQ NYLAWYQQTQ GKSPQLLVYS
AKTLADGVPS RFSGSGSGTQ YSLKINSLQP EDFGSYYCQH FWSTPYTFGG
GTLEIKR
[JOINT-2] AL
[MYC EPITOPE] EQKLISEEDL
[JOINT-3] NSAVD
[HEXAHISTIDINE TAG] HHHHHH [STOP]

【国際公開パンフレット】

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International Bureau

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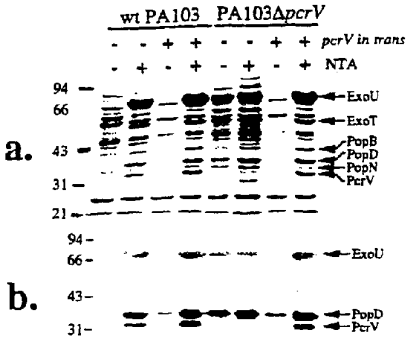
[Continued on next page]

(54) Title: METHOD AND COMPOSITIONS FOR IMMUNIZATION WITH THE PSEUDOMONAS V ANTIGEN



(57) Abstract: A method of inhibiting, moderating or diagnosing *Pseudomonas aeruginosa* infection is disclosed. In one embodiment, this method comprises inoculating a patient with an effective amount of PcrV antigen.

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GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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

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— *without international search report and to be republished upon receipt of that report*

METHOD AND COMPOSITIONS FOR IMMUNIZATION WITH THE PSEUDOMONAS V ANTIGEN

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with United States government support awarded by the following agencies: NIH/NIADA Grant Nos. R01 A131665-08, K04 AI01289-04 and R01 HL59239-02. The United States has certain rights in this invention.

BACKGROUND OF THE INVENTION

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that is capable of causing fatal acute lung infections in critically ill individuals (1). The ability of the bacterium to damage the lung epithelium has been linked with the expression of toxins that are directly injected into eukaryotic cells via a type III-mediated secretion and translocation mechanism (2, 3).

The proteins encoded by the *P. aeruginosa* type III secretion and translocation apparatus demonstrate a high level of amino acid identity with members of the *Yersinia* Yop regulon (4-6). Of all the type III systems discovered in Gram-negative bacteria, only *P. aeruginosa* possesses a homologue to the *Yersinia* V antigen, PcrV (see 6 for review of type III systems). Homologous proteins to the secretion and translocation apparatus are encoded by both plant and animal pathogenic bacteria. These organisms include human pathogens such as *Salmonella typhimurium*, *Shigella flexneri*, *Enteropathogenic E. coli*, *Chlamydia spp.*, and plant pathogens such as *Xanthamonas campestris*, *Pseudomonas syringae*, *Erwinia amylovora* and *Ralstonia solanacearum*. However, only *P. aeruginosa* and *Yersinia* encode the V antigen.

Yahr, et al., 1997, discloses the sequence of the operon encoding PcrV and compares the sequence to the LcrV protein. Thus, the amino acid

sequence of PcrV is known and is available under accession number AF010149 of GenBank.

SUMMARY OF THE INVENTION

The present invention involves methods and compositions developed from our observation that the *Pseudomonas* V antigen can be used to protect animals from a lethal lung infection.

In one embodiment, the present invention is a method of inhibiting *Pseudomonas* infection comprising inoculating a patient with an effective amount of PcrV antigen. In another embodiment, DNA encoding PcrV is used as a gene vaccine.

In one preferred embodiment, the antigen is expressed as a recombinant protein and used to immunize patients at risk.

Preferably, the patient is completely protected from infection.

In another embodiment, the DNA encoding PcrV (called *pcrV*) or a DNA fragment may be used diagnostically to detect *P. aeruginosa* infection.

In another embodiment, the recombinant protein (rPcrV) is used diagnostically to detect antibodies from patients. Patient antibody response to PcrV may be associated with prognosis. Therefore, in this embodiment the recombinant protein is used as a prognostic indicator by measuring the patient's antibody titer.

The present invention also provides a method for inhibiting a *Pseudomonas* infection in an individual by contacting the individual with an effective amount of a PcrV inhibitor, in particular with a PcrV antibody, antibody derivative or fragment, or antibody mimic. PcrV antibodies, antibody derivatives and antibody fragments are also provided.

It is an object of the present invention to actively and passively immunize a patient against Pseudomonas infection.

It is another object of the present invention to diagnostically detect the *P. aeruginosa* infection.

It is another object of the present invention to diagnostically detect antibodies from *Pseudomonas* patients.

Other objects, features and advantages of the present invention will become apparent to one of skill in the art after review of the specification, claims and drawings.

DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are a stained gel (Fig. 1A) and Western blot (Fig. 1B) illustrating the phenotypic analysis of PA103ΔpcrV.

Figs. 2A and 2B are a graph (Fig. 2A) and set of bar graphs (Fig. 2B) illustrating the survival and lung injury of *P. aeruginosa* parental and mutant strains.

Figs. 3A and 3B are a graph (Fig. 3A) and a set of bar graphs (Fig. 3B) illustrating the effect of immunization on survival, lung injury, and bacterial colonization.

Fig. 4 is a graph of the number of animals surviving a challenge with 5 x 10^5 CFU/mouse of strain PA103 after passive administration of polyclonal IgG specific for PcrV, ExoU, PopD or control IgG from an unimmunized animal.

Fig. 5 is a graph (Fig. 5A) and a set of bar graphs (Fig. 5B) illustrating survival and protection from lung injury by concomitant administration of IgG to different bacterial antigens and bacterial challenge. One-way ANOVA for lung injury, $p=0.026$, and lung edema, $p<0.0005$.

Figs. 6A and B are printouts of SEQ ID NOs:1 and 2 with additional explanatory information. Fig. 6A is SEQ ID NO:1. Fig. 6B is SEQ ID NO:2.

Fig. 7 is a printout of SEQ ID NOs:3 and 4 with additional explanatory information.

Fig. 8 is a synthetic recombinant single chain antibody (SCFV-M166) (SEQ ID NOs:5 and 6).

DESCRIPTION OF THE INVENTION

We disclose herein that PcrV has a novel regulatory effect on expression of the type III secreted products, is involved in the translocation of type III toxins, and is the first antigen that protects against lung injury induced by *P. aeruginosa* infection. Vaccination against PcrV prior to the airspace instillation of anti-PcrV IgG not only ensured the survival of challenged animals but also decreased lung inflammation and injury caused by the bacteria.

LcrV, or the V antigen, is a multifunctional protein that regulates secretion/translocation of the Yop effector proteins and plays an extracellular role in pathogenesis by altering the host cytokine response to *Yersinia* infection (7-11). The only known homologue of this critical pathogenic factor is an extracellular protein encoded by *P. aeruginosa*, termed PcrV.

One embodiment of the present invention is a method of moderating or inhibiting a *Pseudomonas* infection by immunizing a patient with an effective amount of the PcrV antigen. By "effective amount" we mean an amount of PcrV antigen effective to show some moderation or inhibition of *Pseudomonas* infection as compared to control subjects or animals who have not been treated with the antigen.

By "moderating" we mean that infection is inhibited by at least fifty percent compared to a non-immunized animal. Preferably, infection is completely prevented. A quantitative assessment of infection would preferably include the examination of the amount of bacteria in the

bloodstream or pleural fluids and/or an examination of lung injury parameters. For example, the absence of bacteria in the bloodstream or pleural fluids would indicate prevention of infection. A reduction in lung injury parameters would indicate that infection is moderated.

Infection could be quantitatively assessed by several other clinical indicators, including the reduction of bacterial load in the sputum, blood or pleural fluids, reduction in the size of the infiltrate, oxygenation improvement, reduction in the length of time on mechanical ventilation, reduction in fever and reduction in white blood cell count.

By "PcrV antigen" we mean that portion or fragment of the PcrV protein that is necessary to invoke an immune response which prevents or moderates infection. We have used the full-length PcrV protein as an antigen to induce protection. Additionally, we have mapped the protective epitope to the fragment comprising amino acids 144-257 of PcrV. To define the epitope, monoclonal antibodies that protected against infection and cytotoxicity were tested for binding to progressively smaller forms of recombinant PcrV. (By "recombinant PcrV" or "rPcrV" we mean the protein produced from a PcrV gene that has been placed in a non-native host.) This protection localized the region.

The PcrV antigen may be most easily obtained by the method we used, commercially available bacterial expression plasmid pet16b from Novagen. The pcrV gene was first cloned from the *P. aeruginosa* chromosome as part of an operon. The coding region was amplified and inserted into two different vectors. One vector is for expression from *P. aeruginosa* as shown in Fig. 1. This is a vector from Herbert Schweizer (reference 19) which we modified to contain an appropriate promoter sequence such that PcrV expression is coordinately regulated with the rest of

the delivery and intoxication apparatus of the bacterium. The second plasmid, pET16b, is for expression and purification purposes from *E. coli*.

The advantage of this system is that we do not have to worry about contaminating *P. aeruginosa* proteins, the protein is produced in great abundance, and there is a one-step purification process. In this situation the PcrV coding region is amplified to be cloned in frame with a histidine tag provided on the pET16b vector. The multiple histidine residues fused to the amino terminus of PcrV allow affinity chromatography using a nickel-NTA column. Therefore, a preferable PcrV antigen is a recombinant version of the natural PcrV protein.

Immunization may be done systemically or intranasally. Immunization of these individuals would preferably start during normal vaccination procedures for other childhood diseases. We would predict long-lived protection with booster doses probably around ages 5 and 10.

In another embodiment, one would use DNA encoding the PcrV protein or the complement of this DNA to diagnostically detect *P. aeruginosa* infection. One would obtain the DNA sequence of the PcrV antigen at GenBank AF010149. The coding region for PcrV is at nucleotides 626-1510. One may also choose to use a fragment of this coding region or complement of this fragment. A successful probe is one that will hybridize specifically to the PcrV DNA and not to other regions.

One would preferably use a hybridization probe of at least 40 continuous nucleotides within the antigen sequence or two primers of at least 25 continuous nucleotides within the sequence. One skilled in the art would appreciate that many standard forms of nucleic acid diagnostic techniques would be suitable, for example, hybridization of the single-stranded 40 nucleotide probe to DNA or RNA extracted from a patient's sputum. In another example, patient's sputum would be used as a source for bacterial

DNA or RNA to serve as a template for the PCR or RT-PCR reaction, respectively.

One would also determine *Pseudomonas aeruginosa* infection in an individual by contacting a sample obtained from the individual with an antibody specific for PcrV and correlating enhanced antibody binding as compared with a control sample with *Pseudomonas aeruginosa* infection in the individual.

In an additional embodiment, the DNA encoding PcrV is used as a gene vaccine using standard molecular biological methods. For example, one could review the following references for techniques known to those of skill in the art: Davis, H.L., et al., "DNA vaccine for hepatitis B: Evidence for immunogenicity in chimpanzees and comparison with other vaccines," Proc. Natl. Acad. Sci. 93:7213-7218, 1996; Barry, M.A., et al., "Protection against mycoplasma infection using expression-library immunization," Nature 377:632-635, 1995; Xiang, Z.Q., et al., "Immune responses to nucleic acid vaccines to rabies virus," Virology 209:569-579, 1995. By "effective amount" of a gene vaccine, we mean an amount of vaccine effective to moderate or eliminate *Pseudomonas* infection or *Pseudomonas* infection symptoms.

The protein or antigen could also be used diagnostically to detect antibodies in patients and, thus, predict the patient's infection status. One would preferably contact a sample obtained from an individual suspected of *Pseudomonas* infection with the PcrV protein or fragment thereof and detect protein/antibody binding. One would then correlate enhanced antibody binding (as compared with a control sample) with *Pseudomonas aeruginosa* infection in the individual. One could also use the PcrV antibody or antibody fragments therapeutically.

In another embodiment, the invention is the use of the antibody sequence (which we report below and in SEQ ID NOs:1-4) to produce

recombinant single chain antibodies that may block PcrV and could also utilize the sequence in gene delivery experiments, where one would deliver eukaryotic vectors that will then lead to the production of single chain antibodies in animals for prolonged periods. The sequence could also be utilized to humanize the murine monoclonal antibody to produce a product that can be utilized in human patient care.

Once the antibody is safe for human use, one could: (a) administer it systemically and (b) administer it into the lungs as either a preventative treatment or as a therapy. In order to use the PcrV antibody in humans, the antibody is preferably "humanized". In general, once the monoclonal antibody is obtained the heavy and light chain variable regions are cloned. These cloned fragments are then inserted into a human antibody backbone (constant regions). Thus, we can control the class of antibody (IgG, IgA, etc.) in addition to providing the binding specificity.

For use in the present invention, the PcrV antibody may be a monoclonal antibody or polyclonal. The antibodies may be human or humanized, particularly for therapeutic applications. Antibody fragments or derivatives, such as an Fab, $F(ab')_2$ or Fv, may also be used. Single-chain antibodies, for example as described in Huston, et al. (Int. Rev. Immunol. 10:195-217, 1993) may also find use in the methods described herein. By "effective amount" of the PcrV antibody or antibody fragment we mean an amount sufficient to moderate or eliminate *Pseudomonas* infection or infection symptoms.

Preferably, human or humanized monoclonal or polyclonal antibodies to PcrV are administered to prevent or treat infections with *P. aeruginosa*. In patients at high risk for *P. aeruginosa* infection, antibodies could be administered for prevention of infection. In addition, antibodies may be administered after the onset of infection to treat the infection. In this case,

antibodies can be administered alone or in combination with antibiotics. Administration of antibodies in conjunction with antibiotics may allow the administration of shorter courses or lower doses of antibiotics, thereby decreasing the risk of emergence of antibiotic-resistant organisms.

We envision at least three types of hypothetical patients: (1) A healthy individual at risk of serious injury or burn (fire fighter, military personnel, police) would be immunized with the vaccine by a methodology (either injection or intranasal) that would give long-lived protection. A booster would be given on admission (intramuscular injection) to the hospital after injury. (2) A patient who is being subjected to mechanical ventilation. (3) A patient who has been genetically diagnosed with cystic fibrosis.

In addition to PcrV antibodies and antibody fragments, small molecule peptidomimetics or non-peptide mimetics can be designed to mimic the action of the PcrV antibodies in inhibiting or modulating *Pseudomonas* infection, presumably by interfering with the action of PcrV. Methods for designing such small molecule mimics are well known (see, for example, Ripka and Rich, Curr. Opin. Chem. Biol. 2:441-452, 1998; Huang, et al., Biopolymers 43:367-382, 1997; al-Obeidi, et al., Mol. Biotechnol. 9:205-223, 1998). Small molecule inhibitors that are designed based on the PcrV antibody may be screened for the ability to interfere with the PcrV-PcrV antibody binding interaction. Candidate small molecules exhibiting activity in such an assay may be optimized by methods that are well known in the art, including for example, *in vitro* screening assays, and further refined in *in vivo* assays for inhibition or modulation of *Pseudomonas* infection by any of the methods described herein or as are well known in the art. Such small molecule inhibitors of PcrV action should be useful in the present method for inhibiting or modulating a *Pseudomonas* infection.

In another aspect of the present invention, PcrV protein may be used to identify a PcrV receptor which may be present in the host cells, particularly in human cells, more particularly in human epithelial cells or macrophages. Identification of a PcrV receptor allows for the screening of small molecule libraries, for example combinatorial libraries, for candidates that interfere with PcrV binding. Such molecules may also be useful in a method to inhibit or modulate a *Pseudomonas* infection.

Our first attempts at receptor identification will be to use PcrV in pull-down experiments. PcrV will be fused to glutathione S-transferase (GST) and attached to column matrix for affinity chromatography of solubilized cellular extracts. Proteins binding specifically to PcrV will be eluted and subjected to amino terminal sequencing for identification. In parallel experiments PcrV will be subjected to yeast two-hybrid analysis. In this case PcrV is fused in frame with the DNA binding domain of Gal4. Once the clone is obtained it will be transformed into a suitable yeast host strain. The yeast strain containing the Gal4PcrV construct will be transformed with a Hela cell cDNA bank cloned in frame with the Gal4 activation domain. Double transformants that complement the ability to utilize histidine and produce beta galactosidase (proteins that interact with PcrV) will be analyzed genetically and at the nucleotide sequence level. In case the receptor is a cellular glycolipid we will utilize an overlay technique where glycolipids are separated by thin-layer chromatography and then probed with radiolabeled bacteria. The binding to specific components will be monitored by autoradiography. Similarly, epithelial and macrophage proteins will be separated by SDS-PAGE, blotted onto nitrocellulose and overlaid with radiolabeled bacteria or labeled PcrV. Again, the protein components to which the bacteria bind are then identified by autoradiography.

Pseudomonas species are known to infect a wide spectrum of hosts within the animal kingdom and even within the plant kingdom. As will be apparent to one of ordinary skill in the art, the compositions and methods disclosed herein may have use across a wide range of organisms in inhibiting or modulating diseases or conditions resulting from infection by a *Pseudomonas* species. The compositions and methods of the present invention are described herein particularly for application to *Pseudomonas aeruginosa* but it is well within the competence of one of ordinary skill in the art to apply the methods taught herein to other species.

EXAMPLES

1. Role of PcrV in Cytotoxicity

To determine the role of PcrV in type III-mediated regulation/secretion, we constructed a nonpolar allele of PcrV and used the construct to replace the wild-type allele in *P. aeruginosa* strain PA103, a strain that is highly cytotoxic *in vitro* (3) and causes lung epithelial damage *in vivo* (12, 13). Cytotoxicity and lung injury are due to the production of a specific cytotoxin, ExoU (3).

PA103Δ*pcrV* was characterized by the expression of several extracellular products that are secreted by the *P. aeruginosa* type III system which include the ExoU cytotoxin (3), PcrV (5), and a protein required for the translocation of toxins, PopD (14). SDS-polyacrylamide gel electrophoresis of concentrated culture supernatants indicated that the parental strain, PA103 is induced for production and secretion of the type III proteins by growth in medium containing a chelator of calcium, nitrilotriacetic acid (NTA) (Fig. 1). When an expression clone encoding PcrV was provided in *trans* in the parental strain, extracellular protein production in response to the presence or absence of NTA is normal. PA103Δ*pcrV* exhibits a calcium blind phenotype;

extracellular protein production is strongly induced in both the presence and absence of NTA. These results suggest that the secretory system is fully functional but deregulated. This deregulated phenotype is in contrast to the calcium independent phenotype reported for an LcrV defective strain which fails to produce the extracellular Yops, grows at 37°C regardless of the presence or absence of calcium, and shows only partial induction of the Yops (7). Complementing PA103Δ*pcrV* with a clone expressing wild-type PcrV restored normal regulation of extracellular protein production in response to NTA induction.

To test the contribution of PcrV to *P. aeruginosa* pathogenesis, two infection models were used. In an *in vitro* model the parental and several mutant derivative strains were compared for their ability to cause cytotoxicity in a CHO cell infection assay (3). The negative controls in this experiment included PA103*popD*::Ω, which has been previously shown to be defective in the translocation of type III virulence determinants (14) and PA103Δ*exoU*, which is non-cytotoxic due to the absence of ExoU production (3, 15).

After a 3 hour infection, CHO cells were unable to exclude trypan blue with the wild-type and Δ*pcrV* strain complemented with a plasmid construct expressing PcrV. Staining did not occur when CHO cells were infected with the negative control strains or with PA103Δ*pcrV* (data not shown). These results suggest that PcrV expression is required for cytotoxicity. Purified recombinant PcrV was not cytotoxic when added exogenously to tissue culture cells. Since secretion of the type III proteins required for translocation was unaffected by the deletion of *pcrV* (Fig. 1A and B), PA103Δ*pcrV* appears to be defective in ExoU translocation.

Figs. 1A and 1B are a stained gel (Fig. 1A) and Western blot (Fig. 1B) illustrating the phenotypic analysis of PA103ΔpcrV. The parental and Δ*pcrV* derivatives, with and without a plasmid expressing PcrV in *trans*, were grown

in the absence or presence of the inducer of type III secretion in *P. aeruginosa*, nitrilotriacetic acid (NTA). The extracellular protein profile (Fig. 1A) was analyzed on a SDS-polyacrylamide gel (10%) stained with Coomassie blue. The migration of the *P. aeruginosa*-encoded type III proteins is indicated to the left and the migration of molecular weight markers is indicated on the right. Fig. 1B is a Western blot of a duplicate gel using antibodies specific for ExoU, PcrV, and PopD and ^{125}I-Protein A to detect bound IgG.

Wild-type and mutant *P. aeruginosa* strains were tested in an acute lung infection model using low and high challenge doses of bacteria. Survival measurements indicated that PcrV and PopD were required to induce a lethal infection (Fig. 2A). In experiments utilizing three independent measurements of lung injury (the flux of labeled albumin from the airspaces of the lung to the bloodstream, the flux of labeled albumin from the airspaces of the lung to the pleural fluids, and the wet/dry ratio, which measures lung edema) the degree of injury caused by PA103Δ*pcrV*, the vector control strain (PA103Δ*pcrV*pUCP18), and PA103*popD*::Ω were no different than the uninfected control animals (Fig. 2B). Complementation of PA103Δ*pcrV* with *pcrV in trans* restored lung injury levels to those measured with the parental strain, PA103. Taken together these data indicate that PcrV expression is required for virulence of *P. aeruginosa* in the acute lung infection model and that part of the function of PcrV appears to be linked to the ability to translocate type III effector proteins into eukaryotic cells.

Figs. 2A and 2B are a graph (Fig. 2A) and set of bar graphs (Fig. 2B) illustrating the survival and lung injury of *P. aeruginosa* parental and mutant strains. Referring to Fig. 2A, mice were challenged with 5 x 10^5 cfu of each of the indicated strains and survival was monitored for one week. Referring to Fig. 2B, lung injury was assessed by the flux of labeled albumin from the

airspaces of the lung to the blood (lung epithelial injury), to the pleural fluid (pleural fluid) or by measuring the wet/dry ratio (lung edema). Two bacterial infectious doses were used as denoted by the solid and striped bars. Significant differences (*p<0.001) between control and test groups was determined by one-way ANOVA and Dunnet multiple comparison tests. The following abbreviations were used: PA103, parental wild-type strain; Δ*pcrV*, PA103Δ*pcrV*; Δ*pcrV*pUCP*pcrV*, PA103Δ*pcrV* complemented with a plasmid expressing *PcrV*; Δ*pcrV*pUCP, PA103Δ*pcrV* with a vector control; *popD*::Ω, PA103*popD*::Ω, a translocation defective strain.

2. Immunization with PcrV

To determine whether immunization with PcrV protected animals from a lethal lung infection, recombinant PcrV (rPcrV) or ExoU (rExoU) were purified as histidine-tagged fusion proteins and used as antigens. Mice were immunized and subsequently challenged via their airspaces with a lethal dose of strain PA103. When survival was measured, both vaccines protected the mice (Fig 3A). When lung injury was assessed, only PcrV vaccinated animals had significantly less epithelial damage and lung edema (Fig. 3B). Animals immunized with the PcrV vaccine also had significantly fewer bacteria in their lungs, suggesting that a blockade of the *Pseudomonas* V antigen may facilitate rapid clearance of bacteria from the lung, protecting the animals from severe epithelial injury (Fig. 3B).

Figs. 3A and 3B are a graph (Fig. 3A) and a set of bar graphs (Fig. 3B) illustrating the effect of immunization on survival, lung injury, and bacterial colonization. Referring to Fig. 3A, mice were immunized (PcrV, n=10; ExoU, n=5; control, n=10) as indicated and challenged with strain PA103 at 5 $x10^5$ CFU/animal. The percent of surviving animals was determined for one week; $p<0.05$ by the Mantel-Cox log rank test. Referring to Fig. 3B, lung injury assessment and bacterial colonization of vaccinated animals 4 hours after

installation of PA103. Lung epithelial injury, lung edema, and bacterial burden; PcrV, n=9; ExoU, n=4; and control, n=8. The final number of bacteria in the lung is indicated as the number on the Y axis x 10^4 CFU. Significant differences (*) for lung injury ($p<0.01$), lung edema ($p<0.05$), and bacterial numbers ($p<0.05$) as determined by Dunnet multiple comparison test. One-way ANOVA for lung injury, p=0.0005; lung edema, p=0.0437; bacterial burden, p=0.0075.

To determine whether therapeutic intervention was possible, mice were passively immunized with preimmune rabbit IgG or rabbit IgG specific for rPcrV, rExoU, or rPopD one hour prior to airspace instillation of PA103 at a concentration of 5 x10^5 CFU/mouse. Antibodies to rPcrV provided complete protection to a lethal infection (Fig. 4). Anti-rExoU IgG provided partial survival, which was significantly different from the administration of control IgG, although all the surviving animals appeared severely ill during the trial. Survival was not improved by the passive transfer of antibodies to another of the type III translocation proteins, PopD. From these results we conclude that antibodies to PcrV are highly protective in the acute lung infection model and that PcrV may be exposed on the bacterial surface or in a soluble form that is available for antibody-antigen interactions.

Fig. 4 is a graph of the number of animals surviving a challenge with 5 x 10^5 CFU/mouse of strain PA103. Animals were pretreated with 100 µg of immune IgG or control IgG from an unimmunized rabbit (rPcrV, preimmune serum). N=10 for each group; *$p<0.05$ versus control group for treatment with anti-PcrV and anti-ExoU IgG preparations by Mantel-Cox log rank test.

If PcrV is accessible for neutralization, then concomitant administration of the bacterial inoculum with anti-rPcrV IgG should completely protect against lung injury and lethality. IgG preparations were mixed with the inoculum (10-fold higher dose than the lethal inoculum) prior to instillation of

the bacteria into the lung and survival was measured. Only anti-rPcrV IgG was protective against this extreme infection (Fig. 5A). Lung injury was measured in animals infected with the normal lethal dose of 5×10^5 bacteria. The efflux of labeled albumin from the airspaces of the lung was only 3% more than uninfected controls (Fig. 5B) after co-administration of anti-rPcrV IgG. The decreased efflux of labeled protein from the lung to the pleural fluids was the same as the uninfected controls when anti-PcrV was included with the inoculum. Curiously lung edema, as measured by the wet/dry ratio, was significantly reduced by the addition of either anti-rPcrV or anti-rPopD. (Fig. 5B). Thus, the concomitant administration of anti-rPcrV IgG with the bacteria was even more effective in normalizing all the lung injury parameters than vaccination. These data support the accessibility of PcrV for antibody-mediated neutralization and document a clinically relevant decrease in lung injury; antibodies to PcrV may serve as therapeutic reagents in the treatment of severe nosocomial pneumonia caused by *Pseudomonas aeruginosa*.

Fig. 5 is a graph (Fig. 5A) and a set of bar graphs (Fig. 5B) illustrating survival and protection from lung injury by concomitant administration of IgG and bacterial challenge. IgG (5 μg) was mixed with either 5×10^6 (for survival assays, n=10 per group) or 5×10^5 (for the measurement of lung injury, n=4 to 6 animals per group) *P. aeruginosa* strain PA103. This mixture was instilled into the lungs and survival (Fig. 5A) or lung injury (Fig. 5B) was assessed. For survival, *$p<0.05$ versus control IgG for anti-PcrV by the Mantel-Cox log rank test; for lung epithelial injury and lung edema *$p<0.05$ versus control IgG by Dunnet multiple comparison test. One-way ANOVA for lung injury, $p=0.026$, and lung edema, $p<0.0005$.

In acute *P. aeruginosa* infections, the net effect of type III-mediated intoxication may be to promote the dissemination of the bacterium beyond the epithelium leading to infection of the pleural fluids, spleen, liver, and

bloodstream. Blood-borne infections with *P. aeruginosa* from either acute ventilator-associated pneumonia or from burn wound infections can result in a 40-80% mortality rate in spite of aggressive antibiotic treatment (16). PcrV must be a component of the type III translocation complex in *P. aeruginosa*, as mutants defective in the production of this protein are unable to intoxicate CHO cells or cause lung epithelial injury even though they are able to produce and secrete the type III effectors and proteins required for translocation. Unlike PopD, which is also necessary for translocation, PcrV is accessible for antibody-mediated neutralization suggesting that antibodies may be useful therapeutic agents in acute infections.

3. Methods for Examples 1 and 2

Construction of a nonpolar insertion in *PcrV* and complementation. A 5.0-kb *Eco*RI-*Nsi*I restriction fragment encoding *pcrGVHpopBD* and flanking sequences was cloned into the allelic replacement vector pNOT19 (17). Two *Not*I sites (one within *pcrG* and one within *popB*) were removed from the inserted sequences by using the Sculptor mutagenesis system (Amersham). An internal *Sst*I restriction fragment was deleted from *pcrV*, resulting in an in-frame deletion of residues 17-221 (pNOTΔ*pcrV*). To select for integration of the plasmid, a gene encoding tetracycline resistance (TcΩ) was cloned into the *Hind*III site of the vector (pNOTΩΔpcrV). The MOB cassette (17) was added as a *Not*I fragment. Selection of merodiploids, resolution of plasmid sequences, and confirmation of allelic replacement was performed as previously described (18). A shuttle plasmid (pUCP, 19) was used to construct a clone to complement the *pcrV* deletion. The coding sequence for PcrV was amplified and cloned behind the control of the ExoS promoter region (20). The transcription of ExoS is coordinately regulated with the operons that control type III secretion and translocation in *P. aeruginosa* (2).

The nucleotide sequence was confirmed for each DNA construct involving site specific mutagenesis, PCR amplification, or in-frame deletion.

SDS-PAGE and Western blot analysis of secreted products. *P. aeruginosa* were grown under inducing (+NTA) or non-inducing conditions (-NTA) for expression of the type III secreted products (18). Cultures were harvested based on optical density measurements at 540 nm and supernatant fractions were concentrated by the addition of a saturated solution of ammonium sulfate to a final concentration of 55%. Each lane of an SDS-polyacrylamide gel (11%) was loaded with 3 µl of a 20-fold concentrated supernatant and stained with Coomassie blue. An identical gel was subjected to Western blot analysis as previously described (3-5) using a cocktail of rabbit antisera, which specifically recognizes ExoU, PopD, and PcrV. Protein A labeled with ^{125}I was used as a secondary reagent to identify bound IgG.

Infection models and lung injury assessments. Chinese Hamster Ovary cells (CHO) were used in an *in vitro* infection model designed to measure cytotoxicity and type III translocation (21). Briefly, a bacterial inoculum was prepared in tissue culture medium without serum. CHO cells, which were propagated in serum containing medium, were washed and infected with various *P. aeruginosa* strains at a multiplicity of infection of 5:1. Cultures were incubated under tissue culture conditions for 3 hours (37°C, 5% CO_2), washed, and stained with trypan blue. Permeability to the dye was determined from phase contrast photographs. Infection with the parental strain PA103, which expresses ExoU, results in trypan blue staining of approximately 80% of the monolayer after 3 hours of incubation and complete destruction of the monolayer at 4-5 hours of incubation. Mouse infections and assessment of lung injury was performed as previously described (16). Briefly, male 8- to 12-week old pathogen-free BALB/c mice were purchased

from Simonsen Laboratories (Gilroy, CA) and housed in barrier conditions. The mice were briefly anesthetized with inhaled Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL) and placed supine, at an angle of approximately 30°. Fifty microliters of the bacterial inoculum was instilled slowly into the left lobe using a modified 24 gauge animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY) inserted into the trachea via the oropharynx. When lung injury assessments were measured, 0.5 μCi of ^{131}I-labeled human serum albumin (Merck-Frosst, Quebec, Canada), 0.05 μg of anhydrous Evans blue in ml of Ringer's lactate with 5% mouse albumin were added to the instillate. After 4 hours of infection, the mice were anesthetized, blood was collected by a carotid arterial puncture and median sternotomies were performed. The lungs, pleural fluids, tracheas, oropharynxes, stomachs, and livers were harvested, and the radioactivity was measured. The percentage of radioactive albumin that left the instilled lungs and entered the circulation or the pleural fluid was calculated by multiplying the counts measured in the terminal blood samples (per ml) times the blood volume (body weight X 0.07). The wet-dry ratios of the lungs were determined by adding 1 ml of water to the lungs and homogenizing the mixture. Homogenates were placed in preweighed aluminum pans and dried to constant weight in an 80°C oven for three days. Lung homogenates were also sequentially diluted and plated on sheep blood agar for quantitative assessment of bacteria.

Production of rabbit antiserum to PcrV, PopD, and ExoU. rPcrV, rPopD, and rExoU were produced as histidine tagged fusion proteins in pET16b and purified by nickel chromatography as previously described (22). Rabbits were injected intradermally (10 sites) with 300 μg of recombinant protein emulsified in Freund's complete adjuvant, boosted with antigen in Freund's incomplete adjuvant, and periodically bled at 7 day intervals. For

passive immunization, the IgG fraction was isolated using Protein A column chromatography (Pierce Chemicals, Rockford, IL). Mice were injected with 100 µg IgG (intraperitoneal injection) 1 hour before challenge with 5 x 10^6 CFU of strain PA103. For active immunization with rPcrV and rExoU, endotoxin was removed from protein preparations by extraction with 1% Triton X-114 (23). Following the extractions, Triton X-114 was removed by Sephacryl S-200 chromatography. All vaccine preparations contained less than 1 ng of endotoxin per 40 µg of recombinant protein as determined by using a limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). BALB/c mice were injected subcutaneously with 10 µg of recombinant proteins in Freund's complete adjuvant. At day 30 the mice were boosted with an additional 10 µg of antigen in Freund's incomplete adjuvant. On day 51 the mice were challenged by instillation of *P. aeruginosa* into their left lungs.

4. Synthesis of Monoclonal Antibodies

Mice were immunized with 10 µg of purified, LPS-free, recombinant PcrV in Freund's complete adjuvant and boosted two weeks later with the same dose of antigen emulsified in Freund's incomplete adjuvant. Immunizations were performed subcutaneously. Spleens were harvested from mice one week after booster doses of PcrV in Freund's incomplete adjuvant.

A single spleen was placed in 5 ml of tissue culture medium without serum, cut into pieces and gently homogenized. Large pieces of tissue were allowed to settle from the homogenate and the supernatant, single-cell suspension was removed and subjected to centrifugation at 1200 rpm for 10 minutes. The pelleted cells were resuspended in 10 ml of a solution to lyse red blood cells for 5 minutes and subsequently underlaid with 10 ml of fetal bovine serum. The material was centrifuged at 1200 rpm for 8 minutes, the

supernatant was discarded and the cells were suspended in 30 ml of medium.

Spleenic cells and myeloma cells (P3x63Ag8.653) were harvested by centrifugation at 1200 rpm for 10 minutes, and each pellet was separately suspended in 10 ml of tissue culture medium. 10^8 spleen cells and 2×10^7 myeloma cells were mixed and pelleted together by centrifugation at 1200 rpm for 6 minutes. The supernatant was removed by aspiration and 1 ml of 35% polyethylene glycol (PEG) was added. The cells were suspended in this solution gently and centrifuged at 1000 rpm for 3 minutes. In some experiments centrifugation was eliminated.

Exactly 8 minutes after the addition of PEG, 25 ml of medium was added and the cells were gently resuspended. Following a 5 minute 1200 rpm centrifugation step, the cell pellet was suspended at a density of 1×10^6 per ml in 30% conditioned medium and 70% complete medium (with serum). The cells were incubated overnight at 37°C. The next day the cells were harvested by centrifugation and suspended in 200 ml of 30% conditioned medium and 70% complete medium with hypoxanthine, aminopterin and thymidine (HAT).

Approximately 0.2 ml of this cell suspension was added per well to ten 96-well plates (12 ml per 96 well plate). The density of the remaining cells was adjusted to 2.5×10^5 per ml and the cells were plated in the 96 well format. Plates were screened microscopically for single colonies and supernatants were subsequently tested for antibody production by enzyme-linked immunosorbent assay using recombinant PcrV as the antigen. Clones producing antibodies reactive to PcrV were subcultured to larger culture dishes and then isotyped.

The binding of antibodies was tested in an enzyme linked immunosorbent assay using recombinant PcrV as the antigen (histidine-

tagged protein) coating the wells. Monoclonal antibodies were also tested in Western blot reactions using a *P. aeruginosa* supernatant containing native PcrV without the histidine tag.

5. Identification of PcrV Antigen

We obtained about three hundred cell lines producing antibodies that bound the tagged PcrV. These initial cell lines were preserved in liquid nitrogen for safekeeping. All cell lines were passaged to isolate stable clones. In conjunction with isolating stable clones we developed in *in vitro* assay as a correlate for protection against intoxication in animal infection models.

The hybridomas that were stable to passage and still produced antibodies reactive to PcrV in ELISA (approximately 80 cell lines) were subsequently tested in a Fluorescence Activated Cell Sorter using the following techniques and assumptions: We reasoned that if antibody is blocking the type III intoxication system, then in the presence of a monoclonal that blocks, fewer cells will be killed by our toxins. We exposed cells to each of the 80 monoclonal antibodies, added toxic bacteria, incubated, and then added a dye that is only permeable to dead cell DNA (propidium iodide). Excess dye was washed away and the cells were harvested, fixed, and analyzed by FACS. Dead cells would be fluorescent since the dye leaked in and stained DNA in the nucleus.

We found that if the cells were incubated with rabbit polyclonal anti-PcrV, mouse polyclonal anti-PcrV, or mab166 and bacteria, fewer cells died than in controls with irrelevant polyclonal antibody (anti-PopD) or the other 78 monoclonal antibodies.

Mab 166 was specifically found to bind to the bacterially encoded type III-secreted factor termed PcrV. PcrV mediates the interaction of *P. aeruginosa* and lung cells to facilitate the translocation of bacterial toxins that cause cellular death. This reaction is postulated to eliminate lung cells that are involved in the innate immune response to *P. aeruginosa*. The killing of these cells leaves the host epithelium open for *P. aeruginosa* colonization

and spread to the pleural fluids and bloodstream. *P. aeruginosa*-encoded antibiotic resistance makes effective treatment unlikely once the bacteria have entered the bloodstream.

The protection afforded by mab 166 pre- and post-bacterial instillation in animal models of acute lung infection with *P. aeruginosa* is significant. To design antibody treatment modalities for intervention in human *P. aeruginosa* infections it will be necessary to produce either a human monoclonal antibody or to immunize at risk patients with the protective epitope of PcrV defined by mab 166. The goal of the work described below is to define the amino acid sequence of PcrV bound by mab 166.

Results

We used a molecular genetic approach to define the amino acid residues bound by mab 166. PcrV possesses 294 amino acids. The approach consisted of deleting parts of the molecule at the nucleotide sequence level using the polymerase chain reaction. Each product was cloned into a protein expression vector in frame with a gene encoding the glutathione S transferase protein. This strategy ensured that deletions encoding small numbers of PcrV amino acids could be detected using Western or dot blot techniques. Control bacterial lysates encoding only glutathione S transferase showed no reactivity to either our anti-PcrV polyclonal or mab 166 monoclonal antibody.

A total of 66 (with one full-length PcrV expression plasmid) clones were constructed, expressed, and tested for reactivity to rabbit polyclonal anti-PcrV antisera. All but one clone bound to anti-PcrV rabbit antibody verifying that the expressed proteins were in-frame with PcrV. The one non-reactive clone was eliminated from the analysis. None of the C-terminal deletions (n = 5 constructs) bound mab 166 suggesting that the epitope was in the C-terminal half of the protein. Only one of the N-terminal truncation

proteins (n = 8 constructs) encoding PcrV amino acids (aa) 139-294 bound to mab 166. This experiment verified our hypothesis that the mab 166 epitope was encoded by the carboxyl terminal half of the protein. The remaining 51 constructs encoded various internal deletions of the molecule. Binding analysis tabulated in Table 1, below, demonstrated that the smallest epitope recognized by mab 166 consists of aa 144-257 of PcrV.

TABLE 1

PcrV Epitope Mapping		
All proteins are amino-terminal tagged GST-PcrV truncates.		
Amino Acids	Binding to pAb	Binding to mAb 166
1-294 (full-length)	yes	yes
(C-term truncates)		
1-46	yes	no
1-76	yes	no
1-134	yes	no
1-172	yes	no
1-75 + 173-294	yes	no
(N-term truncates)		
139-294	yes	yes
148-294	yes	no
159-294	yes	no
164-294	yes	no
194-294	yes	no
261-294	yes	no
269-294	yes	no
278-294	yes	no

Amino Acids	Binding to pAb	Binding to mAb 166
(internal fragments)		
139-191	yes	no
139-195	yes	no
139-234	yes	no
139-243	yes	no
139-256	yes	no
139-257	yes	yes; weak
139-258	yes	yes
139-259	yes	yes
139-260	yes	yes
139-261	yes	yes
139-262	yes	yes
139-263	yes	yes
139-264	yes	yes
139-265	yes	yes
139-266	yes	yes
139-274	yes	yes
139-281	yes	yes
140-266	yes	yes
141-266	yes	yes
142-266	yes	yes
143-266	yes	yes
144-266	yes	yes
145-266	yes	no
146-266	yes	no
147-266	yes	no
148-170	no*	no
148-202	yes	no
159-202	yes	no
159-209	yes	no
159-216	yes	no
159-226	yes	no
159-234	yes	no
164-234	yes	no
164-243	yes	no
164-256	yes	no
164-266	yes	no
164-275	yes	no
164-281	yes	no
194-234	yes	no
194-243	yes	no
194-256	yes	no
194-266	yes	no
194-275	yes	no
194-281	yes	no

Amino Acids	Binding to pAb	Binding to mAb 166
141-258	yes	yes; weak
142-258	NT**	yes
143-258	yes	yes
144-258	yes	yes
141-257	yes	yes
142-257	yes	yes
143-257	yes	yes
144-257	yes; weak	yes; weak

NOTES:
*Truncate 148-170 is the only one that is not recognized by the rabbit polyclonal control antibody.
**NT; Not Tested due to an insufficient amount of bacterial lysate.
-As predicted, pGEX-4T-2 vector control lysates were not recognized by either antibody.
-The smallest epitope of PcrV recognizable by mAb166 appears to consist of amino acids 144-257.

6. Examination of PcrV-Specific Antibody

Methods:

Poly A+ RNA extraction: Hybridoma cell line m166 was cultured in complete Dulbeccos minimum essential medium with 4.5 g/L D-glucose, 10 mM HEPES, 50 μM 2-mercaptoethanol, 3 mM L-glutamine, and 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate. After the cells reached confluent state in a 75 cm^2 flask, the cells were harvested from centrifuging at 600 rpm for 5 minutes. The pellet of the cells was homogenized in 2 mL of TRIzol reagent (Life Technologies, Gaithersburg, MD), and total RNA (100 μg) was extracted after chloroform fractionation, isopropanol precipitation and 70% ethanol wash. Poly A+ RNA (4 μg) was extracted with oligotex mRNA spin-column (Qiagen, Valencia, CA).

RNA oligo-capping: mRNA (250 ng) was incubated with calf intestinal phosphatase at 50°C for 1 hour to dephosphorylate non-mRNA or truncated mRNA. After the reaction, phenol/chloroform extraction and ethanol precipitation was performed and the dephosphorylated RNA was incubated with tobacco acid pyrophosphatase at 37°C for 1 hour to remove the 5'-cap structure from full-length mRNA. After phenol/chloroform extractions and ethanol precipitation, the synthetic RNA oligo (GeneRacer RNA Oligo, Invitrogen, Carlsbad, CA) was ligated to the decapped RNA with T4 RNA

ligase at 37°C for 1 hour. After phenol/chloroform extraction and ethanol precipitation, the RNA was suspended in 13 µL diethylpyrocarbonate-treated water.

Reverse-transcribing mRNA: The RNA-oligo ligated, full-length mRNA (13 µL) was reverse-transcribed with 54 base the pair primer containing a dT tail of 18 nucleotides (GeneRacer Oligo dT, Invitrogen), and Avian myeloblastosis virus reverse transcriptase at 42°C for 1 hour in 20 µL reaction. After the reaction, the sample was diluted 4 times with sterile water.

Amplifying cDNA ends by polymerase chain reaction (PCR): One microliter of the cDNA was used for PCR. The 5' primer from the synthetic RNA oligo sequence (GeneRacer 5' Primer, Invitrogen) and the murine immunoglobulin gamma 2b chain CH1 region specific primer or the murine immunoglobulin kappa chain CL region specific primer were used. The cycling parameters used for the PCR reaction was; 1) 94°C, 2 minutes, 1 cycle, 2) 94°C, 30 seconds and 72°C, 1 minute, 5 cycle, 3) 94°C, 30 seconds, 70°C, 30 seconds, and 72°C, 1 minutes, 5 cycle, 4) 94°C, 30 seconds, 68°C, 30 seconds, and 72°C, 1 minutes, 20 cycle, 5) 72°C, 10 minutes.

Subcloning and DNA sequencing: PCR products (the murine immunoglobulin gamma 2b chain CH1 region derived fragment and the murine immunoglobulin kappa chain CL region derived fragment) were subcloned into the pCRII vector (TOPO cloning, Invitrogen) and submitted to UCSF Molecular Bioresource Center to analyze the DNA sequence.

SEQ ID NO:1 is the DNA sequence of m166 heavy chain mRNA, SEQ ID NO:2 is the amino acid sequence of the m166 heavy chain (IgG II_b), SEQ ID NO:3 is the DNA sequence of the m166 light chain mRNA, and SEQ ID NO:4 is the amino acid sequence of the m166 light chain. Figs. 6A, 6B and 7 examine the sequences and supply more detail.

Commercial Implications

One could use the antibody sequence to produce recombinant single chain antibodies that may block PcrV and could also utilize the sequence in gene delivery experiments, where one would deliver eukaryotic vectors that will then lead to the production of single chain antibodies in animals for prolonged periods. Finally, the sequence could be utilized to humanize the murine monoclonal antibody to produce a product that can be utilized in human patient care. One of skill in the art would look to standard methods such as grafting the antigen binding complementarity determining regions (CDRs) from variable domains of rodent antibodies on to human variable domains in order to create a humanized antibody.

7. Single Chain Antibody Against PcrV

a. Assembling a single chain antibody:

VH gene and VL gene were multiplied by polymerase chain reaction (PCR) with specific primers for each gene. Multiplied VH and VL fragments were assembled with a linker by using PCR with primers. The assembled single chain antibody gene (scFv::m166:VH and VL genes with linker) was transferred into the cloning vector pCR4 Topo (Invitrogen, Carlsbad, CA). Then, the coding sequence of scFv::m166 was subcloned into the *E. coli* expression vector pBAD/gIII (Invitrogen) in LMG194 as the host *E. coli*.

b. Protein induction and purification:

For protein induction, in RM medium containing 0.2% glucose and 100 µg of ampicillin, the transformed *E. coli* was cultured overnight at 37°C in an orbital shaker (200 rpm), and the next day, 5 mL of the cultured *E. coli* was transferred into 500 mL of the same medium and incubated for 3 hours at room temperature at 100 rpm. After L-arabinose was added at the concentration of 0.004%, the *E. coli* was cultured overnight. The third day, the protein was harvested from the periplasmic space of the *E. coli* by

osmotic shock methods. The solution including osmotic shock derived periplasmic protein was dialyzed overnight against the lysis buffer. During the fourth day, the dialyzed solution was applied onto a nickel-NTA column to purify the hexahistidine-tagged single chain antibody. The eluted solution from the nickel column was dialyzed against phosphate buffered saline overnight. On the fifth day, the dialyzed solution was applied to a centrifuge concentrator to make a higher concentration of scFv:m166.

c. The binding test:

The purified single chain antibody (scF::m166) was tested by using an enzyme linked immunosorbent assay against recombinant PcrV and by immunoblot (western blot) against both recombinant PcrV protein and native PcrV of *P. aeruginosa* PA103.

The single chain antibody will allow us to humanize the antibody utilizing phage-display techniques and to improve affinity of the antibody using these techniques. The single chain antibody can be utilized as a diagnostic tool (for histology) but would not be utilized as a therapy. However, the gene for the single chain antibody can be utilized in gene therapy, so that animals would produce single-chain antibodies over an interval, which could lead to protection against *P. aeruginosa* infections.

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CLAIMS

We claim:

1. A method of inhibiting *Pseudomonas aeruginosa* infection comprising inoculating a patient with an effective amount of PcrV antigen.

2. The method of claim 1 wherein the PcrV antigen is a fragment of the PcrV protein, said fragment capable of inducing an immune response specific to the V antigen.

3. The method of claim 1 wherein the patient is inoculated with a gene vaccine comprising DNA encoding PcrV.

4. The method of claim 3 wherein the DNA encodes a fragment of the PcrV protein, said fragment capable of inducing an immune response specific to the PcrV antigen.

5. The method of claim 1 wherein the patient is a human patient.

6. A method of diagnosing *Pseudomonas aeruginosa* infection comprising the step of exposing a patient's sample to a nucleotide probe, wherein the probe hybridizes specifically to a PcrV-encoding nucleic acid and not to other nucleic acids.

7. The method of claim 6 wherein the patient is a human patient.

8. A method of diagnosing a *Pseudomonas aeruginosa* infection comprising the steps of

a) exposing a patient's sample to nucleotide primers designed to amplify the *pcrV* gene,

b) performing a polymerase chain reaction, wherein the *pcrV* gene is amplified if present in the sample, and

c) correlating *Pseudomonas aeruginosa* infection with the presence of an amplified product.

9. The method of claim 8 wherein the patient is a human patient.

10. A method of diagnosing a *Pseudomonas aeruginosa* infection, comprising the steps of:

a) exposing the patient sample to a PcrV antigen, and

b) correlating *Pseudomonas aeruginosa* infection with the presence of a PcrV-specific antibody/antigen complex.

11. A method of inhibiting *Pseudomonas aeruginosa* infection comprising inoculating a patient with an effective amount of a gene vaccine, wherein the gene vaccine encodes PcrV antigen.

12. The method of claim 11 wherein the gene vaccine encodes the entire PcrV protein.

13. The method of claim 11 wherein the gene vaccine encodes a fragment of the PCR PcrV protein, wherein the fragment is capable of inducing an immune response specific to the PcrV antigen.

14. The method of claim 11 wherein the patient is a human patient.

15. A method of treating or preventing *Pseudomonas aeruginosa* infection comprising the steps of obtaining a humanized or human PcrV antibody or antibody fragment, and administering the antibody systemically, wherein the antibody inhibits or prevents *Pseudomonas aeruginosa* infection.

16. A method of treating or preventing *Pseudomonas aeruginosa* infection comprising the steps of obtaining a humanized or human PcrV antibody or antibody fragment and administering the antibody to the lungs as a therapeutic agent.

17. A method of treating a *Pseudomonas aeruginosa* infection comprising the step of inoculating a *Pseudomonas aeruginosa*-infected patient with an effective amount of PcrV antigen.

18. An antibody specific for the PcrV antigen.

19. The antibody of claim 18, wherein the antibody is a monoclonal.

20. The antibody of claim 19, wherein the antibody is mab 166.

21. An anti-PcrV monoclonal antibody or fragment thereof that recognizes an epitope that includes amino acid residues 144 and 257 in the PcrV polypeptide amino acid sequence.

22. An anti-PcrV monoclonal antibody or fragment thereof that recognizes an epitope that includes amino acid residues 144 through 257 in the PcrV polypeptide amino acid sequence.

23. A monoclonal antibody or fragment thereof comprising the CDR's of the light chain polypeptide amino acid sequence shown in Fig. 7.

24. A monoclonal antibody or fragment thereof comprising the CDR's of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.

25. A monoclonal antibody or fragment thereof comprising the CDR's of the light chain polypeptide amino acid sequence shown in Fig. 7 and the CDR's of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.

26. The monoclonal antibody or fragment of any of claims 23 and 25 that further comprises the FR regions of the light chain polypeptide amino acid sequence shown in Fig. 7.

27. The monoclonal antibody or fragment of any of claims 24 and 25 that further comprises the FR regions of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.

28. The monoclonal antibody or fragment of claim 25 that further comprises the FR regions of the heavy chain polypeptide amino acid sequence shown in Fig. 6B and the FR regions of the light chain polypeptide amino acid sequence shown in Fig. 7.

29. The antibody or fragment of any of claims 21-28 that is humanized.

30. The antibody or fragment of any of claims 21-28 that is human.

31. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 and a pharmaceutically acceptable carrier.

32. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 in an amount effective for treating or preventing Pseudomonas infection in a patient.

33. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 in an amount effective for reducing the pathogenicity of Pseudomonas in a patient.

34. A method for treating or preventing Pseudomonas infection in a patient comprising administering to the patient an effective amount of the composition of claim 31 or 32.

35. A method for reducing the pathogenicity of Pseudomonas in a patient comprising administering to the patient an effective amount of the composition of claim 31 or 33.

36. A method for modulating the cytotoxicity of Pseudomonas to a human cell comprising contacting said Pseudomonas with the antibody or fragment of any of claims 21-30 in the presence of the human cell.

37. A nucleic acid encoding the antibody or fragment of any of claims 21-30.

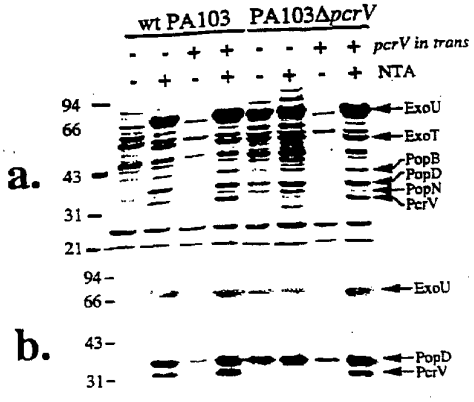
wt PA103
PA103ΔpcrV
pcrV in trans
NTA
a.
b.
94
66
43
31
21
ExoU
ExoT
PopB
PopD
PopN
PcrV

FIG. 1

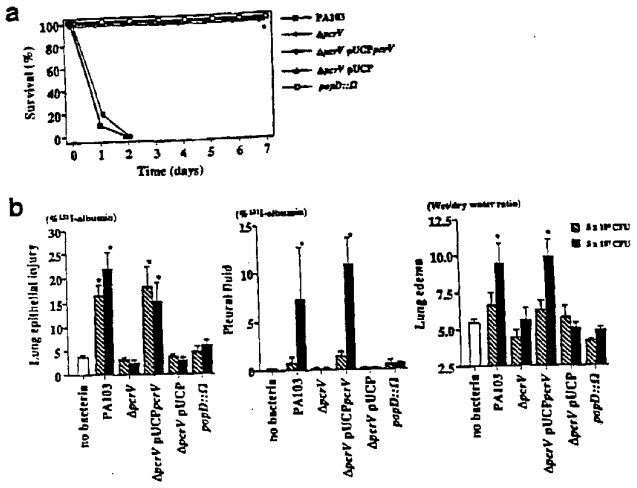
a
Survival (%)
Time (days)
PA103
ΔpcrV
ΔpcrV pUCPpcrV
ΔpcrV pUCP
popD::Ω
b
Lung epithelial injury
Pleural fluid
Lung edema
no bacteria
PA103
ΔpcrV
ΔpcrV pUCPpcrV
ΔpcrV pUCP
popD::Ω

FIG. 2

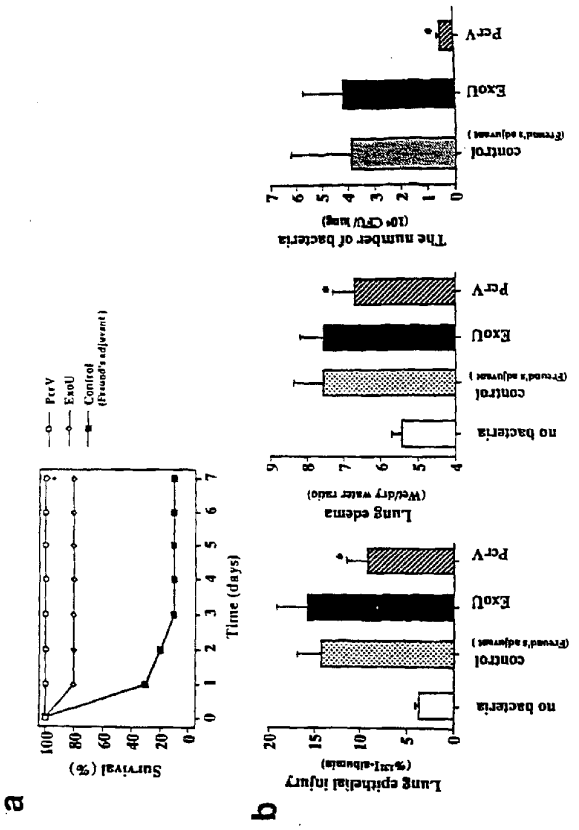
a
Survival (%)
Time (days)
PcrV
ExoU
Control
(Freund's adjuvant)
b
Lung epithelial injury
(%Rel-albumin)
Lung edema
(Wet/dry water ratio)
The number of bacteria
(10⁶ CFU/ lung)
no bacteria
control
(Freund's adjuvant)
ExoU
PcrV

FIG. 3

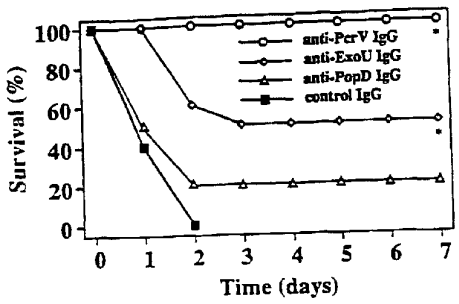
100
80
60
40
20
0
Survival (%)
0 1 2 3 4 5 6 7
Time (days)
anti-PcrV IgG
anti-ExoU IgG
anti-PopD IgG
control IgG

FIG. 4

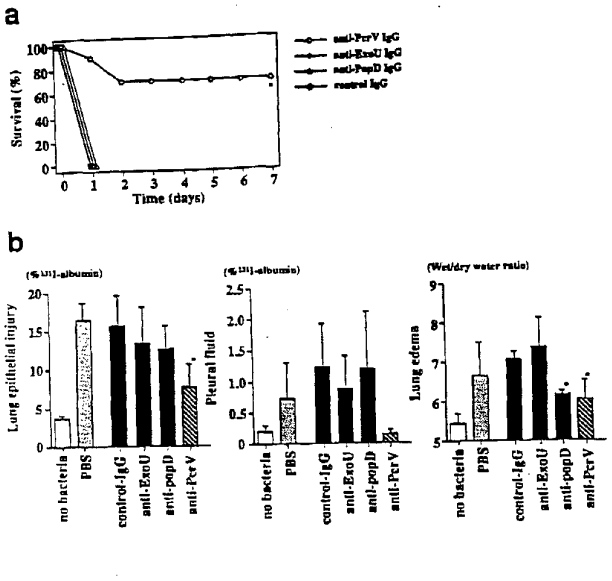
a
Survival (%)
Time (days)
anti-PcrV IgG
anti-ExoU IgG
anti-PopD IgG
control IgG
b
Lung epithelial injury
Pleural fluid
Lung edema
(Wet/dry water ratio)
no bacteria
PBS
control-IgG
anti-ExoU
anti-popD
anti-PcrV

FIG. 5

m166 heavy chain

1. m166 heavy chain (IgG2b) complete mRNA sequence:

(From the transcriptional start point to the polyA-tail)

```
CCATCCTCTT CTCATAGAGC CTCCATCAGA GCATGGCTGT CTTGGGGCTG
CTCTTCTGCC TGGTGACATT CGCAAGGTGT GTCCTATCCC AGGTGCAGCT
GAAGCAGTCA GGACCTGGCC TAGTGCAGCC CTCACAGAGC CTGTCCATCA
CCTGCACAGT CTCTGGTTTC TCATTAACTA GCTATGGTGT ACACTGGGTT
CGTCAGTCTC CAGGAAAGGG TCTGGAGTGG CTGGGAGTGA TATGGAGTGG
TGGAGACACA GACTATAATG CAGCTTTCAT ATCCAGACTG AGCATCAGCA
AGGACAATTC CAAGAGCCAA CTCTTCTTTA AAATGAACAG TCTGCGAGCT
ACTGACACAG CCATATATTA CTGTGCCAGA AATAGAGGGG ATATTTACTA
TGATTTCACT TATGCCATGG ACTACTGGGG TCAAGGAACC TCAGTCACCG
TCTCCTCAGC CAAAACAACA CCCCCATCAG TCTATCCACT GGCCCCTGGG
TGTGGAGATA CAACTGGTTC CTCCGTGACT CTGGGATGCC TGGTCAAGGG
CTACTTCCCT GAGTCAGTGA CTGTGACTTG GAACTCTGGA TCCCTGTCCA
GCAGTGTGCA CACCTTCCCA GCTCTCCTGC AGTCTGGACT CTACACTATG
AGCAGCTCAG TGACTGTCCC CTCCAGCACC TGGCCAAGTC AGACCGTCAC
CTGCAGCGTT GCTCACCCAG CCAGCAGCAC CACGGTGGAC AAAAAACTTG
AGCCCAGCGG GCCCATTTCA ACAATCAACC CCTGTCCTCC ATGCAAGGAG
TGTCACAAAT GCCCAGCTCC TAACCTCGAG GGTGGACCAT CCGTCTTCAT
CTTCCCTCCA AATATCAAGG ATGTACTCAT GATCTCCCTG ACACCCAAGG
TCACGTGTGT GGTGGTGGAC GTGAGCGAGG ATGACCCAGA CGTCCAGATC
AGCTGGTTTG TGAACAACGT GGAAGTACAC ACAGCTCAGA CACAAACCCA
TAGAGAGGAT TACAACAGTA CTATCCGGGT GGTCAGCACC CTCCCCATCC
AGCACCAGGA CTGGATGAGT GGCAAGGAGT TCAAATGCAA GGTCAACAAC
AAAGACCTCC CATCACCCAT CGAGAGAACC ATCTCAAAAA TTAAAGGGCT
AGTCAGAGCT CCACAAGTAT ACATCTTGCC GCCACCAGCA GAGCAGTTGT
CCAGGAAAGA TGTCAGTCTC ACTTGCCTGG TCGTGGGCTT CAACCCTGGA
GACATCAGTG TGGAGTGGAC CAGCAATGGG CATACAGAGG AGAACTACAA
GGACACCGCA CCAGTCCTGG ACTCTGACGG TTCTTACTTC ATATATAGCA
AGCTCAATAT GAAAACAAGC AAGTGGGAGA AAACAGATTC CTTCTCATGC
AACGTGAGAC ACGAGGGTCT GAAAAATTAC TACCTGAAGA AGACCATCTC
CCGGTCTCCG GGTAAATGAG CTCAGCACCC ACAAAGCTCT CAGGTCCTAA
GAGACACTGG CACCCATATC CATGCATCCC TTGTATAAAT AAAGCATCCA
GCAAAGCCTG GTACCATGTA AAAAAAAAAA AAAAAAAA
```

FIG. 6A

2. m166 heavy chain (IgG2b) complete amino acid sequence:

(From the start coden to the stop codon)

```
MAVLGLLFCL VTFPSCVLSQ VQLKQSGPGL VQPSQSLSIT CTVSGFSLTS
YGVHWVRQSP GKGLEWLGVI WSGGDTDYNA AFISRLSISK DNSKSQLFFK
MNSLRAIDTA IYYCARNRGD IYYDFTYAMD YWGQGTSVTV SSAKTTPPSV
YPLAPGCGDT TGSSVTLGCL VKGYFPESVT VTWNSGSLSS SVHTFPALLQ
SGLYTMSSSV TVPSSTWPSQ TVTCSVAHPA SSTTVDKKLE PSGPISTINP
CPPCKECHKC PAPNLEGGPS VFIFPPNIKD VLMISLTPKV TCVVVDVSED
DPDVQISWFV NNVEVHTAQT QTHREDYNST IRVVSTLPIQ HQDWMSGKEF
KCKVNNKDLP SPIERTISKI KGLVRAPQVY ILPPPAEQLS RKDVSLTCLV
VGFNPGDISV EWTSNGHTEE NYKDTAPVLD SDGSYFIYSK LNMKTSKWEK
TDSFSCNVRH EGLKNYYLKK TISRSPGK[STOP]
```

```
[Sig-pep] MAVLGLLFCLVTFPSCVLS

[VH-region]
FR1:  QVQLKQSGPGLVQPSQSLSITCTVSGFSLT
CDR1: SYGVH
FR2:  WVRQSPGKGLEWLG
CDR2: VIWSGGDTDYNAAFIS
FR3:  RLSISKDNSKSQLFFKMNSLRAIDTAIYYCAR
CDR3: NRGDIYYDFTYAMDY
FR4:  WGQGTSVTVSS

[CH-region]
CH:
AKTTPPSVYP LAPGCGDTTG SSVTLGCLVK GYFPESVTVT WNSGSLSSSV
HTFPALLQSG LYTMSSSVTV PSSTWPSQTV TCSVAHPASS TTVDKKLEPS
GPISTINPCP PCKECHKCPA PNLEGGPSVF IFPPNIKDVL MISLTPKVTC
VVVDVSEDDP DVQISWFVNN VEVHTAQTQT HREDYNSTIR VVSTLPIQHQ
DWMSGKEFKC KVNNKDLPSP IERTISKIKG LVRAPQVYIL PPPAEQLSRK
DVSLTCLVVG FNPGDISVEW TSNGHTEENY KDTAPVLDSD GSYFIYSKLN
MKTSKWEKTD SFSCNVRHEG LKNYYLKKTI SRSPGK[STOP]
```

FIG. 6B

m166 light chain

1. m166 light chain (k) complete mRNA sequence:

(From the transcriptional start point to the polyA tail)

```
ACACCCTTTG CTGGAGTCAG AATCACACTG ATCACACACA GTCATGAGTG
TGCTCACTCA GGTCCTGGCG TTGCTGCTGC TGTGGCTTAC AGGTGCCAGA
TGTGACATCC AGATGACTCA GTCTCCAGCC TCCCTATCTG CATCTGTGGG
AGAAACTGTC ACCATCACAT GTCGAGCAAG TGGGAATATT CAAAATTATT
TAGCATGGTA TCAGCAGACA CAGGGAAAAT CTCCTCAGCT CCTGGTCTAT
TCTGCAAAAA CCTTAGCAGA TGGTGTGCCA TCAAGGTTCA GTGGCAGTGG
ATCAGGAACA CAATATTCTC TCAAGATCAA CAGCCTGCAG CCTGAAGATT
TTGGGAGTTA TTACTGTCAA CATTTTTGGA GTACTCCGTA CACGTTCGGA
GGGGGGACCA AGCTGGAAAT AAAACGGGCT GATGCTGCAC CAACTGTATC
CATCTTCCCA CCATCCAGTG AGCAGTTAAC ATCTGGAGGT GCCTCAGTCG
TGTGCTTCTT GAACAACTTC TACCCCAAAG ACATCAATGT CAAGTGGAAG
ATTGATGGCA GTGAACGACA AAATGGCGTC CTGAACAGTT GGACTGATCA
GGACAGCAAA GACAGCACCT ACAGCATGAG CAGCACCCTC ACGTTGACCA
AGGACGAGTA TGAACGACAT AACAGCTATA CCTGTGAGGC CACTCACAAG
ACATCAACTT CACCCATTGT CAAGAGCTTC AACAGGAATG AGTGTTAGAG
ACAAAGGTCC TGAGACGCCA CCACCAGCTC CCCAGCTCCA TCCTATCTTC
CCTTCTAAGG TCTTGGAGGC TTCCCCACAA GCGACCTACC ACTGTTGCGG
TGCTCCAAAC CTCCTCCCCA CCTCCTTCTC CTCCTCCTCC CTTTCCTTGG
CTTTTATCAT GCTAATATTT GCAGAAAATA TTCAATAAAG TGAGTCTTTG
CAAAAAAAAA AAAAAAAAAA AAAAAAAAA
```

2. m166 light chain (k) amino acid complete sequence:

(From the start codon to the stop codon)

```
MSVLTQVLAL LLLWLTGARC DIQMTQSPAS LSASVGETVT ITCRASGNIQ
NYLAWYQQTQ GKSPQLLVYS AKTLADGVPS RFSGSGSGTQ YSLKINSLQP
EDFGSYYCQH FWSTPYTFGG GTKLEIKRAD AAPTVSIFPP SSEQLTSGGA
SVVCFLNNFY PKDINVKWKI DGSERQNGVL NSWTDQDSKD STYSMSSTLT
LTKDEYERHN SYTCEATHKT STSPIVKSFN RNEC[STOP]
```

```
[Sig-pep] MSVLTQVLALLLLWLTGARC

[VL-region]
FR1:  DIQMTQSPASLSASVGETVTITC
CDR1: RASGNIQNYLA
FR2:  WYQQTQGKSPQLLVY
CDR2: SAKTLAD
FR3:  GVPSRFSGSGSGTQYSLKINSLQPEDFGSYYC
CDR3: QHFWSTPYT
FR4:  FGGGTKLEIKR

[CL-region]
CL: ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVL
NSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC[STOP]
```

FIG. 7

Synthetic recombinant single chain antibody (scFv-m166)

1. DNA sequence:

```
ATGAAAAAAC TGCTGTTCGC GATTCCGCTG GTGGTGCCGT TCTATAGCCA
TAGCACCATG GAGCTCGAGC GGCAGGTGCA GCTGAAGCAG TCAGGACCTG
GCCTAGTGCG GCCCTCACAG AGCCTGTCCA TCACCTGCAC AGTCTCTGGT
TTCTCATTAA CTAGCTATGG TGTACACTGG GTTCGTCAGT CTCCAGGAAA
GGGTCTGGAG TGGCTGGGAG TGATATGGAG TGGTGGAGAC ACAGACTATA
ATGCAGCTTT CATATCCAGA CTGAGCATCA GCAAGGACAA TTCCAAGAGC
CAACTCTTCT TTAAAATGAA CAGTCTGCGA GCTACTGACA CAGCCATATA
TTACTGTGCC AGAAATAGAG GGGATATTTA CTATGATTTC ACTTATGCCA
TGGACTACTG GGGTCAAGGA ACCTCAGTCA CCGTCTCCTC AGGTGGAGGC
GGCTCAGGCG GAGGTGGCTC TGGCGGTGGC GGATCGGACA TCCAGATGAC
TCAGTCTCCA GCCTCCCTAT CTGCATCTGT GGGAGAAACT GTCACCATCA
CATGTCGAGC AAGTGGGAAT ATTCAAAATT ATTTAGCATG GTATCAGCAG
ACACAGGGAA AATCTCCTCA GCTCCTGGTC TATTCTGCAA AAACCTTAGC
AGATGGTGTG CCATCAAGGT TCAGTGGCAG TGGATCAGGA ACACAATATT
CTCTCAAGAT CAACAGCCTG CAGCCTGAAG ATTTTGGGAG TTATTACTGT
CAACATTTTT GGAGTACTCC GTACACGTTC GGAGGGGGGA CCAAGCTGGA
AATAAAACGG GCTCTAGAAC AAAAACTCAT CTCAGAAGAG GATCTGAATA
GCGCCGTCGA CCATCATCAT CATCATCATT GA
```

2. Amino acid sequence:

```
MKKLLFAIPL VVPFYSHSTM ELERQVQLKQ SGPGLVRPSQ SLSITCTVSG
FSLTSYGVHW VRQSPGKGLE WLGVIWSGGD TDYNAAFISR LSISKDNSKS
QLFFKMNSLR ATDTAIYYCA RNRGDIYYDF TYAMDYWGQG TSVTVSSGGG
GSGGGGSGGG GSDIQMTQSP ASLSASVGET VTITCRASGN IQNYLAWYQQ
TQGKSPQLLV YSAKTLADGV PSRFSGSGSG TQYSLKINSL QPEDFGSYYC
QHFWSTPYTF GGGTKLEIKR ALEQKLISEE DLNSAVDHHH HHH[STOP]
```

```
[Gene III signal sequence] MKKLLFAIPLVVPFYSHS
[Joint-1]                  TMELER
[m166 heavy chain]
QVQLKQSGPG LVRPSQSLSI TCTVSGFSLT SYGVHWVRQS PGKGLEWLGV
IWSGGDTDYN AAFISRLSIS KDNSKSQLFF KMNSLRATDT AIYYCARNRG
DIYYDFTYAM DYWGQGTSVT VSS
[scFv-linker] GGGGSGGGGSGGGGS
[m166 light chain]
DIQMTQSPAS LSASVGETVT ITCRASGNIQ NYLAWYQQTQ GKSPQLLVYS
AKTLADGVPS RFSGSGSGTQ YSLKINSLQP EDFGSYYCQH FWSTPYTFGG
GTKLEIKR
[Joint-2]                  AL
[Myc epitope]              EQKLISEEDL
[Joint-3]                  NSAVD
[Hexahistidine tag]        HHHHHH [STOP]
```

FIG. 8

SEQUENCE LISTING

<110> Frank, Dara W.
Wiener-Kronish, Jeannine
Yahr, Timothy L.
Sawa, Teiji
Fritz, Robert B.

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Phe Cys Leu Val Thr Phe Pro Ser Cys Val Leu Ser Gln Val Gln Leu
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aag cag tca gga cct ggc cta gtg cag ccc tca cag agc ctg tcc atc 149
Lys Gln Ser Gly Pro Gly Leu Val Gln Pro Ser Gln Ser Leu Ser Ile

25 30 35

acc tgc aca gtc tct ggt ttc tca tta act agc tat ggt gta cac tgg 197
Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val His Trp
40 45 50 55

gtt cgt cag tct cca gga aag ggt ctg gag tgg ctg gga gtg ata tgg 245
Val Arg Gln Ser Pro Gly Lys Gly Leu Glu Trp Leu Gly Val Ile Trp
60 65 70

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Ser Gly Gly Asp Thr Asp Tyr Asn Ala Ala Phe Ile Ser Arg Leu Ser
75 80 85

atc agc aag gac aat tcc aag agc caa ctc ttc ttt aaa atg aac agt 341
Ile Ser Lys Asp Asn Ser Lys Ser Gln Leu Phe Phe Lys Met Asn Ser
90 95 100

ctg cga gct act gac aca gcc ata tat tac tgt gcc aga aat aga ggg 389
Leu Arg Ala Thr Asp Thr Ala Ile Tyr Tyr Cys Ala Arg Asn Arg Gly
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gat att tac tat gat ttc act tat gcc atg gac tac tgg ggt caa gga 437
Asp Ile Tyr Tyr Asp Phe Thr Tyr Ala Met Asp Tyr Trp Gly Gln Gly
120 125 130 135

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Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr Pro Pro Ser Val Tyr
140 145 150

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Pro Leu Ala Pro Gly Cys Gly Asp Thr Thr Gly Ser Ser Val Thr Leu
155 160 165

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Gly Cys Leu Val Lys Gly Tyr Phe Pro Glu Ser Val Thr Val Thr Trp
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Asn Ser Gly Ser Leu Ser Ser Ser Val His Thr Phe Pro Ala Leu Leu
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Gln Ser Gly Leu Tyr Thr Met Ser Ser Ser Val Thr Val Pro Ser Ser
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220 225 230

agc acc acg gtg gac aaa aaa ctt gag ccc agc ggg ccc att tca aca 773
Ser Thr Thr Val Asp Lys Lys Leu Glu Pro Ser Gly Pro Ile Ser Thr
235 240 245

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250 255 260

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Asn Leu Glu Gly Gly Pro Ser Val Phe Ile Phe Pro Pro Asn Ile Lys
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Asp Val Ser Glu Asp Asp Pro Asp Val Gln Ile Ser Trp Phe Val Asn
300 305 310

aac gtg gaa gta cac aca gct cag aca caa acc cat aga gag gat tac 1013
Asn Val Glu Val His Thr Ala Gln Thr Gln Thr His Arg Glu Asp Tyr
315 320 325

aac agt act atc cgg gtg gtc agc acc ctc ccc atc cag cac cag gac 1061
Asn Ser Thr Ile Arg Val Val Ser Thr Leu Pro Ile Gln His Gln Asp
330 335 340

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345 350 355

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360 365 370 375

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Ala Pro Gln Val Tyr Ile Leu Pro Pro Pro Ala Glu Gln Leu Ser Arg
380 385 390

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Asp Thr Ala Pro Val Leu Asp Ser Asp Gly Ser Tyr Phe Ile Tyr Ser
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35 40 45

Thr Ser Tyr Gly Val His Trp Val Arg Gln Ser Pro Gly Lys Gly Leu
50 55 60

Glu Trp Leu Gly Val Ile Trp Ser Gly Gly Asp Thr Asp Tyr Asn Ala

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Ala Phe Ile Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln
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Leu Phe Phe Lys Met Asn Ser Leu Arg Ala Thr Asp Thr Ala Ile Tyr
100 105 110

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Gln Thr His Arg Glu Asp Tyr Asn Ser Thr Ile Arg Val Val Ser Thr
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Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys Glu Phe Lys Cys
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Lys Ile Lys Gly Leu Val Arg Ala Pro Gln Val Tyr Ile Leu Pro Pro
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Pro Ala Glu Gln Leu Ser Arg Lys Asp Val Ser Leu Thr Cys Leu Val
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Val Gly Phe Asn Pro Gly Asp Ile Ser Val Glu Trp Thr Ser Asn Gly
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Ser Gly Ser Gly Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu Gln Pro
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Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His Phe Trp Ser Thr Pro Tyr
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Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala
120 125 130

cca act gta tcc atc ttc cca cca tcc agt gag cag tta aca tct gga 487
Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln Leu Thr Ser Gly
135 140 145

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Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr Pro Lys Asp Ile
150 155 160

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aac agt tgg act gat cag gac agc aaa gac agc acc tac agc atg agc 631
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Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg His Asn Ser Tyr
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Gln Leu Leu Val Tyr Ser Ala Lys Thr Leu Ala Asp Gly Val Pro Ser
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Ser Leu Gln Pro Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His Phe Trp

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Pro Lys Asp Ile Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln
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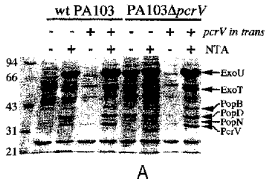
[Continued on next page]

(54) Title: METHOD AND COMPOSITIONS FOR IMMUNIZATION WITH THE PSEUDOMONAS V ANTIGEN

(57) Abstract: A method of inhibiting, moderating or diagnosing *Pseudomonas aeruginosa* infection is disclosed. In one embodiment, this method comprises inoculating a patient with an effective amount of PcrV antigen.



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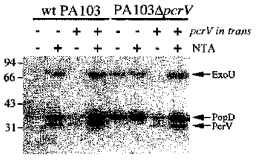

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METHOD AND COMPOSITIONS FOR IMMUNIZATION WITH THE PSEUDOMONAS V ANTIGEN

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with United States government support awarded by the following agencies: NIH/NIADA Grant Nos. R01 A131665-08, K04 AI01289-04 and R01 HL59239-02. The United States has certain rights in this invention.

BACKGROUND OF THE INVENTION

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that is capable of causing fatal acute lung infections in critically ill individuals (1). The ability of the bacterium to damage the lung epithelium has been linked with the expression of toxins that are directly injected into eukaryotic cells via a type III-mediated secretion and translocation mechanism (2, 3).

The proteins encoded by the *P. aeruginosa* type III secretion and translocation apparatus demonstrate a high level of amino acid identity with members of the *Yersinia* Yop regulon (4-6). Of all the type III systems discovered in Gram-negative bacteria, only *P. aeruginosa* possesses a homologue to the *Yersinia* V antigen, PcrV (see 6 for review of type III systems). Homologous proteins to the secretion and translocation apparatus are encoded by both plant and animal pathogenic bacteria. These organisms include human pathogens such as *Salmonella typhimurium*, *Shigella flexneri*, *Enteropathogenic E. coli*, *Chlamydia spp.*, and plant pathogens such as *Xanthamonas campestris*, *Pseudomonas syringae*, *Erwinia amylovora* and *Ralstonia solanacearum*. However, only *P. aeruginosa* and *Yersinia* encode the V antigen.

Yahr, et al., 1997, discloses the sequence of the operon encoding PcrV and compares the sequence to the LcrV protein. Thus, the amino acid

sequence of PcrV is known and is available under accession number AF010149 of GenBank.

SUMMARY OF THE INVENTION

The present invention involves methods and compositions developed from our observation that the *Pseudomonas* V antigen can be used to protect animals from a lethal lung infection.

In one embodiment, the present invention is a method of inhibiting *Pseudomonas* infection comprising inoculating a patient with an effective amount of PcrV antigen. In another embodiment, DNA encoding PcrV is used as a gene vaccine.

In one preferred embodiment, the antigen is expressed as a recombinant protein and used to immunize patients at risk.

Preferably, the patient is completely protected from infection.

In another embodiment, the DNA encoding PcrV (called *pcrV*) or a DNA fragment may be used diagnostically to detect *P. aeruginosa* infection.

In another embodiment, the recombinant protein (rPcrV) is used diagnostically to detect antibodies from patients. Patient antibody response to PcrV may be associated with prognosis. Therefore, in this embodiment the recombinant protein is used as a prognostic indicator by measuring the patient's antibody titer.

The present invention also provides a method for inhibiting a *Pseudomonas* infection in an individual by contacting the individual with an effective amount of a PcrV inhibitor, in particular with a PcrV antibody, antibody derivative or fragment, or antibody mimic. PcrV antibodies, antibody derivatives and antibody fragments are also provided.

It is an object of the present invention to actively and passively immunize a patient against Pseudomonas infection.

It is another object of the present invention to diagnostically detect the *P. aeruginosa* infection.

It is another object of the present invention to diagnostically detect antibodies from *Pseudomonas* patients.

Other objects, features and advantages of the present invention will become apparent to one of skill in the art after review of the specification, claims and drawings.

DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are a stained gel (Fig. 1A) and Western blot (Fig. 1B) illustrating the phenotypic analysis of PA103ΔpcrV.

Figs. 2A and 2B are a graph (Fig. 2A) and set of bar graphs (Fig. 2B) illustrating the survival and lung injury of *P. aeruginosa* parental and mutant strains.

Figs. 3A and 3B are a graph (Fig. 3A) and a set of bar graphs (Fig. 3B) illustrating the effect of immunization on survival, lung injury, and bacterial colonization.

Fig. 4 is a graph of the number of animals surviving a challenge with 5 x 10^5 CFU/mouse of strain PA103 after passive administration of polyclonal IgG specific for PcrV, ExoU, PopD or control IgG from an unimmunized animal.

Fig. 5 is a graph (Fig. 5A) and a set of bar graphs (Fig. 5B) illustrating survival and protection from lung injury by concomitant administration of IgG to different bacterial antigens and bacterial challenge. One-way ANOVA for lung injury, $p=0.026$, and lung edema, $p<0.0005$.

Figs. 6A and B are printouts of SEQ ID NOs:1 and 2 with additional explanatory information. Fig. 6A is SEQ ID NO:1. Fig. 6B is SEQ ID NO:2.

Fig. 7 is a printout of SEQ ID NOs:3 and 4 with additional explanatory information.

Fig. 8 is a synthetic recombinant single chain antibody (SCFV-M166) (SEQ ID NOs:5 and 6).

DESCRIPTION OF THE INVENTION

We disclose herein that PcrV has a novel regulatory effect on expression of the type III secreted products, is involved in the translocation of type III toxins, and is the first antigen that protects against lung injury induced by *P. aeruginosa* infection. Vaccination against PcrV prior to the airspace instillation of anti-PcrV IgG not only ensured the survival of challenged animals but also decreased lung inflammation and injury caused by the bacteria.

LcrV, or the V antigen, is a multifunctional protein that regulates secretion/translocation of the Yop effector proteins and plays an extracellular role in pathogenesis by altering the host cytokine response to *Yersinia* infection (7-11). The only known homologue of this critical pathogenic factor is an extracellular protein encoded by *P. aeruginosa*, termed PcrV.

One embodiment of the present invention is a method of moderating or inhibiting a *Pseudomonas* infection by immunizing a patient with an effective amount of the PcrV antigen. By "effective amount" we mean an amount of PcrV antigen effective to show some moderation or inhibition of *Pseudomonas* infection as compared to control subjects or animals who have not been treated with the antigen.

By "moderating" we mean that infection is inhibited by at least fifty percent compared to a non-immunized animal. Preferably, infection is completely prevented. A quantitative assessment of infection would preferably include the examination of the amount of bacteria in the

bloodstream or pleural fluids and/or an examination of lung injury parameters. For example, the absence of bacteria in the bloodstream or pleural fluids would indicate prevention of infection. A reduction in lung injury parameters would indicate that infection is moderated.

Infection could be quantitatively assessed by several other clinical indicators, including the reduction of bacterial load in the sputum, blood or pleural fluids, reduction in the size of the infiltrate, oxygenation improvement, reduction in the length of time on mechanical ventilation, reduction in fever and reduction in white blood cell count.

By "PcrV antigen" we mean that portion or fragment of the PcrV protein that is necessary to invoke an immune response which prevents or moderates infection. We have used the full-length PcrV protein as an antigen to induce protection. Additionally, we have mapped the protective epitope to the fragment comprising amino acids 144-257 of PcrV. To define the epitope, monoclonal antibodies that protected against infection and cytotoxicity were tested for binding to progressively smaller forms of recombinant PcrV. (By "recombinant PcrV" or "rPcrV" we mean the protein produced from a PcrV gene that has been placed in a non-native host.) This protection localized the region.

The PcrV antigen may be most easily obtained by the method we used, commercially available bacterial expression plasmid pet16b from Novagen. The pcrV gene was first cloned from the *P. aeruginosa* chromosome as part of an operon. The coding region was amplified and inserted into two different vectors. One vector is for expression from *P. aeruginosa* as shown in Fig. 1. This is a vector from Herbert Schweizer (reference 19) which we modified to contain an appropriate promoter sequence such that PcrV expression is coordinately regulated with the rest of

the delivery and intoxication apparatus of the bacterium. The second plasmid, pET16b, is for expression and purification purposes from *E. coli.*

The advantage of this system is that we do not have to worry about contaminating *P. aeruginosa* proteins, the protein is produced in great abundance, and there is a one-step purification process. In this situation the PcrV coding region is amplified to be cloned in frame with a histidine tag provided on the pET16b vector. The multiple histidine residues fused to the amino terminus of PcrV allow affinity chromatography using a nickel-NTA column. Therefore, a preferable PcrV antigen is a recombinant version of the natural PcrV protein.

Immunization may be done systemically or intranasally. Immunization of these individuals would preferably start during normal vaccination procedures for other childhood diseases. We would predict long-lived protection with booster doses probably around ages 5 and 10.

In another embodiment, one would use DNA encoding the PcrV protein or the complement of this DNA to diagnostically detect *P. aeruginosa* infection. One would obtain the DNA sequence of the PcrV antigen at GenBank AF010149. The coding region for PcrV is at nucleotides 626-1510. One may also choose to use a fragment of this coding region or complement of this fragment. A successful probe is one that will hybridize specifically to the PcrV DNA and not to other regions.

One would preferably use a hybridization probe of at least 40 continuous nucleotides within the antigen sequence or two primers of at least 25 continuous nucleotides within the sequence. One skilled in the art would appreciate that many standard forms of nucleic acid diagnostic techniques would be suitable, for example, hybridization of the single-stranded 40 nucleotide probe to DNA or RNA extracted from a patient's sputum. In another example, patient's sputum would be used as a source for bacterial

DNA or RNA to serve as a template for the PCR or RT-PCR reaction, respectively.

One would also determine *Pseudomonas aeruginosa* infection in an individual by contacting a sample obtained from the individual with an antibody specific for PcrV and correlating enhanced antibody binding as compared with a control sample with *Pseudomonas aeruginosa* infection in the individual.

In an additional embodiment, the DNA encoding PcrV is used as a gene vaccine using standard molecular biological methods. For example, one could review the following references for techniques known to those of skill in the art: Davis, H.L., et al., "DNA vaccine for hepatitis B: Evidence for immunogenicity in chimpanzees and comparison with other vaccines," Proc. Natl. Acad. Sci. 93:7213-7218, 1996; Barry, M.A., et al., "Protection against mycoplasma infection using expression-library immunization," Nature 377:632-635, 1995; Xiang, Z.Q., et al., "Immune responses to nucleic acid vaccines to rabies virus," Virology 209:569-579, 1995. By "effective amount" of a gene vaccine, we mean an amount of vaccine effective to moderate or eliminate *Pseudomonas* infection or *Pseudomonas* infection symptoms.

The protein or antigen could also be used diagnostically to detect antibodies in patients and, thus, predict the patient's infection status. One would preferably contact a sample obtained from an individual suspected of *Pseudomonas* infection with the PcrV protein or fragment thereof and detect protein/antibody binding. One would then correlate enhanced antibody binding (as compared with a control sample) with *Pseudomonas aeruginosa* infection in the individual. One could also use the PcrV antibody or antibody fragments therapeutically.

In another embodiment, the invention is the use of the antibody sequence (which we report below and in SEQ ID NOs:1-4) to produce

recombinant single chain antibodies that may block PcrV and could also utilize the sequence in gene delivery experiments, where one would deliver eukaryotic vectors that will then lead to the production of single chain antibodies in animals for prolonged periods. The sequence could also be utilized to humanize the murine monoclonal antibody to produce a product that can be utilized in human patient care.

Once the antibody is safe for human use, one could: (a) administer it systemically and (b) administer it into the lungs as either a preventative treatment or as a therapy. In order to use the PcrV antibody in humans, the antibody is preferably "humanized". In general, once the monoclonal antibody is obtained the heavy and light chain variable regions are cloned. These cloned fragments are then inserted into a human antibody backbone (constant regions). Thus, we can control the class of antibody (IgG, IgA, etc.) in addition to providing the binding specificity.

For use in the present invention, the PcrV antibody may be a monoclonal antibody or polyclonal. The antibodies may be human or humanized, particularly for therapeutic applications. Antibody fragments or derivatives, such as an Fab, $F(ab')_2$ or Fv, may also be used. Single-chain antibodies, for example as described in Huston, et al. (Int. Rev. Immunol. 10:195-217, 1993) may also find use in the methods described herein. By "effective amount" of the PcrV antibody or antibody fragment we mean an amount sufficient to moderate or eliminate *Pseudomonas* infection or infection symptoms.

Preferably, human or humanized monoclonal or polyclonal antibodies to PcrV are administered to prevent or treat infections with *P. aeruginosa*. In patients at high risk for *P. aeruginosa* infection, antibodies could be administered for prevention of infection. In addition, antibodies may be administered after the onset of infection to treat the infection. In this case,

antibodies can be administered alone or in combination with antibiotics. Administration of antibodies in conjunction with antibiotics may allow the administration of shorter courses or lower doses of antibiotics, thereby decreasing the risk of emergence of antibiotic-resistant organisms.

We envision at least three types of hypothetical patients: (1) A healthy individual at risk of serious injury or burn (fire fighter, military personnel, police) would be immunized with the vaccine by a methodology (either injection or intranasal) that would give long-lived protection. A booster would be given on admission (intramuscular injection) to the hospital after injury. (2) A patient who is being subjected to mechanical ventilation. (3) A patient who has been genetically diagnosed with cystic fibrosis.

In addition to PcrV antibodies and antibody fragments, small molecule peptidomimetics or non-peptide mimetics can be designed to mimic the action of the PcrV antibodies in inhibiting or modulating *Pseudomonas* infection, presumably by interfering with the action of PcrV. Methods for designing such small molecule mimics are well known (see, for example, Ripka and Rich, Curr. Opin. Chem. Biol. 2:441-452, 1998; Huang, et al., Biopolymers 43:367-382, 1997; al-Obeidi, et al., Mol. Biotechnol. 9:205-223, 1998). Small molecule inhibitors that are designed based on the PcrV antibody may be screened for the ability to interfere with the PcrV-PcrV antibody binding interaction. Candidate small molecules exhibiting activity in such an assay may be optimized by methods that are well known in the art, including for example, *in vitro* screening assays, and further refined in *in vivo* assays for inhibition or modulation of *Pseudomonas* infection by any of the methods described herein or as are well known in the art. Such small molecule inhibitors of PcrV action should be useful in the present method for inhibiting or modulating a *Pseudomonas* infection.

In another aspect of the present invention, PcrV protein may be used to identify a PcrV receptor which may be present in the host cells, particularly in human cells, more particularly in human epithelial cells or macrophages. Identification of a PcrV receptor allows for the screening of small molecule libraries, for example combinatorial libraries, for candidates that interfere with PcrV binding. Such molecules may also be useful in a method to inhibit or modulate a *Pseudomonas* infection.

Our first attempts at receptor identification will be to use PcrV in pull-down experiments. PcrV will be fused to glutathione S-transferase (GST) and attached to column matrix for affinity chromatography of solubilized cellular extracts. Proteins binding specifically to PcrV will be eluted and subjected to amino terminal sequencing for identification. In parallel experiments PcrV will be subjected to yeast two-hybrid analysis. In this case PcrV is fused in frame with the DNA binding domain of Gal4. Once the clone is obtained it will be transformed into a suitable yeast host strain. The yeast strain containing the Gal4PcrV construct will be transformed with a Hela cell cDNA bank cloned in frame with the Gal4 activation domain. Double transformants that complement the ability to utilize histidine and produce beta galactosidase (proteins that interact with PcrV) will be analyzed genetically and at the nucleotide sequence level. In case the receptor is a cellular glycolipid we will utilize an overlay technique where glycolipids are separated by thin-layer chromatography and then probed with radiolabeled bacteria. The binding to specific components will be monitored by autoradiography. Similarly, epithelial and macrophage proteins will be separated by SDS-PAGE, blotted onto nitrocellulose and overlaid with radiolabeled bacteria or labeled PcrV. Again, the protein components to which the bacteria bind are then identified by autoradiography.

Pseudomonas species are known to infect a wide spectrum of hosts within the animal kingdom and even within the plant kingdom. As will be apparent to one of ordinary skill in the art, the compositions and methods disclosed herein may have use across a wide range of organisms in inhibiting or modulating diseases or conditions resulting from infection by a *Pseudomonas* species. The compositions and methods of the present invention are described herein particularly for application to *Pseudomonas aeruginosa* but it is well within the competence of one of ordinary skill in the art to apply the methods taught herein to other species.

EXAMPLES

1. Role of PcrV in Cytotoxicity

To determine the role of PcrV in type III-mediated regulation/secretion, we constructed a nonpolar allele of PcrV and used the construct to replace the wild-type allele in *P. aeruginosa* strain PA103, a strain that is highly cytotoxic *in vitro* (3) and causes lung epithelial damage *in vivo* (12, 13). Cytotoxicity and lung injury are due to the production of a specific cytotoxin, ExoU (3).

PA103Δ*pcrV* was characterized by the expression of several extracellular products that are secreted by the *P. aeruginosa* type III system which include the ExoU cytotoxin (3), PcrV (5), and a protein required for the translocation of toxins, PopD (14). SDS-polyacrylamide gel electrophoresis of concentrated culture supernatants indicated that the parental strain, PA103 is induced for production and secretion of the type III proteins by growth in medium containing a chelator of calcium, nitrilotriacetic acid (NTA) (Fig. 1). When an expression clone encoding PcrV was provided in *trans* in the parental strain, extracellular protein production in response to the presence or absence of NTA is normal. PA103Δ*pcrV* exhibits a calcium blind phenotype;

extracellular protein production is strongly induced in both the presence and absence of NTA. These results suggest that the secretory system is fully functional but deregulated. This deregulated phenotype is in contrast to the calcium independent phenotype reported for an LcrV defective strain which fails to produce the extracellular Yops, grows at 37°C regardless of the presence or absence of calcium, and shows only partial induction of the Yops (7). Complementing PA103Δ*pcrV* with a clone expressing wild-type PcrV restored normal regulation of extracellular protein production in response to NTA induction.

To test the contribution of PcrV to *P. aeruginosa* pathogenesis, two infection models were used. In an *in vitro* model the parental and several mutant derivative strains were compared for their ability to cause cytotoxicity in a CHO cell infection assay (3). The negative controls in this experiment included PA103*popD*::Ω, which has been previously shown to be defective in the translocation of type III virulence determinants (14) and PA103Δ*exoU*, which is non-cytotoxic due to the absence of ExoU production (3, 15).

After a 3 hour infection, CHO cells were unable to exclude trypan blue with the wild-type and Δ*pcrV* strain complemented with a plasmid construct expressing PcrV. Staining did not occur when CHO cells were infected with the negative control strains or with PA103Δ*pcrV* (data not shown). These results suggest that PcrV expression is required for cytotoxicity. Purified recombinant PcrV was not cytotoxic when added exogenously to tissue culture cells. Since secretion of the type III proteins required for translocation was unaffected by the deletion of *pcrV* (Fig. 1A and B), PA103Δ*pcrV* appears to be defective in ExoU translocation.

Figs. 1A and 1B are a stained gel (Fig. 1A) and Western blot (Fig. 1B) illustrating the phenotypic analysis of PA103ΔpcrV. The parental and Δ*pcrV* derivatives, with and without a plasmid expressing PcrV in *trans*, were grown

in the absence or presence of the inducer of type III secretion in *P. aeruginosa*, nitrilotriacetic acid (NTA). The extracellular protein profile (Fig. 1A) was analyzed on a SDS-polyacrylamide gel (10%) stained with Coomassie blue. The migration of the *P. aeruginosa*-encoded type III proteins is indicated to the left and the migration of molecular weight markers is indicated on the right. Fig. 1B is a Western blot of a duplicate gel using antibodies specific for ExoU, PcrV, and PopD and ^{125}I-Protein A to detect bound IgG.

Wild-type and mutant *P. aeruginosa* strains were tested in an acute lung infection model using low and high challenge doses of bacteria. Survival measurements indicated that PcrV and PopD were required to induce a lethal infection (Fig. 2A). In experiments utilizing three independent measurements of lung injury (the flux of labeled albumin from the airspaces of the lung to the bloodstream, the flux of labeled albumin from the airspaces of the lung to the pleural fluids, and the wet/dry ratio, which measures lung edema) the degree of injury caused by PA103Δ*pcrV*, the vector control strain (PA103Δ*pcrV*pUCP18), and PA103*popD*::Ω were no different than the uninfected control animals (Fig. 2B). Complementation of PA103Δ*pcrV* with *pcrV in trans* restored lung injury levels to those measured with the parental strain, PA103. Taken together these data indicate that PcrV expression is required for virulence of *P. aeruginosa* in the acute lung infection model and that part of the function of PcrV appears to be linked to the ability to translocate type III effector proteins into eukaryotic cells.

Figs. 2A and 2B are a graph (Fig. 2A) and set of bar graphs (Fig. 2B) illustrating the survival and lung injury of *P. aeruginosa* parental and mutant strains. Referring to Fig. 2A, mice were challenged with 5 x 10^5 cfu of each of the indicated strains and survival was monitored for one week. Referring to Fig. 2B, lung injury was assessed by the flux of labeled albumin from the

airspaces of the lung to the blood (lung epithelial injury), to the pleural fluid (pleural fluid) or by measuring the wet/dry ratio (lung edema). Two bacterial infectious doses were used as denoted by the solid and striped bars. Significant differences (*p<0.001) between control and test groups was determined by one-way ANOVA and Dunnet multiple comparison tests. The following abbreviations were used: PA103, parental wild-type strain; Δ*pcrV*, PA103Δ*pcrV*; Δ*pcrV*pUCP*pcrV*, PA103Δ*pcrV* complemented with a plasmid expressing *PcrV*; Δ*pcrV*pUCP, PA103Δ*pcrV* with a vector control; *popD*::Ω, PA103*popD*::Ω, a translocation defective strain.

2. Immunization with PcrV

To determine whether immunization with PcrV protected animals from a lethal lung infection, recombinant PcrV (rPcrV) or ExoU (rExoU) were purified as histidine-tagged fusion proteins and used as antigens. Mice were immunized and subsequently challenged via their airspaces with a lethal dose of strain PA103. When survival was measured, both vaccines protected the mice (Fig 3A). When lung injury was assessed, only PcrV vaccinated animals had significantly less epithelial damage and lung edema (Fig. 3B). Animals immunized with the PcrV vaccine also had significantly fewer bacteria in their lungs, suggesting that a blockade of the *Pseudomonas* V antigen may facilitate rapid clearance of bacteria from the lung, protecting the animals from severe epithelial injury (Fig. 3B).

Figs. 3A and 3B are a graph (Fig. 3A) and a set of bar graphs (Fig. 3B) illustrating the effect of immunization on survival, lung injury, and bacterial colonization. Referring to Fig. 3A, mice were immunized (PcrV, n=10; ExoU, n=5; control, n=10) as indicated and challenged with strain PA103 at 5 $x10^5$ CFU/animal. The percent of surviving animals was determined for one week; $p<0.05$ by the Mantel-Cox log rank test. Referring to Fig. 3B, lung injury assessment and bacterial colonization of vaccinated animals 4 hours after

installation of PA103. Lung epithelial injury, lung edema, and bacterial burden; PcrV, n=9; ExoU, n=4; and control, n=8. The final number of bacteria in the lung is indicated as the number on the Y axis x 10^4 CFU. Significant differences (*) for lung injury ($p<0.01$), lung edema ($p<0.05$), and bacterial numbers ($p<0.05$) as determined by Dunnet multiple comparison test. One-way ANOVA for lung injury, p=0.0005; lung edema, p=0.0437; bacterial burden, p=0.0075.

To determine whether therapeutic intervention was possible, mice were passively immunized with preimmune rabbit IgG or rabbit IgG specific for rPcrV, rExoU, or rPopD one hour prior to airspace instillation of PA103 at a concentration of 5 x10^5 CFU/mouse. Antibodies to rPcrV provided complete protection to a lethal infection (Fig. 4). Anti-rExoU IgG provided partial survival, which was significantly different from the administration of control IgG, although all the surviving animals appeared severely ill during the trial. Survival was not improved by the passive transfer of antibodies to another of the type III translocation proteins, PopD. From these results we conclude that antibodies to PcrV are highly protective in the acute lung infection model and that PcrV may be exposed on the bacterial surface or in a soluble form that is available for antibody-antigen interactions.

Fig. 4 is a graph of the number of animals surviving a challenge with 5 x 10^5 CFU/mouse of strain PA103. Animals were pretreated with 100 µg of immune IgG or control IgG from an unimmunized rabbit (rPcrV, preimmune serum). N=10 for each group; *$p<0.05$ versus control group for treatment with anti-PcrV and anti-ExoU IgG preparations by Mantel-Cox log rank test.

If PcrV is accessible for neutralization, then concomitant administration of the bacterial inoculum with anti-rPcrV IgG should completely protect against lung injury and lethality. IgG preparations were mixed with the inoculum (10-fold higher dose than the lethal inoculum) prior to instillation of

the bacteria into the lung and survival was measured. Only anti-rPcrV IgG was protective against this extreme infection (Fig. 5A). Lung injury was measured in animals infected with the normal lethal dose of 5 x 10^5 bacteria. The efflux of labeled albumin from the airspaces of the lung was only 3% more than uninfected controls (Fig. 5B) after co-administration of anti-rPcrV IgG. The decreased efflux of labeled protein from the lung to the pleural fluids was the same as the uninfected controls when anti-PcrV was included with the inoculum. Curiously lung edema, as measured by the wet/dry ratio, was significantly reduced by the addition of either anti-rPcrV or anti-rPopD. (Fig. 5B). Thus, the concomitant administration of anti-rPcrV IgG with the bacteria was even more effective in normalizing all the lung injury parameters than vaccination. These data support the accessibility of PcrV for antibody-mediated neutralization and document a clinically relevant decrease in lung injury; antibodies to PcrV may serve as therapeutic reagents in the treatment of severe nosocomial pneumonia caused by *Pseudomonas aeruginosa*.

Fig. 5 is a graph (Fig. 5A) and a set of bar graphs (Fig. 5B) illustrating survival and protection from lung injury by concomitant administration of IgG and bacterial challenge. IgG (5 μg) was mixed with either 5 x 10^6 (for survival assays, n=10 per group) or 5 x 10^5 (for the measurement of lung injury, n=4 to 6 animals per group) *P. aeruginosa* strain PA103. This mixture was instilled into the lungs and survival (Fig. 5A) or lung injury (Fig. 5B) was assessed. For survival, *$p<0.05$ versus control IgG for anti-PcrV by the Mantel-Cox log rank test; for lung epithelial injury and lung edema *$p<0.05$ versus control IgG by Dunnet multiple comparison test. One-way ANOVA for lung injury, $p=0.026$, and lung edema, $p<0.0005$.

In acute *P. aeruginosa* infections, the net effect of type III-mediated intoxication may be to promote the dissemination of the bacterium beyond the epithelium leading to infection of the pleural fluids, spleen, liver, and

bloodstream. Blood-borne infections with *P. aeruginosa* from either acute ventilator-associated pneumonia or from burn wound infections can result in a 40-80% mortality rate in spite of aggressive antibiotic treatment (16). PcrV must be a component of the type III translocation complex in *P. aeruginosa*, as mutants defective in the production of this protein are unable to intoxicate CHO cells or cause lung epithelial injury even though they are able to produce and secrete the type III effectors and proteins required for translocation. Unlike PopD, which is also necessary for translocation, PcrV is accessible for antibody-mediated neutralization suggesting that antibodies may be useful therapeutic agents in acute infections.

3. Methods for Examples 1 and 2

Construction of a nonpolar insertion in *PcrV* and complementation. A 5.0-kb *Eco*RI-*Nsi*I restriction fragment encoding *pcrGVHpopBD* and flanking sequences was cloned into the allelic replacement vector pNOT19 (17). Two *Not*I sites (one within *pcrG* and one within *popB*) were removed from the inserted sequences by using the Sculptor mutagenesis system (Amersham). An internal *Sst*I restriction fragment was deleted from *pcrV*, resulting in an in-frame deletion of residues 17-221 (pNOTΔ*pcrV*). To select for integration of the plasmid, a gene encoding tetracycline resistance (TcΩ) was cloned into the *Hind*III site of the vector (pNOTΩΔpcrV). The MOB cassette (17) was added as a *Not*I fragment. Selection of merodiploids, resolution of plasmid sequences, and confirmation of allelic replacement was performed as previously described (18). A shuttle plasmid (pUCP, 19) was used to construct a clone to complement the *pcrV* deletion. The coding sequence for PcrV was amplified and cloned behind the control of the ExoS promoter region (20). The transcription of ExoS is coordinately regulated with the operons that control type III secretion and translocation in *P. aeruginosa* (2).

The nucleotide sequence was confirmed for each DNA construct involving site specific mutagenesis, PCR amplification, or in-frame deletion.

SDS-PAGE and Western blot analysis of secreted products. *P. aeruginosa* were grown under inducing (+NTA) or non-inducing conditions (-NTA) for expression of the type III secreted products (18). Cultures were harvested based on optical density measurements at 540 nm and supernatant fractions were concentrated by the addition of a saturated solution of ammonium sulfate to a final concentration of 55%. Each lane of an SDS-polyacrylamide gel (11%) was loaded with 3 µl of a 20-fold concentrated supernatant and stained with Coomassie blue. An identical gel was subjected to Western blot analysis as previously described (3-5) using a cocktail of rabbit antisera, which specifically recognizes ExoU, PopD, and PcrV. Protein A labeled with ^{125}I was used as a secondary reagent to identify bound IgG.

Infection models and lung injury assessments. Chinese Hamster Ovary cells (CHO) were used in an *in vitro* infection model designed to measure cytotoxicity and type III translocation (21). Briefly, a bacterial inoculum was prepared in tissue culture medium without serum. CHO cells, which were propagated in serum containing medium, were washed and infected with various *P. aeruginosa* strains at a multiplicity of infection of 5:1. Cultures were incubated under tissue culture conditions for 3 hours (37°C, 5% CO_2), washed, and stained with trypan blue. Permeability to the dye was determined from phase contrast photographs. Infection with the parental strain PA103, which expresses ExoU, results in trypan blue staining of approximately 80% of the monolayer after 3 hours of incubation and complete destruction of the monolayer at 4-5 hours of incubation. Mouse infections and assessment of lung injury was performed as previously described (16). Briefly, male 8- to 12-week old pathogen-free BALB/c mice were purchased

from Simonsen Laboratories (Gilroy, CA) and housed in barrier conditions. The mice were briefly anesthetized with inhaled Metofane (methoxyflurane, Pitman-Moore, Mundelein, IL) and placed supine, at an angle of approximately 30°. Fifty microliters of the bacterial inoculum was instilled slowly into the left lobe using a modified 24 gauge animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY) inserted into the trachea via the oropharynx. When lung injury assessments were measured, 0.5 μCi of ^{131}I-labeled human serum albumin (Merck-Frosst, Quebec, Canada), 0.05 μg of anhydrous Evans blue in ml of Ringer's lactate with 5% mouse albumin were added to the instillate. After 4 hours of infection, the mice were anesthetized, blood was collected by a carotid arterial puncture and median sternotomies were performed. The lungs, pleural fluids, tracheas, oropharynxes, stomachs, and livers were harvested, and the radioactivity was measured. The percentage of radioactive albumin that left the instilled lungs and entered the circulation or the pleural fluid was calculated by multiplying the counts measured in the terminal blood samples (per ml) times the blood volume (body weight X 0.07). The wet-dry ratios of the lungs were determined by adding 1 ml of water to the lungs and homogenizing the mixture. Homogenates were placed in preweighed aluminum pans and dried to constant weight in an 80°C oven for three days. Lung homogenates were also sequentially diluted and plated on sheep blood agar for quantitative assessment of bacteria.

Production of rabbit antiserum to PcrV, PopD, and ExoU. rPcrV, rPopD, and rExoU were produced as histidine tagged fusion proteins in pET16b and purified by nickel chromatography as previously described (22). Rabbits were injected intradermally (10 sites) with 300 μg of recombinant protein emulsified in Freund's complete adjuvant, boosted with antigen in Freund's incomplete adjuvant, and periodically bled at 7 day intervals. For

passive immunization, the IgG fraction was isolated using Protein A column chromatography (Pierce Chemicals, Rockford, IL). Mice were injected with 100 µg IgG (intraperitoneal injection) 1 hour before challenge with 5 x 10^5 CFU of strain PA103. For active immunization with rPcrV and rExoU, endotoxin was removed from protein preparations by extraction with 1% Triton X-114 (23). Following the extractions, Triton X-114 was removed by Sephacryl S-200 chromatography. All vaccine preparations contained less than 1 ng of endotoxin per 40 µg of recombinant protein as determined by using a limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). BALB/c mice were injected subcutaneously with 10 µg of recombinant proteins in Freund's complete adjuvant. At day 30 the mice were boosted with an additional 10 µg of antigen in Freund's incomplete adjuvant. On day 51 the mice were challenged by instillation of *P. aeruginosa* into their left lungs.

4. Synthesis of Monoclonal Antibodies

Mice were immunized with 10 µg of purified, LPS-free, recombinant PcrV in Freund's complete adjuvant and boosted two weeks later with the same dose of antigen emulsified in Freund's incomplete adjuvant. Immunizations were performed subcutaneously. Spleens were harvested from mice one week after booster doses of PcrV in Freund's incomplete adjuvant.

A single spleen was placed in 5 ml of tissue culture medium without serum, cut into pieces and gently homogenized. Large pieces of tissue were allowed to settle from the homogenate and the supernatant, single-cell suspension was removed and subjected to centrifugation at 1200 rpm for 10 minutes. The pelleted cells were resuspended in 10 ml of a solution to lyse red blood cells for 5 minutes and subsequently underlaid with 10 ml of fetal bovine serum. The material was centrifuged at 1200 rpm for 8 minutes, the

supernatant was discarded and the cells were suspended in 30 ml of medium.

Spleenic cells and myeloma cells (P3x63Ag8.653) were harvested by centrifugation at 1200 rpm for 10 minutes, and each pellet was separately suspended in 10 ml of tissue culture medium. 10^8 spleen cells and 2 x 10^7 myeloma cells were mixed and pelleted together by centrifugation at 1200 rpm for 6 minutes. The supernatant was removed by aspiration and 1 ml of 35% polyethylene glycol (PEG) was added. The cells were suspended in this solution gently and centrifuged at 1000 rpm for 3 minutes. In some experiments centrifugation was eliminated.

Exactly 8 minutes after the addition of PEG, 25 ml of medium was added and the cells were gently resuspended. Following a 5 minute 1200 rpm centrifugation step, the cell pellet was suspended at a density of 1 x 10^6 per ml in 30% conditioned medium and 70% complete medium (with serum). The cells were incubated overnight at 37°C. The next day the cells were harvested by centrifugation and suspended in 200 ml of 30% conditioned medium and 70% complete medium with hypoxanthine, aminopterin and thymidine (HAT).

Approximately 0.2 ml of this cell suspension was added per well to ten 96-well plates (12 ml per 96 well plate). The density of the remaining cells was adjusted to 2.5 x 10^5 per ml and the cells were plated in the 96 well format. Plates were screened microscopically for single colonies and supernatants were subsequently tested for antibody production by enzyme-linked immunosorbent assay using recombinant PcrV as the antigen. Clones producing antibodies reactive to PcrV were subcultured to larger culture dishes and then isotyped.

The binding of antibodies was tested in an enzyme linked immunosorbent assay using recombinant PcrV as the antigen (histidine-

tagged protein) coating the wells. Monoclonal antibodies were also tested in Western blot reactions using a *P. aeruginosa* supernatant containing native PcrV without the histidine tag.

5. Identification of PcrV Antigen

We obtained about three hundred cell lines producing antibodies that bound the tagged PcrV. These initial cell lines were preserved in liquid nitrogen for safekeeping. All cell lines were passaged to isolate stable clones. In conjunction with isolating stable clones we developed in *in vitro* assay as a correlate for protection against intoxication in animal infection models.

The hybridomas that were stable to passage and still produced antibodies reactive to PcrV in ELISA (approximately 80 cell lines) were subsequently tested in a Fluorescence Activated Cell Sorter using the following techniques and assumptions: We reasoned that if antibody is blocking the type III intoxication system, then in the presence of a monoclonal that blocks, fewer cells will be killed by our toxins. We exposed cells to each of the 80 monoclonal antibodies, added toxic bacteria, incubated, and then added a dye that is only permeable to dead cell DNA (propidium iodide). Excess dye was washed away and the cells were harvested, fixed, and analyzed by FACS. Dead cells would be fluorescent since the dye leaked in and stained DNA in the nucleus.

We found that if the cells were incubated with rabbit polyclonal anti-PcrV, mouse polyclonal anti-PcrV, or mab166 and bacteria, fewer cells died than in controls with irrelevant polyclonal antibody (anti-PopD) or the other 78 monoclonal antibodies.

Mab 166 was specifically found to bind to the bacterially encoded type III-secreted factor termed PcrV. PcrV mediates the interaction of *P. aeruginosa* and lung cells to facilitate the translocation of bacterial toxins that cause cellular death. This reaction is postulated to eliminate lung cells that are involved in the innate immune response to *P. aeruginosa*. The killing of these cells leaves the host epithelium open for *P. aeruginosa* colonization

and spread to the pleural fluids and bloodstream. *P. aeruginosa*-encoded antibiotic resistance makes effective treatment unlikely once the bacteria have entered the bloodstream.

The protection afforded by mab 166 pre- and post-bacterial instillation in animal models of acute lung infection with *P. aeruginosa* is significant. To design antibody treatment modalities for intervention in human *P. aeruginosa* infections it will be necessary to produce either a human monoclonal antibody or to immunize at risk patients with the protective epitope of PcrV defined by mab 166. The goal of the work described below is to define the amino acid sequence of PcrV bound by mab 166.

Results

We used a molecular genetic approach to define the amino acid residues bound by mab 166. PcrV possesses 294 amino acids. The approach consisted of deleting parts of the molecule at the nucleotide sequence level using the polymerase chain reaction. Each product was cloned into a protein expression vector in frame with a gene encoding the glutathione S transferase protein. This strategy ensured that deletions encoding small numbers of PcrV amino acids could be detected using Western or dot blot techniques. Control bacterial lysates encoding only glutathione S transferase showed no reactivity to either our anti-PcrV polyclonal or mab 166 monoclonal antibody.

A total of 66 (with one full-length PcrV expression plasmid) clones were constructed, expressed, and tested for reactivity to rabbit polyclonal anti-PcrV antisera. All but one clone bound to anti-PcrV rabbit antibody verifying that the expressed proteins were in-frame with PcrV. The one non-reactive clone was eliminated from the analysis. None of the C-terminal deletions (n = 5 constructs) bound mab 166 suggesting that the epitope was in the C-terminal half of the protein. Only one of the N-terminal truncation

proteins (n = 8 constructs) encoding PcrV amino acids (aa) 139-294 bound to mab 166. This experiment verified our hypothesis that the mab 166 epitope was encoded by the carboxyl terminal half of the protein. The remaining 51 constructs encoded various internal deletions of the molecule. Binding analysis tabulated in Table 1, below, demonstrated that the smallest epitope recognized by mab 166 consists of aa 144-257 of PcrV.

TABLE 1

PcrV Epitope Mapping		
All proteins are amino-terminal tagged GST-PcrV truncates.		
Amino Acids	Binding to pAb	Binding to mAb 166
1-294 (full-length)	yes	yes
(C-term truncates)		
1-46	yes	no
1-76	yes	no
1-134	yes	no
1-172	yes	no
1-75 + 173-294	yes	no
(N-term truncates)		
139-294	yes	yes
148-294	yes	no
159-294	yes	no
164-294	yes	no
194-294	yes	no
261-294	yes	no
269-294	yes	no
278-294	yes	no

Amino Acids	Binding to pAb	Binding to mAb 166
(internal fragments)		
139-191	yes	no
139-195	yes	no
139-234	yes	no
139-243	yes	no
139-256	yes	no
139-257	yes	yes; weak
139-258	yes	yes
139-259	yes	yes
139-260	yes	yes
139-261	yes	yes
139-262	yes	yes
139-263	yes	yes
139-264	yes	yes
139-265	yes	yes
139-266	yes	yes
139-274	yes	yes
139-281	yes	yes
140-266	yes	yes
141-266	yes	yes
142-266	yes	yes
143-266	yes	yes
144-266	yes	yes
145-266	yes	no
146-266	yes	no
147-266	yes	no
148-170	no*	no
148-202	yes	no
159-202	yes	no
159-209	yes	no
159-216	yes	no
159-226	yes	no
159-234	yes	no
164-234	yes	no
164-243	yes	no
164-256	yes	no
164-266	yes	no
164-275	yes	no
164-281	yes	no
194-234	yes	no
194-243	yes	no
194-256	yes	no
194-266	yes	no
194-275	yes	no
194-281	yes	no

Amino Acids	Binding to pAb	Binding to mAb 166
141-258	yes	yes; weak
142-258	NT**	yes
143-258	yes	yes
144-258	yes	yes
141-257	yes	yes
142-257	yes	yes
143-257	yes	yes
144-257	yes; weak	yes; weak

NOTES:
*Truncate 148-170 is the only one that is not recognized by the rabbit polyclonal control antibody.
**NT; Not Tested due to an insufficient amount of bacterial lysate.
-As predicted, pGEX-4T-2 vector control lysates were not recognized by either antibody.
-The smallest epitope of PcrV recognizable by mAb166 appears to consist of amino acids 144-257.

6. Examination of PcrV-Specific Antibody

Methods:

Poly A+ RNA extraction: Hybridoma cell line m166 was cultured in complete Dulbeccos minimum essential medium with 4.5 g/L D-glucose, 10 mM HEPES, 50 μM 2-mercaptoethanol, 3 mM L-glutamine, and 10% heat-inactivated fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate. After the cells reached confluent state in a 75 cm^2 flask, the cells were harvested from centrifuging at 600 rpm for 5 minutes. The pellet of the cells was homogenized in 2 mL of TRIzol reagent (Life Technologies, Gaithersburg, MD), and total RNA (100 μg) was extracted after chloroform fractionation, isopropanol precipitation and 70% ethanol wash. Poly A+ RNA (4 μg) was extracted with oligotex mRNA spin-column (Qiagen, Valencia, CA).

RNA oligo-capping: mRNA (250 ng) was incubated with calf intestinal phosphatase at 50°C for 1 hour to dephosphorylate non-mRNA or truncated mRNA. After the reaction, phenol/chloroform extraction and ethanol precipitation was performed and the dephosphorylated RNA was incubated with tobacco acid pyrophosphatase at 37°C for 1 hour to remove the 5'-cap structure from full-length mRNA. After phenol/chloroform extractions and ethanol precipitation, the synthetic RNA oligo (GeneRacer RNA Oligo, Invitrogen, Carlsbad, CA) was ligated to the decapped RNA with T4 RNA

ligase at 37°C for 1 hour. After phenol/chloroform extraction and ethanol precipitation, the RNA was suspended in 13 µL diethylpyrocarbonate-treated water.

Reverse-transcribing mRNA: The RNA-oligo ligated, full-length mRNA (13 µL) was reverse-transcribed with 54 base the pair primer containing a dT tail of 18 nucleotides (GeneRacer Oligo dT, Invitrogen), and Avian myeloblastosis virus reverse transcriptase at 42°C for 1 hour in 20 µL reaction. After the reaction, the sample was diluted 4 times with sterile water.

Amplifying cDNA ends by polymerase chain reaction (PCR): One microliter of the cDNA was used for PCR. The 5' primer from the synthetic RNA oligo sequence (GeneRacer 5' Primer, Invitrogen) and the murine immunoglobulin gamma 2b chain CH1 region specific primer or the murine immunoglobulin kappa chain CL region specific primer were used. The cycling parameters used for the PCR reaction was; 1) 94°C, 2 minutes, 1 cycle, 2) 94°C, 30 seconds and 72°C, 1 minute, 5 cycle, 3) 94°C, 30 seconds, 70°C, 30 seconds, and 72°C, 1 minutes, 5 cycle, 4) 94°C, 30 seconds, 68°C, 30 seconds, and 72°C, 1 minutes, 20 cycle, 5) 72°C, 10 minutes.

Subcloning and DNA sequencing: PCR products (the murine immunoglobulin gamma 2b chain CH1 region derived fragment and the murine immunoglobulin kappa chain CL region derived fragment) were subcloned into the pCRII vector (TOPO cloning, Invitrogen) and submitted to UCSF Molecular Bioresource Center to analyze the DNA sequence.

SEQ ID NO:1 is the DNA sequence of m166 heavy chain mRNA, SEQ ID NO:2 is the amino acid sequence of the m166 heavy chain (IgG II_b), SEQ ID NO:3 is the DNA sequence of the m166 light chain mRNA, and SEQ ID NO:4 is the amino acid sequence of the m166 light chain. Figs. 6A, 6B and 7 examine the sequences and supply more detail.

Commercial Implications

One could use the antibody sequence to produce recombinant single chain antibodies that may block PcrV and could also utilize the sequence in gene delivery experiments, where one would deliver eukaryotic vectors that will then lead to the production of single chain antibodies in animals for prolonged periods. Finally, the sequence could be utilized to humanize the murine monoclonal antibody to produce a product that can be utilized in human patient care. One of skill in the art would look to standard methods such as grafting the antigen binding complementarity determining regions (CDRs) from variable domains of rodent antibodies on to human variable domains in order to create a humanized antibody.

7. Single Chain Antibody Against PcrV

a. Assembling a single chain antibody:

VH gene and VL gene were multiplied by polymerase chain reaction (PCR) with specific primers for each gene. Multiplied VH and VL fragments were assembled with a linker by using PCR with primers. The assembled single chain antibody gene (scFv::m166:VH and VL genes with linker) was transferred into the cloning vector pCR4 Topo (Invitrogen, Carlsbad, CA). Then, the coding sequence of scFv::m166 was subcloned into the *E. coli* expression vector pBAD/gIII (Invitrogen) in LMG194 as the host *E. coli*.

b. Protein induction and purification:

For protein induction, in RM medium containing 0.2% glucose and 100 µg of ampicillin, the transformed *E. coli* was cultured overnight at 37°C in an orbital shaker (200 rpm), and the next day, 5 mL of the cultured *E. coli* was transferred into 500 mL of the same medium and incubated for 3 hours at room temperature at 100 rpm. After L-arabinose was added at the concentration of 0.004%, the *E. coli* was cultured overnight. The third day, the protein was harvested from the periplasmic space of the *E. coli* by

osmotic shock methods. The solution including osmotic shock derived periplasmic protein was dialyzed overnight against the lysis buffer. During the fourth day, the dialyzed solution was applied onto a nickel-NTA column to purify the hexahistidine-tagged single chain antibody. The eluted solution from the nickel column was dialyzed against phosphate buffered saline overnight. On the fifth day, the dialyzed solution was applied to a centrifuge concentrator to make a higher concentration of scFv:m166.

c. The binding test:

The purified single chain antibody (scF::m166) was tested by using an enzyme linked immunosorbent assay against recombinant PcrV and by immunoblot (western blot) against both recombinant PcrV protein and native PcrV of *P. aeruginosa* PA103.

The single chain antibody will allow us to humanize the antibody utilizing phage-display techniques and to improve affinity of the antibody using these techniques. The single chain antibody can be utilized as a diagnostic tool (for histology) but would not be utilized as a therapy. However, the gene for the single chain antibody can be utilized in gene therapy, so that animals would produce single-chain antibodies over an interval, which could lead to protection against *P. aeruginosa* infections.

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CLAIMS

We claim:

1. A method of inhibiting *Pseudomonas aeruginosa* infection comprising inoculating a patient with an effective amount of PcrV antigen.

2. The method of claim 1 wherein the PcrV antigen is a fragment of the PcrV protein, said fragment capable of inducing an immune response specific to the V antigen.

3. The method of claim 1 wherein the patient is inoculated with a gene vaccine comprising DNA encoding PcrV.

4. The method of claim 3 wherein the DNA encodes a fragment of the PcrV protein, said fragment capable of inducing an immune response specific to the PcrV antigen.

5. The method of claim 1 wherein the patient is a human patient.

6. A method of diagnosing *Pseudomonas aeruginosa* infection comprising the step of exposing a patient's sample to a nucleotide probe, wherein the probe hybridizes specifically to a PcrV-encoding nucleic acid and not to other nucleic acids.

7. The method of claim 6 wherein the patient is a human patient.

8. A method of diagnosing a *Pseudomonas aeruginosa* infection comprising the steps of

a) exposing a patient's sample to nucleotide primers designed to amplify the *pcrV* gene,

b) performing a polymerase chain reaction, wherein the *pcrV* gene is amplified if present in the sample, and

c) correlating *Pseudomonas aeruginosa* infection with the presence of an amplified product.

9. The method of claim 8 wherein the patient is a human patient.

10. A method of diagnosing a *Pseudomonas aeruginosa* infection, comprising the steps of:

a) exposing the patient sample to a PcrV antigen, and

b) correlating *Pseudomonas aeruginosa* infection with the presence of a PcrV-specific antibody/antigen complex.

11. A method of inhibiting *Pseudomonas aeruginosa* infection comprising inoculating a patient with an effective amount of a gene vaccine, wherein the gene vaccine encodes PcrV antigen.

12. The method of claim 11 wherein the gene vaccine encodes the entire PcrV protein.

13. The method of claim 11 wherein the gene vaccine encodes a fragment of the PCR PcrV protein, wherein the fragment is capable of inducing an immune response specific to the PcrV antigen.

14. The method of claim 11 wherein the patient is a human patient.

15. A method of treating or preventing *Pseudomonas aeruginosa* infection comprising the steps of obtaining a humanized or human PcrV antibody or antibody fragment, and administering the antibody systemically, wherein the antibody inhibits or prevents *Pseudomonas aeruginosa* infection.

16. A method of treating or preventing *Pseudomonas aeruginosa* infection comprising the steps of obtaining a humanized or human PcrV antibody or antibody fragment and administering the antibody to the lungs as a therapeutic agent.

17. A method of treating a *Pseudomonas aeruginosa* infection comprising the step of inoculating a *Pseudomonas aeruginosa*-infected patient with an effective amount of PcrV antigen.

18. An antibody specific for the PcrV antigen.

19. The antibody of claim 18, wherein the antibody is a monoclonal.

20. The antibody of claim 19, wherein the antibody is mab 166.

21. An anti-PcrV monoclonal antibody or fragment thereof that recognizes an epitope that includes amino acid residues 144 and 257 in the PcrV polypeptide amino acid sequence.

22. An anti-PcrV monoclonal antibody or fragment thereof that recognizes an epitope that includes amino acid residues 144 through 257 in the PcrV polypeptide amino acid sequence.

23. A monoclonal antibody or fragment thereof comprising the CDR's of the light chain polypeptide amino acid sequence shown in Fig. 7.

24. A monoclonal antibody or fragment thereof comprising the CDR's of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.

25. A monoclonal antibody or fragment thereof comprising the CDR's of the light chain polypeptide amino acid sequence shown in Fig. 7 and the CDR's of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.

26. The monoclonal antibody or fragment of any of claims 23 and 25 that further comprises the FR regions of the light chain polypeptide amino acid sequence shown in Fig. 7.

27. The monoclonal antibody or fragment of any of claims 24 and 25 that further comprises the FR regions of the heavy chain polypeptide amino acid sequence shown in Fig. 6B.

28. The monoclonal antibody or fragment of claim 25 that further comprises the FR regions of the heavy chain polypeptide amino acid sequence shown in Fig. 6B and the FR regions of the light chain polypeptide amino acid sequence shown in Fig. 7.

29. The antibody or fragment of any of claims 21-28 that is humanized.

30. The antibody or fragment of any of claims 21-28 that is human.

31. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 and a pharmaceutically acceptable carrier.

32. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 in an amount effective for treating or preventing Pseudomonas infection in a patient.

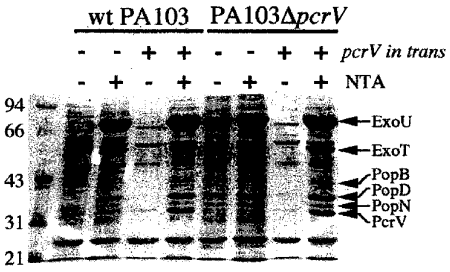
33. A pharmaceutical composition comprising the antibody or fragment of any of claims 21-30 in an amount effective for reducing the pathogenicity of Pseudomonas in a patient.

34. A method for treating or preventing Pseudomonas infection in a patient comprising administering to the patient an effective amount of the composition of claim 31 or 32.

35. A method for reducing the pathogenicity of Pseudomonas in a patient comprising administering to the patient an effective amount of the composition of claim 31 or 33.

36. A method for modulating the cytotoxicity of Pseudomonas to a human cell comprising contacting said Pseudomonas with the antibody or fragment of any of claims 21-30 in the presence of the human cell.

37. A nucleic acid encoding the antibody or fragment of any of claims 21-30.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PA103ΔpcrV
- - + + - - + + pcrV in trans
- + - + - + - + NTA
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66
43
31
21
ExoU
ExoT
PopB
PopD
PopN
PcrV

FIG. 1A

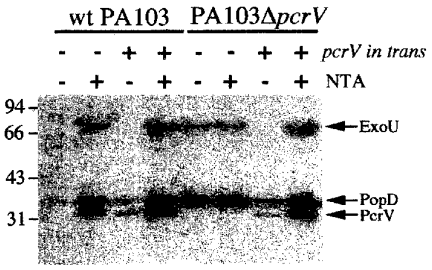

wt PA103
PA103ΔpcrV
- - + + - - + + pcrV in trans
- + - + - + - + NTA
94
66
43
31
ExoU
PopD
PcrV

FIG. 1B

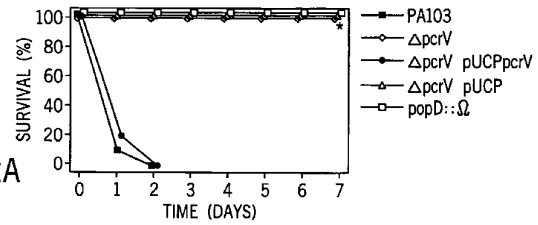


FIG. 2A

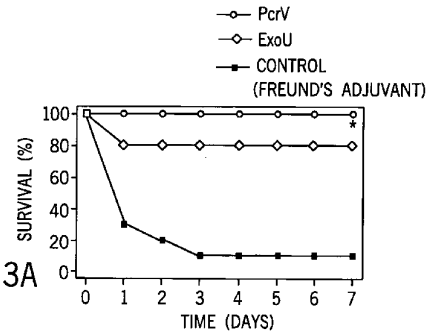


FIG. 3A

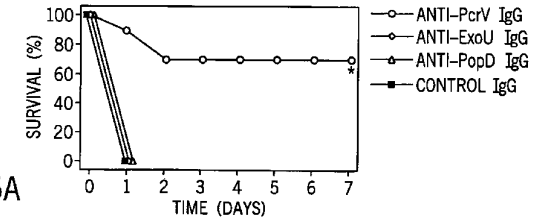
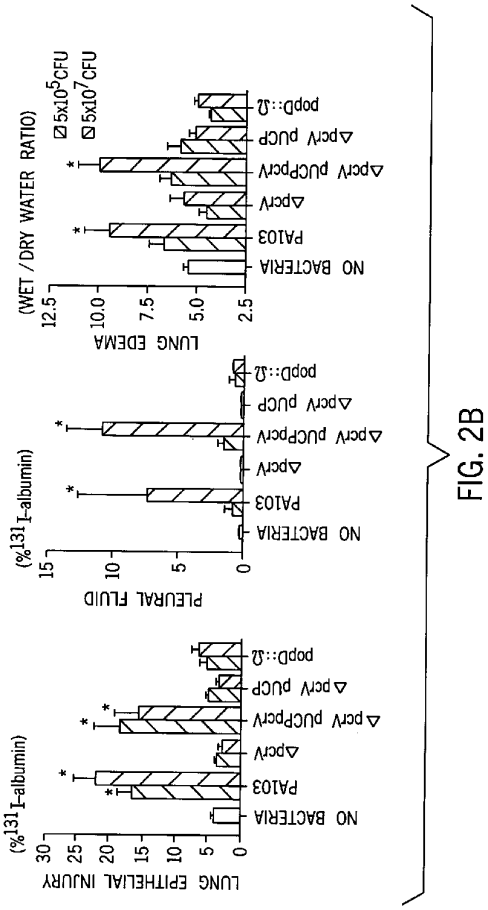


FIG. 5A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(%131I-albumin)
30
25
20
15
10
5
0
NO BACTERIA
PA103
ΔpcrV
ΔpcrV pUCPpcrV
ΔpcrV pUCP
popD::Ω
PLEURAL FLUID
(%131I-albumin)
15
10
5
0
NO BACTERIA
PA103
ΔpcrV
ΔpcrV pUCPpcrV
ΔpcrV pUCP
popD::Ω
LUNG EDEMA
(WET / DRY WATER RATIO)
12.5
10.0
7.5
5.0
2.5
NO BACTERIA
PA103
ΔpcrV
ΔpcrV pUCPpcrV
ΔpcrV pUCP
popD::Ω
5x10^5 CFU
5x10^7 CFU

FIG. 2B

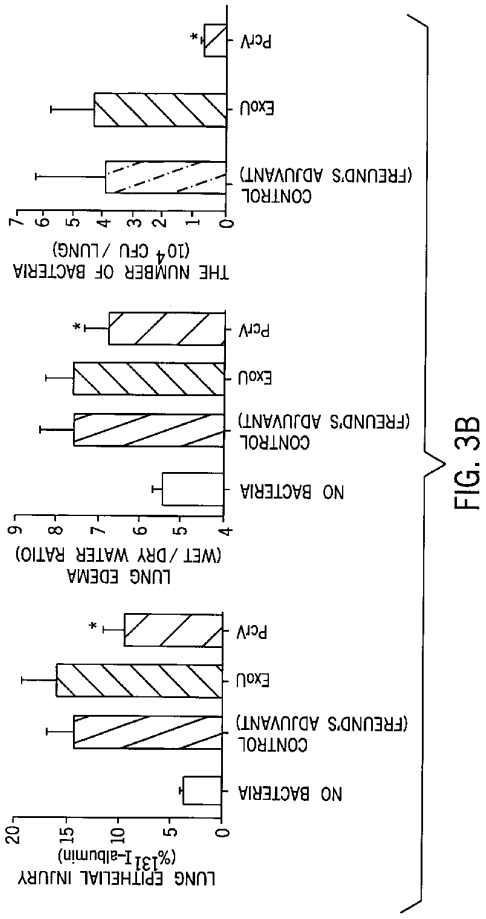
LUNG EPITHELIAL INJURY
(%131I-albumin)
0
5
10
15
20
NO BACTERIA
CONTROL
(FREUND'S ADJUVANT)
ExoU
PcrV
*
LUNG EDEMA
(WET / DRY WATER RATIO)
4
5
6
7
8
9
NO BACTERIA
CONTROL
(FREUND'S ADJUVANT)
ExoU
PcrV
*
THE NUMBER OF BACTERIA
(10^4 CFU / LUNG)
0
1
2
3
4
5
6
7
CONTROL
(FREUND'S ADJUVANT)
ExoU
PcrV
*

FIG. 3B

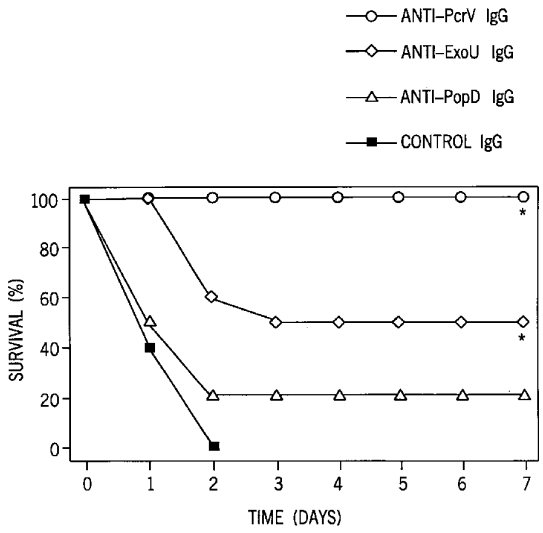
ANTI-PcrV IgG
ANTI-ExoU IgG
ANTI-PopD IgG
CONTROL IgG
SURVIVAL (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
TIME (DAYS)
*
*

FIG. 4

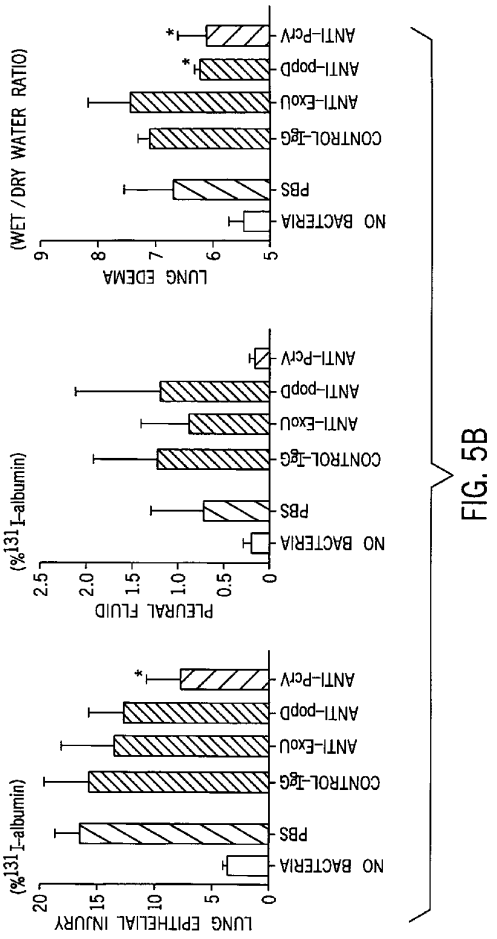
LUNG EPITHELIAL INJURY
(%131I-albumin)
NO BACTERIA
PBS
CONTROL-IgG
ANTI-ExoU
ANTI-popD
ANTI-PcrV
PLEURAL FLUID
(%131I-albumin)
LUNG EDEMA
(WET / DRY WATER RATIO)

FIG. 5B

m166 HEAVY CHAIN
1. m166 HEAVY CHAIN (IgG2b) COMPLETE mRNA SEQUENCE:

(FROM THE TRANSCRIPTIONAL START POINT TO THE POLY A-TAIL)

CCATCCTCTT CTCATAGAGC CTCCATCAGA GCATGGCTGT CTTGGGGCTG
CTCTTCTGCC TGGTGACATT CCCAAGCTGT GTCCTATCCC AGGTGCAGCT
GAAGCAGTCA GGACCTGGCC TAGTGCAGCC CTCACAGAGC CTGTCCATCA
CCTGCACAGT CTCTGGTTTC TCATTAACTA GCTATGGTGT ACACTGGGTT
CGTCAGTCTC CAGGAAAGGG TCTGGAGTGG CTGGGAGTGA TATGGAGTGG
TGGAGACACA GACTATAATG CAGCTTTCAT ATCCAGACTG AGCATCAGCA
AGGACAATTC CAAGAGCCAA CTCTTCTTTA AAATGAACAG TCTGCGAGCT
ACTGACACAG CCATATATTA CTGTGCCAGA AATAGAGGGG ATATTTACTA
TGATTTCACT TATGCCATGG ACTACTGGGG TCAAGGAACC TCAGTCACCG
TCTCCTCAGC CAAAACAACA CCCCCATCAG TCTATCCACT GGCCCCTGGG
TGTGGAGATA CAACTGGTTC CTCCGTGACT CTGGGATGCC TGGTCAAGGG
CTACTTCCCT GAGTCAGTGA CTGTGACTTG GAACTCTGGA TCCCTGTCCA
GCAGTGTGCA CACCTTCCCA GCTCTCCTGC AGTCTGGACT CTACACTATG
AGCAGCTCAG TGACTGTCCC CTCCAGCACC TGGCCAAGTC AGACCGTCAC
CTGCAGCGTT GCTCACCCAG CCAGCAGCAC CACGGTGGAC AAAAAACTTG
AGCCCAGCGG GCCCATTTCA ACAATCAACC CCTGTCCTCC ATGCAAGGAG

FIG. 6A-1

TGTCACAAAT GCCCAGCTCC TAACCTCGAG GGTGGACCAT CCGTCTTCAT
CTTCCCTCCA AATATCAAGG ATGTACTCAT GATCTCCCTG ACACCCAAGG
TCACGTGTGT GGTGGTGGAT GTGAGCGAGG ATGACCCAGA CGTCCAGATC
AGCTGGTTTG TGAACAACGT GGAAGTACAC ACAGCTCAGA CACAAACCCA
TAGAGAGGAT TACAACAGTA CTATCCGGGT GGTCAGCACC CTCCCCATCC
AGCACCAGGA CTGGATGAGT GGCAAGGAGT TCAAATGCAA GGTCAACAAC
AAAGACCTCC CATCACCCAT CGAGAGAACC ATCTCAAAAA TTAAAGGGCT
AGTCAGAGCT CCACAAGTAT ACATCTTGCC GCCACCAGCA GAGCAGTTCT
CCAGGAAAGA TGTCAGTCTC ACTTGCCTGG TCGTGGGCTT CAACCCTGGA
GACATCAGTG TGGAGTGGAC CAGCAATGGG CATACAGAGG AGAACTACAA
GGACACCGCA CCAGTCCTGG ACTCTGACGG TTCTTACTTC ATATATAGCA
AGCTCAATAT GAAAACAAGC AAGTGGGAGA AAACAGATTC CTTCTCATGC
AACGTGAGAC ACGAGGGTCT GAAAAATTAC TACCTGAAGA AGACCATCTC
CCGGTCTCCG GGTAAATGAG CTCAGCACCC ACAAAGCTCT CAGGTCCTAA
GAGACACTGG CACCCATATC CATGCATCCC TTGTATAAAT AAAGCATCCA
GCAAAGCCTG GTACCATGTA AAAAAAAAAA AAAAAAAA

FIG. 6A-2

2. m166 HEAVY CHAIN (IgG2b) COMPLETE AMINO ACID SEQUENCE:

(FROM THE START CODEN TO THE STOP CODON)

MAVLGLLFCL VTFPSCVLSC VQLKQSGPGL VQPSQSLSIT CTVSGFSLTS
YGVHWVRQSP GKGLEWLGVI WSGGDTDYNA AFISRLSISK DNSKSQLFFK
MNSLRATDTA IYYCARNRGD IYYDFTYAMD YWGQGTSVTV SSAKTTPPSV
YPLAPGCGDT TGSSVTLGCL VKGYFPESVT VTWNSGSLSS SVHTFPALLQ
SGLYTMSSSV TVPSSTWPSQ TVTCSVAHPA SSTTVDKKLE PSGPISTINP
CPPCKECHKC PAPNLEGGPS VFIFPPNIKD VLMISLTPKV TCVVVDVSED
DPDVQISWFV NNVEVHTAQT QTHREDYNST IRVVSTLPIQ HQDWMSGKEF
KCKVNNKDLP SPIERTISKI KGLVRAPQVY ILPPPAEQLS RKDVSLTCLV
VGFNPGDISV EWTSNGHTEE NYKDTAPVLD SDGSYFIYSK LNMKTSDWEK
TDSFSCNVRH EGLKNYYLKK TISRSPGK[STOP]

FIG. 6B-1

[SIG-PEP] MAVLGLLFCLVTFPSCVLS

[VH-REGION]
FR1: QVQLKQSGPGLVQPSQSLSITCTVSGFSLT
CDR1: SYGVH
FR2: WVRQSPGKGLEWLG
CDR2: VIWSGGDTDYNAAFIS
FR3: RLSISKDNSKSQLFFKMNSLRATDTAIYYCAR
CDR3: NRGDIYYDFTYAMDY
FR4: WGQGTSVTVSS

[CH-REGION]
CH:

AKTTPPSVYP LAPGCGDTTG SSVTLGCLVK GYFPESVTVT WNSGSLSSSV
HTFPALLQSG LYTMSSSVTV PSSTWPSQTV TCSVAHPASS TTVDKKLEPS
GPISTINPCP PCKECHKCPA PNLEGGPSVF IFPPNIKDVL MISLTPKVTC
VVVDVSEDDP DVQISWFVNN VEVHTAQTQT HREDYNSTIR VVSTLPIQHQ
DWMSGKEFKC KVNNKDLPSP IERTISKIKG LVRAPQVYIL PPPAEQLSRK
DVSLTCLVVG FNPGDISVEW TSNGHTEENY KDTAPVLDSD GSYFIYSKLN
MKTSKWEKTD SFSCNVRHEG LKNYYLKKTI SRSPGK[STOP]

FIG. 6B-2

m166 LIGHT CHAIN
1. m166 LIGHT CHAIN (k) COMPLETE mRNA SEQUENCE:

(FROM THE TRANSCRIPTIONAL START POINT TO THE POLY A-TAIL)

ACACCCTTTG CTGGAGTCAG AATCACACTG ATCACACACA GTCATGAGTG
TGCTCACTCA GGTCCTGGCG TTGCTGCTGC TGTGGCTTAC AGGTGCCAGA
TGTGACATCC AGATGACTCA GTCTCCAGCC TCCCTATCTG CATCTGTGGG
AGAAACTGTC ACCATCACAT GTCGAGCAAG TGGGAATATT CAAAATTATT
TAGCATGGTA TCAGCAGACA CAGGGAAAAT CTCCTCAGCT CCTGGTCTAT
TCTGCAAAAA CCTTAGCAGA TGGTGTGCCA TCAAGGTTCA GTGGCAGTGG
ATCAGGAACA CAATATTCTC TCAAGATCAA CAGCCTGCAG CCTGAAGATT
TTGGGAGTTA TTACTGTCAA CATTTTTGGA GTACTCCGTA CACGTTCGGA
GGGGGGACCA AGCTGGAAAT AAAACGGGCT GATGCTGCAC CAACTGTATC
CATCTTCCCA CCATCCAGTG AGCAGTTAAC ATCTGGAGGT GCCTCAGTCG

FIG. 7A

TGTGCTTCTT GAACAACTTC TACCCCAAAG ACATCAATGT CAAGTGGAAG
ATTGATGGCA GTGAACGACA AAATGGCGTC CTGAACAGTT GGACTGATCA
GGACAGCAAA GACAGCACCT ACAGCATGAG CAGCACCCTC ACGTTGACCA
AGGACGAGTA TGAACGACAT AACAGCTATA CCTGTGAGGC CACTCACAAG
ACATCAACTT CACCCATTGT CAAGAGCTTC AACAGGAATG AGTGTTAGAG
ACAAAGGTCC TGAGACGCCA CCACCAGCTC CCCAGCTCCA TCCTATCTTC
CCTTCTAAGG TCTTGGAGGC TTCCCCACAA GCGACCTACC ACTGTTGCGG
TGCTCCAAAC CTCCTCCCCA CCTCCTTCTC CTCCTCCTCC CTTTCCTTGG
CTTTTATCAT GCTAATATTT GCAGAAAATA TTCAATAAAG TGAGTCTTTG
CAAAAAAAAA AAAAAAAAAA AAAAAAAAA

FIG. 7B

2. m166 LIGHT CHAIN (k) AMINO ACID COMPLETE SEQUENCE:

(FROM THE START CODEN TO THE STOP CODON)

MSVLTQVLAL LLLWLTGARC DIQMTQSPAS LSASVGETVT ITCRASGNIQ
NYLAWYQQTQ GKSPQLLVYS AKTLADGVPS RFSGSGSGTQ YSLKINSLQP
EDFGSYYCQH FWSTPYTFGG GTKLEIKRAD AAPTVSIFPP SSEQLTSGGA
SVVCFLNNFY PKDINVKWKI DGSERQNGVL NSWTDQDSKD STYSMSSTLT
LTKDEYERHN SYTCEATHKT STSPIVKSFN RNEC[STOP]

[SIG-PEP] MSVLTQVLALLLLWLTGARC

[VL-REGION]
FR1: DIQMTQSPASLSASVGETVTITC
CDR1: RASGNIQNYLA
FR2: WYQQTQGKSPQLLVY
CDR2: SAKTLAD
FR3: GVSRFSGSGSGTQSLKINSLQPEDFGSYYC
CDR3: QHFWSTPYT
FR4: FGGGTKLEIKR

[CL-REGION]
CL: ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVL
NSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC[STOP]

FIG. 7C

SYNTHETIC RECOMBINANT SINGLE CHAIN ANTIBODY (scFv-m166)
1. DNA SEQUENCE:

ATGAAAAAAC TGCTGTTCGC GATTCCGCTG GTGGTGCCGT TCTATAGCCA
TAGCACCATG GAGCTCGAGC GGCAGGTGCA GCTGAAGCAG TCAGGACCTG
GCCTAGTGCG GCCCTCACAG AGCCTGTCCA TCACCTGCAC AGTCTCTGGT
TTCTCATTAA CTAGCTATGG TGTACACTGG GTTCGTCAGT CTCCAGGAAA
GGGTCTGGAG TGGCTGGAG TGATATGGAG TGGTGGAGAC ACAGACTATA
ATGCAGCTTT CATATCCAGA CTGAGCATCA GCAAGGACAA TTCCAAGAGC
CAACTCTTCT TTAAAATGAA CAGTCTGCGA GCTACTGACA CAGCCATATA
TTACTGTGCC AGAAATAGAG GGGATATTTA CTATGATTTC ACTTATGCCA
TGGACTACTG GGGTCAAGGA ACCTCAGTCA CCGTCTCCTC AGGTGGAGGC
GGCTCAGGCG GAGGTGGCTC TGGCGGTGGC GGATCGGACA TCCAGATGAC
TCAGTCTCCA GCCTCCCTAT CTGCATCTGT GGGAGAAACT GTCACCATCA
CATGTCGAGC AAGTGGGAAT ATTCAAAATT ATTTAGCATG GTATCAGCAG
ACACAGGGAA AATCTCCTCA GCTCCTGGTC TATTCTGCAA AAACCTTAGC
AGATGGTGTG CCATCAAGGT TCAGTGGCAG TGGATCAGGA ACACAATATT
CTCTCAAGAT CAACAGCCTG CAGCCTGAAG ATTTTGGGAG TTATTACTGT
CAACATTTTT GGAGTACTCC GTACACGTTC GGAGGGGGGA CCAAGCTGGA
AATAAAACGG GCTCTAGAAC AAAAACTCAT CTCAGAAGAG GATCTGAATA
GCGCCGTCGA CCATCATCAT CATCATCATT GA

FIG. 8A

2. AMINO ACID SEQUENCE:

MKKLLFAIPL	VVPFYSHSTM	ELERQVQLKQ	SGPGLVRPSQ	SLSITCTVSG
FSLTSYGVHW	VRQSPGKGLE	WLGVIWSGGD	TDYNAAFISR	LSISKDNSKS
QLFFKMNSLR	ATDTAIYYCA	RNRGDIYYDF	TYAMDYWGQG	TSVTVSSGGG
GSGGGGSGGG	GSDIQMTQSP	ASLSASVGET	VTITCRASGN	IQNYLAWYQQ
TQGKSPQLLV	YSAKTLADGV	PSRFSGSGSG	TQYSLKINSL	QPEDFGSYYC
QHFWSTPYTF	GGGTKLEIKR	ALEQKLISEE	DLNSAVDHHH	HHH[STOP]

FIG. 8B

[GENE III SIGNAL SEQUENCE] MKKLLFAIPLVVPFYSHS
[JOINT-1] TMELER
[m166 HEAVY CHAIN]
QVQLKQSGPG LVRPSQSLSI TCTVSGFSLT SYGVHWVRQS PGKGLEWLGV
IWSGGDTDYN AAFISRLSIS KDNSKSQLFF KMNSLRATDT AIYYCARNRG
DIYYDFTYAM DYWGQGTSVT VSS
[scFv-LINKER] GGGGSGGGGSGGGGS
[m166 LIGHT CHAIN]
DIQMTQSPAS LSASVGETVT ITCRASGNIQ NYLAWYQQTQ GKSPQLLVYS
AKTLADGVPS RFSGSGSGTQ YSLKINSLQP EDFGSYYCQH FWSTPYTFGG
GTLEIKR
[JOINT-2] AL
[MYC EPITOPE] EQKLISEEDL
[JOINT-3] NSAVD
[HEXAHISTIDINE TAG] HHHHHH [STOP]

FIG. 8C

SEQUENCE LISTING

<110> Frank, Dara W.
Wiener-Kronish, Jeannine
Yahr, Timothy L.
Sawa, Teiji
Fritz, Robert B.

<120> Method of and compositions for immunization with the pseudomonas V antigen

<130> 650053.91487

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(54) Title: METHOD AND COMPOSITIONS FOR IMMUNIZATION WITH THE PSEUDOMONAS V ANTIGEN

(57) Abstract: A method of inhibiting, moderating or diagnosing *Pseudomonas aeruginosa* infection is disclosed. In one embodiment, this method comprises inoculating a patient with an effective amount of PerV antigen.

【国際調査報告】

INTERNATIONAL SEARCH REPORT

Inte >nal Application No
PCT/US 02/02382

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/104 A61K48/00 G01N33/569 C07K16/12 A61K39/40 A61P31/04

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 33872 A (MCW RES FOUND INC) 15 June 2000 (2000-06-15)	1-19
A	the whole document	20-37
X	SAWA T ET AL: "Active and passsive immunisation with the Pseudomonas V antigen protects against type III intoxication and lung injury" NATURE MEDICINE, NATURE AMERICA, NEW YORK, US, vol. 5, no. 4, 1999, pages 392-398, XP002142838 ISSN: 1078-8956	1,18
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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 September 2002	25/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Noë, V

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

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/02382

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HOLDER I A ET AL: "ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, THE SOCIETY, WASHINGTON, DC, US" ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, THE SOCIETY, WASHINGTON, DC, US, no. 100, 2000, pages 279-280, XP008007075 ISSN: 1060-2011 abstract	1,17
X	KATAOKA T ET AL: "THE NUCLEOTIDE SEQUENCES OF REARRANGED AND GERM LINE IMMUNO GLOBULIN HEAVY CHAIN VARIABLE REGION GENES OF A MOUSE MYELOMA MC-101 AND EVALUATION OF HEAVY CHAIN VARIABLE REGION GENES IN MOUSE" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 257, no. 1, 1982, pages 277-285, XP002213468 ISSN: 0021-9258 Sequence corresponds with EMBL accession number aaa38515.1 figure 8	24,26
T	FRANK DARA W ET AL: "Generation and characterization of a protective monoclonal antibody to Pseudomonas aeruginosa PcrV." JOURNAL OF INFECTIOUS DISEASES, vol. 186, no. 1, 2002, pages 64-73, XP008007095 ISSN: 0022-1899 the whole document	15-37

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

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 02/02382

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

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

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

Although claims 1-5, 11-17, 34-36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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

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

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

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

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

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

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

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

Remark on Protest

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

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

INTERNATIONAL SEARCH REPORT
ɪrmation on patent family members

Inte ɔnal Application No
PCT/US 02/02382

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2318536 A1	15-06-2000
		EP 1049488 A1	08-11-2000
		WO 0033872 A2	15-06-2000
		US 6309651 B1	30-10-2001

Form PCT/ISA/210 (patent family annex) (July 1992)

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Ａ６１Ｐ 37/02	Ａ６１Ｐ 37/02		４Ｃ０８５
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Ｃ１２Ｎ 1/20	Ｃ１２Ｎ 1/20	Ｚ	
Ｃ１２Ｎ 15/09	Ｃ１２Ｑ 1/06		
Ｃ１２Ｑ 1/06	Ｃ１２Ｑ 1/68	Ａ	
Ｃ１２Ｑ 1/68	Ｇ０１Ｎ 33/53	Ｍ	
Ｇ０１Ｎ 33/53	Ｇ０１Ｎ 33/569	Ｆ	
Ｇ０１Ｎ 33/569	Ｇ０１Ｎ 33/577	Ｂ	
Ｇ０１Ｎ 33/577	Ａ６１Ｋ 37/02		
// Ｃ１２Ｐ 21/08	Ｃ１２Ｎ 15/00	ＺＮＡＡ	
	Ｃ１２Ｐ 21/08		

(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,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,OM,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZM,ZW

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Ｆターム(参考) 4B024 AA01 AA11 BA31 BA53 BA61 CA02 CA09 CA20 DA02 DA03
DA06 EA04 GA11 GA25 HA11 HA13 HA14 HA17
4B063 QA01 QA18 QA19 QQ02 QQ06 QQ43 QQ53 QQ79 QQ91 QR08
QR32 QR35 QR39 QR42 QR48 QR56 QR62 QS16 QS25 QS33
QS34 QS36 QX01 QX02
4B064 AG27 CA10 CA20 CC01 CC24 DA01 DA13
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4C085 AA02 BA19 BB11 BB23 CC02 CC05 CC07 CC21 CC23 EE01
GG01 GG10
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专利名称(译)	用假单胞菌V抗原免疫的方法和组合物		
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[标]申请(专利权)人(译)	加利福尼亚大学董事会		
申请(专利权)人(译)	每次MC重影喻研究胡恩日庸股份有限公司忘川 加州大学圣地亚哥大学的Rejentsu		
[标]发明人	フランクダラダブリュー ウィーナークロニッシジーニン イヤーティモシーエル サワテイジ フリッツロバートビー		
发明人	フランク, ダラ ダブリュー. ウィーナー-クロニッシ, ジーニン イヤー, ティモシー エル. サワ, テイジ フリッツ, ロバート, ビー.		
IPC分类号	G01N33/53 A61K38/00 A61K39/00 A61K39/104 A61K39/395 A61K48/00 A61P31/04 A61P37/02 C07K14/12 C07K16/12 C12N1/20 C12N15/09 C12P21/08 C12Q1/06 C12Q1/68 G01N33/569 G01N33/577		
CPC分类号	A61K39/104 A61K2039/505 A61K2039/53 A61P31/04 A61P37/02 C07K16/1214 C07K2317/565 C07K2317/567 C07K2317/622 C12Q1/689 C12Q2600/156 G01N33/56911 G01N2333/21		
FI分类号	A61K39/00.H A61K39/395.D A61K39/395.N A61K48/00 A61P31/04 A61P37/02 C07K14/12 C12N1/20.Z C12Q1/06 C12Q1/68.A G01N33/53.M G01N33/569.F G01N33/577.B A61K37/02 C12N15/00.ZNA.A C12P21/08		
F-TERM分类号	4B024/AA01 4B024/AA11 4B024/BA31 4B024/BA53 4B024/BA61 4B024/CA02 4B024/CA09 4B024/CA20 4B024/DA02 4B024/DA03 4B024/DA06 4B024/EA04 4B024/GA11 4B024/GA25 4B024/HA11 4B024/HA13 4B024/HA14 4B024/HA17 4B063/QA01 4B063/QA18 4B063/QA19 4B063/QQ02 4B063/QQ06 4B063/QQ43 4B063/QQ53 4B063/QQ79 4B063/QQ91 4B063/QR08 4B063/QR32 4B063/QR35 4B063/QR39 4B063/QR42 4B063/QR48 4B063/QR56 4B063/QR62 4B063/QS16 4B063/QS25 4B063/QS33 4B063/QS34 4B063/QS36 4B063/QX01 4B063/QX02 4B064/AG27 4B064/CA10 4B064/CA20 4B064/CC01 4B064/CC24 4B064/DA01 4B064/DA13 4B065/AA42X 4B065/AC20 4B065/BA14 4B065/BA30 4B065/CA44 4B065/CA46 4C084/AA02 4C084/AA07 4C084/AA13 4C084/AA14 4C084/BA01 4C084/BA02 4C084/BA08 4C084/BA21 4C084/BA22 4C084/BA23 4C084/BA35 4C084/CA04 4C084/CA53 4C084/MA56 4C084/MA66 4C084/NA10 4C084/NA14 4C084/ZA592 4C084/ZB352 4C085/AA02 4C085/BA19 4C085/BB11 4C085/BB23 4C085/CC02 4C085/CC05 4C085/CC07 4C085/CC21 4C085/CC23 4C085/EE01 4C085/GG01 4C085/GG10 4H045/AA11 4H045/AA20 4H045/AA30 4H045/BA10 4H045/CA11 4H045/DA76 4H045/EA29 4H045/EA52 4H045/FA72 4H045/FA74		
代理人(译)	川崎孝雄 Toshizo饭		
优先权	09/770916 2001-01-26 US 60/264795 2001-01-29 US		
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摘要(译)

铜绿假单胞菌公开了一种抑制，调节或诊断感染的方法。在一个实施方案中，该方法包括用有效量的PcrV抗原接种患者。

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A61K 39/00	A61K 39/00	H	4B024
A61K 38/00	A61K 39/395	D	4B063
A61K 39/395	A61K 39/395	N	4B064
A61K 48/00	A61K 48/00		4B065
A61P 31/04	A61P 31/04		4C084

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(33) 優先権主張国	米国 (US)
(31) 優先権主張番号	60/264,795
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(33) 優先権主張国	米国 (US)

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最終頁に続く

(54)【発明の名称】シュードモナス外毒素Aを用いる免疫化のための方法および組成物