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最終頁に続く

(54)【発明の名称】核酸粘膜免疫

(57)【要約】
例えば、複製欠損遺伝子送達ビヒクル（特に、複製欠損アルファウイルスベクターおよび粒子）を使用する抗原の粘膜送達が記載される。粘膜アジュバントおよびＨＩＶ由来の１以上の抗原を含む組成物もまた記載される。粘膜免疫レジメン後に粘膜免疫応答、局所的免疫応答および／または全身免疫応答を誘導する際のこれらの遺伝子送達ビヒクルの使用もまた提供される。本発明は、抗原に対して免疫応答を生成する方法を含む。特定の実施形態において、この方法は、被験体の標的細胞に、少なくとも１つの抗原（またはその改変形態）をコードするポリヌクレオチドを含む複製欠損遺伝子送達ビヒクル（またはベクター）を、粘膜投与する工程を包含し、ここで、この抗原（またはその改変形態）は、その被験体中で免疫応答を刺激し得る。

【特許請求の範囲】
【請求項１】
被験体における免疫応答を生成する方法であって、該方法は、少なくとも１つの第１の抗原またはその改変形態をコードするポリヌクレオチドを含む第１の複製欠損遺伝子送達ビヒクルを標的細胞に粘膜投与する工程を包含し、該第１の抗原またはその改変形態が、粘膜に投与されたとき、該被験体における免疫応答を刺激し得る、方法。
【請求項２】
請求項１に記載の方法であって、前記粘膜投与が鼻腔内投与である、方法。
【請求項３】
請求項１に記載の方法であって、前記粘膜投与が直腸内投与である、方法。
【請求項４】
請求項１に記載の方法であって、前記粘膜投与が膣内投与である、方法。
【請求項５】
請求項１～４のいずれか１項に記載の方法であって、前記少なくとも１つの抗原が、性感染病原体に由来する、方法。
【請求項６】
請求項５に記載の方法であって、前記性感染病原体が細菌である、方法。
【請求項７】
請求項６に記載の方法であって、前記細菌が、淋病菌、クラミジアおよび梅毒からなる群より選択される、方法。
【請求項８】
請求項５に記載の方法であって、前記性感染病原体がウイルスである、方法。
【請求項９】
請求項８に記載の方法であって、前記ウイルスが、ＨＩＶ、ＨＢＶ、ＨＳＶ、ＨＣＶ、およびＨＰＶからなる群より選択される、方法。
【請求項１０】
請求項９に記載の方法であって、前記ウイルスがＨＩＶ－１である、方法。
【請求項１１】
請求項１～１０のいずれか１項に記載の方法であって、前記遺伝子送達ビヒクルが、非ウイルスベクターである、方法。
【請求項１２】
請求項１～１０のいずれか１項に記載の方法であって、前記ポリヌクレオチドが、特定のキャリアを使用して送達される、方法。
【請求項１３】
請求項１２に記載の方法であって、前記ポリヌクレオチドが、金粒子またはタングステン粒子でコーティングされ、該コーティングされた粒子が、遺伝子銃を使用して前記被験体に送達される、方法。
【請求項１４】
請求項１２に記載の方法であって、前記ポリヌクレオチドが、リポソーム調製物中にカプセル化される、方法。
【請求項１５】
請求項１～１０のいずれか１項に記載の方法であって、前記遺伝子送達ビヒクルが、ウイルスベクターである、方法。
【請求項１６】
請求項１５に記載の方法であって、前記ウイルスベクターがレトロウイルスベクターである、方法。
【請求項１７】
請求項１５に記載の方法であって、前記ウイルスベクターがアデノウイルスベクターである、方法。
【請求項１８】

請求項１５に記載の方法であって、前記ウイルスベクターがポックスウイルスベクターである、方法。
【請求項１９】
請求項１５に記載の方法であって、前記ウイルスベクターがピコルナウイルスベクターである、方法。
【請求項２０】
請求項１５に記載の方法であって、前記ウイルスベクターがアルファウイルスベクターである、方法。
【請求項２１】
請求項２０に記載の方法であって、前記アルファウイルスベクターが、シンドビスベクターである、方法。
【請求項２２】
請求項２０に記載の方法であって、前記アルファウイルスベクターが、セムリキ森林ウイルスベクター、ベネズエラウマ脳炎ウイルスベクター、およびロスリバーウイルスベクターからなる群より選択される、方法。
【請求項２３】
請求項２０に記載の方法であって、前記アルファウイルスベクターが、２以上のアルファウイルス由来の配列を含む、方法。
【請求項２４】
請求項１～２３のいずれか１項に記載の方法であって、前記被験体が哺乳動物である、方法。
【請求項２５】
請求項２４に記載の方法であって、前記哺乳動物がヒトである、方法。
【請求項２６】
請求項２０に記載の方法であって、前記アルファウイルスベクターが、抗原提示細胞に送達される、方法。
【請求項２７】
請求項２６に記載の方法であって、前記抗原提示細胞が樹状細胞である、方法。
【請求項２８】
請求項２７に記載の方法であって、前記樹状細胞がヒト樹状細胞である、方法。
【請求項２９】
請求項１～２８のいずれか１項に記載の方法であって、前記標的細胞が、インビボでの感染細胞である、方法。
【請求項３０】
請求項１～２９のいずれか１項に記載の方法であって、前記抗原が、ＨＬＡクラスＩ拘束免疫応答を誘発する、方法。
【請求項３１】
請求項３０に記載の方法であって、前記抗原が、ＨＬＡクラスＩＩ拘束免疫応答をさらに誘発する、方法。
【請求項３２】
請求項１～３１のいずれか１項に記載の方法であって、標的細胞に投与する工程の前または後に、クラスＩ　ＭＨＣタンパク質もしくはクラスＩＩ　ＭＨＣタンパク質、またはこれらの組み合わせ、またはＣＤ３、ＩＣＡＭ－１、ＬＦＡ－３、またはそれらのアナログからなる群より選択されるタンパク質のいずれかをコードする核酸分子を、標的細胞に導入する工程を包含する、方法。
【請求項３３】
請求項１～３２のいずれか１項に記載の方法であって、少なくとも１つの第２の遺伝子送達ビヒクルを投与する工程をさらに包含し、該第２の遺伝子送達ビヒクルが、少なくとも１つの第２の抗原もしくはその改変形態または免疫調節因子をコードするポリヌクレオチドを含む、方法。

【請求項３４】
請求項３３に記載の方法であって、前記第２の遺伝子送達ビヒクルが、粘膜投与される、方法。
【請求項３５】
請求項３３に記載の方法であって、前記第２の遺伝子送達ビヒクルが、非粘膜的に投与される、方法。
【請求項３６】
請求項１～３５のいずれか１項に記載の方法であって、１以上のポリペプチドを前記被験体に投与する工程をさらに包含する、方法。
【請求項３７】
請求項３６に記載の方法であって、前記ポリペプチドが、少なくとも１つの第２の抗原またはその改変形態を含む、方法。
【請求項３８】
請求項３６に記載の方法であって、前記ポリペプチドが、免疫調節因子を含む、方法。
【請求項３９】
請求項３６に記載の方法であって、前記少なくとも１つのポリペプチドが、粘膜投与される、方法。
【請求項４０】
請求項３６に記載の方法であって、前記少なくとも１つのポリペプチドが、非粘膜的に投与される、方法。
【発明の詳細な説明】
【技術分野】
【０００１】
（技術分野）
本発明は、一般的に、粘膜免疫に関し、例えば、遺伝子送達系を用いた粘膜免疫に関し、特に、複製欠損ベクターおよび／または組換えアルファウイルスのベクターおよび粒子の粘膜送達に関する。さらに、種々の経路の粘膜免疫の後に潜在的な粘膜局所免疫応答および粘膜全身免疫応答を誘導するためのこれらの系の使用もまた、記載される。
【背景技術】
【０００２】
（発明の背景）
種々の病原に対して粘膜免疫を引き起こすワクチンの開発が望ましい。多くの疾患を引き起こす病原が、粘膜表面を介して伝達される。例えば、後天性免疫不全症候群（ＡＩＤＳ）は、現代の医療が直面している最も大きな健康の脅威のうちの１つと認識され、そして世界中でのＨＩＶの性的伝達が、ＡＩＤＳの主要な原因である。今までのところ、ＡＩＤＳに対する治療もワクチンも存在しない。
【０００３】
１９８３年～１９８４年に、３つのグループが、ＡＩＤＳの疑わしい病因因子を独立して同定した。例えば、Ｂａｒｒｅ－Ｓｉｎｏｕｓｓｉら（１９８３）Ｓｃｉｅｎｃｅ ２２０：８６８－８７１；Ｍｏｎｔａｇｎｉｅｒら、Ｈｕｍａｎ Ｔ－Ｃｅｌｌ Ｌｅｕｋｅｍｉａ Ｖｉｒｕｓｅｓ（Ｇａｌｌｏ，Ｅｓｓｅｘ ＆ Ｇｒｏｓｓ，編，１９８４）；Ｖｉｌｍｅｒら（１９８４）Ｔｈｅ Ｌａｎｃｅｔ １：７５３；Ｐｏｐｏｖｉｃら（１９８４）Ｓｃｉｅｎｃｅ ２２４：４９７－５００；Ｌｅｖｙら（１９８４）Ｓｃｉｅｎｃｅ ２２５：８４０－８４２を参照のこと。これらの単離物は、リンパ節症関連ウイルス（ＬＡＶ）、ヒトＴ細胞リンパ親和性ウイルスＩＩＩ型（ＨＴＬＶ－ＩＩＩ）、またはＡＩＤＳ関連レトロウイルス（ＡＲＶ）とさまざまに呼ばれた。これらの単離物全てが、同じウイルスの株であり、そして後に、総称してヒト免疫不全ウイルス（ＨＩＶ）と命名された。関連するＡＩＤＳ原因ウイルスの単離によって、本来ＨＩＶと呼ばれたこれらの株は、現在、ＨＩＶ－１と称され、そして関連するウイルスは、ＨＩＶ－２と称される。例えば、Ｇｕｙａｄｅｒら（１９８７）Ｎａｔｕｒｅ ３２６：６６２－６６９；Ｂｒｕ

ｎ－Ｖｅｚｉｎｅｔら（１９８６）Ｓｃｉｅｎｃｅ ２３３：３４３－３４６；Ｃｌａｖｅｌら（１９８６）Ｎａｔｕｒｅ ３２４：６９１－６９５を参照のこと。従って、世界中でＨＩＶ感染を処置および／または予防するために適切な組成物および方法に対する必要性が、当該分野に存在する。

【０００４】

ＨＩＶウイルスに関する大量の情報が集められ、そしてワクチン開発のためのいくつかの標的（ＨＩＶによってコードされるｅｎｖ遺伝子産物、Ｇａｇ遺伝子産物、ｐｏｌ遺伝子産物およびｔａｔ遺伝子産物を含む）が、調べられている。ＨＩＶをコードするネイティブなポリヌクレオチドおよびＨＩＶをコードする合成のポリヌクレオチドを用いた免疫もまた、例えば、共有に係るＰＣＴ／ＵＳ９９／３１２４５およびその中に引用される参考文献中に記載されるように、記載されている。さらに、ＨＩＶをコードするポリヌクレオチドは、ワクチンを同定するための種々の試みにおいて投与されている。（例えば、Ｂａｇａｒａｚｚｉら（１９９９）Ｊ．Ｉｎｆｅｃｔ．Ｄｉｓ．１８０：１３５１－１３５５；Ｗａｎｇら（１９９７）Ｖａｃｃｉｎｅ １５：８２１－８２５を参照のこと）。ＨＩＶのマトリックス／キャプシドドメインを保有する複製可能な（ｒｅｐｌｉｃａｔｉｏｎ－ｃｏｍｐｅｔｅｎｔ）ベネズエラウマ脳炎（ＶＥＥ）アルファウイルスベクターは、動物に皮下投与されて、ＣＴＬ応答を誘発し得た（Ｃａｌｅｙら（１９９７）Ｊ．Ｖｉｒｏｌ．７１：３０３１－３０３８）。さらに、シンドビスウイルス由来のアルファウイルスベクターもまた、動物においてＨＩＶ ｇａｇ特異的応答を誘発することが示された（Ｇａｒｄｎｅｒら（２０００）Ｊ．Ｖｉｒｏｌ．７４：１１８４９－１１８５７）。同様に、ＨＩＶペプチドはまた、動物被験体に投与されている（Ｓｔａａｔｓら（１９９７）ＡＩＤＳ Ｒｅｓ Ｈｕｍ Ｒｅｔｒｏｖｉｒｕｓｅｓ １３：９４５－９５２；Ｂｅｌｙａｋｏｖ（１９９８）Ｊ．Ｃｌｉｎ．Ｉｎｖｅｓｔ．１０２：２０７２）。

【０００５】

組換えアルファウイルスベクターおよびアルファウイルスに基づく層状真核生物ベクター系もまた、記載されている。（例えば、米国特許第６，０１５，６８６号；同第６，０１５，６９４号；同第５，８４３，７２３号を参照のこと）。Ｈａｒｉｈａｒａｎら（１９９８）Ｊ．Ｖｉｒｏｌ．７２：９５０－９５８は、単純疱疹ウイルス（ＨＳＶ）１型の糖タンパク質Ｂを発現するｐＳＩＮベクターを用いた単独の筋内免疫が、広いスペクトルの免疫応答（ウイルス特異的抗体、細胞傷害性Ｔ細胞、および２つの異なるマウスモデルにおける致死的ウイルスチャレンジからの防御）を誘導したことを報告した。Ｐｏｌｏら（１９９９）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９６：４５９８－４６０３は、ｐＳＩＮプラスミドベクターではなくＳＩＮレプリコンベクター粒子を用いた免疫後の、ＨＳＶチャレンジモデルにおける類似の防御を報告した。Ｔｓｕｊｉら（１９９８）Ｊ．Ｖｉｒｏｌ．７２：６９０－６９７は、Ｐｌａｓｍｏｄｉｕｍ ｙｏｅｌｉｉサーカムスポロゾイト（ｃｉｒｃｕｍｓｐｏｒｏｚｏｉｔｅ）タンパク質またはインフルエンザウイルスの核タンパク質の、Ｉ型主要組織適合遺伝子複合体の制限された９マーエピトープを発現する組換えＳＩＮの、マウスでの皮下投与が、大きなエピトープ特異的ＣＤ８（＋）Ｔ細胞応答を誘導し、そしてマラリアもしくはインフルエンザＡウイルスでの感染に対する高い程度の防御を提供したことを、報告した。

【０００６】

しかし、これらの研究および他の研究にもかかわらず、粘膜チャレンジに対して防御し得る粘膜免疫ストラテジーのための複製欠損遺伝子送達ビヒクルの有用性は、十分には規定されていない。従って、種々の性的に伝達される病原の処置および予防に対する組成物および方法に対する必要性は残ったままである。

【発明の開示】

【課題を解決するための手段】

【０００７】

（発明の要旨）

本明細書中に開示されるのは、種々の適用（例えば、粘膜免疫を含む）における使用に適

した遺伝子送達系（例えば、組換えアルファウイルスベクター）であり、そしてさらに、本明細書中の開示は、他の関連する利点を提供する。簡単に述べると、本発明は、１つ以上の性的に伝達される疾患病原と関連する抗原を発現するベクター構築物および粒子、ならびに、特に、防御粘膜免疫レジメンにおいて、これらを作製および利用する方法を含む。好ましくは、ベクターは、例えば、複製欠損アルファウイルスベクター（例えば、シンドビス由来の複製欠損アルファウイルスベクター）である。本発明者らは、免疫後チャレンジに対する抗原特異的防御が、この抗原を発現する遺伝子送達ビヒクル（例えば、アルファウイルスベクター）の粘膜投与後に誘導され得ることを実証した。

【０００８】

１つの局面において、本発明は、抗原に対して免疫応答を生成する方法を含む。特定の実施形態において、この方法は、被験体の標的細胞に、少なくとも１つの抗原（またはその改変形態）をコードするポリヌクレオチドを含む複製欠損遺伝子送達ビヒクル（またはベクター）を、粘膜投与する工程を包含し、ここで、この抗原（またはその改変形態）は、その被験体中で免疫応答を刺激し得る。特定の実施形態において、標的細胞は、粘膜局所組織および／または粘膜全身組織中にある。粘膜投与は、例えば、鼻腔内投与、経口投与、直腸内投与、および／または膣内投与であり得る。好ましくは、性的に伝達される病原に対して、粘膜投与は、直腸経路によるかまたは膣内経路による。特定の実施形態において、少なくとも１つの抗原が、例えば、性的に伝達される病原（例えば、細菌病原（例えば、淋病、クラミジア、および梅毒）またはウイルス病原（例えば、ＨＩＶ、ＨＢＶ、ＨＳＶ、ＨＣＶおよびＨＰＶ））に由来する。特定の実施形態において、抗原は、ＨＬＡクラスＩ－制限免疫応答を誘発し、そして任意に、ＨＬＡクラスＩＩ－制限免疫応答もまた誘発する。

【０００９】

他の局面において、この方法は、免疫増強サイトカイン、リンホカイン、ケモカインなどをコードする遺伝子の送達を含む。これらの遺伝子は、目的の抗原を保有する同じ遺伝子送達ビヒクル中（例えば、アルファウイルスレプリコン粒子）に挿入され得るか、または１つ以上の異なる遺伝子送達ビヒクル上に保有され得る。特定の実施形態において、抗原は、ＨＬＡクラスＩ－制限免疫応答を誘発し、そして任意に、ＨＬＡクラスＩＩ－制限免疫応答もまた誘発する。

【００１０】

遺伝子送達ビヒクル（またはベクター）は、例えば、非ウイルスベクター；；微粒子キャリア（例えば、そのポリヌクレオチドでコーティングされ、そして遺伝子銃を用いて送達される、金粒子またはタングステン粒子）；リポソーム調製物；ウイルスベクター；レトロウイルスベクター；またはアルファウイルス由来のベクターであり得る。特定の局面において、例えば、シンドビスウイルス由来のベクター、セムリキ森林ウイルス由来のベクター、ベネズエラウマ脳炎ウイルス由来のベクター、ロス川ウイルス由来のベクターまたは多数の異なるアルファウイルスから誘導されたベクターキメラ（例えば、ＳＩＮ－ＶＥＥキメラ）のような、アルファウイルス由来のベクターが、使用される。本明細書中に記載される任意の遺伝子送達ベクター（例えば、アルファウイルスベクター）は、例えば、樹状細胞のような抗原提示細胞（ＡＰＣ）に送達され得る。特定の実施形態において、被験体および／または細胞は、哺乳動物（例えば、ヒト）である。本明細書中に記載される任意の方法において、標的細胞は、インビボで感染され得る。さらに、本明細書中に記載される任意の方法において、標的細胞に投与する工程の前または後に、クラスＩ　ＭＨＣタンパク質もしくはクラスＩＩ　ＭＨＣタンパク質のいずれか、またはこれらの組み合わせをコードする核酸分子、あるいはＣＤ３、ＩＣＡＭ－１、ＬＦＡ－３またはこれらのアナログからなる群より選択されるタンパク質をコードする核酸分子がまた、標的細胞に投与され得る。さらに、粘膜ワクチン接種のための上記の遺伝子送達ビヒクルは、１つ以上のさらなる免疫原性組成物（遺伝子送達ビヒクル、ポリペプチド、タンパク質、ケモカイン、サイトカインなど）と組合わせて使用され得、ここで、１つ以上のさらなる組成物が、粘膜経路または非粘膜経路によって送達される。

【００１１】
本発明のこれらの局面および他の局面は、以下の詳細な説明および添付の図面を参照してより明確になる。さらに、特定の手順または組成物（例えば、プラスミドなど）をより詳細に記載する種々の参考文献が、以下に記載される。これらの参考文献は、本明細書中に参考としてその全体が援用される。
【００１２】
（発明の詳細な説明）
本発明の実施は、他に示されない限り、当業者の範囲内の従来の化学方法、生化学方法、分子生物学方法、免疫学方法および薬理学方法を利用する。このような技術は、文献中に完全に説明されている。例えば、Ｒｅｍｉｎｇｔｏｎ’ｓ Ｐｈａｒｍａｃｅｕｔｉｃａｌ Ｓｃｉｅｎｃｅｓ，第１８版（Ｅａｓｔｏｎ，Ｐｅｎｎｓｙｌｖａｎｉａ：Ｍａｃｋ Ｐｕｂｌｉｓｈｉｎｇ Ｃｏｍｐａｎｙ，１９９０）；Ｍｅｔｈｏｄｓ Ｉｎ Ｅｎｚｙｍｏｌｏｇｙ（Ｓ．ＣｏｌｏｗｉｃｋおよびＮ．Ｋａｐｌａｎ編，Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ，Ｉｎｃ．）；ならびにＨａｎｄｂｏｏｋ ｏｆ Ｅｘｐｅｒｉｍｅｎｔａｌ Ｉｍｍｕｎｏｌｏｇｙ，第Ｉ～ＩＶ巻（Ｄ．Ｍ．ＷｅｉｒおよびＣ．Ｃ．Ｂｌａｃｋｗｅｌｌ編，１９８６，Ｂｌａｃｋｗｅｌｌ Ｓｃｉｅｎｔｉｆｉｃ Ｐｕｂｌｉｃａｔｉｏｎｓ）；Ｓａｍｂｒｏｏｋら，Ｍｏｌｅｃｕｌａｒ Ｃｌｏｎｉｎｇ：Ａ Ｌａｂｏｒａｔｏｒｙ Ｍａｎｕａｌ（第２版、１９８９）；Ｓｈｏｒｔ Ｐｒｏｔｏｃｏｌｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ，第４版（Ａｕｓｕｂｅｌら編、１９９９、Ｊｏｈｎ Ｗｉｌｅｙ ＆ Ｓｏｎｓ）；Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ Ｔｅｃｈｎｉｑｕｅｓ：Ａｎ Ｉｎｔｅｎｓｉｖｅ Ｌａｂｏｒａｔｏｒｙ Ｃｏｕｒｓｅ（Ｒｅａｍら編，１９９８，Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ）；ＰＣＲ（Ｉｎｔｒｏｄｕｃｔｉｏｎ ｔｏ Ｂｉｏｔｅｃｈｎｉｑｕｅｓ Ｓｅｒｉｅｓ）、第２版（Ｎｅｗｔｏｎ ＆ Ｇｒａｈａｍ編，１９９７，Ｓｐｒｉｎｇｅｒ Ｖｅｒｌａｇ）；ＰｅｔｅｒｓおよびＤａｌｒｙｍｐｌｅ，Ｆｉｅｌｄｓ Ｖｉｒｏｌｏｇｙ（第２版）Ｆｉｅｌｄｓら（編）Ｂ．Ｎ．Ｒａｖｅｎ Ｐｒｅｓｓ，Ｎｅｗ Ｙｏｒｋ，ＮＹを参照のこと。
【００１３】
本明細書中に引用される全ての刊行物、特許および特許出願は、（上記であるか下記であるかにかかわらず）本明細書中によって参考としてその全体が援用される。
【００１４】
本明細書および添付の特許請求の範囲で使用される場合、単数形「ａ」、「ａｎ」および「ｔｈｅ」は、その文脈が明らかに他を示さない限り、複数形の参照を含む。従って、例えば、「抗原（単数形）」に対する言及は、２つ以上のこのような因子の混合物を含む。
【００１５】
本発明を示す前に、本明細書の後ろに使用される特定の用語の定義を最初に示すことが、本発明の理解に役立ち得る。
【００１６】
「遺伝子移入」または「遺伝子送達」は、宿主細胞へ目的のＤＮＡを確実に挿入するための方法または系をいう。このような方法は、組込まれていない移入ＤＮＡの一過性発現、染色体外複製および移入されたレプリコンの発現（例えば、エピソーム）、または宿主細胞のゲノムＤＮＡへの移入された遺伝子材料の組み込みを引き起こし得る。遺伝子送達発現ベクターとしては、アルファウイルス、ポックスウイルス、およびワクシニアウイルス由来のベクターが挙げられるが、これらに限定されない。免疫のために使用する場合、このような遺伝子送達発現ベクターは、ワクチンまたはワクチンベクターと称され得る。
【００１７】
用語「複製欠損（ｒｅｐｌｉｃａｔｉｏｎ－ｄｅｆｅｃｔｉｖｅ）」および「複製不能（ｒｅｐｌｉｃａｒｉｏｎ－ｉｎｃｏｍｐｅｔｅｎｔ）」は、標的細胞に投与された後にさらなる感染性のウイルス粒子を作製も増殖もしない遺伝子送達ビヒクル（例えば、ウイルスベクター）をいうために、交換可能に使用される。当業者に明らかであるように、投与されるベクターまたは粒子中に含まれるポリヌクレオチド（例えば、ＲＮＡ）は、増殖ま

たは複製し得るが、新しい子孫のベクターまたはウイルス粒子は形成されず、導入後に細胞から細胞へと広がらない。

【００１８】

「アルファウイルスベクター構築物」は、目的の配列または遺伝子の発現を指向し得るアセンブリをいう。例えば、米国特許第６，０１５，６９５号；米国特許第６，０１５，６８６号；米国特許第５，８４２，７２３号、およびＷＯ９７／３８０８７に記載されるように、ベクター構築物は、アルファウイルスの転写を開始し得る５’配列、ならびに発現された場合に、生物学的に活性なアルファウイルス非構造タンパク質をコードする配列（例えば、ＮＳＰ１、ＮＳＰ２、ＮＳＰ３、およびＮＳＰ４）、およびアルファウイルスＲＮＡポリメラーゼ認識配列を含むべきである。さらに、ベクター構築物は、ウイルス連結プロモーター領域を含むべきであり、この領域は、特定の実施形態において、サブゲノムフラグメントのウイルス転写を妨害するか、増大するかまたは低下させるために、改変され得る。このベクターはまた、生存可能なウイルスベクター粒子の産生を可能にするのに十分なサイズの核酸分子、インビトロもしくはインビボにおいてｃＤＮＡからウイルスＲＮＡの合成を開始し得る５’プロモーター、ならびに１つ以上の制限部位、複数の抗原を発現するための手段（例えば、ＩＲＥＳエレメント）およびポリアデニル化配列を含み得る。アルファウイルスベクター構築物を構成する任意の配列は、１つ以上のアルファウイルスから誘導され得る。ＲＮＡ分子の場合、アルファウイルスベクター構築物もまた、「ＲＮＡレプリコン」と称され得る。

【００１９】

「構造タンパク質発現カセット」は、アルファウイルスの構造タンパク質を発現し得る、組換え産生される分子をいう。この発現カセットは、プロモーターおよびアルファウイルス構造タンパク質をコードする配列を含む必要がある。必要に応じて、この発現カセットは、転写終結部位、スプライス認識部位、およびポリアデニル化付加部位を含み得る。好ましいプロモーターとしては、ＣＭＶプロモーターおよびアデノウイルスＶＡ１ＲＮＡプロモーター、ならびにアルファウイルスサブゲノム連結領域プロモーターが挙げられる。さらに、発現カセットは、Ｎｅｏ、ＳＶ２　Ｎｅｏ、ハイグロマイシン、フレオマイシン、ヒスチジノールおよびＤＨＦＲのような選択マーカーを含み得る。

【００２０】

「組換えアルファウイルス粒子」は、アルファウイルスベクター構築物を含むキャプシドをいう。好ましくは、アルファウイルスキャプシドは、ウイルスコードタンパク質（例えば、エンベロープタンパク質）が埋め込まれる脂質二重層（例えば、細胞膜）内に含まれる。本発明のアルファウイルスベクター構築物を含む種々のベクターが、アルファウイルス粒子内に含まれ得る。さらに、組換えアルファウイルス粒子は、多数の異なるアルファウイルス由来のエレメントを含むキメラであり得る（例えば、ＶＥＥ由来のキャプシドタンパク質および／またはエンベロープタンパク質を有する、ＳＩＮ由来のＲＮＡベクター構築物）（共有に係る米国特許第６，３２９，２０１号もまた参照のこと）。

【００２１】

用語「ポリペプチド」および「タンパク質」は、アミノ酸残基のポリマーをいい、そして最少の長さの産物に限定されない。従って、ペプチド、オリゴペプチド、ダイマー、マルチマーなどは、この定義内に含まれる。全長タンパク質およびそのフラグメントの両方が、この定義によって含まれる。この用語はまた、ポリペプチドの発現後修飾、例えば、グリコシル化、アセチル化、リン酸化などを含む。さらに、本発明の目的に関して、「ポリペプチド」は、そのタンパク質が所望の活性を維持する限りは、ネイティブな配列に対する欠失、付加および置換（一般的に、天然において保存的である）などの改変を含むタンパク質をいう。これらの改変は、意図的であり得るか（例えば、部位指向型変異誘発を介して）、または偶発的であり得る（例えば、タンパク質を産生する宿主の変異もしくはＰＣＲ増幅に起因するエラーを介して）。

【００２２】

「抗原」は、体液性抗原特異的応答および／または細胞性抗原特異的応答を生成するよう

に宿主の免疫系を刺激する１つ以上のエピトープ（線状、コンフォメーショナルのいずれか、または両方）を含む分子をいう。この用語は、用語「免疫原」と交換可能に使用される。通常、Ｂ細胞エピトープは、少なくとも５アミノ酸を含むが、これは３～４アミノ酸ほど小さいものでもあり得る。Ｔ細胞エピトープ（例えば、ＣＴＬエピトープ）は、少なくとも約７～９アミノ酸を含み、そしてヘルパーＴ細胞エピトープは、少なくとも１２～２０アミノ酸を含む。通常、エピトープは、約７アミノ酸と１５アミノ酸との間のアミノ酸（例えば、９、１０、１２、または１５アミノ酸）を含む。用語「抗原」は、サブユニット抗原（すなわち、その抗原が天然で会合する生物全体から、隔離または分離されている抗原）、および殺傷されたか、弱毒化されたかまたは不活性化された、細菌、ウイルス、真菌、寄生虫または他の微生物の両方を示す。抗体（例えば、抗イディオタイプ抗体）またはそのフラグメント、および合成ペプチドミモトープ（ｍｉｍｏｔｏｐｅ）（これは、抗原または抗原性決定基を模倣し得る）もまた、本明細書中に使用されるような抗原の定義の下に捕えられる。同様に、インビボにおいて（例えば、遺伝子治療およびＤＮＡ免疫適用において）抗原または抗原性決定基を発現するオリゴヌクレオチドまたはポリヌクレオチドもまた、本明細書において抗原の定義内に含まれる。

【００２３】

本発明の目的に関して、抗原は、以下により完全に記載されるように、任意のいくつかの既知のウイルス、細菌、寄生虫および真菌から誘導され得る。この用語はまた、種々の腫瘍抗原のいずれかを意図する。好ましくは、抗原は、性的に伝達される病原体（例えば、ウイルスまたは細菌）から誘導される。さらに、本発明の目的に関して、「抗原」は、本明細書中に規定されるように、そのタンパク質が免疫学的応答を誘発する能力を維持する限りは、ネイティブな配列に対する欠失、付加および置換（一般的に、天然において保存的である）のような改変を含むタンパク質をいう。これらの改変は、意図的であり得るか（例えば、部位指向型変異誘発を介して）、または偶発的であり得る（例えば、抗原を産生する宿主の変異を介して）。

【００２４】

抗原または組成物に対する「免疫学的応答」は、目的の組成物中に存在する抗原に対する体液性免疫応答および／または細胞性免疫応答の被験体における発生である。本発明の目的に関して、「体液性免疫応答」は、抗体分子によって媒介される免疫応答をいい、一方、「細胞性免疫応答」は、Ｔリンパ球および／もしくは他の白血球によって媒介される免疫応答である。細胞性免疫の１つの重要な局面は、細胞溶解性Ｔ細胞（「ＣＴＬ」）による抗原特異的応答を含む。ＣＴＬは、主要組織適合遺伝子複合体（ＭＨＣ）によってコードされるタンパク質と関連して提示され、そして細胞の表面上に発現されるペプチド抗原に対して、特異性を有する。ＣＴＬは、細胞内微生物の破壊またはこのような微生物で感染された細胞の溶解を、誘導および促進するように補助するように作用する。細胞性免疫の別の局面は、ヘルパーＴ細胞による抗原特異的応答を含む。ヘルパーＴ細胞は、その表面上にＭＨＣ分子と関連するペプチド抗原を提示する細胞に対して、非特異的なエフェクター細胞の機能を刺激し、そしてその非特異的なエフェクター細胞の活性に焦点をあわせるように補助する。「細胞性免疫応答」はまた、サイトカイン、ケモカイン、ならびに活性化Ｔ細胞および／または他の白血球によって産生される他のこのような分子の産生をいい、これには、ＣＤ４＋　Ｔ細胞、およびＣＤ８＋　Ｔ細胞由来の分子が挙げられる。さらに、ケモカイン応答は、投与された抗原に応答して種々の白血球または内皮細胞によって誘導され得る。

【００２５】

細胞性免疫応答を惹起する組成物またはワクチンは、その細胞表面でのＭＨＣ分子と会合した抗原の提示によって、脊椎動物被験体を感作するために役立ち得る。その細胞媒介性免疫応答は、その表面に抗原を提示する細胞にかまたはその細胞付近に対する。さらに、抗原特異的Ｔリンパ球が、免疫された宿主のさらなる防御を可能にするために生成され得る。

【００２６】

特定の抗原が細胞媒介性免疫応答を刺激する能力は、多数のアッセイにより決定され得、そのようなアッセイは、例えば、リンパ増殖（リンパ球活性化）アッセイ、ＣＴＬ細胞傷害性細胞アッセイ、または感作された被験体におけるその抗原に特異的なＴリンパ球についてのアッセイである。そのようなアッセイは、当該分野で周知である。例えば、Ｅｒｉｃｋｓｏｎら、Ｊ．Ｉｍｍｕｎｏｌ．（１９９３）１５１：４１８９－４１９９；Ｄｏｅら、Ｅｕｒ．Ｊ．Ｉｍｍｕｎｏｌ．（１９９４）２４：２３６９－２３７６を参照のこと。細胞媒介性免疫応答を測定する最近の方法としては、細胞内サイトカインまたはＴ細胞集団によるサイトカイン分泌の測定（例えば、ＥＬＩＳＰＯＴ技術による）、あるいはエピトープ特異的Ｔ細胞の測定（例えば、テトラマー技術による）（ＭｃＭｉｃｈａｅｌ，Ａ．Ｊ．およびＯ’Ｃａｌｌａｇｈａｎ，Ｃ．Ａ．，Ｊ．Ｅｘｐ．Ｍｅｄ．１８７（９）：１３６７－１３７１，１９９８；Ｍｃｈｅｙｚｅｒ－Ｗｉｌｌｉａｍｓ，Ｍ．Ｇ．ら、Ｉｍｍｕｎｏｌ．Ｒｅｖ．１５０：５－２１，１９９６；Ｌａｌｖａｎｉ，Ａ．ら、Ｊ．Ｅｘｐ．Ｍｅｄ．１８６：８５９－８６５，１９９７により概説される）が、挙げられる。

【００２７】

従って、本明細書中で使用される免疫学的応答は、ＣＴＬの生成および／またはヘルパーＴ細胞の生成もしくは活性化を、刺激する応答であり得る。ケモカインおよび／またはサイトカインの生成もまた、刺激され得る。目的の抗原は、抗体媒介性免疫応答も惹起し得る。従って、免疫学的応答は、以下の効果のうちの１つ以上を包含し得る：Ｂ細胞による抗体の生成；ならびに／あるいは目的の組成物またはワクチン中に存在する抗原に特異的に指向されるサプレッサーＴ細胞、細胞傷害性Ｔ細胞、もしくはヘルパーＴ細胞および／またはγδＴ細胞の活性化。これらの応答は、感染性を中和し、そして／または抗体－補体依存性細胞傷害または抗体依存性細胞傷害（ＡＤＣＣ）を媒介して、免疫された宿主に対して防御を提供するように作用し得る。そのような応答は、当該分野で周知である、標準的免疫アッセイおよび中和アッセイを使用して、決定され得る。

【００２８】

「免疫学的組成物」は、被験体へのその組成物の投与が、目的の抗原性分子に対する体液性免疫応答および／または細胞性免疫応答の発生を被験体において生じる、抗原性分子を含む組成物である。その免疫原性組成物は、レシピエント被験体中に直接（例えば、注射によって、吸入によって、経口投与経路によって、鼻内投与経路によって、または他の任意の粘膜（例えば、直腸内もしくは膣内）投与経路によって）導入され得る。

【００２９】

「サブユニットワクチン」によって、目的の病原体（例えば、ウイルス、細菌、寄生生物、または真菌）に由来する抗原に由来するかまたはその抗原と相同である、選択された１つ以上の抗原（しかし、すべての抗原ではない）を含む、ワクチン組成物が意味される。そのような組成物は、インタクトな病原性細胞も病原性粒子も、またはそのような細胞もしくは粒子の溶解物も実質的に含まない。従って、「サブユニットワクチン」は、その病原体から少なくとも部分的に精製された（好ましくは、実質的に精製された）免疫原性ポリペプチド、またはそのアナログから調製され得る。従って、そのサブユニットワクチン中に含まれる抗原を得る方法としては、標準的精製技術、組換え生成、または合成生成が挙げられ得る。

【００３０】

「免疫調節因子」とは、免疫応答を調節可能な分子（例えば、タンパク質）をさす。免疫調節因子の非限定的例としては、リンホカイン（サイトカインとしても公知）（例えば、ＩＬ－６、ＴＧＦ－β、ＩＬ－１、ＩＬ－２、ＩＬ－３など）；およびケモカイン（例えば、分泌タンパク質（例えば、マクロファージ阻害因子））が、挙げられる。特定のサイトカイン（例えば、ＴＲＡＮＣＥ、ｆｌｔ－３Ｌ、および分泌形態のＣＤ４０Ｌ）は、ＡＰＣの免疫刺激能力を増強可能である。本発明の実施において単独でかまたは組み合わせて使用され得るサイトカインの非限定的例としては、インターロイキン２（ＩＬ－２）、幹細胞因子（ＳＣＦ）、インターロイキン３（ＩＬ－３）、インターロイキン６（ＩＬ－

６）、インターロイキン１２（ＩＬ－１２）、Ｇ－ＣＳＦ、顆粒球マクロファージコロニー刺激因子（ＧＭ－ＣＳＦ）、インターロイキン１α（ＩＬ－１α）、インターロイキン１１（ＩＬ－１１）、ＭＩＰ－１γ、白血球阻害因子（ＬＩＦ）、ｃ－ｋｉｔリガンド、トロンボポエチン（ＴＰＯ）、ＣＤ４０リガンド（ＣＤ４０Ｌ）、腫瘍壊死因子関連活性化誘導性サイトカイン（ＴＲＡＮＣＥ）およびｆｌｔ３リガンド（ｆｌｔ－３Ｌ）が、挙げられる。サイトカインは、いくつかの供給業者（例えば、Ｇｅｎｚｙｍｅ（Ｆｒａｍｉｎｇｈａｍ，ＭＡ）、Ａｍｇｅｎ（Ｔｈｏｕｓａｎｄ　Ｏａｋｓ，ＣＡ）、Ｒ＆Ｄ　Ｓｙｓｔｅｍｓおよびｉｍｍｕｎｅｘ（Ｓｅａｔｔｌｅ，ＷＡ））から市販されている。これらの分子の多くの配列は、例えば、ＧｅｎＢａｎｋデータベースから入手可能である。通常は明示的に記載されないが、野生型サイトカインまたは精製サイトカイン（例えば、組換え生成されたサイトカインまたはその変異体）と類似する生物学的活性を有する分子、およびこれらの分子をコードする核酸が、本発明の趣旨および範囲内で使用されることが意図される。

【００３１】

「被験体」によって、脊椎動物門の任意のメンバーが意味され、そのメンバーとしては、限定はしないが、以下が挙げられる：ヒトおよび他の霊長類（非ヒト霊長類（例えば、チンパンジーおよび他の類人猿およびサル種））；農場動物（例えば、ウシ、ヒツジ、ブタ、ヤギ、およびウマ）；家庭動物（例えば、イヌおよびネコ）；実験室動物（齧歯類（例えば、マウス、ラットおよびモルモットを含む）；鳥類（家禽、野生鳥類、および猟鳥類（例えば、ニワトリ、シチメンチョウ、および他のキジ類鳥類、ガンカモ科など））。この用語は、特定の齢を示さない。従って、成体個体および新生個体の両方が包含されることが、意図される。上記の系は、上記の任意の脊椎動物種における使用について意図される。なぜなら、これらの脊椎動物のすべての免疫系は、同様に作動するからである。

【００３２】

「薬学的に受容可能な」または「薬理学的に受容可能な」によって、生物学的にも他のいかなる様式にも望ましくないのではない物質が意味され、すなわち、その物質は、処方物または組成物中にて、いかなる望ましくない生物学的効果を引き起こすことも、その物質が含まれる組成物の成分のいずれかと有害な様式で相互作用することもなく、個体に投与され得る。

【００３３】

（Ａ．遺伝子送達系）

（１．アルファウイルスベクターおよび粒子）

上記のように、本発明は、アルファウイルスベクター構築物、そのような構築物を含むアルファウイルス粒子、ならびにそのようなベクター構築物および粒子を生成する方法および粘膜免疫においてそのようなベクター構築物および粒子を利用する方法を提供する。アルファウイルスベースの複製欠損レプリコン粒子を、粘膜ワクチン送達系として使用することは、多数の利点を提供する。おそらく、複製欠損ウイルスベクターで免疫する最も重要な局面は、免疫部位での遺伝子産物の制御である。複製ベクター（報告されている、生きた弱毒化アルファウイルスベネズエラウマ脳炎ベース（ＶＥＥ）ベクター（Ｃａｌｅｙら（１９９７）Ｊ．Ｖｉｒｏｌ．７１：３０３１－３０３８；Ｄａｖｉｓら（１９９６）Ｊ．Ｖｉｒｏｌ．７０：３７８１－３７８７）を含む）での免疫は、その宿主全体にわたるそのベクターの播種を生じる。対照的に、複製欠損ベクターでの免疫は、局所発現を生じ、新たに増殖した子孫ベクターの拡散を伴わない。さらに、複製の欠損は、免疫部位での望ましくない炎症応答の危険を減少させる（Ｖｉｌｌａｃｒｅｓ（２０００）Ｖｉｒｏｌｏｇｙ　２７０：５４）。

【００３４】

本発明における使用に適したアルファウイルスベクターは、例えば、米国特許第６，０１５，６８６号；同第６，０１５，６９４号；同第５，７８９，２４５号；および同第５，８４２，７２３号に記載されるような、任意の手段によって構築され得る。簡単に述べると、上記のベクター構築物および粒子を調製する際に使用するために適切なアルファウイ

ルスをコードする配列が、天然に存在する供給源または寄託機関（例えば、Ａｍｅｒｉｃａｎ Ｔｙｐｅ Ｃｕｌｔｕｒｅ Ｃｏｌｌｅｃｔｉｏｎ，Ｒｏｃｋｖｉｌｌｅ，Ｍａｒｙｌａｎｄ）から、本明細書中に提供される開示を考慮して容易に入手され得る。適切なアルファウイルスの代表的例としては、アウラウイルス（ＡＴＣＣ ＶＲ－３６８）、ベバルウイルス（ＡＴＣＣ ＶＲ－６００；ＡＴＣＣ ＶＲ－１２４０）、カバソ（Ｃａｂａｓｓｏｕ）ウイルス（ＡＴＣＣ ＶＲ－９２２）、チクングニヤウイルス（ＡＴＣＣ ＶＲ－６４；ＡＴＣＣ ＶＲ－１２４１）、東部ウマ脳脊髄炎ウイルス（ＡＴＣＣ ＶＲ－６５，ＡＴＣＣ ＶＲ－１２４２）、フォートモルガン（Ｆｏｒｔ Ｍｏｒｇａｎ）ウイルス（ＡＴＣＣ ＶＲ－９２４）、ゲタウイルス（ＡＴＣＣ ＶＲ－３６９；ＡＴＣＣ ＶＲ－１２４３）、キジラガチ（Ｋｙｚｙｌａｇａｃｈ）ウイルス（ＡＴＣＣ ＶＲ－９２７）、マヤロウイルス（ＡＴＣＣ ＶＲ－６６）、マヤロウイルス（ＡＴＣＣ ＶＲ－１２７７）、ミッデルブルグウイルス（ＡＴＣＣ ＶＲ－３７０）、ムカンボウイルス（ＡＴＣＣ ＶＲ－５８０；ＡＴＣＣ ＶＲ－１２４４）、ンヅム（Ｎｄｕｍｕ）ウイルス（ＡＴＣＣ ＶＲ－３７１）、ピクスナウイルス（ＡＴＣＣ ＶＲ－３７２；ＡＴＣＣ ＶＲ－１２４５）、ロスリバーウイルス（ＡＴＣＣ ＶＲ－３７３、ＡＴＣＣ ＶＲ－１２４６）、セムリキ森林ウイルス（ＡＴＣＣ ＶＲ－６７、ＡＴＣＣ ＶＲ－１２４７）、シンドビスウイルス（ＡＴＣＣ ＶＲ－６８、ＡＴＣＣ ＶＲ－１２４８）、トネート（Ｔｏｎａｔｅ）ウイルス（ＡＴＣＣ ＶＲ－９２５）、トリニティウイルス（ＡＴＣＣ ＶＲ－４６９）、ユナウイルス（ＡＴＣＣ ＶＲ－３７４）、ベネズエラウマ脳脊髄炎ウイルス（ＡＴＣＣ ＶＲ－６９）、ベネズエラウマ脳脊髄炎ウイルス（ＡＴＣＣ ＶＲ－９２３、ＡＴＣＣ ＶＲ－１２５０、ＡＴＣＣ ＶＲ－１２４９、ＡＴＣＣ ＶＲ－５３２）、西部ウマ脳脊髄炎ウイルス（ＡＴＣＣ ＶＲ－７０；ＡＴＣＣ ＶＲ－１２５１；ＡＴＣＣ ＶＲ－６２２；ＡＴＣＣ ＶＲ－１２５２）、ワタロワウイルス（ＡＴＣＣ ＶＲ－９２６）、およびＹ－６２－３３（ＡＴＣＣ ＶＲ－３７５）が、挙げられる。１つ以上のアルファウイルスから得られる配列が、同じベクターおよび／または粒子中で使用され得る。本発明の１つの好ましい局面において、野生型アルファウイルスをコードする配列が、シンドビスウイルスから得られる。

【００３５】

このアルファウイルスベクター成分は、代表的には、そのウイルスの構造タンパク質遺伝子のうちの１つ以上が１つ以上の目的遺伝子（例えば、抗原）により置換されている、自己複製ＲＮＡ（レプリコン）を含む。ＲＮＡベースのベクター（アルファウイルスベクターを含む）はまた、「レプリコン」としても公知である。なぜなら、それらのベクターは、ＲＮＡ自己増幅および高レベル発現に必要なレプリカーゼ機能を保持し、かつプラスミドＤＮＡでのトランスフェクション後（Ｄｕｂｅｎｓｋｙら（１９９６）Ｊ．Ｖｉｒｏｌ．７０：５０８－５１９）またはウイルス様粒子の感染後に、インビボで放出され得るからである。アルファウイルス構築物はまた、代表的には、不活化したか、タンデムであるか、または改変された、ウイルス接合部領域を含む。適切なアルファウイルスベクターを構築するための他の改変および方法は、例えば、米国特許第６，０１５，６８６号およびＷＯ ９７／３８０８７に記載される。

【００３６】

アルファウイルスベクター構築物のレプリコンＲＮＡは、物理的手段によって標的細胞に直接送達され得るか、または最初に粒子へとパッケージングされ得る。一般的に、パッケージングに必要な構造タンパク質は、ヘルパー構築物によってかまたはパッケージング細胞株によって、トランスで供給される。その構造タンパク質は、同種（例えば、そのベクター構築物と同じアルファウイルス由来）であり得；異種（例えば、そのベクター構築物と異なるアルファウイルス由来）であり得；そしてハイブリッド（例えば、複数のアルファウイルスのエレメントを含む）であり得る。重要なことに、そのレプリコンＲＮＡのみが、その粒子中にパッケージされる。なぜなら、ヘルパーＲＮＡは、代表的には、キャプシド化に必要なｃｉｓ作動性パッケージング配列を欠くからである。従って、培養中でかまたはインビボで標的細胞に感染する、アルファウイルス粒子が生成され得、そしてその

アルファウイルス粒子は、高レベルに目的遺伝子を発現し得る。しかし、それらのアルファウイルス粒子は、他の細胞への拡散し得るウイルス粒子を生成するのに必要なアルファウイルスゲノムの重要な部分を欠くという点で、欠損性である。このようにして、そのレプリコンＲＮＡが適切な宿主細胞中に導入される場合、そのレプリコンＲＮＡは、自己増幅し、そして目的遺伝子が、サブゲノムｍＲＮＡから発現される。しかし、新規なビリオン粒子は、構造タンパク質遺伝子が存在しないので、インビボではアセンブルされない。

【００３７】

種々のアルファウイルスレプリコンの間での直接的な比較研究は何ら実施されていないが、天然の細胞親和性の差異が存在することが、公知である。例えば、リンパ親和性ＶＥＥは、マウスＤＣを形質導入することが最近示された（ＭａｃＤｏｎａｌｄら（２０００）Ｊ．Ｖｉｒｏｌ．７４：９１４－９２２）一方で、ＳＩＮおよびＳＦＶは、リンパ親和性ではない。ヒトＤＣの感染が、アルファウイルスまたはそれに由来するベクターについて最近示された（Ｇａｒｄｎｅｒら（２０００）Ｊ．Ｖｉｒｏｌ．７４：１１８４９－１１８５７；ＷＯ　００／６１７７２）。Ｇａｒｄｎｅｒは、未成熟ヒトＤＣにおける増殖のために非常に効率が良いＳＩＮ改変体を同定した。そのＳＩＮ改変体についてのヒトＤＣ親和性の遺伝的決定要因が、糖タンパク質Ｅ２における単一アミノ酸置換にマッピングされており、この情報を使用して、ヒトＤＣを標的とするために使用され得るアルファウイルスレプリコン粒子が、生成され得る。インビトロおよびインビボの両方でレプリコンに感染したＤＣの詳細な特徴付けによって、そのレプリコン感染細胞が、その発生能力および抗原提示能力を維持することが示された。このことは、感染性疾患および悪性疾患に対するワクチン適用のためのＤＣ標的化ＳＩＮレプリコンの潜在的有用性を示した。

【００３８】

（２．さらなる遺伝子送達ビヒクル）

上記のように、粘膜遺伝子免疫は、局所免疫応答および全身免疫応答の両方を惹起することが示されている。しかし、復帰変異体および潜在的感染の危険に関連する安全性の問題によって、これらの系の有用性が制限される。さらに、複製ウイルスベースのベクターは、投与部位から宿主全体に迅速に拡散し得、そして他の部位で望ましくない炎症応答を引き起こし得る。従って、本発明は、その宿主において複製欠損性であり、従って、感染性状態に戻る可能性を持たない、ウイルスベース遺伝子送達系および非ウイルスベース遺伝子送達系を記載する。従って、本発明は、任意の複製欠損遺伝子移入ビヒクルを使用する、粘膜免疫のための組成物および方法を包含する。

【００３９】

多数のウイルスベースの系が、哺乳動物細胞中への遺伝子移入のために開発されている。例えば、レトロウイルスは、遺伝子送達系のための便利な基本骨格を提供する。選択された配列が、当該分野で公知の技術を使用して、ベクター中に挿入され得、そしてレトロウイルス粒子中にパッケージングされ得る。その後、その組換えウイルスは、単離され得、そして被験体の細胞へと、インビボまたはエキソビボのいずれかで送達され得る。多数のレトロウイルス系が記載されている（米国特許第５，２１９，７４０号；ＭｉｌｌｅｒおよびＲｏｓｍａｎ，ＢｉｏＴｅｃｈｎｉｑｕｅｓ（１９８９）７：９８０－９９０；Ｍｉｌｌｅｒ，Ａ．Ｄ．，Ｈｕｍａｎ　Ｇｅｎｅ　Ｔｈｅｒａｐｙ（１９９０）１：５－１４；Ｓｃａｒｐａら、Ｖｉｒｏｌｏｇｙ（１９９１）１８０：８４９－８５２；Ｂｕｒｎｓら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９３）９０：８０３３－８０３７；ならびにＢｏｒｉｓ－ＬａｗｒｉｅおよびＴｅｍｉｎ，Ｃｕｒ．Ｏｐｉｎ．Ｇｅｎｅｔ．Ｄｅｖｅｌｏｐ．（１９９３）３：１０２－１０９）。

【００４０】

多数のアデノウイルスベクターもまた、記載されている。宿主ゲノム中に組み込むレトロウイルスとは異なり、アデノウイルスは、染色体外に維持され、それにより、挿入変異に関連する危険が最小になる（Ｈａｊ－ＡｈｍａｄおよびＧｒａｈａｍ，Ｊ．Ｖｉｒｏｌ．（１９８６）５７：２６７－２７４；Ｂｅｔｔら、Ｊ．Ｖｉｒｏｌ．（１９９３）６７：５９１１－５９２１；Ｍｉｔｔｅｒｅｄｅｒら、Ｈｕｍａｎ　Ｇｅｎｅ　Ｔｈｅｒａｐｙ

（１９９４）５：７１７－７２９；Ｓｅｔｈら、Ｊ．Ｖｉｒｏｌ．（１９９４）６８：９３３－９４０：Ｂａｒｒら、Ｇｅｎｅ Ｔｈｅｒａｐｙ（１９９４）１：５１－５８；Ｂｅｒｋｎｅｒ，Ｋ．Ｌ．，Ｂｉｏ Ｔｅｃｈｎｉｑｕｅｓ（１９８８）６：６１６－６２９；ならびにＲｉｃｈら、Ｈｕｍａｎ Ｇｅｎｅ Ｔｈｅｒａｐｙ（１９９３）４；４６１－４７６）。

【００４１】

さらに、種々のアデノ随伴ウイルス（ＡＡＶ）ベクター系が、遺伝子送達のために開発されている。ＡＡＶベクターは、当該分野で周知の技術を使用して容易に構築され得る。例えば、米国特許第５，１７３，４４１号および同第５，１３９，９４１号；国際公開番号ＷＯ９２／０１０７０（１９９２年１月２３日公開）およびＷＯ９３／０３７６９（１９９３年３月４日公開）；Ｌｅｂｋｏｗｓｋｉら、Ｍｏｌｅｃ．Ｃｅｌｌ．Ｂｉｏｌ．（１９８８）８：３９８８－３９９６；Ｖｉｎｃｅｎｔら、Ｖａｃｃｉｎｅｓ ９０（１９９０）（Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ Ｌａｂｏｒａｔｏｒｙ Ｐｒｅｓｓ）；Ｃａｒｔｅｒ，Ｂ．Ｊ．，Ｃｕｒｒｅｎｔ Ｏｐｉｎｉｏｎ ｉｎ Ｂｉｏｔｅｃｈｎｏｌｏｇｙ（１９９２）３：５３３－５３９；Ｍｕｚｙｃｚｋａ，Ｎ．，Ｃｕｒｒｅｎｔ Ｔｏｐｉｃｓ ｉｎ Ｍｉｃｒｏｂｉｏｌ．ａｎｄ Ｉｍｍｕｎｏｌ．（１９９２）１５８：９７－１２９；Ｋｏｔｉｎ，Ｒ．Ｍ．，Ｈｕｍａｎ Ｇｅｎｅ Ｔｈｅｒａｐｙ（１９９４）５：７９３－８０１；ＳｈｅｌｌｉｎｇおよびＳｍｉｔｈ，Ｇｅｎｅ Ｔｈｅｒａｐｙ（１９９４）１：１６５－１６９；ならびにＺｈｏｕら、Ｊ．Ｅｘｐ．Ｍｅｄ．（１９９４）１７９：１８６７－１８７５を参照のこと。

【００４２】

ポリヌクレオチドを粘膜によってか他の様式で送達するために有用な別のベクター系は、Ｓｍａｌｌ，Ｊｒ．，Ｐ．Ａ．ら（１９９７年１０月１４日発行の米国特許第５，６７６，９５０号（本明細書中に参考として援用される））により記載される腸内投与される組換えポックスウイルスワクチン、ならびにワクシニアウイルスおよび鳥類ポックスウイルスである。例として、本願遺伝子を発現するワクシニアウイルス組換え体は、以下のように構築され得る。特定の合成Ｇａｇ／抗原コード配列をコードするＤＮＡが、まず、その配列がワクシニアプロモーター配列および隣接ワクシニアＤＮＡ配列（例えば、チミジンキナーゼ（ＴＫ）をコードする配列）と隣接するように、適切なベクター中に挿入される。その後、このベクターが、細胞をトランスフェクトするために使用され、この細胞が、ワクシニアに同時感染される。そのワクシニアプロモーター＋目的のコード配列をコードする遺伝子をそのウイルスゲノム中に挿入するように、相同組換えが作用する。生じるＴＫ組換え体は、５－ブロモデオキシウリジンの存在下でその細胞を培養し、そしてその５－ブロモデオキシウリジンに対して耐性であるウイルスプラークを選ぶことによって、選択され得る。

【００４３】

あるいは、鳥類ポックスウイルス（例えば、鶏痘ウイルスおよびカナリアポックスウイルス）もまた、本願遺伝子を送達するために使用され得る。哺乳動物病原体由来の免疫原を発現する組換え鳥類ポックスウイルスは、非鳥類種に投与された場合に防御免疫を付与することが公知である。鳥類ポックスベクターの使用は、ヒトおよび他の哺乳動物種において特に望ましい。なぜなら、その鳥類ポックス属のメンバーは、感受性の鳥類種においてのみ生産的複製をし得、従って、哺乳動物細胞において感染しないからである。組換え鳥類ポックスウイルスを生成するための方法は、当該分野で公知であり、そしてワクシニアウイルスの生成に関して上記したような、遺伝子組換えを使用する。例えば、ＷＯ９１／１２８８２；ＷＯ ８９／０３４２９；およびＷＯ９２／０３５４５を参照のこと。ピコルナウイルス由来ベクターもまた、使用され得る（例えば、米国特許第５，６１４，４１３号および同第６，０６３，３８４号を参照のこと）。

【００４４】

分子結合体化ベクター（例えば、Ｍｉｃｈａｅｌら、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．（１９９３）２６８：６８６６－６８６９およびＷａｇｎｅｒら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ

．Ｓｃｉ．ＵＳＡ（１９９２）８９：６０９９－６１０３に記載されるアデノウイルスキメラベクター）もまた、遺伝子送達のために使用され得る。

【００４５】

ワクシニアベースの感染系／トランスフェクション系は、宿主細胞において目的のコード配列（例えば、合成Ｇａｇ／ＨＣＶコア発現カセット）の誘導性一過性発現を提供するために使用され得る。この系において、細胞は、まず、バクテリオファージＴ７　ＲＮＡポリメラーゼをコードするワクシニアウイルス組換え体で、インビトロ感染される。このポリメラーゼは、Ｔ７プロモーターを保有するテンプレートのみを転写する点で、精巧な特異性を示す。感染後、細胞は、Ｔ７プロモーターにより駆動される目的ポリヌクレオチドでトランスフェクトされる。そのワクシニアウイルス組換え体から細胞質において発現されるポリメラーゼは、トランスフェクトされたＤＮＡをＲＮＡへと転写し、その後、そのＲＮＡが、宿主の翻訳機構によってタンパク質へと翻訳される。この方法は、大量のＲＮＡおよびその翻訳産物の高レベルで一過性の細胞質生成を提供する。例えば、Ｅｌｒｏｙ－ＳｔｅｉｎおよびＭｏｓｓ，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９９０）８７：６７４３－６７４７；Ｆｕｅｒｓｔら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９８６）８３：８１２２－８１２６を参照のこと。

【００４６】

（３．増幅系）

ワクシニアウイルス組換え体もしくは鳥類ポックスウイルス組換え体での感染、または他のウイルスベクターを使用する遺伝子送達のための別のアプローチとして、宿主細胞への導入後に高レベル発現をもたらす、増幅系が使用され得る。詳細には、Ｔ７　ＲＮＡポリメラーゼのコード領域の前に存在するＴ７　ＲＮＡポリメラーゼプロモーターが、操作され得る。このテンプレートに由来するＲＮＡの翻訳は、Ｔ７　ＲＮＡポリメラーゼを生成し、このＴ７　ＲＮＡポリメラーゼは、その後、より多くのテンプレートを転写する。同時に、発現がこのＴ７プロモーターの制御下にあるｃＤＮＡが、存在する。従って、この増幅テンプレートＲＮＡの翻訳から生成されたＴ７　ＲＮＡポリメラーゼのいくらかは、所望の遺伝子の転写をもたらす。いくらかのＴ７　ＲＮＡポリメラーゼが、その増幅を開始するために必要であるので、Ｔ７　ＲＮＡポリメラーゼは、そのテンプレートとともに、その転写反応を開始するために細胞中に導入され得る。このポリメラーゼは、タンパク質としてか、またはそのＲＮＡポリメラーゼをコードするプラスミド上で、導入され得る。Ｔ７系および細胞を形質転換するためのその細胞についてのさらなる考察に関して、例えば、国際公開ＷＯ９４／２６９１１；ＳｔｕｄｉｅｒおよびＭｏｆｆａｔ，Ｊ．Ｍｏｌ．Ｂｉｏｌ．（１９８６）１８９：１１３－１３０；ＤｅｎｇおよびＷｏｌｆｆ，Ｇｅｎｅ（１９９４）１４３：２４５－２４９；Ｇａｏら、Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ｒｅｓ．Ｃｏｍｍｕｎ．（１９９４）２００：１２０１－１２０６；ＧａｏおよびＨｕａｎｇ，Ｎｕｃ．Ａｃｉｄｓ．Ｒｅｓ．（１９９３）２１：２８６７－２８７２；Ｃｈｅｎら、Ｎｕｃ．Ａｃｉｄｓ　Ｒｅｓ．（１９９４）２２：２１１４－２１２０；ならびに米国特許第５，１３５，８５５号を参照のこと。

【００４７】

（４．リポソーム送達ビヒクル／脂質送達ビヒクル）

目的のポリヌクレオチドはまた、ウイルスベクターを用いずに送達され得る。例えば、被験体または被験体由来の細胞へ送達する前に、リポソーム中にＤＮＡまたはＲＮＡとしてパッケージングされ得る。脂質カプセル化は、一般的には、核酸に安定に結合するかまたは核酸を安定に捕捉し、そして保持することができるリポソームを使用して達成される。濃縮ＤＮＡ対脂質調製物の比は、変動し得るが、ほぼ１：１（ｍｇＤＮＡ：μｍｏｌ脂質）以上の脂質である。核酸の送達のためのキャリアとしてのリポソームの使用の概説に関して、ＨｕｇおよびＳｌｅｉｇｈｔ，Ｂｉｏｃｈｉｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ．（１９９１）１０９７：１－１７；Ｓｔｒａｕｂｉｎｇｅｒら、Ｍｅｔｈｏｄｓ　ｏｆ　Ｅｎｚｙｍｏｌｏｇｙ（１９８３）Ｖｏｌ．１０１，ｐｐ．５１２－５２７を参照のこと。

【００４８】

本発明における使用のためのリポソーム調製物としては、カチオン性（正に荷電した）調製物、アニオン性（負に荷電した）調製物および中性調製物が挙げられ、カチオン性リポソームが、特に好ましい。カチオン性リポソームは、プラスミドＤＮＡ（Ｆｅｌｇｎｅｒら，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９８７）８４：７４１３－７４１６）、ｍＲＮＡ（Ｍａｌｏｎｅら，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９８９）８６：６０７７－６０８１）、および精製転写因子（Ｄｅｂｓら，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．（１９９０）２６５：１０１８９－１０１９２）の機能的形態での細胞内送達を媒介することが示された。

【００４９】

カチオン性リポソームは、容易に入手可能である。例えば、Ｎ［１－２，３－ジオレオイルオキシ）プロピル］－Ｎ，Ｎ，Ｎ－トリエチルアンモニウム（ＤＯＴＭＡ）リポソームは、商標Ｌｉｐｏｆｅｃｔｉｎの下で、ＧＩＢＣＯ ＢＲＬ，Ｇｒａｎｄ Ｉｓｌａｎｄ，ＮＹから入手可能である（Ｆｅｌｇｎｅｒら，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９８７）８４：７４１３－７４１６もまた参照のこと）。他の市販の脂質としては、ＤＤＡＢ／ＤＯＰＥおよびＤＯＴＡＰ／ＤＯＰＥ（Ｂｏｅｒｈｉｎｇｅｒ）が挙げられる。他のカチオン性リポソームは、当該分野で周知の技術を用いて、容易に入手可能な材料から調製され得る。例えば、ＤＯＴＡＰ（１，２－ビス（オレオイルオキシ）－３－（トリメチルアンモニオ）プロパン）リポソームの合成の説明に関して、Ｓｚｏｋａら，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９７８）７５：４１９４－４１９８；ＰＣＴ公開番号ＷＯ ９０／１１０９２を参照のこと。カチオン性微粒子が、当該分野で公知の技術を使用して、容易に利用可能な材料から調製され得る。例えば、共有に係るＷＯ９１／１３６５９９を参照のこと。

【００５０】

同様に、アニオン性リポソームおよび中性リポソームは、（例えば、Ａｖａｎｔｉ Ｐｏｌａｒ Ｌｉｐｉｄｓ（Ｂｉｒｍｉｎｇｈａｍ，ＡＬ）から）容易に入手可能であるか、または容易に入手可能な材料を用いて容易に調製され得る。このような材料としては、とりわけ、ホスファチジルコリン、コレステロール、ホスファチジルエタノールアミン、ジオレオイルホスファチジルコリン（ＤＯＰＣ）、ジオレオイルホスファチジルグリセロール（ＤＯＰＧ）、ジオレオイルホスファチジルエタノールアミン（ＤＯＰＥ）が挙げられる。これらの材料はまた、ＤＯＴＭＡ出発材料およびＤＯＴＡＰ出発材料と適切な比で混合され得る。これらの材料を用いてリポソームを作製するための方法は、当該分野で周知である。

【００５１】

リポソームは、多重膜リポソーム（ＭＬＶ）、小さな単膜リポソーム（ＳＵＶ）、または大きな単膜リポソーム（ＬＵＶ）を包含し得る。種々のリポソーム－核酸複合体が、当該分野で公知の方法を使用して調製される。例えば、Ｓｔｒａｕｂｉｎｇｅｒら，ＭＥＴＨＯＤＳ ＯＦ ＩＭＭＵＮＯＬＯＧＹ（１９８３），第１０１巻，５１２－５２７頁；Ｓｚｏｋａら，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９７８）７５：４１９４－４１９８；Ｐａｐａｈａｄｊｏｐｏｕｌｏｓら，Ｂｉｏｃｈｉｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ（１９７５）３９４：４８３；Ｗｉｌｓｏｎら，Ｃｅｌｌ（１９７９）１７：７７；ＤｅａｍｅｒおよびＢａｎｇｈａｍ，Ｂｉｏｃｈｉｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ（１９７６）４４３：６２９；Ｏｓｔｒｏら，Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ｒｅｓ．Ｃｏｍｍｕｎ．（１９７７）７６：８３６；Ｆｒａｌｅｙら，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９７９）７６：３３４８；ＥｎｏｃｈおよびＳｔｒｉｔｔｍａｔｔｅｒ，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９７９）７６：１４５；Ｆｒａｌｅｙら，Ｊ Ｂｉｏｌ．Ｃｈｅｍ．（１９８０）２５５：１０４３１；ＳｚｏｋａおよびＰａｐａｈａｄｊｏｐｏｕｌｏｓ，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ（１９７８）７５：１４５；ならびにＳｃｈａｅｆｅｒ－Ｒｉｄｄｅｒら，Ｓｃｉｅｎｃｅ（１９８２）２１５：１６６を参照のこと。

【００５２】

このＤＮＡおよび／またはタンパク質抗原はまた、Ｐａｐａｈａｄｊｏｐｏｕｌｏｓら、Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ．（１９７５）３９４：４８３－４９１により記載されるものと類似する渦巻形脂質組生物中で送達され得る。米国特許第４，６６３，１６１号および同第４，８７１，４８８号を参照のこと。

【００５３】

（５．粒子状キャリア）

この組成物はまた、粒子状キャリアにカプセル化され得るか、吸着され得るか、または粒子状キャリアと結合し得る。このようなキャリアは、免疫系に対し選択された抗原の多数のコピーを提示し、局所的なリンパ節での抗原の移動、捕捉および保持を促進する。この粒子は、マクロファージおよび樹状細胞のような専門的（ｐｒｏｆｅｓｓｉｏｎ）抗原提示細胞により取り込まれ得るか、ならびに／またはサイトカイン放出の刺激のような他の機構を通して抗原提示を増強し得る。粒子状キャリアの例として、ポリメチルメタクリレートポリマーに由来するもの、ならびにポリ（ラクチド）およびＰＬＧとして知られるポリ（ラクチド－コ－グリコリド）に由来する微粒子が挙げられる。例えば、Ｊｅｆｆｅｒｙら、Ｐｈａｒｍ．Ｒｅｓ．（１９９３）１０：３６２－３６８；ＭｃＧｅｅ ＪＰら、Ｊ Ｍｉｃｒｏｅｎｃａｐｓｕｌ．１４（２）：１９７－２１０，１９９７；Ｏ’Ｈａｇａｎ ＤＴら、Ｖａｃｃｉｎｅ １１（２）：１４９－５４，１９９３を参照のこと。

【００５４】

さらに、他の粒子状システムおよびポリマーは、目的の遺伝子のインビボまたはエキソビボでの送達のために使用され得る。例えば、ポリリジン、ポリアルギニン、ポリオルニチン、スペルミン、スペルミジンならびにこれらの分子の複合体のようなポリマーは、目的の核酸を移入するために有用である。同様に、ＤＥＡＥデキストラン媒介型トランスフェクション、リン酸カルシウム沈殿または他の不溶性の無機塩（例えば、リン酸ストロンチウム）を使用する沈殿、ベントナイトおよびカオリンを含む珪酸アルミニウム、酸化クロム、ケイ酸マグネシウム、タルクなどは、本発明の方法での使用が見出される。例えば、遺伝子移入に有用な送達システムの概説としては、Ｆｅｌｇｎｅｒ，Ｐ．Ｌ．，Ａｄｖａｎｃｅｄ Ｄｒｕｇ Ｄｅｌｉｖｅｒｙ Ｒｅｖｉｅｗｓ（１９９０）５：１６３－１８７を参照のこと。ペプトイド（Ｚｕｃｋｅｒｍａｎ，Ｒ．Ｎ．ら、米国特許第５，８３１，００５号，１９９８年１１月３日発行）はまた、本発明の構築物の送達のために有用であり得る。

【００５５】

さらに、金またはタングステンのような粒子状キャリアを使用する微粒子銃（ｂｉｏｌｉｓｔｉｃ）送達システムは、本発明の合成発現カセットの送達のために特に有用である。この粒子は、送達されるべき合成発現カセットで覆われ、そして一般に、減圧雰囲気下で、「遺伝子銃」から黒色火薬発射を使用して高速に加速される。例えば、このような技術およびそのために有用な装置について記載は、例えば、米国特許第４，９４５，０５０号；同第５，０３６，００６号；同第５，１００，７９２号；同第５，１７９，０２２号；同第５，３７１，０１５号；および同第５，４７８，７４４号を参照のこと。さらに、針のない注入システムも使用され得る（Ｄａｖｉｓ，Ｈ．Ｌ．ら、Ｖａｃｃｉｎｅ １２：１５０３－１５０９，１９９４；Ｂｉｏｊｅｃｔ，Ｉｎｃ．，Ｐｏｒｔｌａｎｄ，ＯＲ）。

【００５６】

（Ｂ．抗原）

本明細書中に記載される任意の遺伝子送達ビヒクルは、１つ以上の異種遺伝子産物、特にポリペプチド抗原をコードする１つ以上の異種配列を含み得る。実質的に任意の異種遺伝子産物またはポリペプチドが、使用され得る。本発明の実施において特に有用な抗原として、性感染病原体あるいは粘膜表面を介して感染または伝達される他のウイルスに由来するポリペプチド抗原が挙げられる。性感染病原体およびそれに由来する抗原の非限定的な代表例として、細菌性病原体（例えば、クラミジア、淋病および梅毒）およびウイルス性病原体（例えば、ヒト免疫不全ウイルス（「ＨＩＶ」）、Ｂ型肝炎ウイルスおよびＣ型肝

炎ウイルス（それぞれ「ＨＢＶ」および「ＨＣＶ」）、ヒトパピローマウイルス（「ＨＰＶ」）、単純ヘルペスウイルス（「ＨＳＶ」）など）に由来する抗原が挙げられる。粘膜表面を介して伝達され得る他のウイルスの非限定的な例としては、ライノウイルス、インフルエンザ、ＲＳウイルス（ＲＳＶ）、パラインフルエンザウイルス（ＰＩＶ）などが挙げられる。本発明の文脈の中で使用される場合、「免疫原性部分」とは、適切な条件の下で、免疫応答（すなわち、細胞媒介型または体液性）を引き起こすことが可能な、それぞれの抗原部分のことをいう。「部分」とは、大きさが可変のものであり得るか、好ましくは、少なくとも９アミノ酸長であり、そして抗原全体を含み得る。細胞仲介型免疫応答は、主要組織適合遺伝子複合体（「ＭＨＣ」）クラスＩ提示、ＭＨＣクラスＩＩ提示またはその両方を介して媒介され得る。

【００５７】

ＨＩＶの遺伝子は、プロウイルスＤＮＡの中央領域に位置し、そして少なくとも９つのタンパク質をコードし、これらのタンパク質は３つの主要なクラスに分けられる：（１）主要な構造タンパク質、Ｇａｇ、ＰｏｌおよびＥｎｖ；（２）調節タンパク質、ＴａｔおよびＲｅｖならびに（３）補助タンパク質、Ｖｐｕ、Ｖｐｒ、ＶｉｆおよびＮｅｆ。$HIV_{SF2}$から得られる抗原と関連して本明細書中で例示されるが、他のＨＩＶ改変体から得られる配列は、本明細書の教示に従って類似の様式で操作され得る。このような他の改変体として、限定しないが、単離体$HIV_{IIIb}$、$HIV_{SF2}$、$HIV-1_{SF162}$、$HIV-1_{SF170}$、$HIV_{LAV}$、$HIV_{LAI}$、$HIV_{MN}$、$HIV-1_{CM235}$、$HIV-1_{US4}$、多様なサブタイプに由来する他のＨＩＶ－１株（例えば、サブタイプＡ～Ｇ、およびＯ）、ＨＩＶ－２株および多様なサブタイプ（例えば、$HIV-2_{UC1}$および$HIV-2_{UC2}$）、ならびにサル免疫不全ウイルス（ＳＩＶ）から得られるＧａｐタンパク質コード配列が挙げられる。（例えば、Ｖｉｒｏｌｏｇｙ，第３版（Ｗ．Ｋ．Ｊｏｋｌｉｋ編　１９８８）；Ｆｕｎｄａｍｅｎｔａｌ　Ｖｉｒｏｌｏｇｙ，第２版（Ｂ．Ｎ．ＦｉｅｌｄｓおよびＤ．Ｍ．Ｋｎｉｐｅ編　１９９１）；Ｖｉｒｏｌｏｇｙ，第３版（これらおよび他の関連するウイルスの記載に関してＦｉｅｌｄｓ，ＢＮ，ＤＭ　Ｋｎｉｐｅ，ＰＭ　Ｈｏｗｌｅｙ編，１９９６，Ｌｉｐｐｉｎｃｏｔｔ－Ｒａｖｅｎ，Ｐｈｉｌａｄｅｌｐｈｉａ，ＰＡ）を参照のこと。

【００５８】

当業者には明白なように、上記の抗原の種々の免疫原性部分が、本明細書中に記載されるように投与される場合、免疫応答を誘導するために組み合わされ得る。さらに、抗原をコードする遺伝子送達ビヒクルはまた、１つ以上のさらなる遺伝子送達ビヒクルおよび／またはポリペプチドと組み合わせて使用され得る。

【００５９】

さらに、ＨＩＶのオープンリーディングフレームについて異なる地理的地域（ｇｅｏｇｒａｐｈｉｃ　ｒｅｇｉｏｎ）に見出される大きな免疫学的可変性に起因して抗原の特定の組み合わせは、特定の地理的地域において投与するために好まれ得る。簡単に言うと、ＨＩＶの少なくとも８つの異なるサブタイプが同定されとおり、そしてそのうちサブタイプＢウイルスは、北アメリカ、ラテンアメリカおよびカリブ海、ヨーロッパ、日本ならびにオーストラリアでより流行している。ほぼ全てのサブタイプが、サハラ以南のアフリカに存在し、サブタイプＡおよびＤは中央アフリカおよび東アフリカで優勢であり、そしてサブタイプＣは、南アフリカで優勢である。サブタイプＣはまた、インドで流行しており、そして最近、南ブラジルで同定された。サブタイプＥは、はじめにタイで同定され、そしてまた、中央アフリカ共和国にも存在する。サブタイプＦは、はじめにブラジルおよびルーマニアで記載された。最も最近記載されたサブタイプは、Ｇであり、ロシアおよびガボンで見出され、そしてサブタイプＨは、ザイールおよびカメルーンで見出された。グループＯウイルスは、また、カメルーンおよびガボンにおいても見出された。従って、当業者には明らかなように、投与する地理的地域において流行している、特定のＨＩＶサブタイプに適切な投与のためのベクターを構築することが、一般的に好ましい。特定の地域のサブタイプは、２次元２重免疫拡散法、またはその地域の中の個体から単離されたＨＩＶゲ

ノム（またはそのフラグメント）の配列決定によって決定され得る。

【００６０】

上記のように、種々のＧａｇ抗原およびＥｎｖ抗原もまた、ＨＩＶによって提示される。ＨＩＶ－１　Ｇａｇタンパク質は、会合、粒子の放出後のビリオンの成熟およびウイルス複製における侵入後早期段階（ｅａｒｌｙ　ｐｏｓｔ－ｅｎｔｒｙ　ｓｔｅｐ）を含むウイルスの生活環の多くの段階に関与する。ＨＩＶ－１　Ｇａｇタンパク質の役割は、多様かつ複雑である（Ｆｒｅｅｄ，Ｅ．Ｏ．（１９９８）Ｖｉｒｏｌｏｇｙ　２５１：１－１５）。

【００６１】

本発明のＥｎｖコード配列は、ＨＩＶにコードされる以下のポリペプチドのポリヌクレオチド配列を含むが、それらに限定されない：ｇｐ１６０、ｇｐ１４０およびｇｐ１２０（例えば、ＨＩＶ－１$_{ＳＦ２}$（「ＳＦ２」）Ｅｎｖポリペプチドについての記載に関して米国特許第５,７９２，４５９号を参照のこと）。ＨＩＶ－１の外膜タンパク質は、約１６０ｋＤの糖タンパク質である（ｇｐ１６０）。宿主細胞へのウイルス感染の間に、ｇｐ１６０は、宿主細胞プロテアーゼによって切断され、ｇｐ１２０および内在性膜タンパク質、ｇｐ４１を形成する。このｇｐ４１部分は、ビリオンの２重膜に固着される（そして２重膜にまたがる）が、ｇｐ１２０セグメントは、周囲の環境中に突き出る。ｇｐ１２０とｇｐ４１との間に共有結合による付着は存在しないので、遊離型ｇｐ１２０は、ビリオンおよび感染した細胞の表面から放出される。従って、ｇｐ１６０は、ｇｐ１２０およびｇｐ４１についてコードする配列を含む。このポリペプチドｇｐ４１は、オリゴマー形成ドメイン（ＯＤ）および膜貫通ドメイン（ＴＭ）を含むいくつかのドメインを含む。ネイティブのエンベロープにおいて、オリゴマー形成ドメインは、３つのｇｐ４１ポリペプチドが、３量体構造を形成するための非共有結合性会合のために必要とされる：ｇｐ４１三量体（およびそれ自体）との非共有結合性相互作用を介して、ｇｐ１２０ポリペプチドはまた、３量体構造に組織化される。切断部位は、ｇｐ１２０についてのポリペプチド配列とｇｐ４１に対応するポリペプチド配列との間にほぼ存在する。この切断部位は、この部位での切断を予防するために変異され得る。得られるｇｐ１４０ポリペプチドは、ｇｐ４１の膜貫通ドメインが欠失した、ｇｐ１６０の短縮型形態に対応する。このｇｐ１４０ポリペプチドは、ｇｐ４１部分のオリゴマー形成ドメインの存在によって、単量体形態およびオリゴマー形態（すなわち３量体）の両方で存在し得る。切断部位が切断を予防するために変異され、そしてｇｐ４１の膜貫通部分が欠失している状況では、得られるポリペプチド産物は、「変異した」ｇｐ１４０と呼ばれ得る。当業者に明らかなように、切断部位は、種々の方法によって変異され得る（例えば、ＷＯ００／３９３０２もまた参照のこと）。

【００６２】

上記のように、ＨＩＶ抗原の少なくとも１つの免疫原性部分が、遺伝子送達ビヒクルの中に取り込まれ得、そして粘膜免疫のために使用され得る。ビヒクル（例えば、アルファウイルスベクター構築物）の中に取り込まれた免疫原性部分は、様々な長さのものであり得るが、概して好ましくは、この部分は少なくとも９アミノ酸長であり、そして抗原全体を含み得る。特定の配列の免疫原性は、しばしば予想することが困難であるが、Ｔ細胞エピトープは、可能性のあるＴ－ヘルパー部位およびＣＴＬ部位についてコード領域を走査するために、ＴＳＩＴＥＳ（ＭｅｄＩｍｍｕｎｅ，Ｍａｒｙｌａｎｄ）のようなコンピュータアルゴリズムを利用して、予測され得る。この分析から、ペプチドが合成され、インビトロ細胞傷害性アッセイでの標的として使用される。しかし、他のアッセイもまた、利用され得、例えば、ＥＬＩＳＡ（これは、新たに導入されたベクターに対する抗体の存在を検出する）、ならびに、Ｔヘルパー細胞について試験するアッセイ（例えば、γ－インターフェロンアッセイ、ＩＬ－２産生アッセイおよび増殖アッセイ）が挙げられる。

【００６３】

免疫原性部分はまた、他の方法によって選択され得る。例えば、ＨＬＡ　Ａ２．１トランスジェニックマウスは、ウイルス抗原のヒトＴ細胞認識のためのモデルとして有用である

ことが示されてきた。簡単に言うと、インフルエンザウイルスシステムおよびＢ型肝炎ウイルスシステムにおいて、マウスＴ細胞レセプターレパートリーは、ヒトＴ細胞によって認識される同一の抗原決定基を認識する。両方のシステムにおいて、ＨＬＡ　Ａ２．１トランスジェニックマウスにおいて生じるＣＴＬ応答は、ＨＬＡ　Ａ２．１ハプロタイプのヒトＣＴＬによって認識されるエピトープと事実上同一のエピトープに関する（Ｖｉｔｉｅｌｌｏら（１９９１）Ｊ．Ｅｘｐ．Ｍｅｄ．１７３：１００７－１０１５；Ｖｉｔｉｅｌｌｏら（１９９２）Ａｂｓｔｒａｃｔ　ｏｆ　Ｍｏｌｅｃｕｌａｒ　Ｂｉｏｌｏｇｙ　ｏｆ　Ｈｅｐａｔｉｔｉｓ　Ｂ　Ｖｉｒｕｓ　Ｓｙｍｐｏｓｉａ）。

【００６４】

ＨＩＶのさらなる免疫原性部分は、コード配列を種々の位置にて（例えば、ＨＩＶゲノムの種々のドメイン由来の１つ以上のエピトープを含むように）短縮することによって得られ得る。上記のように、このようなドメインは、Ｇａｇ、Ｇａｇ－ｐｏｌｙｍｅｒａｓｅ、Ｇａｇ－ｐｒｏｔｅａｓｅ、ｒｅｖｅｒｓｅ　ｔｒａｎｓｃｒｉｐｔａｓｅ（ＲＴ）、ｉｎｔｅｇｒａｓｅ（ＩＮ）およびＥｎｖのような構造的ドメインを含む。これらの構造的ドメインは、しばしばさらにポリペプチド（例えば、ｐ５５、ｐ２４、ｐ６（Ｇａｇ）；ｐ１６０、ｐ１０、ｐ１５、ｐ３１、ｐ６５（ｐｏｌ、ｐｒｏｔ、ＲＴおよびＩＮ）；ならびにｇｐ１６０、ｇｐ１２０およびｇｐ４１（Ｅｎｖ））に再分割される。ＨＩＶのさらなるエピトープおよび他の性感染疾患は既知であるか、または当該分野で公知の方法を使用して容易に決定され得る。さらに本発明には、例えば、ＰＣＴ／ＵＳ９９／３１２４５；ＰＣＴ／ＵＳ９９／３１２７３およびＰＣＴ／ＵＳ９９／３１２７２に記載されるようなポリペプチドの分子改変体が含まれる。

【００６５】

他の性感染性または粘膜感染性の、ウイルス性および細菌性の両方の疾患はまた、本明細書中に記載の組成物および方法を使用して扱われ得る。例えば、ＨＳＶの場合、ＨＳＶ抗原（例えば、ｇＢ、ｇＤ）を発現するベクターによる膣の免疫は、膣に対して保護を提供し得るようである。従って、ＨＳＶに由来する１つ以上の抗原は、ＨＳＶ感染の処置および予防のために本明細書中に記載されるように使用され得る。

【００６６】

（１．異種遺伝子産物を運ぶベクターの調製）

ある実施形態において、１つ以上の抗原をコードする配列は、ウイルスベクターまたは粒子のような、遺伝子送達ビヒクルの中に組み込まれる。本明細書中に提供される開示を考慮した当業者に明らかなように、種々のウイルスベクターのパッケージングの効率、それゆえ種々のウイルスベクターのウイルスの力価、ーは、パッケージングされる配列のサイズにある程度に依存する。従って、パッケージングの効率および生存可能なベクター粒子（例えば、アルファウイルスベクター粒子）の生成を増加させるために、さらなる非コード配列は、ベクター構築物に付加され得る。

【００６７】

本明細書中に記載されるベクターまたは得られた粒子の特定の適用において、１より多くの異種遺伝子の発現が、望まれる。例えば、ＨＩＶのような性感染疾患を処置するために、ベクターまたは粒子の複数回の投与が、あるいは１より多くの遺伝子産物を発現するベクターまたは粒子の投与が、必要とされ得る。さらに、免疫原性は、抗原および免疫調節物質（例えば、サイトカイン、リンホカイン、ケモカインなど）の両方をコードすることによって、さらに増強され得る。従って、本発明の１つの実施形態において、ウイルスベクター（例えば、アルファウイルスベクター）は、適切なシグナル（例えば、リボソーム読み過し、またはシストロン間の内部リボソーム侵入部位（ＩＲＥＳ））を配置することによって構築され得る。あるいは、複数のサブゲノム（ｓｕｂｇｅｎｏｍｉｃ）連結領域プロモーター（例えば、アルファウイルスに由来する）が、利用され得る。さらに、ベクター構築物は、種々の粘膜感染（例えば、性感染）病原体の全てまたは免疫原性部分を（別々に、または１つの構築物として）発現し得る。

【００６８】

本発明の１つの局面の範囲内において、ＨＩＶ抗原の免疫原性部分の発現を指向するベクター構築物（例えば、アルファウイルスベクターおよび粒子）が、提供される。プロウイルスとしても既知のＨＩＶ－１の完全な形態は、約９．８キロベースの長さである。（例えば、Ｍｕｅｓｉｎｇら（１９８５）Ｎａｔｕｒｅ３１３：４５０－４８を参照のこと）。プロウイルスの両方の末端は、長い末端反復配列（ＬＴＲ）として既知の反復配列に隣接する。

【００６９】

上記のタンパク質（例えば抗原）をコードする配列は、種々の供給源から容易に得られ得、このような供給源として、例えば、Ａｍｅｒｉｃａｎ Ｔｙｐｅ Ｃｕｌｔｕｒｅ Ｃｏｌｌｅｃｔｉｏｎ（ＡＴＣＣ，Ｒｏｃｋｖｉｌｌｅ，ＭＤ）のような寄託機関、またはＢｒｉｔｉｓｈ Ｂｉｏ－Ｔｅｃｈｎｏｌｏｇｙ Ｌｉｍｉｔｅｄ（Ｃｏｗｌｅｙ，Ｏｘｆｏｒｄ，Ｅｎｇｌａｎｄ）のような商業的な供給源が挙げられる。Ｂ型肝炎ウイルスをコードする分子的にクローン化されたゲノムの代表的な例は、Ａｍｅｒｉｃａｎ Ｔｙｐｅ Ｃｕｌｔｕｒｅ Ｃｏｌｌｅｃｔｉｏｎ（ＡＴＣＣ，Ｒｏｃｋｖｉｌｌｅ，ＭＤ）のような供給源から入手され得る。例えば、ＡＴＣＣ Ｎｏ．４５０２０は、ｐＢＲ３２２（Ｍｏｒｉａｒｔｙら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ７８：２６０６－２６１０，１９８１）のＢａｍ ＨＩ部位におけるＢ型肝炎の総ゲノムＤＮＡ（純粋なＤａｎｅ粒子から抽出される）（Ｂｌｕｍら、ＴＩＧ ５（５）：１５４－１５８，１９８９の図３を参照のこと）を含む。

【００７０】

あるいは、目的のポリペプチドをコードする配列は、ポリメラーゼ連鎖反応（ＰＣＲ）によって生成され得る。Ｍｕｌｌｉｓら（１９８７）Ｍｅｔｈｏｄｓ Ｅｎｚｙｍｏｌ．１５５：３３５－３５０；ＰＣＲ Ｐｒｏｔｏｃｏｌｓ，Ａ Ｇｕｉｄｅ ｔｏ Ｍｅｔｈｏｄｓ ａｎｄ Ａｐｐｌｉｃａｔｉｏｎｓ，Ｉｎｎｉｓら（編）Ｈａｒｃｏｕｒｔ Ｂｒａｃｅ Ｊｏｖａｎｏｖｉｃｈ Ｐｕｂｌｉｓｈｅｒｓ，ＮＹ（１９９４））．この技術は、ＤＮＡの所望の領域を複製するために、ＤＮＡポリメラーゼ（通常、熱安定性のＤＮＡポリメラーゼ）を使用する。複製されるべきＤＮＡ領域は、複製反応を開始するための所望のＤＮＡの反対の末端および反対の鎖に相補的な具体的配列オリゴヌクレオチドによって同定される。繰り返される連続的な複製のサイクルは、使用されるプライマー対によって定められたＤＮＡフラグメントの増幅を生じる。多数のパラメータが、反応の成功に影響する。これらのパラメータの中には、アニーリング温度およびアニーリング時間、伸長時間、$Mg^{2+}$およびＡＴＰ濃度、ｐＨ、ならびにプライマーと、テンプレートとデオキシリボヌクレオチドとの相対濃度がある。

【００７１】

一旦、所望のタンパク質についてのコード配列が、調製されるかまたは単離されると、そのような配列は、任意の適切なベクターまたはレプリコンの中にクローン化され得る。多数のクローニングベクターが、当業者に既知であり、そして適切なクローニングベクターの選択は、選択の問題である。他の配列に対する連結は、当該分野で公知の標準的な手順を使用して実施される。

【００７２】

（２．ポリペプチド調製）

同様に、選択されたコード配列は、発現のための任意の適切な発現ベクターの中にクローン化され得る。この発現産物は、必要に応じて、粘膜投与に先立ち精製され得る。簡単に言うと、これらのタンパク質をコードするポリヌクレオチドは、適切な発現系の中で発現され得る発現ベクターの中に導入され得る。種々の細菌、酵母、哺乳動物、昆虫および植物の発現系が、当該分野で利用可能であり、そして任意のそのような発現系が、使用され得る。必要に応じて、これらのタンパク質をコードするポリヌクレオチドが、無細胞翻訳系において翻訳され得る。このような方法は、当該分野で周知である。タンパク質はまた、固相タンパク質合成によって構築され得る。所望の場合、ポリペプチドはまた、アミノ酸リンカーまたはシグナル配列ならびにタンパク質の精製に有用なリガンド（例えば、グ

ルタチオン－Ｓ－トランスフェラーゼおよびブドウ球菌プロテイン質Ａ）のような他のアミノ酸配列を含み得る。あるいは、目的の抗原は、商業的な供給源から購入され得る。

【００７３】

（Ｃ．送達）

本明細書中に記載される組成物（例えば、遺伝子送達ビヒクルおよび任意のポリペプチド抗原）は、任意の適切な手段（例えば、静脈内に、筋肉内に、腹腔内に、皮下に、経口的に、直腸に、眼内に、鼻腔内に）または種々の物理的な方法（例えば、リポフェクション（Ｆｅｌｇｎｅｒら（１９８９）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８４：７４１３－７４１７）、直接的なＤＮＡ注入（Ａｃｓａｄｉら（１９９１）Ｎａｔｕｒｅ ３５２：８１５－８１８）；微粒子銃（Ｗｉｌｌｉａｍｓら（１９９１）ＰＮＡＳ ８８：２７２６－２７３０）；いくつかの型リポソーム（例えば、Ｗａｎｇら（１９８７）ＰＮＡＳ ８４：７８５１－７８５５を参照のこと）；$CaPO_4$（Ｄｕｂｅｎｓｋｙら（１９８４）ＰＮＡＳ ８１：７５２９－７５３３）；ＤＮＡリガンド（Ｗｕら（１９８９）Ｊ．ｏｆ Ｂｉｏｌ．Ｃｈｅｍ．２６４：１６９８５－１６９８７）；ポリペプチドのみの投与；核酸のみの投与（ＷＯ９０／１１０９２）；または死滅したアデノウイルスに連結したＤＮＡの投与（Ｃｕｒｉｅｌら（１９９２）、Ｈｕｍ．Ｇｅｎｅ Ｔｈｅｒ．３：１４７－１５４）；ポリリジンのようなポリカチオン化合物を介して、レセプター特異的リガンドを利用して；ならびにセンダイウイルスまたはアデノウイルスのようなソラレン不活性化ウイルス）を使用して送達され得る。さらに、真核生物層状ベクター開始系（ｉｎｉｔｉａｔｉｏｎ ｓｙｓｔｅｍ）は、直接的に（すなわち、インビボに）投与されてもよく、または取り出された細胞に投与され（エキソビボ）、そして続いて戻されてもよい。

【００７４】

好ましい実施形態において、遺伝子送達ビヒクルおよび任意のポリペプチド含有組成物は、粘膜に投与される通りである。粘膜送達の方法は、当該分野で公知であり、例えば、Ｒｅｍｉｎｇｔｏｎ（上記）に記載される通りである。直腸および膣への組成物の送達は、性感染病原体の場合に特に好ましい。なぜなら、この様式では、最初に病原体に曝された細胞への接近を提供するからである。同様に、鼻腔内への投与は、例えば、鼻腔の粘膜を介して感染するライノウイルスのような、疾患において好まれ得る。いくつかの場合、鼻腔内への投与は、膣の粘膜に免疫を誘導し得、そして経口の免疫は、直腸粘膜への免疫を誘導し得る。さらに、種々の経路の粘膜投与の組み合わせおよび／または種々の経路による全身的な投与は、最適な防御および保護を誘導するために使用され得る（病原体が侵入する部位ならびに粘膜性の病原体が分散した全身の部位の両方において）。さらに、粘膜への投与は、シリンジまたは他の投与デバイスの必要性をなくする。投薬処置は、単回用量スケジュールまたは複数回用量スケジュールであり得る。

【００７５】

ある実施形態において、複製欠損ベクターは、核酸免疫などによって標準的遺伝子送達プロトコルを使用して投与される。遺伝子送達のための方法は、当該分野で公知である。例えば、米国特許第５，３９９，３４６号，同第５，５８０，８５９号，同第５，５８９，４６６号を参照のこと。アルファウイルス組成物は、脊椎動物被験体に直接的にか、あるいは被験体由来の細胞にエキソビボで送達され、そして細胞を被験体に再移植するかのいずれかにより送達され得る。好ましい実施形態において、この組成物は、インビボで送達される。インビボでの複製欠損組成物の送達は、概して、当該分野で公知の任意の手段（例えば、従来のシリンジ、Ｂｉｏｊｅｃｔ（登録商標）のような針なしの（ｎｅｅｄｌｅｓｓ）デバイスまたはＡｃｃｅｌｌ（登録商標）遺伝子送達システム（ＰｏｗｄｅｒＪｅｃｔ Ｔｅｃｈｎｏｌｏｇｉｅｓ，Ｉｎｃ．，Ｏｘｆｏｒｄ，Ｉｎｃ．，Ｏｘｆｏｒｄ，Ｅｎｇｌａｎｄ）のような遺伝子銃のいずれかを使用する注入によって）を使用して成し遂げられ得る。この構築物は、皮下に、上皮に、皮内に、筋肉内に、静脈内に、粘膜に（例えば、鼻に、直腸に、および膣に）、腹腔内に、経口的に、またはそれらの組み合わせのいずれかによって送達（例えば、注射）され得る。

【００７６】
本発明の他の局面の範囲内において、本明細書中に記載される遺伝子送達システム（組換えアルファウイルスベクターまたは組換えアルファウイルス粒子を含む）の投与のための方法が、提供される。簡単に言うと、ウイルスベクター投与の最終形態は、通常、特定の治療適用に依存し、ベクター効力を増加させる最良の形態、および最も簡便な投与経路である。概して、この実施形態は、例えば、以下によって送達されるように、設計され得る組成物を含む：（１）血流への直接的な注入；（２）特定の組織または腫瘍への直接的な注入；（３）経口投与；（４）経鼻吸入；（５）粘膜組織への直接的な適用（例えば、鼻腔内に、直腸内に、および／または膣内に）；および／または（６）形質導入された自己細胞の動物へのエキソビボ投与。従って、治療用のアルファウイルスベクターは、このベクターが以下となるような様式によって投与され得る：（ａ）正常で健康な細胞に形質導入し得、そして細胞を、治療用のタンパク質または因子（これは、全身にまたは局所的に分泌される）の生産株へと形質転換にする、（ｂ）異常細胞または欠損細胞を形質転換して、この細胞を、正常に機能する表現型へと形質転換する、（ｃ）異常細胞が破壊されるようにこの異常細胞を形質転換する、および／または（ｄ）免疫応答を操作するために、細胞を形質導入する。
【００７７】
本明細書中に開示された組成物は、単独で投与され得るか、あるいは１つ以上のさらなる遺伝子送達ビヒクルおよび／または１つ以上のタンパク質とともに投与され得る。このような実施形態において、複数の組成物が、任意の順序で、例えば、遺伝子送達ビヒクルの後にタンパク質で；複数遺伝子送達ビヒクルの後に複数タンパク質の投与によって；タンパク質投与の後に単一のまたは複数の遺伝子送達ビヒクルの投与によって；同時投与によって；などで投与され得る。従って、タンパク質および核酸の混合物は、同一のまたは異なるビヒクル、および、同一のまたは異なる投与の形態を使用して、投与され得る。
【００７８】
ある実施形態において、直接的な送達は、一般的に、上記のように、従来のシリンジまたは遺伝子銃（例えば、Ａｃｃｅｌｌ（登録商標）遺伝子送達システム（ＰｏｗｄｅｒＪｅｃｔ　Ｔｅｃｈｎｏｌｏｇｉｅｓ，Ｉｎｃ．，Ｏｘｆｏｒｄ，Ｅｎｇｌａｎｄ））を使用する注入によって、ウイルス性ベクターを用いてかまたは用いずになされる。
【００７９】
従って、注入は、皮下に、上皮に、皮内に、粘膜内に（例えば、鼻に、直腸に、口におよび膣に）、腹腔内に、静脈内に、または筋肉内のいずれかに投与され得る。他の投与形態として、肺への投与、坐剤、針なしの注入、経皮的な（ｔｒａｎｓｃｕｔａｎｅｏｕ）適用および経皮性の（ｔｒａｎｓｄｅｒｍｉｃ）適用が挙げられる。投薬処置は、単回用量スケジュールまたは複数用量スケジュールであり得る。上記のように、核酸の投与はまた、ペプチドまたは他の物質の投与と組み合わされ得る。
【００８０】
（Ｄ．薬学的組成物）
本発明はまた、複製欠損ベクター（例えば、組換えアルファウイルス構築物または組換えアルファウイルス粒子）を薬学的に受容可能なキャリア、希釈剤、またはレシピエントと組み合わせて含む薬学的組成物を含む。さらに、アジュバントのような他の成分もまた、存在し得る。米国特許第６，０１５，６９４号に、より十分に記載されるように、安定性でかつ容易に投与可能な免疫原性化合物は、特に、冷蔵および／または従来の投与手段（シリンジなど）が、簡単に利用できない第三世界の国々において、必要とされる。
【００８１】
ある実施形態において、ポリペプチドもまた、含有され得る。活性成分として免疫原性ポリペプチドを含む免疫原性化合物の調製は、当業者に公知である。代表的には、このような免疫原性化合物は、液体中に溶液または懸濁液のいずれかとして注入可能物として調製され；注入の前に液体中に溶解または懸濁液するに適切な固体形態もまた、調製され得る。調製物はまた、乳化され得るか、またはリポソーム中にタンパク質がカプセル化され得

る。
【００８２】
本発明の組成物は、好ましくは、薬学的に受容可能なキャリアを含む。このキャリアは、それ自体が宿主に有害な抗体の産生を誘導するべきでない。薬学的に受容可能なキャリアは、当業者に周知である。適切なキャリアは、代表的には、タンパク質、多糖類、ポリ乳酸、ポリグリコール酸、重合体のアミノ酸、アミノ酸コポリマー、脂質凝集物（例えば、油滴またはリポソーム）、および不活性ウイルス粒子のような、巨大で、ゆっくりと代謝される高分子である。粒子状キャリアの例として、ポリメチルメタクリルレートポリマーに由来するもの、ならびにポリ（ラクチド）およびポリ（ラクチド－コ－グリコリド）（ＰＬＧとして知られる）に由来する巨大粒子が挙げられる。例えば、Ｊｅｆｆｅｒｙら、Ｐｈａｒｍ．Ｒｅｓ．（１９９３）１０：３６２－３６８；ＭｃＧｅｅら（１９９７）Ｊ　Ｍｉｃｒｏｅｎｃａｐｓｕｌ．１４（２）：１９７－２１０；Ｏ’Ｈａｇａｎら（１９９３）Ｖａｃｃｉｎｅ　１１（２）：１４９－５４を参照のこと。このようなキャリアは、当業者に周知である。さらに、これらのキャリアは、免疫刺激因子（「アジュバント」）として機能し得る。さらに、抗原は、細菌のトキソイド（例えば、ジフテリア、破傷風、コレラなどに由来するトキソイド）ならびにＥ．ｃｏｌｉに由来するトキシンと結合され得る。
【００８３】
薬学的に受容可能な塩（例えば、無機酸塩（例えば、塩酸塩、臭化水素酸塩、リン酸塩または硫酸塩）、および有機酸の塩（例えば、アセテート、プロピオネート、マロネートまたはベンゾエート））はまた、本発明の組成物に使用され得る。特に有用なタンパク質基質は、血清アルブミン、キーホールリンペットヘモシアニン、免疫グロブリン分子、サイログロブリン、オボアルブミン、破傷風トキシドおよび当業者に周知の他のタンパク質である。本発明の組成物はまた、液体または賦形剤（例えば、水、生理食塩水、グリセロール、デキストロース、エタノールなど）を単独でかまたは組合わせて、ならびに基質（例えば、保湿剤、乳化剤、またはｐＨ緩衝化剤）を含み得る。リポソームはまた、本発明の組成物に対するキャリアとして使用され得、このようなリポソームは、上記されている。
【００８４】
簡単には、ウイルス粒子に関して、複製欠損ベクター（上記で粒子ともいわれる）は粗製形態または精製形態のいずれかで貯蔵され得る。貯蔵の方法および条件は、米国特許第６，０１５，６９４号に記載されている。
【００８５】
さらに、本明細書中に記載される組成物は、種々の賦形剤、アジュバント、キャリア、補助物質、調節剤などを含み得る。好ましくは、組成物は、免疫学的応答をマウントするに十分な抗原量を含む。適切な有効量は、当業者によって決定され得る。このような量は、比較的広範であり、慣用的試験を介して決定され得、そして一般に、抗原当たり、約０．１μｇ～約１０００μｇ、より好ましくは、約１μｇ～約３００μｇの粒子のオーダーの量である。
【００８６】
このようなアジュバントとしては、以下が挙げられるがこれらに限定されない：（１）アルミニウム塩（ミョウバン）（例えば、水酸化アルミニウム、リン酸アルミニウム、硫酸アルミニウムなど）；（２）水中油エマルジョン処方物（他の特定の免疫賦活剤（例えば、ムラミルペプチド（以下を参照のこと）または細菌性細胞壁成分）ありまたはなし）（例えば、（ａ）微小流動化剤（ｍｉｃｒｏｆｌｕｉｄｉｚｅｒ）（例えば、モデル１１０Ｙ微小流動化剤（Ｍｉｃｒｏｆｌｕｉｄｉｃｓ，Ｎｅｗｔｏｎ，ＭＡ））を使用してミクロン以下の粒子中に処方されるＭＦ５９（国際公開番号ＷＯ９０／１４８３７）（５％スクアレン、０．５％Ｔｗｅｅｎ８０および０．５％　Ｓｐａｎ８５（必要に応じて種々の量のＭＴＰ－ＰＥ（以下を参照のこと）を含有するが、必要ではない）を含有する）、（ｂ）ミクロン以下のエマルジョンに微小流動化されているかまたはより大きいサイズのエマルジョンを生成するようにボルテックスされているかのいずれかであるＳＡＦ（１０％

スクアレン、０．４％Ｔｗｅｅｎ８０、５％プルロニックブロックポリマーＬ１２１およびｔｈｒ－ＭＤＰ（以下を参照のこと）を含有する）、および（ｃ）Ｒｉｂｉ™アジュバント系（ＲＡＳ）（Ｒｉｂｉ　Ｉｍｍｕｎｏｃｈｅｍ，Ｈａｍｉｌｔｏｎ，ＭＴ）（２％スクアレン、０．２％Ｔｗｅｅｎ８０、ならびにモノホスホリピドＡ（ＭＰＬ）、トレハロースジミコレート（ＴＤＭ）および細胞壁骨格（ＣＷＳ）からなる群由来の１つ以上の細菌性細胞壁成分（好ましくは、ＭＰＬ＋ＣＷＳ（Ｄｅｔｏｘ™）を含有する）；（３）サポニンアジュバント（例えば、Ｓｔｉｍｕｌｏｎ™（Ｃａｍｂｒｉｄｇｅ　Ｂｉｏｓｃｉｅｎｃｅ，Ｗｏｒｃｅｓｔｅｒ，ＭＡ））が使用され得るか、またはそれから生成される粒子（例えば、ＩＳＣＯＭ（免疫賦活複合体））；（４）フロイント完全アジュバント（ＣＦＡ）およびフロイント不完全アジュバント（ＩＦＡ）；（５）サイトカイン（例えば、インターロイキン（ＩＬ－１、ＩＬ－２など）、マクロファージコロニー刺激因子（Ｍ－ＣＳＦ）、腫瘍壊死因子（ＴＮＦ）、βケモカイン（ＭＩＰ、１－α　Ｒａｎｔｅｓ、１－β　Ｒａｎｔｅｓなど）；（６）細菌性ＡＤＰリボシル化毒素（例えば、コレラ毒素（ＣＴ）、百日咳毒素（ＰＴ）またはＥ．ｃｏｌｉ熱不安定毒素（ＬＴ）の解毒された変異体（特に、ＬＴ－Ｋ６３（ここで、野生型アミノ酸６３位がリジンに置換されている）、ＬＴ－Ｒ７２（ここで、野生型アミノ酸７２位がアルギニンに置換されている）、ＣＴ－Ｓ１０９（ここで、野生型アミノ酸１０９位がセリンに置換されている）、およびＰＴ－Ｋ９／Ｇ１２９（ここで、野生型アミノ酸９位がリジンに、そして１２９位がグリシンに置換されている）（例えば、国際公開番号ＷＯ９３／１３２０２；ＷＯ９２／１９２６５；ＷＯ９５／１７２１１；ＷＯ９８／１８９２８およびＷＯ０１／２２９９３）；および（７）この組成物の有効性を増強するように免疫賦活剤として作用する他の物質。

【００８７】

ムラミルペプチドとしては、以下が挙げられるがこれらに限定されない：Ｎ－アセチル－ムラミル－Ｌ－トレオニル－Ｄ－イソグルタミン（ｔｈｒ－ＭＤＰ）、Ｎ－アセチル－ノルムラミル－Ｌ－アラニル－Ｄ－イソグルタミン（ｉｓｏｇｌｕａｔｍｅ）（ｎｏｒ－ＭＤＰ）、Ｎ－アセチルムラミル－Ｌ－アラニル－Ｄ－イソグルタミニル－Ｌ－アラニン－２－（１’－２’－ジパルミトイル－ｓｎ－グリセロ－３－ヒドロキシ（ｈｕｙｄｒｏｘｙ）ホスホリルオキシ）－エチルアミン（ＭＴＰ－ＰＥ）など。

【００８８】

本明細書中に記載される薬学的組成物の投与は、任意の適切な経路によってであり得る（例えば、項Ｃを参照のこと）。特に好ましくは、粘膜（例えば、直腸および／または膣）投与である。投薬処置は、単回用量スケジュールまたは複数回用量スケジュールであり得る。複数用量スケジュールは、ワクチン接種の初期経過が１～１０回の別々の用量で、続いて、免疫応答を維持および／または強化するために選択されるその後の時間間隔（例えば、第２の用量について１～４ヶ月）を開けて与えられる他の用量、そして必要ならば、続いて数ヵ月後の用量、である。投薬レジメンはまた、少なくとも一部は、モダリティーの有効性、使用されるワクチン送達、被験体の必要性および開業医の判断への依存によって決定される。

【００８９】

なお他の実施形態において、複数の投与（例えば、プライム－ブースト型投与）は、有利に使用される。例えば、目的の抗原を発現する組換えアルファウイルス粒子が投与される（例えば、ＩＶＡＧまたはＩＲ）。続いて、抗原が、例えば、ポリペプチド抗原および適切なアジュバントを含む組成物中で投与される。あるいは、抗原は、遺伝子送達ビヒクルの前に投与される。複数のポリペプチドおよび複数の遺伝子の送達ビヒクル投与（任意の順番で）もまた、使用され得る。

【００９０】

（Ｅ．複製欠損粒子および抗原性ポリペプチドを利用する方法）

本発明の特定の局面において、組成物および方法が、性感染疾患を予防、抑制、安定化または逆転し得る組成物（例えば、アルファウイルスベクター構築物）を粘膜投与するため

に提供される。このような疾患の代表的な例としては、細菌感染、ウイルス感染、特に、性感染疾患（以下が挙げられるがこれらに限定されない：ＨＩＶ、ＨＢＶ、ＨＴＬＶ　Ｉ、ＨＴＬＶ　ＩＩ、ＨＰＶ、ＨＳＶ、ＨＣＶ、クラミジア、淋病および／または梅毒）が挙げられる。

【００９１】

より具体的には、本発明の１つの局面において、組成物および方法が、性感染病原性因子に対する免疫応答（体液性または細胞媒介性のいずれか）を刺激するために提供される。その結果、この病原性因子は、殺傷されるかまたは阻害される。病原性因子の代表的な例としては、細菌及びウイルスが挙げられる。

【００９２】

本発明の１つの実施形態において、病原性因子は、ウイルスであり、そして方法が、特定の免疫応答を刺激するため、およびウイルス伝播を阻害するために提供される。この方法は、抗原またはその改変形態の発現を、（１）ウイルス抗原に対する免疫応答を開始させるか、または（２）ウイルス相互作用に必要とされる細胞レセプターを占有することによりウイルス伝播を予防するかのいずれかを行い得る感受性標的細胞を指向するベクター構築物を送達するように設計された組換えアルファウイルス粒子を使用することによる。タンパク質をコードするベクター核酸の発現は、一過性でも時間とともに安定でもよい。免疫応答が病原性抗原に対して刺激される場合、組換えアルファウイルスは、好ましくは、抗原の改変形態を発現するように設計される。この抗原は、免疫応答を刺激し、そしてネイティブな抗原と比較して低減した病原性を有する。この免疫応答は、細胞が正確な様式で抗原を提示する場合（すなわち、アクセサリー分子（例えば、ＣＤ３、ＩＣＡＭ－１、ＩＣＡＭ－２、ＬＦＡ－１またはそのアナログ（例えば、Ａｌｔｍａｎｎら、Ｎａｔｕｒｅ　３３８：５１２，１９８９））を伴うＭＨＣクラスＩ分子および／またはクラスＩＩ分子に関して）に達成される。アルファウイルスベクターで感染された細胞は、免疫応答を効率的にすることが予期される。なぜなら、アルファウイルスベクターは、本当のウイルス感染を密に模倣するからであり、そしてアルファウイルスベクターは、（ａ）複製しない細胞を感染し得、（ｂ）宿主細胞ゲノムに組み込まれず、（ｃ）生命を脅かすいずれの疾患にも関連せず、そして（ｄ）高レベルの異種タンパク質を発現する、からである。これらの差異のために、アルファウイルスベクターは、健康な個体に対するワクチン用途に使用され得る安全なウイルスベクターであると、容易に考えられ得る。

【００９３】

本発明のこの局面はさらに、同様の様式で機能することが予期され得る他の系を超える利点を有する。ここで、提示（ｐｒｅｓｅｎｔｅｒ）細胞は、完全に生存可能かつ健常であり、そして異種遺伝子と比較して低レベルのウイルス抗原が発現される。これは、異なる利点を示す。なぜなら、発現される抗原性エピトープは、抗原についての遺伝子のサブフラグメントを組換えアルファウイルスに選択的にクローニングすることによって変更され、免疫優性エピトープより重要でない免疫原性エピトープに対する応答を導き得る。このようなアプローチは、複数のエピトープを有するペプチドの発現に拡張され得、ここで、１つ以上のエピトープが異なるタンパク質由来である。さらに、本発明のこの局面は、これらのペプチドフラグメントの細胞内合成およびＭＨＣクラスＩ分子との結合を介して、抗原性エピトープ、および遺伝子のサブフラグメントによってコードされる抗原のペプチドフラグメントに対して指向される細胞傷害性Ｔリンパ球（ＣＴＬ）の効果的な刺激を可能にする。このアプローチは、ＣＴＬ誘導に対する主要な免疫優性エピトープをマッピングするのに利用され得る。

【００９４】

本明細書中に記載される粘膜投与に適切な組成物（例えば、アルファウイルス構築物および粒子）は、１つ以上の阻害緩和剤（例えば、アンチセンスなどを発現するウイルス性阻害遺伝子または細菌性阻害遺伝子）を含み得る。阻害緩和剤についての議論は、例えば、米国特許第６，０１５，６８６号に記載され、これは、抗原および阻害緩和剤（例えば、アンチセンスｔａｔなどを発現する）がどのように同時発現および／または設計されて、

感染に必要とされるタンパク質（例えば、ＣＤ４）を過剰発現し得るのかを記載する。この方法において、比較的少数のベクター感染ＨＩＶ耐性細胞は、遊離ウイルスまたはウイルス性感染細胞との、複数の非生産性融合事象に対して「シンク（ｓｉｎｋ）」または「磁石（ｍａｇｎｅｔ）」として作用する。ＨＩＶの場合、相互作用の２つの因子は、ｇｐ１２０／ｇｐ４１エンベロープタンパク質およびＣＤ４レセプター分子である。よって、適切なブロッカーは、病原性効果を生じることなくＨＩＶ侵入をブロックするＨＩＶ ｅｎｖアナログ、またはＣＤ４レセプターアナログのいずれかを発現するベクター構築物である。ＣＤ４アナログは、分泌され、そして近隣の細胞を保護するように機能するが、ｇｐ１２０／ｇｐ４１は、ベクター含有細胞のみを保護するように細胞内にのみ分泌または産生される。安定性または補体溶解を増強するためにヒト免疫グロブリン重鎖または他の成分をＣＤ４に付加することが、有利であり得る。このようなハイブリッド可溶性ＣＤ４をコードするアルファウイルスベクターを宿主へ送達することによって、安定なハイブリッド分子の連続的な供給を生じる。処置の効率は、疾患進行の通常の指標（抗体レベル、ウイルス抗原産生、感染性ＨＩＶレベル、または非特異的感染のレベルを含む）を測定することによりアッセイされ得る。

【００９５】

このような転写サプレッサータンパク質は、ウイルス特異的トランス活性化タンパク質（上記のような）によって発現が刺激される任意のウイルス特異的転写プロモーターを使用して、組織培養物中において選択され得る。ＨＩＶの特定の場合において、ＨＩＶ ｔａｔタンパク質、およびＨＩＶプロモーターによって駆動されるＨＳＶＴＫ遺伝子を発現する細胞株は、ＡＣＶの存在下で死滅する。しかし、一連の変異ｔａｔ遺伝子がこの系に導入される場合、適切な特性を用いる（すなわち、野生型ｔａｔの存在下でのＨＩＶプロモーターからの転写を抑制する）変異体は、増殖し、そして選択される。次いで、変異遺伝子は、これらの細胞から再度単離され得る。複数コピーの条件付き致死的ベクター／ｔａｔ系を含有する細胞株を使用して、生存細胞クローンがこれらの遺伝子における内因性変異によって生じないことを保証し得る。次いで、無作為に変異誘発されたｔａｔ遺伝子の一群が、「回収可能な（ｒｅｓｃｕａｂｌｅ）」アルファウイルスベクター（すなわち、変異体ｔａｔタンパク質を発現し、そして細菌性複製起点、ならびに増殖および細菌における選択についての薬物耐性マーカーを含むベクター）を使用してこれらの細胞に導入される。このことは、評価されるべき多くの無作為な変異を可能にし、そして引き続く所望の変異体細胞株の分子クローニングを容易にする。この手順を使用して、潜在的抗ウイルス治療のための種々のウイルス性転写アクチベーター／ウイルス性プロモーター系における変異を同定および利用し得る。

【００９６】

本明細書中に記載される遺伝子送達ビヒクルに使用され得るさらなる阻害緩和剤としては、所望の細胞中で異種導入遺伝子の発現が低減（抑制）され得る系が挙げられ、これは、例えば、ベクター中にＴＯＰ結合リガンドおよびパッケージングエレメントを含むことによるビリオンパッケージングプロセスの間を含む。例えば、共有に係る米国出願番号０９／６０８，７３０号に記載されるように、これらの方法は、他の細胞型全てにおける導入遺伝子の高レベル発現および翻訳の能力を維持しながら、ビリオン産生細胞における導入遺伝子翻訳の抑制を可能にする。これらの系はまた、種々のウイルスベクターに適用可能であり、そしてさらに、他のビリオン産生系（例えば、ｃｒｅ－ｌｏｘ系におけるアデノウイルスビリオン構築物を含む）と合わせられ得る。

【００９７】

以下の実施例は、例示の目的で提示され、限定の目的で提示されるのではない。

【実施例】

【００９８】

（実施例１：材料および方法）

（マウスおよび細胞株）

雌性Ｂａｌｂ／ｃマウスを、Ｃｈａｒｌｅｓ Ｒｉｖｅｒ Ｂｒｅｅｄｉｎｇ Ｌａｂｏ

ｒａｔｏｒｉｅｓから購入し、このマウスは、研究の開始時に６～８週齢であった。繊維芽細胞株ＳｖＢａｌｂ（Ｈ－２$^{d}$）を、標的細胞として使用した。この細胞株は、クラスＩＭＨＣ分子を発現するが、クラスＩＩＭＨＣ分子を発現せず、よってＣＤ８$^{+}$細胞に対して指向されるが、ＣＤ４$^{+}$細胞に対しては指向されない。

【００９９】

（材料）

ｐ７ｇは、Ｈ－２Ｋ$^{d}$で拘束されたＨＩＶ－１$_{ＳＦ２}$ｐ２４ｇａｇ　ＣＴＬエピトープであり、そして９マーの合成ペプチドである：（ａａ、１９９－ＡＭＱＭＬＫＥＴＩ－２０７）（２１）。このエピトープを、Ｒｅｓｅａｒｃｈ　Ｇｅｎｅｔｉｃｓ（Ｈｕｎｔｓｖｉｌｌｅ　ＡＬ）によるＦｍｏｃ固相法（例えば、Ｍａｔｈｉｏｗｉｔｚら、Ｎａｔｕｒｅ　３８６：４１０－４１４を参照のこと）を使用して遊離アミンＮ末端および遊離酸Ｃ末端で合成した。

【００１００】

（免疫）

５匹の雌性Ｂａｌｂ／ｃマウスの群を、各ワクチン経路または免疫経路に使用し、そして屠殺の際に、組織をプールした。データは、同様または同一の結果を有する代表的な２～４個のこのようなデータ点として示される。全ての免疫を、２～３週の間隔で３～４回行った。ＩＮ免疫を、ＰＢＳ中で懸濁された２５μｌ容量中に２．５Ｅ１０$^{6}$のＳＩＮ粒子を用いて行った。ＩＮ免疫を、麻酔なしで行った。１２．５μｌ容量中に２．５Ｅ１０$^{6}$を用いて、ＩＶＡＧ免疫およびＩＲ免疫を、麻酔したマウスにて行い、このマウスを、横臥で２０分間維持した。ＩＭ免疫を、５０μｌ容量中に２．５Ｅ１０$^{6}$のＳＩＮ粒子を用いて大腿部筋肉にて行った。このマウスを、最終免疫１週間後に屠殺した。

【００１０１】

（血清および組織の収集）

後部眼窩叢（ｒｅｔｒｏ　ｏｒｂｉｔａｌ　ｐｌｅｘｕｓ）を介して、屠殺の１日前にマウスから採血し、そしてＥＬＩＳＡアッセイのために血清を分離した。頸部リンパ節（ＣＬＮ）、腸骨リンパ節（ＩＬＮ）、膣／子宮粘膜組織（ＶＵＭ）および脾臓（ＳＰ）を群当たり５匹のマウスから回収およびプールし、そして単一の細胞懸濁物を、標準的$^{51}$Ｃｒ放出ＣＴＬアッセイ（ＶＵＭ以外）、ＩＦＮ－γ－分泌細胞（ＩＦＮＳＣ）を検出するためのｇａｇエピトープ特異的ＥＬＩＳＰＯＴ、または抗体分泌細胞（ＡＳＣ）を検出するためのｇａｇ－ｐ５５特異的ＥＬＩＳＰＯＴに使用した。

【００１０２】

（単一細胞懸濁物の調製）

５匹のマウスの群を、上記のように、ＩＮ経路、ＩＭ経路、ＩＲ経路またはＩＶＡＧ経路を介して２～３週間の間隔で３回免疫した。最終免疫の１週間後、５匹の免疫したマウスの群由来のＳＰ、ＣＬＮおよびＩＬＮの各々を、回収およびプールした。ＳＰ組織、ＣＬＮ組織およびＩＬＮ組織を、２５０μｍのポアー直径を有するナイロンメッシュを通して裂き、そして培地（ＥＬＩＳＰＯＴアッセイ培地：１０％ＦＣＳ、抗生物質、ＨｅｐｅｓおよびＬ－グルタミンを含有するＲＰＭＩ（完全ＲＰＭＩ）；$^{51}$Ｃｒ放出アッセイ培地）中で３回洗浄し、計数し、そしてＥＬＩＳＰＯＴアッセイまたは$^{51}$Ｃｒ放出アッセイについてのウェル中に播種した。群当たり５匹のマウスのプールから膣、子宮および子宮角全体を取り出すことによる改変を伴う、Ｈｏｌｍｇｒｅｎ（例えば、ＬｙｃｋｅおよびＨｏｌｍｇｒｅｎ（１９８６）Ｉｍｍｕｎｏｌｏｇｙ　５９：３０１－３０８を参照のこと）によって記載される方法に基づいて、ＶＵＭ由来の単一の細胞懸濁物を調製した。子宮角を縦に切断し、そして膣組織および子宮組織を一緒に、５ｍｍ片に刻んだ。次いで、組織片を、１０％ＦＣＳおよび５ｍＭ　Ｈｅｐｅｓを含みＣａ＋＋およびＭｇ＋＋を含まないＨＢＳＳ（完全ＨＢＳＳ）で３回洗浄した。次いで、この小片を、３７℃で連続的に攪拌しながら、完全ＨＢＳＳ中１ｍｇ／ｍｌ　コラゲナーゼ／ディスパーゼ（ｄｉｓｐａｓｅ）＋０．５ｍｇ／ｍｌ　ＤＮａｓｅで３０分間１回、そして完全ＲＰＭＩ中コラゲナーゼ＋０．５ｍｇ／ｍｌ　ＤＮａｓｅで４５分間２回酵素処理した。各酵素処理に続いて

、放出された細胞を回収し、完全ＲＰＭＩで２回洗浄した。各酵素処理から回収した細胞懸濁物を、プールおよび計数した。この方法は、慣用的に５匹のマウス当たり最少でも$10^7$個の生存単核細胞（ＭＮＣ）の回収を生じた。

【０１０３】

（$^{51}$Ｃｒ放出ＣＴＬアッセイ）

組織由来の単一細胞懸濁物を、上記のように調製し、そしてウェル当たり$5\times10^6$細胞で２４ウェルディッシュにて培養した。これらの細胞のうち、$1\times10^6$個を、１０ｍＭの濃度の合成ｐ７ｇペプチド（アミノ酸１９４～２１３）で、３７℃で１時間感作させ、次いで洗浄し、そして残りの$4\times10^6$個の未処理細胞とともに同時培養した。これらの細胞を、以下の脾細胞（Ｓｐｌｅｎｏｃｙｔｅ）培養培地２ｍｌ中でのバルク培養として刺激した：１０％熱非働化ウシ胎仔血清（Ｈｙｃｌｏｎｅ，Ｌｏｇａｎ，Ｕｔａｈ，ＵＳＡ）、１００Ｕ／ｍｌ　ペニシリン、１００ｇ／ｍｌ　ストレプトマイシン、１０ｍｌ／Ｌの１００ｍＭピルビン酸ナトリウム、および５０Ｍ　２－メルカプトエタノールを補充した、１００ｍＭのＬ－グルタミンを含むＲＰＭＩ１６４０（Ｇｉｂｃｏ，Ｇｒａｎｄ　Ｉｓｌａｎｄ，Ｎｅｗ　Ｙｏｒｋ，ＵＳＡ）／α－Ｍｅｍ（Ｌ－グルタミン、デオキシリボヌクレオシドまたはリボヌクレオシドを含む最少必須培地α培地）（１：１）。さらに、５％　Ｒａｔ　Ｔ－Ｓｔｉｍ　ＩＬ２（Ｒａｔ　Ｔ－Ｓｔｉｍ；Ｃｏｌｌａｂｏｒａｔｉｖｅ　Ｂｉｏｍｅｄｉｃａｌ　Ｐｒｏｄｕｃｔｓ，Ｂｅｄｆｏｒｄ，Ｍａｓｓａｃｈｕｓｅｔｔｓ，ＵＳＡ）を、ＩＬ２の供給源として使用し、そして細胞を培養する直前に培養培地に添加した。

【０１０４】

６～７日の刺激期間後に、細胞を回収し、そして$^{51}$クロム放出アッセイにおけるエフェクターとして使用した。約$10^6$個のＳＶ／Ｂａｌｂ標的細胞を、５０Ｃｉの$^{51}$Ｃｒを含有する２００μｌの培地中で、１Ｍの濃度での正確なペプチドｐ７ｇまたはミスマッチ細胞標的対（ネガティブコントロールとして）とともに、６０分間インキュベートし、そして洗浄した。エフェクター細胞を、$5\times10^3$個の標的細胞とともに、９６ウェル組織培養プレート（丸底またはＶ底）中２００μｌの培養培地にて種々のエフェクター対標的比で４時間培養した。２連のウェルからの平均ｃｐｍを使用して、本明細書中に示されるような特異的放出パーセントを計算した。

【０１０５】

（ＥＬＩＳＰＯＴアッセイ）

群当たり５匹のマウス由来のプールしたＣＬＮおよびＳＰからの単一の細胞懸濁物を、モノクローナルラット抗マウス抗ＩＦＮ－γ（Ｐｈａｒｍｉｎｇｅｎ）およびｐ５５で予めコートし、そして１０％ウシ胎仔血清、５ｍＭ　Ｈｅｐｅｓおよび抗生物質を含有する完全ＲＰＭＩ培地（ｐＨ７．２）でブロックしたニトロセルロースまたはｐｖｄｆプレート（Ｍｉｌｉｐｏｒｅ）上に添加した。ｐ５５特異的抗体分泌細胞（ＡＳＣ）全体を検出するために、細胞を３７℃で一晩インキュベートした後、プレートを、ＰＢＳ／０．０５％Ｔｗｅｅｎ（Ｐ／Ｔ）で洗浄した。ビオチン化ヤギ抗ＩｇＨ＋Ｌ（Ｓｏｕｔｈｅｒｎ　Ｂｉｏｔｅｃｈｎｏｌｏｇｙ　Ａｓｓｏｃｉａｔｅｓ，Ｂｉｒｍｉｎｇｈａｍ，Ａｌａｂａｍａ）を、ＰＢＳ／１％正常ヤギ血清に１：７０００希釈で添加し、そして室温（ＲＴ）で２時間インキュベートした。プレートを、Ｐ／Ｔで洗浄し、そして１：１０００希釈でのアビジン－ペルオキシダーゼ（Ｐｈａｒｍｉｎｇｅｎ）とともに３７℃で１時間インキュベートした。プレートを、Ｐ／Ｔで洗浄し、そしてスポットを、Ｔｒｉｓ－ＨＣｌ緩衝液にＤＡＢを３０分間添加することにより可視化した。プレートを、脱イオン水で洗浄し、風乾した。スポットを、群当たりおよび組織当たり２連のウェルから手動で低倍率下で計数した。この結果は、１０，０００，０００個の細胞当たりのＡＳＣとして示され、そして同様の結果を有する少なくとも２つの実験の代表例である。

【０１０６】

ｇａｇ由来のｐ７ｇペプチド、またはポリクローナルＴ細胞活性化についてのポジティブコントロールとしての抗ＣＤ３（Ｐｈａｒｍｉｎｇｅｎ）および抗ＣＤ２８（Ｐｈａｒｍ

ｉｎｇｅｎ）、またはネガティブコントロールとしての培地のみの存在下で、細胞を一晩インキュベートした後のＩＦＮ－γ分泌細胞の検出について、プレートを洗浄し、そしてビオチン化抗ＩＦＮ－γ（Ｐｈａｒｍｉｎｇｅｎ）を、ＰＢＳ／０．１％ＢＳＡ／０．０２％Ｔｗｅｅｎに添加し、Ｒ／Ｔで２時間インキュベートした。プレートをＰ／Ｔで洗浄し、そして１：１０００希釈でのアビジン－ペルオキシダーゼ（Ｐｈａｒｍｉｎｇｅｎ）とともに、３７℃で１時間インキュベートした。プレートをＰ／Ｔで洗浄し、そしてスポットを、Ｔｒｉｓ－ＨＣｌ緩衝液にＤＡＢを３０分間添加することにより可視化した。プレートを、脱イオン水（Ｈ２Ｏ）で洗浄し、風乾した。スポットを、Ａｌｐｈａ Ｉｎｎｏｔｅｃｈ Ｃｏｒｐｏｒａｔｉｏｎ（Ｓａｎ Ｌｅａｎｄｒｏ，ＣＡ）からのソフトウェアを使用する自家製自動化ＥＬＩＳＰＯＴリーダーを用いて計数した。

【０１０７】

（ＥＬＩＳＡアッセイ）

ＨＩＶ－１ ｐ５５ｇａｇ特異的血清ＩｇＧ力価を、標準的ＥＬＩＳＡアッセイによって定量した。簡単には、ＥＬＩＳＡプレート（Ｎｕｎｃ Ｍａｘｉｓｏｒｐによる９６ウェルＵ底）を、５μｇ／ウェルのｐ５５タンパク質でコートした。１×ＰＢＳ＋０．０３％ Ｔｗｅｅｎ ２０（Ｓｉｇｍａ）で洗浄した後、ウェルをブロックし、そしてサンプルを、連続希釈で、アッセイ希釈剤（１×ＰＢＳ＋５％ヤギ血清（Ｇｉｂｃｏ Ｂｒｌ）＋０．０３％ Ｔｗｅｅｎ ２０（Ｓｉｇｍａ）で作製した）に添加した。標準血清を、定量化目的で、各アッセイに含ませた。サンプルおよび標準血清を、３７℃で１時間インキュベートし、ＰＢＳ／０．０３％ Ｔｗｅｅｎで洗浄した。次いで、サンプルを、ヤギ抗マウスＩｇＧ－ＨＲＰ（Ｃａｌｔａｇ）の１：４００００希釈とともにインキュベートし、そしてテトラメチルベンジジン（ＴＭＢ－Ｋｉｒｋｅｇａａｒｄ ａｎｄ Ｐｅｒｒｙ）で１５分間発色させ、次いで、２Ｎ ＨＣｌで停止した。各ウェルの最適密度を、Ｔｉｔｅｒｔｅｋを使用して４５０ｎｍで測定した。

【０１０８】

（実施例２：ワクシニアウイルス（ＶＶ）－ｇａｇでのチャレンジの前のＳＩＮ－ｇａｇでの粘膜免疫対全身性免疫後のＣＴＬ応答）

粘膜免疫または全身性免疫の種々の経路を介して、局所的ｇａｇ特異的ＣＴＬ応答および全身性ｇａｇ特異的ＣＴＬ応答を誘導するＳＩＮ－ｇａｇ粒子の能力を、評価した。マウスを、２．５Ｅ１０$^{6}$のＳＩＮ－ｇａｇ粒子を用いて鼻内（ＩＮ）または筋肉内（ＩＭ）で、および１０$^{7}$のＳＩＮ－ｇａｇ粒子を用いて膣内（ＩＶＡＧ）または直腸内（ＩＲ）で免疫し、そして最終免疫の７日後に屠殺した。全身性のｇａｇ特異的ＣＴＬ応答を決定するために、ＣＬＮ、ＩＬＮ、ＶＵＭおよびＳＰ由来の単一細胞懸濁物を調製し、そしてｇａｇ特異的ＩＦＮ－γ、ＥＬＩＳＰＯＴアッセイを行った。ＩＮ免疫後のＣＬＮでの局所的なｇａｇ特異的ＣＴＬ応答を観察した。逆に、ＩＶＡＧ免疫およびＩＲ免疫後の、ＩＬＮまたはＶＵＭでの局所的なＣＴＬ応答は、観察されなかった（図１）。さらに、ＩＮ免疫およびＩＭ免疫後のＳＰにおける全身性ｇａｇ特異的ＣＴＬ応答が見られたが、ＩＶＡＧ免疫およびＩＲ免疫後では見られなかった（図２）。ＥＬＩＳＰＯＴアッセイにより、ＩＦＮ－γ分泌細胞の数として検出されるＣＴＬ応答の溶解性活性を評価するために、ＳＩＮ－ｇａｇ粒子でのＩＮ免疫後のＣＬＮ細胞およびＳＰ細胞に対して、標準$^{51}$Ｃｒ放出アッセイを行った。ＳＩＮ－ｇａｇでのＩＮ免疫後のＣＬＮおよびＳＰにおける局所的および全身性の溶解性ＣＴＬ活性を、観察した（図３）。よって、ＳＩＮ－ｇａｇでのＩＮまたはＩＭでの免疫は、局所的および全身性ＣＴＬ応答を誘導したが、ＩＶＡＧまたはＩＲでの免疫では誘導しなかった。

【０１０９】

（実施例３：ＶＶ－ｇａｇでのチャレンジ後のＳＩＮ－ｇａｇでの粘膜免疫対全身性免疫後のＣＴＬ応答）

粘膜免疫および全身性免疫ならびに局所チャレンジの後の局所的ＣＴＬ応答および全身性ＣＴＬ応答を、決定した（また、Ｖａｊｄｙら（２００１）Ｊｏｕｒｎａｌ ｏｆ Ｉｎｆｅｃｔｉｏｕｓ Ｄｉｓｅａｓｅ １８４：１６１３を参照のこと）。マウスを、２．

５Ｅ１０$^{6}$のＳＩＮ－ｇａｇ粒子を用いてＩＮまたはＩＭで免疫し、そして最終免疫の１～３週間後に、ＨＩＶ－１　ｐ５５－ｇａｇを発現する１０$^{7}$～１０$^{8}$ｐｆｕのワクシニアウイルス（ＶＶ）を用いてＩＲまたはＩＶＡＧでチャレンジし、そして最終免疫の５日後に屠殺した。以下のコントロールを使用した：ナイーブなマウスの群を、ＩＶＡＧでチャレンジした、ナイーブなマウスの群を、ＩＲでチャレンジした、マウスの群を、ＳＩＮ－ｇａｇを用いてＩＭ、ＩＮ、ＩＲまたはＩＶＡＧで免疫し、そしてＨＩＶ－１エンベロープｇｐ１６０（ＶＶ－ｅｎｖ）を発現するワクシニアウイルスを用いてＩＶＡＧでチャレンジした。

【０１１０】

ＳＩＮ－ｇａｇでのＩＭ免疫またはＩＮ免疫、およびＶＶ－ｇａｇでの膣内チャレンジの後、局所的、ＣＬＮ、および全身性、ＳＰ、リンパ組織における強力なｇａｇ特異的ＣＴＬ応答を検出した。重要なことに、強力なＣＴＬ応答をまた、ＥＬＩＳＰＯＴアッセイによって測定されるように、離れた粘膜エフェクター部位（ＶＵＭ）、および離れた誘導性部位（ＩＬＮ）で検出した（図４）。コントロール群は、局所的ＣＴＬ応答も全身性ＣＴＬ応答も示さなかった（図５）。よって、ＳＩＮ－ｇａｇを用いる大腿部筋肉での全身性注射および鼻粘膜への粘膜投与、その後のＶＶ－ｇａｇでの膣内チャレンジは、鼻粘膜の局所的な誘導性部位（ＣＬＮ）のみならず膣内粘膜の離れた誘導性部位（ＩＬＮ）およびエフェクター部位（ＶＵＭ）でもまた強力なＣＴＬ応答を誘導した。さらに、ＳＩＮ－ｇａｇでのＩＮ免疫およびＩＭ免疫の後のＶＶ－ｇａｇでの膣内チャレンジは、強力な全身性ＣＴＬ応答を誘導した。

【０１１１】

局所免疫および局所チャレンジの後の局所的ＣＴＬ応答および全身性ＣＴＬ応答を決定するために、マウスを、１０$^{7}$のＳＩＮ－ｇａｇ粒子を用いてＩＶＡＧまたはＩＲで免疫し、そして１０$^{7}$ｐｆｕのＶＶ－ｇａｇを用いてＩＶＡＧでチャレンジした。ＳＩＮ－ｇａｇでのＩＲ免疫およびＶＶ－ｇａｇでのＩＶＡＧチャレンジの後、ＩＬＮおよびＳＰのそれぞれにおける局所的ＣＴＬ応答および全身性ＣＴＬ応答を、ＩＦＮ－γ　ＥＬＩＳＰＯＴアッセイによって測定されるように、検出した（図５）。逆に、ＳＩＮ－ｇａｇを用いてＩＶＡＧで免疫し、ＶＶ－ｇａｇを用いてＩＶＡＧでチャレンジしたマウスは、ＩＬＮおよびＶＵＭで局所的に強力なＣＴＬ応答を示したが、ＳＰでは低い全身性ＣＴＬ応答を示した（図５）。コントロール群は、局所的ＣＴＬ応答も全身性ＣＴＬ応答も示さなかった。ＥＬＩＳＰＯＴ技術によって検出されたＣＴＬ応答の溶解活性を確認するために、標準的Ｃｒ放出アッセイを、ＳＩＮ－ｇａｇを用いてＩＲまたはＩＶＡＧで免疫し、ＶＶ－ｇａｇを用いてＩＶＡＧでチャレンジしたマウスから単離したＩＬＮおよびＳＰを用いて行い、同様の結果を得た。よって、ＳＩＮ－ｇａｇ粒子での膣内免疫および直腸免疫は、粘膜エフェクター部位および誘導性部位にて局所的ＣＴＬ応答を誘導する。さらに、ＳＩＮ－ｇａｇ粒子での直腸（膣ではない）免疫後のＶＶ－ｇａｇでのＩＶＡＧチャレンジは、全身性リンパ組織で強力なＣＴＬ応答を引き起こした。

【０１１２】

（実施例４）

（ＶＶ－ＧＡＧを使用した粘膜チャレンジからの防御）

局所性および全身性のＣＴＬ応答の存在が種々のチャレンジからの粘膜防御と相関があった否かを決定するために、ｇａｇ発現ワクシニアウイルスチャレンジモデルを使用した。ワクシニアは、直腸内、および種々の非経口経路においける接種に続いて卵巣に感染し、そして複製することがこれまで示されてきた。ＳＩＮ－ｇａｇを用いる、ＩＲ、ＩＶＡＧ、ＩＮ，またはＩＭでの免疫の二週間後または三週間後に、全ての群のマウスを、ＩＶＡＧで１０$^{7}$ｐｆｕのＶＶ－ｇａｇを用いてチャレンジした。ＶＶ－ｇａｇでのＩＶＡＧチャレンジの５日後、これらのマウスを、屠殺し、そして、ｐｆｕアッセイを、これらの卵巣について実施した。以下のコントロールを使用した：ＩＶＡＧにてチャレンジしたナイーブマウス、ＩＲにてチャレンジしたナイーブマウス、ＳＩＮ－ｇａｇを用いてＩＭ、ＩＮ、ＩＲ、またはＩＶＡＧで免疫し、そしてＨＩＶ－１エンベロープｇｐ１６０（ＶＶ－

ｅｎｖ）を発現したワクシニアウイルスでＩＶＡＦチャレンジしたマウスの群。

【０１１３】

ＳＩＮ－ｇａｇを用いてＩＶＡＧまたはＩＲで免疫したマウスは、これらの卵巣においてｐｆｕが検出されなかった点で、ＶＶ－ｇａｇを用いるそれぞれＩＶＡＧおよびＩＲでのチャレンジに対して防御された（図６）。さらに、ＳＩＮ－ｇａｇを用いてＩＮで免疫したマウスおよびＶＶ－ｇａｇを用いてＩＶＡＧでチャレンジしたマウスは、部分的にのみ防御されていた（図６）。重要なことに、ＩＭで免疫したマウスおよびＩＶＡＧでチャレンジしたマウスは、防御されていなかった（図ＶＩ）。ＶＶ－ｇａｇを用いてＩＲまたはＩＶＡＧでチャレンジしたナイーブマウスは、それらの卵巣において、高い数値のｐｆｕを有した（図６）。さらに、ＳＩＮ－ｇａｇを用いて、ＩＭ、ＩＮ、ＩＲ、またはＩＶＡＧで免疫したマウス、およびＶＶ－ｅｎｖを用いてＩＶＡＧでチャレンジされたマウスはまた、それらの卵巣において高いｐｆｕ力価を有し、そして、この防御が抗原特異的であることを実証した（図ＶＩ）。重要なことに、ＳＩＮ－ｇａｇを用いてＩＭで免疫したマウスおよびＶＶ－ｇａｇを用いて腹膜でチャレンジしたマウスは、部分的に防御され、そして、ＩＭ免疫によって、粘膜チャレンジに対する防御行われないが、全身性のチャレンジに対していくらかの防御を与えることを実証した（データは示さず）。従って、局所／粘膜性であるが、遠位／粘膜性または全身性ではない免疫によって、このマウスを局所／粘膜性のチャレンジに対して防御した。

【０１１４】

（実施例５）

（シンドビスウイルスに基づくレプリコン粒子で局所免疫した後の、膣粘膜におけるＨＩＶ－１　ＧＡＧ特異的$CD8^+$Ｔ細胞応答および膣ウイルスチャレンジからの防御）

５匹のＢＡＬＢ／ｃマウスの群を、各々のワクチンまたは免疫経路について使用し、そして、この組織を、屠殺時にプールした。マウスを、２週間の間隔で種々の経路によって３回免疫し、２～３週間休止して、$10^7$プラーク形成単位（ＰＦＵ）のＶＶ－ｇａｇまたはＶＶ－ｇｐ１６０を使用して、膣内（ＩＶＡＧ）または直腸内（ＩＲ）にてチャレンジした（Ｇａｒｄｎｅｒら　（２０００）　Ｊ　Ｖｉｒｏｌ　７４：　１１８４９－５７）。　Ｇａｒｄｎｅｒ　ら　（２０００），　前出に記載されるように、ＳＩＮ－ｇａｇ粒子を調製した。鼻腔内（ＩＮ）免疫を、ＰＢＳ中に懸濁される、２５μｌの容量の２．５×$10^6$のＳＩＮ－ｇａｇ粒子を使用して、麻酔なしで実施した。その　ＳＩＮ－ｇａｇ粒子およびＶＶ－ｇａｇウイルスを、麻酔したマウスにＩＶＡＧまたはＩＲで、１２．５μｌの容量にて適用し、その後、これらのマウスを、２０分間、背面横臥姿勢（ｄｏｒｓａｌ　ｒｅｃｕｍｂｅｎｃｙ）で維持した。筋内（ＩＭ）免疫を、５０μｌの容量の中の２．５ｘ$10^6$のＳＩＮ粒子を用いて、大腿筋において実施した。これらのマウスを、組織採取のために、ＶＶチャレンジの５日後に、屠殺した。

【０１１５】

頸部リンパ節（ＣＬＮ）、ＩＬＮ、膣／子宮の粘膜組織（ＶＵＭ）および脾臓（ＳＰ）を、１群当たり５匹のマウスから採取し、そして、プールした。単一細胞懸濁物を、ＨＩＶ－１　ｇａｇ由来のｐ７ｇペプチドに特異的なＩＦＮ－γ－分泌細胞（ＩＦＮＳＣ）を検出するためのＥＬＩＳＰＯＴアッセイのために使用した。ＶＶ－ｇａｇを使用して、膣または直腸でのチャレンジの５日後、５匹の免疫したマウスの群の各々に由来する、ＳＰ、ＣＬＮおよびＩＬＮを回収、そしてプールした。ＳＰ、ＣＬＮ、およびＩＬＮの組織を、ポア直径２５０μｍのナイロンメッシュを介して細かく裂き、そして、以下の培地中で３回洗浄した：ＥＬＩＳＰＯＴアッセイ培地：ＥＬＩＳＰＯＴアッセイのために、計数して、ウェルに播種した１０％　ＦＣＳ、抗生物質、ＨＥＰＥＳおよびＬ－グルタミン　を含有するＲＰＭＩ（完全ＲＰＭＩ）。単一細胞懸濁物を、Ｊｏｈａｎｓｓｏｎら（１９９８）　Ｉｎｆｅｃｔ．Ｉｍｍｕｎ　６６：　５１４－５２０に記載の方法に基づいてＶＵＭから調製した。この方法によって、慣用的に、５匹のマウス当たり最小$10^7$個、最小９０％　生存の単核細胞（ＭＮＣ）の回収を得た。

【０１１６】

ＨＩＶ－１　ｇａｇ由来のｐ７ｇペプチドは、ＣＤ８$^+$Ｔ細胞によって認識されることが示されてきた（ＤｏｅおよびＷａｌｋｅｒ（１９９６）ＡＩＤＳ　１０：　７９３－９４）。また、ＤＮＡ免疫の後の、ＣＤ４$^+$細胞およびＣＤ８$^+$細胞のｐ７ｇ特異的ＩＦＮ－γ細胞内サイトカイン染色の後に、ペプチドは、ＣＤ８$^+$Ｔ細胞によってのみ認識され、かつ、ＣＤ４$^+$Ｔ細胞によっては認識されなかったことが実証された。群毎の５マウスからの、プールされたＶＵＭ、ＩＬＮ、ＣＬＮまたはＳＰに由来する単一細胞懸濁液を調製し、そして、１ｍｌ当たり１０$^7$～３×１０$^7$の濃度に調整した。各細胞調製物からの１００μｌを、９６ウェルニトロセルロースプレートまたはｐｖｄｆプレート（Ｍｉｌｉｐｏｒｅ）の第１の列に、二連で添加し、２倍の連続希釈を実行した。３７℃で一晩インキュベートした後に、これらのプレートを、洗浄し、そしてビオチン化した抗ＩＦＮ－γ（ＰｈａｒＭｉｎｇｅｎ）を添加した。次いで、これらのプレートを、室温で２時間インキュベートし、そして、洗浄した。次いで、このプレートに、アビジン－ペルオキシダーゼ（ＰｈａｒＭｉｎｇｅｎ）を添加し、そして、３７℃で３０分間インキュベートし、そして、洗浄した。これらのプレートを、３０分間、アミノエチルカルバゾール溶液（Ｓｉｇｍａ）で、発色させた。３つの独立した実験の結果を、二連で、１群につき５匹のマウスのプールから最低で４ウェルに由来する１０００万の単核細胞（ＭＮＣ）当たりのＩＦＮ－γ分泌細胞の平均（±ＳＤ）として提供する。

【０１１７】

（Ａ．ＳＩＮ－ｇａｇを用いた粘膜または全身の免疫の後の、粘膜性および全身性ＣＤ８$^+$Ｔ細胞応答、その後の、ワクシニアウイルスｇａｇを用いた膣チャレンジ）

免疫後のウイルスチャレンジは、免疫応答を増強するだけではなく、免疫応答と防御との間の相関関係を得ることを可能にする。従って、マウスを、ＩＮ経路、ＩＭ経路、ＩＲ経路、またはＩＶＡＧ経路を介してＳＩＮ－ｇａｇで初回抗原刺激し、その後、ＶＶ－ｇａｇを使用してチャレンジした。この粘膜および全身のＣＴＬ応答ならびに卵巣におけるＶＶ－ｇａｇ複製からの防御を測定した。ＩＮ、ＩＭ、そして、ＩＶＡＧで免疫したマウスを、ＩＶＡＧでチャレンジしたが、その一方で、ＩＲで免疫したマウスを、ＩＲまたはＩＶＡＧでチャレンジした。ＩＦＮ－γＥＬＩＳＰＯＴアッセイによって測定したときに、ＩＮまたはＩＭで免疫してＩＶＡＧでチャレンジしたマウスのＶＵＭ、ＩＬＮおよびＳＰにおける粘膜および全身のＣＤ８$^+$Ｔ細胞応答の結果を、それぞれ、図１１Ａおよび図１１Ｂに示す。ＣＤ８$^+$Ｔ細胞応答を、いずれの群のＣＬＮにおいても検出し得なかった。これらの応答は、ｇａｇ特異的であった。なぜならば、ＶＶ－ｇａｇを用いた、ナイーブマウスのＩＶＡＧチャレンジまたはＩＲチャレンジ、あるいは、ＶＶ－ｇｐ１６０を使用したＳＩＮ－ｇａｇ免疫したマウスのＩＶＡＧチャレンジまたはＩＲチャレンジは、ＣＤ８$^+$細胞応答を誘導しなかったからであるからである。これらのデータによって、ＩＮまたはＩＭで免疫したマウスの膣ＶＶチャレンジによって、ＶＵＭおよびＩＬＮにおけるＣＤ$^{8+}$Ｔ細胞応答が誘導されたことが示される。

【０１１８】

ＳＩＮ－ｇａｇでのＩＲ免疫、その後のＶＶ－ｇａｇを用いるＩＶＡＧチャレンジの後に、最も高いＣＤ８$^+$Ｔ細胞応答が、ＩＬＮおよびＳＰにおいて見出され、そして、比較的低い応答がＶＵＭにおいて見出された（図１１Ｃ）。同様に、ＳＩＮ－ｇａｇを使用するＩＶＡＧ免疫、その後のＶ－ｇａｇを用いたＩＶＡＧチャレンジの後に、最も高いＣＤ８$^+$Ｔ細胞応答がＩＬＮおよびＳＰにおいて見出され、そして、相対的に低い応答が、ＶＵＭにおいて見出された（図１１Ｄ）。ＩＶＡＧまたはＩＲでの免疫およびチャレンジの後で観察したＣＤ８$^+$Ｔ細胞応答は、ＩＮ免疫およびＩＭ免疫の後にＩＶＡＧチャレンジした後で観察されるＣＤ８$^+$Ｔ細胞応答と比較して、一般的に１０分の１である。

【０１１９】

（Ｂ．ＳＩＮ－ｇａｇを用いた、粘膜免疫、その後のワクシニアウイルス－ｇａｇを用いた膣チャレンジの後の局所性のウイルス複製からの防御）

ＩＶＡＧまたはＩＲでのウイルスチャレンジ後の、種々の経路を介して初回抗原刺激した、マウスの卵巣におけるワクシニアウイルス複製からの防御をまた、標準的なＰＦＵアッ

セイを使用して試験した。図１２において示されるように、ＳＩＮ－ｇａｇを使用してＩＭで初回抗原刺激したマウスは、ＶＶ－ｇａｇを使用したＩＶＡＧでのチャレンジから防御されなかった。さらに、ＩＮで初回抗原刺激した１９匹のマウスのうちの５匹は、それらの卵巣においてＶＶ複製の徴候を示さなかった。対照的に、ＶＶ－ｇａｇを用いるＩＶＡＧまたはＩＲのチャレンジの後に、ＳＩＮ－ｇａｇを用いてＩＶＡＧまたはＩＲで免疫したマウスは、ワクシニアウイルス複製がそれらの卵巣において検出されなかったという点で、ＶＶ－ｇａｇを使用したＩＶＡＧ（図１２）およびＩＲでのチャレンジに対して完全に防御された。このコントロールの、ナイーブマウスは、ＶＶ－ｇａｇを用いたＩＲまたはＩＶＡＧでのチャレンジ後に、それらの卵巣において高いレベルのＶＶを有した（図１２）。また、ＳＩＮ－ｇａｇを使用して、ＩＮ、ＩＭ、ＩＶＡＧおよびＩＲで免疫し、そしてＶＶ－ｇｐ１６０を用いてＩＶＡＧでチャレンジしたコントロールマウスは、それらの卵巣において高いレベルのＶＶを有していた（図１２）。これは、この防御がｇａｇ特異的であったことを実証する。従って、ＳＩＮ－ｇａｇレプリコン粒子を使用した、遠位粘膜または全身の免疫ではなく、ＳＩＮ－ｇａｇレプリコン粒子を使用した、局所的粘膜免疫が、膣でのウイルスチャレンジに対する最大の防御を与えた。

【０１２０】

従って、粘膜性のＩＶＡＧまたはＩＲでの免疫は、膣でのウイルスチャレンジに対する防御を与えた。さらに、このデータによって、アルファウイルスに基づくレプリコンが、このような局所性の防御を誘導するために有効な機構を提供することが示される。ＳＩＮ－ｇａｇレプリコン粒子を使用する免疫、ＶＶ－ｇａｇを用いるその後の局所粘膜チャレンジの、遠位粘膜（ＩＮ）経路、局所粘膜（ＩＶＡＧおよびＩＲ）経路および全身（ＩＭ）経路の比較によって、ＩＮおよびＩＭでの免疫経路は、膣粘膜（ＶＵＭ）、排出（ｄｒａｉｎｇ）リンパ節（ＩＬＮ）、およびＳＰにおけるより高いｇａｇ特異的粘膜ＣＤ８$^+$Ｔ細胞媒介性応答を誘導することが実証された。しかし、ＩＶＡＧおよびＩＲの免疫は、ＶＶ－ｇａｇを用いる、膣でのウイルスチャレンジに対して最大防御を与えた。さらに、これらの応答は、ｇａｇ特異的であった。なぜならば、ＳＩＮ－ｇａｇを用いる免疫、その後のＶＶ－ｇｐ１６０を用いる膣のチャレンジは、ＣＤ８$^+$Ｔ細胞応答を分泌するｇａｇ特異的ＩＦＮ－γのいずれも誘導せず、卵巣におけるＶＶ複製においても何ら影響を与えなかったからである。ＶＶ－ｇａｇを用いる、ナイーブマウスのＩＶＡＧチャレンジはまた、検出可能なｇａｇ特異的ＩＦＮ－γＣＤ８$^+$　Ｔ細胞応答のいずれも誘導しなかった。従って、ＩＦＮ－γＣＤ８$^+$　Ｔ細胞応答の誘導は、ｇａｇ特異的であり、そして、ＳＩＮ－ｇａｇ免疫に続く、適応性特異的応答、その後のの結果として存在した。ＶＶ－ｇａｇチャレンジはまた、以下の事実に示されるように、体液性応答によって媒介されなかった：（ＳＩＮ－ｇａｇレプリコンを使用する、粘膜免疫の２週間後、ＥＬＩＳＡによって測定されるような）ｇａｇ特異的血清力価は、検出不能な抗ｇａｇ血清であった。従って、この観察された防御は、ｇａｇ特異的Ｔ細胞媒介性応答の結果であった。

【０１２１】

要約すると、これらの結果によって、このＳＩＮレプリコン送達システムを、ＨＩＶに対する、ＣＤ８$^+$Ｔ細胞応答および防御免疫を誘導するために粘膜的に適用し得ることが実証される。これらの結果は、一般的に性交感染症病原体についての重要な理解、ならびに、子宮頸部癌、結腸癌、および肺癌に対する治療遺伝子療法についての重要な理解を有する。

【０１２２】

（実施例６）

（局所性および全身性のリンパ組織におけるＨＩＶ－１　ＧＡＧを発現する細胞の同定）

粘膜免疫の後に、局所性リンパ組織および全身性リンパ組織においてＳＩＮ－ｇａｇ粒子の取り込みおよび発現に関連する細胞を、単回用量のＳＩＮ－ｇａｇ粒子を使用して、マウスをＩＮで免疫することにより同定した。鼻腔関連リンパ組織（ＮＡＬＴ）、ＣＬＮおよびＳＰ由来の凍結切片を、ｇａｇに対する抗体を使用して染色した。免疫後第１日目で、ＮＡＬＴ（図９）およびＣＬＮ（図１０）にて局所的に、ならびに、ＳＰ（図１１）に

おいて全身的にの両方で、ｇａｇを発現する多くの細胞を見出したが、圧倒的に殆どの細胞が、ＳＰに局在しているようであった。ｇａｇを発現する細胞を同定するために、細胞は、ＡＰＣ活性を有する単球系統細胞に対するマーカーとしてのＣＤ１１ｂまたはＣＤ１１ｃと、Ｂ細胞についてのマーカーとしてのＢ２２０とを用いて、二重に染色した。大部分のｇａｇ発現細胞は、ＣＤ１１ｂを同時発現したが、その一方、僅かなgag発現細胞のみが、CD１１ｃを同時発現した。重要なことには、B２２０$^{+}$細胞は、ｇａｇと同時発現せず、そして、このＣＤ１１ｂ$^{+}$またはＣＤ１１ｃ$^{+}$のｇａｇ発現細胞は、濾胞外（ｅｘｔｒａ－ｆｏｌｌｉｃｕｌａｒ）Ｔ細胞領域に存在した。従って、免疫おける、それに続く、初期時点において、ＣＤ１１ｂ$^{+}$細胞は、局所リンパ組織および全身性リンパ組織の両方でｇａｇを発現するＡＰＣ能力を有する主要な細胞である。

【０１２３】

（実施例７）

（鼻腔内免疫後の血清中の体液性応答）

ＳＩＮレプリコンが抗体産生を誘導する能力がまた、決定された。

【０１２４】

（Ａ．ＨＩＶ由来抗原）

ｇａｇを発現するＳＩＮレプリコンを使用した、３回のＩＮ免疫の２週間後に、血清力価を、ＥＬＩＳＡによって測定した（図１２）。さらに、初回抗原刺激－追加免疫実験を実施した。第１に、ＳＩＮ－ｇａｇを使用する３回のＩＮ免疫の後に、マウスを、ＩＮで、４週間の間隔にて３回、ｐ２４＋ＬＴＫ６３を使用して追加免疫した。さらに、動物を、ＨＩＶエンベロープｇｐ１４０を発現するＳＩＮを使用して初回抗原刺激し、そして、Ｏｇｐ１４０＋粘膜アジュバントＬＴＲ７２およびＣｐＧを使用して追加免疫する実験を実施した。これらの特定の場合において、低レベルまたはのゼロレベルの抗体力価が観察され（図１０）、これは、ｇａｇを使用する膣または直腸での免疫の後に観察された防御が抗体媒介性でないことを示した。

【０１２５】

（Ｂ．インフルエンザ由来抗原）

インフルエンザポリペプチド（ＨＡ）を発現するＳＩＮレプリコンを使用する、１以上の粘膜（例えば、ＩＮ、ＩＶＡＧ、ＩＲ）免疫の２週間後に、血清力価を、ＥＬＩＳＡによって測定する。さらに、初回抗原刺激－追加免疫実験を実施する。第１に、ＳＩＮ－ＨＡマウスを用いた、粘膜免疫の後に、マウスを、ＨＡ＋ＬＴＫ６３を使用して１回以上粘膜で追加免疫する。さらに、動物を、ＨＡ抗原を発現するＳＩＮを使用して初回抗原刺激し、そして、ＨＡポリペプチド＋粘膜アジュバントＬＴＲ７２およびＣｐＧを使用して追加免疫する実験を実施する。粘膜に投与されたときに、ＳＩＮ－ＨＡレプリコンが、抗体産生を誘導する。

【０１２６】

（実施例８）

（アルファウイルスレプリコン粒子キメラの粘膜送達の後の免疫応答の誘導）

粘膜送達後の抗原特異的な免疫応答を誘導するためのアルファウイルスレプリコン粒子キメラの能力を実証するために、上記の実験と類似の鼻腔内免疫実験を実施した。具体的には、ＳＩＮとＶＥＥとの間のレプリコン粒子キメラを、米国特許出願第６０／２９５，４５１号に記述されたように、構築し、その結果、ＳＩＮレプリコンＲＮＡを、ＶＥＥエンベロープ糖タンパク質中に、組み込むか（ＳＩＮ／ＶＥＥ）、または、ＶＥＥレプリコンＲＮＡを、ＳＩＮエンベロープ糖タンパク質に組み込む（ＶＥＥ／ＳＩＮ）ことをした。ＨＩＶ　ｐ５５　ｇａｇ抗原をコードするこれらのレプリコン粒子、ならびに、また同じＨＩＶ　ｇａｇ抗原をコードするＳＩＮレプリコン粒子およびＶＥＥレプリコン粒子はまた、鼻腔内でマウスを免疫するのに使用した。

【０１２７】

これらのレプリコン粒子を、第０日、第１４日、および第２８日の免疫スケジュールで、２５μｌ中の２．５×１０$^{6}$粒子の用量にて３回投与した。最終免疫の後の第２週間目に

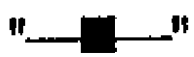
おいて、脾臓を取出し、ｇａｇ特異的細胞応答を、ＩＦＮ－γ　ＥＬＩＳＰＯＴアッセイによって決定した。図１３において示されているように、これらのレプリコン粒子調製物の各々は、免疫原性においていくつかの変更を有する、ｇａｇ特異的応答を誘導した。

【図面の簡単な説明】

【０１２８】

【図１】図１は、$^{51}$Ｃｒ放出アッセイにおいて測定した、ｇａｇ発現ＳＩＮレプリコン粒子を用いて鼻腔内免疫した後の頸部リンパ節における局所性ＨＩＶ－１　ｇａｇ特異的ＣＴＬ応答を示すグラフである。「－－◆－－」は、ＳＩＮ　ＤＣ＋動物の第１の群を示し；

【化１】



は、ＳＩＮ　ＤＣ＋動物の第２の群を示し；「－－□－－」は、ＳＩＮ　ＤＣ－を示し；そして「－－◆－－」は、筋内送達されたコントロールプラスミド（ＣＭＶプロモーターを用いて）を示す。

【図２】図２は、$^{51}$Ｃｒ放出アッセイにおいて測定した、ＳＩＮレプリコン粒子を用いて鼻腔内免疫した後の脾臓における全身性ｇａｇ特異的ＣＴＬ応答を示すグラフである。「－－◆－－」は、ＳＩＮ　ＤＣ＋動物の第１の群を示し；

【化２】



は、ＳＩＮ　ＤＣ＋動物の第２の群を示し；「－－□－－」は、ＳＩＮ　ＤＣ－動物を示し；そして「－－●－－」は、筋内送達されたコントロールプラスミド（ＣＭＶプロモーターを用いて）を示す。

【図３】図３は、ＳＩＮ粒子を用いた鼻腔免疫後の局所性リンパ組織および末梢リンパ組織における、ＩＮＦ－γ分泌細胞の数を示すグラフである。

【図４】図４は、$^{51}$Ｃｒ放出アッセイにおいて測定した、ＳＩＮレプリコン粒子の直腸内（ＩＲ）投与または膣内（ＩＶＡＧ）投与後の直腸粘膜および膣粘膜に流れる腸骨リンパ節における、局所性ＨＩＶ－１　ｇａｇ特異的ＣＴＬ応答を示すグラフである。「－－◆－－」は、ＳＩＮの直腸内投与、続くワクシニアウイルス（ＶＶ）での直腸内（ＩＲ）チャレンジ後の、第１群における応答を示し；

【化３】



は、ＳＩＮの膣内投与、続くＶＶでの膣内チャレンジを示し；「－▲－」は、ＳＩＮのＩＲ投与、続くＶＶでのチャレンジ後の、第２の群における応答を示し；そして「－Ｘ－」は、筋内送達されたコントロールプラスミド（ＣＭＶプロモーターを用いて）を示す。

【図５】図５は、$^{51}$Ｃｒ放出アッセイにおいて測定した、ＳＩＮレプリコン粒子の直腸内（ＩＲ）投与または膣内（ＩＶＡＧ）投与後の脾臓における、全身性ＨＩＶ－１　ｇａｇ特異的ＣＴＬ応答を示すグラフである。「－－◆－－」は、ＳＩＮレプリコンの直腸内投与、続くワクシニアウイルス（ＶＶ）での直腸内チャレンジ後の、第１の動物群における応答を示し；

【化４】



は、ＳＩＮの膣内投与、続くＶＶでの膣内チャレンジを示し；

【化５】

"—■—"

は、免疫なしでＶＶで膣内チャレンジした動物を示し；

【化６】

"—■—"

は、ＳＩＮレプリコンの直腸内投与、続くＳＩＮレプリコンの直腸内投与後の、動物における応答を示し；「－●－」は、ＳＩＮレプリコンの膣内投与、続くＳＩＮレプリコンの膣内投与後の、動物における応答を示し；

【化７】

"—■—"

は、ＳＩＮレプリコンの鼻腔投与、続くＳＩＮレプリコンの膣内投与後の、動物における応答を示す。

【図６】図６は、ＳＩＮ粒子を用いた直腸内（ＩＲ）免疫および膣内（ＩＶＡＧ）免疫、ならびにワクシニアウイルス（ＶＶ）でのＩＲチャレンジおよびＩＶＡＧチャレンジ後の、脾臓組織におけるＩＦＮ－γ分泌細胞を示すグラフである。

【図７】図７は、ＳＩＮ粒子を用いた直腸内（ＩＲ）免疫および膣内（ＩＶＡＧ）免疫、ならびにワクシニアウイルス（ＶＶ）でのＩＲチャレンジおよびＩＶＡＧチャレンジ後の、腸骨リンパ節におけるＩＦＮ－γ分泌細胞を示すグラフである。

【図８】図８は、ＳＩＮ－ｇａｇ粒子の膣送達および直腸送達後の、ワクシニアウイルス（ＶＶ）力価を示すグラフである。

【図９】図９は、ｇａｇ特異的血清力価を示すグラフである。左から右に、バーは、動物の力価を示す：ＳＩＮ－ｇａｇで初回免疫、および追加免疫なし；ＳＩＮ－ｇａｇで初回免疫、ならびにｐ２４ポリペプチドおよびＬＴＫ６３アジュバントを用いて追加免疫；ナイーブな動物；ならびに、初回免疫なしだが、ｐ２４およびＬＴＫ６３アジュバントを用いて引続く追加免疫。

【図１０】図１０は、ｇｐ１２０特異的血清力価を示すグラフである。左から右に、バーは、動物の力価を示す：ＳＩＮ－ｇｐ１４０で初回免疫、および追加免疫なし；ＳＩＮ－ｇａｇで初回免疫、ならびにｇｐ１４０ポリペプチドおよびＬＴＫ７２アジュバントおよびＣｐＧを用いて追加免疫；ナイーブな動物；ならびに、初回免疫なしだが、ｇｐ１４０ポリペプチドおよびＬＴＫ７２アジュバントおよびＣｐＧを用いて引続く追加免疫。

【図１１Ａ】図１１（パネルＡ～Ｄ）は、ＳＩＮ－ｇａｇ粒子を用いたＩＮ（Ａ）免疫、ＩＭ（Ｂ）免疫、ＩＲ（Ｃ）免疫およびＩＶＡＧ（Ｄ）免疫、続くＶＶ－ｇａｇを用いたＩＶＡＧチャレンジ後の、局所性細胞性免疫応答および全身性細胞性免疫応答の誘導を示すグラフである。ＩＮ免疫およびＩＭ免疫は、ＩＲ免疫およびＩＶＡＧ免疫と比較して、ＶＵＭおよびＩＬＮにおいて、数倍高いｇａｇ特異的ＩＦＮ－γ分泌細胞を誘導した。マウスを、２．５×１０$^{6}$個のＳＩＮ－ｇａｇ粒子を用いてＩＮまたはＩＭで、ならびに１０$^{7}$個のＳＩＮ－ｇａｇ粒子を用いてＩＲまたはＩＶＡＧで３回免疫し、３週間後、これらを、１０$^{7}$ｐｆｕのＶＶ－ｇａｇを用いて膣内チャレンジし、５日後に屠殺した。結果を、３回の独立した実験の１０００万個の単核細胞（ＭＮＣ）当たりのｐ７ｇ特異的ＩＦＮ－γを分泌する細胞の平均数　±　ＳＤとして示す。

【図１１Ｂ】図１１（パネルＡ～Ｄ）は、ＳＩＮ－ｇａｇ粒子を用いたＩＮ（Ａ）免疫、ＩＭ（Ｂ）免疫、ＩＲ（Ｃ）免疫およびＩＶＡＧ（Ｄ）免疫、続くＶＶ－ｇａｇを用いたＩＶＡＧチャレンジ後の、局所性細胞性免疫応答および全身性細胞性免疫応答の誘導を示すグラフである。ＩＮ免疫およびＩＭ免疫は、ＩＲ免疫およびＩＶＡＧ免疫と比較して、ＶＵＭおよびＩＬＮにおいて、数倍高いｇａｇ特異的ＩＦＮ－γ分泌細胞を誘導した。マウスを、２．５×１０$^{6}$個のＳＩＮ－ｇａｇ粒子を用いてＩＮまたはＩＭで、ならびに１０$^{7}$個のＳＩＮ－ｇａｇ粒子を用いてＩＲまたはＩＶＡＧで３回免疫し、３週間後、これらを、１０$^{7}$ｐｆｕのＶＶ－ｇａｇを用いて膣内チャレンジし、５日後に屠殺した。結果

を、３回の独立した実験の１０００万個の単核細胞（ＭＮＣ）当たりのｐ７ｇ特異的ＩＦＮ－γを分泌する細胞の平均数　±　ＳＤとして示す。
【図１１Ｃ】図１１（パネルＡ～Ｄ）は、ＳＩＮ－ｇａｇ粒子を用いたＩＮ（Ａ）免疫、ＩＭ（Ｂ）免疫、ＩＲ（Ｃ）免疫およびＩＶＡＧ（Ｄ）免疫、続くＶＶ－ｇａｇを用いたＩＶＡＧチャレンジ後の、局所性細胞性免疫応答および全身性細胞性免疫応答の誘導を示すグラフである。ＩＮ免疫およびＩＭ免疫は、ＩＲ免疫およびＩＶＡＧ免疫と比較して、ＶＵＭおよびＩＬＮにおいて、数倍高いｇａｇ特異的ＩＦＮ－γ分泌細胞を誘導した。マウスを、２．５×$10^6$個のＳＩＮ－ｇａｇ粒子を用いてＩＮまたはＩＭで、ならびに$10^7$個のＳＩＮ－ｇａｇ粒子を用いてＩＲまたはＩＶＡＧで３回免疫し、３週間後、これらを、$10^7$ｐｆｕのＶＶ－ｇａｇを用いて膣内チャレンジし、５日後に屠殺した。結果を、３回の独立した実験の１０００万個の単核細胞（ＭＮＣ）当たりのｐ７ｇ特異的ＩＦＮ－γを分泌する細胞の平均数　±　ＳＤとして示す。
【図１１Ｄ】図１１（パネルＡ～Ｄ）は、ＳＩＮ－ｇａｇ粒子を用いたＩＮ（Ａ）免疫、ＩＭ（Ｂ）免疫、ＩＲ（Ｃ）免疫およびＩＶＡＧ（Ｄ）免疫、続くＶＶ－ｇａｇを用いたＩＶＡＧチャレンジ後の、局所性細胞性免疫応答および全身性細胞性免疫応答の誘導を示すグラフである。ＩＮ免疫およびＩＭ免疫は、ＩＲ免疫およびＩＶＡＧ免疫と比較して、ＶＵＭおよびＩＬＮにおいて、数倍高いｇａｇ特異的ＩＦＮ－γ分泌細胞を誘導した。マウスを、２．５×$10^6$個のＳＩＮ－ｇａｇ粒子を用いてＩＮまたはＩＭで、ならびに$10^7$個のＳＩＮ－ｇａｇ粒子を用いてＩＲまたはＩＶＡＧで３回免疫し、３週間後、これらを、$10^7$ｐｆｕのＶＶ－ｇａｇを用いて膣内チャレンジし、５日後に屠殺した。結果を、３回の独立した実験の１０００万個の単核細胞（ＭＮＣ）当たりのｐ７ｇ特異的ＩＦＮ－γを分泌する細胞の平均数　±　ＳＤとして示す。
【図１２】図１２は、ＳＩＮ－ｇａｇ粒子を用いた膣免疫または直腸免疫後の、ＶＶ－ｇａｇを用いた膣チャレンジからの卵巣の保護を示す。マウスを、２．５×$10^6$個のＳＩＮ－ｇａｇ粒子を用いてＩＮまたはＩＭで、ならびに$10^7$個のＳＩＮ－ｇａｇ粒子を用いてＩＲまたはＩＶＡＧで３回免疫し、３週間後、$10^7$ｐｆｕのＶＶ－ｇａｇを用いて膣内チャレンジし、５日後に屠殺した。卵巣を収集し、そしてＶＶ力価の決定のための標準的なｐｆｕアッセイを実行した。各点は、プラーク形成単位の数／卵巣／各動物を示す。コントロールとしてのナイーブマウスを、ＶＶ－ｇａｇを用いてチャレンジし、そしてＳＩＮ－ｇａｇ粒子を用いて免疫したマウスを、ＶＶ－ｇｐ１６０を用いてチャレンジし、そして両方の場合において、高いｐｆｕ力価が卵巣において明らかであった。
【図１３】図１３は、アルファウイルスベクター粒子を用いた鼻腔免疫後の免疫学的応答（ＩＦＮ－γ　ＥＬＩＳＰＯＴアッセイによって測定）の誘導を示すグラフである。棒グラフによって示される実際の数は、以下の通りである：ＳＩＮ－ｇａｇレプリコン粒子は、１１５４（±４９９）を与えた；ＶＥＥ－ｇａｇレプリコン粒子は、１５３０（±４２５）を与えた；ＳＩＮ／ＶＥＥ－ｇａｇは１４０（±１４０）を与え；そしてＶＥＥ／ＳＩＮ－ｇａｇは２５８６（±７６２）を与えた。

【図１】

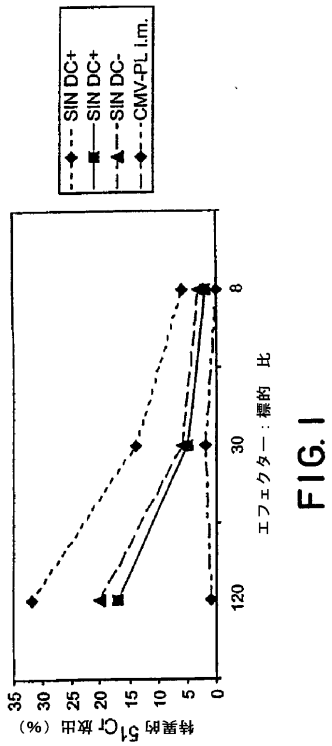



FIG. 1

【図２】

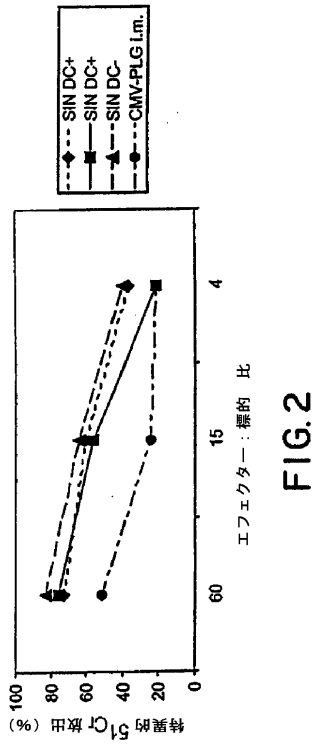



FIG. 2

【図３】

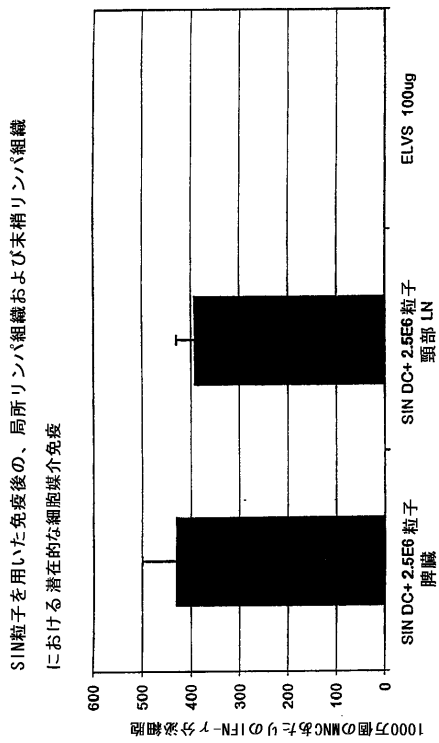



FIG. 3

【図４】

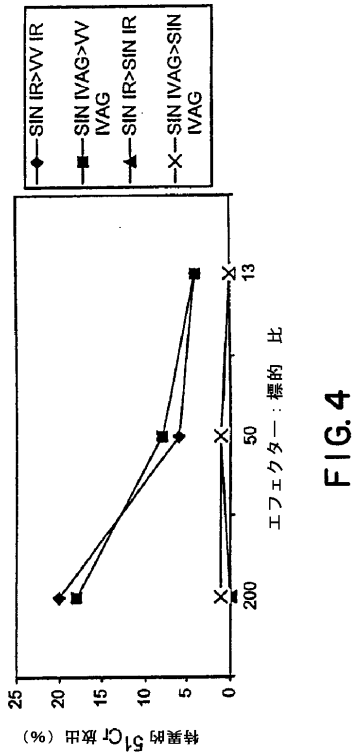



FIG. 4

【図５】

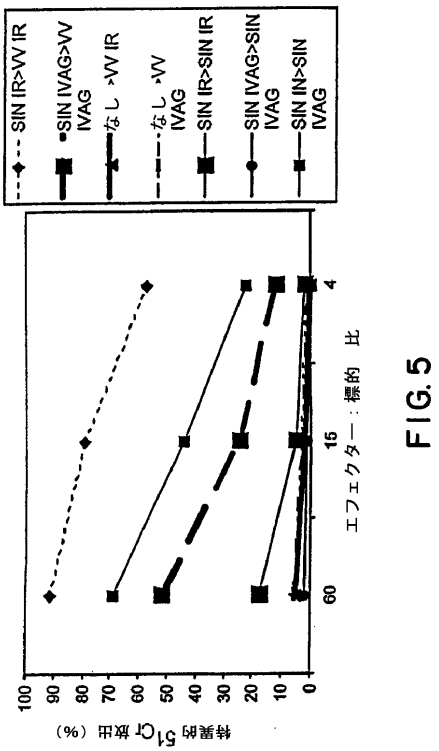



FIG.5

【図６】

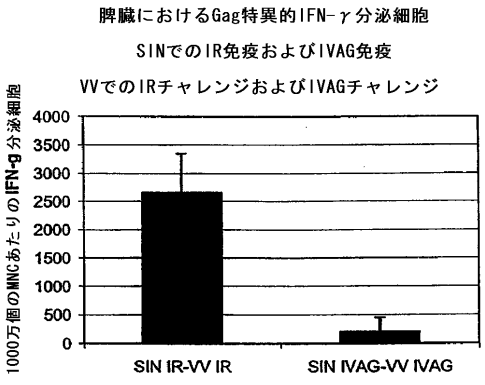



FIG.6

【図７】

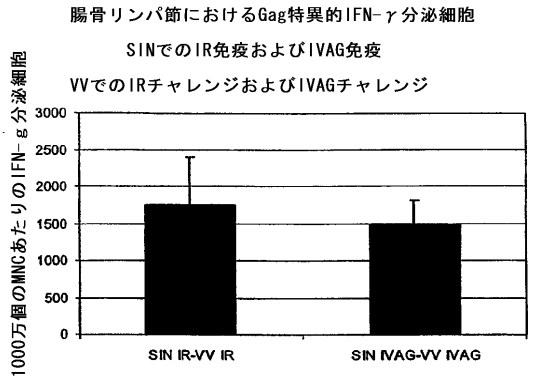



FIG.7

【図８】

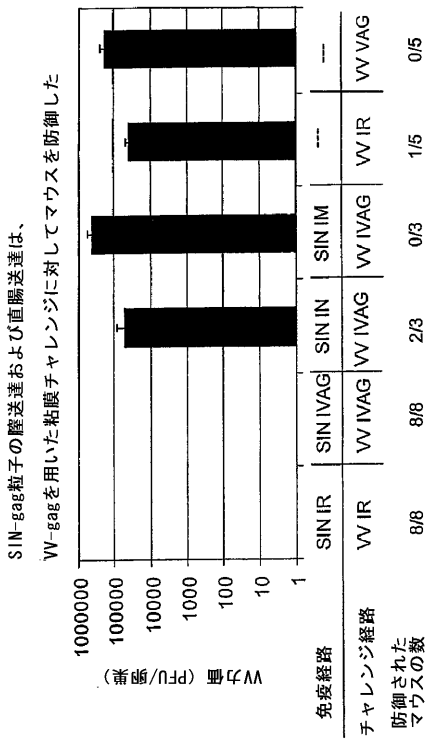



FIG.8

【図９】

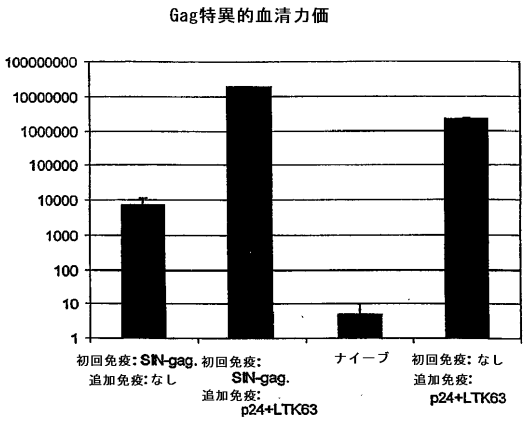



FIG. 9

【図１０】

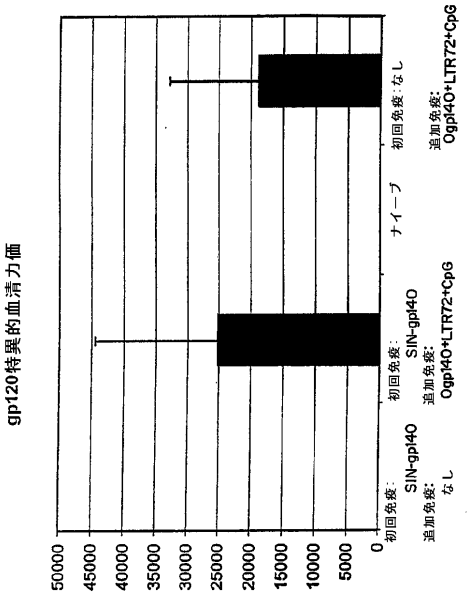



FIG. 10

【図１１Ａ】

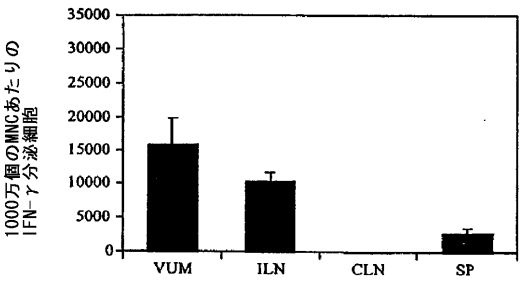



FIG. 11A

【図１１Ｂ】

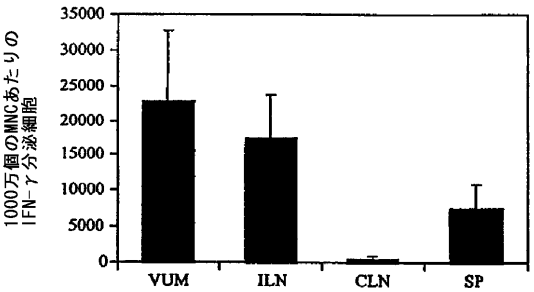



FIG. 11B

【図１１Ｃ】

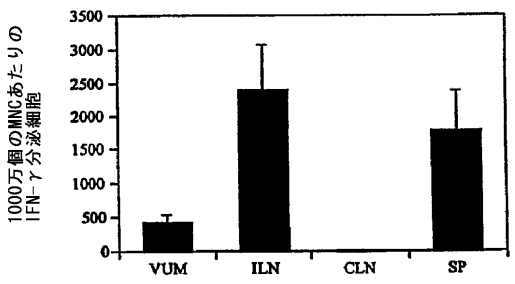



FIG. 11C

【図１１Ｄ】

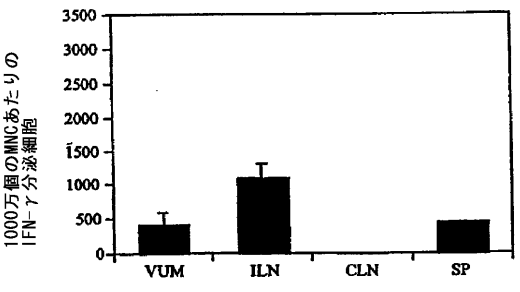



FIG. 11D

【図１２】

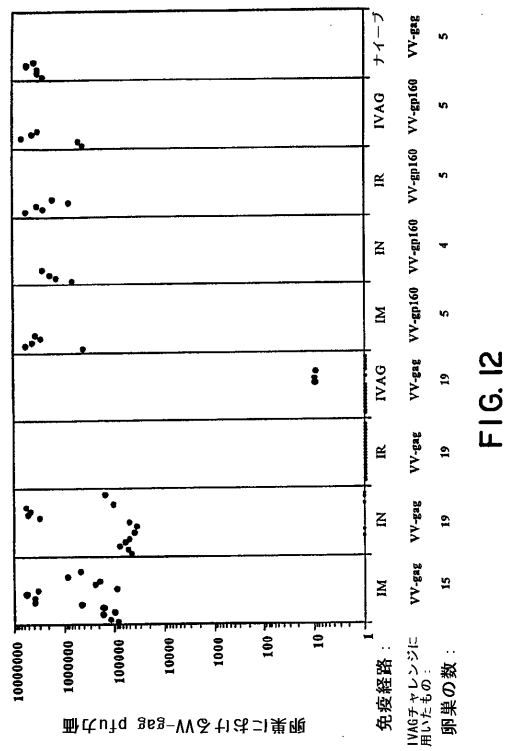



FIG. 12

【図１３】

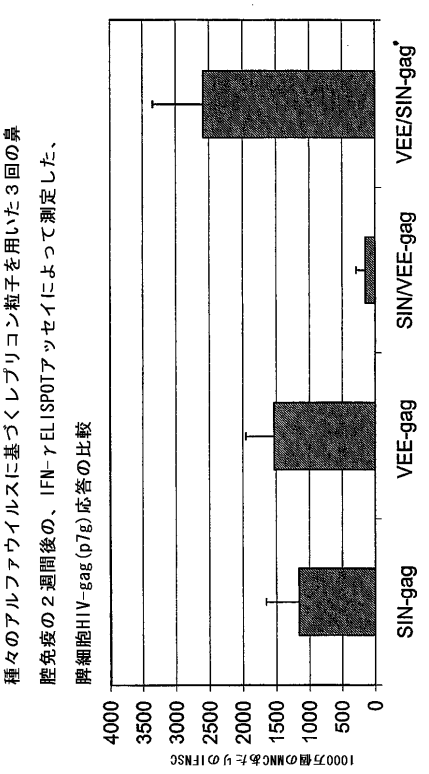



FIG. 13

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(54) Title: NUCLEIC ACID MUCOSAL IMMUNIZATION

(57) Abstract: Mucosal delivery of antigens using, for example, a replication-defective gene delivery vehicle, particularly replication-defective alphavirus vectors and particles, is described. Also described are compositions comprising a mucosal adjuvant and one or more antigens derived from HIV. Also provided is the use of these gene delivery vehicles in inducing mucosal, local and/or systemic immune responses following mucosal immunization regimes.

# NUCLEIC ACID MUCOSAL IMMUNIZATION

Technical Field

The present invention relates generally to mucosal immunization, for example, mucosal immunization using gene delivery systems. In particular to mucosal delivery of replication-defective vectors and/or recombinant alphavirus vectors and particles. Further, use of these systems for inducing potent mucosal, local and systemic immune responses following various routes of mucosal immunization is also described.

Background of the Invention

Development of vaccines which invoke mucosal immunity against various pathogens would be desirable. Many disease-causing pathogens are transmitted through mucosal surfaces. For example, acquired immune deficiency syndrome (AIDS) is recognized as one of the greatest health threats facing modern medicine and worldwide sexual transmission of HIV is the leading cause of AIDS. There are, as yet, no cures or vaccines for AIDS.

In 1983-1984, three groups independently identified the suspected etiological agent of AIDS. See, e.g., Barre-Sinoussi et al. (1983) Science 220:868-871; Montagnier et al., in Human T-Cell Leukemia Viruses (Gallo, Essex & Gross, eds., 1984); Vilmer et al. (1984) The Lancet 1:753; Popovic et al. (1984) Science 224:497-500; Levy et al. (1984) Science 225:840-842. These isolates were variously called lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type III (HTLV-III), or AIDS-associated retrovirus (ARV). All of these isolates are strains of the same virus, and were later collectively named Human Immunodeficiency Virus (HIV). With the isolation of a related AIDS-causing virus, the strains originally called HIV are now termed HIV-1 and the related virus is called HIV-2 See, e.g., Guyader et al. (1987) Nature 326:662-669; Brun-Vezinet et al. (1986) Science 233:343-346; Clavel et al. (1986) Nature 324:691-695. Consequently, there is a need in the art for compositions and methods suitable for treating and/or preventing HIV infection worldwide.

A great deal of information has been gathered about the HIV virus, and several targets for vaccine development have been examined including the *env*, *Gag*, *pol* and *tat* gene products encoded by HIV. Immunization with native and synthetic HIV-encoding polynucleotides has also been described, as described for example, in co-owned PCT/US99/31245 and references cited therein. In addition, polynucleotides encoding HIV have been administered in various attempts to identify a vaccine. (*See, e.g.*, Bagarazzi et al. (1999) *J. Infect. Dis.* 180:1351-1355; Wang et al. (1997) *Vaccine* 15:821-825). A replication-competent Venezuelan equine encephalitis (VEE) alphavirus vector carrying the matrix/capsid domain of HIV could elicit CTL responses has been administered subcutaneously in animals (Caley et al. (1997) *J. Virol.* 71:3031-3038). In addition, alphavirus vectors derived from Sindbis virus has also been shown to elicit HIV gag-specific responses in animals (Gardner et al. (2000) *J. Virol.* 74:11849-11857). Similarly, HIV peptides have also been administered to animal subjects. (Staats et al. (1997) *AIDS Res Hum Retroviruses* 13:945-952; Belyakov (1998) *J.Clin.Invest.* 102: 2072).

Recombinant alphavirus vectors and layered eukaryotic vector systems based on alphaviruses have also been described. (See, e.g., U.S. Patent Nos. 6,015,686; 6,015,694; 5,843,723). Hariharan et al. (1998) *J. Virol.* 72:950-958 reported that a single intramuscular immunization with pSIN vectors expressing the glycoprotein B of herpes simplex virus (HSV) type 1 induced a broad spectrum of immune responses, including virus-specific antibodies, cytotoxic T cells, and protection from lethal virus challenge in two different murine models. Polo et al. (1999) *Proc. Natl. Acad. Sci. USA* 96:4598-4603 reported similar protection in HSV challenge models following immunization with SIN replicon vectors particles rather than pSIN plasmid vectors. Tsuji et al. (1998) *J. Virol.* 72:690-697 reported that subcutaneous administration in mice of recombinant SIN expressing a class I major histocompatibility complex-restricted 9-mer epitope of the *Plasmodium yoelii* circumsporozoite protein or the nucleoprotein of influenza virus induces a large epitope-specific CD8(+) T-cell response and provides a high degree of protection against infection with malaria or influenza A virus.

However, despite these and other studies, the utility of replication-defective gene delivery vehicles for mucosal immunization strategies that can protect against

mucosal challenge has not been sufficiently defined. Thus, there remains a need for compositions and methods directed to treatment and prevention of various sexually transmitted pathogens.

Summary of the Invention

Disclosed herein are gene delivery systems (*e.g.*, recombinant alphavirus vectors) which are suitable for use in a variety of applications, including for example, mucosal immunization, and further provides other related advantages. Briefly stated, the invention includes vector constructs and particles expressing antigens associated with one or more sexually transmitted disease pathogens, as well as methods of making and utilizing the same, particularly in protective mucosal immunization regimes. Preferably, the vectors are replication-defective, for example alphavirus vectors such as those derived from Sindbis. The present inventors have demonstrated that antigen-specific protection against post-immunization challenge can be induced following mucosal administration of gene delivery vehicles (*e.g.*, alphavirus vectors) expressing the antigen.

In one aspect, the invention includes a method of generating an immune response against an antigen. In certain embodiments, the method comprises mucosally administering to target cells of a subject, a replication-defective gene delivery vehicle (or vector) comprising a polynucleotide encoding at least one antigen (or modified form thereof), wherein the antigen (or modified form thereof) is capable of stimulating an immune response in the subject. In certain embodiments, the target cells are in mucosal, local and/or systemic tissues. Mucosal administration can be, for example, intranasal, oral, intrarectal, and/or intravaginal administration. Preferably, for sexually transmitted pathogens, mucosal administration is by the intrarectal or intravaginal route. In certain embodiments, at least one antigen is derived, for example, from a sexually transmitted pathogen such as bacterial pathogen (*e.g.*, gonorrhea, chlamydia and syphilis) or a viral pathogen (*e.g.*, HIV, HBV, HSV, HCV and HPV). In certain embodiments, the antigen(s) elicit(s) an HLA class I-restricted immune response and, optionally, also elicits an HLA Class II-restricted immune response.

In other aspects, the methods include delivery of genes encoding immune-enhancing cytokines, lymphokines, chemokines and the like. These genes can be inserted into the same gene delivery vehicle carrying the antigen(s) of interest (*e.g.*, alphavirus replicon particle) or can be carried on one or more different gene delivery vehicles. In certain embodiments, the antigen(s) elicit(s) an HLA class I-restricted immune response and, optionally, also elicits an HLA Class II-restricted immune response.

The gene delivery vehicle (or vector) can be, for example, a nonviral vector; a particulate carrier (*e.g.*, gold or tungsten particles coated with the polynucleotide and delivered using a gene gun); a liposome preparation; viral vector; a retroviral vector; or an alphavirus-derived vector. In certain aspects, an alphavirus-derived vector, for example a vector derived from Sindbis virus, Semliki Forest virus, Venezuelan Equine Encephalitis virus, Ross River virus or vector chimeras derived from any number of different alphaviruses (*e.g.*, SIN-VEE chimeras) are used. Any of the gene delivery vectors described herein (*e.g.*, alphavirus vector) can be delivered, for example, to antigen presenting cells (APCs) such as dendritic cells. In certain embodiments, the subject and/or the cells is a mammal, for example a human. In any of the methods described herein, the target cells can be infected *in vivo*. Further, in any of the methods described herein, prior or subsequent to the step of administering to target cells, a nucleic acid molecule which encodes either Class I or Class II MHC protein, or combinations thereof, or a protein selected from the group consisting of CD3, ICAM-1, LFA-3 or analogues thereof can also be administered to the target cells. Furthermore, the above-described gene delivery vehicle for mucosal vaccination may be used in combination with one or more additional immunogenic compositions (gene delivery vehicle, polypeptide, protein, chemokine, cytokine, etc.) in which the one or more additional compositions are delivered by a mucosal or non-mucosal route(s).

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.). These references are incorporated herein by reference in their entirety.

Brief Description of the Drawings

Figure 1 is a graph depicting local HIV-1 gag-specific CTL responses in cervical lymph nodes following intranasal immunization with gag-expressing SIN replicon particles as measured in a $^{51}Cr$ release assay. "--♦--" depicts a first group of SIN DC+ animals; "—■—" depicts a second group of SIN DC+ animals; "--□--" depicts SIN DC-; and "— -♦- —" depicts a control plasmid (with CMV promoter) delivered intramuscularly.

Figure 2 is a graph depicting systemic gag-specific CTL responses in spleen following intranasal administration with SIN replicon particles as measured in a $^{51}Cr$ release assay. "--♦--" depicts a first group of SIN DC+ animals; "—■—" depicts a second group of SIN DC+ animals; "--□--" depicts SIN DC- animals; and "— -●- —" depicts a control plasmid (with CMV promoter) delivered intramuscularly.

Figure 3 is a graph depicting number of INF-γ secreting cells in local and peripheral lymphoid tissue following intranasal immunization with SIN particles.

Figure 4 is a graph depicting local HIV-1 gag-specific CTL responses in iliac lymph nodes draining the rectal and vaginal mucosa following intrarectal (IR) or intra-vaginal (IVAG) administration of SIN replicon particles, as measured in a $^{51}Cr$ release assay. "--♦--" depicts responses in a first group after intrarectal administration of SIN followed by intrarectal (IR) challenge with vaccinia virus (VV); "—■—" depicts intravaginal administration of SIN followed by intravaginal challenge with VV; "—▲—" depicts responses in a second group following IR administration of SIN followed by challenge with VV; and "—X—" depicts a control plasmid (with CMV promoter) delivered intramuscularly.

Figure 5 is a graph depicting systemic HIV-1 gag-specific CTL responses in spleen following intra-rectal (IR) or intra-vaginal (IVAG) administration of SIN replicon particles, as measured in a $^{51}Cr$ release assay. "--♦--" depicts responses in a first group of animals after intrarectal administration of SIN replicons followed by intrarectal challenge with vaccinia virus (VV); "▬■▬" depicts intravaginal administration of SIN followed by intravaginal challenge with VV; "— ▬ —" depicts animals challenged intravaginally with VV with no immunization; "—■—" depicts

responses in animals after intrarectal administration of SIN replicons followed by intrarectal administration of SIN replicons; "—●—" depicts responses in animals after intravaginal administration of SIN replicons followed by intravaginal administration of SIN replicons; "—■—" depicts responses in animals after intranasal administration of SIN replicons followed by intravaginal administration of SIN replicons;

Figure 6 is a graph depicting IFN-γ secreting cells in spleen tissue following intrarectal (IR) and intravaginal (IVAG) immunization with SIN particles and IR and IVAG challenge with vaccinia virus (VV).

Figure 7 is a graph depicting IFN-γ secreting cells in iliac lymph nodes following intrarectal (IR) and intravaginal (IVAG) immunization with SIN particles and IR and IVAG challenge with vaccinia virus (VV).

Figure 8 is a graph depicting vaccinia virus (VV) titer following vaginal and rectal delivery of SIN-gag particles.

Figure 9 is a graph depicting gag-specific serum titers. From left to right, the bars show titers in animals: primed with SIN-gag and no boost; primed with SIN-gag and boosted with p24 polypeptide and LTK63 adjuvant; naïve animals; and no prime but subsequent boost with p24 and LTK63 adjuvant.

Figure 10 is a graph depicting gp120-specific serum titers. From left to right, the bars show titers in animals: primed with SIN-gp140 and no boost; primed with SIN-gag and boosted with gp-140 polypeptide and LTK72 adjuvant and CpG; naïve animals; and no prime but subsequent boost with gp-140 polypeptide and LTK72 adjuvant and CpG.

Figure 11, panels A to D, are graphs depicting Induction of local and systemic cellular immune responses after IN (A), IM (B), IR (C) and IVAG (D) immunizations with SIN-gag particles followed by IVAG challenge with VV-gag. IN and IM immunizations induced several fold higher numbers of gag-specific IFN-γ secreting cells in VUM and ILN compared to IR and IVAG immunizations. The mice were immunized 3 times IN or IM with $2.5 \times 10^6$ SIN-gag particles and IR or IVAG with $10^7$ SIN-gag particles, three weeks later they were challenged vaginally with $10^7$ pfu of VV-gag, and sacrificed 5 days later. The results are shown as average number of p7g-

specific IFN-γ secreting cells per 10 million mononuclear cells (MNC) of three independent experiments ± SD.

Figure 12 depicts Protection in ovaries from vaginal challenge with VV-gag following vaginal or rectal immunizations with SIN-gag particles. The mice were immunized 3 times IN or IM with $2.5x10^6$ SIN-gag particles and IR or IVAG with $10^7$ SIN-gag particles, three weeks later they were challenged vaginally with $10^7$ pfu of VV-gag, and sacrificed 5 days later. Ovaries were collected and standard pfu assay for determination of VV titers was performed. Each dot represents the numbers of plaque forming units per ovary/each mouse. As control naïve mice were challenged with VV-gag and mice immunized with SIN-gag particles were challenged with VV-gp160 and in both cases high pfu titers were evident in the ovaries.

Figure 13 is a graph depicting the induction of an immunological response (as measured by IFN-γ ELISPOT assay) following intranasal immunization with alphavirus vector particles. Actual numbers represented by the bar graph are as follows: SIN-gag replicon particles gave 1154 (± 499); VEE-gag replicon particles 1530 (± 425); SIN/VEE-gag 140 (± 140); and VEE/SIN-gag 2586 (± 762).

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Short Protocols in Molecular Biology*, 4th ed. (Ausubel et al. eds., 1999, John Wiley & Sons); *Molecular Biology Techniques: An Intensive Laboratory Course*, (Ream et al., eds., 1998, Academic Press); *PCR (Introduction to Biotechniques Series)*, 2nd ed. (Newton & Graham eds., 1997, Springer Verlag); Peters and Dalrymple, *Fields Virology* (2d ed), Fields et al. (eds.), B.N. Raven Press, New York, NY.

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more such agents.

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Gene transfer" or "gene delivery" refers to methods or systems for reliably inserting DNA of interest into a host cell. Such methods can result in transient expression of non-integrated transferred DNA, extrachromosomal replication and expression of transferred replicons (e.g., episomes), or integration of transferred genetic material into the genomic DNA of host cells. Gene delivery expression vectors include, but are not limited to, vectors derived from alphaviruses, pox viruses and vaccinia viruses. When used for immunization, such gene delivery expression vectors may be referred to as vaccines or vaccine vectors.

The terms "Replication-defective" and "replication-incompetent" are used interchangeably to refer to a gene-delivery vehicle such as a viral vector, that does not make or propogate additional infectious, viral particles after being administered to a target cell. As will be apparent to those of skill in the art, the polynucleotides (*e.g.*, RNA) contained in the administered vector or particles may amplify or replicate, however, new progeny vector or viral particles are not formed and do not spread from cell to cell after administration.

"Alphavirus vector construct" refers to an assembly that is capable of directing the expression of a sequence(s) or gene(s) of interest. As described, for example, in U.S. Patent No. 6,015,695; U.S. Patent No. 6,015,686; U.S. Patent No. 5,842,723, and WO 97/38087, the vector construct should include a 5' sequence which is capable of initiating transcription of an alphavirus, as well as sequence(s) which, when expressed, code for biologically active alphavirus non-structural proteins (*e.g.*, NSP1, NSP2, NSP3, and NSP4), and an alphavirus RNA polymerase recognition sequence. In addition, the vector construct should include a viral junction promoter region that may, in certain embodiments, be modified in order to prevent, increase, or reduce viral

transcription of the subgenomic fragment. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable viral vector particles, a 5' promoter which is capable of initiating the synthesis of viral RNA *in vitro* or *in vivo* from cDNA, as well as one or more restriction sites, means for expressing multiple antigens (*e.g.*, IRES element) and a polyadenylation sequence. Any of the sequences making up the alphavirus vector construct may be derived from one or more alphaviruses. As an RNA molecule, the alphavirus vector construct may also be referred to as an "RNA replicon."

"Structural protein expression cassette" refers to a recombinantly produced molecule that is capable of expressing alphavirus structural protein(s). The expression cassette must include a promoter and a sequence encoding alphavirus structural protein(s). Optionally, the expression cassette may include transcription termination, splice recognition, and polyadenylation addition sites. Preferred promoters include the CMV and adenovirus VA1RNA promoters, as well as alphavirus subgenomic junction region promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, hygromycin, phleomycin, histidinol, and DHFR.

"Recombinant alphavirus particle" refers to a capsid that contains an alphavirus vector construct. Preferably, the alphavirus capsid is contained within a lipid bilayer, such as a cell membrane, in which viral encoded proteins (*e.g.*, envelope proteins) are embedded. A variety of vectors may be contained within the alphavirus particle, including the alphavirus vector constructs of the present invention. In addition, the recombinant alphavirus particle may be a chimera, containing elements from any number of different alphaviruses (*e.g.*, RNA vector construct from SIN with capsid and/or envelope proteins from VEE). (See, also co-owned U.S. Patent No. 6,329,201).

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native

sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the proteins or errors due to PCR amplification.

An "antigen" refers to a molecule containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Normally, a B-cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids, and a helper T-cell epitope at least about 12-20 amino acids. Normally, an epitope will include between about 7 and 15 amino acids, such as, 9, 10, 12 or 15 amino acids. The term "antigen" denotes both subunit antigens, (i.e., antigens which are separate and discrete from a whole organism with which the antigen is associated in nature), as well as, killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein. Similarly, an oligonucleotide or polynucleotide that expresses an antigen or antigenic determinant *in vivo*, such as in gene therapy and DNA immunization applications, is also included in the definition of antigen herein.

For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi, as described more fully below. The term also intends any of the various tumor antigens. Preferably, the antigens are derived from a sexually transmitted pathogen, for example a virus or a bacteria. Furthermore, for purposes of the present invention, an "antigen" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the antigens.

An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to an antigen present in the composition of interest. For purposes of the present invention, a "humoral immune

response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells. In addition, a chemokine response may be induced by various white blood or endothelial cells in response to an administered antigen.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376. Recent methods of measuring cell-mediated immune response include measurement of intracellular cytokines or cytokine secretion by T-cell populations (*e.g.*, by ELISPOT technique), or by measurement of epitope specific T-cells (e.g., by the tetramer technique)(reviewed by McMichael, A.J., and O'Callaghan, C.A., *J. Exp. Med.* 187(9):1367-1371, 1998; Mcheyzer-Williams, M.G., et al, *Immunol. Rev.* 150:5-21, 1996; Lalvani, A., et al, *J. Exp. Med.* 186:859-865, 1997).

Thus, an immunological response as used herein may be one that stimulates the production of CTLs, and/or the production or activation of helper T- cells. The production of chemokines and/or cytokines may also be stimulated. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor, cytotoxic, or helper T-cells and/or γδ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

An "immunogenic composition" is a composition that comprises an antigenic molecule where administration of the composition to a subject results in the development in the subject of a humoral and/or a cellular immune response to the antigenic molecule of interest. The immunogenic composition can be introduced directly into a recipient subject, such as by injection, inhalation, oral, intranasal or any other mucosal (*e.g.*, intra-rectally or intra-vaginally) route of administration.

By "subunit vaccine" is meant a vaccine composition that includes one or more selected antigens but not all antigens, derived from or homologous to, an antigen from a pathogen of interest such as from a virus, bacterium, parasite or fungus. Such a composition is substantially free of intact pathogen cells or pathogenic particles, or the lysate of such cells or particles. Thus, a "subunit vaccine" can be prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or analogs thereof. The method of obtaining an antigen included in the subunit vaccine can thus include standard purification techniques, recombinant production, or synthetic production.

An "immuno-modulatory factor" refers to a molecule, for example a protein that is capable of modulating an immune response. Non-limiting examples of immunomodulatory factors include lymphokines (also known as cytokines), such as IL-6, TGF-β, IL-1, IL-2, IL-3, etc.); and chemokines (*e.g.*, secreted proteins such as macrophage inhibiting factor). Certain cytokines, for example TRANCE, flt-3L, and a

secreted form of CD40L are capable of enhancing the immunostimulatory capacity of APCs. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1α), interleukin-11 (IL-11), MIP-1γ, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO), CD40 ligand (CD40L), tumor necrosis factor-related activation-induced cytokine (TRANCE) and flt3 ligand (flt-3L). Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). The sequences of many of these molecules are also available, for example, from the GenBank database. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or mutants thereof) and nucleic acid encoding these molecules are intended to be used within the spirit and scope of the invention.

By "subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual in a formulation or composition without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

A. GENE DELIVERY SYSTEMS

1. Alphavirus Vectors and Particles

As noted above, the present invention provides alphavirus vector constructs, alphavirus particles containing such constructs, as well as methods for making and utilizing such vector constructs and particles in mucosal immunizations. The use of alphavirus based, replication-defective replicon particles, as a mucosal vaccine delivery system provides a number of advantages. Perhaps the most important aspect of immunization with a replication-defective virus vector is the control of gene product at the site of immunization. Immunization with replicating vectors, including the reported live, attenuated alphavirus Venezuelan equine encephalitis-based (VEE) vectors (Caley et al. (1997) *J. Virol.* 71:3031-3038; Davis et al. (1996) *J. Virol.* 70:3781-3787), results in the dissemination of the vector throughout the host. In contrast, immunization with a replication-defective vector results in the local expression, without the spread of newly propagated progeny vector. Moreover, the deficiency in replication reduces the risk of unwarranted inflammatory responses at sites of immunization (Villacres (2000) *Virology*, 270:54).

Alphavirus vectors suitable for use in the present invention can be constructed by any means, for example, as described in U.S. Patent Nos: 6,015,686; 6,015,694; 5,789,245 and 5,842,723. Briefly, sequences encoding alphavirus suitable for use in preparing the above-described vector constructs and particles may be readily obtained given the disclosure provided herein from naturally-occurring sources, or from depositories (*e.g.*, the American Type Culture Collection, Rockville, Maryland). Representative examples of suitable alphaviruses include Aura (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mayaro virus (ATCC VR-1277), Middleburg (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248), Tonate (ATCC VR-925), Triniti (ATCC VR-469), Una (ATCC VR-374),

Venezuelan equine encephalomyelitis (ATCC VR-69), Venezuelan equine encephalomyelitis virus (ATCC VR-923, ATCC VR-1250 ATCC VR-1249, ATCC VR-532), Western equine encephalomyelitis (ATCC VR-70, ATCC VR-1251, ATCC VR-622, ATCC VR-1252), Whataroa (ATCC VR-926), and Y-62-33 (ATCC VR-375). Sequences obtained from one or more alphaviruses can be used in the same vector and/or particle. Within one preferred aspect of the present invention, the sequences that encode wild-type alphavirus are obtained from a Sindbis virus.

The alphavirus vector component typically includes self-amplifying RNA (replicon) in which one or more of the structural protein genes of the virus are replaced by one or more genes of interest (*e.g.*, antigens). RNA-based vectors, including alphavirus vectors, are also known as "replicons" because they retain the replicase functions necessary for RNA self-amplification and high-level expression, and can be launched *in vivo* following transfection with plasmid DNA (Dubensky et al. (1996) *J. Virol.* 70:508-519) or infection with virus-like particles. Alphavirus vector constructs will also typically contain inactivated, tandem or modified viral junction regions. Other modifications and methods of constructing suitable alphavirus vectors are described, for example, in U.S. Patent No. 6,015,686 and WO 97/38087.

The replicon RNA of the alphavirus vector construct may be delivered directly to the target cells by physical means or first packaged into particles. Generally, structural proteins necessary for packaging are supplied *in trans* by helper constructs or by packaging cell lines. The structural proteins may be homologous (*e.g.*, derived from the same alphavirus as the vector construct); heterologous (*e.g.*, derived from an alphavirus different from the vector construct); and hybrid (*e.g.*, containing elements of multiple alphaviruses). Importantly, only the replicon RNA is packaged into the particle, as the helper RNAs typically lack the *cis*-acting packaging sequence required for encapsidation. Thus, alphavirus particles can be generated which infect target cells in culture or *in vivo*, and can express the gene of interest to high level; however, they are defective in that they lack critical portions of the alphavirus genome necessary to produce virus particles which could spread to other cells. In this way, when the replicon RNA is introduced into a suitable host cell, it self-amplifies, and the gene of interest is expressed from the subgenomic mRNA. However, no new virion particles are assembled *in vivo* due to the absence of structural protein genes.

Although no direct comparative studies among the various alphavirus replicons have been performed, differences in natural cell tropism are known to exist. For example, the lymphotropic VEE recently was shown to transduce murine DC (MacDonald et al. (2000) *J. Virol.* 74:914-922), while SIN and SFV are not lymphotropic. Infection of human DC has recently been demonstrated for alphaviruses or their derived vectors (Gardner et al. (2000) *J. Virol.* 74:11849-11857; WO 00/61772). Gardner identified SIN variants that are highly efficient for growth in immature human DC. The genetic determinant of human DC tropism for the SIN variant has been mapped to a single amino acid substitution in glycoprotein E2, and using this information, alphavirus replicon particles that can be used to target human DC can be generated. Detailed characterization of replicon infected DC both *in vitro* and *in vivo* revealed that the replicon-infected cells maintained their developmental and antigen presenting capabilities, indicating the potential utility of the DC-targeted SIN replicons for vaccine applications against infectious and malignant disease.

2. Additional Gene Delivery Vehicles

As noted above, mucosal genetic immunization has been shown to elicit both local and systemic immune responses. However, safety issues associated with revertants and potential infection hazards limits the utility of these systems. Moreover, replicating virus-based vector may spread rapidly throughout the host from the site of administration and cause unwarranted inflammatory responses at other sites. Therefore, the present invention describes viral and non-viral based genetic delivery systems that are replication-defective in the host and, accordingly, do not bear the possibility of reverting to an infectious state. Thus, the invention includes compositions and methods of mucosal immunization using any replication-defective gene transfer vehicle.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. Selected sequences can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (U.S. Patent No. 5,219,740; Miller and Rosman, *BioTechniques* (1989) 7:980-990; Miller, A.D., *Human Gene Therapy* (1990) 1:5-14;

Scarpa et al., *Virology* (1991) 180:849-852; Burns et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:8033-8037; and Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* (1993) 3:102-109.

A number of adenovirus vectors have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) 57:267-274; Bett et al., *J. Virol.* (1993) 67:5911-5921; Mittereder et al., *Human Gene Therapy* (1994) 5:717-729; Seth et al., *J. Virol.* (1994) 68:933-940; Barr et al., *Gene Therapy* (1994) 1:51-58; Berkner, K.L. *BioTechniques* (1988) 6:616-629; and Rich et al., *Human Gene Therapy* (1993) 4:461-476).

Additionally, various adeno-associated virus (AAV) vector systems have been developed for gene delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al., *Molec. Cell. Biol.* (1988) 8:3988-3996; Vincent et al., *Vaccines 90* (1990) (Cold Spring Harbor Laboratory Press); Carter, B.J. *Current Opinion in Biotechnology* (1992) 3:533-539; Muzyczka, N. *Current Topics in Microbiol. and Immunol.* (1992) 158:97-129; Kotin, R.M. *Human Gene Therapy* (1994) 5:793-801; Shelling and Smith, *Gene Therapy* (1994) 1:165-169; and Zhou et al., *J. Exp. Med.* (1994) 179:1867-1875.

Another vector system useful for delivering polynucleotides, mucosally and otherwise, is the enterically administered recombinant poxvirus vaccines described by Small, Jr., P.A., et al. (U.S. Patent No. 5,676,950, issued October 14, 1997, herein incorporated by reference) as well as the vaccinia virus and avian poxviruses. By way of example, vaccinia virus recombinants expressing the genes can be constructed as follows. The DNA encoding the particular synthetic Gag/antigen coding sequence is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells that are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the coding sequences of interest into the viral genome. The resulting

TK⁻recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the genes. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an avipox vector is particularly desirable in human and other mammalian species since members of the avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, e.g., WO 91/12882; WO 89/03429; and WO 92/03545. Picornavirus-derived vectors can also be used. (See, e.g., U.S. Patent Nos. 5,614,413 and 6,063,384).

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al., *J. Biol. Chem.* (1993) 268:6866-6869 and Wagner et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery.

A vaccinia based infection/transfection system can be conveniently used to provide for inducible, transient expression of the coding sequences of interest (for example, a synthetic Gag/HCV-core expression cassette) in a host cell. In this system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA that is then translated into protein by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, e.g., Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA* (1990) 87:6743-6747; Fuerst et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:8122-8126.

3. Amplification Systems

As an alternative approach to infection with vaccinia or avipox virus recombinants, or to the delivery of genes using other viral vectors, an amplification system can be used that will lead to high level expression following introduction into host cells. Specifically, a T7 RNA polymerase promoter preceding the coding region for T7 RNA polymerase can be engineered. Translation of RNA derived from this template will generate T7 RNA polymerase that in turn will transcribe more template. Concomitantly, there will be a cDNA whose expression is under the control of the T7 promoter. Thus, some of the T7 RNA polymerase generated from translation of the amplification template RNA will lead to transcription of the desired gene. Because some T7 RNA polymerase is required to initiate the amplification, T7 RNA polymerase can be introduced into cells along with the template(s) to prime the transcription reaction. The polymerase can be introduced as a protein or on a plasmid encoding the RNA polymerase. For a further discussion of T7 systems and their use for transforming cells, see, e.g., International Publication No. WO 94/26911; Studier and Moffatt, *J. Mol. Biol.* (1986) 189:113-130; Deng and Wolff, *Gene* (1994) 143:245-249; Gao et al., *Biochem. Biophys. Res. Commun.* (1994) 200:1201-1206; Gao and Huang, *Nuc. Acids Res.* (1993) 21:2867-2872; Chen et al., *Nuc. Acids Res.* (1994) 22:2114-2120; and U.S. Patent No. 5,135,855.

4. Liposomal/Lipid Delivery Vehicles

The polynucleotide of interest can also be delivered without a viral vector. For example, packaged as DNA or RNA in liposomes prior to delivery to the subject or to cells derived therefrom. Lipid encapsulation is generally accomplished using liposomes that are able to stably bind or entrap and retain nucleic acid. The ratio of condensed DNA to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger et al., in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations, with

cationic liposomes particularly preferred. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416); mRNA (Malone et al., *Proc. Natl. Acad. Sci. USA* (1989) 86:6077-6081); and purified transcription factors (Debs et al., *J. Biol. Chem.* (1990) 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416). Other commercially available lipids include (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Cationic microparticles can be prepared from readily available materials using techniques known in the art. See, *e.g.,* co-owned WO 01/136599.

Similarly, anionic and neutral liposomes are readily available, such as, from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphoshatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilammelar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger et al., in METHODS OF IMMUNOLOGY (1983), Vol. 101, pp. 512-527; Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; Papahadjopoulos et al., *Biochim. Biophys. Acta* (1975) 394:483; Wilson et al., *Cell* (1979) 17:77); Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629; Ostro et al., *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley et al., *Proc. Natl. Acad. Sci. USA* (1979) 76:3348); Enoch and

Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) 76:145); Fraley et al., *J. Biol. Chem.* (1980) 255:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) 75:145; and Schaefer-Ridder et al., *Science* (1982) 215:166.

The DNA and/or protein antigen(s) can also be delivered in cochleate lipid compositions similar to those described by Papahadjopoulos et al., *Biochem. Biophys. Acta.* (1975) 394:483-491. See, also, U.S. Patent Nos. 4,663,161 and 4,871,488.

5. Particulate Carriers

The compositions may also be encapsulated, adsorbed to, or associated with, particulate carriers. Such carriers present multiple copies of a selected antigen to the immune system and promote migration, trapping and retention of antigens in local lymph nodes. The particles can be taken up by profession antigen presenting cells such as macrophages and dendritic cells, and/or can enhance antigen presentation through other mechanisms such as stimulation of cytokine release. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee JP, et al., *J Microencapsul.* 14(2):197-210, 1997; O'Hagan DT, et al., *Vaccine* 11(2):149-54, 1993.

Furthermore, other particulate systems and polymers can be used for the *in vivo* or *ex vivo* delivery of the gene of interest. For example, polymers such as polylysine, polyarginine, polyornithine, spermine, spermidine, as well as conjugates of these molecules, are useful for transferring a nucleic acid of interest. Similarly, DEAE dextran-mediated transfection, calcium phosphate precipitation or precipitation using other insoluble inorganic salts, such as strontium phosphate, aluminum silicates including bentonite and kaolin, chromic oxide, magnesium silicate, talc, and the like, will find use with the present methods. See, e.g., Felgner, P.L., *Advanced Drug Delivery Reviews* (1990) 5:163-187, for a review of delivery systems useful for gene transfer. Peptoids (Zuckerman, R.N., et al., U.S. Patent No. 5,831,005, issued November 3, 1998) may also be used for delivery of a construct of the present invention.

Additionally, biolistic delivery systems employing particulate carriers such as gold and tungsten, are especially useful for delivering synthetic expression cassettes of

the present invention. The particles are coated with the synthetic expression cassette(s) to be delivered and accelerated to high velocity, generally under a reduced atmosphere, using a gun powder discharge from a "gene gun." For a description of such techniques, and apparatuses useful therefore, see, e.g., U.S. Patent Nos. 4,945,050; 5,036,006; 5,100,792; 5,179,022; 5,371,015; and 5,478,744. Also, needle-less injection systems can be used (Davis, H.L., et al, *Vaccine* **12**:1503-1509, 1994; Bioject, Inc., Portland, OR).

B. ANTIGENS

Any of the gene delivery vehicles described herein can contain one or more heterologous sequences encoding one or more heterologous gene products, particularly polypeptide antigens. Virtually any heterologous gene product or polypeptide can be used. Antigens that are particularly useful in the practice of the present invention include polypeptide antigens derived from sexually transmitted pathogens or other viruses that infect or are transmitted through mucosal surfaces. Non-limiting representative examples of sexually transmitted pathogens and antigens derived therefrom include antigens derived from bacterial pathogens (*e.g.*, chlamydia, gonorrhea and syphilis) and viral pathogens (*e.g.*, Human Immmunodeficiency Virus ("HIV"), Hepatitis B and C Virus ("HBV" and "HCV", respectively), Human Papiloma Virus ("HPV"), Herpes Simplex Virus ("HSV"), and the like). Non-limiting examples of other viruses that may be transmitted via mucosal surfaces include rhinovirus, influenza, respiratory syncytial virus (RSV), parainfluenza virus (PIV), and the like. As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen that is capable, under the appropriate conditions, of causing an immune response (*i.e.*, cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins divided into three major classes: (1) the major structural proteins, Gag, Pol, and Env; (2) the regulatory proteins, Tat and Rev and (3) the

accessory proteins, Vpu, Vpr, Vif, and Nef. Although exemplified herein with relation to antigens obtained from $HIV_{SF2}$, sequence obtained from other HIV variants may be manipulated in similar fashion following the teachings of the present specification. Such other variants include, but are not limited to, Gag protein encoding sequences obtained from the isolates $HIV_{IIIb}$, $HIV_{SF2}$, HIV-$1_{SF162}$, HIV-$1_{SF170}$, $HIV_{LAV}$, $HIV_{LAI}$, $HIV_{MN}$, HIV-$1_{CM235}$, HIV-$1_{US4}$, other HIV-1 strains from diverse subtypes (e.g., subtypes, A through G, and O), HIV-2 strains and diverse subtypes (e.g., HIV-$2_{UC1}$ and HIV-$2_{UC2}$), and simian immunodeficiency virus (SIV). (See, e.g., Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991); *Virology*, 3rd Edition (Fields, BN, DM Knipe, PM Howley, Editors, 1996, Lippincott-Raven, Philadelphia, PA; for a description of these and other related viruses).

As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described antigens may be combined in order to induce an immune response when administered as described herein. Further, the antigen-encoding gene delivery vehicles can also be used in combination with one or more additional gene delivery vehicles and/or polypeptides.

In addition, due to the large immunological variability that is found in different geographic regions for the open reading frame of HIV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, at least eight different subtypes of HIV have been identified and, of these, subtype B viruses are more prevalent in North America, Latin America and the Caribbean, Europe, Japan and Australia. Almost every subtype is present in sub-Saharan Africa, with subtypes A and D predominating in central and eastern Africa, and subtype C in southern Africa. Subtype C is also prevalent in India and it has been recently identified in southern Brazil. Subtype E was initially identified in Thailand, and is also present in the Central African Republic. Subtype F was initially described in Brazil and in Romania. The most recent subtypes described are G, found in Russia and Gabon, and subtype H, found in Zaire and in Cameroon. Group O viruses have been identified in Cameroon and also in Gabon. Thus, as will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration that is appropriate to the particular HIV subtype that is prevalent in the geographical region of

administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, by sequencing the HIV genome (or fragments thereof) isolated from individuals within that region.

As described above, also presented by HIV are various *Gag* and *Env* antigens. HIV-1 *Gag* proteins are involved in many stages of the life cycle of the virus including, assembly, virion maturation after particle release, and early post-entry steps in virus replication. The roles of HIV-1 *Gag* proteins are numerous and complex (Freed, E.O. (1998) *Virology* 251:1-15).

*Env* coding sequences of the present invention include, but are not limited to, polynucleotide sequences encoding the following HIV-encoded polypeptides: gp160, gp140, and gp120 (see, e.g., U.S. Patent No. 5,792,459 for a description of the HIV-$1_{SF2}$ ("SF2") Env polypeptide). The envelope protein of HIV-1 is a glycoprotein of about 160 kD (gp160). During virus infection of the host cell, gp160 is cleaved by host cell proteases to form gp120 and the integral membrane protein, gp41. The gp41 portion is anchored in (and spans) the membrane bilayer of virion, while the gp120 segment protrudes into the surrounding environment. As there is no covalent attachment between gp120 and gp41, free gp120 is released from the surface of virions and infected cells. Thus, gp160 includes the coding sequences for gp120 and gp41. The polypeptide gp41 is comprised of several domains including an oligomerization domain (OD) and a transmembrane spanning domain (TM). In the native envelope, the oligomerization domain is required for the non-covalent association of three gp41 polypeptides to form a trimeric structure: through non-covalent interactions with the gp41 trimer (and itself), the gp120 polypeptides are also organized in a trimeric structure. A cleavage site (or cleavage sites) exists approximately between the polypeptide sequences for gp120 and the polypeptide sequences corresponding to gp41. This cleavage site(s) can be mutated to prevent cleavage at the site. The resulting gp140 polypeptide corresponds to a truncated form of gp160 where the transmembrane spanning domain of gp41 has been deleted. This gp140 polypeptide can exist in both monomeric and oligomeric (*i.e.* trimeric) forms by virtue of the presence of the oligomerization domain in the gp41 moiety. In the situation where the cleavage site has been mutated to prevent cleavage and the transmembrane portion of gp41 has been deleted the resulting polypeptide

product can be designated "mutated" gp140. As will be apparent to those in the field, the cleavage site can be mutated in a variety of ways. (See, also, WO 00/39302).

As noted above, at least one immunogenic portion of an HIV antigen may be incorporated into a gene delivery vehicle and used for mucosal immunization. The immunogenic portion(s) incorporated into the vehicle (*e.g.*, alphavirus vector construct) may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITES (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA, which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays and proliferation assays.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T cell receptor repertoire recognizes the same antigenic determinants recognized by human T cells. In both systems, the CTL response generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al. (1991) *J. Exp. Med. 173*:1007-1015; Vitiello et al. (1992) *Abstract of Molecular Biology of Hepatitis B Virus Symposia*).

Additional immunogenic portions of the HIV may be obtained by truncating the coding sequence at various locations including, for example, to include one or more epitopes from the various domains of the HIV genome. As noted above, such domains include structural domains such as *Gag, Gag-polymerase, Gag-protease, reverse transcriptase (RT), integrase (IN)* and *Env.* The structural domains are often further subdivided into polypeptides, for example, p55, p24, p6 (*Gag*); p160, p10, p15, p31, p65 (*pol, prot, RT and IN)*; and gp160, gp120 and gp41 (*Env*). Additional epitopes of HIV and other sexually transmitted diseases are known or can be readily determined using methods known in the art. Also included in the invention are molecular variants

of such polypeptides, for example as described in PCT/US99/31245; PCT/US99/31273 and PCT/US99/31272.

Other sexually or mucosally transmitted diseases, both viral and bacterial, can also be addressed using the compositions and methods described herein. For example, it appears that in the case of HSV, vaginal immunization with vector expressing HSV antigens (*e.g.*, gB, gD) may provide protection against vaginal. Thus, one or more antigens derived from HSV can be used in as described herein to treat and prevent HSV infection.

1. Preparation of Vectors Carrying Heterologous Gene Products

In certain embodiments, sequences encoding one or more antigens are incorporated into a gene delivery vehicle such as viral vector or particle. As will be evident to one of ordinary skill in the art given the disclosure provided herein, the efficiency of packaging and hence, viral titer, of various viral vectors is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production of viable vector particles (*e.g.*, alphavirus vector particles), additional non-coding sequences may be added to the vector construct.

In certain applications of the vectors or resulting particles described herein, the expression of more than one heterologous gene is desired. For example, in order to treat sexually transmitted disease such as HIV, multiple administrations of vectors or particles, or administration of vectors or particles expression more than one gene product may be required. In addition, immunogenicity may be further enhanced by encoding both an antigen and an immunomodulator (*e.g.*, cytokine, lymphokine, chemokine, etc.). Therefore, within one embodiment of the invention viral vectors (*e.g.*, alphavirus vectors) may be constructed by placing appropriate signals, such as ribosome readthrough or internal ribosome entry sites (IRES) between cistrons. Alternatively, multiple subgenomic junction region promoters (*e.g.*, derived from alphavirus) can be utilized. Further, a vector construct may express (either separately or as one construct) all or immunogenic portions of various mucosally transmitted (*e.g.*, sexually transmitted) pathogens.

Within one aspect of the invention, vector constructs (*e.g.*, alphavirus vectors and particles) are provided which direct the expression of immunogenic portions of HIV

antigens. The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilobases in length. (see, e.g., Muesing et al. (1985) *Nature* 313:450-48). Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs).

Sequences that encode the above-described proteins (*e.g.* antigens) may be readily obtained from a variety of sources, including for example, depositories such as the American Type Culture Collection (ATCC, Rockville, MD), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford, England). Representative examples of molecularly cloned genomes that encode the hepatitis B virus may be obtained from sources such as the American Type Culture Collection (ATCC, Rockville, MD). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see* Figure 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the *Bam* HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA 78*:2606-2610, 1981).

Alternatively, sequences encoding the polypeptide of interest can be generated by the polymerase chain reaction (PCR). Mullis et al. (1987) *Methods Enzymol.* 155:335-350; *PCR Protocols, A Guide to Methods and Applications*, Innis et al (eds) Harcourt Brace Jovanovich Publishers, NY (1994)). This technique uses DNA polymerase, usually a thermostable DNA polymerase, to replicate a desired region of DNA. The region of DNA to be replicated is identified by oligonucleotides of specified sequence complementary to opposite ends and opposite strands of the desired DNA to prime the replication reaction. Repeated successive cycles of replication result in amplification of the DNA fragment delimited by the primer pair used. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, $Mg^{2+}$ and ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides.

Once coding sequences for desired proteins have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Ligations to other sequences are performed using standard procedures, known in the art.

2. Polypeptide Preparation

Similarly, the selected coding sequences can be cloned into any suitable expression vector for expression. The expressed product can optionally be purified prior to mucosal administration. Briefly, a polynucleotide encoding these proteins can be introduced into an expression vector that can be expressed in a suitable expression system. A variety of bacterial, yeast, mammalian, insect and plant expression systems are available in the art and any such expression system can be used. Optionally, a polynucleotide encoding these proteins can be translated in a cell-free translation system. Such methods are well known in the art. The proteins also can be constructed by solid phase protein synthesis. If desired, the polypeptides also can contain other amino acid sequences, such as amino acid linkers or signal sequences, as well as ligands useful in protein purification, such as glutathione-S-transferase and staphylococcal protein A. Alternatively, antigens of interest can be purchased from commercial sources.

C. DELIVERY

The compositions (*e.g.*, gene delivery vehicles and optional polypeptide antigens) described herein can be delivered using any suitable means (*e.g.*, intravenously, intramuscularly, intraperitoneally, subcutaneously, orally, rectally, intraocularly, intranasally), or by various physical methods such as lipofection (Felgner et al. (1989) *Proc. Natl. Acad. Sci. USA* 84:7413-7417), direct DNA injection (Acsadi et al. (1991) *Nature* 352:815-818); microprojectile bombardment (Williams et al. (1991) *PNAS* 88:2726-2730); liposomes of several types (*see, e.g.*, Wang et al. (1987) *PNAS* 84:7851-7855); $CaPO_4$ (Dubensky et al. (1984) *PNAS* 81:7529-7533); DNA ligand (Wu et al (1989) *J. of Biol. Chem.* 264:16985-16987); administration of polypeptides alone; administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al. (1992), *Hum. Gene Ther.* 3:147-154); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. In addition, the eukaryotic layered vector initiation systems may either be administered directly (*i.e., in vivo*), or to cells that have been removed (*ex vivo*), and subsequently returned.

In a preferred embodiment, the gene delivery vehicles and optional polypeptide-containing compositions are administered mucosally. Methods of mucosal delivery are known in the art, for example as described in *Remington's, supra*. Delivery of the compositions rectally and vaginally is particularly preferred in the case of sexually transmitted pathogens, as this mode of administration provides access to the cells first exposed to the pathogens. Similarly, intranasal administration may be preferred in diseases, like rhinovirus, that infect through nasal mucosa. In some instances, intranasal administration may induce immunity in the vaginal mucosa and oral immunization may induce immunity in the rectal mucosa. Moreover, combinations of various routes of mucosal administration and/or various routes of systemic administration can be used in order to induce optimal immunity and protection (both at the site the pathogen enters as well as at systemic sites where a mucosal pathogen has spread to. Additionally, mucosal administration eliminates the need for syringes or other administration devices. Dosage treatment may be a single dose schedule or a multiple dose schedule.

In certain embodiments, the replication-defective vectors are administered via nucleic acid immunization or the like using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Patent Nos. 5,399,346, 5,580,859, 5,589,466. Alphavirus compositions can be delivered either directly to the vertebrate subject or, alternatively, delivered *ex vivo*, to cells derived from the subject and the cells reimplanted in the subject. In preferred embodiments, the compositions are delivered *in vivo*. Delivery of replication-defective compositions *in vivo* can generally be accomplished using any means known in the art, for example, by injection using either a conventional syringe, needless devices such as Bioject® or a gene gun, such as the Accell® gene delivery system (PowderJect Technologies, Inc., Oxford, England). The constructs can be delivered (*e.g.*, injected) either subcutaneously, epidermally, intradermally, intramuscularly, intravenous, mucosally (such as nasally, rectally and vaginally), intraperitoneally, orally or combinations thereof.

Within other aspects of the present invention, methods are provided for administering the gene delivery systems described herein, including recombinant alphavirus vectors or particles. Briefly, the final mode of viral vector administration usually relies on the specific therapeutic application, the best mode of increasing vector potency, and the most convenient route of administration. Generally, this embodiment

includes compositions which can be designed to be delivered by, for example, (1) direct injection into the blood stream; (2) direct injection into a specific tissue or tumor; (3) oral administration; (4) nasal inhalation; (5) direct application to mucosal tissues (*e.g.*, intranasally, intrarectally and/or intravaginally); and/or (6) *ex vivo* administration of transduced autologous cells into the animal. Thus the therapeutic alphavirus vector can be administered in such a fashion such that the vector can (a) transduce a normal healthy cell and transform the cell into a producer of a therapeutic protein or agent which is secreted systemically or locally, (b) transform an abnormal or defective cell, transforming the cell into a normal functioning phenotype, (c) transform an abnormal cell so that it is destroyed, and/or (d) transduce cells to manipulate the immune response.

The compositions disclosed herein can be administered alone or can be administered with one or more additional gene delivery vehicles and/or one or more proteins. In such embodiments, the multiple compositions can be administered in any order, for example gene delivery vehicle followed by protein; multiple gene delivery vehicles followed by multiple protein administrations; protein administration(s) followed by single or multiple gene delivery vehicle administration; concurrent administration; and the like. Thus, a mixture of protein and nucleic acid can be administered, using the same or different vehicles and the same or different modes of administration.

In certain embodiments, direct delivery will generally be accomplished with or without viral vectors, as described above, by injection using either a conventional syringe or a gene gun, such as the Accell® gene delivery system (PowderJect Technologies, Inc., Oxford, England).

Thus, injection can be either subcutaneously, epidermally, intradermally, intramucosally such as nasally, rectally, orally and vaginally, intraperitoneally, intravenously, or intramuscularly. Other modes of administration include pulmonary administration, suppositories, needle-less injection, transcutaneous and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. As noted above, administration of nucleic acids may also be combined with administration of peptides or other substances.

D. PHARMACEUTICAL COMPOSITIONS

The present invention also includes pharmaceutical compositions comprising a replication-defective vector (*e.g.*, recombinant alphavirus construct or alphavirus particle) in combination with a pharmaceutically acceptable carrier, diluent, or recipient. Further, other ingredients, such as adjuvants, may also be present. As described more fully in U.S. Patent No. 6,015,694, storage stable and easy administerable immunogenic compositions are particularly needed in Third World countries where refrigeration and/or traditional administration means (syringes, etc.) are not readily available.

In certain embodiments, polypeptides may also be included. The preparation of immunogenic compounds that contain immunogenic polypeptide(s) as active ingredients is known to those skilled in the art. Typically, such immunogenic compounds are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified, or the protein encapsulated in liposomes.

Compositions of the invention preferably comprise a pharmaceutically acceptable carrier. The carrier should not itself induce the production of antibodies harmful to the host. Pharmaceutically acceptable carriers are well known to those in the art. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee et al. (1997) *J Microencapsul.* 14(2):197-210; O'Hagan et al. (1993) *Vaccine* 11(2):149-54. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as toxoid from diphtheria, tetanus, cholera, etc., as well as toxins derived from *E. coli*.

Pharmaceutically acceptable salts can also be used in compositions of the invention, for example, mineral salts such as hydrochlorides, hydrobromides,

phosphates, or sulfates, as well as salts of organic acids such as acetates, proprionates, malonates, or benzoates. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those of skill in the art. Compositions of the invention can also contain liquids or excipients, such as water, saline, glycerol, dextrose, ethanol, or the like, singly or in combination, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents. Liposomes can also be used as a carrier for a composition of the invention, such liposomes are described above.

Briefly, with regard to viral particles, replication-defective vectors (also referred to above as particles) may be preserved either in crude or purified forms. Preservation methods and conditions are described in U.S. Patent No. 6,015,694.

Further, the compositions described herein can include various excipients, adjuvants, carriers, auxiliary substances, modulating agents, and the like. Preferably, the compositions will include an amount of the antigen sufficient to mount an immunological response. An appropriate effective amount can be determined by one of skill in the art. Such an amount will fall in a relatively broad range that can be determined through routine trials and will generally be an amount on the order of about 0.1 μg to about 1000 μg, more preferably about 1 μg to about 300 μg, of particle/antigen.

Such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid

A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), beta chemokines (MIP, 1-alpha, 1-beta Rantes, etc.); (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. W093/13202; W092/19265; WO 95/17211; WO 98/18928 and WO 01/22993); and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acteyl-normuramyl-L-alanyl-D-isogluatme (nor-MDP), N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(l'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

Administration of the pharmaceutical compositions described herein may be by any suitable route (see, *e.g.*, Section C). Particularly preferred is mucosal (*e.g.*, rectal and/or vaginal) administration. Dosage treatment may be a single dose schedule or a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals, chosen to maintain and/or reinforce the immune response, for example at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the potency of the modality, the vaccine delivery employed, the need of the subject and be dependent on the judgment of the practitioner.

In yet other embodiments, a multiple administrations (*e.g.*, prime-boost type administration) will be advantageously employed. For example, recombinant

alphavirus particles expressing the antigen(s) of interest are administered (*e.g.*, IVAG or IR). Subsequently, the antigen(s) are administered, for example in compositions comprising the polypeptide antigen(s) and a suitable adjuvant. Alternatively, antigens are administered prior to gene delivery vehicles. Multiple polypeptide and multiple gene delivery vehicle administrations (in any order) may also be employed.

E. METHODS FOR UTILIZING REPLICATION-DEFECTIVE PARTICLES AND ANTIGENIC POLYPEPTIDES

Within certain aspects of the present invention, compositions and methods are provided for mucosally administering a composition (*e.g.*, an alphavirus vector construct) that is capable of preventing, inhibiting, stabilizing or reversing sexually transmitted diseases. Representative examples of such diseases include bacterial and viral infections, particularly sexually transmitted infections, including but not limited to HIV, HBV HTLV I, HTLV II, HPV, HSV, HCV, chlamydia, gonorrhea, and/or syphilis.

More specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a sexually transmitted pathogenic agent, such that the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents include bacteria and viruses.

Within one embodiment of the invention the pathogenic agent is a virus, and methods are provided for stimulating a specific immune response and inhibiting viral spread by using recombinant alphavirus viral particles designed to deliver a vector construct that directs the expression of an antigen or modified form thereof to susceptible target cells capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the vector nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant alphavirus is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I

and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogues thereof (*e.g.*, Altmann et al., *Nature 338*:512, 1989). Cells infected with alphavirus vectors are expected to do this efficiently because they closely mimic genuine viral infection and because they: (a) are able to infect non-replicating cells, (b) do not integrate into the host cell genome, (c) are not associated with any life threatening diseases, and (d) express high levels of heterologous protein. Because of these differences, alphavirus vectors can easily be thought of as safe viral vectors that can be used on healthy individuals for vaccine use.

This aspect of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy and low levels of viral antigens, relative to heterologous genes, are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant alphavirus, leading to responses against immunogenic epitopes that may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

The compositions (*e.g.*, alphavirus constructs and particles) suitable for mucosal administration described herein may include one or more inhibitory palliative, *e.g.*, a viral or bacterial inhibitory gene which express anti-sense or the like. A discussion of inhibitory palliatives are described, for example, in U.S. Patent No. 6,015,686 which describes how antigens and inhibitory palliatives (*e.g.*, expressing anti-sense tat, etc.) can be co-expressed and/or designed to overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells. In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker

would be a vector construct expressing either an HIV *env* analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of an alphavirus vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule. Efficacy of treatment can be assayed by measuring the usual indicators of disease progression, including antibody level, viral antigen production, infectious HIV levels, or levels of nonspecific infections.

Such a transcriptional repressor protein may be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are introduced to the system, a mutant with the appropriate properties (*i.e.*, represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector/tat system may be used to assure that surviving cell clones are not caused by endogenous mutations in these genes. A battery of randomly mutagenized tat genes is then introduced into these cells using a "rescuable" alphavirus vector (*i.e.*, one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

Additional inhibitory palliatives that may be used in the gene delivery vehicles described herein include systems wherein expression of the heterologous transgene may be reduced (suppressed) in desired cells, including during the virion packaging process, for example by including a TOP-binding ligand and packaging elements in the vector. As described, for example, in co-owned U.S. Serial Number 09/608,730, these methods

allow for the suppression of transgene translation in virion producing cells while maintaining the capacity for high level expression and translation of the transgene in all other cell types. These systems are also applicable to a variety of viral vectors and, in addition, can be combined with other virion production systems, including, for example, adenovirus virion construction in a cre-lox system.

The following examples are offered by way of illustration, and not by way of limitation.

## EXAMPLE 1
## MATERIALS AND METHODS

*Mice and Cell lines*

Female Balb/c mice were purchased from Charles River Breeding Laboratories and were at the age of 6 to 8 weeks at the onset of the studies. The fibroblast cell line SvBalb ($H\text{-}2^d$) was used as target cells. This cell line expresses class I but not class II MHC molecules and is therefore directed against $CD8^+$ but not $CD4^+$ cells.

*Materials*

p7g is an $H\text{-}2K^d$ restricted $HIV\text{-}1_{SF2}$p24gag CTL epitope and is a synthetic 9 mer peptide: (aa, 199-AMQMLKETI-207).(21) This peptide was synthesized with free amine N termini and free acid C termini using Fmoc solid phase methods by Research Genetics (Huntsville AL) (see, e.g., Mathiowitz et al. *Nature* 386:410-414).

*Immunizations*

Groups of 5 female Balb/C mice were used for each vaccine or immunization route and the tissues were pooled upon sacrifice. The data are presented as representative of 2-4 such data points with similar or identical results. All immunizations were performed 3-4 times at 2-3 week intervals. IN immunizations were performed with $2.5E10^6$ SIN particles in a volume of 25 µl suspended in PBS. IN immunizations were performed without anesthesia. IVAG and IR immunizations with $2.5E10^6$ in a volume of 12.5 µl were performed on anesthetized mice that were kept in

dorsal recumbency for 20 minutes. IM immunizations were performed in thigh muscle with 2.5E10$^6$ SIN particles in a volume of 50 µl The mice were sacrificed one week following the final immunization.

*Sera and Tissue Collection*

Mice were bled through the retro orbital plexus one day prior to sacrifice and the sera separated for ELISA assays. Cervical lymph nodes (CLN), iliac lymph nodes (ILN), vaginal/uteral mucosal tissues (VUM) and spleens (SP) were harvested and pooled from 5 mice per group and single cell suspensions were used for a standard $^{51}$Cr-release CTL assay (except VUM), a gag epitope specific ELISPOT to detect IFN-γ-secreting cells (IFNSC) or gag-p55 specific ELISPOT to detect antibody secreting cells (ASC).

*Preparation of single cell suspensions*

Groups of 5 mice were immunized 3 times as described above through the IN, IM, IR or IVAG routes with 2-3 week intervals. One week following the final immunization SP, CLN and ILN from groups of 5 immunized mice each were harvested and pooled. SP, CLN and ILN tissues were teased through a nylon mesh with pore diameter 250 µm and washed three times in media (ELISPOT assay media: RPMI containing 10% FCS, antibiotics, Hepes and L-glutamin (complete RPMI); $^{51}$Cr-release assay media), counted and seeded into wells for ELISPOT or $^{51}$Cr-release assay. Single cell suspensions from VUM were prepared based on the method described by Holmgren (see, for example, Lycke and Holmgren (1986) *Immunology* 59:301-308) with modifications by removing the entire vagina, uterus and uteral horns from pools of 5 mice per group. The uteral horns were cut longitudinally and together with vaginal and uteral tissues were diced into 5 mm pieces. The tissue pieces were then washed three times in HBSS without Ca++ and Mg++ containing 10% FCS and 5 mM Hepes (complete HBSS). The pieces were then treated enzymatically under agitation at 37°C sequentially once with 1 mg/ml Collagenase/Dispase plus 0.5 mg/ml DNase in complete HBSS for 30 min. and twice with collagenase plus 0.5 mg/ml DNase in complete RPMI for 45 min. Following each enzymatic treatment released cells were recovered and washed twice with complete RPMI. The cell suspensions recovered from

each enzymatic treatment were pooled and counted. This method routinely resulted in recovery of a minimum of $10^7$ viable mononuclear cells (MNC) per five mice.

*$^{51}$Cr-release CTL Assay*

Single cell suspensions from the tissues were prepared as described above and were cultured in a 24-well dish at $5x10^6$ cells per well. Of these cells, $1X10^6$ were sensitized with synthetic p7g peptide (amino acids 194-213) at a concentration of 10 mM for 1 hour at 37°C and then washed and co-cultured with the remaining $4x10^6$ untreated cells. The cells were stimulated as a bulk culture in 2 ml of Splenocyte culture medium: RPMI 1640 with 100 mM L-glutamine (Gibco, Grand Island, New York, USA)/-Mem (Minimum Essential Medium Alpha Medium with L-glutamine, deoxyribonucleosides or ribonucleosides) (1:1) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah, USA), 100 U/ml penicillin, 100 g/ml streptomycin, 10 ml/L of 100 mM sodium pyruvate and 50 M 2-mercaptoethanol. In addition, 5% Rat T-Stim IL2 (Rat T-Stim; Collaborative Biomedical Products, Bedford, Massachusetts, USA) was used as a source of IL2 and was added to the culture media just before the cells were to be cultured.

After a stimulation period of 6 to 7 days, the cells were collected and used as effectors in a $^{51}$Chromium release assay. Approximately $10^6$ SV/Balb target cells were incubated in 200 µl of medium containing 50 Ci of $^{51}$Cr and with the correct peptide p7g, or a mismatched cell-target pair as the negative control at a concentration of 1 M for 60 min and washed. Effector cells were cultured with $5x10^3$ target cells at various effector to target ratios in 200 µl of culture medium in 96-well tissue culture plates (round or v-bottom) for 4 hr. The average cpm from duplicate wells was used to calculate percent specific release as presented here.

*ELISPOT assays*

Single cell suspensions from pooled CLN and SP from 5 mice per group were added onto nitrocellulose or pvdf plates (Milipore) precoated with a monoclonal rat anti-mouse anti-IFN-γ (Pharmingen) and p55 and blocked with complete RPMI medium at pH 7.2, containing 10% fetal calf serum, 5mM Hepes, and antibiotics. For detection of total p55-specific antibody secreting cells (ASC), following overnight

incubation of cells at 37°C, the plates were washed with PBS/0.05% Tween (P/T). Biotinylated goat anti-IgH+L (Southern Biotechnology Associates, Birmingham, Alabama) was added at 1:7000 dilution in PBS/1% normal goat serum and incubated at room temperature (RT) for 2 hours. The plates were washed with P/T and incubated for 1 hr. at 37°C with Avidin-peroxidase at 1:1000 dilution (Pharmingen). The plates were washed with P/T and the spots were visualized by adding DAB in Tris-HCl buffer for 30 minutes. The plates were washed with de-ionized water and air-dried. The spots were counted manually under low magnification from duplicate wells per group and per tissue. The results are presented as ASC per 10 million cells and are representative of at least two experiments with similar results.

For detection of IFN-γ secreting cells following overnight incubation of cells in the presence of gag-derived p7g peptide, or anti-CD3 (Pharmingen) and anti-CD28 (Pharmingen) as positive control for polyclonal T cell activation, or media only as negative control, the plates were washed and biotinylated anti-IFN-γ (Pharmingen) was added in PBS/0.1% BSA/0.02% Tween and incubated at R/T for 2 hours. The plates were washed with P/T and incubated for 1 hr. at 37°C with Avidin-peroxidase (Pharmingen) at 1:1000 dilution. The plates were washed with P/T and the spots were visualized by adding DAB in Tris-HCl buffer for 30 minutes. The plates were washed with de-ionized H2O and air-dried. The spots were counted with an in house developed automated ELISPOT reader using software from Alpha Innotech Corporation (San Leandro, CA).

*ELISA assays*

HIV-1 p55 gag specific serum IgG titers were quantified by a standard ELISA assay. Briefly, ELISA plates (96 well U bottom by Nunc Maxisorp) were coated with p55 protein at 5 μg/well. After washing with 1X PBS + 0.03% Tween 20 (Sigma), the wells were blocked and samples were added in serial dilutions in an assay diluent made up of 1X PBS + 5% goat serum (Gibco Brl) + 0.03% Tween 20 (Sigma). A standard serum was included in each assay for quantitation purposes. The samples and standard sera were incubated at 37°C for one hour and washed with PBS/0.03% Tween. The samples were then incubated with a 1:40000 dilution of a goat anti-mouse IgG-HRP (Caltag) and developed with tetramethylbenzidine (TMB-Kirkegaard and Perry) for 15

minutes and then stopped with 2N HCl. The optical density of each well was measured using Titertek at 450 nm.

EXAMPLE 2

CTL RESPONSES FOLLOWING MUCOSAL VS. SYSTEMIC IMMUNIZATIONS WITH SIN-GAG BEFORE CHALLENGE WITH VACCINIA VIRUS (VV)-GAG

The ability of SIN-gag particles to induce local and systemic gag-specific CTL responses was assessed through various routes of mucosal or systemic immunization. Mice were immunized intranasally (IN) or intramuscularly (IM) with $2.5E10^6$ SIN-gag particles and intravaginally (IVAG) or intrarectally (IR) with $10^7$ SIN-gag particles and sacrificed 7 days following the final immunization. To determine systemic gag-specific CTL responses, single cell suspensions from CLN, ILN, VUM and SP were prepared and a gag-specific IFN-γ. ELISPOT assay was performed. Local gag-specific CTL responses in CLN following IN immunizations were observed. In contrast, no local CTL responses ILN or in VUM, following IVAG and IR immunizations were seen (Figure 1). Moreover, systemic gag-specific CTL responses in SP following IN and IM immunizations were found, but not following IVAG and IR immunizations, (Figure 2). To assess the lytic activity of the CTL responses that were detected as the number of IFN-γ secreting cells by the ELISPOT assay, a standard $^{51}Cr$-release assay on CLN and SP cells following IN immunizations with SIN-gag particles was performed. Local and systemic lytic CTL activity in CLN and SP following IN immunizations with SIN-gag (Figure 3) was observed. Thus, IN or IM, but not IVAG or IR, immunizations with SIN-gag induced local and systemic CTL responses.

EXAMPLE 3

CTL RESPONSES FOLLOWING MUCOSAL VS. SYSTEMIC IMMUNIZATIONS WITH SIN-GAG AFTER CHALLENGE WITH VV-GAG

Local and systemic CTL responses following mucosal and systemic immunizations and local challenge were determined. (see, also, Vajdy et al. (2001) Journal of Infectious Disease 184:1613). Mice were immunized IN or IM with $2.5E10^6$ SIN-gag particles and 1-3 weeks following the final immunization were challenged IR or IVAG with $10^{7-8}$ pfu of Vaccinia virus (VV) expressing HIV-1 p55-gag and

sacrificed 5 days following the final immunization. The following controls were used: a group of naïve mice were challenged IVAG, a group of naïve mice were challenged IR, groups of mice were immunized IM, IN, IR or IVAG with SIN-gag and challenged IVAG with Vaccinia virus expressing HIV-1 envelope gp160 (VV-env).

Following IM or IN immunizations with SIN-gag and vaginal challenge with VV-gag a potent gag-specific CTL responses in local, CLN, and systemic, SP, lymphoid tissues was detected. Importantly, potent CTL responses were also detected in the distant mucosal effector site, VUM as well as in the distant inductive site, ILN as measured by the ELISPOT assay (Figure 4). None of the control groups demonstrated local or systemic CTL responses (Figure 5). Thus, systemic injection in thigh muscle as well as mucosal administration onto the nasal mucosa with SIN-gag followed by vaginal challenge with VV-gag induced potent CTL responses not only in the local inductive site of the nasal mucosa (CLN), but also in the distant inductive (ILN) and effector (VUM) sites of the vaginal mucosa. Moreover, both IN and IM immunizations with SIN-gag followed by vaginal challenge with VV-gag induced potent systemic CTL responses.

To determine local and systemic CTL responses following local immunization and local challenge, mice were immunized IVAG or IR with $10^7$ SIN-gag particles and challenged IVAG with $10^7$ pfu VV-gag. Following IR immunizations with SIN-gag and IVAG challenge with VV-gag, local as well as systemic CTL responses in ILN and SP respectively, were detected, as measured by the IFN-γ ELISPOT assay (Figure 5). In contrast, mice immunized IVAG with SIN-gag and challenged IVAG with VV-gag demonstrated strong CTL responses locally in ILN and VUM but low CTL responses systemically in SP (Figure 5). None of the control groups demonstrated any local or systemic CTL responses. To confirm the lytic activity of the CTL responses detected by the ELISPOT technique, a standard Cr-release assay was performed with ILN and SP isolated from mice immunized IR or IVAG with SIN-gag and challenged IVAG with VV-gag and similar results were obtained. Thus, vaginal and rectal immunizations with SIN-gag particles induce local CTL responses in the mucosal effector and inductive sites. Furthermore, rectal, but not vaginal, immunization with SIN-gag particles followed by IVAG challenge with VV-gag evoked strong CTL responses systemic lymphoid tissue.

EXAMPLE 4

PROTECTION FROM MUCOSAL CHALLENGE WITH VV-GAG

To determine whether the presence of local and systemic CTL responses correlated with mucosal protection from various challenge a gag-expressing vaccinia virus challenge model was used. Vaccinia has been shown to infect and replicate in ovaries following intra-rectal, and various parenteral routes of inoculation. Two or three weeks following IR, IVAG, IN or IM immunizations with SIN-gag all groups of mice were challenged IVAG with $10^7$ pfu of VV-gag. Five days following IVAG challenge with VV-gag, the mice were sacrificed and a pfu assay performed on their ovaries. The following controls were used: naïve mice challenged IVAG, naïve mice challenged IR, groups of mice immunized IM, IN, IR or IVAG with SIN-gag and challenged IVAG with Vaccinia virus expressing HIV-1 envelope gp160 (VV-env).

Mice immunized IVAG or IR with SIN-gag were protected against IVAG and IR challenge with VV-gag respectively, in that no pfu was detected in their ovaries (Figure 6). Moreover, mice immunized IN with SIN-gag and challenged IVAG with VV-gag were only partially protected (Figure 6). Importantly, mice immunized IM and challenged IVAG were not protected (Figure VI). The naïve mice that were challenged with VV-gag IR or IVAG had high numbers of pfu in their ovaries (Figure 6). Moreover, the mice that were immunized IM, IN, IR or IVAG with SIN-gag and challenged IVAG with VV-env also had high pfu titers in their ovaries, demonstrating that the protection was antigen-specific (Figure VI). Importantly, mice immunized IM with SIN-gag and challenged intra-peritoneally with VV-gag were partially protected demonstrating that although IM immunization does not protect against mucosal challenge it does offer some degree of protection against systemic challenge (data not shown). Thus, local/mucosal, but not distant/mucosal or systemic, immunization protected the mice against local/mucosal challenge.

EXAMPLE 5

HIV-1 GAG-SPECIFIC CD8+ T CELL RESPONSES IN THE VAGINAL MUCOSA AND PROTECTION FROM VAGINAL VIRAL CHALLENGE FOLLOWING LOCAL IMMUNIZATIONS WITH SINDBIS VIRUS-BASED REPLICON PARTICLES

Groups of 5 BALB/c mice were used for each vaccine or immunization route and the tissues were pooled upon sacrifice. Mice were immunized by various routes 3

times at 2 week intervals, rested for 2-3 weeks and then challenged intra-vaginally (IVAG) or intra-rectally (IR) with $10^7$ plaque-forming units (PFU) of VV-gag or VV-gp160 (Gardner et al. (2000) J Virol 74:11849-57. SIN-gag particles were prepared as described in Gardner et al. (2000), *supra*. Intra-nasal (IN) immunizations were performed without anesthesia with $2.5x10^6$ SIN-gag particles in a volume of 25 µl suspended in PBS. The SIN-gag particles and the VV-gag virus were applied on anesthetized mice IVAG or IR in a volume of 12.5 µl following which the mice were kept in dorsal recumbency for 20 minutes. Intra-muscular (IM) immunizations were performed in thigh muscle with $2.5x10^6$ SIN particles in a volume of 50 µl. The mice were sacrificed 5 days following VV challenge for tissue collection.

Cervical lymph nodes (CLN), ILN, vaginal/uteral mucosal tissues (VUM) and spleens (SP) were harvested and pooled from 5 mice per group. Single cell suspensions were used for an ELISPOT assay to detect IFN-γ-secreting cells (IFNSC) specific for the p7g peptide derived from HIV-1 gag. Five days following vaginal or rectal challenge with VV-gag SP, CLN and ILN from groups of 5 immunized mice each were harvested and pooled. SP, CLN and ILN tissues were teased through a nylon mesh with the pore diameter of 250 µm and washed three times in media (ELISPOT assay media: RPMI containing 10% FCS, antibiotics, HEPES and L-glutamin (complete RPMI) counted and seeded into wells for ELISPOT assay. Single cell suspensions were prepared from VUM based on the method described by Johansson et al. (1998) *Infect. Immun* 66:514-520. This method routinely resulted in recovery of a minimum of $10^7$, minimum 90% viable, mononuclear cells (MNC) per five mice.

The p7g peptide derived from HIV-1 gag has been shown to be recognized by $CD8^+$ T cells (Doe & Walker (1996) *AIDS* 10:793-94). It was also demonstrated that peptide was only recognized by $CD8^+$ T cells and not by $CD4^+$ T cells, following p7g-specific IFN-γ intracellular cytokine staining of $CD4^+$ and $CD8^+$ cells after DNA immunizations. Single cell suspensions from pooled VUM, ILN, CLN or SP from 5 mice per group were prepared and adjusted to concentrations of $10^7$ to $3\times10^7$ per ml. 100 µl from each cell preparation was added onto the first row of 96 well nitrocellulose or pvdf plates (Milipore) in duplicates and 2-fold serial dilutions were performed. Following overnight incubation at 37°C the plates were washed and biotinylated anti-

IFN-γ (PharMingen) was added. The plates were then incubated at room temperature for 2 hours and washed. The plates were then added Avidin-peroxidase (PharMingen) and incubated for 30 min. at 37°C and washed. The plates were developed with aminoethyl carbazole solution (Sigma) for 30 min. Results from 3 independent experiments are presented as mean (±SD) of IFN-γ secreting cells per 10 million mononuclear cells (MNC) from a minimum of 4 wells from pools of 5 mice per group in duplicates.

**A. Mucosal and systemic CD8+ T cell responses after mucosal or systemic immunizations with SIN-gag followed by vaginal challenge with Vaccinia virus-gag**

Viral challenge following immunization not only enhances the immune response but also allows a correlation to be drawn between the immune response and protection. Therefore, mice were primed with SIN-gag through the IN, IM, IR or IVAG routes and then challenged with VV-gag. The mucosal and systemic CTL responses as well as protection from VV-gag replication in ovaries was measured. The mice immunized IN, IM and IVAG were challenged IVAG, while the mice that were immunized IR were challenged IR or IVAG. Results of mucosal and systemic $CD8^+$ T cell responses in VUM, ILN and SP of mice immunized IN or IM and challenged IVAG as measured by the IFN-γ ELISPOT assay are shown in Figures 11A and 11B, respectively. No $CD8^+$ T cell responses were detectable in CLN of either group. These responses were gag-specific since IVAG or IR challenge of naïve mice with VV-gag, or IVAG or IR challenge of SIN-gag immunized mice with VV-gp160 did not induce $CD8^+$ T cell responses. These data show that vaginal VV challenge of IN or IM immunized mice induced $CD8^+$ T cell responses in VUM and ILN.

After IR immunization with SIN-gag followed by IVAG challenge with VV-gag the highest $CD8^+$ T cell responses were found in ILN and SP, and relatively low responses were found in VUM (Figure 11C). Similarly, after IVAG immunization with SIN-gag followed by IVAG challenge with VV-gag, the highest $CD8^+$ T cell responses were found in ILN and SP, and relatively low responses in VUM (Figure 11D). The $CD8^+$ T cell responses observed after IVAG or IR immunizations and challenge were generally 10-fold lower compared to the $CD8^+$ T cell responses observed after IN and IM immunizations followed by IVAG challenge.

**B. Protection from local viral replication after mucosal immunizations with SIN-gag followed by vaginal challenge with Vaccinia virus-gag**

Protection from vaccinia virus replication in the ovaries of mice primed through various routes following IVAG or IR virus challenge, using a standard PFU assay was also tested. As shown in Figure 12, mice primed IM with SIN-gag were not protected from challenge with IVAG VV-gag. In addition, 5 of the 19 mice primed IN showed no evidence of VV replication in their ovaries. In contrast, following IVAG or IR challenge with VV-gag, mice primed IVAG or IR with SIN-gag were completely protected against IVAG (Figure 12) and IR challenge with VV-gag respectively, in that no vaccinia virus replication was detected in their ovaries. The control, naïve mice had high levels of VV in their ovaries after IR or IVAG challenge with VV-gag (Figure 12). Also, the control mice that were immunized IN, IM, IVAG and IR with SIN-gag and challenged IVAG with VV-gp160 had high levels of VV in their ovaries (Figure 12), demonstrating that the protection was gag-specific. Thus, local mucosal immunization, but not distant mucosal or systemic immunization, with SIN-gag replicon particles conferred maximum protection against vaginal viral challenge.

Thus, mucosal IVAG or IR immunization conferred protection against vaginal viral challenge. In addition, the data indicate that alphavirus based replicons provide an effective mechanism to induce such local protection. Comparison of the distant mucosal (IN), local mucosal (IVAG and IR) and systemic (IM) routes of immunization with SIN-gag replicon particles, followed by local mucosal challenge with VV-gag, demonstrated that the IN and IM immunization routes induced higher gag-specific mucosal $CD8^+$ T cell mediated responses in the vaginal mucosa (VUM), the draining lymph nodes (ILN), and in SP. However, IVAG and IR immunizations conferred maximum protection against vaginal challenge with VV-gag. Furthermore, the responses were gag-specific since immunization with SIN-gag followed by vaginal challenge with VV-gp160 did not induce any gag-specific IFN-γ secreting $CD8^+$ T cell responses, nor had any impact on VV replication in ovaries. IVAG challenge of naïve mice with VV-gag also failed to induce any detectable gag-specific IFN-γ $CD8^+$ T cell responses. Therefore, the induction of IFN-γ $CD8^+$ T cell responses was gag-specific and as a result of adaptive specific responses following SIN-gag immunization. VV-

gag challenge was also not mediated by humoral responses as indicated by the fact that gag-specific serum titers (as measured by ELISA two weeks after mucosal immunizations with SIN-gag replicons) were undetectable anti-gag serum. Thus, the observed protection was a result of gag-specific T cell-mediated responses.

In summary, the results demonstrate that the SIN replicon delivery system can be applied mucosally for the induction of $CD8^+$ T cell responses and protective immunity against HIV. These results have important implications for sexually transmitted pathogens in general as well as for therapeutic gene therapy against cervical, colon, and lung cancers.

## EXAMPLE 6

### IDENTIFICATION OF CELLS EXPRESSING HIV-1 GAG IN LOCAL AND SYSTEMIC LYMPHOID TISSUES

Cells that are involved in the uptake and expression of SIN-gag particles in local and systemic lymphoid tissues following mucosal immunization were identified by immunizing mice IN with a single dose of SIN-gag particles. Frozen sections from nasal associated lymphoid tissue (NALT), CLN and SP were stained with an antibody against gag. At 1 day post immunization many cells expressing gag both locally in NALT (Figure 9) and CLN (Figure 10) as well as systemically in SP (Figure 11) were found, although by far most cells appeared to be localized in SP. To identify the cells that express gag, cells were double strained with CD11b or CD11c as markers for monocyte lineage cells with potential APC activity, and B220 a maker for B cells. Most of the gag-expressing cells co-expressed CD11b, while only few co-expressed CD11c. Importantly, no $B220^+$ cells co-expressed gag and the $CD11b^+$ or $CD11c^+$ gag-expressing cells were in extra-follicular T-cell areas. Thus, at early time points following in immunizations $CD11b^+$ cells are the major cells with APC potential to express gag in both local and systemic lymphoid tissues.

## EXAMPLE 7

### HUMORAL RESPONSES IN SERA FOLLOWING INTRANASAL IMMUNIZATIONS

The ability of SIN replicons to induce antibody production was also determined.

A. HIV-derived antigens

Two weeks following 3 IN immunizations with SIN replicons expressing gag, serum titers were measured by ELISA (Figure 12). Additionally, prime-boost experiments were conducted. First, following 3 IN immunizations with SIN-gag mice were boosted IN three times at 4 weeks intervals with p24 plus LTK63. Additionally, experiments were conducted in which animals were primed with SIN expressing HIV envelope gp140 and boosted with Ogp140 plus the mucosal adjuvant LTR72 and CpG. In these particular cases, low or no levels of antibody titers were observed (Figure 10), indicating that the protection observed following vaginal or rectal immunizations with gag was not antibody-mediated.

B. Influenza-derived antigens

Two weeks following 1 or more mucosal (*e.g.*, IN, IVAG, IR) immunizations with SIN replicons expressing influenza polypeptide(s) (HA), serum titers are measured by ELISA. Additionally, prime-boost experiments are conducted. First, following mucosal immunizations with SIN-HA mice are boosted mucosally one or more times with HA plus LTK63. Additionally, experiments are conducted in which animals were primed with SIN expressing HA antigens and boosted with HA polypeptides plus the mucosal adjuvant LTR72 and CpG. SIN-HA replicons will induce antibody production when administered mucosally.

## EXAMPLE 8

## INDUCTION OF IMMUNE RESPONSES FOLLOWING MUCOSAL DELIVERY OF ALPHAVIRUS REPLICON PARTICLE CHIMERAS

To demonstrate the ability of alphavirus replicon particle chimeras to induce antigen specific immune responses following mucosal delivery, intranasal immunization experiments similar to those described above were performed. Specifically, replicon particle chimeras between SIN and VEE, as described in U.S. Serial Number 60/295,451, were constructed such that SIN replicon RNA was packaged within VEE envelope glycoproteins (SIN/VEE) or VEE replicon RNA was packaged

within SIN envelope glycoproteins (VEE/SIN). These replicon particles encoding HIV p55 gag antigen, as well as SIN replicon particles and VEE replicon particles also encoding the same HIV gag antigen were used to immunize mice intranasally.

The replicon particles were administered three times, at a dose of $2.5 \times 10^6$ particles in 25 ul, with an immunization schedule of days 0,14, and 28. At two weeks following the final immunization, spleens were removed and gag-specific cellular responses were determined by IFN-γ ELISPOT assay. As shown in Figure 13, each of the replicon particle preparations induced gag-specific responses, with some variation in immunogenicity.

Claims

What is claimed is:

1. A method of generating an immune response in a subject, comprising mucosally administering to target cells a first replication-defective gene delivery vehicle comprising a polynucleotide encoding at least one first antigen or modified form thereof, said first antigen or modified form thereof being capable of stimulating an immune response in the subject when administered mucosally.

2. The method of claim 1, wherein the mucosal administration is intranasal.

3. The method of claim 1, wherein the mucosal administration is intrarectal.

4. The method of claim 1, wherein the mucosal administration is intravaginal.

5. The method of any of claims 1 to 4, wherein the at least one antigen is derived from a sexually transmitted pathogen.

6. The method of claim 5, wherein the sexually transmitted pathogen is a bacteria.

7. The method of claim 6, wherein the bacteria is selected from the group consisting of gonorrhea, chlamydia and syphilis.

8. The method of claim 5, wherein the sexually transmitted pathogen is a virus.

9. The method of claim 8, wherein the virus is selected from the group consisting of HIV, HBV, HSV, HCV and HPV.

10. The method of claim 9, wherein the virus is HIV-1.

11. The method of any of claims 1 to 10, wherein the gene delivery vehicle is a nonviral vector.

12. The method of any of claims 1 to 10, wherein said polynucleotide is delivered using a particulate carrier.

13. The method of claim 12, wherein said polynucleotide is coated on a gold or tungsten particle and said coated particle is delivered to said subject using a gene gun.

14. The method of claim 12, wherein said polynucleotide is encapsulated in a liposome preparation.

15. The method of any of claims 1 to 10, wherein said gene delivery vehicle is a viral vector.

16. The method of claim 15, wherein said viral vector is a retroviral vector.

17. The method of claim 15, wherein said viral vector is an adenoviral vector.

18. The method of claim 15, wherein said viral vector is a poxvirus vector.

19. The method of claim 15, wherein said viral vector is a picornavirus vector.

20. The method of claim 15, wherein said viral vector is an alphavirus vector.

21. The method of claim 20, wherein said alphavirus vector is a Sindbis vector.

22. The method of claim 20, wherein said alphavirus vector is selected from the group consisting of Semliki Forest virus, Venezuelan equine encephalitis virus and Ross River virus vector.

23. The method of claim 20, wherein said alphavirus vector comprises sequences from two or more alphaviruses.

24. The method of any of claims 1 to 23, wherein the subject is a mammal.

25. The method of claim 24, wherein the mammal is a human.

26. The method of claim 20, wherein the alphavirus vector is delivered to antigen presenting cells.

27. The method of claim 26, wherein the antigen presenting cells are dendritic cells.

28. The method of claim 27, wherein the dendritic cells are human.

29. The method of any of claims 1 to 28, wherein the target cells are infected *in vivo*.

30. The method of any of claims 1 to 29, wherein the antigen elicits an HLA class I-restricted immune response.

31. The method of claim 30, wherein the antigen further elicits an HLA Class II-restricted immune response.

32. The method of any of claims 1 to 31, including, prior or subsequent to the step of administering to target cells, introducing into target cells a nucleic acid molecule which encodes either Class I or Class II MHC protein, or combinations thereof, or a protein selected from the group consisting of CD3, ICAM-1, LFA-3 or analogues thereof.

33. The method of any of claims 1 to 32, further comprising the step of administering at least one second gene delivery vehicle, said second gene delivery vehicle comprising polynucleotides encoding at least one second antigen or modified form thereof or an immunomodulatory factor.

34. The method of claim 33, wherein the second gene delivery vehicle is administered mucosally.

35. The method of claim 33, wherein the second gene delivery vehicle is administered non-mucosally.

36. The method of any of claims 1 to 35, further comprising the step of administering one or polypeptides to the subject.

37. The method of claim 36, wherein the polypeptides comprise at least one second antigen or modified form thereof.

38. The method of claim 36, wherein the polypeptides comprise an immunomodulatory factor.

39. The method of claim 36, wherein at least one of the polypeptides is administered mucosally.

40. The method of claim 36, wherein at least one of the polypeptides is administered non-mucosally.

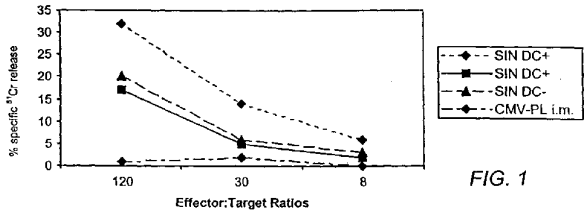

35
30
25
20
15
10
5
0
% specific $^{51}$Cr release
120
30
8
Effector:Target Ratios
SIN DC+
SIN DC+
SIN DC-
CMV-PL i.m.

FIG. 1

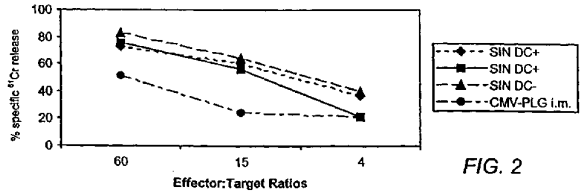

100
80
60
40
20
0
% specific $^{51}$Cr release
60
15
4
Effector:Target Ratios
SIN DC+
SIN DC+
SIN DC-
CMV-PLG i.m.

FIG. 2

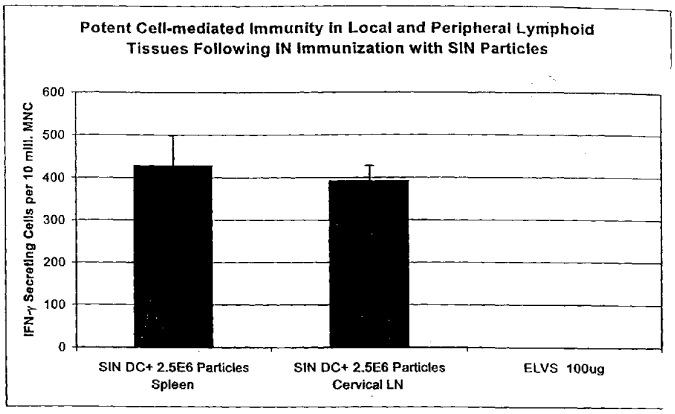

Potent Cell-mediated Immunity in Local and Peripheral Lymphoid Tissues Following IN Immunization with SIN Particles
IFN-γ Secreting Cells per 10 mill. MNC
600
500
400
300
200
100
0
SIN DC+ 2.5E6 Particles Spleen
SIN DC+ 2.5E6 Particles Cervical LN
ELVS 100ug

*FIG. 3*

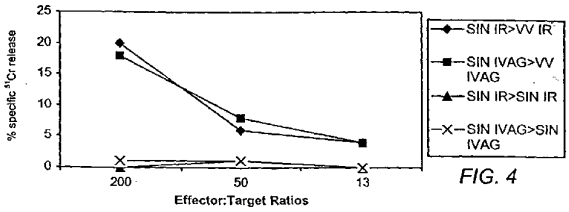

25
20
15
10
5
0
% specific $^{51}$Cr release
200
50
13
Effector:Target Ratios
SIN IR>VV IR
SIN IVAG>VV IVAG
SIN IR>SIN IR
SIN IVAG>SIN IVAG

FIG. 4

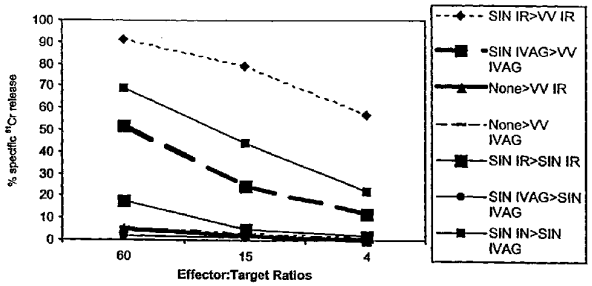

100
90
80
70
60
50
40
30
20
10
0
% specific $^{51}$Cr release
60
15
4
Effector:Target Ratios
SIN IR>VV IR
SIN IVAG>VV IVAG
None>VV IR
None>VV IVAG
SIN IR>SIN IR
SIN IVAG>SIN IVAG
SIN IN>SIN IVAG

FIG. 5

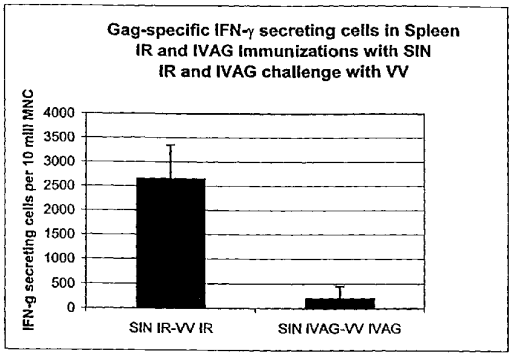

Gag-specific IFN-γ secreting cells in Spleen
IR and IVAG Immunizations with SIN
IR and IVAG challenge with VV
IFN-g secreting cells per 10 mill MNC
4000
3500
3000
2500
2000
1500
1000
500
0
SIN IR-VV IR
SIN IVAG-VV IVAG

*FIG. 6*

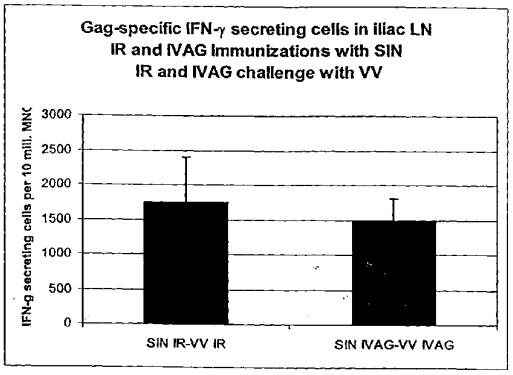

Gag-specific IFN-γ secreting cells in iliac LN
IR and IVAG immunizations with SIN
IR and IVAG challenge with VV
IFN-g secreting cells per 10 mill. MNC
3000
2500
2000
1500
1000
500
0
SIN IR-VV IR
SIN IVAG-VV IVAG

FIG. 7

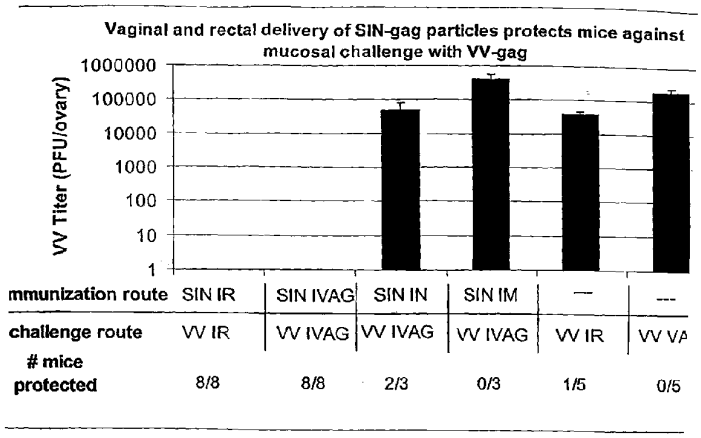

Vaginal and rectal delivery of SIN-gag particles protects mice against mucosal challenge with VV-gag
VV Titer (PFU/ovary)
1000000
100000
10000
1000
100
10
1
mmunization route
SIN IR
SIN IVAG
SIN IN
SIN IM
—
---
challenge route
VV IR
VV IVAG
VV IVAG
VV IVAG
VV IR
VV VA
# mice protected
8/8
8/8
2/3
0/3
1/5
0/5

FIG. 8

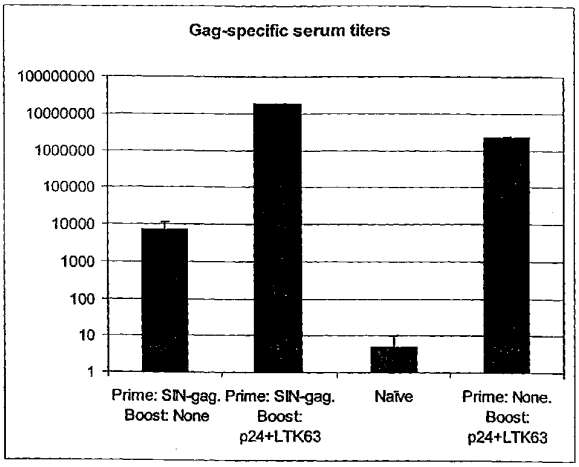

Gag-specific serum titers
100000000
10000000
1000000
100000
10000
1000
100
10
1
Prime: SIN-gag. Boost: None
Prime: SIN-gag. Boost: p24+LTK63
Naïve
Prime: None. Boost: p24+LTK63

*FIG. 9*

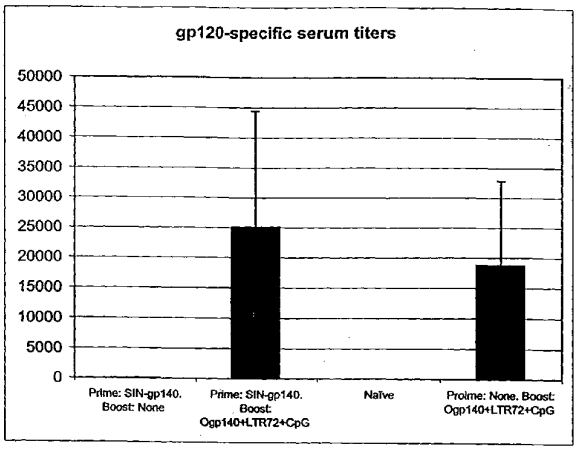

gp120-specific serum titers
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Prime: SIN-gp140. Boost: None
Prime: SIN-gp140. Boost: Ogp140+LTR72+CpG
Naïve
Proime: None. Boost: Ogp140+LTR72+CpG

*FIG. 10*

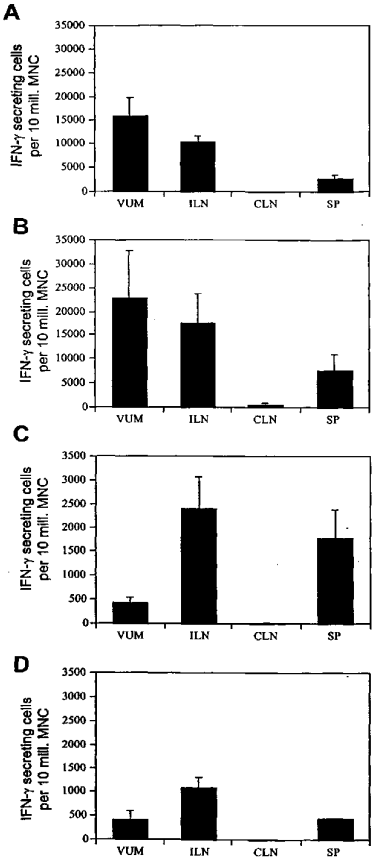

A
IFN-γ secreting cells per 10 mill. MNC
35000
30000
25000
20000
15000
10000
5000
0
VUM
ILN
CLN
SP
B
IFN-γ secreting cells per 10 mill. MNC
35000
30000
25000
20000
15000
10000
5000
0
VUM
ILN
CLN
SP
C
IFN-γ secreting cells per 10 mill. MNC
3500
3000
2500
2000
1500
1000
500
0
VUM
ILN
CLN
SP
D
IFN-γ secreting cells per 10 mill. MNC
3500
3000
2500
2000
1500
1000
500
0
VUM
ILN
CLN
SP

Figure 11

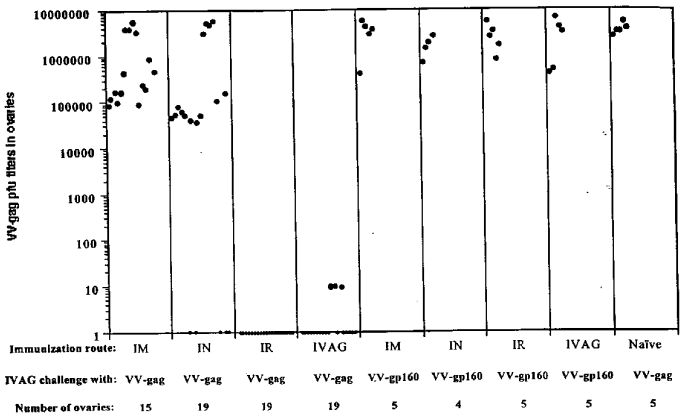
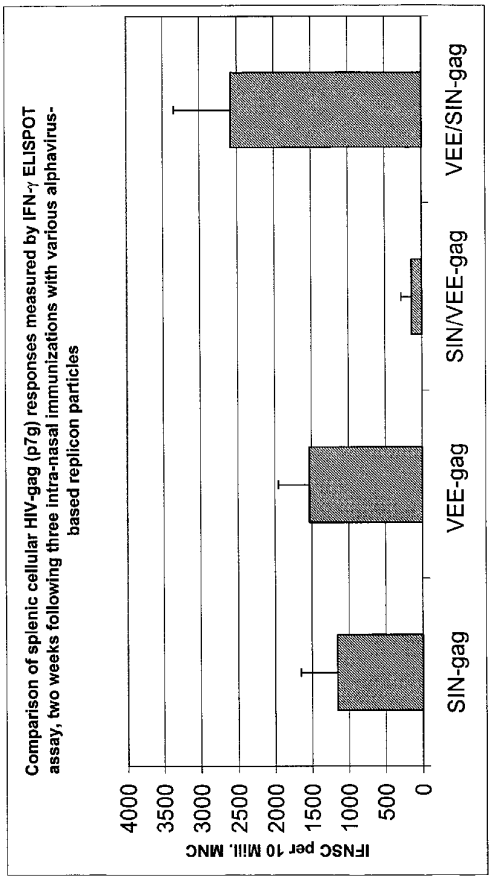

VV-gag pfu titers in ovaries
10000000
1000000
100000
10000
1000
100
10
1
Immunization route: IM IN IR IVAG IM IN IR IVAG Naïve
IVAG challenge with: VV-gag VV-gag VV-gag VV-gag VV-gp160 VV-gp160 VV-gp160 VV-gp160 VV-gag
Number of ovaries: 15 19 19 19 5 4 5 5 5

Figure 12

FIGURE 13


Comparison of splenic cellular HIV-gag (p7g) responses measured by IFN-γ ELISPOT assay, two weeks following three intra-nasal immunizations with various alphavirus-based replicon particles
IFNSC per 10 Mill. MNC
4000
3500
3000
2500
2000
1500
1000
500
0
SIN-gag
VEE-gag
SIN/VEE-gag
VEE/SIN-gag

【国際公開パンフレット（コレクトバージョン）】

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(54) Title: NUCLEIC ACID MUCOSAL IMMUNIZATION

(57) Abstract: Mucosal delivery of antigens using, for example, a replication-defective gene delivery vehicle, particularly replication-defective alphavirus vectors and particles, is described. Also described are compositions comprising a mucosal adjuvant and one or more antigens derived from HIV. Also provided is the use of these gene delivery vehicles in inducing mucosal, local and/or systemic immune responses following mucosal immunization regimes.

# NUCLEIC ACID MUCOSAL IMMUNIZATION

Technical Field

The present invention relates generally to mucosal immunization, for example, mucosal immunization using gene delivery systems. In particular to mucosal delivery of replication-defective vectors and/or recombinant alphavirus vectors and particles. Further, use of these systems for inducing potent mucosal, local and systemic immune responses following various routes of mucosal immunization is also described.

Background of the Invention

Development of vaccines which invoke mucosal immunity against various pathogens would be desirable. Many disease-causing pathogens are transmitted through mucosal surfaces. For example, acquired immune deficiency syndrome (AIDS) is recognized as one of the greatest health threats facing modern medicine and worldwide sexual transmission of HIV is the leading cause of AIDS. There are, as yet, no cures or vaccines for AIDS.

In 1983-1984, three groups independently identified the suspected etiological agent of AIDS. See, e.g., Barre-Sinoussi et al. (1983) Science 220:868-871; Montagnier et al., in Human T-Cell Leukemia Viruses (Gallo, Essex & Gross, eds., 1984); Vilmer et al. (1984) The Lancet 1:753; Popovic et al. (1984) Science 224:497-500; Levy et al. (1984) Science 225:840-842. These isolates were variously called lymphadenopathy-associated virus (LAV), human T-cell lymphotropic virus type III (HTLV-III), or AIDS-associated retrovirus (ARV). All of these isolates are strains of the same virus, and were later collectively named Human Immunodeficiency Virus (HIV). With the isolation of a related AIDS-causing virus, the strains originally called HIV are now termed HIV-1 and the related virus is called HIV-2 See, e.g., Guyader et al. (1987) Nature 326:662-669; Brun-Vezinet et al. (1986) Science 233:343-346; Clavel et al. (1986) Nature 324:691-695. Consequently, there is a need in the art for compositions and methods suitable for treating and/or preventing HIV infection worldwide.

A great deal of information has been gathered about the HIV virus, and several targets for vaccine development have been examined including the *env*, *Gag*, *pol* and *tat* gene products encoded by HIV. Immunization with native and synthetic HIV-encoding polynucleotides has also been described, as described for example, in co-owned PCT/US99/31245 and references cited therein. In addition, polynucleotides encoding HIV have been administered in various attempts to identify a vaccine. (*See, e.g.*, Bagarazzi et al. (1999) *J. Infect. Dis.* 180:1351-1355; Wang et al. (1997) *Vaccine* 15:821-825). A replication-competent Venezuelan equine encephalitis (VEE) alphavirus vector carrying the matrix/capsid domain of HIV could elicit CTL responses has been administered subcutaneously in animals (Caley et al. (1997) *J. Virol.* 71:3031-3038). In addition, alphavirus vectors derived from Sindbis virus has also been shown to elicit HIV gag-specific responses in animals (Gardner et al. (2000) *J. Virol.* 74:11849-11857). Similarly, HIV peptides have also been administered to animal subjects. (Staats et al. (1997) *AIDS Res Hum Retroviruses* 13:945-952; Belyakov (1998) *J.Clin.Invest.* 102: 2072).

Recombinant alphavirus vectors and layered eukaryotic vector systems based on alphaviruses have also been described. (See, e.g., U.S. Patent Nos. 6,015,686; 6,015,694; 5,843,723). Hariharan et al. (1998) *J. Virol.* 72:950-958 reported that a single intramuscular immunization with pSIN vectors expressing the glycoprotein B of herpes simplex virus (HSV) type 1 induced a broad spectrum of immune responses, including virus-specific antibodies, cytotoxic T cells, and protection from lethal virus challenge in two different murine models. Polo et al. (1999) *Proc. Natl. Acad. Sci. USA* 96:4598-4603 reported similar protection in HSV challenge models following immunization with SIN replicon vectors particles rather than pSIN plasmid vectors. Tsuji et al. (1998) *J. Virol.* 72:690-697 reported that subcutaneous administration in mice of recombinant SIN expressing a class I major histocompatibility complex-restricted 9-mer epitope of the *Plasmodium yoelii* circumsporozoite protein or the nucleoprotein of influenza virus induces a large epitope-specific CD8(+) T-cell response and provides a high degree of protection against infection with malaria or influenza A virus.

However, despite these and other studies, the utility of replication-defective gene delivery vehicles for mucosal immunization strategies that can protect against

mucosal challenge has not been sufficiently defined. Thus, there remains a need for compositions and methods directed to treatment and prevention of various sexually transmitted pathogens.

Summary of the Invention

Disclosed herein are gene delivery systems (*e.g.*, recombinant alphavirus vectors) which are suitable for use in a variety of applications, including for example, mucosal immunization, and further provides other related advantages. Briefly stated, the invention includes vector constructs and particles expressing antigens associated with one or more sexually transmitted disease pathogens, as well as methods of making and utilizing the same, particularly in protective mucosal immunization regimes. Preferably, the vectors are replication-defective, for example alphavirus vectors such as those derived from Sindbis. The present inventors have demonstrated that antigen-specific protection against post-immunization challenge can be induced following mucosal administration of gene delivery vehicles (*e.g.*, alphavirus vectors) expressing the antigen.

In one aspect, the invention includes a method of generating an immune response against an antigen. In certain embodiments, the method comprises mucosally administering to target cells of a subject, a replication-defective gene delivery vehicle (or vector) comprising a polynucleotide encoding at least one antigen (or modified form thereof), wherein the antigen (or modified form thereof) is capable of stimulating an immune response in the subject. In certain embodiments, the target cells are in mucosal, local and/or systemic tissues. Mucosal administration can be, for example, intranasal, oral, intrarectal, and/or intravaginal administration. Preferably, for sexually transmitted pathogens, mucosal administration is by the intrarectal or intravaginal route. In certain embodiments, at least one antigen is derived, for example, from a sexually transmitted pathogen such as bacterial pathogen (*e.g.*, gonorrhea, chlamydia and syphilis) or a viral pathogen (*e.g.*, HIV, HBV, HSV, HCV and HPV). In certain embodiments, the antigen(s) elicit(s) an HLA class I-restricted immune response and, optionally, also elicits an HLA Class II-restricted immune response.

In other aspects, the methods include delivery of genes encoding immune-enhancing cytokines, lymphokines, chemokines and the like. These genes can be inserted into the same gene delivery vehicle carrying the antigen(s) of interest (*e.g.*, alphavirus replicon particle) or can be carried on one or more different gene delivery vehicles. In certain embodiments, the antigen(s) elicit(s) an HLA class I-restricted immune response and, optionally, also elicits an HLA Class II-restricted immune response.

The gene delivery vehicle (or vector) can be, for example, a nonviral vector; a particulate carrier (*e.g.*, gold or tungsten particles coated with the polynucleotide and delivered using a gene gun); a liposome preparation; viral vector; a retroviral vector; or an alphavirus-derived vector. In certain aspects, an alphavirus-derived vector, for example a vector derived from Sindbis virus, Semliki Forest virus, Venezuelan Equine Encephalitis virus, Ross River virus or vector chimeras derived from any number of different alphaviruses (*e.g.*, SIN-VEE chimeras) are used. Any of the gene delivery vectors described herein (*e.g.*, alphavirus vector) can be delivered, for example, to antigen presenting cells (APCs) such as dendritic cells. In certain embodiments, the subject and/or the cells is a mammal, for example a human. In any of the methods described herein, the target cells can be infected *in vivo*. Further, in any of the methods described herein, prior or subsequent to the step of administering to target cells, a nucleic acid molecule which encodes either Class I or Class II MHC protein, or combinations thereof, or a protein selected from the group consisting of CD3, ICAM-1, LFA-3 or analogues thereof can also be administered to the target cells. Furthermore, the above-described gene delivery vehicle for mucosal vaccination may be used in combination with one or more additional immunogenic compositions (gene delivery vehicle, polypeptide, protein, chemokine, cytokine, etc.) in which the one or more additional compositions are delivered by a mucosal or non-mucosal route(s).

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.). These references are incorporated herein by reference in their entirety.

Brief Description of the Drawings

Figure 1 is a graph depicting local HIV-1 gag-specific CTL responses in cervical lymph nodes following intranasal immunization with gag-expressing SIN replicon particles as measured in a $^{51}Cr$ release assay. "--♦--" depicts a first group of SIN DC+ animals; "—■—" depicts a second group of SIN DC+ animals; "--□--" depicts SIN DC-; and "— -♦- —" depicts a control plasmid (with CMV promoter) delivered intramuscularly.

Figure 2 is a graph depicting systemic gag-specific CTL responses in spleen following intranasal administration with SIN replicon particles as measured in a $^{51}Cr$ release assay. "--♦--" depicts a first group of SIN DC+ animals; "—■—" depicts a second group of SIN DC+ animals; "--□--" depicts SIN DC- animals; and "— -●- —" depicts a control plasmid (with CMV promoter) delivered intramuscularly.

Figure 3 is a graph depicting number of INF-γ secreting cells in local and peripheral lymphoid tissue following intranasal immunization with SIN particles.

Figure 4 is a graph depicting local HIV-1 gag-specific CTL responses in iliac lymph nodes draining the rectal and vaginal mucosa following intrarectal (IR) or intra-vaginal (IVAG) administration of SIN replicon particles, as measured in a $^{51}Cr$ release assay. "--♦--" depicts responses in a first group after intrarectal administration of SIN followed by intrarectal (IR) challenge with vaccinia virus (VV); "—■—" depicts intravaginal administration of SIN followed by intravaginal challenge with VV; "—▲—" depicts responses in a second group following IR administration of SIN followed by challenge with VV; and "—X—" depicts a control plasmid (with CMV promoter) delivered intramuscularly.

Figure 5 is a graph depicting systemic HIV-1 gag-specific CTL responses in spleen following intra-rectal (IR) or intra-vaginal (IVAG) administration of SIN replicon particles, as measured in a $^{51}Cr$ release assay. "--♦--" depicts responses in a first group of animals after intrarectal administration of SIN replicons followed by intrarectal challenge with vaccinia virus (VV); "▬■▬" depicts intravaginal administration of SIN followed by intravaginal challenge with VV; "—▬—" depicts animals challenged intravaginally with VV with no immunization; "—■—" depicts

responses in animals after intrarectal administration of SIN replicons followed by intrarectal administration of SIN replicons; "—●—" depicts responses in animals after intravaginal administration of SIN replicons followed by intravaginal administration of SIN replicons; "—■—" depicts responses in animals after intranasal administration of SIN replicons followed by intravaginal administration of SIN replicons;

Figure 6 is a graph depicting IFN-γ secreting cells in spleen tissue following intrarectal (IR) and intravaginal (IVAG) immunization with SIN particles and IR and IVAG challenge with vaccinia virus (VV).

Figure 7 is a graph depicting IFN-γ secreting cells in iliac lymph nodes following intrarectal (IR) and intravaginal (IVAG) immunization with SIN particles and IR and IVAG challenge with vaccinia virus (VV).

Figure 8 is a graph depicting vaccinia virus (VV) titer following vaginal and rectal delivery of SIN-gag particles.

Figure 9 is a graph depicting gag-specific serum titers. From left to right, the bars show titers in animals: primed with SIN-gag and no boost; primed with SIN-gag and boosted with p24 polypeptide and LTK63 adjuvant; naïve animals; and no prime but subsequent boost with p24 and LTK63 adjuvant.

Figure 10 is a graph depicting gp120-specific serum titers. From left to right, the bars show titers in animals: primed with SIN-gp140 and no boost; primed with SIN-gag and boosted with gp-140 polypeptide and LTK72 adjuvant and CpG; naïve animals; and no prime but subsequent boost with gp-140 polypeptide and LTK72 adjuvant and CpG.

Figure 11, panels A to D, are graphs depicting Induction of local and systemic cellular immune responses after IN (A), IM (B), IR (C) and IVAG (D) immunizations with SIN-gag particles followed by IVAG challenge with VV-gag. IN and IM immunizations induced several fold higher numbers of gag-specific IFN-γ secreting cells in VUM and ILN compared to IR and IVAG immunizations. The mice were immunized 3 times IN or IM with $2.5 \times 10^6$ SIN-gag particles and IR or IVAG with $10^7$ SIN-gag particles, three weeks later they were challenged vaginally with $10^7$ pfu of VV-gag, and sacrificed 5 days later. The results are shown as average number of p7g-

specific IFN-γ secreting cells per 10 million mononuclear cells (MNC) of three independent experiments ± SD.

Figure 12 depicts Protection in ovaries from vaginal challenge with VV-gag following vaginal or rectal immunizations with SIN-gag particles. The mice were immunized 3 times IN or IM with $2.5x10^6$ SIN-gag particles and IR or IVAG with $10^7$ SIN-gag particles, three weeks later they were challenged vaginally with $10^7$ pfu of VV-gag, and sacrificed 5 days later. Ovaries were collected and standard pfu assay for determination of VV titers was performed. Each dot represents the numbers of plaque forming units per ovary/each mouse. As control naïve mice were challenged with VV-gag and mice immunized with SIN-gag particles were challenged with VV-gp160 and in both cases high pfu titers were evident in the ovaries.

Figure 13 is a graph depicting the induction of an immunological response (as measured by IFN-γ ELISPOT assay) following intranasal immunization with alphavirus vector particles. Actual numbers represented by the bar graph are as follows: SIN-gag replicon particles gave 1154 (± 499); VEE-gag replicon particles 1530 (± 425); SIN/VEE-gag 140 (± 140); and VEE/SIN-gag 2586 (± 762).

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Short Protocols in Molecular Biology*, 4th ed. (Ausubel et al. eds., 1999, John Wiley & Sons); *Molecular Biology Techniques: An Intensive Laboratory Course*, (Ream et al., eds., 1998, Academic Press); *PCR (Introduction to Biotechniques Series)*, 2nd ed. (Newton & Graham eds., 1997, Springer Verlag); Peters and Dalrymple, *Fields Virology* (2d ed), Fields et al. (eds.), B.N. Raven Press, New York, NY.

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of two or more such agents.

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Gene transfer" or "gene delivery" refers to methods or systems for reliably inserting DNA of interest into a host cell. Such methods can result in transient expression of non-integrated transferred DNA, extrachromosomal replication and expression of transferred replicons (e.g., episomes), or integration of transferred genetic material into the genomic DNA of host cells. Gene delivery expression vectors include, but are not limited to, vectors derived from alphaviruses, pox viruses and vaccinia viruses. When used for immunization, such gene delivery expression vectors may be referred to as vaccines or vaccine vectors.

The terms "Replication-defective" and "replication-incompetent" are used interchangeably to refer to a gene-delivery vehicle such as a viral vector, that does not make or propogate additional infectious, viral particles after being administered to a target cell. As will be apparent to those of skill in the art, the polynucleotides (*e.g.*, RNA) contained in the administered vector or particles may amplify or replicate, however, new progeny vector or viral particles are not formed and do not spread from cell to cell after administration.

"Alphavirus vector construct" refers to an assembly that is capable of directing the expression of a sequence(s) or gene(s) of interest. As described, for example, in U.S. Patent No. 6,015,695; U.S. Patent No. 6,015,686; U.S. Patent No. 5,842,723, and WO 97/38087, the vector construct should include a 5' sequence which is capable of initiating transcription of an alphavirus, as well as sequence(s) which, when expressed, code for biologically active alphavirus non-structural proteins (*e.g.*, NSP1, NSP2, NSP3, and NSP4), and an alphavirus RNA polymerase recognition sequence. In addition, the vector construct should include a viral junction promoter region that may, in certain embodiments, be modified in order to prevent, increase, or reduce viral

transcription of the subgenomic fragment. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable viral vector particles, a 5' promoter which is capable of initiating the synthesis of viral RNA *in vitro* or *in vivo* from cDNA, as well as one or more restriction sites, means for expressing multiple antigens (*e.g.*, IRES element) and a polyadenylation sequence. Any of the sequences making up the alphavirus vector construct may be derived from one or more alphaviruses. As an RNA molecule, the alphavirus vector construct may also be referred to as an "RNA replicon."

"Structural protein expression cassette" refers to a recombinantly produced molecule that is capable of expressing alphavirus structural protein(s). The expression cassette must include a promoter and a sequence encoding alphavirus structural protein(s). Optionally, the expression cassette may include transcription termination, splice recognition, and polyadenylation addition sites. Preferred promoters include the CMV and adenovirus VA1RNA promoters, as well as alphavirus subgenomic junction region promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, hygromycin, phleomycin, histidinol, and DHFR.

"Recombinant alphavirus particle" refers to a capsid that contains an alphavirus vector construct. Preferably, the alphavirus capsid is contained within a lipid bilayer, such as a cell membrane, in which viral encoded proteins (*e.g.*, envelope proteins) are embedded. A variety of vectors may be contained within the alphavirus particle, including the alphavirus vector constructs of the present invention. In addition, the recombinant alphavirus particle may be a chimera, containing elements from any number of different alphaviruses (*e.g.*, RNA vector construct from SIN with capsid and/or envelope proteins from VEE). (See, also co-owned U.S. Patent No. 6,329,201).

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native

sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the proteins or errors due to PCR amplification.

An "antigen" refers to a molecule containing one or more epitopes (either linear, conformational or both) that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is used interchangeably with the term "immunogen." Normally, a B-cell epitope will include at least about 5 amino acids but can be as small as 3-4 amino acids. A T-cell epitope, such as a CTL epitope, will include at least about 7-9 amino acids, and a helper T-cell epitope at least about 12-20 amino acids. Normally, an epitope will include between about 7 and 15 amino acids, such as, 9, 10, 12 or 15 amino acids. The term "antigen" denotes both subunit antigens, (i.e., antigens which are separate and discrete from a whole organism with which the antigen is associated in nature), as well as, killed, attenuated or inactivated bacteria, viruses, fungi, parasites or other microbes. Antibodies such as anti-idiotype antibodies, or fragments thereof, and synthetic peptide mimotopes, which can mimic an antigen or antigenic determinant, are also captured under the definition of antigen as used herein. Similarly, an oligonucleotide or polynucleotide that expresses an antigen or antigenic determinant *in vivo*, such as in gene therapy and DNA immunization applications, is also included in the definition of antigen herein.

For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi, as described more fully below. The term also intends any of the various tumor antigens. Preferably, the antigens are derived from a sexually transmitted pathogen, for example a virus or a bacteria. Furthermore, for purposes of the present invention, an "antigen" refers to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the ability to elicit an immunological response, as defined herein. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts that produce the antigens.

An "immunological response" to an antigen or composition is the development in a subject of a humoral and/or a cellular immune response to an antigen present in the composition of interest. For purposes of the present invention, a "humoral immune

response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells. In addition, a chemokine response may be induced by various white blood or endothelial cells in response to an administered antigen.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376. Recent methods of measuring cell-mediated immune response include measurement of intracellular cytokines or cytokine secretion by T-cell populations (*e.g.*, by ELISPOT technique), or by measurement of epitope specific T-cells (e.g., by the tetramer technique)(reviewed by McMichael, A.J., and O'Callaghan, C.A., *J. Exp. Med.* 187(9):1367-1371, 1998; Mcheyzer-Williams, M.G., et al, *Immunol. Rev.* 150:5-21, 1996; Lalvani, A., et al, *J. Exp. Med.* 186:859-865, 1997).

Thus, an immunological response as used herein may be one that stimulates the production of CTLs, and/or the production or activation of helper T- cells. The production of chemokines and/or cytokines may also be stimulated. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor, cytotoxic, or helper T-cells and/or γδ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

An "immunogenic composition" is a composition that comprises an antigenic molecule where administration of the composition to a subject results in the development in the subject of a humoral and/or a cellular immune response to the antigenic molecule of interest. The immunogenic composition can be introduced directly into a recipient subject, such as by injection, inhalation, oral, intranasal or any other mucosal (*e.g.*, intra-rectally or intra-vaginally) route of administration.

By "subunit vaccine" is meant a vaccine composition that includes one or more selected antigens but not all antigens, derived from or homologous to, an antigen from a pathogen of interest such as from a virus, bacterium, parasite or fungus. Such a composition is substantially free of intact pathogen cells or pathogenic particles, or the lysate of such cells or particles. Thus, a "subunit vaccine" can be prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or analogs thereof. The method of obtaining an antigen included in the subunit vaccine can thus include standard purification techniques, recombinant production, or synthetic production.

An "immuno-modulatory factor" refers to a molecule, for example a protein that is capable of modulating an immune response. Non-limiting examples of immunomodulatory factors include lymphokines (also known as cytokines), such as IL-6, TGF-β, IL-1, IL-2, IL-3, etc.); and chemokines (*e.g.*, secreted proteins such as macrophage inhibiting factor). Certain cytokines, for example TRANCE, flt-3L, and a

secreted form of CD40L are capable of enhancing the immunostimulatory capacity of APCs. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1α), interleukin-11 (IL-11), MIP-1γ, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO), CD40 ligand (CD40L), tumor necrosis factor-related activation-induced cytokine (TRANCE) and flt3 ligand (flt-3L). Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). The sequences of many of these molecules are also available, for example, from the GenBank database. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or mutants thereof) and nucleic acid encoding these molecules are intended to be used within the spirit and scope of the invention.

By "subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The system described above is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual in a formulation or composition without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

A. GENE DELIVERY SYSTEMS

1. Alphavirus Vectors and Particles

As noted above, the present invention provides alphavirus vector constructs, alphavirus particles containing such constructs, as well as methods for making and utilizing such vector constructs and particles in mucosal immunizations. The use of alphavirus based, replication-defective replicon particles, as a mucosal vaccine delivery system provides a number of advantages. Perhaps the most important aspect of immunization with a replication-defective virus vector is the control of gene product at the site of immunization. Immunization with replicating vectors, including the reported live, attenuated alphavirus Venezuelan equine encephalitis-based (VEE) vectors (Caley et al. (1997) *J. Virol.* 71:3031-3038; Davis et al. (1996) *J. Virol.* 70:3781-3787), results in the dissemination of the vector throughout the host. In contrast, immunization with a replication-defective vector results in the local expression, without the spread of newly propagated progeny vector. Moreover, the deficiency in replication reduces the risk of unwarranted inflammatory responses at sites of immunization (Villacres (2000) *Virology*, 270:54).

Alphavirus vectors suitable for use in the present invention can be constructed by any means, for example, as described in U.S. Patent Nos: 6,015,686; 6,015,694; 5,789,245 and 5,842,723. Briefly, sequences encoding alphavirus suitable for use in preparing the above-described vector constructs and particles may be readily obtained given the disclosure provided herein from naturally-occurring sources, or from depositories (*e.g.*, the American Type Culture Collection, Rockville, Maryland). Representative examples of suitable alphaviruses include Aura (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mayaro virus (ATCC VR-1277), Middleburg (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248), Tonate (ATCC VR-925), Triniti (ATCC VR-469), Una (ATCC VR-374),

Venezuelan equine encephalomyelitis (ATCC VR-69), Venezuelan equine encephalomyelitis virus (ATCC VR-923, ATCC VR-1250 ATCC VR-1249, ATCC VR-532), Western equine encephalomyelitis (ATCC VR-70, ATCC VR-1251, ATCC VR-622, ATCC VR-1252), Whataroa (ATCC VR-926), and Y-62-33 (ATCC VR-375). Sequences obtained from one or more alphaviruses can be used in the same vector and/or particle. Within one preferred aspect of the present invention, the sequences that encode wild-type alphavirus are obtained from a Sindbis virus.

The alphavirus vector component typically includes self-amplifying RNA (replicon) in which one or more of the structural protein genes of the virus are replaced by one or more genes of interest (*e.g.*, antigens). RNA-based vectors, including alphavirus vectors, are also known as "replicons" because they retain the replicase functions necessary for RNA self-amplification and high-level expression, and can be launched *in vivo* following transfection with plasmid DNA (Dubensky et al. (1996) *J. Virol.* 70:508-519) or infection with virus-like particles. Alphavirus vector constructs will also typically contain inactivated, tandem or modified viral junction regions. Other modifications and methods of constructing suitable alphavirus vectors are described, for example, in U.S. Patent No. 6,015,686 and WO 97/38087.

The replicon RNA of the alphavirus vector construct may be delivered directly to the target cells by physical means or first packaged into particles. Generally, structural proteins necessary for packaging are supplied *in trans* by helper constructs or by packaging cell lines. The structural proteins may be homologous (*e.g.*, derived from the same alphavirus as the vector construct); heterologous (*e.g.*, derived from an alphavirus different from the vector construct); and hybrid (*e.g.*, containing elements of multiple alphaviruses). Importantly, only the replicon RNA is packaged into the particle, as the helper RNAs typically lack the *cis*-acting packaging sequence required for encapsidation. Thus, alphavirus particles can be generated which infect target cells in culture or *in vivo*, and can express the gene of interest to high level; however, they are defective in that they lack critical portions of the alphavirus genome necessary to produce virus particles which could spread to other cells. In this way, when the replicon RNA is introduced into a suitable host cell, it self-amplifies, and the gene of interest is expressed from the subgenomic mRNA. However, no new virion particles are assembled *in vivo* due to the absence of structural protein genes.

Although no direct comparative studies among the various alphavirus replicons have been performed, differences in natural cell tropism are known to exist. For example, the lymphotropic VEE recently was shown to transduce murine DC (MacDonald et al. (2000) *J. Virol.* 74:914-922), while SIN and SFV are not lymphotropic. Infection of human DC has recently been demonstrated for alphaviruses or their derived vectors (Gardner et al. (2000) *J. Virol.* 74:11849-11857; WO 00/61772). Gardner identified SIN variants that are highly efficient for growth in immature human DC. The genetic determinant of human DC tropism for the SIN variant has been mapped to a single amino acid substitution in glycoprotein E2, and using this information, alphavirus replicon particles that can be used to target human DC can be generated. Detailed characterization of replicon infected DC both *in vitro* and *in vivo* revealed that the replicon-infected cells maintained their developmental and antigen presenting capabilities, indicating the potential utility of the DC-targeted SIN replicons for vaccine applications against infectious and malignant disease.

2. Additional Gene Delivery Vehicles

As noted above, mucosal genetic immunization has been shown to elicit both local and systemic immune responses. However, safety issues associated with revertants and potential infection hazards limits the utility of these systems. Moreover, replicating virus-based vector may spread rapidly throughout the host from the site of administration and cause unwarranted inflammatory responses at other sites. Therefore, the present invention describes viral and non-viral based genetic delivery systems that are replication-defective in the host and, accordingly, do not bear the possibility of reverting to an infectious state. Thus, the invention includes compositions and methods of mucosal immunization using any replication-defective gene transfer vehicle.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. Selected sequences can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (U.S. Patent No. 5,219,740; Miller and Rosman, *BioTechniques* (1989) 7:980-990; Miller, A.D., *Human Gene Therapy* (1990) 1:5-14;

Scarpa et al., *Virology* (1991) 180:849-852; Burns et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:8033-8037; and Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* (1993) 3:102-109.

A number of adenovirus vectors have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) 57:267-274; Bett et al., *J. Virol.* (1993) 67:5911-5921; Mittereder et al., *Human Gene Therapy* (1994) 5:717-729; Seth et al., *J. Virol.* (1994) 68:933-940; Barr et al., *Gene Therapy* (1994) 1:51-58; Berkner, K.L. *BioTechniques* (1988) 6:616-629; and Rich et al., *Human Gene Therapy* (1993) 4:461-476).

Additionally, various adeno-associated virus (AAV) vector systems have been developed for gene delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski et al., *Molec. Cell. Biol.* (1988) 8:3988-3996; Vincent et al., *Vaccines 90* (1990) (Cold Spring Harbor Laboratory Press); Carter, B.J. *Current Opinion in Biotechnology* (1992) 3:533-539; Muzyczka, N. *Current Topics in Microbiol. and Immunol.* (1992) 158:97-129; Kotin, R.M. *Human Gene Therapy* (1994) 5:793-801; Shelling and Smith, *Gene Therapy* (1994) 1:165-169; and Zhou et al., *J. Exp. Med.* (1994) 179:1867-1875.

Another vector system useful for delivering polynucleotides, mucosally and otherwise, is the enterically administered recombinant poxvirus vaccines described by Small, Jr., P.A., et al. (U.S. Patent No. 5,676,950, issued October 14, 1997, herein incorporated by reference) as well as the vaccinia virus and avian poxviruses. By way of example, vaccinia virus recombinants expressing the genes can be constructed as follows. The DNA encoding the particular synthetic Gag/antigen coding sequence is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells that are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the coding sequences of interest into the viral genome. The resulting

TK recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the genes. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an avipox vector is particularly desirable in human and other mammalian species since members of the avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, e.g., WO 91/12882; WO 89/03429; and WO 92/03545. Picornavirus-derived vectors can also be used. (See, e.g., U.S. Patent Nos. 5,614,413 and 6,063,384).

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al., *J. Biol. Chem.* (1993) 268:6866-6869 and Wagner et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery.

A vaccinia based infection/transfection system can be conveniently used to provide for inducible, transient expression of the coding sequences of interest (for example, a synthetic Gag/HCV-core expression cassette) in a host cell. In this system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA that is then translated into protein by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, e.g., Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA* (1990) 87:6743-6747; Fuerst et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:8122-8126.

3. Amplification Systems

As an alternative approach to infection with vaccinia or avipox virus recombinants, or to the delivery of genes using other viral vectors, an amplification system can be used that will lead to high level expression following introduction into host cells. Specifically, a T7 RNA polymerase promoter preceding the coding region for T7 RNA polymerase can be engineered. Translation of RNA derived from this template will generate T7 RNA polymerase that in turn will transcribe more template. Concomitantly, there will be a cDNA whose expression is under the control of the T7 promoter. Thus, some of the T7 RNA polymerase generated from translation of the amplification template RNA will lead to transcription of the desired gene. Because some T7 RNA polymerase is required to initiate the amplification, T7 RNA polymerase can be introduced into cells along with the template(s) to prime the transcription reaction. The polymerase can be introduced as a protein or on a plasmid encoding the RNA polymerase. For a further discussion of T7 systems and their use for transforming cells, see, e.g., International Publication No. WO 94/26911; Studier and Moffatt, *J. Mol. Biol.* (1986) 189:113-130; Deng and Wolff, *Gene* (1994) 143:245-249; Gao et al., *Biochem. Biophys. Res. Commun.* (1994) 200:1201-1206; Gao and Huang, *Nuc. Acids Res.* (1993) 21:2867-2872; Chen et al., *Nuc. Acids Res.* (1994) 22:2114-2120; and U.S. Patent No. 5,135,855.

4. Liposomal/Lipid Delivery Vehicles

The polynucleotide of interest can also be delivered without a viral vector. For example, packaged as DNA or RNA in liposomes prior to delivery to the subject or to cells derived therefrom. Lipid encapsulation is generally accomplished using liposomes that are able to stably bind or entrap and retain nucleic acid. The ratio of condensed DNA to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger et al., in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations, with

cationic liposomes particularly preferred. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416); mRNA (Malone et al., *Proc. Natl. Acad. Sci. USA* (1989) 86:6077-6081); and purified transcription factors (Debs et al., *J. Biol. Chem.* (1990) 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416). Other commercially available lipids include (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Cationic microparticles can be prepared from readily available materials using techniques known in the art. See, *e.g.,* co-owned WO 01/136599.

Similarly, anionic and neutral liposomes are readily available, such as, from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphoshatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilammelar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger et al., in METHODS OF IMMUNOLOGY (1983), Vol. 101, pp. 512-527; Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; Papahadjopoulos et al., *Biochim. Biophys. Acta* (1975) 394:483; Wilson et al., *Cell* (1979) 17:77); Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629; Ostro et al., *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley et al., *Proc. Natl. Acad. Sci. USA* (1979) 76:3348); Enoch and

Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) 76:145); Fraley et al., *J. Biol. Chem.* (1980) 255:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) 75:145; and Schaefer-Ridder et al., *Science* (1982) 215:166.

The DNA and/or protein antigen(s) can also be delivered in cochleate lipid compositions similar to those described by Papahadjopoulos et al., *Biochem. Biophys. Acta.* (1975) 394:483-491. See, also, U.S. Patent Nos. 4,663,161 and 4,871,488.

5. Particulate Carriers

The compositions may also be encapsulated, adsorbed to, or associated with, particulate carriers. Such carriers present multiple copies of a selected antigen to the immune system and promote migration, trapping and retention of antigens in local lymph nodes. The particles can be taken up by profession antigen presenting cells such as macrophages and dendritic cells, and/or can enhance antigen presentation through other mechanisms such as stimulation of cytokine release. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee JP, et al., *J Microencapsul.* 14(2):197-210, 1997; O'Hagan DT, et al., *Vaccine* 11(2):149-54, 1993.

Furthermore, other particulate systems and polymers can be used for the *in vivo* or *ex vivo* delivery of the gene of interest. For example, polymers such as polylysine, polyarginine, polyornithine, spermine, spermidine, as well as conjugates of these molecules, are useful for transferring a nucleic acid of interest. Similarly, DEAE dextran-mediated transfection, calcium phosphate precipitation or precipitation using other insoluble inorganic salts, such as strontium phosphate, aluminum silicates including bentonite and kaolin, chromic oxide, magnesium silicate, talc, and the like, will find use with the present methods. See, e.g., Felgner, P.L., *Advanced Drug Delivery Reviews* (1990) 5:163-187, for a review of delivery systems useful for gene transfer. Peptoids (Zuckerman, R.N., et al., U.S. Patent No. 5,831,005, issued November 3, 1998) may also be used for delivery of a construct of the present invention.

Additionally, biolistic delivery systems employing particulate carriers such as gold and tungsten, are especially useful for delivering synthetic expression cassettes of

the present invention. The particles are coated with the synthetic expression cassette(s) to be delivered and accelerated to high velocity, generally under a reduced atmosphere, using a gun powder discharge from a "gene gun." For a description of such techniques, and apparatuses useful therefore, see, e.g., U.S. Patent Nos. 4,945,050; 5,036,006; 5,100,792; 5,179,022; 5,371,015; and 5,478,744. Also, needle-less injection systems can be used (Davis, H.L., et al, *Vaccine* **12**:1503-1509, 1994; Bioject, Inc., Portland, OR).

B. ANTIGENS

Any of the gene delivery vehicles described herein can contain one or more heterologous sequences encoding one or more heterologous gene products, particularly polypeptide antigens. Virtually any heterologous gene product or polypeptide can be used. Antigens that are particularly useful in the practice of the present invention include polypeptide antigens derived from sexually transmitted pathogens or other viruses that infect or are transmitted through mucosal surfaces. Non-limiting representative examples of sexually transmitted pathogens and antigens derived therefrom include antigens derived from bacterial pathogens (*e.g.*, chlamydia, gonorrhea and syphilis) and viral pathogens (*e.g.*, Human Immmunodeficiency Virus ("HIV"), Hepatitis B and C Virus ("HBV" and "HCV", respectively), Human Papiloma Virus ("HPV"), Herpes Simplex Virus ("HSV"), and the like). Non-limiting examples of other viruses that may be transmitted via mucosal surfaces include rhinovirus, influenza, respiratory syncytial virus (RSV), parainfluenza virus (PIV), and the like. As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen that is capable, under the appropriate conditions, of causing an immune response (*i.e.*, cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins divided into three major classes: (1) the major structural proteins, Gag, Pol, and Env; (2) the regulatory proteins, Tat and Rev and (3) the

accessory proteins, Vpu, Vpr, Vif, and Nef. Although exemplified herein with relation to antigens obtained from $HIV_{SF2}$, sequence obtained from other HIV variants may be manipulated in similar fashion following the teachings of the present specification. Such other variants include, but are not limited to, Gag protein encoding sequences obtained from the isolates $HIV_{IIIb}$, $HIV_{SF2}$, HIV-$1_{SF162}$, HIV-$1_{SF170}$, $HIV_{LAV}$, $HIV_{LAI}$, $HIV_{MN}$, HIV-$1_{CM235}$, HIV-$1_{US4}$, other HIV-1 strains from diverse subtypes (e.g., subtypes, A through G, and O), HIV-2 strains and diverse subtypes (e.g., HIV-$2_{UC1}$ and HIV-$2_{UC2}$), and simian immunodeficiency virus (SIV). (See, e.g., Virology, 3rd Edition (W.K. Joklik ed. 1988); *Fundamental Virology*, 2nd Edition (B.N. Fields and D.M. Knipe, eds. 1991); *Virology*, 3rd Edition (Fields, BN, DM Knipe, PM Howley, Editors, 1996, Lippincott-Raven, Philadelphia, PA; for a description of these and other related viruses).

As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described antigens may be combined in order to induce an immune response when administered as described herein. Further, the antigen-encoding gene delivery vehicles can also be used in combination with one or more additional gene delivery vehicles and/or polypeptides.

In addition, due to the large immunological variability that is found in different geographic regions for the open reading frame of HIV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, at least eight different subtypes of HIV have been identified and, of these, subtype B viruses are more prevalent in North America, Latin America and the Caribbean, Europe, Japan and Australia. Almost every subtype is present in sub-Saharan Africa, with subtypes A and D predominating in central and eastern Africa, and subtype C in southern Africa. Subtype C is also prevalent in India and it has been recently identified in southern Brazil. Subtype E was initially identified in Thailand, and is also present in the Central African Republic. Subtype F was initially described in Brazil and in Romania. The most recent subtypes described are G, found in Russia and Gabon, and subtype H, found in Zaire and in Cameroon. Group O viruses have been identified in Cameroon and also in Gabon. Thus, as will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration that is appropriate to the particular HIV subtype that is prevalent in the geographical region of

administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, by sequencing the HIV genome (or fragments thereof) isolated from individuals within that region.

As described above, also presented by HIV are various *Gag* and *Env* antigens. HIV-1 *Gag* proteins are involved in many stages of the life cycle of the virus including, assembly, virion maturation after particle release, and early post-entry steps in virus replication. The roles of HIV-1 *Gag* proteins are numerous and complex (Freed, E.O. (1998) *Virology* 251:1-15).

*Env* coding sequences of the present invention include, but are not limited to, polynucleotide sequences encoding the following HIV-encoded polypeptides: gp160, gp140, and gp120 (see, e.g., U.S. Patent No. 5,792,459 for a description of the HIV-$1_{SF2}$ ("SF2") Env polypeptide). The envelope protein of HIV-1 is a glycoprotein of about 160 kD (gp160). During virus infection of the host cell, gp160 is cleaved by host cell proteases to form gp120 and the integral membrane protein, gp41. The gp41 portion is anchored in (and spans) the membrane bilayer of virion, while the gp120 segment protrudes into the surrounding environment. As there is no covalent attachment between gp120 and gp41, free gp120 is released from the surface of virions and infected cells. Thus, gp160 includes the coding sequences for gp120 and gp41. The polypeptide gp41 is comprised of several domains including an oligomerization domain (OD) and a transmembrane spanning domain (TM). In the native envelope, the oligomerization domain is required for the non-covalent association of three gp41 polypeptides to form a trimeric structure: through non-covalent interactions with the gp41 trimer (and itself), the gp120 polypeptides are also organized in a trimeric structure. A cleavage site (or cleavage sites) exists approximately between the polypeptide sequences for gp120 and the polypeptide sequences corresponding to gp41. This cleavage site(s) can be mutated to prevent cleavage at the site. The resulting gp140 polypeptide corresponds to a truncated form of gp160 where the transmembrane spanning domain of gp41 has been deleted. This gp140 polypeptide can exist in both monomeric and oligomeric (*i.e.* trimeric) forms by virtue of the presence of the oligomerization domain in the gp41 moiety. In the situation where the cleavage site has been mutated to prevent cleavage and the transmembrane portion of gp41 has been deleted the resulting polypeptide

product can be designated "mutated" gp140. As will be apparent to those in the field, the cleavage site can be mutated in a variety of ways. (See, also, WO 00/39302).

As noted above, at least one immunogenic portion of an HIV antigen may be incorporated into a gene delivery vehicle and used for mucosal immunization. The immunogenic portion(s) incorporated into the vehicle (*e.g.*, alphavirus vector construct) may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITES (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA, which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays and proliferation assays.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T cell receptor repertoire recognizes the same antigenic determinants recognized by human T cells. In both systems, the CTL response generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al. (1991) *J. Exp. Med. 173*:1007-1015; Vitiello et al. (1992) *Abstract of Molecular Biology of Hepatitis B Virus Symposia*).

Additional immunogenic portions of the HIV may be obtained by truncating the coding sequence at various locations including, for example, to include one or more epitopes from the various domains of the HIV genome. As noted above, such domains include structural domains such as *Gag, Gag-polymerase, Gag-protease, reverse transcriptase (RT), integrase (IN)* and *Env*. The structural domains are often further subdivided into polypeptides, for example, p55, p24, p6 (*Gag*); p160, p10, p15, p31, p65 (*pol, prot, RT and IN*); and gp160, gp120 and gp41 (*Env*). Additional epitopes of HIV and other sexually transmitted diseases are known or can be readily determined using methods known in the art. Also included in the invention are molecular variants

of such polypeptides, for example as described in PCT/US99/31245; PCT/US99/31273 and PCT/US99/31272.

Other sexually or mucosally transmitted diseases, both viral and bacterial, can also be addressed using the compositions and methods described herein. For example, it appears that in the case of HSV, vaginal immunization with vector expressing HSV antigens (*e.g.*, gB, gD) may provide protection against vaginal. Thus, one or more antigens derived from HSV can be used in as described herein to treat and prevent HSV infection.

1. Preparation of Vectors Carrying Heterologous Gene Products

In certain embodiments, sequences encoding one or more antigens are incorporated into a gene delivery vehicle such as viral vector or particle. As will be evident to one of ordinary skill in the art given the disclosure provided herein, the efficiency of packaging and hence, viral titer, of various viral vectors is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production of viable vector particles (*e.g.*, alphavirus vector particles), additional non-coding sequences may be added to the vector construct.

In certain applications of the vectors or resulting particles described herein, the expression of more than one heterologous gene is desired. For example, in order to treat sexually transmitted disease such as HIV, multiple administrations of vectors or particles, or administration of vectors or particles expression more than one gene product may be required. In addition, immunogenicity may be further enhanced by encoding both an antigen and an immunomodulator (*e.g.*, cytokine, lymphokine, chemokine, etc.). Therefore, within one embodiment of the invention viral vectors (*e.g.*, alphavirus vectors) may be constructed by placing appropriate signals, such as ribosome readthrough or internal ribosome entry sites (IRES) between cistrons. Alternatively, multiple subgenomic junction region promoters (*e.g.*, derived from alphavirus) can be utilized. Further, a vector construct may express (either separately or as one construct) all or immunogenic portions of various mucosally transmitted (*e.g.*, sexually transmitted) pathogens.

Within one aspect of the invention, vector constructs (*e.g.*, alphavirus vectors and particles) are provided which direct the expression of immunogenic portions of HIV

antigens. The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilobases in length. (see, e.g., Muesing et al. (1985) *Nature* 313:450-48). Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs).

Sequences that encode the above-described proteins (*e.g.* antigens) may be readily obtained from a variety of sources, including for example, depositories such as the American Type Culture Collection (ATCC, Rockville, MD), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford, England). Representative examples of molecularly cloned genomes that encode the hepatitis B virus may be obtained from sources such as the American Type Culture Collection (ATCC, Rockville, MD). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see* Figure 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the *Bam* HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981).

Alternatively, sequences encoding the polypeptide of interest can be generated by the polymerase chain reaction (PCR). Mullis et al. (1987) *Methods Enzymol.* 155:335-350; *PCR Protocols, A Guide to Methods and Applications*, Innis et al (eds) Harcourt Brace Jovanovich Publishers, NY (1994)). This technique uses DNA polymerase, usually a thermostable DNA polymerase, to replicate a desired region of DNA. The region of DNA to be replicated is identified by oligonucleotides of specified sequence complementary to opposite ends and opposite strands of the desired DNA to prime the replication reaction. Repeated successive cycles of replication result in amplification of the DNA fragment delimited by the primer pair used. A number of parameters influence the success of a reaction. Among them are annealing temperature and time, extension time, $Mg^{2+}$ and ATP concentration, pH, and the relative concentration of primers, templates, and deoxyribonucleotides.

Once coding sequences for desired proteins have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Ligations to other sequences are performed using standard procedures, known in the art.

2. Polypeptide Preparation

Similarly, the selected coding sequences can be cloned into any suitable expression vector for expression. The expressed product can optionally be purified prior to mucosal administration. Briefly, a polynucleotide encoding these proteins can be introduced into an expression vector that can be expressed in a suitable expression system. A variety of bacterial, yeast, mammalian, insect and plant expression systems are available in the art and any such expression system can be used. Optionally, a polynucleotide encoding these proteins can be translated in a cell-free translation system. Such methods are well known in the art. The proteins also can be constructed by solid phase protein synthesis. If desired, the polypeptides also can contain other amino acid sequences, such as amino acid linkers or signal sequences, as well as ligands useful in protein purification, such as glutathione-S-transferase and staphylococcal protein A. Alternatively, antigens of interest can be purchased from commercial sources.

C. DELIVERY

The compositions (*e.g.,* gene delivery vehicles and optional polypeptide antigens) described herein can be delivered using any suitable means (*e.g.*, intravenously, intramuscularly, intraperitoneally, subcutaneously, orally, rectally, intraocularly, intranasally), or by various physical methods such as lipofection (Felgner et al. (1989) *Proc. Natl. Acad. Sci. USA* 84:7413-7417), direct DNA injection (Acsadi et al. (1991) *Nature* 352:815-818); microprojectile bombardment (Williams et al. (1991) *PNAS* 88:2726-2730); liposomes of several types (*see, e.g.,* Wang et al. (1987) *PNAS* 84:7851-7855); $CaPO_4$ (Dubensky et al. (1984) *PNAS* 81:7529-7533); DNA ligand (Wu et al (1989) *J. of Biol. Chem.* 264:16985-16987); administration of polypeptides alone; administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al. (1992), *Hum. Gene Ther.* 3:147-154); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. In addition, the eukaryotic layered vector initiation systems may either be administered directly (*i.e., in vivo*), or to cells that have been removed (*ex vivo*), and subsequently returned.

In a preferred embodiment, the gene delivery vehicles and optional polypeptide-containing compositions are administered mucosally. Methods of mucosal delivery are known in the art, for example as described in *Remington's, supra*. Delivery of the compositions rectally and vaginally is particularly preferred in the case of sexually transmitted pathogens, as this mode of administration provides access to the cells first exposed to the pathogens. Similarly, intranasal administration may be preferred in diseases, like rhinovirus, that infect through nasal mucosa. In some instances, intranasal administration may induce immunity in the vaginal mucosa and oral immunization may induce immunity in the rectal mucosa. Moreover, combinations of various routes of mucosal administration and/or various routes of systemic administration can be used in order to induce optimal immunity and protection (both at the site the pathogen enters as well as at systemic sites where a mucosal pathogen has spread to. Additionally, mucosal administration eliminates the need for syringes or other administration devices. Dosage treatment may be a single dose schedule or a multiple dose schedule.

In certain embodiments, the replication-defective vectors are administered via nucleic acid immunization or the like using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Patent Nos. 5,399,346, 5,580,859, 5,589,466. Alphavirus compositions can be delivered either directly to the vertebrate subject or, alternatively, delivered *ex vivo*, to cells derived from the subject and the cells reimplanted in the subject. In preferred embodiments, the compositions are delivered *in vivo*. Delivery of replication-defective compositions *in vivo* can generally be accomplished using any means known in the art, for example, by injection using either a conventional syringe, needless devices such as Bioject® or a gene gun, such as the Accell® gene delivery system (PowderJect Technologies, Inc., Oxford, England). The constructs can be delivered (*e.g.*, injected) either subcutaneously, epidermally, intradermally, intramuscularly, intravenous, mucosally (such as nasally, rectally and vaginally), intraperitoneally, orally or combinations thereof.

Within other aspects of the present invention, methods are provided for administering the gene delivery systems described herein, including recombinant alphavirus vectors or particles. Briefly, the final mode of viral vector administration usually relies on the specific therapeutic application, the best mode of increasing vector potency, and the most convenient route of administration. Generally, this embodiment

includes compositions which can be designed to be delivered by, for example, (1) direct injection into the blood stream; (2) direct injection into a specific tissue or tumor; (3) oral administration; (4) nasal inhalation; (5) direct application to mucosal tissues (*e.g.*, intranasally, intrarectally and/or intravaginally); and/or (6) *ex vivo* administration of transduced autologous cells into the animal. Thus the therapeutic alphavirus vector can be administered in such a fashion such that the vector can (a) transduce a normal healthy cell and transform the cell into a producer of a therapeutic protein or agent which is secreted systemically or locally, (b) transform an abnormal or defective cell, transforming the cell into a normal functioning phenotype, (c) transform an abnormal cell so that it is destroyed, and/or (d) transduce cells to manipulate the immune response.

The compositions disclosed herein can be administered alone or can be administered with one or more additional gene delivery vehicles and/or one or more proteins. In such embodiments, the multiple compositions can be administered in any order, for example gene delivery vehicle followed by protein; multiple gene delivery vehicles followed by multiple protein administrations; protein administration(s) followed by single or multiple gene delivery vehicle administration; concurrent administration; and the like. Thus, a mixture of protein and nucleic acid can be administered, using the same or different vehicles and the same or different modes of administration.

In certain embodiments, direct delivery will generally be accomplished with or without viral vectors, as described above, by injection using either a conventional syringe or a gene gun, such as the Accell® gene delivery system (PowderJect Technologies, Inc., Oxford, England).

Thus, injection can be either subcutaneously, epidermally, intradermally, intramucosally such as nasally, rectally, orally and vaginally, intraperitoneally, intravenously, or intramuscularly. Other modes of administration include pulmonary administration, suppositories, needle-less injection, transcutaneous and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. As noted above, administration of nucleic acids may also be combined with administration of peptides or other substances.

D. PHARMACEUTICAL COMPOSITIONS

The present invention also includes pharmaceutical compositions comprising a replication-defective vector (*e.g.*, recombinant alphavirus construct or alphavirus particle) in combination with a pharmaceutically acceptable carrier, diluent, or recipient. Further, other ingredients, such as adjuvants, may also be present. As described more fully in U.S. Patent No. 6,015,694, storage stable and easy administerable immunogenic compositions are particularly needed in Third World countries where refrigeration and/or traditional administration means (syringes, etc.) are not readily available.

In certain embodiments, polypeptides may also be included. The preparation of immunogenic compounds that contain immunogenic polypeptide(s) as active ingredients is known to those skilled in the art. Typically, such immunogenic compounds are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified, or the protein encapsulated in liposomes.

Compositions of the invention preferably comprise a pharmaceutically acceptable carrier. The carrier should not itself induce the production of antibodies harmful to the host. Pharmaceutically acceptable carriers are well known to those in the art. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; McGee et al. (1997) *J Microencapsul.* 14(2):197-210; O'Hagan et al. (1993) *Vaccine* 11(2):149-54. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as toxoid from diphtheria, tetanus, cholera, etc., as well as toxins derived from *E. coli*.

Pharmaceutically acceptable salts can also be used in compositions of the invention, for example, mineral salts such as hydrochlorides, hydrobromides,

phosphates, or sulfates, as well as salts of organic acids such as acetates, proprionates, malonates, or benzoates. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those of skill in the art. Compositions of the invention can also contain liquids or excipients, such as water, saline, glycerol, dextrose, ethanol, or the like, singly or in combination, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents. Liposomes can also be used as a carrier for a composition of the invention, such liposomes are described above.

Briefly, with regard to viral particles, replication-defective vectors (also referred to above as particles) may be preserved either in crude or purified forms. Preservation methods and conditions are described in U.S. Patent No. 6,015,694.

Further, the compositions described herein can include various excipients, adjuvants, carriers, auxiliary substances, modulating agents, and the like. Preferably, the compositions will include an amount of the antigen sufficient to mount an immunological response. An appropriate effective amount can be determined by one of skill in the art. Such an amount will fall in a relatively broad range that can be determined through routine trials and will generally be an amount on the order of about 0.1 μg to about 1000 μg, more preferably about 1 μg to about 300 μg, of particle/antigen.

Such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid

A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), beta chemokines (MIP, 1-alpha, 1-beta Rantes, etc.); (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. W093/13202; W092/19265; WO 95/17211; WO 98/18928 and WO 01/22993); and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acteyl-normuramyl-L-alanyl-D-isogluatme (nor-MDP), N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(l'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

Administration of the pharmaceutical compositions described herein may be by any suitable route (see, *e.g.*, Section C). Particularly preferred is mucosal (*e.g.*, rectal and/or vaginal) administration. Dosage treatment may be a single dose schedule or a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals, chosen to maintain and/or reinforce the immune response, for example at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the potency of the modality, the vaccine delivery employed, the need of the subject and be dependent on the judgment of the practitioner.

In yet other embodiments, a multiple administrations (*e.g.*, prime-boost type administration) will be advantageously employed. For example, recombinant

alphavirus particles expressing the antigen(s) of interest are administered (*e.g.*, IVAG or IR). Subsequently, the antigen(s) are administered, for example in compositions comprising the polypeptide antigen(s) and a suitable adjuvant. Alternatively, antigens are administered prior to gene delivery vehicles. Multiple polypeptide and multiple gene delivery vehicle administrations (in any order) may also be employed.

E. METHODS FOR UTILIZING REPLICATION-DEFECTIVE PARTICLES AND ANTIGENIC POLYPEPTIDES

Within certain aspects of the present invention, compositions and methods are provided for mucosally administering a composition (*e.g.*, an alphavirus vector construct) that is capable of preventing, inhibiting, stabilizing or reversing sexually transmitted diseases. Representative examples of such diseases include bacterial and viral infections, particularly sexually transmitted infections, including but not limited to HIV, HBV HTLV I, HTLV II, HPV, HSV, HCV, chlamydia, gonorrhea, and/or syphilis.

More specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a sexually transmitted pathogenic agent, such that the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents include bacteria and viruses.

Within one embodiment of the invention the pathogenic agent is a virus, and methods are provided for stimulating a specific immune response and inhibiting viral spread by using recombinant alphavirus viral particles designed to deliver a vector construct that directs the expression of an antigen or modified form thereof to susceptible target cells capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the vector nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant alphavirus is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I

and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogues thereof (*e.g.*, Altmann et al., *Nature 338*:512, 1989). Cells infected with alphavirus vectors are expected to do this efficiently because they closely mimic genuine viral infection and because they: (a) are able to infect non-replicating cells, (b) do not integrate into the host cell genome, (c) are not associated with any life threatening diseases, and (d) express high levels of heterologous protein. Because of these differences, alphavirus vectors can easily be thought of as safe viral vectors that can be used on healthy individuals for vaccine use.

This aspect of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy and low levels of viral antigens, relative to heterologous genes, are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant alphavirus, leading to responses against immunogenic epitopes that may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

The compositions (*e.g.*, alphavirus constructs and particles) suitable for mucosal administration described herein may include one or more inhibitory palliative, *e.g.*, a viral or bacterial inhibitory gene which express anti-sense or the like. A discussion of inhibitory palliatives are described, for example, in U.S. Patent No. 6,015,686 which describes how antigens and inhibitory palliatives (*e.g.*, expressing anti-sense tat, etc.) can be co-expressed and/or designed to overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells. In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker

would be a vector construct expressing either an HIV *env* analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of an alphavirus vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule. Efficacy of treatment can be assayed by measuring the usual indicators of disease progression, including antibody level, viral antigen production, infectious HIV levels, or levels of nonspecific infections.

Such a transcriptional repressor protein may be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are introduced to the system, a mutant with the appropriate properties (*i.e.*, represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector/tat system may be used to assure that surviving cell clones are not caused by endogenous mutations in these genes. A battery of randomly mutagenized tat genes is then introduced into these cells using a "rescuable" alphavirus vector (*i.e.*, one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

Additional inhibitory palliatives that may be used in the gene delivery vehicles described herein include systems wherein expression of the heterologous transgene may be reduced (suppressed) in desired cells, including during the virion packaging process, for example by including a TOP-binding ligand and packaging elements in the vector. As described, for example, in co-owned U.S. Serial Number 09/608,730, these methods

allow for the suppression of transgene translation in virion producing cells while maintaining the capacity for high level expression and translation of the transgene in all other cell types. These systems are also applicable to a variety of viral vectors and, in addition, can be combined with other virion production systems, including, for example, adenovirus virion construction in a cre-lox system.

The following examples are offered by way of illustration, and not by way of limitation.

## EXAMPLE 1
## MATERIALS AND METHODS

*Mice and Cell lines*

Female Balb/c mice were purchased from Charles River Breeding Laboratories and were at the age of 6 to 8 weeks at the onset of the studies. The fibroblast cell line SvBalb ($H\text{-}2^d$) was used as target cells. This cell line expresses class I but not class II MHC molecules and is therefore directed against $CD8^+$ but not $CD4^+$ cells.

*Materials*

p7g is an $H\text{-}2K^d$ restricted $HIV\text{-}1_{SF2}$p24gag CTL epitope and is a synthetic 9 mer peptide: (aa, 199-AMQMLKETI-207).(21) This peptide was synthesized with free amine N termini and free acid C termini using Fmoc solid phase methods by Research Genetics (Huntsville AL) (see, e.g., Mathiowitz et al. *Nature* 386:410-414).

*Immunizations*

Groups of 5 female Balb/C mice were used for each vaccine or immunization route and the tissues were pooled upon sacrifice. The data are presented as representative of 2-4 such data points with similar or identical results. All immunizations were performed 3-4 times at 2-3 week intervals. IN immunizations were performed with $2.5E10^6$ SIN particles in a volume of 25 µl suspended in PBS. IN immunizations were performed without anesthesia. IVAG and IR immunizations with $2.5E10^6$ in a volume of 12.5 µl were performed on anesthetized mice that were kept in

dorsal recumbency for 20 minutes. IM immunizations were performed in thigh muscle with $2.5E10^6$ SIN particles in a volume of 50 µl The mice were sacrificed one week following the final immunization.

*Sera and Tissue Collection*

Mice were bled through the retro orbital plexus one day prior to sacrifice and the sera separated for ELISA assays. Cervical lymph nodes (CLN), iliac lymph nodes (ILN), vaginal/uteral mucosal tissues (VUM) and spleens (SP) were harvested and pooled from 5 mice per group and single cell suspensions were used for a standard $^{51}Cr$-release CTL assay (except VUM), a gag epitope specific ELISPOT to detect IFN-γ-secreting cells (IFNSC) or gag-p55 specific ELISPOT to detect antibody secreting cells (ASC).

*Preparation of single cell suspensions*

Groups of 5 mice were immunized 3 times as described above through the IN, IM, IR or IVAG routes with 2-3 week intervals. One week following the final immunization SP, CLN and ILN from groups of 5 immunized mice each were harvested and pooled. SP, CLN and ILN tissues were teased through a nylon mesh with pore diameter 250 µm and washed three times in media (ELISPOT assay media: RPMI containing 10% FCS, antibiotics, Hepes and L-glutamin (complete RPMI); $^{51}Cr$-release assay media), counted and seeded into wells for ELISPOT or $^{51}Cr$-release assay. Single cell suspensions from VUM were prepared based on the method described by Holmgren (see, for example, Lycke and Holmgren (1986) *Immunology* 59:301-308) with modifications by removing the entire vagina, uterus and uteral horns from pools of 5 mice per group. The uteral horns were cut longitudinally and together with vaginal and uteral tissues were diced into 5 mm pieces. The tissue pieces were then washed three times in HBSS without Ca++ and Mg++ containing 10% FCS and 5 mM Hepes (complete HBSS). The pieces were then treated enzymatically under agitation at 37°C sequentially once with 1 mg/ml Collagenase/Dispase plus 0.5 mg/ml DNase in complete HBSS for 30 min. and twice with collagenase plus 0.5 mg/ml DNase in complete RPMI for 45 min. Following each enzymatic treatment released cells were recovered and washed twice with complete RPMI. The cell suspensions recovered from

each enzymatic treatment were pooled and counted. This method routinely resulted in recovery of a minimum of $10^7$ viable mononuclear cells (MNC) per five mice.

*$^{51}$Cr-release CTL Assay*

Single cell suspensions from the tissues were prepared as described above and were cultured in a 24-well dish at $5x10^6$ cells per well. Of these cells, $1X10^6$ were sensitized with synthetic p7g peptide (amino acids 194-213) at a concentration of 10 mM for 1 hour at 37°C and then washed and co-cultured with the remaining $4x10^6$ untreated cells. The cells were stimulated as a bulk culture in 2 ml of Splenocyte culture medium: RPMI 1640 with 100 mM L-glutamine (Gibco, Grand Island, New York, USA)/-Mem (Minimum Essential Medium Alpha Medium with L-glutamine, deoxyribonucleosides or ribonucleosides) (1:1) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah, USA), 100 U/ml penicillin, 100 g/ml streptomycin, 10 ml/L of 100 mM sodium pyruvate and 50 M 2-mercaptoethanol. In addition, 5% Rat T-Stim IL2 (Rat T-Stim; Collaborative Biomedical Products, Bedford, Massachusetts, USA) was used as a source of IL2 and was added to the culture media just before the cells were to be cultured.

After a stimulation period of 6 to 7 days, the cells were collected and used as effectors in a $^{51}$Chromium release assay. Approximately $10^6$ SV/Balb target cells were incubated in 200 µl of medium containing 50 Ci of $^{51}$Cr and with the correct peptide p7g, or a mismatched cell-target pair as the negative control at a concentration of 1 M for 60 min and washed. Effector cells were cultured with $5x10^3$ target cells at various effector to target ratios in 200 µl of culture medium in 96-well tissue culture plates (round or v-bottom) for 4 hr. The average cpm from duplicate wells was used to calculate percent specific release as presented here.

*ELISPOT assays*

Single cell suspensions from pooled CLN and SP from 5 mice per group were added onto nitrocellulose or pvdf plates (Milipore) precoated with a monoclonal rat anti-mouse anti-IFN-γ (Pharmingen) and p55 and blocked with complete RPMI medium at pH 7.2, containing 10% fetal calf serum, 5mM Hepes, and antibiotics. For detection of total p55-specific antibody secreting cells (ASC), following overnight

incubation of cells at 37°C, the plates were washed with PBS/0.05% Tween (P/T). Biotinylated goat anti-IgH+L (Southern Biotechnology Associates, Birmingham, Alabama) was added at 1:7000 dilution in PBS/1% normal goat serum and incubated at room temperature (RT) for 2 hours. The plates were washed with P/T and incubated for 1 hr. at 37°C with Avidin-peroxidase at 1:1000 dilution (Pharmingen). The plates were washed with P/T and the spots were visualized by adding DAB in Tris-HCl buffer for 30 minutes. The plates were washed with de-ionized water and air-dried. The spots were counted manually under low magnification from duplicate wells per group and per tissue. The results are presented as ASC per 10 million cells and are representative of at least two experiments with similar results.

For detection of IFN-γ secreting cells following overnight incubation of cells in the presence of gag-derived p7g peptide, or anti-CD3 (Pharmingen) and anti-CD28 (Pharmingen) as positive control for polyclonal T cell activation, or media only as negative control, the plates were washed and biotinylated anti-IFN-γ (Pharmingen) was added in PBS/0.1% BSA/0.02% Tween and incubated at R/T for 2 hours. The plates were washed with P/T and incubated for 1 hr. at 37°C with Avidin-peroxidase (Pharmingen) at 1:1000 dilution. The plates were washed with P/T and the spots were visualized by adding DAB in Tris-HCl buffer for 30 minutes. The plates were washed with de-ionized H2O and air-dried. The spots were counted with an in house developed automated ELISPOT reader using software from Alpha Innotech Corporation (San Leandro, CA).

*ELISA assays*

HIV-1 p55 gag specific serum IgG titers were quantified by a standard ELISA assay. Briefly, ELISA plates (96 well U bottom by Nunc Maxisorp) were coated with p55 protein at 5 μg/well. After washing with 1X PBS + 0.03% Tween 20 (Sigma), the wells were blocked and samples were added in serial dilutions in an assay diluent made up of 1X PBS + 5% goat serum (Gibco Brl) + 0.03% Tween 20 (Sigma). A standard serum was included in each assay for quantitation purposes. The samples and standard sera were incubated at 37°C for one hour and washed with PBS/0.03% Tween. The samples were then incubated with a 1:40000 dilution of a goat anti-mouse IgG-HRP (Caltag) and developed with tetramethylbenzidine (TMB-Kirkegaard and Perry) for 15

minutes and then stopped with 2N HCl. The optical density of each well was measured using Titertek at 450 nm.

EXAMPLE 2

CTL RESPONSES FOLLOWING MUCOSAL VS. SYSTEMIC IMMUNIZATIONS WITH SIN-GAG BEFORE CHALLENGE WITH VACCINIA VIRUS (VV)-GAG

The ability of SIN-gag particles to induce local and systemic gag-specific CTL responses was assessed through various routes of mucosal or systemic immunization. Mice were immunized intranasally (IN) or intramuscularly (IM) with $2.5E10^6$ SIN-gag particles and intravaginally (IVAG) or intrarectally (IR) with $10^7$ SIN-gag particles and sacrificed 7 days following the final immunization. To determine systemic gag-specific CTL responses, single cell suspensions from CLN, ILN, VUM and SP were prepared and a gag-specific IFN-γ. ELISPOT assay was performed. Local gag-specific CTL responses in CLN following IN immunizations were observed. In contrast, no local CTL responses ILN or in VUM, following IVAG and IR immunizations were seen (Figure 1). Moreover, systemic gag-specific CTL responses in SP following IN and IM immunizations were found, but not following IVAG and IR immunizations, (Figure 2). To assess the lytic activity of the CTL responses that were detected as the number of IFN-γ secreting cells by the ELISPOT assay, a standard $^{51}Cr$-release assay on CLN and SP cells following IN immunizations with SIN-gag particles was performed. Local and systemic lytic CTL activity in CLN and SP following IN immunizations with SIN-gag (Figure 3) was observed. Thus, IN or IM, but not IVAG or IR, immunizations with SIN-gag induced local and systemic CTL responses.

EXAMPLE 3

CTL RESPONSES FOLLOWING MUCOSAL VS. SYSTEMIC IMMUNIZATIONS WITH SIN-GAG AFTER CHALLENGE WITH VV-GAG

Local and systemic CTL responses following mucosal and systemic immunizations and local challenge were determined. (see, also, Vajdy et al. (2001) Journal of Infectious Disease 184:1613). Mice were immunized IN or IM with $2.5E10^6$ SIN-gag particles and 1-3 weeks following the final immunization were challenged IR or IVAG with $10^{7-8}$ pfu of Vaccinia virus (VV) expressing HIV-1 p55-gag and

sacrificed 5 days following the final immunization. The following controls were used: a group of naïve mice were challenged IVAG, a group of naïve mice were challenged IR, groups of mice were immunized IM, IN, IR or IVAG with SIN-gag and challenged IVAG with Vaccinia virus expressing HIV-1 envelope gp160 (VV-env).

Following IM or IN immunizations with SIN-gag and vaginal challenge with VV-gag a potent gag-specific CTL responses in local, CLN, and systemic, SP, lymphoid tissues was detected. Importantly, potent CTL responses were also detected in the distant mucosal effector site, VUM as well as in the distant inductive site, ILN as measured by the ELISPOT assay (Figure 4). None of the control groups demonstrated local or systemic CTL responses (Figure 5). Thus, systemic injection in thigh muscle as well as mucosal administration onto the nasal mucosa with SIN-gag followed by vaginal challenge with VV-gag induced potent CTL responses not only in the local inductive site of the nasal mucosa (CLN), but also in the distant inductive (ILN) and effector (VUM) sites of the vaginal mucosa. Moreover, both IN and IM immunizations with SIN-gag followed by vaginal challenge with VV-gag induced potent systemic CTL responses.

To determine local and systemic CTL responses following local immunization and local challenge, mice were immunized IVAG or IR with $10^7$ SIN-gag particles and challenged IVAG with $10^7$ pfu VV-gag. Following IR immunizations with SIN-gag and IVAG challenge with VV-gag, local as well as systemic CTL responses in ILN and SP respectively, were detected, as measured by the IFN-γ ELISPOT assay (Figure 5). In contrast, mice immunized IVAG with SIN-gag and challenged IVAG with VV-gag demonstrated strong CTL responses locally in ILN and VUM but low CTL responses systemically in SP (Figure 5). None of the control groups demonstrated any local or systemic CTL responses. To confirm the lytic activity of the CTL responses detected by the ELISPOT technique, a standard Cr-release assay was performed with ILN and SP isolated from mice immunized IR or IVAG with SIN-gag and challenged IVAG with VV-gag and similar results were obtained. Thus, vaginal and rectal immunizations with SIN-gag particles induce local CTL responses in the mucosal effector and inductive sites. Furthermore, rectal, but not vaginal, immunization with SIN-gag particles followed by IVAG challenge with VV-gag evoked strong CTL responses systemic lymphoid tissue.

EXAMPLE 4

PROTECTION FROM MUCOSAL CHALLENGE WITH VV-GAG

To determine whether the presence of local and systemic CTL responses correlated with mucosal protection from various challenge a gag-expressing vaccinia virus challenge model was used. Vaccinia has been shown to infect and replicate in ovaries following intra-rectal, and various parenteral routes of inoculation. Two or three weeks following IR, IVAG, IN or IM immunizations with SIN-gag all groups of mice were challenged IVAG with $10^7$ pfu of VV-gag. Five days following IVAG challenge with VV-gag, the mice were sacrificed and a pfu assay performed on their ovaries. The following controls were used: naïve mice challenged IVAG, naïve mice challenged IR, groups of mice immunized IM, IN, IR or IVAG with SIN-gag and challenged IVAG with Vaccinia virus expressing HIV-1 envelope gp160 (VV-env).

Mice immunized IVAG or IR with SIN-gag were protected against IVAG and IR challenge with VV-gag respectively, in that no pfu was detected in their ovaries (Figure 6). Moreover, mice immunized IN with SIN-gag and challenged IVAG with VV-gag were only partially protected (Figure 6). Importantly, mice immunized IM and challenged IVAG were not protected (Figure VI). The naïve mice that were challenged with VV-gag IR or IVAG had high numbers of pfu in their ovaries (Figure 6). Moreover, the mice that were immunized IM, IN, IR or IVAG with SIN-gag and challenged IVAG with VV-env also had high pfu titers in their ovaries, demonstrating that the protection was antigen-specific (Figure VI). Importantly, mice immunized IM with SIN-gag and challenged intra-peritoneally with VV-gag were partially protected demonstrating that although IM immunization does not protect against mucosal challenge it does offer some degree of protection against systemic challenge (data not shown). Thus, local/mucosal, but not distant/mucosal or systemic, immunization protected the mice against local/mucosal challenge.

EXAMPLE 5

HIV-1 GAG-SPECIFIC CD8+ T CELL RESPONSES IN THE VAGINAL MUCOSA AND PROTECTION FROM VAGINAL VIRAL CHALLENGE FOLLOWING LOCAL IMMUNIZATIONS WITH SINDBIS VIRUS-BASED REPLICON PARTICLES

Groups of 5 BALB/c mice were used for each vaccine or immunization route and the tissues were pooled upon sacrifice. Mice were immunized by various routes 3

times at 2 week intervals, rested for 2-3 weeks and then challenged intra-vaginally (IVAG) or intra-rectally (IR) with $10^7$ plaque-forming units (PFU) of VV-gag or VV-gp160 (Gardner et al. (2000) J Virol 74:11849-57. SIN-gag particles were prepared as described in Gardner et al. (2000), *supra*. Intra-nasal (IN) immunizations were performed without anesthesia with $2.5x10^6$ SIN-gag particles in a volume of 25 µl suspended in PBS. The SIN-gag particles and the VV-gag virus were applied on anesthetized mice IVAG or IR in a volume of 12.5 µl following which the mice were kept in dorsal recumbency for 20 minutes. Intra-muscular (IM) immunizations were performed in thigh muscle with $2.5x10^6$ SIN particles in a volume of 50 µl. The mice were sacrificed 5 days following VV challenge for tissue collection.

Cervical lymph nodes (CLN), ILN, vaginal/uteral mucosal tissues (VUM) and spleens (SP) were harvested and pooled from 5 mice per group. Single cell suspensions were used for an ELISPOT assay to detect IFN-γ-secreting cells (IFNSC) specific for the p7g peptide derived from HIV-1 gag. Five days following vaginal or rectal challenge with VV-gag SP, CLN and ILN from groups of 5 immunized mice each were harvested and pooled. SP, CLN and ILN tissues were teased through a nylon mesh with the pore diameter of 250 µm and washed three times in media (ELISPOT assay media: RPMI containing 10% FCS, antibiotics, HEPES and L-glutamin (complete RPMI) counted and seeded into wells for ELISPOT assay. Single cell suspensions were prepared from VUM based on the method described by Johansson et al. (1998) *Infect. Immun* 66:514-520. This method routinely resulted in recovery of a minimum of $10^7$, minimum 90% viable, mononuclear cells (MNC) per five mice.

The p7g peptide derived from HIV-1 gag has been shown to be recognized by $CD8^+$ T cells (Doe & Walker (1996) *AIDS* 10:793-94). It was also demonstrated that peptide was only recognized by $CD8^+$ T cells and not by $CD4^+$ T cells, following p7g-specific IFN-γ intracellular cytokine staining of $CD4^+$ and $CD8^+$ cells after DNA immunizations. Single cell suspensions from pooled VUM, ILN, CLN or SP from 5 mice per group were prepared and adjusted to concentrations of $10^7$ to $3\times10^7$ per ml. 100 µl from each cell preparation was added onto the first row of 96 well nitrocellulose or pvdf plates (Milipore) in duplicates and 2-fold serial dilutions were performed. Following overnight incubation at 37°C the plates were washed and biotinylated anti-

IFN-γ (PharMingen) was added. The plates were then incubated at room temperature for 2 hours and washed. The plates were then added Avidin-peroxidase (PharMingen) and incubated for 30 min. at 37°C and washed. The plates were developed with aminoethyl carbazole solution (Sigma) for 30 min. Results from 3 independent experiments are presented as mean (±SD) of IFN-γ secreting cells per 10 million mononuclear cells (MNC) from a minimum of 4 wells from pools of 5 mice per group in duplicates.

**A. Mucosal and systemic CD8+ T cell responses after mucosal or systemic immunizations with SIN-gag followed by vaginal challenge with Vaccinia virus-gag**

Viral challenge following immunization not only enhances the immune response but also allows a correlation to be drawn between the immune response and protection. Therefore, mice were primed with SIN-gag through the IN, IM, IR or IVAG routes and then challenged with VV-gag. The mucosal and systemic CTL responses as well as protection from VV-gag replication in ovaries was measured. The mice immunized IN, IM and IVAG were challenged IVAG, while the mice that were immunized IR were challenged IR or IVAG. Results of mucosal and systemic $CD8^+$ T cell responses in VUM, ILN and SP of mice immunized IN or IM and challenged IVAG as measured by the IFN-γ ELISPOT assay are shown in Figures 11A and 11B, respectively. No $CD8^+$ T cell responses were detectable in CLN of either group. These responses were gag-specific since IVAG or IR challenge of naïve mice with VV-gag, or IVAG or IR challenge of SIN-gag immunized mice with VV-gp160 did not induce $CD8^+$ T cell responses. These data show that vaginal VV challenge of IN or IM immunized mice induced $CD8^+$ T cell responses in VUM and ILN.

After IR immunization with SIN-gag followed by IVAG challenge with VV-gag the highest $CD8^+$ T cell responses were found in ILN and SP, and relatively low responses were found in VUM (Figure 11C). Similarly, after IVAG immunization with SIN-gag followed by IVAG challenge with VV-gag, the highest $CD8^+$ T cell responses were found in ILN and SP, and relatively low responses in VUM (Figure 11D). The $CD8^+$ T cell responses observed after IVAG or IR immunizations and challenge were generally 10-fold lower compared to the $CD8^+$ T cell responses observed after IN and IM immunizations followed by IVAG challenge.

**B. Protection from local viral replication after mucosal immunizations with SIN-gag followed by vaginal challenge with Vaccinia virus-gag**

Protection from vaccinia virus replication in the ovaries of mice primed through various routes following IVAG or IR virus challenge, using a standard PFU assay was also tested. As shown in Figure 12, mice primed IM with SIN-gag were not protected from challenge with IVAG VV-gag. In addition, 5 of the 19 mice primed IN showed no evidence of VV replication in their ovaries. In contrast, following IVAG or IR challenge with VV-gag, mice primed IVAG or IR with SIN-gag were completely protected against IVAG (Figure 12) and IR challenge with VV-gag respectively, in that no vaccinia virus replication was detected in their ovaries. The control, naïve mice had high levels of VV in their ovaries after IR or IVAG challenge with VV-gag (Figure 12). Also, the control mice that were immunized IN, IM, IVAG and IR with SIN-gag and challenged IVAG with VV-gp160 had high levels of VV in their ovaries (Figure 12), demonstrating that the protection was gag-specific. Thus, local mucosal immunization, but not distant mucosal or systemic immunization, with SIN-gag replicon particles conferred maximum protection against vaginal viral challenge.

Thus, mucosal IVAG or IR immunization conferred protection against vaginal viral challenge. In addition, the data indicate that alphavirus based replicons provide an effective mechanism to induce such local protection. Comparison of the distant mucosal (IN), local mucosal (IVAG and IR) and systemic (IM) routes of immunization with SIN-gag replicon particles, followed by local mucosal challenge with VV-gag, demonstrated that the IN and IM immunization routes induced higher gag-specific mucosal $CD8^+$ T cell mediated responses in the vaginal mucosa (VUM), the draining lymph nodes (ILN), and in SP. However, IVAG and IR immunizations conferred maximum protection against vaginal challenge with VV-gag. Furthermore, the responses were gag-specific since immunization with SIN-gag followed by vaginal challenge with VV-gp160 did not induce any gag-specific IFN-γ secreting $CD8^+$ T cell responses, nor had any impact on VV replication in ovaries. IVAG challenge of naïve mice with VV-gag also failed to induce any detectable gag-specific IFN-γ $CD8^+$ T cell responses. Therefore, the induction of IFN-γ $CD8^+$ T cell responses was gag-specific and as a result of adaptive specific responses following SIN-gag immunization. VV-

gag challenge was also not mediated by humoral responses as indicated by the fact that gag-specific serum titers (as measured by ELISA two weeks after mucosal immunizations with SIN-gag replicons) were undetectable anti-gag serum. Thus, the observed protection was a result of gag-specific T cell-mediated responses.

In summary, the results demonstrate that the SIN replicon delivery system can be applied mucosally for the induction of $CD8^+$ T cell responses and protective immunity against HIV. These results have important implications for sexually transmitted pathogens in general as well as for therapeutic gene therapy against cervical, colon, and lung cancers.

EXAMPLE 6

IDENTIFICATION OF CELLS EXPRESSING HIV-1 GAG IN LOCAL AND SYSTEMIC LYMPHOID TISSUES

Cells that are involved in the uptake and expression of SIN-gag particles in local and systemic lymphoid tissues following mucosal immunization were identified by immunizing mice IN with a single dose of SIN-gag particles. Frozen sections from nasal associated lymphoid tissue (NALT), CLN and SP were stained with an antibody against gag. At 1 day post immunization many cells expressing gag both locally in NALT (Figure 9) and CLN (Figure 10) as well as systemically in SP (Figure 11) were found, although by far most cells appeared to be localized in SP. To identify the cells that express gag, cells were double strained with CD11b or CD11c as markers for monocyte lineage cells with potential APC activity, and B220 a maker for B cells. Most of the gag-expressing cells co-expressed CD11b, while only few co-expressed CD11c. Importantly, no $B220^+$ cells co-expressed gag and the $CD11b^+$ or $CD11c^+$ gag-expressing cells were in extra-follicular T-cell areas. Thus, at early time points following in immunizations $CD11b^+$ cells are the major cells with APC potential to express gag in both local and systemic lymphoid tissues.

EXAMPLE 7

HUMORAL RESPONSES IN SERA FOLLOWING INTRANASAL IMMUNIZATIONS

The ability of SIN replicons to induce antibody production was also determined.

A. HIV-derived antigens

Two weeks following 3 IN immunizations with SIN replicons expressing gag, serum titers were measured by ELISA (Figure 12). Additionally, prime-boost experiments were conducted. First, following 3 IN immunizations with SIN-gag mice were boosted IN three times at 4 weeks intervals with p24 plus LTK63. Additionally, experiments were conducted in which animals were primed with SIN expressing HIV envelope gp140 and boosted with Ogp140 plus the mucosal adjuvant LTR72 and CpG. In these particular cases, low or no levels of antibody titers were observed (Figure 10), indicating that the protection observed following vaginal or rectal immunizations with gag was not antibody-mediated.

B. Influenza-derived antigens

Two weeks following 1 or more mucosal (*e.g.*, IN, IVAG, IR) immunizations with SIN replicons expressing influenza polypeptide(s) (HA), serum titers are measured by ELISA. Additionally, prime-boost experiments are conducted. First, following mucosal immunizations with SIN-HA mice are boosted mucosally one or more times with HA plus LTK63. Additionally, experiments are conducted in which animals were primed with SIN expressing HA antigens and boosted with HA polypeptides plus the mucosal adjuvant LTR72 and CpG. SIN-HA replicons will induce antibody production when administered mucosally.

## EXAMPLE 8

## INDUCTION OF IMMUNE RESPONSES FOLLOWING MUCOSAL DELIVERY OF ALPHAVIRUS REPLICON PARTICLE CHIMERAS

To demonstrate the ability of alphavirus replicon particle chimeras to induce antigen specific immune responses following mucosal delivery, intranasal immunization experiments similar to those described above were performed. Specifically, replicon particle chimeras between SIN and VEE, as described in U.S. Serial Number 60/295,451, were constructed such that SIN replicon RNA was packaged within VEE envelope glycoproteins (SIN/VEE) or VEE replicon RNA was packaged

within SIN envelope glycoproteins (VEE/SIN). These replicon particles encoding HIV p55 gag antigen, as well as SIN replicon particles and VEE replicon particles also encoding the same HIV gag antigen were used to immunize mice intranasally.

The replicon particles were administered three times, at a dose of $2.5 \times 10^6$ particles in 25 ul, with an immunization schedule of days 0,14, and 28. At two weeks following the final immunization, spleens were removed and gag-specific cellular responses were determined by IFN-γ ELISPOT assay. As shown in Figure 13, each of the replicon particle preparations induced gag-specific responses, with some variation in immunogenicity.

Claims

What is claimed is:

1. A method of generating an immune response in a subject, comprising mucosally administering to target cells a first replication-defective gene delivery vehicle comprising a polynucleotide encoding at least one first antigen or modified form thereof, said first antigen or modified form thereof being capable of stimulating an immune response in the subject when administered mucosally.

2. The method of claim 1, wherein the mucosal administration is intranasal.

3. The method of claim 1, wherein the mucosal administration is intrarectal.

4. The method of claim 1, wherein the mucosal administration is intravaginal.

5. The method of any of claims 1 to 4, wherein the at least one antigen is derived from a sexually transmitted pathogen.

6. The method of claim 5, wherein the sexually transmitted pathogen is a bacteria.

7. The method of claim 6, wherein the bacteria is selected from the group consisting of gonorrhea, chlamydia and syphilis.

8. The method of claim 5, wherein the sexually transmitted pathogen is a virus.

9. The method of claim 8, wherein the virus is selected from the group consisting of HIV, HBV, HSV, HCV and HPV.

10. The method of claim 9, wherein the virus is HIV-1.

11. The method of any of claims 1 to 10, wherein the gene delivery vehicle is a nonviral vector.

12. The method of any of claims 1 to 10, wherein said polynucleotide is delivered using a particulate carrier.

13. The method of claim 12, wherein said polynucleotide is coated on a gold or tungsten particle and said coated particle is delivered to said subject using a gene gun.

14. The method of claim 12, wherein said polynucleotide is encapsulated in a liposome preparation.

15. The method of any of claims 1 to 10, wherein said gene delivery vehicle is a viral vector.

16. The method of claim 15, wherein said viral vector is a retroviral vector.

17. The method of claim 15, wherein said viral vector is an adenoviral vector.

18. The method of claim 15, wherein said viral vector is a poxvirus vector.

19. The method of claim 15, wherein said viral vector is a picornavirus vector.

20. The method of claim 15, wherein said viral vector is an alphavirus vector.

21. The method of claim 20, wherein said alphavirus vector is a Sindbis vector.

22. The method of claim 20, wherein said alphavirus vector is selected from the group consisting of Semliki Forest virus, Venezuelan equine encephalitis virus and Ross River virus vector.

23. The method of claim 20, wherein said alphavirus vector comprises sequences from two or more alphaviruses.

24. The method of any of claims 1 to 23, wherein the subject is a mammal.

25. The method of claim 24, wherein the mammal is a human.

26. The method of claim 20, wherein the alphavirus vector is delivered to antigen presenting cells.

27. The method of claim 26, wherein the antigen presenting cells are dendritic cells.

28. The method of claim 27, wherein the dendritic cells are human.

29. The method of any of claims 1 to 28, wherein the target cells are infected *in vivo*.

30. The method of any of claims 1 to 29, wherein the antigen elicits an HLA class I-restricted immune response.

31. The method of claim 30, wherein the antigen further elicits an HLA Class II-restricted immune response.

32. The method of any of claims 1 to 31, including, prior or subsequent to the step of administering to target cells, introducing into target cells a nucleic acid molecule which encodes either Class I or Class II MHC protein, or combinations thereof, or a protein selected from the group consisting of CD3, ICAM-1, LFA-3 or analogues thereof.

33. The method of any of claims 1 to 32, further comprising the step of administering at least one second gene delivery vehicle, said second gene delivery vehicle comprising polynucleotides encoding at least one second antigen or modified form thereof or an immunomodulatory factor.

34. The method of claim 33, wherein the second gene delivery vehicle is administered mucosally.

35. The method of claim 33, wherein the second gene delivery vehicle is administered non-mucosally.

36. The method of any of claims 1 to 35, further comprising the step of administering one or polypeptides to the subject.

37. The method of claim 36, wherein the polypeptides comprise at least one second antigen or modified form thereof.

38. The method of claim 36, wherein the polypeptides comprise an immunomodulatory factor.

39. The method of claim 36, wherein at least one of the polypeptides is administered mucosally.

40. The method of claim 36, wherein at least one of the polypeptides is administered non-mucosally.

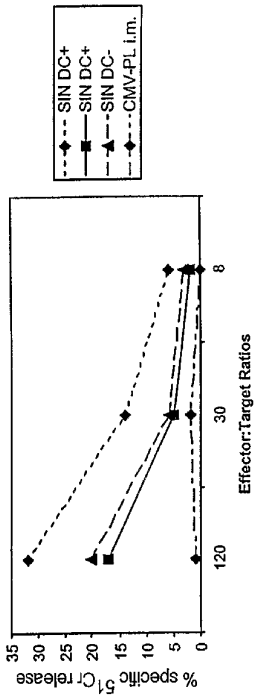
SIN DC+
SIN DC+
SIN DC-
CMV-PL i.m.
% specific 51Cr release
35
30
25
20
15
10
5
0
120
30
8
Effector:Target Ratios

FIG. 1

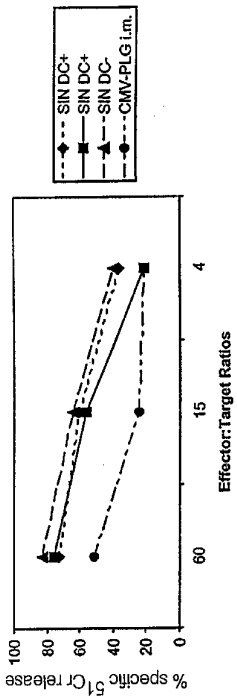
SIN DC+
SIN DC+
SIN DC-
CMV-PLG i.m.
% specific 51Cr release
100
80
60
40
20
0
60
15
4
Effector:Target Ratios

FIG. 2

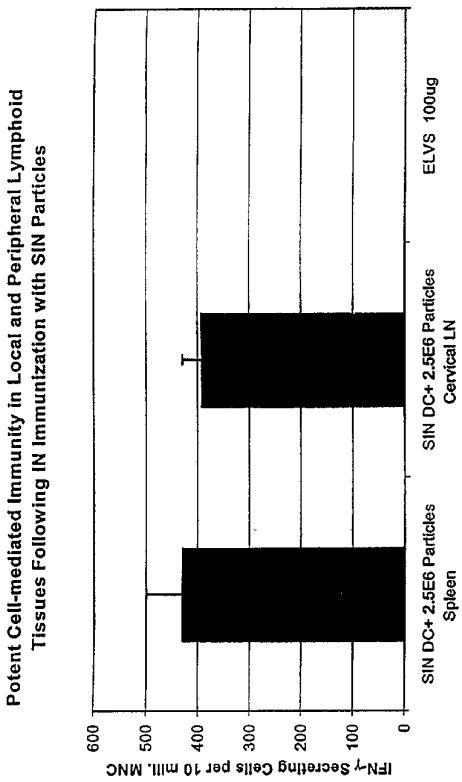
Potent Cell-mediated Immunity in Local and Peripheral Lymphoid Tissues Following IN Immunization with SIN Particles
IFN-γ Secreting Cells per 10 mill. MNC
600
500
400
300
200
100
0
SIN DC+ 2.5E6 Particles Spleen
SIN DC+ 2.5E6 Particles Cervical LN
ELVS 100ug

FIG. 3

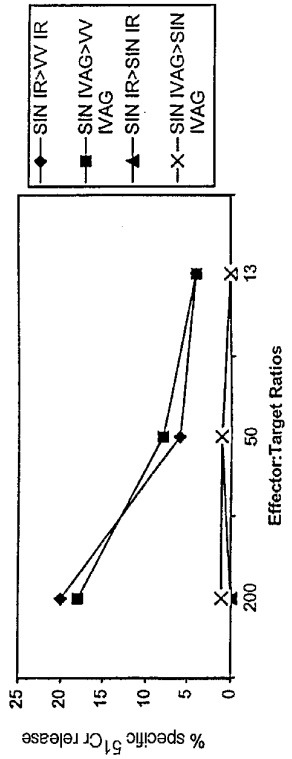
SIN IR>VV IR
SIN IVAG>VV IVAG
SIN IR>SIN IR
SIN IVAG>SIN IVAG
% specific 51Cr release
25
20
15
10
5
0
200
50
13
Effector:Target Ratios

FIG. 4

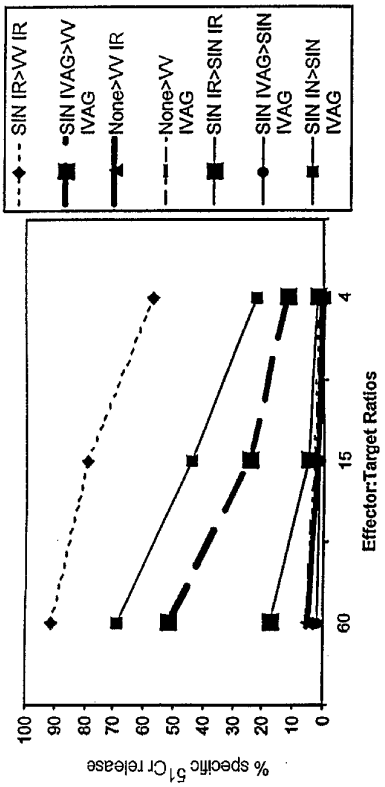
SIN IR>VV IR
SIN IVAG>VV IVAG
None>VV IR
None>VV IVAG
SIN IR>SIN IR
SIN IVAG>SIN IVAG
SIN IN>SIN IVAG
% specific 51Cr release
100
90
80
70
60
50
40
30
20
10
0
60
15
4
Effector:Target Ratios

FIG. 5

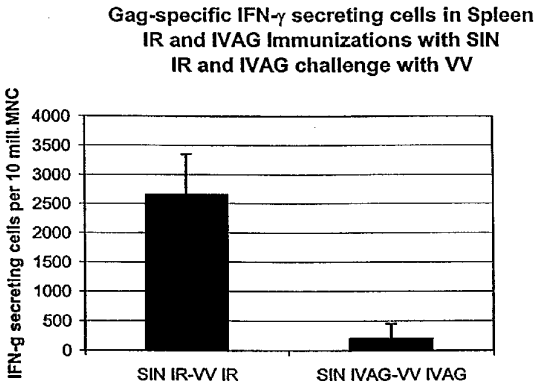
Gag-specific IFN-γ secreting cells in Spleen
IR and IVAG Immunizations with SIN
IR and IVAG challenge with VV
IFN-g secreting cells per 10 mill MNC
4000
3500
3000
2500
2000
1500
1000
500
0
SIN IR-VV IR
SIN IVAG-VV IVAG

FIG. 6

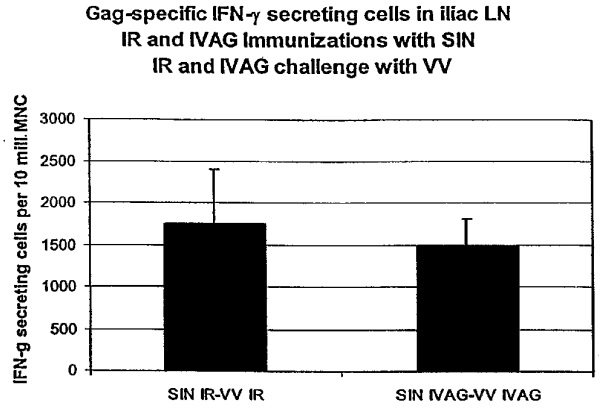
Gag-specific IFN-γ secreting cells in iliac LN
IR and IVAG Immunizations with SIN
IR and IVAG challenge with VV
IFN-g secreting cells per 10 mill MNC
3000
2500
2000
1500
1000
500
0
SIN IR-VV IR
SIN IVAG-VV IVAG

FIG. 7

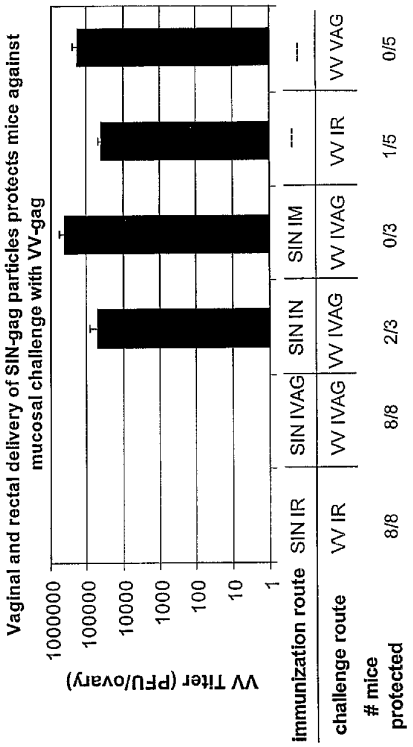
Vaginal and rectal delivery of SIN-gag particles protects mice against mucosal challenge with VV-gag
VV Titer (PFU/ovary)
1000000
100000
10000
1000
100
10
1
immunization route
SIN IR
SIN IVAG
SIN IN
SIN IM
---
---
challenge route
VV IR
VV IVAG
VV IVAG
VV IVAG
VV IR
VV VAG
# mice protected
8/8
8/8
2/3
0/3
1/5
0/5

FIG. 8

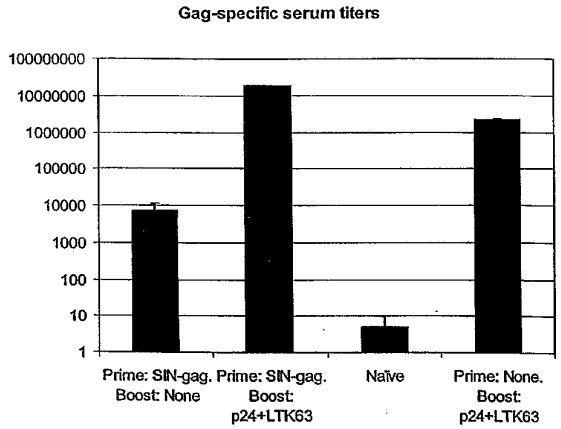
Gag-specific serum titers
100000000
10000000
1000000
100000
10000
1000
100
10
1
Prime: SIN-gag. Boost: None
Prime: SIN-gag. Boost: p24+LTK63
Naïve
Prime: None. Boost: p24+LTK63

FIG. 9

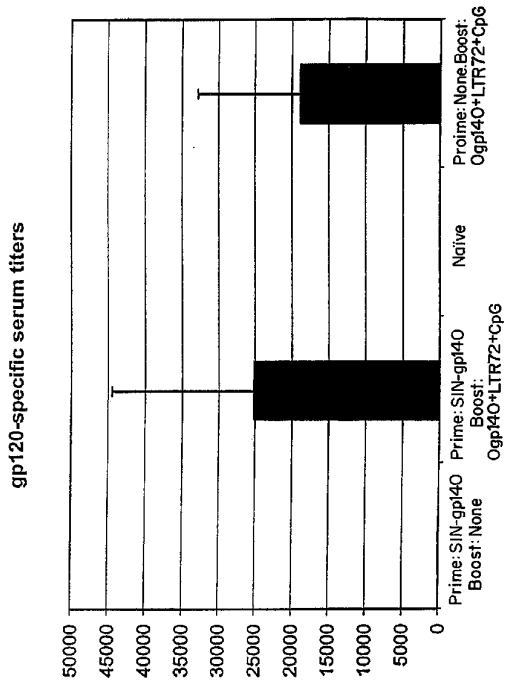
gp120-specific serum titers
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Prime: SIN-gp140
Boost: None
Prime: SIN-gp140
Boost:
Ogp140+LTR72+CpG
Naïve
Proime: None Boost:
Ogp140+LTR72+CpG

FIG. 10

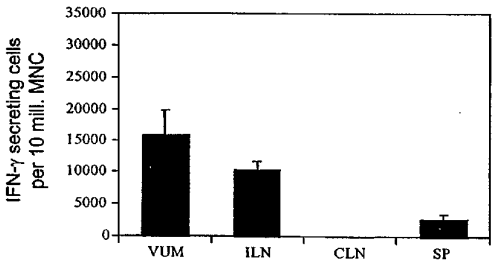


FIG. IIA

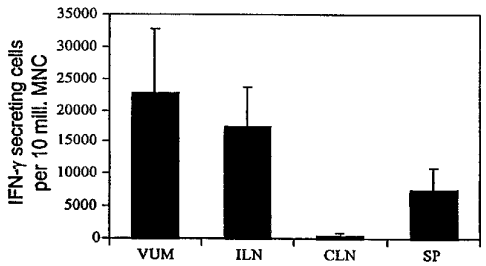


FIG. IIB

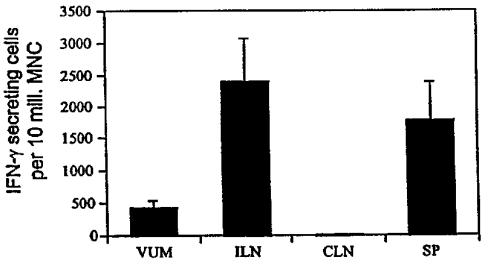


FIG. IIC

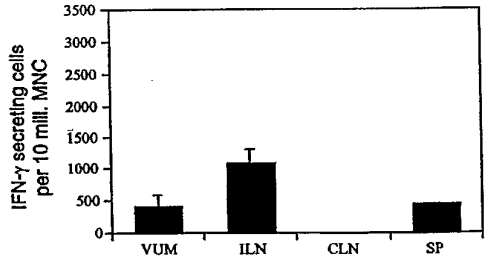


FIG. IID

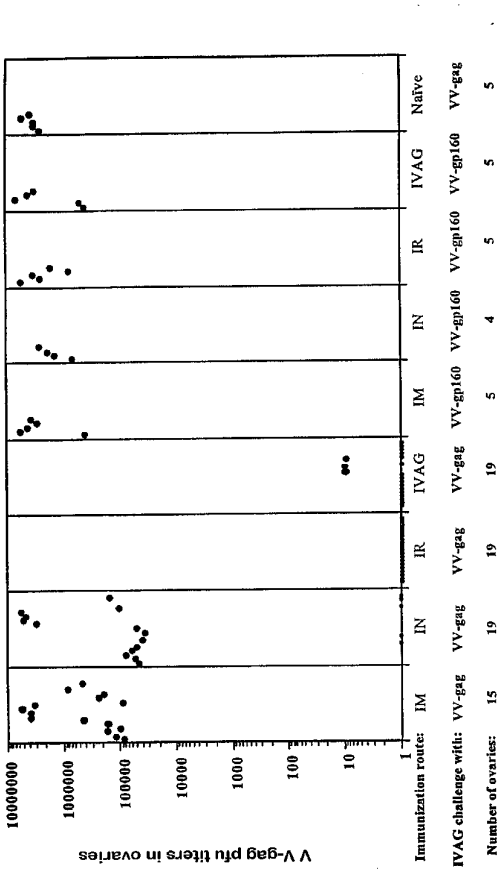
V V-gag pfu titers in ovaries
10000000
1000000
100000
10000
1000
100
10
1
Immunization route:
IM
IN
IR
IVAG
IM
IN
IR
IVAG
IM
IN
IR
IVAG
Naive
IVAG challenge with:
VV-gag
VV-gag
VV-gag
VV-gag
VV-gp160
VV-gp160
VV-gp160
VV-gag
VV-gag
Number of ovaries:
15
19
19
19
5
4
5
5
5
5

FIG. 12

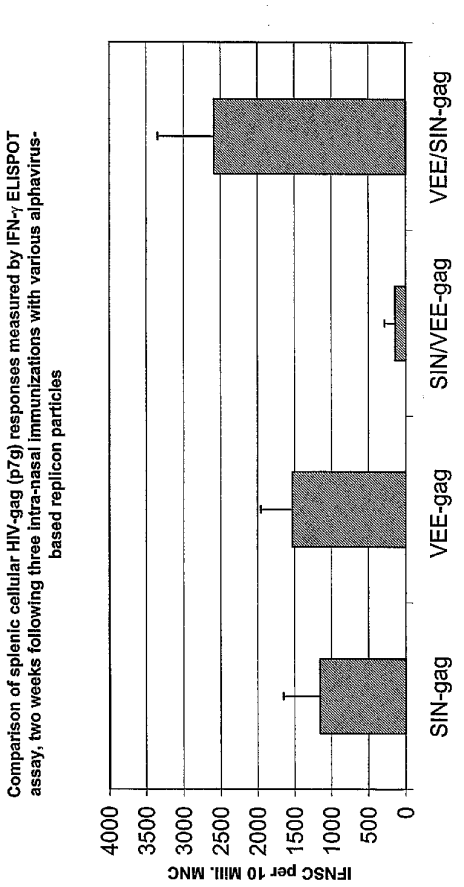
Comparison of splenic cellular HIV-gag (p7g) responses measured by IFN-γ ELISPOT assay, two weeks following three intra-nasal immunizations with various alphavirus-based replicon particles
IFNSC per 10 Mill. MNC
4000
3500
3000
2500
2000
1500
1000
500
0
SIN-gag
VEE-gag
SIN/VEE-gag
VEE/SIN-gag

FIG. 13

【国際公開パンフレット（コレクトバージョン）】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agents: **DOLLARD, Anne, S.** et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662 (US).

(81) Designated States *(national)*: 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.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, 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).

**Published:**
*with international search report*

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

WO 02/080982 A3

(54) Title: NUCLEIC ACID MUCOSAL IMMUNIZATION

(57) Abstract: Mucosal delivery of antigens using, for example, a replication-defective gene delivery vehicle, particularly replication-defective alphavirus vectors and particles, is described. Also described are compositions comprising a mucosal adjuvant and one or more antigens derived from HIV. Also provided is the use of these gene delivery vehicles in inducing mucosal, local and/or systemic immune responses following mucosal immunization regimes.

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/01031

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/21 A61K39/00 A61P31/18

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

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)
WPI Data, PAJ, EPO-Internal, BIOSIS, MEDLINE, EMBASE, SCISEARCH, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | BELYAKOV IGOR M ET AL: "Induction of a mucosal cytotoxic T-lymphocyte response by intrarectal immunization with a replication-deficient recombinant vaccinia virus expressing human immunodeficiency virus 89.6 envelope protein." JOURNAL OF VIROLOGY, vol. 72, no. 10, October 1998 (1998-10), pages 8264-8272, XP002212653 ISSN: 0022-538X page 8264 abstract --- -/-- | 1,5-19, 24,25, 29-40 |

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

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

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 6 September 2002 | 20. 11. 2002 |

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

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

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 02/01031

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KANO M. ET AL: "Induction of SIV-specific immune responses by using recombinant sendai viral vector" 7TH CONFERENCE ON RETROVIRUSES AND OPPORTUNISTIC INFECTIONS, [Online] 30 January 2000 (2000-01-30) - 2 February 2000 (2000-02-02), XP002212654 Retrieved from the Internet: <URL:http://www.retroconference.org/2000/abstracts/807.htm> [retrieved on 2002-09-05] | 1,2, 5-19,24, 25,29-40 |
| Y | abstract 807 | 20-23, 26-28 |
| Y | VILLACRES MARIA C ET AL: "Maintenance of CD8+ T-cell memory following infection with recombinant Sindbis and vaccinia viruses." VIROLOGY, vol. 270, no. 1, 25 April 2000 (2000-04-25), pages 54-64, XP002212655 ISSN: 0042-6822 page 54 abstract | 20-23, 26-28 |
| A | CALEY IAN J ET AL: "Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector." JOURNAL OF VIROLOGY, vol. 71, no. 4, 1997, pages 3031-3038, XP002212656 ISSN: 0022-538X page 3031, left-hand column, paragraph 1 -page 3032, left-hand column, paragraph 3 | |
| P,X | DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 2001 VAJDY MICHAEL ET AL: "Human immunodeficiency virus type 1 Gag-specific vaginal immunity and protection after local immunizations with Sindbis virus-based replicon particles." Database accession no. PREV200200304040 XP002212657 cited in the application abstract & JOURNAL OF INFECTIOUS DISEASES, vol. 184, no. 12, 2001, pages 1613-1616, ISSN: 0022-1899 | 1,2,5-40 |

-/--

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

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/01031

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | DATABASE BIOSIS [Online]<br>BIOSCIENCES INFORMATION SERVICE,<br>PHILADELPHIA, PA, US;<br>8 March 2001 (2001-03-08)<br>VAJDY MICHAEL SEJR ET AL: "Potent mucosal HIV-1 specific immunity and protection from vaginal viral challenge following mucosal immunizations with non-replicating Sindbis virus-based particles."<br>Database accession no. PREV200100258491<br>XP002212658<br>abstract<br>& FASEB JOURNAL,<br>vol. 15, no. 5, 8 March 2001 (2001-03-08), page A1192<br>Annual Meeting of the Federation of American Societies for Experimental Biology on Experimental Biology 2001;Orlando, Florida, USA; March 31-April 04, 2001<br>ISSN: 0892-6638<br>--- | 1,2,5-40 |
| A | CAIRNS JS AND SARVER N: "New viral vectors for HIV vaccine delivery"<br>AIDS RESEARCH AND HUMAN RETROVIRUSES, MARY ANN LIEBERT, US,<br>vol. 14, no. 17,<br>20 November 1998 (1998-11-20), pages 1501-1508, XP002133046<br>ISSN: 0889-2229<br>page 1505, left-hand column, paragraph 3 -right-hand column, paragraph 3<br>----- | |

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

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 02/01031

**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 1-40 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 dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

see additional sheet

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

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

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

1, 5-40 (all partially); 2 (completely)

**Remark on Protest**

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

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

International Application No. PCT/US 02/01031

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1, 5-40 (all partially); 2 (completely)

A method of generating an immune response in a subject by administrating via a mucosal route a replication defective gene delivery vehicle comprising a polynucleotide encoding a first antigen, and wherein said mucosal route is intranasal.

2. Claims: 1, 5-40 (all partially); 3 (completely)

A method of generating an immune response in a subject by administrating via a mucosal route a replication defective gene delivery vehicle comprising a polynucleotide encoding a first antigen, and wherein said mucosal route is intrarectal.

3. Claims: 1, 5-40 (all partially); 4 (completely)

A method of generating an immune response in a subject by administrating via a mucosal route a replication defective gene delivery vehicle comprising a polynucleotide encoding a first antigen, and wherein said mucosal route is intravaginal.

フロントページの続き

| (51)Int.Cl.[7] | FI | テーマコード（参考） |
|---|---|---|
| Ａ６１Ｋ 39/118 | Ａ６１Ｋ 39/12 | |
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| Ａ６１Ｋ 39/21 | Ａ６１Ｋ 39/245 | |
| Ａ６１Ｋ 39/245 | Ａ６１Ｋ 39/29 | |
| Ａ６１Ｋ 39/29 | Ａ６１Ｐ 1/16 | |
| Ａ６１Ｐ 1/16 | Ａ６１Ｐ 31/04 | |
| Ａ６１Ｐ 31/04 | Ａ６１Ｐ 31/12 | |
| Ａ６１Ｐ 31/12 | Ａ６１Ｐ 31/18 | |
| Ａ６１Ｐ 31/18 | Ａ６１Ｐ 31/22 | |
| Ａ６１Ｐ 31/22 | Ａ６１Ｐ 37/04 | |
| Ａ６１Ｐ 37/04 | Ａ６１Ｐ 43/00 １２１ | |
| Ａ６１Ｐ 43/00 | Ａ６１Ｋ 37/02 | |

(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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アメリカ合衆国 カリフォルニア ９４７０４, バークレイ, リージェント ストリート ２６２１ビー

Ｆターム(参考) 4C084 AA01 AA02 AA13 CA01 CA04 DC50 MA02 MA56 NA14 ZA751 ZA752 ZB091 ZB092 ZB331 ZB332 ZB351 ZB352 ZC551 ZC552 ZC751 ZC752
4C085 AA03 BA16 BA45 BA46 BA51 BA69 BA78 BA89 BA92 CC07 CC08 EE01 GG10
4C087 AA01 AA02 BC83 CA12 MA02 MA56 NA14 ZA75 ZB09 ZB33 ZB35 ZC55 ZC75