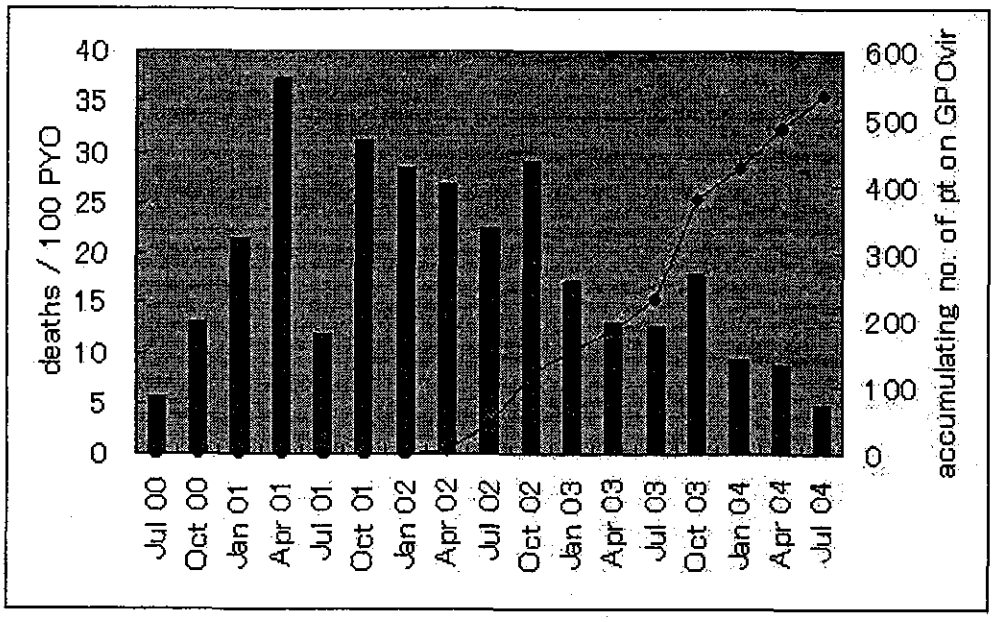
図1　ランパン病院 HIV 外来を受診した患者の死亡率の推移



棒グラフは，3 ヶ月ごとの死亡率（左 Y 軸）を示している．折れ線グラフは，GPOvir 治療を開始した患者の累積数を示している．

厚生労働科学研究費補助金（エイズ対策研究事業）

平成16年度分担研究報告書

研究課題　：　HIV-1 サブタイプ B'感染性分子クローンの樹立とその性状の解析

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研究要旨
東南アジアおよび中国における代表的な流行株のひとつであるサブタイプB'の感染性分子クローン、95MMB106.22を樹立した。このウイルスは、もとの分離株同様 CXCR4 と CCR5 の両方をコレセプターとして使用した。また PHA 刺激した PBMC や MAGIC5 でよく増殖し、中和試験や薬剤耐性試験の試薬として有用である。しかし、分離株が増殖する様々なT細胞株やマクロファージでは増殖を認めなかった。T 細胞株指向性ウイルスである NL432、マクロファージ指向性ウイルスである AD8 との組換体を用いた解析から、もとの分離株とのこれらの性質の差が、vpr 途中から nef5'側の間に原因があることが明らかになった。この領域を組換えることにより、ワクチンモデルとして有用なより広い宿主域を持つウイルスを得ることができると考えられた。

A．研究目的

これまでウイルス学研究に用いられてきた HIV-1 感染性分子クローンのほとんどがサブタイプ B（欧米型サブタイプ B）であった。我々は、東南アジア諸国における HIV-1 流行株がサブタイプは B'（アジア型サブタイプ B)、C、CRF01_AE とその組換体であることを報告してきた。中国雲南省においてもサブタイプ B' とその組換体が流行の中心である。また近年、中国内陸部の売血者におけるサブタイプ B' の流行が報告された。サブタイプは B' の感染性分子クローンの樹立は、サブタイプ間組換体の出現機構等東南アジアにおいて流行している HIV-1 のウイルス学的研究や、この地域における将来のワクチン開発にとって有用であり、今回、ミャンマーにおいて収集したサブタイプ B' 分離株から感染性分子クローンの樹立を試みた。

B．研究方法

分離株 B106 は、ミャンマーの首都マンダレーにおいて 1995 年に IDU による感染者から収集した全血から PBMC 共培養法により分離した。

ゲノム全長のクローンを得るため、分離株 B106 感染 PBMC から DNA を抽出、精製し、これを鋳型として、ゲノムの 5' LTR から pbs までの約 800 bp および pbs から 3' LTR までの約 9000 bp を PCR 法によって増幅後、2LTR form のゲノムがより安定にクローニングできるよう開発した pBR322 ベースの TA クローニングベクター pBRTA2 にクローニングした。系統樹解析により、サブタイプB'であることを確認した。pbs の NarI サイトを用いてこれらのフラグメントを結合させ、ゲノム完全長を含むプラスミドを作製した。さらに多くのクローンをスクリーニングするため、NarI から nef に存在する XhoI サイトまでの領域を、新たに PCR で増幅した DNA と置き換えたゲノム完全長のクローンを多数作製した。

感染性のスクリーニングは、得られたプラスミドをそれぞれ HeLa 細胞に transfect し、ウイルスの産生を確認した後、あらかじめ PHA で刺激した PBMC に感染させ、感染性を検討する方法で行った。coreceptor usage は、CD4 と CXCR4 または CCR5 を発現させた NP2 細胞におけるシンシチウム形成とウイルス産生を指標として決定した。T細胞株への感染性は、MT2、M8166、H9、Molt4、CEMx174、PM1、Jarkat 細胞で検討した。 マクロファージへの感染性の検討は、末梢血由来 CD14 陽性細胞を M-CSF で1週間誘導したもので行った。

C．研究結果

まず、gag p17 および env C2/V3 領域の系統解

析でサブタイプ B' と同定されていた B106 の pbs から 3' LTR までの約 9000 bp のクローン B106.3 の全塩基配列を決定して系統解析を行い、B106.3 がサブタイプ B' であることを確認した（図１）。

このクローンの構造蛋白のコーディング領域がすべて確認できたので、 5' LTR を結合させてゲノム全長のクローンを作製し感染性を検討したところ、PBMC への感染性は有していなかったが、transfection によりこれまでに樹立した感染性分子クローンと同等量のウイルスが産生されたので、LTR が active であると考えて、この全長のクローンの gag から nef までの領域を、新たに PCR で増幅した DNA と置き換えたクローンを作製し、スクリーニングを続けた。その結果 PBMC に感染しウイルスを産生する感染性分子クローン 95MM-B106.22 を得た（図２）。

このウイルスは、薬剤耐性試験でよく用いられる MAGIC5 細胞でも増殖した（図３）。NP2 細胞を使ってこのクローンの coreceptor 利用能を分離株 B106 と比較検討したところ、このクローンは、分離株同様 CXCR4、CCR5 のいずれを発現する細胞でも細胞変性を伴いながらよく増殖し、いわゆる X4/R5 virus であることが分かった（図４）。しかしながら、分離株がよく増殖するＴ細胞株やマクロファージには感染しなかった。

Ｔ細胞株への感染性の欠陥の原因となる領域を同定するために、Ｔ細胞株に高い感染性を示すサブタイプ B 感染性分子クローン NL432 とのキメラクローンを作製して M8166 細胞への感染性を検討したところ（図５）、NL432 由来の LTR と B106.22 由来の pbs から nef 5' 領域を持つクローン (NL/B106) は、B106.22 同様Ｔ細胞株には感染性を持たなかったが、逆に B106.22 由来の LTR と NL432 由来の pbs から nef 5' 領域を持つクローン (B106/NL) は、NL432 と同様の kinetics でシンシチウム形成を伴いながらよく増殖した。NL432 由来の領域を vpr 途中から nef5' だけにし、他の領域は B106.22 由来のクローン（B106/Nlenv）でも、NL432 と同等の kinetics でよく増殖したので、Ｔ細胞株への感染性に関して、B106.22 のこの領域に欠陥の原因があることがわかった。

またマクロファージへの感染性の欠陥の原因となる領域を同定するために、マクロファージに高い感染性を示すサブタイプ B 感染性分子クローン AD8 とのキメラクローンを作製してマクロファージへの感染性を検討した（図６）。B106.22 および NL432 の vpr 途中から nef5' を AD8 と置換したクローン（B106/AD8env 、 NL/AD8env） は 、 AD8 と比べて kinetics は遅いものの、マクロファージで増殖することができた。この結果から、AD8 のマクロファージでの増殖能の高さを決定する要因がこの領域以外にも存在するが、少なくともこの領域を置換することで、マクロファージへの感染性を付与することができることがわかった。。

D．考察

昨年報告した CRF08_BC の感染性分子クローンの場合と同様に、LTR が active なクローンをベースに２つの LTR に挟まれた領域をクローニングしスクリーニングする方法を取ったことで、効率良く感染性分子クローンを樹立することに成功した。

今回樹立したクローンは PBMC、MAGIC5 細胞でよく増殖するので、臨床分離株の薬剤耐性試験や中和試験などウイルス学的性状を調べるための研究に有用である。一方、分離株がよく増殖する Ｔ 細胞株やマクロファージには感染性を示さなかった。NL432 や AD8 との組換え体を用いた感染実験の結果、vpr 途中から nef5' までの領域に欠陥があり、ここを新しい PCR 産物と置換することで、より広い宿主域を持つクローンを樹立できる可能性があることを明らかにできたので、今後この領域を新しく調整したフラグメントと置換したクローンをスクリーニングし、分離株と同様の広い宿主域を持つクローンの樹

立を目指す。

さらに今後、中国におけるもう一つの重要な流行株である CRF07_BC についても、有用な感染性分子クローンの樹立を目指す。

E．結論

今回樹立したサブタイプ B'の感染性分子クローン B106.22 は、PBMC や MAGIC5 を用いたサブタイプ特異的なウイルス学的特性の解析に用いることは可能だが、今後 vpr 途中から nef5'側の領域を改変することにより、より宿主域の広い感染性分子クローンが得られる可能性があることを見いだした。このことは、将来のワクチン開発や、SHIV を用いた HIV-1 感染モデルの樹立にとって重要な知見であった。

F．健康危険情報

なし。

G．研究発表

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H．知的財産権の出願・登録状況

該当なし。

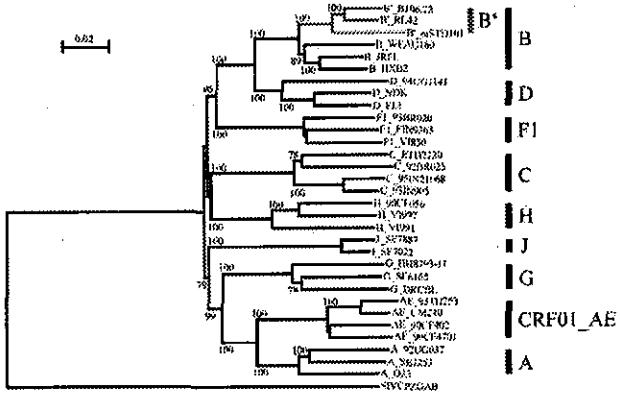

図1　B106 クローンの系統樹解析

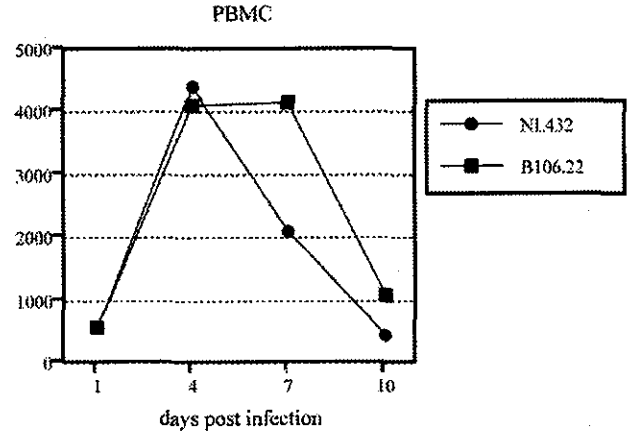

図2　B106.22 の PBMC における増殖

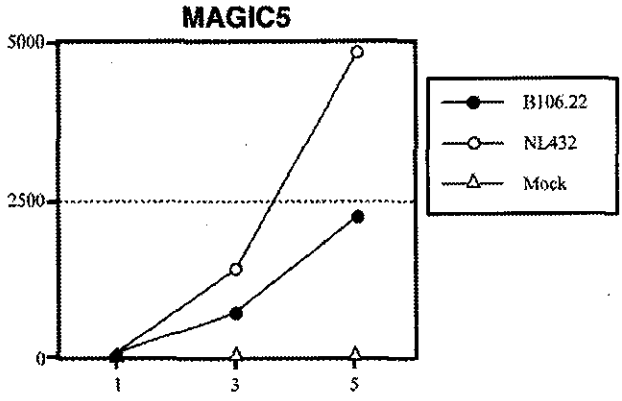

図3　B106.22 の MAGIC5 細胞における増殖

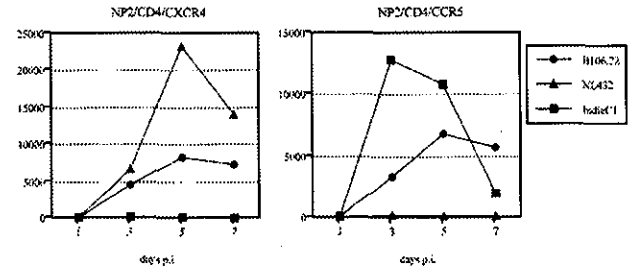

図4　B106.22 の NP2 細胞における増殖

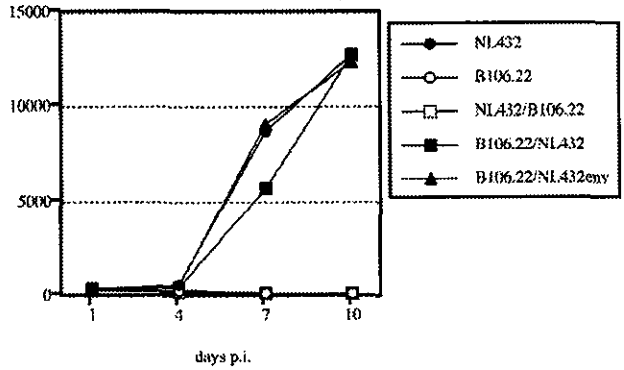

図5　B106.22 と NL432 のキメラウイルスの M8166 細胞における増殖

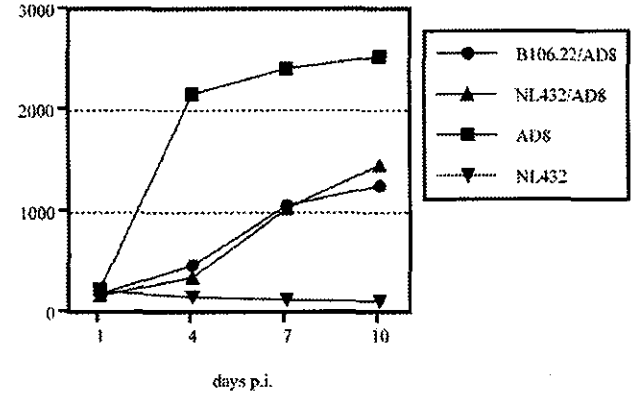

図6　B106.22 と AD8 のキメラウイルスのマクロファージにおける増殖

厚生労働科学研究費補助金（エイズ対策研究事業）
平成１６年度分担研究報告書

研究課題：タイ型 HIV-1 ヴァリアント CRF01_AE の高度薬剤耐性変異に関する分子進化学的解析とそれを用いたレトロウイルス組換え解析系の開発の試み

分担研究者：椎野　禎一郎（国立感染症研究所エイズ研究センター・主任研究官）

研究要旨

我々は、タイに起源をもつ CRF01_AE の国内の家族内感染例［NH1（父親）→NH2（母親）→NH3（子供）］のうち HAART 中に薬剤耐性の兆候を現した NH3 より、特殊な多剤耐性変異ウイルスを分離した。この多剤耐性は、逆転写酵素のフィンガー領域に起った 11 アミノ酸からなる挿入変異（69Ins）によって引き起こされていることが明らかとなった。NH3 より経時的に採取した検体を用いた逆転写酵素領域の分子進化学的解析から、この高度多剤耐性形質は 69Ins を含むフィンガー領域の変異群（M41L, 69Ins, T69I）とパーム領域の変異群（L210W, T215Y）間の遺伝子組換えによって生まれた可能性がある。高度多剤耐性の表現型を利用してフィンガー領域とパーム領域の間で組換えが生じたウイルスを選択できることに注目し、組換えウイルスのみを選択培養することによって、この領域に生じた交叉の位置を正確にマップすることを目指している。この目的のため、L210W, T215Y を持った CRF01_AE 感染性クローンのフィンガーとパーム領域間の約 400 ヌクレオチドの領域に存在する 20 個の制限酵素サイトに、それぞれアミノ酸配列を変えないように変異を導入した変異ウイルスクローンを作成し、その感染性を確かめた。このウイルスと、M41L, 69Ins, T69I を持った CRF01_AE 感染性クローンの共感染によって得られた組換えウイルスの大量解析の手法を確立するため、いくつかの実験を行った。

A. 研究目的

HIV-1 には、塩基配列によって系統的に分類可能なサブタイプのほかにサブタイプ間の組換えで生じたと思われるウイルスが存在する。こうした組換えウイルスの中には、従来のサブタイプと同様に感染を広げている型（circulating recombinant form: CRF）も見つかっている。特に東南アジアから東アジア地域ではこうした CRF が疫学的に重要な型となっており、HIV ゲノムが感染過程で組換えを起こす機構に注目が集まっている。最近、武部らはミャンマーや中国雲南省で組換えパターンの大きな多型性を持つ HIV-1 が感染した集団を見出した。こうした多型性は、HIV-1 の組換えがある条件下ではかなりの頻度で生じることを示している。これらのウイルスのゲノム構造には、組換えの際に交叉

が生じるサイトに共通点があるように見える。本研究は、HIV-1 の組換えの起きるゲノム上の位置を遺伝学的に解析し、ウイルスゲノムの組換えの機構を知ることを目的とする。

B. 研究方法

組換えの位置と頻度を遺伝学的に知るためには、ゲノム上に組換えが起こったことを示すマーカー遺伝子を置く必要がある。また、配列レベルでの解析を容易にするためには組換えを観察するゲノム領域は短いほうが良いが、１回の塩基配列解析で解析可能な領域で交叉が起きる確率は、非常に低いことが予測される。そのため、必要に応じて組換えを起こしたウイルスのみを的確に選択できる系が要求される。本研究では、疫学的調査で明確な組換えが観察されている逆転写酵素領域（RT 領域）に注目し、マーカー遺伝子として制限酵素切断サイトに同義置換変異を導入する。さらに、RT の抗ウイルス剤耐性変異の組み合わせを応用して、400bp 弱の領域に組換えが生じた時のみ次代のウイルスが採取できるような系を構築する（図１）。こうした組換え HIV-1 を用いて、さまざまな条件下での組換えのパターンを観察する。

実験に利用するウイルス株は，佐藤らによって作成された CRF01_AE の感染性クローン（93JP-NH1 ）を改変したものを用いた。我々は、このクローンと同系統のウイルス株に感染し抗ウイルス剤療法を受けた感染者（NH3）の血液検体の提供を受け、その RT 領域の変異を塩基配列と薬剤耐性の両面から観察した。薬剤治療後の検体にある耐性ウイルス株は、佐藤らによって報告された活性部位の大きな挿入配列と点突然変異（M41L, D67N, K70R, L210W, T215Y）によって、複数の NRTI に対して高い耐性を獲得している。多重薬剤に対する高耐性を発現するためには、これらの変異のすべてがそろう必要がある。挿入配列は、K70R の近傍に位置するため、M41L, K70R と挿入配列のみを持つウイルス株と、L210W, T215Y のみを持つウイルス株を作成し、両者を複数の NRTI 存在下で共感染させれば、これらの突然変異のおよそ 400bp の間に組換えが生じるか、または複数の点突然変異が生じた場合のみ、感染が成立する。組換えと新たな点突然変異を区別するために、これらのウイルス株では、K70R の 30bp 下流と L210W の 7bp 上流に存在する SacI の切断部位を同義置換で切断不能に改変した。さらに、L210W, T215Y を持つクローンを元に、400bp の中間部に存在し同義置換での改変が可能な 17 箇所の制限酵素切断部位をすべて改変したウイルスを作成した。これらのウイルスを用いた組換え交配実験を行うための、感染条件の探索・スクリーニング・構造解析の手法を確立している。

C. 研究結果

NH3 の経時採取検体の塩基配列解析（図２）は、多剤薬剤耐性ウイルスの出現以前に、既知の耐性変異を持つ一連のウイルスが存在していたことを示唆している。特に、挿入配列 (Ins69) と M41L,T69I を持つウイルスと、L210W, T215Y を持つウイルスはいずれも PBMC 中にその痕跡を認めることができる。こうしたウイルスが共感染することによって、多剤耐性変異株が発生した可能性が高い。

感染者 NH3 の血清中および PBMC より見出された RT 領域の塩基配列を元に、NH3 に存在した耐性変異の組み合わせを持つ９種類の 93JP-NH1 変異株を作成した。これらのウイルスの NRTI に対する耐性を MAGIC5 への感染性を用いて調べた結果、点突然変異は単独で AZT と 3TC への耐性獲得に効果があること、挿入変異は点

突然変異の存在するウイルスにおいて耐性を若干上昇させることがわかった（表 1）。前半の変異（M41L, D67N, 挿入配列, T69I）を持つウイルスと、後半の変異（L210W, T215Y）を持つウイルスは、いずれも AZT には野生型の約 6 倍、3TC は 3～4 倍、ddI は約 3 倍の耐性を示した。また、41L・挿入変異・69I・210W・215Y の５つの変異を持つ場合には、AZT, 3TC への極めて高い耐性が発現され、同時に d4T, ddI, ddC への耐性が獲得されることがわかった。

210W・215Y の２つの NRTI 耐性変異を持つクローンを元に、挿入変異部位（79）と 210W の間にある 17 箇所の制限酵素切断部位をすべて欠失させた変異クローン（NH1-WYDRS）を作成した（図３）。変異クローンは、93JPNH1 と同様の感染性と細胞指向性を持っていた（図４～７）。さらに、この制限酵素切断部位欠失クローンと、M41L, D67N, 挿入配列, T69I を持つ感染性クローン(NH1-LI)を HeLa 細胞に co-transfection し、NH1-WYDRS／NH1-LI 混合株を作成した。RT-PCR と制限酵素解析によって、この株内には NH1-WYDRS と NH1-LI のゲノムがおよそ 4:6 で含まれていることが確認できた（図 8)。

組換えウイルスゲノムを大量に構造解析するため、96well-microtiter plate を用いた細胞・上清からの RT 領域の RT-PCR～クローニング～塩基配列決定の手順を確立した。この過程で、従来の RT-PCR を用いたクローニング手法は、RT-PCR の配列複製の正確性が低いことと人為的な組換えが生じることから、実験結果の精度が目的にそぐわないことが明らかとなった（図８)。前者の問題は、信頼性の高い PCR 試薬を利用することで解決することがわかった。後者に関しては、DNA をテンプレートとした実験でも確認された（図９）ことから、PCR 反応中に生じていることがわかった。この組換えの機序は、部分的に増幅した断片が次の増幅過程に介在して生じる現象と推測し、伸長反応の条件を検討したところ、10 分間の反応を行うことで回避できることがわかった（図 10)。

NH1-WYDRS／NH1-LI 混合株を MT2 細胞に感染させて３週間後の培養液中のウイルスについて、薬剤感受性と RT 領域の遺伝子型を調べたところ、2.5μM の AZT 存在下で組換えウイルスが検出できた（図１１)。

１．D. 考察

組換え交配実験の親株とする予定のウイルスの薬剤耐性は、AZT, 3TC, ddI で野生型より高い耐性を示しているが、すべての変異がそろったウイルスに比べるとその耐性は明らかに低い。また、すべての変異がそろったウイルスは、d4T や ddC に対しても耐性を示す。したがって、複数の NRTI の濃度を注意深く設定することで、400bp の中間配列に組換えの生じたウイルスのみを効率的に選択することが可能と思われる。一方、こうした耐性は親株に少数の点突然変異が加わることでも獲得可能かもしれない。しかし、すべての NRTI に対する耐性を点突然変異のみで獲得するためには、多くの塩基置換が起こる必要がある。したがって、多剤薬剤耐性を選択の指標とすれば、点突然変異による残存ウイルスを除ける可能性が高い。さらに本実験では、耐性変異加えて制限酵素切断部位の欠失変異を設けているため、これを用いた選択を併用することで、点突然変異と組換えウイルスを区別することが可能である。このシステムを完成させ、組換えの生じる位置の詳細なマッピングを行いたい。

E. 結論

感染者 NH3 の体内には、過去に HAART 中に蓄積した点突然変異や単独の挿入変異を持つ耐性ウイルスが数種類存在していた。これらの変異ウイルスで組換えが生じることによりフィンガー側の変異（M41L, 挿入配列, T69I）とパーム側の変異（L210W, T215Y）を持つ多剤耐性ウイルスが生まれた可能性がある。フィンガー側の変異またはパーム側の変異のみを持つウイルスは、両方の変異を併せ持つウイルスに比べて明らかに薬剤感受性が高く、この感受性の差を選択実験に利用できる。41L・挿入変異・69I の３つの NRTI 耐性変異を持ち 69I と 210W の間にある同義置換での改変が可能な 17 箇所の制限酵素切断部位をすべて改変した変異クローンと、210W・215Y の２つの NRTI 耐性変異と直前の SacI サイトの欠失変異を持つ変異クローンを作成し、その感染性が元のウイルスと変らないことを確かめた。さらに、これらのクローンを種々の細胞に重複感染させ、組換えウイルスを効率よく得る方法について検討した。また、組換えウイルスの大量ゲノム解析を行うための手法を開発した。

なし

F. 健康危険情報

なし

G. 研究発表

論文発表

学会発表

なし

H. 知的財産権の出願・登録状況

平成１６年度 研究成果の刊行に関する一覧表

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Gene Therapy (2004) 11, 628–635

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RESEARCH ARTICLE

DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration

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Delivery of foreign genes to the digestive tract mucosa by oral administration of nonreplicating gene transfer vectors would be a very useful method for vaccination and gene therapy. However, there have been few reports on suitable vectors. In the present study, we found that plasmid DNA can be packaged in vitro into a virus-like particle (VLP) composed of open reading frame 2 of hepatitis E virus, which is an orally transmissible virus, and that these VLPs can deliver this foreign DNA to the intestinal mucosa in vivo. The delivery of plasmid DNA to the mucosa of the small intestine was confirmed by the results of immunohistochemical analyses using an expression plasmid encoding human immunodeficiency virus env (HIV env) gp120. After oral administration of VLPs loaded with HIV env cDNA, significant levels of specific IgG and IgA to HIV env in fecal extracts and sera were found. Moreover, mice used in this study exhibited cytotoxic T-lymphocyte responses specific to HIV env in the spleen, Payer's patches and mesenteric lymph nodes. These findings suggest that VLPs derived from orally transmissible viruses can be used as vectors for delivery of genes to mucosal tissue by oral administration for the purpose of DNA vaccination and gene therapy.
Gene Therapy (2004) **11**, 628–635. doi:10.1038/sj.gt.3302193
Published online 19 February 2004

Keywords: *VLP; oral DNA vaccine; CTL; HIV; mucosal immunity*

Introduction

The successful outcome of novel gene therapies and DNA vaccinations largely depends on the development of effective delivery systems.[1] In human applications, both the efficacy and safety of any delivery system used for gene transfer are major concerns. It has been shown that tissue-specific gene transfer by a viral vector could be achieved naturally and effectively through cell specificity of the virus receptors.[2] However, there is a risk of vector toxicity through viral infection of the host cells. Also, the limited sizes of transgenes often present a serious obstacle. Nonviral vectors, such as liposomes, are safer but do not have a cell-specific targeting component and have limited transduction both *in vitro* and *in vivo*. This limitation has been partly overcome by the development of molecular conjugates consisting of cell-specific ligands that confer cell specificity to nonviral vectors.[3,4]

The development of a system for delivering genes to or conferring immunity to mucosal tissue by oral administration would provide a convenient means for effective treatment or prevention of various human diseases, including cancers, infectious diseases and immunological disorders.[5] Since many pathogenic viruses and bacteria establish their initial infections through the mucosal surface, vaccine strategies that can stimulate mucosal immunity have been widely studied (reviewed in Ogra *et al*[6]). However, there are several difficulties in oral immunization with nonreplicating molecules, such as low pH in the stomach, the presence of proteolytic enzymes in the digestive tract and the presence of physical as well as biochemical barriers associated with the mucosal surface itself.[6]

Among the various nonreplicating molecules, a virus-like particle (VLP), an empty particle with a structure similar to that of an authentic virus particle, offers the possibility of a new approach for vaccine development.[7] It is expected that the VLP structure will provide resistance to severe environments in the digestive tracts and enable specific binding to the mucosal surface if an appropriate VLP is chosen.[8] However, VLPs can induce immune responses to themselves, and this is a problem for using VLPs as a vaccine vector to carry foreign DNA. A system using polyoma virus VP1 VLPs as a carrier of DNA by intranasal administration has been reported.[9] These VLPs work as an adjuvant, since DNA vaccine can induce immune responses by intranasal administration without VLPs. Hepatitis E virus (HEV) is an unclassified calicivirus-like, positive-strand RNA virus that causes

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Received 27 March 2003; accepted 21 October 2003

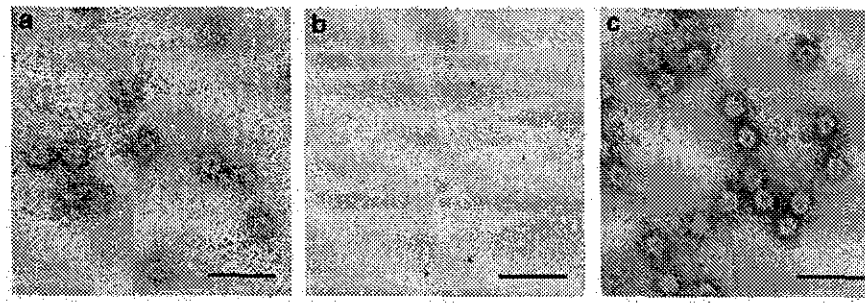

Figure 1 Electron micrographs of HEV-VLPs: (a) purified HEV-VLPs before treatment; (b) disassembled HEV-VLPs after treatment of VLPs with EGTA and DTT; and (c) refolded HEV-VLPs in the presence of $CaCl_2$, DMSO and DNA. Bars represent 50 nm.

human acute hepatitis by fecal–oral transmission. HEV first infects epithelial cells of the small intestine and then reaches the liver through the portal vein. It has recently been reported that overexpression of a part of open reading frame 2 (ORF2) in a baculovirus expression system results in the assembly of this protein into a VLP.[10] We have also reported that VLPs carrying foreign epitopes elicit strong mucosal and systemic immune responses to both the VLPs and exogenous epitopes without the requirement of any kind of adjuvant when orally administered to mice.[11]

Since infection with human immunodeficiency virus (HIV) most likely occurs through exposure of mucosal tissue to the virus, HIV-specific immune responses at mucosal sites are critical for the initial control of infection. Therefore, a nonreplicating vaccine vector that elicits mucosal immunity by oral administration would be a powerful HIV vaccine. In the present study, we found that unrelated plasmid constructs can be encapsulated into HEV-VLPs and delivered to the intestinal mucosa by oral administration. HIV DNA vaccine-loaded HEV-VLPs can elicit mucosal and systemic cellular as well as humoral immune responses by oral administration.

Results

In vitro refolding of VLPs

The HEV-VLPs produced by a recombinant baculovirus system were disassembled by the removal of calcium ions (Figure 1b). When calcium ions were supplemented to the disrupted VLPs in the presence of plasmid DNA, the DNA was encapsulated into the refolded VLPs (Figure 1c). No significant morphological difference due to the VLP disassembling–refolding process was observed under an electron microscope.

Density shifts of VLPs and amount of plasmid DNA after DNA encapsulation

Plasmid DNA encapsulation in the refolded VLPs was confirmed by CsCl equilibrium gradient centrifugation. VLP density is greater when loaded with a DNA plasmid. A heavier density gradient peak was present only when DNA was incorporated into the VLPs (Figure 2d). A single lighter density peak was produced for VLPs alone (Figure 2a), refolded VLPs (Figure 2b) and intact VLPs in the presence of plasmid DNA (Figure 2c). Despite the various sizes of plasmid DNA used for

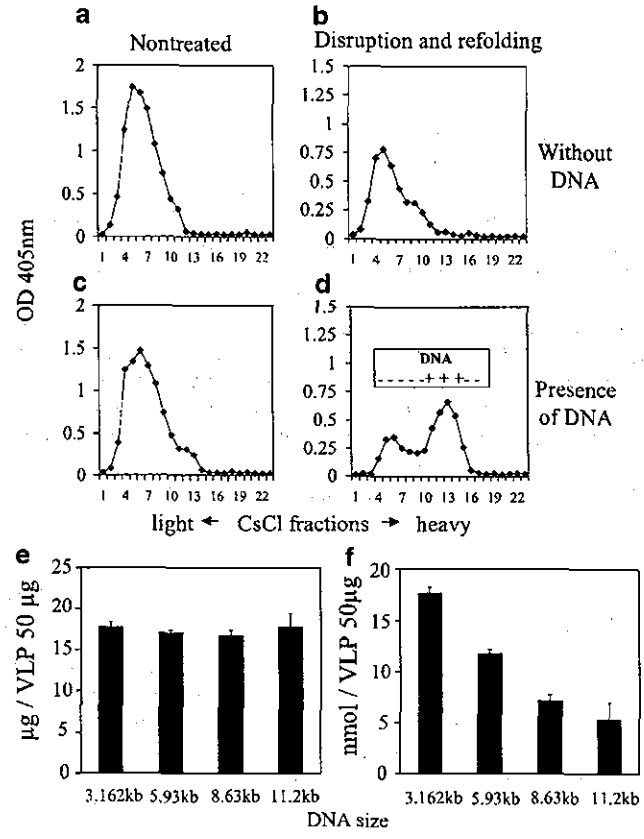

Figure 2 CsCl gradient profiles of intact and refolded VLPs. No DNA added: (a) intact; (b) refolded. DNA added: (c) intact; (d) refolded. The amount of DNA encapsulated in VLPs is expressed as μg (e) and molality (f) per 50 μg VLP protein.

encapsulation, the amounts of plasmid in VLPs were almost the same (17–19 μg per 50 μg of HEV-VLPs) (Figure 2e and f). A solution with a high concentration of plasmid DNA showed high viscosity, and VLPs including DNA were not obtained for general use in experiments. Based on these results, we used this amount (1 mg/ml) as the optimal concentration (data not shown).

Gene transfer by HEV-VLPs

Initially, four cell lines derived from mice, rabbits, monkeys and humans were studied for their ability to

transfer genes *in vitro*. The fluorescence of GFP-expressing cells was observed under a fluorescence microscope. Although the percentages of fluorescence-positive cells were not so high (11.2% of NIH3T3 cells, 19.6% of RK-13 cells, 21.0% of COS-7 cells and 20.1% of HepG2 cells), all of the cell lines used in this study showed positive reactions (Figure 3). In contrast, no fluorescence-positive cells were observed when the cells were incubated with plasmid DNA alone or intact VLPs in the presence of plasmid DNA (data not shown). We next tried gene transduction *in vivo*. Mice that had orally received a vaccine of DNA expressing HIV env gp120 of the NL432 strain (pJWNL432) that was encapsulated in VLPs were killed 2 days after immunization, and the expression of HIV env protein in the digestive tract was examined. HIV env protein was found in epithelial cells of the small intestine by immunohistochemistry (Figure 4), indicating that the HEV structure necessary for the entry of HEV into target cells had been preserved in refolded VLPs and that the DNA encapsulated in HEV-VLPs had been delivered to intestinal tissues.

Systemic and mucosal HIV-specific humoral immune responses in mice that had orally received a vaccine of HIV DNA encapsulated in VLPs

Mice were orally or subcutaneously immunized four times at 1-week intervals with pJWNL432 either naked or encapsulated in HEV-VLPs. The serum levels of HIV env-specific IgG antibodies in mice that had received loaded VLPs were significantly higher than those in mice that had received naked DNA ($P<0.05$ at 12 wpi, Figure 5a and e). Moreover, specific IgA was detected at high levels in sera of mice that had received loaded VLPs but not in sera of mice that had been immunized subcutaneously ($P<0.05$ at 12 wpi, Figure 5b and f). HIV env-specific IgA was only detected in fecal extracts of mice that had orally received pJWNL432-encapsulated HEV-VLPs (Figure 5d and h). No specific IgG was detected in any of the fecal samples (Figure 5c and g). The levels of HIV env-specific IgG antibodies detected in sera from subcutaneously and orally immunized mice were the same (Figure 5a and e). HEV-specific IgA was detected in both sera and fecal extracts of mice that had been orally administered VLP but not in sera or fecal extracts of mice that had been immunized subcutaneously (Figure 5j and l). Both orally and subcutaneously immunized mice showed HEV-specific IgG in sera (Figure 5i) and fecal extracts (Figure 5k).

Elicitation of HIV-specific cytotoxic T lymphocytes at systemic and mucosal sites by oral administration of a vaccination of HIV DNA encapsulated in VLPs

Cytotoxic T lymphocyte (CTL) responses in the spleen, mesenteric lymph nodes (MLN) and Payer's patches (PP) were investigated at 5 weeks after the first immunization. Mice that had orally received pJWNL432 encapsulated in HEV-VLPs showed HIV env epitope-specific CTL responses in the spleen, MLN and PP, whereas cells from the same tissues in mice that had received naked DNA vaccine did not show any CTL activity (Figure 6a). The P18 peptide is a dominant HIV env CTL and Th cell epitope in BALB/c mice and is restricted to the H-2D^d allele. These effector cell functions derived from our experiments were inhibited by either anti-CD8 or -H-2D^d monoclonal antibody (mAb) (Figure 6b,c), indicating that oral immunization of mice with a vaccine of HIV env DNA-encapsulated HEV-VLPs elicited CD8$^+$ and MHC class I-restricted CTLs both locally and systemically.

Discussion

A large number of pathogens gain access to the human body via mucosa such as oral, nasal or genital mucosa. The best defense against these predominantly mucosal

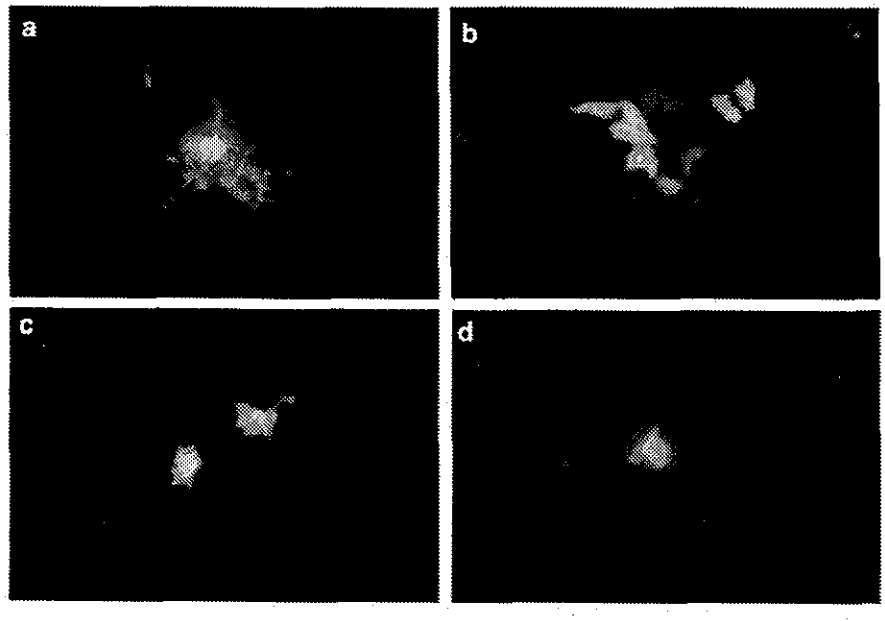

Figure 3 Expression of GFP in cells transfected with plasmid DNA encapsulated in HEV-VLPs: (a) NIH/3T3 cells (mouse); (b) RK-13 cells (rabbit); (c) COS-7 cells (monkey); and (d) HepG2 cells (human).

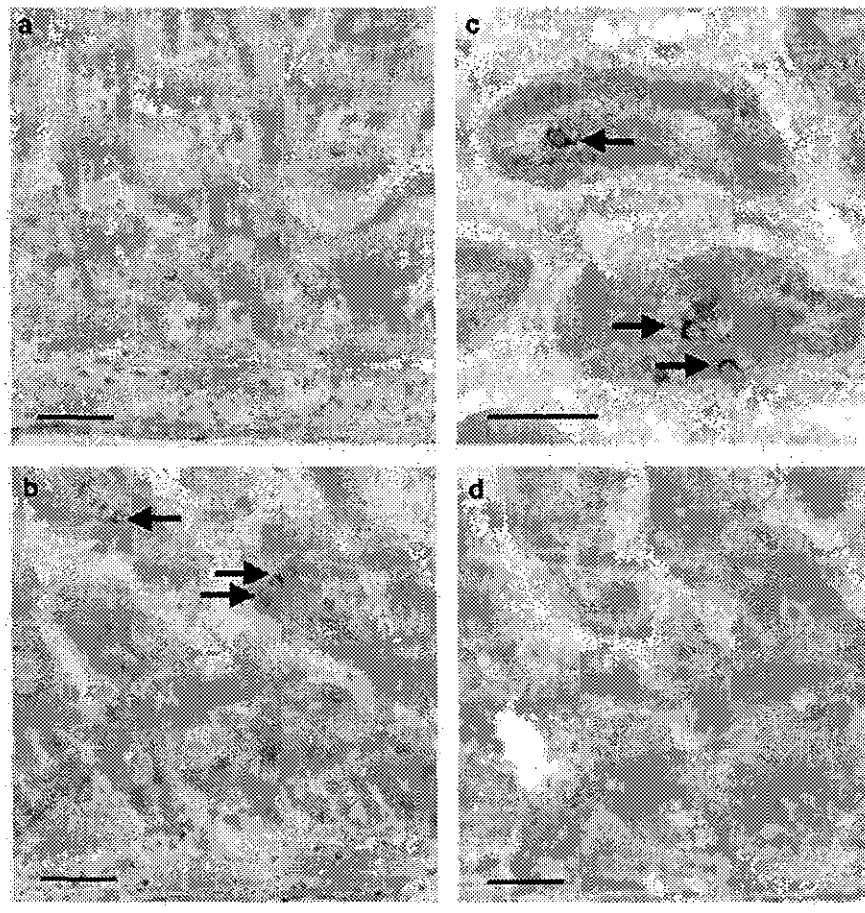

Figure 4 Immunostaining of serial sections of small intestine tissue from mice 2 days after oral administration of pJWNL432-encapsulated VLPs. HIV env proteins were observed in epithelial cells (arrow) (b, c), and control mAb did not show any positive reactions (d). Control mice were also administered pJWNL432 without VLP encapsulation (a). Bar marker represents 50 μm.

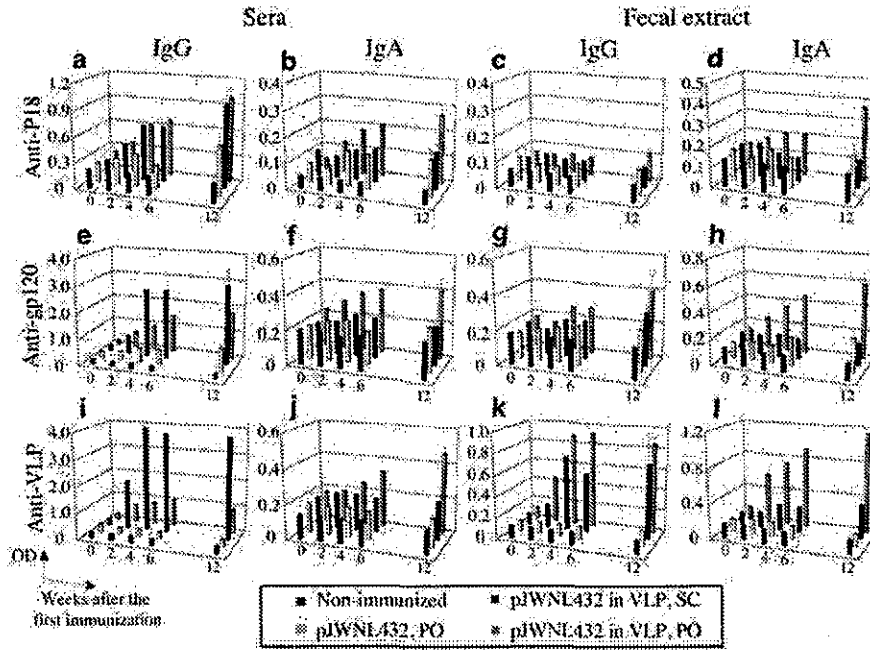

Figure 5 IgG (a, c, e ,g, i and k) and IgA (b, d, f, h, j and l) levels in sera (a, b, e, f, i and j) and fecal extracts (c, d, g, h, k and l) of immunized mice. Mice were orally (■) or subcutaneously (▨) administered pJWNL432 encapsulated in VLP or naked (■). Symbols indicate HIV env-specific antibody levels. Background levels to HIV env in nonimmunized mice (▩) are also shown. The IgG and IgA antibody levels are expressed as OD at dilutions of 1:100 and 1:2 for serum and fecal extracts, respectively. The mean OD values ±s.e.s were obtained from five mice/group.

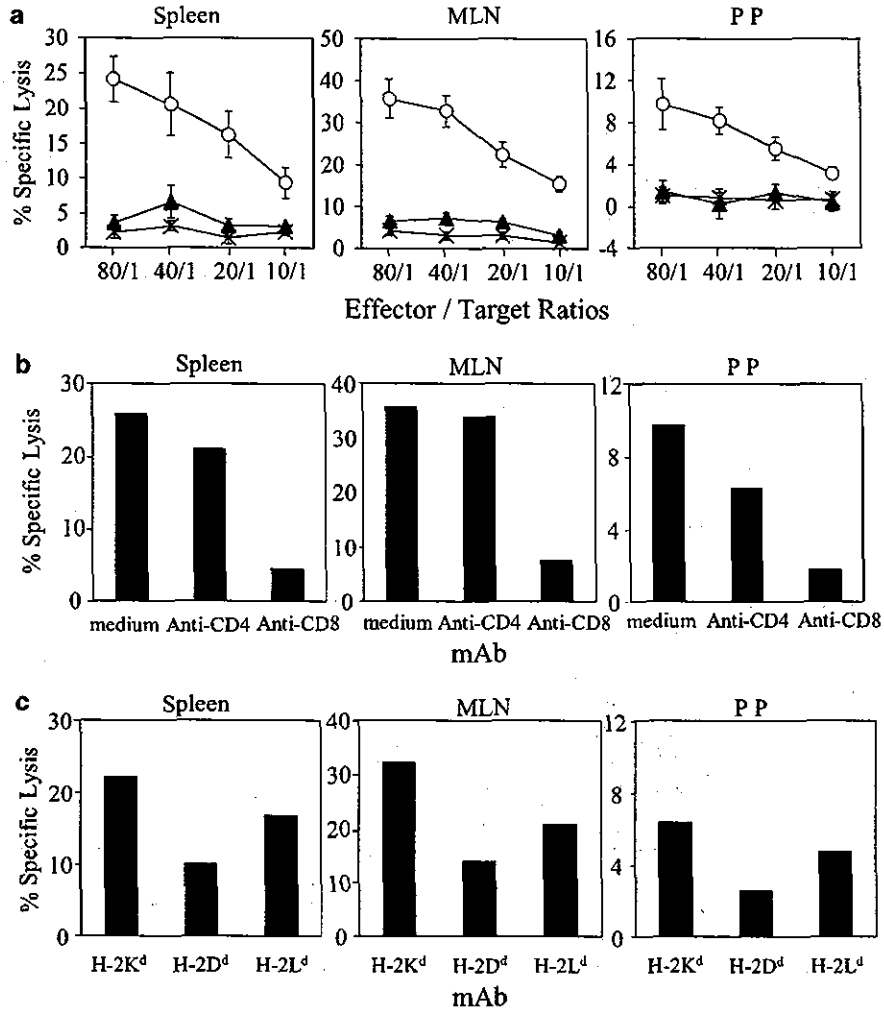

Figure 6 Spleen, MLN and PP cells from mice orally administered pJWNL432-encapsulated VLPs elicited CTL. (a) Mice were orally administered pJWNL432 encapsulated in VLPs (circles) or naked (triangles). Results for nonimmunized controls are also shown (×). (b) Effector cells obtained from the spleen, MLN and PP cells of mice orally administered pJWNL432-encapsulated VLPs are mediated CD8+ cells. Lytic activities of effector cells were assessed in the presence of anti CD4 mAb, anti-CD8 mAb or medium. Effector:target ratio was 80:1. (c) HIV env-specific lysis was restricted by MHC class I. Effector cells were examined for P18-specific lytic activities in the presence of anti-H-2K^d, anti-H-2D^d or H-2L^d mAb. The percentage of P18-specific lysis was calculated as (% lysis of target cells labeled with P18)−(% lysis of target cells labeled with control peptide). Each value is the mean percentage of the specific lysis values obtained from five mice.

pathogens is mucosal vaccines that are capable of inducing both systemic and mucosal immunity. Recent evidence has shown that DNA vaccination can confer protection against a number of infectious agents, including viruses and bacteria, although peripheral immunization with naked DNA is less than optimal for stimulating mucosal immunity.[12,13] In fact, it is quite difficult to induce both mucosal and systemic immune responses by oral administration of naked DNA. This study demonstrated that an orally administered DNA vaccine encapsulated in an orally transmissible virus-derived VLP induced both mucosal and systemic immunity.

The delivery of a DNA vaccine for induction of mucosal immune responses is usually achieved by gene transfer to the upper nasopharynx-associated lymphoid tissue (NALT), upper airway, salivary glands and tonsils.[5,14] Despite its obvious convenience, oral administration is rarely successful, since it is quite difficult to protect plasmid DNA from the environment in the digestive tract. The efficacy of orally delivered DNA vaccine to NALT is improved by encapsulating plasmid DNA in poly (lactide-coglycolide) (PLG) microparticles for protection against the gastric environment.[15,16] The immune responses to particle-borne DNA immunizations by means such as utilization of a gene gun or PLG differ from those to DNA immunizations without particles.[13] It is thought that the microparticles are actively taken up by cells such as macrophages or M cells of PP of the small intestine and thus facilitate the presentation of antigens to local immune systems.[15,17] This mechanism is the same as that of gene gun immunization of a DNA vaccine, that is, phagocytic cells such as macrophages or dendritic cells take up plasmid DNA delivered by a gene gun. The delivered gene is expressed only in these cells.[18] Similarly, only mucosal immunity was induced in mice by oral administration of DNA-encapsulated PLG microparticles.[15,16] It is likely that the mechanism underlying immune recognition of

HEV-VLP infection is similar to that of direct intramuscular or subcutaneous DNA immunization without the use of particles. Protein expressed by HEV-VLP-infected cells is recognized by the immune surveillance system, resulting in the elicitation of Ag-specific immune responses. We showed in this study that genes could be expressed in epithelial cells in the small intestine after delivery by HEV-VLPs (Figure 4). It is plausible that HEV-VLPs, which are derived from an orally transmissible virus, were incorporated into HEV-permissive epithelial cells in the small intestine, because they retained structures and properties similar to those of HEV particles, producing an infection similar to that induced naturally.[19] The Ag-expressing cells might be recognized by intraepithelial lymphocytes or submucous antigen-presenting cells by the same mechanism as that in the case of general virus infection.

An HEV-VLP has several advantages as a vector of DNA. Firstly, in our experience, large amounts can be easily obtained from standard cultivation protocols compared with the amounts of other VLPs obtained. The yield of purified HEV-VLPs collected from a culture supernatant of 50–100 μg/ml is more than 100 times greater than that of other VLPs. Secondly, the outcome of gene delivery in humans can be predicted using conventional laboratory animals, since HEV naturally infects various animals as well as humans through the same infectious route and target cells.[10,20] Thirdly, HEV-VLPs are stable at room temperature. Fourthly, anti-HEV immune responses had no effect on DNA administration in the present study, and this might be related to the neutralizing antibody for preventing infection with HEV. Neutralizing antibodies to HEV for inhibiting infection have not yet been found. This is also the case for HCV. The mechanism by which HEV is eliminated by antibodies is thought to be antibody-dependent cell-mediated cytotoxicity (ADCC). The effect of induction of immune responses to DNA vaccine in our system is not clear. Thus, HEV-VLPs are an attractive vaccine vector in developing countries because these VLP can be preserved without the requirement of any particular equipment. Finally, we have reported that an HEV-VLP can carry foreign amino-acid sequences as a part of the ORF2 protein exposed on the particle surface without any morphological or biological alteration.[10] Liposomal vectors resembling retroviral envelopes endowed with targeting molecules for gene delivery have been reported. The vicronectin receptor, $\alpha_v\beta_3$-integrin, is commonly upregulated on malignant melanoma cells, and liposome carrying an Arg-Gly-Asp (RGD) integrin-binding motif has been used for a system to deliver DNA to these tumor cells.[21] It has also been reported that targeting DNA to M cells by intranasal administration for the induction of mucosal and systemic responses can be achieved by formulating DNA with polylysine linked to viral adhesion.[22] It may be possible to design chimeric ORF2 proteins carrying these targeting molecules to retarget HEV-VLP to particular cell types.

Oral vaccination has obvious advantages for a field trial in a large-scale public health vaccination program.[23] From a practical standpoint, oral administration is less stressful for vaccine recipients and does not require professional skill for the vaccine administration. Moreover, delivery of vaccines via the intestinal tract is considered to be inherently safer than systemic injection. Encouraging results of phase I trials using Norwalk virus VLPs have recently been reported.[24] Trials using DNA vaccines for infectious and malignancy diseases have also been conducted.[25] The results of the present study suggest that oral administration of DNA vaccine encapsulated in oral transmissible virus VLPs, HEV-VLPs, is effective for inducing both humoral and cellular immunity locally as well as systematically. HEV-VLPs might be useful not only for vaccination but also as a vector in human gene therapy.

Materials and methods

Mice

BALB/c female mice were purchased from Clea Japan (Tokyo, Japan) and were housed in the Laboratory Animal Center of Mie University School of Medicine during the experimental period.

Peptide synthesis

The peptides used in this study were the HIV env CTL epitope (HIV 308-322, RIQRGPGRAFVTIGK; P18)[26] and a control peptide (HCV nonstructural protein 5 CTL epitope MSYSWTGALVTPCAAE; P17).[27]

Plasmid DNA

A highly efficient mammalian expression vector, pJW4303,[28] was used for efficient expression of HIV env gp120 of the NL432 strain.[29] Various sizes of plasmid DNA were also used for the *in vitro* packaging experiment (3.162 kb: pUC118; 5.93 kb: pJW322; 8.63 kb: pJWSIVenv; 11.2 kb: pABWN).

Production and purification of HEV-VLPs

HEV-VLPs were produced and purified by previously described methods.[10,11] Briefly, Tn5 cells maintained in Excel 405 serum-free medium (JRH, KS) were infected with the recombinant baculovirus expressing HEV-ORF2 at an m.o.i. of >5 and cultured for 6 days. The supernatant was harvested and the recombinant baculovirus in the supernatant was pelleted by ultracentrifugation at 10 000 *g* for 30 min at 4°C. The VLPs in the supernatant were collected by further ultracentrifugation at 100 000 *g* for 2 h at 4°C. Pelleted VLPs were then resuspended in 10 mM potassium-[2-(*N*-morpholino) ethanesulfonic acid] (MES) buffer (pH 6.2) and purified on a CsCl equilibrium density gradient. The purified HEV-VLPs were spun down and resuspended in potassium-MES buffer and kept at 4°C.

DNA packaging

Plasmid DNA was encapsulated into HEV-VLPs according to a previously described procedure.[30] Purified VLPs (50 μg) were disrupted by incubation in 180 μl of a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA and 20 mM dithiothreitol. Following 30 min of incubation at room temperature, 200 μg (20 μl) of each plasmid in 50 mM Tris-HCl buffer (pH 7.5) and 150 mM NaCl was added. The disrupted VLP preparation was refolded by incubation for 1 h with increasing concentrations of $CaCl_2$ up to a final concentration of 5 mM. VLPs were pelleted by ultracentrifugation and resuspended in 10 mM potassium-MES buffer (pH 6.2). At each step, the VLP structure formation was confirmed by electron

microscopy after negative staining, as described previously.[11] To estimate the amounts of encapsulated plasmid DNA, refolded and purified VLPs were treated with 10 IU benzonase (SIGMA-ALDRICH, Irvin, UK) for 1 h at 20°C to remove DNA on the surfaces of VLPs and disrupted with EGTA (1 mM). Absorbance of the supernatant was measured for detection of plasmid DNA contents.

Density analysis of refolded VLPs

Refolded VLPs were separated on a CsCl equilibrium density gradient and fractioned into 0.2 ml aliquots. HEV-VLPs in each fraction were detected by ELISA as previously described,[10] as well as DNA contents.

Gene transfer in mammalian cells

Four cell lines (NIH/3T3 (mouse), RK13 (rabbit), COS-7 (monkey), HepG2 (human)) were used in transfection experiments. Sterilized coverslips were placed in six-well plates, and 5×10^5 cells per well were seeded in the plates. After overnight culture, cells were washed twice with a medium, and about 1 μg of VLP-encapsulated EGFP expression vector (BD Bioscience Clontech, CA, USA) diluted with 0.5 ml medium was added. After 2 h of incubation at 37°C, VLPs were removed. Cells were then incubated for 48 h at 37°C. At the end of the culture period, cells were removed from the culture medium and washed three times with PBS. Coverslips were then mounted onto microscope slide glasses. Fluorescence of the GFP-expressing cells was observed under a fluorescence microscope.

Immunization

Mice were orally immunized four times with 50 μg protein of HEV-VLP/DNA (pJWNL432) complex or 20 μg naked pJWNL432 DNA in 100 μl of potassium-MES buffer at 1 week intervals.

Immunohistochemical analysis

At 2 days after oral immunization, the mice were killed and tissues were collected. Cryostat sections were air-dried and incubated in 0.5% HIO_4 for 10 min to quench endogenous peroxidase activity. The sections were further pretreated with chicken anti-mouse IgG antibody (Chemicon International, Inc., CA, USA) to prevent nonspecific reactions of a secondary antibody. The sections were then incubated with an HIV env-specific mAb (HIV-1 IIIB gp120 mAb (902)), which was obtained through the AIDS Research and Reference Reagent Program,[31] for 30 min at 37°C. The bound antibodies were visualized with a biotinated secondary antibody, HRP-labeled avidin–biotin complex (ABC-peroxidase staining kit, Elite Vector Lab. Inc., CA, USA) and 3.3′-diaminobenzidine tetrachloride with 0.01% H_2O_2. Sections were slightly counterstained with hematoxylin. An mAb (A1/3D1, ANOGEN, Canada) against hepatitis C virus core, which is same isotype to 902, was used as a control.

ELISA

Serum and fecal samples were collected at 0 (preimmunization), 2, 4, 6 and 12 weeks after the first immunization. Feces were suspended in ice-cold PBS at 200 mg/ml, and the centrifuge supernatant was used as fecal extract. Culture plates (96-well) were coated with purified HEV-VLPs or synthesized oligopeptides (P18) at a concentration of 10 or 100 μg/well, respectively, overnight at 4°C followed by 30 min of blocking with PBS containing 0.1% FBS and 0.05% Tween 20. To determine the anti-HIV env gp120 antibody responses, CV-1 cells were seeded in 96-well plates and infected with recombinant Sendai virus expressing HIV env gp120 of NL432 strain (SeV gp120),[32] and then the plates were incubated at 37°C. At 3 days after infection, plates were washed and fixed with PBS containing 10% formalin for 10 min. Test samples were added to each well and incubated at room temperature for 1 h. For detection of anti-HIV env gp120 antibody, test samples were reacted with wild-type Sendai virus-infected CV-1 cells before addition to the wells to eliminate the nonspecific antibody. Biotin-labeled anti-mouse IgG (Vector, CA, USA) or IgA (CALTAG, CA, USA) was used as the detection antibody. Following 1 h incubation, the plates were washed and further incubated with avidin-HRP (Vector, CA, USA). The reaction was developed using an ABTS substrate (Roch Diagnostic, Mannheim, Germany).

Generation of CTL effector cells

Effector cells were derived from spleen, MLN and PP cells as precursor CTLs. Aliquots of 5×10^6 spleen cells were co-cultured with 2.5×10^6 mitomycin C-treated autologous spleen cells labeled with a peptide at 37°C in a CO_2 incubator. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

Target cells, A20.2J cells (2×10^6), were incubated at 37°C in a 5% CO_2 atmosphere with 10 μg/ml of P18 or control peptide for 16 h. The target cells were then washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as ((experimental release−spontaneous release)/(100% release−spontaneous release)) × 100. All the experiments were performed at least four times, and each experimental group consisted of five mice.

Blocking of cytolysis

^{51}Cr-labeled target cells (10^6 cells) were preincubated at 4°C for 1 h with anti-H-2 K^d, D^d or L^d mAb (Meiji Institute of Health Science Ltd., Tokyo, Japan) (1 μg/ml), and effector cells were then added. In a separate experiment, effector cells (10^7 cells) were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) (10 μg/ml) at 4°C for 1 h, and then the labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

Statistical analysis

Statistical analysis was performed using Mann–Whitney's U test and Kruskal–Wallis test. Values are expressed as means ± s.d.s. A 95% confidence limit was taken as significant ($P < 0.05$).

Acknowledgements

This work was supported by Health Science Research Grants from the Ministry of Health, Labor and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Pediatrics International (2004) **46**, 236–244

Feature Article

Molecular epidemiology of HIV: Tracking AIDS pandemic

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Abstract

Background: Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic is a global threat to maternal and child health, especially in developing countries. It is estimated that 800 000 children are infected and 580 000 children die of AIDS-related illnesses every year. Molecular epidemiology has been a useful tool in analyzing the origin of HIV and tracking the course of global HIV spread. This article provides an overview of recent advances in the field of molecular epidemiology of HIV across the world, and discuss the biological implications.

Methods: Based on the near full-length or partial nucleotide sequence information, the phylogeny and recombinant structure of HIV strains are analyzed. Using genotype classification of HIV as a molecular marker, the origin and the genesis of HIV epidemic are investigated.

Results: The HIV-1 group M, a major HIV group responsible for current AIDS pandemic, began its expansion in human population approximately 70 years ago and diversified rapidly over time, now comprising a number of different subtypes and circulating recombinant forms (CRF). Of note, recent studies revealed that new recombinant strains are arising continually, becoming a powerful force in the spread of HIV-1 across the globe.

Conclusion: Global dissemination of HIV is a dramatic and deadly example of recent genome emergence and expansion. Molecular epidemiological investigation is expected to provide information critical for prevention and future vaccine strategies.

Key words acquired immunodeficiency syndrome, circulating recombinant form, genetic variability, human immunodeficiency virus, molecular epidemiology, recombination, subtype, vertical transmission.

The human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) pandemic continues to expand globally at a rate of 14 000 new infections every day. The Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimated that more than 60 million individuals were infected with HIV worldwide by the end of the year 2002. Of these, approximately 25 million patients have already died, and 42 million people were living with HIV/AIDS.[1] It is known that HIV transmits through blood and sexual contacts and via mother-to-infant. Vertical infection occurs during pregnancy and delivery as well as through breast milk at an estimated rate of 25–30%. UNAIDS estimates that close to 3 million children under 15 years of age are now living with HIV/AIDS worldwide: 800 000 children are become infected and 580 000 children die of AIDS every year (Table 1).[2] Of approximately 14 000 new HIV infections which occur each day, 2000 are in children under 15 years of age and approximately 5000 are women in their reproductive age (Table 1), mostly in developing countries in Africa, Asia and Latin America (Table 2).[2] The HIV/AIDS epidemic has a serious impact in the health of mothers and children.

Molecular epidemiological investigations have been a powerful tool in analyzing the origin of HIV and in tracking the global spread of this pathogen. Since HIV-1 group M began its expansion in the human population early in the 20th century (1931, 95% confidence interval: 1915–1941),[4] it has diversified rapidly, now comprising a number of different subtypes and circulating recombinant forms (CRFs).[3–5]

Classification of human immunodeficiency virus

Phylogenetic analyses of globally circulating viral strains have identified three distinct groups of HIV-1 (M, N, and O),

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Received 7 October 2003.