表 1　HIV-DNA・RNA 量の変動

症例	日付	HIV-DNA	HIV-RNA
70066	07.9.4	23	2698
	08.3.18	5	感度未満
	08.9.4	25	感度未満
70084	07.9.6	437	229
	08.1.10	372	661
	08.7.24	959	20
70141	07.9.25	81	48
	08.3.18	40	感度未満
	08.8.26	252	3
70347	07.11.22	60	感度未満
	08.1.17	58	感度未満
	08.11.25	28	感度未満

単位はいずれもcopies/106 CD4+ lymphocytes

図 1　HIV-DNA 量と HIV-RNA 量の相関

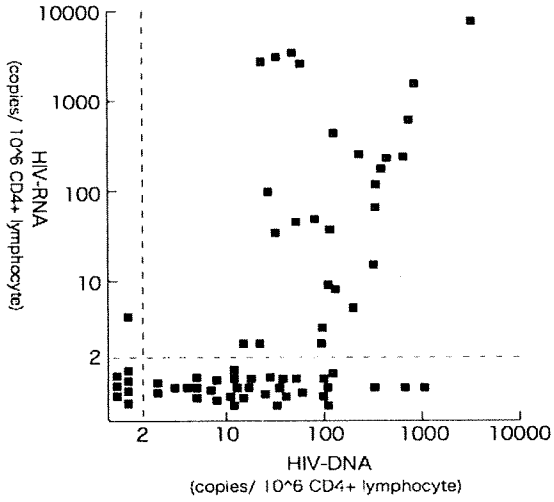


図 2　HIV-DNA 量と A.I. の相関

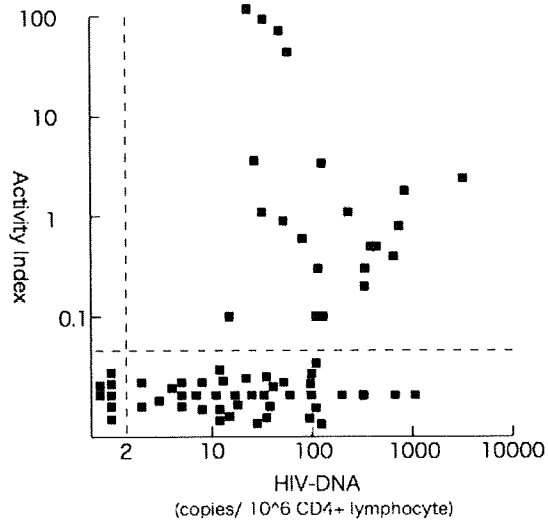


図 3　HIV-DNA 量と ART 導入前の CD4 数

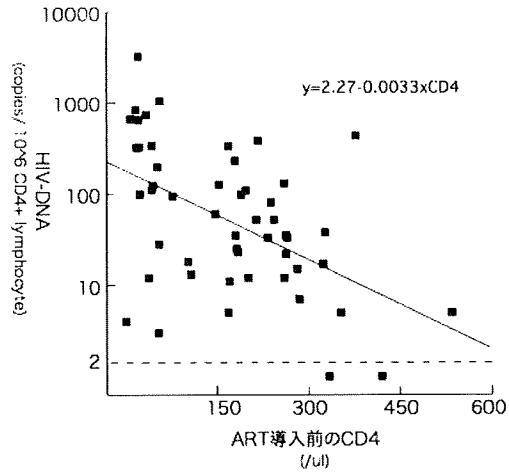
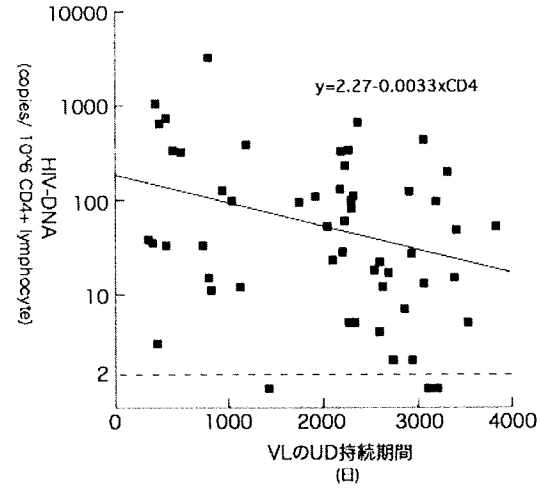


図 4　HIV-DNA 量と VL の UD 持続期間



厚生労働科学研究費補助金(エイズ研究事業)
分担研究報告書

末梢 CD4 陽性 T リンパ球中の残存プロウイルス量とその活動指数は治療中断の指標となりうるかを明らかにする研究

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研究要旨

本研究は、HAART 著効患者の HIV-1 残存プロウイルス量(HIV-DNA)とその転写活性能(HIV-mRNA)から患者の HIV 感染病態を把握し、これらのパラメーターが治療中断のエビデンスとして利用できるかを検討することを目的とする。今年度は昨年度までの HAART 反応良好群 23 症例に加え、新たに 4 症例を追加し計 27 例について解析を行った。HIV-DNA 低値(<2 コピー/10^6cells)、activity index 低値(very low)の症例は 7 例であった。7 例中 4 例は感染早期に HAART を開始した症例であった。感染早期治療導入例で経時的変化を検討したところ、HIV-DNA、HIV-mRNA、activity Index は経時的に減少する傾向があり 2 年以内に HIV-RNA、activity index ともに感度以下になっていた。これらの患者で治療中断が可能かどうかについては、今後の検討が必要である。

A. 研究目的

多剤併用療法(HAART)により HIV-1 感染者の予後は大きく改善した。現在「HAART は一生継続」がコンセンサスになっているが、長期内服に伴う副作用や、薬剤耐性 HIV が問題になってきており、抗 HIV 薬を一生にわたり服用することは、患者および社会へ大きな負担をかけることになる。この視点からも、治療経過が良好な患者で HAART 中断を判定できるエビデンスの開発と実用化が待たれている。本研究は、HAART 著効患者の HIV-1 残存リザバーサイズ(HIV-DNA)とその転写活性能(HIV-mRNA)から患者の HIV 感染病態を把握し、これらのパラメーターが治療中断のエビデンスとして利用できるかを検討することを最終的な目的とする。今年度は、治療経過良好な症例で HIV-DNA および HIV-mRNA を測定し、解析、検討を行った。

B. 研究方法

対象は当院免疫感染症科を受診中で、抗 HIV 療法施行中の HIV-1 感染者のうち血中 HIV-1 RNA 量が検出限度(50copies/ml)以下を維持している患者。Informed Consent のもとに採血を行い、HIV-1 プロウイルス(HIV-DNA)と HIV-1 mRNA の定量を行う。測定は名古屋医療センターにて行うため検体をクール便にて名古屋医療センターに送付する。なお、この研究は、当院倫理委員会の承認を得ている(平成 18 年 7 月)。

C. 研究結果

1. 「末梢 CD4 陽性 T リンパ球中の残存プロウイルス量とその活動指数」測定対象症例

治療経過良好な症例として昨年度までの 23 名に加え 4 名を追加し、計 27 名（うち 4 名は感染早期に治療開始、3 名は複数回測定)にて、HIV-DNA, HIV-mRNA の測定を行った。症例の内訳は、男性 22 名、女性 5 名、血友病 4 名、性感染 23 名、感染早期の HAART 導入例 4 名、平均年齢 38.5 歳(20-63 歳)、測定時の平均 CD4 陽性 T 細胞数 674/μl(382-1215/μl)、 HIV-RNA 感度以下を持

続している期間 4.0 年(0.1-9.5 年)であった。HIV-DNA 量は、平均 212 コピー/10^6cells(0-1720/μl)、HIV-mRNA は、平均 468 コピー/10^6cells(0-7381/10^6cells)、activity index (HIV-DNA/HIV-mRNA)は 7.5 (very low-142)と症例により様々な値を示した。治療中断対象症例になり得る HIV-DNA 低値(<2 コピー/10^6cells)、activity index 低値(very low)の症例は、今回の検討では 7 例、認められた。

2.感染早期に HAART 導入した症例での検討

感染早期に HAART を開始した 4 例中 1 例は、初回測定時 (HAART 導入後 3 ヵ月、血清中の HIV-RNA<50 コピー/ml 到達後 1 ヵ月)に HIV-DNA、HIV-mRNA はいずれも検出感度以下であり activity Index は very low であった。残りの 3 例は、初回測定後 (各々HAART 導入後 8、6、12 ヵ月、血清中の HIV-RNA<50 コピー/ml 到達後 6、3、6 ヵ月)、約 6 ヶ月ごとに 2-3 回、測定した。初回測定時は 3 例とも HIV-DNA は陽性 (19、36、218 コピー/ml)、HIV-mRNA は 2 例で陽性 (318、1707、検出感度以下)、activity Index は 2 例は 16.8、および 46.9 と比較的高値を示し、1 例は very low であった。しかし 2 回目以後の測定にていずれの症例とも多少の測定値の誤差はあるが血清中の HIV-RNA<50 コピー/ml 到達後 2 年以内に HIV-DNA、HIV-mRNA はいずれも検出感度以下となり activity Index も very low となった。

D.考察

今回の検討では、治療反応良好群にて測定を行ったが、HIV-DNA、HIV-mRNA、activity Index は、症例により様々な値をとり、一定の傾向は認められなかった。しかし、感染早期に HAART 導入した症例では、同一症例内で経時的に測定すると治療の経過とともに HIV-DNA、HIV-mRNA、activity Index は低下した。また、以前施行した治療中断例での検討においては、成功例では HIV-DNA は低値であった。しかし CD4 陽性 T リンパ球だけではなく monocyte 系を始め他の細胞も HIV-1 リザバーとなり得ることを考慮すると、末梢 CD4 陽性 T リンパ球中の HIV-DNA、HIV-mRNA、activity Index だけを治療中断の指標とするのは危険だと考えられる。しかし感染早期に HAART 導入した症例や、一部の症例では CD4 陽性 T リンパ球以外の HIV-1 リザバーが少ない可能性があり、そのような症例では末梢 CD4 陽性 T リンパ球中の HIV-DNA は治療中断の指標となる可能性がある。今後、症例数を増やし検討していく必要がある。

E.結論

- 当院通院中の HIV 感染者 27 名において検討を行った結果、7 名が HIV-DNA 低値、activity Index 低値であり、治療中断対象症例になり得る。
- 急性期に HAART を開始した症例も治療中断対象症例になり得る。
- 感染早期に HAART を導入した症例を含め、一部の症例では末梢 CD4 陽性 T リンパ球中の HIV-DNA、Activity Index は治療中断の指標となる可能性がある。
- 治療中断に関しては末梢 CD4 陽性 T リンパ球中の HIV-DN、Activity Index 以外のパラメーターも検討する必要がある。

F.健康危険情報

特記事項なし

G.研究発表

1.論文発表

1. Minami R, Takahama S, Ando H, Miyamura T, Suematsu E, and Yamamoto M. Human herpesvirus 8 DNA load in the leukocytes correlates with thrombocytopenia in HIV-1 infected individuals.″ which you submitted to AIDS Res Hum Retroviruses. (in press)
2. 南 留美, HAART による脂質代謝異常と高分子アディポネクチンの関連, Focus on HIV/AIDS, 2008

2.学会発表

1. High molecular weight form of adiponectin in antiretroviral drug-induced dyslipidemia based on in vivo and in vitro analyses. Minami R, Takahama S, Ando H, Yamamoto M, XVII International AIDS Conference, 3-8 August, 2008, Mexico CIty
2. 抗HIV剤の肝細胞，HCV感染肝細胞における脂質代謝への影響，南 留美、高濱 宗一郎、安藤 仁，山本 政弘，第20回日本エイズ学会，11月26-28日，大阪
3. プロテアーゼ阻害剤が骨代謝に及ぼす影響，高濱宗一郎，南 留美，安藤 仁，山本 政弘，第20回日本エイズ学会，11月26-28日，大阪
4. 社会的背景の複雑な患者の退院調整を振り返って一発達遅滞の患者の一事例を通じて一，長与由紀子，城崎真弓，辻 麻理子，本松 由紀，首藤美奈子，安藤 仁，南 留美，山本政弘，第20回日本エイズ学会，11月26-28日，大阪
5. 2003-2007年の新規HIV-1感染者における薬剤耐性頻度の動向，杉浦 亙，潟永博之，吉田繁，南 留美，山本 政弘ら，第20回日本エイズ学会，11月26-28日，大阪

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金(エイズ対策研究事業)
分担研究報告書
末梢 CD4 陽性 T リンパ球中の残存プロウイルス量とその活動指数は治療中断の指標となりうるかを明らかにする研究

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研究要旨

HAART 中断後も HIV ウイルス量が測定限界以下を維持している症例を経験した。本例は本研究の対象適格例の中の一例（血液製剤による感染）である。42 歳男性。平成 4 年より抗 HIV 療法が開始された。HCV の重複感染があり、H15 年に IFN 療法を受けたが failure。関節置換術を行うにあたり抗 HIV 療法の一時中断が必要となった。中断前 VD4 は 591/μ l、VL は＜50 コピー。中断後 5 ヶ月時点で VL の増加はみられていない。過去に種々の理由で HAART 中断が行われた症例はすべて、中断後 VL は上昇、CD4 の減少が観察されていたが、当施設としてはじめて中断可能例の存在が確認された。今後慎重に経過観察が必要である。

A. 研究目的

1990 年代後半から始まった核酸系逆転写酵素阻害剤（NRTI）とプロテアーゼ阻害剤（PI）もしくは非核酸系逆転写酵素阻害剤（NNRTI）を組み合わせた HIV に対する多剤併用療法（HAART）は非常に有用であり、HIV 感染症は予後が確実に改善し、慢性疾患としてとらえられるようになった。HAART により HIV の増殖をほぼ完全に抑え続けても、生体内から HIV を駆逐するためには数十年かかるといわれており治癒に導くには HAART を一生涯継続する必要性が強調されている。他方 HAART の長期服用による弊害として代謝性副作用や服用疲れによるアドヒアランスの低下、それに由来する耐性ウイルスの出現などが問題になっており、もし薬剤中断についての安全性を示す証拠が得られれば、感染者の負担（精神的、肉体的、社会的）の軽減に大いに寄与するに違いない。

本研究の目的は HAART 著効患者を対象にして末梢 CD4 陽性 T リンパ球中に残存しているプロウイルスコピー数と全長 HIV-1 mRNA を定量し、1 コピー当りのプロウイルスの HIV-1mRNA 転写能（活動指数）を算出し活動指数と残存プロウイルス量が HAART 中断のエビデンスになるかを検討することである。今回対象適格症例の中に HAART 中断を行った後も HIV ウイルス量が測定限界以下に抑制され続けている症例を経験したので報告する。

B. 研究方法

研究対象：HAART により血漿 HIV ウイルス量（VL)が測定限界以下に抑制され、かつ CD4 リンパ球数の回復が目覚しい HAART 著効例である。これに加え、様々な経緯で治療中断を行なった成功例と失敗例も対象とする。CD4 陽性細胞の精製：EDTA 加末梢血より StemSep14052 を用い精製。DNA と RNA の抽出および精製：DNA はキアゲン Blood mini kit を用いて抽出・精製する。RNA はトリゾールにて抽出する。リアルタイム PCR 法による定量：主任研究者らにより開発された高感度リアルタイム PCR 法の検出限界は 2 コピー/10^6 細胞である。この方法により、末梢 CD4 陽性 T リンパ球中に残存している HIV-1 プロウイルスコピー数と全長 HIV-1mRNA のコピー数を高感度で定量する。プロウイルスの活動度：残

存プルウイルス１コピー当りの HIV-1mRNA 転写活性を活動指数(全長 HIV-1mRNA コピー数/プロウイルスコピー数) で表現する。塩基配列の決定：プライマーやプローブとのミスマッチにより定量値が過小評価される可能性があるのでプライマー、プローブ領域の塩基配列を決定した上で定量値の妥当性を評価する。必要に応じてプロウイルスの全長塩基配列を決定する。今回の症例についてはプロウイルス活動度の測定はできなかったが臨床的背景について記す。

(倫理面への配慮)

本研究を進める上で患者の協力は不可欠である。研究の必要性と意義を十分説明し理解と協力を得ることを前提とする。施設倫理委員会に研究計画書を提出し、審査・承認を得た上で研究を開始する。研究参加同意書には患者の自筆でサインをお願いし、同意書原本は主治医もしくは施設担当責任者の下で保管することを義務とする。検査結果は個人情報保護の観点から漏出しないよう厳重に管理する。

C. 研究結果

症例：42 歳、男性。血友病 A (重症型)。血液製剤による感染で HCV を重複感染している。平成 4 年より AZT/3TC で治療開始され、平成 9 年 6 月当科初診時、AZT/ddC を服用中で治療されており、ＣＤ４陽性細胞数は 460/μl、同年 9 月より 3TC/d4T に変更後 HIV ウイルス量 <400 コピー/ml で以後も良好にコントロールされた。抗 HIV 薬はその後も 2 剤のままで治療された。平成 15 年 C 型肝炎に対して PEG-IFN+リバビリン療法が行なわれたが failure。HIV 感染症の動態は不変 (CD4>500/μl、VL<50 コピー/ml)。平成 20 年 10 月、関節置換術を希望し抗 HIV 療法中止した (CD4：591/μl、VL<50 コピー/ml)。その後 CD4 は 11 月 493、12 月 449、1 月 365、2 月 384、3 月 317 と減少傾向にあるが、VL は測定限界以下を維持している。

D. 考察

研究適格症例の中で HAART 中断後も VL が測定限界以下を維持している症例をはじめて経験した。本例の病態として、まさしく今回対象適格症例であり、プロウイルス活動指数が低い中断可能症例であったことが考えられるが、もともと長期無発症例であって HAART を行わなくてもウイルス量増加しないタイプであった可能性もある。注意すべきは VL は抑制されているものの、CD4 陽性リンパ球数が漸減している印象があることである。体内の HIV の動態について１００%把握できないことは常に考慮すべきであり、今後注意深い慎重な経過観察が必要である。

E. 健康危険情報

なし

F. 研究発表

1. 論文発表

なし

2. 学会発表

なし

G. 知的財産権の出願・登録状況

1. 特許出願

なし

2. 実用新案特許

なし

3. その他

なし

厚生労働科学研究費補助金（エイズ対策研究事業）
研究報告書

プロウイルス活動指数測定法の一般検査化のための基礎検討

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研究要旨

昨年度に引き続き、プロウイルス活動指数測定法を一般検査化するための基礎検討を行った。具体的には、標準 HIV-1 gag RNA を作成し、ツーステップ RT・Real-Time PCR による HIV-1 mRNA 定量系の基礎検討を実施した。その結果、チューブあたり 10 の 1 乗のオーダーから 10 の 8 乗のオーダーにわたって概ね良好な検量線が得られ、再現性においてもチューブあたりで設定した最小値である 12.5 コピー添加時の Cv 値が 1.998%と良好な結果が得られた。実検体の測定に向けては、今後、更なる検討が必要と思われるが、本年度の検討により、プロウイルス活動指数測定法の一般検査化に向けてさらに前進したと言える。

A. 研究目的

研究班の目的は、血中 HIV-1 RNA 量(viral load)が検出限度以下となった HAART 著効例を対象に、CD4 陽性 T 細胞内の HIV-1 DNA 量と HIV-1 mRNA 量を定量し、プロウイルスの活動指数(HIV-1 mRNA 量／HIV-1 DNA 量)が治療中断の指標として利用できるか否かを明らかにすることであり、我々は、プロウイルス活動指数測定法を一般検査化するための基礎検討を行ってきた。昨年度は、HIV-1 DNA 定量系の高い感度を維持したまま、実験系を簡略化することに成功したが、ワンステップ RT・Real-Time PCR による HIV-1 mRNA 定量系の構築においては、感度が不十分との結論に至った。そこで本年度は、ツーステップ RT・Real-Time PCR による HIV-1 mRNA 定量系について基礎検討を実施した。

B. 研究方法

【標準 HIV-1 gag RNA の作成と保存】HIV-1 gag 遺伝子を含むプラスミドを、制限酵素を用いて直線化し、T7 RNA ポリメラーゼを用いたインビトロ転写反応により HIV-1 gag RNA を産生した。インビトロ転写反応産物を精製後、50ng/μl *E.coli* total RNA

を添加したRNase free waterに溶解し、標準HIV-1 gag RNAとした。この標準HIV-1 gag RNAは、4℃で少なくとも13週間まで安定に保存できることを確認済みである。

【ツーステップRT・Real-Time PCR】

Transcriptor Reverse Transcriptase (Roche)を用いて逆転写反応を行い、RNAをcDNAへ変換した。Real-Time PCR試薬には、QuantiTect Probe PCR Kit (QIAGEN)を用い、装置としてABI PRISM 7900HT (Applied Biosystems)を使用した。

C. 研究結果

標準HIV-1 gag RNAを用いた反応曲線を図1に示した。チューブあたり10の1乗のオーダーから10の8乗のオーダーまで反応シグナルが得られ、概ね良好な検量線が得られた(図2)。10回の測定により再現性を調べた結果、チューブあたりで設定した最小値である12.5コピーにおいても、Cv値が1.998%と良好な再現性が得られた(図3)。

D. 考察

本年度は、標準HIV-1 gag RNAを作成し、ツーステップRT・Real-Time PCRによるHIV-1 mRNA定量系について、基礎検討を実施した。その結果、チューブあたり10の1乗のオーダーから10の8乗のオーダーまで概ね良好な検量線が得られ、チューブあたりで設定した最小値である12.5コピーにおいても、Cv値が1.998%と良好な再現性が得られた。このツーステップRT・Real-Time PCRによるHIV-1 mRNA定量系の広い測定範囲と高い感度は、昨年度のワンステップRT・Real-Time PCRでは得られなかったものであり、一般検査化に向けて大きく前進したと言える。ただし、追加で実施した血漿検体を用いた測定結果では、比較に用いたCobas TaqMan HIV-1(Automatic)の測定値と乖離した結果が得られており、実検体の測定に向けては、更なる検討が必要と思われる。

E. 結論

標準HIV-1 gag RNAを作成し、ツーステップRT・Real-Time PCRによるHIV-1 mRNA定量系の基礎検討を実施した。その結果、広い測定範囲と高い感度を持ち合わせていることが分かった。実検体の測定に向けては、今後、更なる検討が必要と思われるが、本年度の検討により、プロウイルス活動指数測定法の一般検査化に向けてさらに前進したと言える。

F. 健康危険情報

なし

G. 研究発表

なし

H. 知的財産権の出願・登録状況

なし

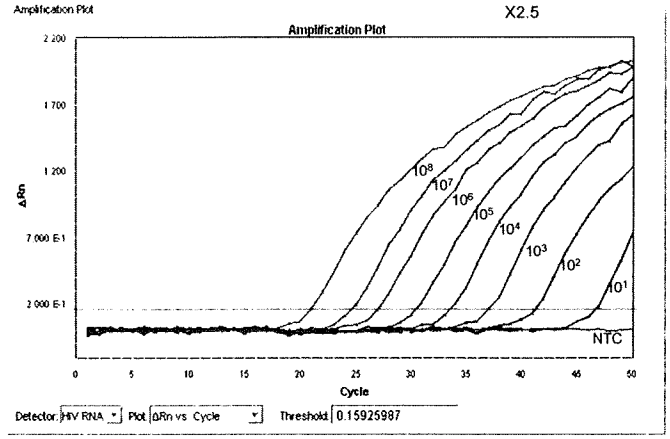

図1 反応曲線

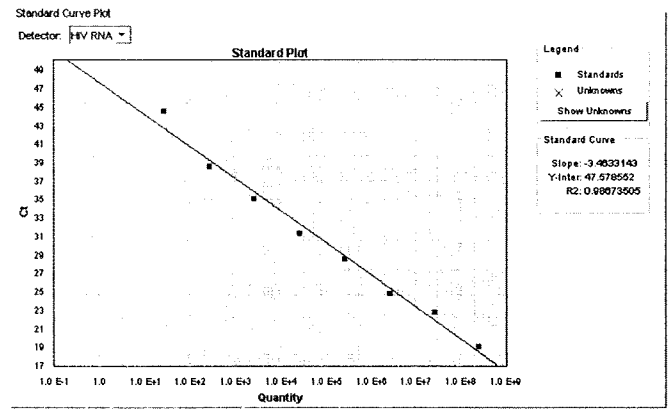

図2 検量線

Real-time PCR											
	Ct値(copies/tube)										
回数	12.5	25	37.5	50	62.5	75	87.5	100	112.5	125	137.5
1	42.24	42.07	41.80	41.16	40.34	39.92	40.26	38.76	39.58	39.14	38.64
2	43.01	43.06	42.07	41.58	40.17	39.65	39.04	40.03	39.47	38.46	36.94
3	43.60	42.49	41.70	41.92	41.31	40.19	40.19	39.45	40.08	38.10	37.63
4	42.61	43.71	41.77	40.37	41.58	39.87	40.37	39.42	37.77	37.81	38.24
5	43.08	42.48	41.73	41.53	40.93	39.99	40.47	38.66	38.09	38.50	36.63
6	42.91	42.86	42.48	40.22	40.13	39.91	40.64	39.53	39.02	39.06	37.50
7	41.57	42.82	41.66	41.89	41.09	40.20	38.99	39.54	38.57	38.82	38.70
8	44.22	41.19	41.67	41.09	40.30	40.64	39.21	38.89	38.58	38.82	38.61
9	44.44	43.95	42.48	41.36	40.27	40.08	39.21	39.35	38.46	37.71	38.07
10	43.30	42.44	42.93	41.26	41.03	40.09	39.38	38.62	38.62	38.38	38.55
	10	10	10	10	10	10	10	10	10	10	10
平均値	43.098	42.708	42.029	41.237	40.714	40.053	39.776	39.224	38.824	38.483	37.950
SD	0.861	0.790	0.149	0.570	0.532	0.263	0.661	0.467	0.711	0.494	0.746
2SD	1.722	1.579	0.897	1.141	1.063	0.526	1.323	0.933	1.422	0.988	1.491
CV	1.998	1.849	1.067	1.383	1.305	0.656	1.663	1.189	1.831	1.283	1.965
2SD	11.376	11.128	11.131	10.096	39.651	39.528	38.453	38.291	37.402	37.495	36.459
-2SD	14.820	14.287	42.926	42.378	41.777	40.579	41.099	40.157	40.246	39.470	39.441

図3 再現性の検討

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
Xiao P, Usami O, Suzuki Y, Ling H, Shimizu N, Hoshino H, Zhuang M, Ashino Y, Gu H, Hattori T.	Characterization of a CD4-independent clinical HIV-1 that can efficiently infect human hepatocytes through chemokine (C-X-C motif) receptor 4.	AIDS	22	1749-1757	2008
Bi X, Suzuki Y, Gatanaga H, Oka S.	High frequency and proliferation of CD4+FOXP3+ regulatory T cells in HIV-1 infected patients with low CD4 count.	Eur. J. Immunol.	39	301-309	2009
Minami R, Yamamoto M, Takahama S, Ando H, Miyamura T, Suematsu E.	Human herpesvirus 8 DNA load in the leukocytes correlates with the platelet counts in HIV type 1-infected individuals.	AIDS Res. Hum. Retroviruses	25	1-8	2009
南 留美	HAARTによる脂質代謝異常と高分子アディポネクチンの関連	Focus on HIV/AIDS			2008
Ibe S, Hattori J, Fujisaki S, Shigemi U, Fujisaki S, Shimizu K, Nakamura K, Kazumi T, Yokomaku Y, Mamiya N, Hamaguchi M, Kaneda T.	Trend of drug-resistant HIV type 1 emergence among therapy-naive patients in Nagoya, Japan: an 8-year surveillance from 1999 to 2006.	AIDS Res. Hum. Retroviruses	24	7-14	2008

Takahashi M, Kudaka Y, Okumura N, Hirano A, Banno K, Kaneda T.	Pharmacokinetic parameters of lopinavir determined by moment analysis in Japanese HIV type 1-infected patients.	AIDS Res. Hum. Retroviruses	24	114-115	2008
Ibe S, Shigemi U, Sawaki K, Fujisaki S, Hattori J, Yokomaku Y, Mamiya N, Hamaguchi M, Kaneda T.	Analysis of near full-length genomic sequences of drug-resistant HIV-1 spreading among therapy-naïve individuals in Nagoya, Japan: amino acid mutations associated with viral replication activity.	AIDS Res. Hum. Retroviruses	24	1121-1125	2008
Takahashi M, Konishi M, Kudaka Y, Okumura N, Hirano A, Terahata N, Banno K, Kaneda T.	A conventional LC-MS method developed for the determination of plasma raltegravir concentrations.	Biol. Pharm. Bull.	31	1601-1604	2008
Fujisaki S, Ibe S, Hattori J, Shigemi U, Fujisaki S, Shimizu K, Nakamura K, Yokomaku Y, Mamiya N, Utsumi M, Hamaguchi M, Kaneda T.	An 11-Year Surveillance of HIV Type 1 Subtypes in Nagoya, Japan.	AIDS Res. Hum. Retroviruses	25	15-21	2009

Ⅳ．研究成果の刊行物・別刷

Characterization of a CD4-independent clinical HIV-1 that can efficiently infect human hepatocytes through chemokine (C-X-C motif) receptor 4

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Objective: HIV-1 isolates are prominently CD4-dependent and, to date, only a few laboratory-adapted CD4-independent strains have been reported. Therefore, whether CD4-independent viruses may exist in HIV-1-infected patients has remained unclear. Here, we report the successful isolation of a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia patient in the late-stage of AIDS with extremely low CD4 cell count (CD4 = 1/μl). We characterized this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

Design and methods: To determine coreceptor usage and CD4-independent infection, the HIV-1 envelope (Env)-pseudotypes and Env-chimeric viruses were used.

Results: SDA-1 was able to infect $CD4^-$ cell lines through either chemokine (C-X-C motif) receptor 4 or CCR5. It still maintained the ability to infect $CD4^+$ cells through multiple coreceptors of chemokine (C-X-C motif) receptor 4, chemokine (C-C motif) receptor 5, chemokine (C-C motif) receptor 3 and chemokine (C-C motif) receptor 8. Productive infection by SDA-1 was noted in both CD4-negative hepatoma cells and primary cultured human hepatocytes. Moreover, we demonstrated that SDA-1 could efficiently infect human hepatocytes on both static and mitotic phases through chemokine (C-X-C motif) receptor 4, without inducing apoptotic cell death.

Conclusion: The present study provides evidence that emergence of CD4-independent HIV-1 virus *in vivo* may occur in HIV-1-infected patients. In addition, these results shed light on the mechanisms involved in liver damage in HIV-1-infected individuals, which could have important implications concerning the range of mutability and the pathogenesis of AIDS.

AIDS 2008, **22**:1749–1757

Keywords: CD4-independence, HIV-1, human hepatocytes, human hepatoma cells

Introduction

The entry of HIV-1 into target cells requires interaction of the viral envelope (Env) with CD4 and a chemokine coreceptor [1,2]. Macrophage-tropic HIV-1 viruses primarily use chemokine (C-C motif) receptor 5 (CCR5) (R5) as a coreceptor, whereas T-cell-tropic viruses use chemokine (C-X-C motif) receptor 4

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Received: 17 January 2008; revised: 21 April 2008; accepted: 9 May 2008.

DOI:10.1097/QAD.0b013e328308937c

(CXCR4) (X4). Dual-tropic viruses (R5X4) use both coreceptors [3]. A few rare viruses can also use alternative coreceptors such as chemokine (C-C motif) receptor 1 (CCR1), chemokine (C-C motif) receptor 2b (CCR2b), chemokine (C-C motif) receptor 8 (CCR8), chemokine (C-X-C motif) receptor 6 (CXCR6), G protein-coupled receptor 1 (GPR1) or GPR15/Bob for entry into coreceptor-transfected $CD4^+$ cell lines [4]. Whatever the coreceptor specificity of an HIV-1 isolate, an interaction with CD4 is always the first step in a chain of events leading to fusion of the viral envelope with the cellular membrane. However, previous studies have shown that SIV [5] and HIV-2 [6] can also infect cells independently of CD4.

In contrast to SIV and HIV-2, HIV-1 CD4-independent viruses are rarely isolated. To date, only a few laboratory CD4-independent HIV-1 variants [7–10] have been reported. Therefore, whether such viruses may exist in HIV-1-infected patients has remained unclear. However, several studies [11–14] have shown that HIV-1-DNA and p24, a core HIV-1 antigen, were detected in CD4-negative cells or tissues such as brain, kidney and liver in HIV-infected individuals, suggesting the possibility that low levels of CD4-independent variants exist *in vivo*. Among such $CD4^-$ cells or tissues, liver is an important organ in determining the prognosis of HIV-1-infected patients. End-stage liver disease is becoming a frequent cause of death in HIV-1-infected hospitalized patients [15–17]. Although the cause of liver injury in HIV-1 patients might be multifactorial, such as hepatitis B virus (HCV) and hepatitis B virus (HBV) coinfection and the side effects of antiretroviral therapy, a number of reports have documented that histological liver abnormalities occurred solely as a result of HIV-1 infection [13,18,19]. Nonetheless, few attempts have been made to elucidate the mechanisms of the liver damage in HIV-1-infected individuals.

In this study, we successfully isolated a CD4-independent clinical HIV-1 strain, designated SDA-1, from the viral quasispecies of a therapy-naive HIV-1 and *Pneumocystis jirovecii* pneumonia (PJP) patient in the late stage of AIDS with extremely low CD4 cell numbers. We characterized the phenotype of this virus and further explored whether it could infect or induce pathological effects in human hepatocytes.

Materials and methods

Patient's information

A 53-year-old Japanese man infected with HIV-1 was admitted to Tohoku University Hospital owing to prolonged fever and severe dyspnea in 2000. His plasma viral load and CD4 cell count at the time of admission was 220 000 copies/ml and 1 cell/μl, respectively. He was diagnosed with PJP, and his clinical stage was classified as IV-C3 [20]. The onset and route of HIV-1 infection were unknown. No evidence of coinfection with HBV or HCV in this patient was found. The patient was treated with trimethoprim and sulfamethoxazole (TMP–SMX) and highly active antiretroviral therapy (HAART). His condition deteriorated rapidly and he died 33 days after admission. Consent for autopsy was denied by the patient's family.

Before HAART, plasma samples and peripheral blood mononuclear cells (PBMC) were collected from this patient and cryopreserved in liquid nitrogen until use. The institutional Ethics Committee approved this study and written informed consent was obtained from the patient.

Virus isolation

HIV-1 isolation was achieved by using an in-vitro short-term phytohemagglutinin (PHA)–PBMC coculture method. Briefly, cryopreserved PBMC (2×10^6) from the patient were cocultivated with PHA-stimulated PBMC (5×10^6) from an HIV-1 seronegative healthy donor. The culture was maintained in RPMI-1640 (Invitrogen, California, USA) containing 10% fetal calf serum and 5 U/ml of recombinant interleukin-2 (IL-2) (Sigma, St. Louis, Missouri, USA). Proliferation of HIV-1 was examined by measuring p24 antigen in the cell culture supernatant using a p24 ELISA kit (RETRO-TEK, ZeptoMetrix Corp., New York, USA). The virus stocks were kept at −80 °C until use.

Amplification of *env* and sequence analysis

The full-length HIV-1 *env* genes were amplified by limiting dilution nested PCR from proviral PBMC DNA or plasma RNA as previously described [21,22]. To avoid artificial recombination and resampling of the viral genomes, independent nested PCR reactions were carried out per specimen [23,24].

The first round PCR was conducted with a F5852–R8935 primer pair (F5852, 5′-TAGAGCCCTGGAAG CATCCAGGAAG, HIV-1 HXB2 nucleotide position 5852–5876; R8935, 5′-TTGCTACTTGTGATTGCT CCATGT, HXB2 nucleotide position 8912–8935). The second round PCR was performed with a F5957–R8903 primer pair (F5957, 5′-GATCGAATTCTAGGCATC TCCTATGGCAGGAAGAAG, HXB2 nucleotide position 5957–5982, containing an additional *Eco*RI site (underlined) to facilitate cloning; R8903, 5′-AGCT CTC GAGGTCTCGAGATACTGCTCCCACCC, HXB2 nucleotide position 8881–8903, containing an additional *Xho*I site (underlined)). The purified PCR products were subcloned into the *Eco*RI and *Xho*I sites of the pSM-HXB2 plasmid. All correctly oriented *env* clones were then screened for biological function [22] followed by sequencing and phylogenetic analysis as previously described [25,26].

Cell lines and cell culture

All the cell lines, unless otherwise specifically mentioned, were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum. Human glioma NP-2-CD4$^+$ cells transfected with a variety of chemokine receptors as indicated [27] were maintained in medium containing 500 μg/ml of G-418 (Promega, Wisconsin, USA) and 1 μg/ml of puromycin (Sigma). Human CD4-negative osteosarcoma (HOS) cells expressing either CXCR4 or CCR5 [28] were cultured in medium containing 1 μg/ml of puromycin. Human hepatoma cells Huh-7 and Hep-G2 [29] were obtained through the Cell Resource Center for Biomedical Research, Tohoku University, Japan. Human primary cultured hepatocytes (p-hepatocytes, BD Bioscience, California, USA) were maintained on BD Matrigel with Hepato-STIM hepatocyte culture medium (BD Bioscience).

Reagents and antibodies

The CXCR4 antagonist AMD3100 [30], and the CCR5 antagonist TAK-779 [31] were provided by the NIH AIDS Research and Reference Reagent Programme and Takeda Chemical Industries, Ltd., Osaka, Japan, respectively. Recombinant human soluble CD4 (sCD4) was from ImmunoDiagnostics, Inc. (Woburn, Massachusetts, USA). Antialbumin-fluorescein isothiocyanate (FITC) antibody was from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Anticytokeratin-18–phycoerythrin and antialpha fetoprotein (AFP)–FITC antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). Anti-HIV-1-p24 (clone KC57)-FITC antibody was from Beckman Coulter. All other antibodies were from BD Pharmingen (San Diego, California, USA).

Pseudotyped virus infection assay

The HIV-1 Env-pseudotypes were generated as previously described [32]. Briefly, 293T cells (5×10^6 cells/10 cm-dish) were transfected with 5 μg of luciferase-expressing pNL4-3-Luc-R$^-$E$^-$ [33] or green fluorescent protein (GFP)-expressing pNL4-3-GFP [34] plasmid in combination with 10 μg of one of the *env*-expressing plasmids, pSM-SDA-1, pSM-HXB2 (X4), pSM-ADA (R5), or pSM-89.6 (R5X4). The vesicular stomatitis virus-G pseudotypes were also prepared [35].

For infection assays of luciferase-pseudotypes (luc-p), 10 ng p24 of luc-p were added into each well of 24-well plates (5×10^4 cells/well). After 12 h infection, the cells were washed and incubated for an additional 36 h at 37 °C. The cells were then lysed using a Luciferase Assay kit (Promega) and the luciferase activity was examined by a luminometer (Lumat 9507, Germany). To determine the effects of various reagents related to the viral receptors, target cells were preexposed for 1 h with the indicated concentration of the antagonists, or the antibodies. For GFP-pseudotypes (GFP-p) infection, target cells were infected with 10 ng p24 of GFP-p virus for 48 h and fixed by 5% paraformaldehyde. Infectivities were visualized under a Zeiss LSM510 confocal microscopy and DIC images with a 512 × 512 resolution were acquired.

Chimeric viruses

All *env* recombinant chimeric viruses in this study were generated in the background of pNL43, an X4-tropic HIV-1 infectious clone [36]. Briefly, the fragment of pNL43 containing *Eco*RI (nt 5743–5748) and *Kpn*I (nt 6343–6348) was amplified by PCR with a F5671–R6472 primer pair (F5671, 5′-GGCTCCATAACTTAGGA CAAC, pNL43 nucleotide position 5671–5691; R6472, 5′-TACTTCTTGTGGGTTGGGGTC, pNL43 position 6452–6472), followed by insertion into the pSM–SDA-1 using *Eco*RI and *Kpn*I. The new *Eco*RI–*Xho*I fragment (3155 bp) covering the entire SDA-1 *env* gene was then replaced with the equivalent region of pNL43 to construct the Env-chimeric virus NL43_SDA-1. Similarly, Env-chimeras of ADA (NL43_ADA), 89.6 (NL43_89.6) or truncated *env* (NL43_env (-)) were created, respectively. All Env-chimeric viruses were prepared by transfecting 293T cells as described above. For infection assays, 100 ng p24 of the chimeric viruses or virus stock supernatants were added in each well of 24-well plates (5×10^4 cells/well). After 2 h adsorption, the cells were washed and incubated for 48 h. Viral replication was monitored by p24 antigen production.

Flow cytometry and apoptosis assay

We performed cell-surface staining for CD4, CXCR4 and CCR5 by flow cytometry. To determine the purification and differentiation of p-hepatocytes, we tested the specific markers using antialbumin–FITC, anti-AFP–FITC and anticytokeratin-18–phycoerythrin antibodies. Appropriate class matched antibodies were used in each experiment. To detect the proliferation and intracellular p24, p-hepatocytes were fixed and permeabilized using a Cytofix-Cytoperm kit (BD Bioscience). Subsequently, the cells were stained with anti-Ki-67–phycoerythrin and antip24–FITC antibodies. Apoptosis of the p-hepatocytes was determined using the Apoptosis Detection kit I (BD Pharmingen). Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson, New Jersey, USA). All Data were acquired and analyzed using Cell Quest software (BD Bioscience).

Nucleotide sequence accession number

The GenBank accession number for the sequence determined in this study is AY902478 (SDA-1).

Results

Evaluation of SDA-1 viral quasispecies

In an attempt to isolate CD4-independent clinical HIV-1 strain(s), we performed virus isolation from a

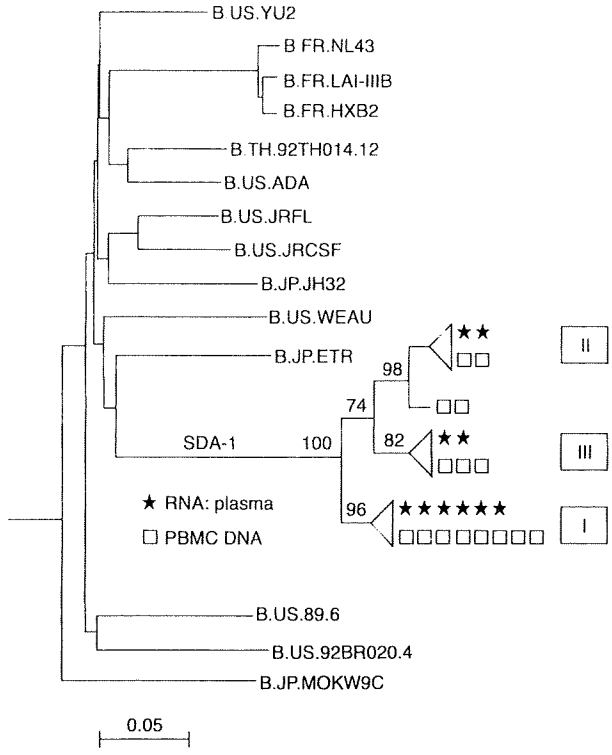

Fig. 1. Evolution of SDA-1 *env* quasispecies in plasma and PBMC. Phylogenetic analysis of newly characterized, SDA-1 gp120 *env* nucleotide sequences obtained from plasma ($n = 10$) and PBMC ($n = 15$) with representative sequences of HIV-1 subtype B. Numbers at branch nodes refer to the percentage of bootstrap values and symbols indicate individual clones.

therapy-naive HIV-1 and PJP patient with extremely low CD4 cell number, and successfully isolated the virus (peak of p24, 500 ng) from this patient and designated it SDA-1. To assess the quasispecies diversity present in *vivo*, we analyzed the SDA-1 *env* clones derived from plasma RNA and PBMC. As shown in Fig. 1, SDA-1 is grouped within the HIV-1 subtype B reference sequences. Within SDA-1's sequence cluster, three phylogenetic forms were identified. Supported by a significant bootstrap value (96%), form I was the predominant quasispecies, representing 70% of all sequences. Two minor quasispecies (forms II and III) had similar structures but differed in the position of the first breakpoint. The mean distances between major and minor quasispecies did not differ significantly from the sequence heterogeneity. Furthermore, the quasispecies diversities between plasma and PBMC were similar within each form, and were all below 5.0%.

Multicoreceptor usage and CD4-independent entry of SDA-1

To determine the receptor usage of SDA-1, we randomly selected five clones from the predominant quasispecies and generated Env-pseudotypes and Env-chimeric viruses as representatives. As a control, the Envs from a variety of HIV-1 subtypes with X4 (HXB2), R5 (ADA), and R5X4 (89.6) tropism were used. Utilizing luciferase-pseudotypes (luc-p), we first examined the coreceptor usage of SDA-1. We found that in the presence of CD4, all representative SDA-1 Env-pseudotypes were able to use efficiently both CXCR4 and CCR5, with additional moderate usage of CCR3 and CCR8 (Fig. 2a).

We next investigated whether SDA-1 Envs are capable of inducing CD4-independent infection. We found that SDA-1 Envs mediated entry into both HOS–CXCR4 and HOS–CCR5. However, the infectivities of SDA-1 for HOS–CXCR4 were approximately 2.5-fold higher than that for HOS–CCR5 (Fig. 2b). In stark contrast, none of the other types of luc-p viruses entered either of those cells. Furthermore, we evaluated the ability of SDA-1 Envs in mediating cell–cell fusion, a dye-transfer cell–cell fusion assay [37] was used with HOS–CXCR4 and HOS–CCR5 cells. Only in the cells expressing SDA-1 Envs (effector cells) did cell–cell fusion with CD4-negative, CXCR4- or CCR5-positive HOS cells (target cells) occur (data not shown).

In addition to the results with HOS–CXCR4 and CCR5, preexposure of HOS cells to Leu-3A, a CD4 monoclonal antibody (mAb) that recognizes the gp120 binding site on CD4 [38], failed to block SDA-1 infection. In contrast, pretreatment with antagonists for CXCR4 or CCR5 effectively inhibited infection (Table 1). Furthermore, the infectivities of SDA-1 on HOS–CXCR4 and HOS–CCR5 were enhanced by preexposure of the virus to sCD4 indicating that the binding of SDA-1 Env to CD4 induces further conformational changes in gp120 to fully expose the chemokine receptor binding domain. Collectively, SDA-1 Envs mediated the CD4-independent infection via both CXCR4 and CCR5.

Having clarified that SDA-1 is a CD4-independent isolate, we next investigated what types of $CD4^-$ cells are able to support SDA-1's entry. We focused first on human liver-derived cell lines, as the mechanisms of the liver damage in HIV-1-infected individuals are still unclear.

Two hepatoma cell lines, Huh-7 and Hep-G2, were used as targets. We first examined the expression of the receptors on the cell surface by flow cytometry and found that both CXCR4 and CCR5 were expressed on Huh-7 and Hep-G2 cells. In contrast, CD4 was not detected on either, which was confirmed by RT–PCR (data not shown). We then evaluated whether SDA-1 can enter into hepatoma cells with luc-p viruses. We found that only SDA-1 luc-p viruses efficiently infected Huh-7; however, its infectivity was marginal in Hep-G2 (Fig. 2c). Previous studies have shown that few HIV-1 variants can infect $CD8^+$ cells using CD8 as receptor [10,39]. Therefore, we further explored receptors used by

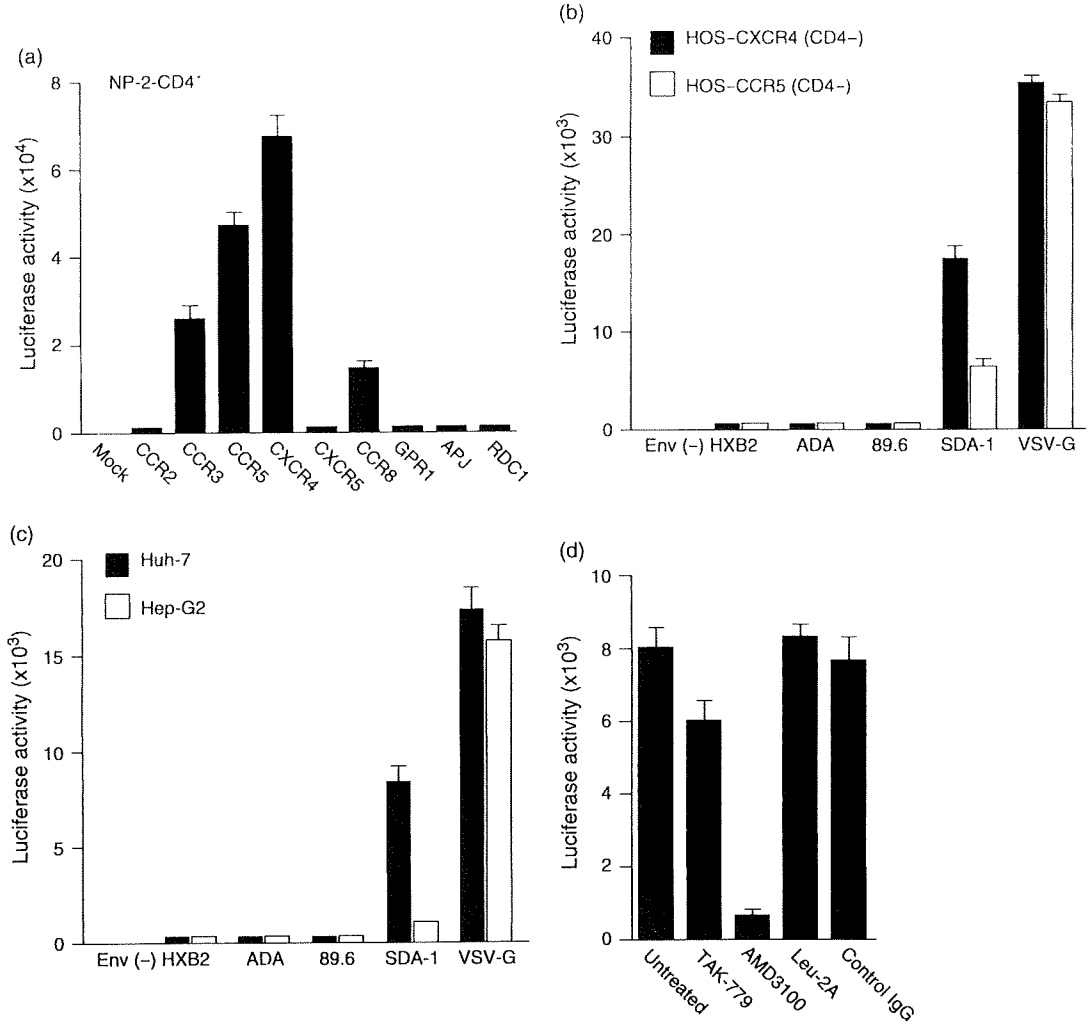

Fig. 2. Multicoreceptor usage and CD4-independent entry of SDA-1. (a) SDA-1 Envs mediate entry of $CD4^+$ cells using multiple coreceptors. NP-2-$CD4^+$ cells coexpressing one of the indicated chemokine receptors were exposed to SDA-1 luc-p viruses for 48 h and the luciferase activities were measured. (b) SDA-1 Envs mediate entry of $CD4^-$ cell lines through either CXCR4 or CCR5. The HOS cells ($CD4^-$) expressing either CXCR4 or CCR5 were exposed to the indicated HIV-1 luc-p viruses or VSV-G for 48 h, after which the infectivities were determined. (c) Entry of SDA-1 into $CD4^-$ human hepatoma cells. Huh-7 and Hep-G2 were exposed to the indicated HIV-1 luc-p viruses or VSV-G. Infectivities were determined at 48 h. (d) Effects of receptor-related antagonists or antibodies on the entry of SDA-1 entry into Huh-7 cells. Interaction of SDA-1 luc-p viruses with Huh-7 cells was tested in the absence or presence of AMD3100 (1.0 μM), TAK-779 (100 nM), anti-CD8 Leu-2A antibody (30 μg/ml) or class-matched control antibody (30 μg/ml). Results shown (a–d) are means of triplicate experiments. Bars, standard deviation. IgG, immunoglobulin G; VSV, vesicular stomatitis virus.

SDA-1 for entry into hepatoma cells. As shown in Fig. 2d, preexposure of Huh-7 to anti-CD8 Leu-2A mAb, as well as the CCR5 antagonist, TAK-779, failed to block SDA-1 infection of Huh-7, whereas anti-CXCR4 with AMD3100 effectively suppressed the infectivity. These results suggested that SDA-1 enters Huh-7 cells principally via CXCR4.

Replication of SDA-1 in human hepatoma cells

Although SDA-1 luc-p viruses infected some cells independently of CD4 cells, it was necessary to determine whether SDA-1 can replicate in those $CD4^-$ cells, particularly in hepatoma cells. For this purpose, we constructed NL43-based Env-chimeric viruses described above. We then examined whether the chimeric viruses were able to replicate in $CD4^-$ cells. As shown in Fig. 3a, the SDA-1 Env-chimeric viruses replicated efficiently in HOS–CXCR4 and HOS–CCR5 cells to similar levels. In contrast, none of the other Env-chimeric viruses infected either of those cells lines. Furthermore, we examined whether SDA-1 Env-chimeric viruses could replicate in hepatoma cells. As shown in Fig. 3b, high levels of NL43-SDA-1 replication were observed in Huh-7 cells. However, marginal replication was detected

Table 1. Inhibition of SDA-1 by blocking reagents in CD4⁻ cells.

Reagent	% Inhibition	
	HOS-CXCR4	HOS-CCR5
Medium	0	0
Control mAb (30 μg/ml)	0	0
Leu-3A (30 μg/ml)	10	12
Soluble CD4 (10 μg/ml)	225[a]	120[a]
AMD3100 (1.0 μM)	99	0
TAK-779 (100 nM)	0	97

CCR5, chemokine (C-C motif) receptor 5; CXCR4, chemokine (C-X-C motif) receptor 4; HOS, Human CD4-negative osteosarcoma; mAb, monoclonal antibody.
[a]Enhancement of entry.

in Hep-G2 cells. Although both Huh-7 and Hep-G2 cells are derived from human hepatoma, many potential host factors [40] could influence HIV replication, which for the most part remain unknown. Similarly, only Huh-7 cells, but not Hep-G2 cells, were susceptible to HCV [41,42]. These reasons may be related to the difference between Huh-7 and Hep-G2 regarding the level of replication by SDA-1.

SDA-1 replicates in both proliferating and static hepatocytes

To investigate further whether normal human hepatocytes could sustain entry and replication of SDA-1, p-hepatocytes were used for the following experiments. Among the three specific markers of human hepatocytes, both albumin and cytokeratin-18, but not alpha-fetoprotein were detected in the p-hepatocytes suggesting that the hepatocytes we used were well differentiated (data not shown). We also found that CXCR4 was expressed on the surface of p-hepatocytes. In contrast, neither CD4 nor CCR5 was detected on the p-hepatocyte surface or by real-time PCR (RT–PCR) (data not shown).

We next explored whether SDA-1 can enter p-hepatocytes by using GFP-p. As shown in Fig. 4a, only SDA-1 GFP-p viruses gave GFP-positive cells in p-hepatocytes, whereas other HIV-1 GFP-p viruses did not. The GFP-positive cells showed spindle-like shapes suggesting that the infection occurred in the p-hepatocytes but not in the contaminating lymphocytes. Furthermore, we studied whether SDA-1 can replicate in the p-hepatocytes. As shown in Fig. 4b, the p-hepatocytes were productively infected by the SDA-1 Env-chimeric viruses and SDA-1 virus stock itself but not by the other HIV-1 Env-chimeric viruses. Moreover, we found that AMD3100 inhibited the replication of SDA-1 in p-hepatocytes in a dose-dependent manner (Fig. 4c) indicating that the infection of p-hepatocytes by SDA-1 was mediated through CXCR4.

A previous study [19] reported that the HIV-1 gp120 *env* directly caused hepatocyte death by signaling through CXCR4 *in vitro*; however, most studies were performed using the hepatoma Huh-7 cells not hepatocytes, therefore, it may not really reflect the nature of liver damage. To explore the pathological effects of HIV-1 CD4-independent infection on hepatocytes, we exposed p-hepatocytes to the SDA-1 and analyzed cell viability. We found that the viability of the p-hepatocytes in cells cultured with or without SDA-1 Env-chimeric viruses was comparable (96%, P was not significant) indicating that HIV-1 CD4-independent infection rarely induces hepatocyte death via an apoptotic process (data not shown). To further examine whether the infection or replication of SDA-1 is limited only to a certain number of p-hepatocytes or whether the infectivity or replication is influenced by the cell cycle, we studied the intracellular expression by flow cytometry of p24 and Ki-67 [43], a marker strictly associated with cell proliferation, in the HIV-1-infected p-hepatocytes. As shown in Fig. 4d, we found that 32.49% of p-hepatocytes were infected by SDA-1. However, there was no significant difference in

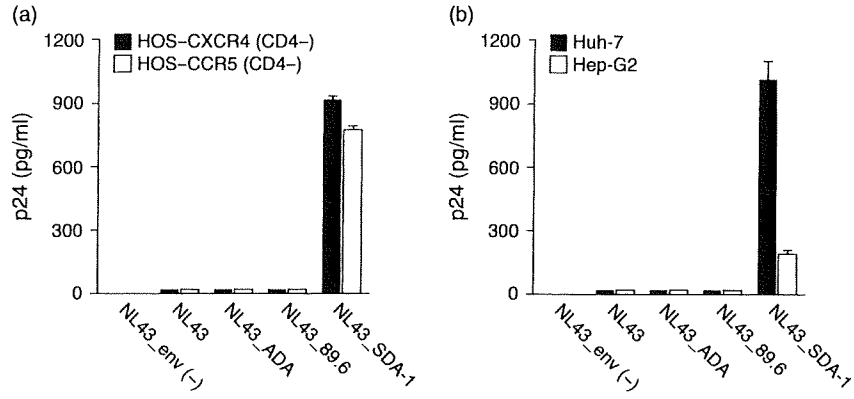

Fig. 3. CD4-independent infection of SDA-1 Env-chimeric viruses. The HOS cells (CD4⁻) expressing either CXCR4 or CCR5 (a) and two CD4⁻ human hepatoma cells (b) were incubated with the indicated HIV-1 Env-chimeric viruses. Virus replication was then monitored by p24 antigen production on day 3. Results shown (a, b) are means of triplicate experiments. Bars, standard deviation.

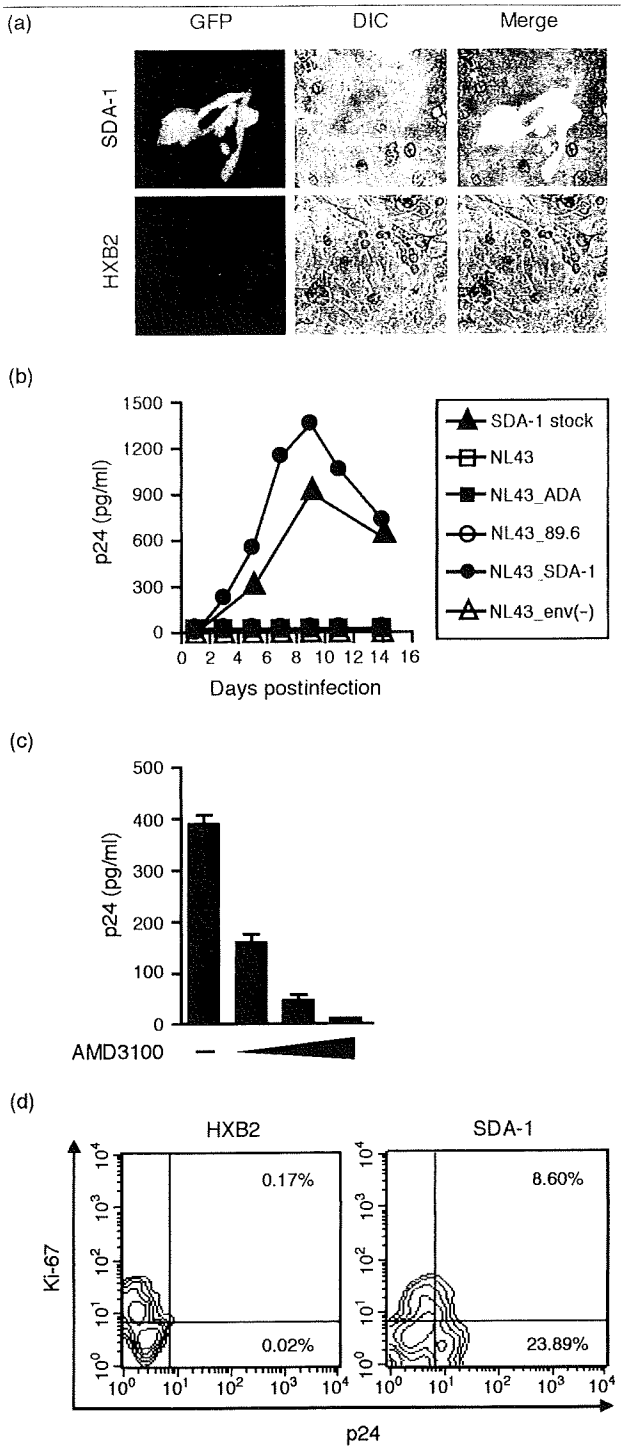

Fig. 4. SDA-1 enters and replicates in CD4$^-$ human p-hepatocytes. (a) Entry of SDA-1 into p-hepatocytes. The p-hepatocytes were exposed to the indicated HIV-1 GFP-p viruses for 48 h. Infectivity was determined as GFP$^+$ cells by confocal microscopy. (b) Replication of SDA-1 Env-chimeric viruses and SDA-1 virus stock in human p-hepatocytes. (c) SDA-1 infects p-hepatocytes through CXCR4. The inhibitory effects of AMD 3100 (0.1, 0.3 and 1.0 μM) on SDA-1 Env-chimeric viruses infection of p-hepatocytes were studied. Results shown are means of triplicate experiments. Bars, SD. (d) SDA-1 replicates in both proliferating and static human p-hepatocytes. Intracellular stainings of HIV-1-infected p-hepatocytes for p24 and Ki-67 were analyzed by flow cytometry. CXCR4, chemokine (C-X-C motif) receptor 4; GFP-p, GFP-pseudotypes.

percentage of p24 expression between Ki-67$^+$ (31%) and Ki-67$^-$ p-hepatocytes (33.1%), suggesting that SDA-1 efficiently enters and replicates in both proliferating and static hepatocytes.

Considering that SDA-1 can infect hepatocytes *in vitro*, it would have been interesting to determine whether the patient's liver was infected *in vivo*. However, consent for a liver biopsy was denied by the patient's family. There was no evidence of liver dysfunction. When virus was isolated from this patient; however, liver damage [an aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio ≥ 1] was observed at the end of the clinical stage. Although the cause of liver injury was unclear, our present data suggest that CD4-independent HIV-1 infection may lead to hepatocellular damage.

Discussion

In this study, we characterized a quasispecies of a CD4-independent HIV-1 isolate, termed SDA-1, which was able to utilize either CXCR4 or CCR5 in the absence of CD4. Moreover, we demonstrated that SDA-1 efficiently entered and replicated in Huh-7 hepatoma cells and normal human hepatocytes, through CXCR4, without inducing apoptotic cell death.

Many SIV and HIV-2 isolates can infect cells without CD4, at least to some extent. However, CD4-independent HIV-1 viruses have been rarely isolated and, so far, only a few laboratory-adapted CD4-independent HIV-1 variants have been reported. It must be noted that CD4-independent HIV-1 variants, isolated *in vitro* by passage through cells lacking CD4, have been shown to be more sensitive to neutralizing antibodies than CD4-dependent viruses [44,45]. Therefore, we might hypothesize that the emergence of a quasispecies of HIV-1 with a reduced requirement for CD4 is likely to be at a low abundance relative to the more common CD4$^+$ strains. However, with disease progression, HIV-1 variants with reduced affinity for CD4 and with increased affinity for chemokine receptor could evolve and become more robust in the viral quasispecies, disseminate in a variety of CD4$^-$ tissues *in vivo* under conditions of both reduced immunological pressure and a dramatically reduced pool of target CD4$^+$ cells concomitant with high levels of virus replication. It will be important to search the viral quasispecies in other patients, especially in the later stages of HIV-1 disease for the existence of similar CD4-independent HIV-1 variants and expanded cellular tropism.