Table 2. C 型慢性肝炎治療ガイドライン 2006

初回投与	genotype 1	genotype 2
〈高ウイルス量〉 1 Meq/ml 以上 100 KIU/ml 以上 300 fmol/l 以上	peg-IFNα-2b +ribavirin 併用療法 (48 週間)	peg-IFNα-2b +ribavirin 併用療法 (24 週間)
〈低ウイルス量〉 1 Meq/ml 未満 100 KIU/ml 未満 300 fmol/l 未満	IFN 単独療法(24 週間) peg-IFNα-2a 単独療法(24～48 週間)	IFN 単独療法(8～24 週間) peg-IFNα-2a 単独療法(24～48 週間)
再投与	genotype 1	genotype 2
〈高ウイルス量〉 1 Meq/ml 以上 100 KIU/ml 以上 300 fmol/l 以上	peg-IFNα-2b +ribavirin 併用療法 (48 週間)	peg-IFNα-2b +ribavirin 併用療法 (24 週間)
〈低ウイルス量〉 1 Meq/ml 未満 100 KIU/ml 未満 300 fmol/l 未満	peg-IFNα-2b +ribavirin 併用療法 (24 週間)	peg-IFNα-2b +ribavirin 併用療法 (24 週間)

与するかが著効率に大きく関係すると思われるので，保険上の問題はあるが，可能ならば 48 週間を超えて 72 週間あるいは 96 週間の治療を行えば，著効率はさらに上がるものと思われる．

肝細胞癌○

現在本邦では年間約 35,000 人が原発性肝癌で死亡しているが，原発性肝癌の 95% を占める肝細胞癌は，慢性肝疾患(主に肝硬変)をベースに発症することがほとんどで，その 90% は C 型肝炎ウイルスまたは B 型肝炎ウイルスの持続感染者からの発症である．残る 10% はアルコール長期多飲者，自己免疫性肝炎，原発性胆汁性肝硬変患者などであるが，近年メタボリック症候群に関連した非アルコール性脂肪性肝炎(NASH)患者からの肝細胞癌の発生が注目されている．

肝細胞癌の治療法としては，以前から肝切除術，経皮的エタノール注入療法(PEI，PEIT)や経皮的マイクロ波凝固療法(PMCT)などの超音波ガイド下経皮的局所療法，経カテーテル的肝動脈化学塞栓療法(TACE, TAE)などがあるが，2000 年前後より，経皮的ラジオ波焼灼術(RFA，PRFA)，皮下埋め込み型リザーバーポートを介した肝動注化学療法(HAI)，そして生体肝移植術が行われるようになり，早期の肝細胞癌，門脈内腫瘍栓を有する高度進行肝癌，また進行した肝硬変のため肝癌に対する積極的な治療が施行できない肝予備能低下例のいずれに対しても，従来とは比較にならない良好な治療効果が得られている．本稿では，これら最近施行されるようになった 3 つの治療法について述べる．

1．経皮的ラジオ波焼灼術(RFA，PRFA)

従来から行われていた経皮的エタノール注入療法(PEI)は液体(エタノール)を注入するため，腫瘍内に均一に拡散せず，隔壁や被膜を通過できないため，腫瘍の残存とそれに伴う局所再発が問題であった．関らが発表した経皮的マイクロ波凝固療法(PMCT)は，挿入した針から熱を発生させ，腫瘍を熱凝固させる画期的な治療法であったが，一回の治療で得られる腫瘍の壊死範囲は直径 2 cm 程度であった[9]．一方 Rossi らが報告した経皮的ラジオ波焼灼術(RFA)は，PMCT より一回に得

られる壊死範囲が大きいことから，わが国でも1999 年以降多くの施設で施行されるようになった[10]．RFA は 2004 年 4 月にようやく保険収載となり，現在も RFA 針の開発，改良が進んでいる．

現在 RFA 一回の治療で得られる腫瘍壊死範囲は直径 3 cm 程度であり，従来の PEI の一般的な治療対象といわれていた「直径 3 cm，3 個以内」の肝細胞癌に対しては RFA のよい適応と考えられる．とくに 2 cm 以下の症例に関しては局所再発率，生存率のいずれも肝切除術とまったく同等の成績が得られており，手術侵襲の少ない RFA を選択するケースが増えている．近年，multiple-detector row CT(MDCT)などの画像診断用機器の急速な進歩により，詳細な肝細胞癌の情報が得られるようになり，治療前後の画像を三次元的に検討することも可能になっている．最近の RFA 治療の成績の向上は，これら画像診断能の進歩に負う部分が大きいことも見逃せない．ちなみに当科における RFA の局所再発率は，3 年で 4% と十分満足できる結果が得られている．

2．リザーバー肝動注化学療法(HAI)

以前は vp3，vp4 の門脈腫瘍栓が出現した症例や肝右葉全体を占める塊状型，びまん型肝細胞癌症例に対しては，内科的治療はまったく奏効せず，仮に手術を行っても術後早期に肝動脈造影で花が咲いたような同時多発再発を認めることが多く，予後不良であると考えられていた．しかしながら，近年施行されるようになった HAI により，著効する症例がしばしばみられ，中には vp4 の門脈腫瘍栓合併塊状型肝細胞癌で余命 2〜3ヵ月と考えられた症例が，HAI により tumor free となり，3 年以上無再発で生存する症例もみられている．

HAI はまず固有肝動脈，あるいはそれより末梢の肝動脈へのカテーテルの留置を行う．胃十二指腸動脈など肝臓以外の臓器への抗癌薬の流出が予想される場合は，コイルなどによる血行改変術が必要である．大腿動脈刺入部側のカテーテルの端はリザーバーポートに接続し，皮下トンネルを通して，腹部の皮下に埋設する．

HAI のレジメンは fluorouracil(5-FU) を用いたものが一般的で，現段階では low dose FP と呼ばれる低容量の 5-FU と cisplatin(CDDP)の併用療法と，5-FU と IFN の併用療法の両者が有力視されている．low dose FP 療法は HAI 施行当初から行われているレジメンで，多くの施設で行われており，ある一定の効果が示されているが，消化器症状や腎毒性などの CDDP の副作用が問題となることがある[11]．一方，5-FU/IFN 併用療法は，抗癌薬としての副作用は比較的軽く反復投与がしやすいこと，とくに C 型肝炎，肝硬変患者に対しては肝炎の鎮静化も見込めるため，肝予備能の保持が期待できることが有利な点であるが，IFN の副作用である発熱やうつ状態の出現，また肝癌治療に対して IFN は保険収載されていないことが大きな問題となる[12]．現在 5-FU/IFN 療法は治験進行中であり，保険治療認可が待たれる．

low dose FP 療法，5-FU/IFN 療法とも，一般に週 5 日連日投与後 2 日休薬，これを 2〜4 週間継続するのを 1 クールとし，2〜3 クール施行後効果判定を行って，著明な治療効果が得られる症例はさらに治療継続，あるいは切除術や経皮的局所療法などの根治療法が行われる．当科における治療成績は，奏効率 40%，1 年生存率 48% であった．ただし，明らかに治療に反応しない症例も半数程度存在し，奏効例の 1 年生存率 100% に対し，非奏効例は 6ヵ月生存率 44% と明らかな差を認めた．現段階ではどのような症例に対して HAI が有効であるかは不明であるが，治療開始前のインフォームドコンセントをとる際に注意が必要である．奏効例を 1 例示すが，左葉の塊状型肝癌，門脈左枝への腫瘍栓を認めた症例に対し，5-FU/IFN 療法を 2 クール施行した．腫瘍は著明に縮小し，腫瘍栓もほぼ消失している．

3．肝 移 植

2004 年から B 型，C 型のウイルス性肝硬変や肝細胞癌症例に対しても，生体肝移植術が保険適用となり，生体肝移植症例が増加してきている[13]．

肝細胞癌症例に対する生体肝移植術施行時に問

題となるのが，術後の肝癌の再発である．高度進行症例に対して肝移植術を行った場合，術後早期に移植肝への癌の再発を認めることが欧米の報告から知られている．このためイタリアのグループより「Milan Criteria」という基準が提唱された[14]．これは術前の肝細胞癌が単発なら5 cm 以下，多発なら3 cm，3 個以内で，門脈侵襲などの脈管侵襲，遠隔転移を認めない症例を指し，「Milan Criteria」に合致した症例は「Milan Criteria」から逸脱した症例に比して有意に無再発生存率，生存率ともに良好であったことを示した．このデータに倣い，本邦においては「Milan Criteria」を満たす肝癌症例のみ保険適用となっている．

従来，肝予備能がわるく，肝癌に対する治療が困難であった child C 症例のみが移植の適応と考えられていたが，「Milan Criteria」合致肝細胞癌症例の保険適用の導入に伴い，肝移植対象として検討される肝細胞癌症例が増えてきている．すなわち，将来的には child C になる可能性が高い child B 症例で待機期間中に肝癌が「Milan Criteria」を超えてしまいそうな症例，さらには肝予備能は正常であるが，度重なる局所治療・TACE にもかかわらず，次回再発時には「Milan Criteria」を超えそうな症例は，肝予備能が比較的温存されていても肝移植の適応として考えられるようになってきている．現在の当院第二外科における，「Milan Criteria」合致例での肝癌肝移植症例の 3 年生存率は 83% である．比較的良好なデータであるが，症例の蓄積によりこの数値はさらに向上すると思われるため，とくに初回治療後に再発した症例は繰り返し内科的治療を継続するべきか，「Milan Criteria」を超える前に移植に踏み切るべきか慎重な検討が必要である．移植をするにせよ，しないにせよ，肝移植という選択肢があるということは，あらかじめ患者家族および患者本人にインフォームドコンセントを行う必要がある．

ただし，脳死肝移植がほとんど行われていない今日の状況では，肝移植を選択する場合ほぼ生体肝移植しか可能性がないこと，生体肝移植を行うためには健康なドナーにメスを入れねばならず，100% 安全とはいいきれないこと，B 型肝硬変，C 型肝硬変患者の場合，移植後肝炎の再発の可能性があり，HBV については核酸アナログと抗 HBV 免疫グロブリンの投与でほぼ制御可能であるが，HCV については術後に C 型肝炎の再燃が 100% に近い割合で起こり，肝炎の再発が術後の生存率を明らかに低下させるため，IFN 治療の必要があることなどを，患者やドナーらに十分説明して理解してもらわなければならない．

おわりに○

以上，最近大きく変化している肝疾患に対する治療について述べたが，今後もしばらくは肝炎，肝細胞癌患者に対する治療のニーズは高いと考えられ，新たな治療法の開発・改良が行われるものと思われる．

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連　載

● 薬の知識

バラクルード® 0.5 mg 錠(エンテカビル)

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バラクルード® 0.5 mg 錠(エンテカビル)は2006年9月に発売になったB型慢性肝炎，B型肝硬変に対する経口の逆転写酵素阻害薬であり，B型慢性肝疾患に対する同様の核酸アナログとしては，ゼフィックス® (ラミブジン)，ヘプセラ® (アデフォビル)に次いで本邦で3剤目となる(図1).

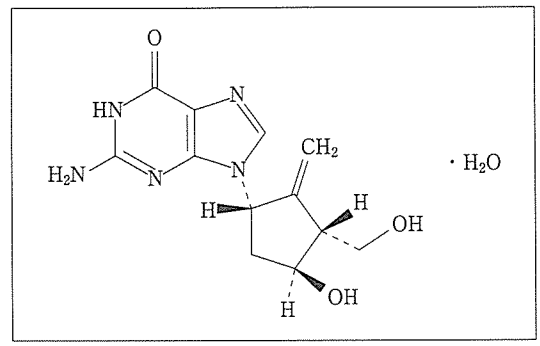

図1　エンテカビルの構造式

Ⅰ．逆転写酵素阻害薬の作用機序

これらの逆転写酵素阻害薬の作用機序についてはすでによく知られているが，不完全2本鎖DNAウイルスであるB型肝炎ウイルス(HBV)のウイルスゲノムは，肝細胞の核内でいったん完全2本鎖DNAとなり，宿主のRNA polymeraseでプレゲノムRNAとなった後，ウイルス由来の逆転写酵素を用いてHBVゲノムDNAを複製する.

エンテカビルなどの逆転写酵素阻害薬は，宿主のゲノムDNAを構成するデオキシアデノシン，デオキシグアノシンなどのヌクレオシドに構造的に非常に類似した核酸アナログと呼ばれる物質である．エンテカビルはHBVプレゲノムRNAの逆転写反応中に伸長されるHBVゲノムDNA中に取り込まれ，隣のヌクレオシドとの共有結合にねじれを生じさせることによりDNA伸長反応が止まり，ウイルスゲノムの複製を抑制している(図2)．(実際には，DNA伸長反応はエンテカビル結合の2-3塩基後に停止している.)ちなみにエンテカビルはグアノシンの核酸アナログ，ラミブジンはシチジン，アデフォビルはアデノシンの核酸アナログである.

また，この逆転写反応に先立って，HBVの逆転写酵素がプレゲノムRNAと結合して

Key words : B型肝炎ウイルス，逆転写酵素阻外薬，核酸アナログ，B型慢性肝炎

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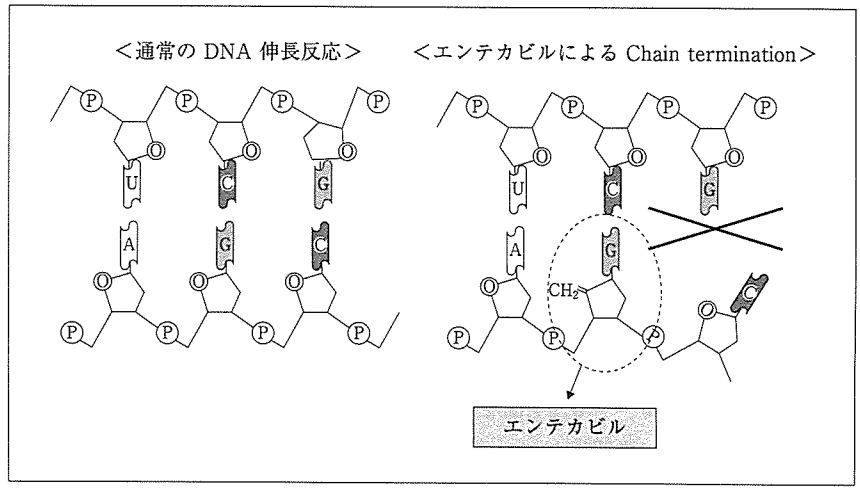

図2　エンテカビルの作用機序

DNA 伸長反応が始まる，「プライミング」という反応が起きるが，エンテカビルはこのプライミング阻害活性をもつ点が，ラミブジンと異なる点で，エンテカビルのほうがより強い抗ウイルス活性をもつ根拠の一つとなっている．

II．エンテカビルの国内第二相試験

エンテカビルの国内第二相試験では，核酸アナログ未使用慢性 B 型肝炎患者に対してラミブジン 100 mg/day とエンテカビル 0.01，0.1，0.5 mg/day 投与の用量反応性試験が行われ，エンテカビル 0.5 mg/day 投与群はラミブジン 100 mg/day 投与群に比して優位に HBV DNA を抑制することが示された．

さらにエンテカビル 0.5 mg/day 48 週間投与の国内第二相試験では，高い HBV DNA 減少率と，ALT 正常化率を認め，投与終了後の肝生検の組織において肝線維化の著明な改善を認めた．

これらの結果を基に，核酸アナログ未使用患者に対してバラクルード 0.5 mg/day で投与されることになった．なお欧米では，肝組織の改善度でもエンテカビルがラミブジンより優るという報告もある．

III．ラミブジン耐性株に対するエンテカビルの投与

これらの核酸アナログは，投与開始後数週間で速やかに HBV DNA，ALT の低下が見込める一方で，内服中止すると大部分の症例で再燃が認められる点，また HBV ゲノム DNA の変異による耐性株の出現する点が大きな問題である．とくにラミブジンにおける耐性株の出現率の高さは深刻で，当科のデータでも投与開始 4〜5 年でほぼ半数の患者に耐性株が出現している．ラミブジン耐性株は，HBV polymerase の逆転写酵素領域の YMDD モチーフと呼ばれるアミノ酸配列が YIDD もしくは YVDD に変異することにより起こる（M 204 I，M 204 V と表記）．アデフォビル長期投与による耐性株の出現頻度は，ラミブジンに比して明らかに低いが，2 年で 3%，4 年で 18%の変異株の出現を認めている（A 181 T または N 236 T）．

エンテカビルは，国外でも発売されて間もないため，長期投与のデータはまだないが，核酸

アナログ未使用症例では，投与2年後で現在までのところ，変異株の出現を認めていない．しかしながら，すでにM204IまたはM204Vの変異をもつラミブジン耐性株に対するエンテカビルの投与においては，1年で7%，2年で9%のエンテカビル耐性株の出現を認めており，ラミブジン耐性例に対してはエンテカビル投与に際して，十分な注意が必要である(T184G，S202IまたはM250V)．なおラミブジン耐性例に対しては，バラクルード0.5 mgを1日に2錠(1.0 mg/day)投与が推奨されている．

Ⅳ．今後のB型慢性肝疾患に対する治療方針

今後のB型慢性肝疾患に対する治療方針であるが，厚生労働省「B型およびC型肝炎ウイルスの感染者に対する標準化に関する臨床的研究班」(熊田博光班長)による「B型慢性肝炎治療の新ガイドライン2006」によると，35歳以上の，ALT値が1.5倍以上を持続する症例，あるいは35歳未満でも肝病変の進行した症例では，核酸アナログの投与が推奨されている．核酸アナログ未使用例に関しては，抗ウイルス効果が高く，変異株の出現の可能性の低いエンテカビル(バラクルード)が今後第一選択となることは異論のないところであろう(2006年10月現在，本邦ではアデフォビルの単独投与は認可されていない)．

一方，すでにラミブジン耐性を有する患者に対しては，従来どおりラミブジンとアデフォビルの併用を行うか，エンテカビル単独投与に変更するかについては，まだ結論が出ておらず，今後の長期投与成績の結果を待つ必要がある．

もともと，逆転写酵素阻害薬は，レトロウイルスであるHIVに対して開発されたものをHBVにも使用開始されたという経緯があり，欧米ではさらに数種類の抗HIV用逆転写酵素阻害薬が，HBVに対しても抗ウイルス効果をもつという報告がある．

現在，本邦で市販されている3種類のHBVに対する核酸アナログのなかでは，単剤投与としてはエンテカビルの変異株出現率がもっとも低いが，長期投与に伴う変異株の出現の可能性を考えると，将来的には抗HIV療法と同様に数種類の薬剤によるカクテル療法についても検討が必要になるかもしれない．

Ⅴ．投与法

最後に投与法であるが，本剤は食事の影響により吸収率が低下するので，空腹時(食後2時間以降かつ次の食事の2時間以上前)に内服する必要があり，現実的には食後2時間以上経った就寝前の内服がよいと思われる．

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Peptide-Loaded Dendritic-Cell Vaccination Followed by Treatment Interruption for Chronic HIV-1 Infection: A Phase 1 Trial

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Immune response enhanced by therapeutic HIV-1 vaccine may control viral proliferation after discontinuation of highly active antiretroviral therapy (HAART). Although which strategies for therapeutic vaccination are feasible remains controversial, application of dendritic cells (DCs) as a vaccine adjuvant represents a promising approach to improving deteriorated immune function in HIV-1-infected individuals. The safety and efficacy of DC-based vaccine loaded with HIV-1-derived cytotoxic T lymphocytes (CTL) peptides were thus investigated in this study. Autologous DCs loaded with seven CTL peptides with HLA-A*2402 restriction were immunized to four HIV-1-infected individuals under HAART. In terms of safety, peptide-loaded DCs were well tolerated, and only mild local and general symptoms were observed during vaccine administration. ELISPOT assays to detect IFN-γ production in $CD8^+$ lymphocytes revealed a limited breadth of responses to immunized peptides in two of four participants, but no response in the remaining two participants. Differences in immunological response might be attributable to the fact that responders displayed higher nadir CD4 counts before starting HAART and were immunized with a larger number of DCs per reactive peptide than non-responders. Discontinuation of HAART after vaccination failed to lower viral set points compared to those before starting HAART. This early outcome warrants further exploration to elucidate the therapeutic value of vaccination with DCs in HIV-1 infection. ***J. Med. Virol. 78: 711–718, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: HIV-1; vaccine; HAART; treatment interruption

INTRODUCTION

Although highly active antiretroviral therapy (HAART) has significantly improved prognosis for HIV-1 infection, life-long therapy remains a requirement for continuous viral suppression [Ramratnam et al., 2000; Siliciano et al., 2003]. Long-term toxicity of HAART is therefore of medical concern and the economic cost of HAART has become a major social problem. These issues have facilitated attempts at strategic or structured treatment interruption (STI). However, successful results have not been obtained in patients starting HAART in the chronic phases, as HIV-1-specific immunity is already exhausted at the moment of treatment interruption [Oxenius et al., 2002; Fagard et al., 2003; Kaufmann et al., 2004].

Several lines of evidence have revealed that cytotoxic T lymphocytes (CTLs) play a critical role in control of HIV-1 proliferation, and that maintenance of CTL function during chronic infection requires the presence of $CD4^+$ helper T cells [Borrow et al., 1994; Koup et al., 1994; McMichael and Rowland-Jones, 2001]. However, HIV-1 selectively infects and destroys HIV-1-specific $CD4^+$ T cells, and causes quantitative and qualitative

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TABLE I. Baseline Subject Characteristics

	Subject			
	1	2	3	4
Age (years)	39	46	52	40
Sex	M	M	M	M
Duration of known seropositivity (months)	66	39	46	67
Duration of HAART (months)	65	37	42	64
HAART menu[a]	AZT + 3TC + EFV	AZT + 3TC + EFV	AZT + 3TC + EFV	d4T + 3TC + NFV
CD4 counts (/μl)				
Before HAART	164	216	50	2
At enrollment	453	658	330	340
Viral load (copies/ml)				
At enrollment	<50	<50	<50	<50
Preparation of vaccine				
Number of PBMCs obtained by leukopheresis	7.6×10^9	9.4×10^9	6.8×10^9	9.7×10^9
Number of DCs used for each vaccination	$0.7–1.2 \times 10^7$	$0.9–1.4 \times 10^7$	$1.0–1.5 \times 10^7$	$1.2–1.8 \times 10^7$
Amino acid sequences of epitope portions[b]				
Gag28	3R	3R, 7L[c]	3R	3R, 7V[c]
Gag296	wt	wt	wt	wt
Nef138	2F	5C[c]	2F	2F
Env584	4G[c]	4Q	4K, 7R, 11L[c]	wt and 4K[c]

[a]AZT, azidothymidine; 3TC, lamivudine; EFV, efavirenz; NFV, nelfinavir.
[b]wt (wild-type) represents amino acid sequences are identical to those of SF-2. Others represent amino acid positions of substitution and the substituted amino acids (see Table II).
[c]These substitutions were not included in peptides used in this study.

impairment of HIV-1-specific immunity, as shown by us and other groups [Watanabe et al., 2001; Kawamura et al., 2003]. When HAART is started and viral proliferation is controlled, destruction of $CD4^+$ T cells stops and naive lymphocytes are provided from the thymus. The immune system, however, is unable to produce and maintain HIV-1-specific immunity due to a loss of antigen stimuli under HAART. Treatment interruption in patients who start HAART in the chronic phase thus results in unfavorable outcomes.

Given this pathogenesis of HIV-1 infection, a therapeutic HIV-1 vaccine that is administered during HAART and potentiates HIV-1-specific immunity would theoretically offer a feasible strategy for achieving better viral control after STI. To test this hypothesis, we conducted a phase 1 clinical trial in which autologous dendritic cells (DCs) loaded with HIV-1-derived CTL epitope peptides were administered to four HIV-1-infected individuals and HAART was discontinued thereafter. DCs were used as highly specialized antigen-presenting cells that not only restore qualitative impairment of CTLs, but also stimulate naive $CD8^+$ T cells newly provided from the thymus during HAART [Banchereau et al., 2000]. We report herein the safety and efficacy of this DC-based therapeutic vaccine in addition to clinical outcomes after interruption of HAART.

MATERIALS AND METHODS

Participants

Subjects comprised four men with chronic HIV-1 infection and HLA genotype A*2402. All participants were under HAART with undetectable viral loads (VL; <50 copies/ml) for ≥1 year before enrollment. The institutional ethics committee approved this clinical trial and all participants provided written informed consent. Baseline characteristics are summarized in Table I.

Synthetic Peptides

Clinical-grade synthetic peptides (Table II) used for vaccination and ELISPOT assay were purchased from Multiple Peptide Systems (San Diego, CA). Gag(1–115) comprises a pool of 12- to 17-mer peptides with 10 amino acid overlaps that cover the whole Gag protein (subtype B consensus sequence) but do not include peptides containing Gag28 and Gag296 epitopes. Gag overlapping peptides were purchased from Operon Biotechnologies (Huntsville, AL). CMV-pp65 [Kuzushima et al., 2001] and EBV-TL9 [Lee et al., 1997] are both HLA-A24-restricted epitopes derived from Cytomegalovirus and Epstein-Barr virus and were purchased from Sigma-Genosys Japan (Ishikari, Japan).

TABLE II. A*2402-restricted CTL Epitope Peptides Used in This Study

Protein	Epitope	Amino acid position	Peptides used in this study	
			Designation	Sequence
Gag	Gag28	28–36	Gag28-wt	KYKLKHIVW
			Gag28-3R	KYRLKHIVW
	Gag296	296–306	Gag296	RDYVDRFYKTL
Nef	Nef138	138–147	Nef138-wt	RYPLTFGWCF
			Nef138-2F	RFPLTFGWCF
Env	Env584	584–594	Env584-wt	RYLRDQQLLGI
			Env584-4Q	RYLQDQQLLGI

RNA Extraction, PCR Amplification, and Sequencing

Viral RNA was extracted from plasma and subjected to first and second polymerase chain reaction (PCR), as described previously [Furutsuki et al., 2004]. PCR primers for Nef and Env epitope portions have been described previously [Furutsuki et al., 2004], and other primers are listed below (all nucleotide positions are in accordance with the HIV-1 SF2 strain).

For Gag28 epitope, 1st PCR primer set: forward: 5′-CGCAGGACTCGGCTTGCTGAAG-3′ (691-712) reverse: 5′-GCTATGTCACTTCCCCTTGGTTC-3′ (1506-1484). For Gag28 epitope, 2nd PCR primer set: forward: 5′-GAGAGAGATGGGTGCGAGAGC-3′ (784-804) reverse: 5′-TCTCTAAAGCTTCCTTGGTGTC-3′ (1097-1076). For Gag296 epitope, 1st PCR primer set: forward: 5′-AAGTAATACCCATGTTTTCAG-3′ (1296-1316) reverse: 5′-CTAAAATTGCCTCTCTGCATC-3′ (1947-1927). For Gag296 epitope, 2nd PCR primer set: forward: 5′-CCAGATGAGAGAACCAAGG-3′ (1474-1492) reverse: 5′-ATCTGGGTTTGCATTTTGG-3′ (1783-1765). For reverse transcriptase region, 1st primer set: forward: 5′-ATGATAGGGGGAATTGGAGGTTT-3′ (2393-2415) reverse: 5′-TACTTCTGTTAGTGCTTTGGTTCC-3′ (3422-3399). For reverse transcriptase region, 2nd primer set #1: forward: 5′-GACCTACACCTGTCAACATAATTGG-3′ (2492-2516) reverse: 5′-TAATCCCTGCATAAATCTGACTTGC-3′ (3379-3355). For reverse transcriptase region, 2nd primer set #2: forward: 5′-GTACTTTAAATTTCCCCATTAGTCC-3′ (2543-2567) reverse: 5′-CAGTCCAGCTGTCTTTTTCTGGC-3′ (3316-3294). For protease region, 1st primer set: forward: 5′-AGACAGGYTAATTTTTTAGGGA-3′ (2074-2095) reverse: 5′-TATGGATTTTCAGGCCCAATTTTTGA-3′ (2716-2691). For protease region, 2nd primer set: forward: 5′-AGAGCCAACAGCCCCACCAG-3′ (2155-2174) reverse: 5′-ACTTTTGGGCCATCCATTCC-3′ (2618-2599).

Purified PCR products were either directly sequenced or subcloned into pGEM-T vectors (Promega, Madison, WI) and sequenced using an ABI Prism dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) on a Perkin-Elmer ABI-377 sequencer.

Preparation of DCs

Leukocytes fractions were collected from participants by leukopheresis of blood samples, and peripheral blood mononuclear cells (PBMCs) were purified through a ficoll-hypaque gradient. Obtained PBMCs were aliquoted into cryotubes, and stored at −150°C until use. For induction of immature DCs, frozen PBMCs were thawed, suspended in PBS and incubated in plastic dishes for 30 min at 37°C. Adherent cells were then cultured in RPMI medium (HyClone, Logan, UT) containing 10% human AB serum (COSMO BIO, Tokyo, Japan), 50 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ), and 50 ng/ml of recombinant human interleukin 4 (PeproTech). After 6–7 days of culture, TNF-α (PeproTech) (50 ng/ml) and 7 CTL epitope peptides (10 μM each) were added and peptide-loaded mature DCs were harvested the next day. Cells were resuspended in 1 ml of saline and kept on ice until inoculation into participants. All procedures were conducted in a dedicated facility based on GCP as defined by the Japanese Ministry of Health, Labor, and Welfare.

Vaccination and Interruption of HAART

DCs loaded with HIV-1-specific epitope peptides were injected subcutaneously into axillary areas six times every 2 weeks. After the 6th vaccination, HAART was discontinued and clinical, immunological, and virological consequences were observed every week. If the HAART regimen contained efavirenz, nevirapine, or lamivudine, these antiretroviral agents were changed at least 2 weeks before treatment interruption to other agents with shorter half-lives. HAART was restarted when participants met any of the following criteria: VL > 50,000 copies/ml; VL > 5,000 copies/ml on three consecutive measurements; or CD4 counts <200/μl on two consecutive measurements.

ELISPOT Assay

For ELISPOT assay, PBMCs were aliquoted to 96-well multiscreen plates precoated with 5 μg/ml anti-IFN-γ monoclonal antibody (mAb) 1-D1K (Mabtech, Nacka Stand, Sweden). Peptides were added at concentrations of 10^{-6} M and incubated for 18 hr at 37°C. After washing wells, 100 μl of 1 μg/ml biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech) was added and incubated at room temperature for 90 min. After unbound mAb was removed, 100 μl of 1:1,000 diluted streptavidin-alkaline phosphatase conjugate (Mabtech) was added and incubated at room temperature for 60 min. Spots were developed using an alkaline phophatase conjugate substrate kit (BIO-RAD, Hercules, CA), and counted using a KS Elispot compact (Carl Zeiss, Oberkochen, Germany). Assays were conducted in triplicate and results were represented as mean numbers of spots per 10^6 PBMCs. When the number of spots was more than three-times the number in controls (PBMCs cultured without peptides), the response was considered significant.

RESULTS

Selection of CTL Epitope Peptides Derived From HIV-1

As immunogens for vaccination, we used HIV-1-derived peptides that were known to elicit strong CTL response and were restricted to HLA-A*2402 expressed in approximately 70% of the Japanese population (allele frequency of A*2402; 36.5%) [Tanaka et al., 1996]. The selected CTL epitope portions with A*2402 restriction were amino acid positions from 28 to 36 of Gag(Gag28), from 296 to 306 of Gag(Gag296), from 138 to 147 of Nef (Nef138), and from 584 to 594 of Env (Env584) (Table II). Whereas amino acid sequences in the Gag296 epitope portion have been shown to be conserved, other

three-epitope portions have been reported to display amino acid mutations [Ikeda-Moore et al., 1997, 1998; Dorrell et al., 1999; Furutsuki et al., 2004]. Thus, for the three-epitope portions, both wild-type peptides (Gag28-wt, Nef138-wt, and Env584-wt) and one of the representative mutant peptides (Gag28-3R, Nef138-2F, and Env584-4Q) were selected (Table II). Sequence analysis of HIV-1 derived from the four enrolled participants revealed that at least two of four epitope portions displayed amino acid sequences identical to immunized peptides (Table I).

DC-Based Vaccine Administration and Treatment Interruption

The four men enrolled in this study displayed undetectable VL under HAART (Table I). Leukopheresis was used to collect $6.8–9.7 \times 10^9$ PBMCs from each participant, and $0.7–1.8 \times 10^7$ mature DCs were harvested for each vaccination without contamination by pathogens or reactivation of autologous HIV-1. Peptides were either loaded to DCs by mixture (Subjects 1 and 2) or separately (Subjects 3 and 4), and peptide-loaded DCs were injected subcutaneously to areas near the axilla in two to three divided doses. During the course of six vaccinations in the four participants, subcutaneous bleeding (n = 1), erhythema at the injection site (n = 1), and general malaise (n = 1) were reported as local and generalized adverse events, all of which were non-serious and resolved without specific treatment (Table III).

Serum VLs were examined every week after treatment interruption and became positive above the detection limit of 50 copies/ml in all four participants, in weeks 3, 3, 1, and 2, respectively (Table III; Fig. 1). Subject 4 experienced fever at 38°C, myalgia, skin rash, and cervical lymph node swelling at 1 week after interruption, accompanied by mild liver dysfunction and thrombocytopenia, mimicking acute retroviral syndrome, and subsiding spontaneously within 2 weeks. All participants met criteria to restart HAART (at weeks 8, 4, 5, and 3, respectively) and VLs had been suppressed to undetectable levels by 11–30 weeks after restart of original HAART regimens. Differences between peak VL after treatment interruption and VL before start of HAART did not exceed 0.5 in $\log_{10}$ scale in all four participants (Table III). CD4 counts decreased after discontinuation of HAART in all participants to the level of approximately 200/μl (Fig. 1). After restarting HAART, CD4 counts in Subjects 2 and 4 gradually recovered, but those in Subjects 1 and 3 fluctuated at lower levels than prior to treatment interruption despite successful viral control by restarted HAART.

Immunological Analysis of Vaccines

HIV-1-specific CTL response to immunized peptides was evaluated by ELISPOT assay to detect IFN-γ-producing cells. Unseparated PBMCs were used for the assay, as preliminary experiments showed that IFN-γ production responding to both immunized peptides and control peptides was only seen in the $CD8^+$ population (data not shown). Significant responses to Nef138-wt were observed in Subjects 1 and 2, with weak responses to Nef138-2F in Subject 1 after the 5th vaccination (Fig. 2; black bars). Response in Subject 2 was

TABLE III. Clinical Outcomes of Vaccine Administration and Interruption of Antiretroviral Therapy

	Subject			
	1	2	3	4
CD4 counts (/μl)				
At 1st vaccination	512	310	520	428
Nadir after treatment interruption	257	252	181	213
Decrease in CD4 counts[a]	−255	−58	−339	−215
Viral load ($\log_{10}$ [copies/ml])				
Before start of HAART	4.08	5.15	4.15	5.23
Peak after treatment interruption	4.00	5.04	4.58	5.26
Reduction of VLs[b]	0.08	0.11	−0.43	−0.03
First detectable VLs after interruption (weeks)	3	3	1	2
Duration of interruption (weeks)	10	18	6	5
Adverse events				
During vaccination	Subcutaneous bleeding at injection site		General malaise	Erythema at injection site
After interruption of HAART				Fever, lymph node swelling, thrombocytopenia, elevated liver enzyme
HAART during 2 weeks before interruption	AZT + ddC + NFV	AZT + ddC + NFV	AZT + ABC + NFV	d4T + ABC + NFV
Drug-resistant mutations				
Reverse transcriptase region	None	None	None	None
Protease region	None	None	M36J (4w) none (6w)	None

[a]CD4 count at nadir after treatment interruption subtracted from count at 1st vaccination.
[b]VL before start of HAART subtracted from VL at peak after treatment interruption.

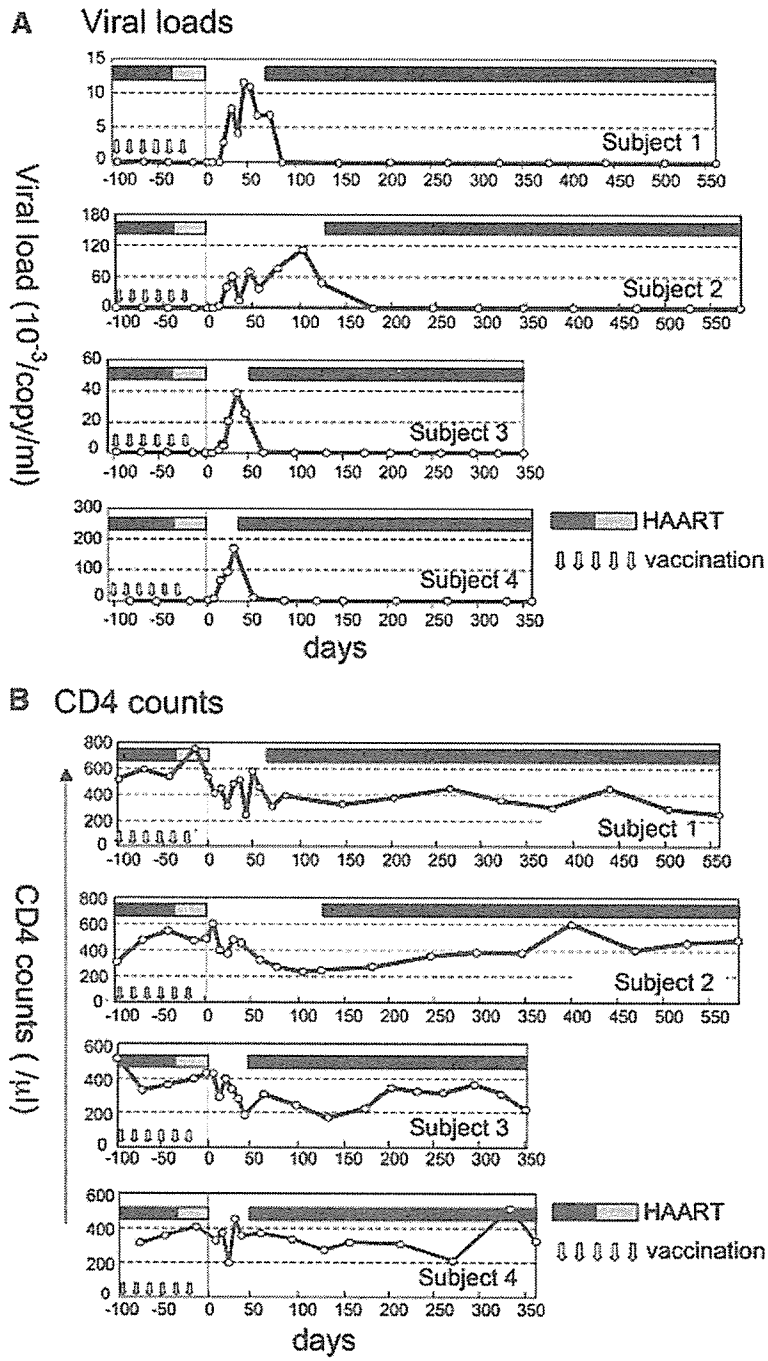

Fig. 1. Clinical courses of the four subjects. Viral loads (A) and CD4 counts (B) of the four subjects during vaccination (downward arrows) are described together with duration of treatment interruption and restart of HAART. Black bars represent duration under original HAART regimens and gray bars represent duration under alternative HAART regimens to avoid drug resistance.

specifically induced by DC-based vaccine, as no responses to control peptides of Gag(1–115), CMV-pp65, or EBV-TL9 were detected. In Subject 1, however, response was also observed to control peptides of Gag(1–115) and EBV-TL9 after the 5th vaccination, suggesting that this response to Nef138-wt and Nef138-2F included non-specific stimuli by DC injection. When HAART was discontinued and autologous virus rebounded, specific responses in Subjects 1 and 2 were induced to Nef138-wt and Nef138-2F in addition to Gag(1–115), whereas

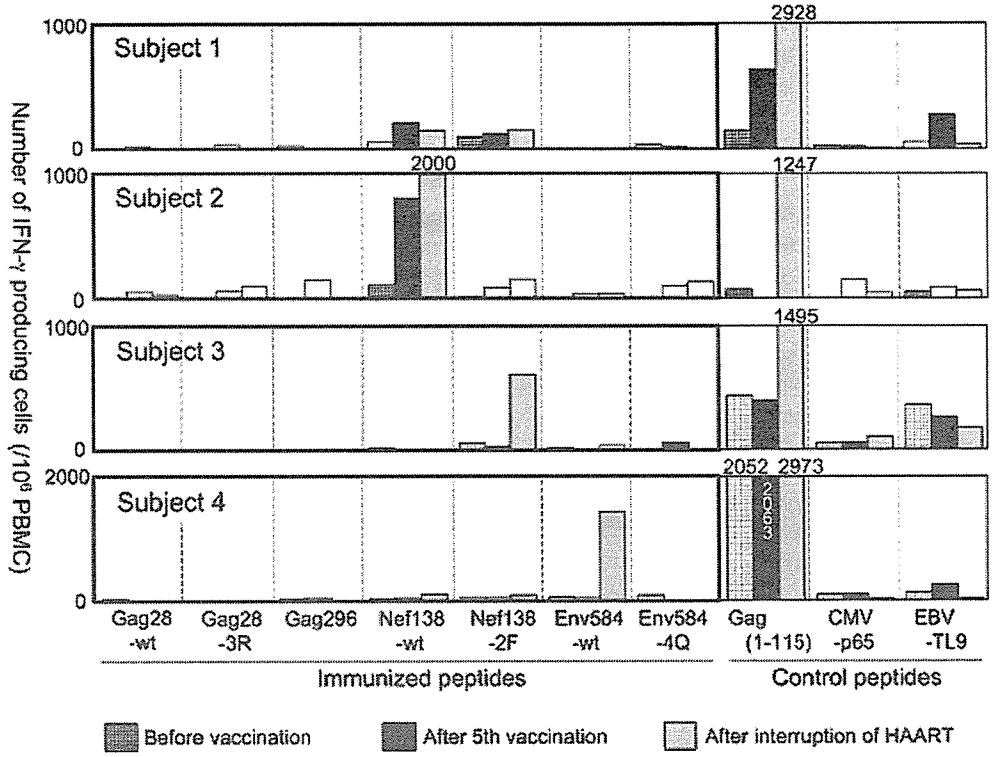

Fig. 2. Peptide-specific responses in PBMCs of vaccines. PBMCs collected before vaccination (shaded bars), after 5th vaccination (black bars) and 4 weeks after treatment interruption (gray bars), were incubated with immunized peptides; Gag28-wt, Gag28-3R, Gag296, Nef138-wt, Nef138-2F, Env584-wt, and Env584-4Q in addition to control peptides; Gag(1–115), CMV-pp65, and EBV-TL9. Response to each peptide was analyzed using ELISPOT assay detecting IFN-γ-producing cells. Specific response was calculated by subtracting number of IFN-γ-producing cells without peptides from number of cells with each peptide, and the subtracted number of cells was represented as per 10^5 PBMCs. When absolute numbers of IFN-γ-producing cells with peptides were less than three times numbers of cells without peptides, the response was considered as background and represented with white bars. To show details of data, some bars are scaled out and the specific response is represented by actual numbers of spots per 10^5 PBMCs.

no significant responses were observed for other immunized peptides.

The limited breadth of response in Subjects 1 and 2 to immunized peptides raised the possibility that differences in avidity between immunized peptides and HLA-A*2402 molecules affected the results because seven peptides were loaded by mixture into DCs for these participants. Avidity of the seven peptides was thus tested using a T2-A24 stabilization assay [Foung et al., 1986; Kuzushima et al., 2001], revealing that Env584-wt, Env584-4Q, Nef138-wt, and Nef138-2F bind HLA-A*2402 with relatively high avidity, whereas Gag28-3R binds with moderate avidity, and both Gag296 and Gag28-wt bind with low avidity (data not shown). Based on this result, each peptide for Subjects 3 and 4 was incubated with DCs separately ($\sim 1.4 \times 10^6$ DCs/peptide) and used for vaccination. However, injection of separately-loaded DCs did not induce specific response to any of the seven immunized peptides, despite the fact that rebound of autologous HIV-1 after treatment interruption induced strong responses to Nef138-2F, Env584, and Gag(1–115). We also conducted tetramer-binding assay using Nef138-wt-tetramer and ELISPOT assay using autologous DCs as antigen-presenting cells to amplify IFN-γ production, but could not find peptide-specific population or response in Subjects 3 and 4 (data not shown).

Drug-Resistance Mutations

Since one of the concerns regarding interruption of HAART is the potential emergence of drug-resistance mutations, we sequenced reverse transcriptase and protease genes of HIV-1 derived from plasma before start of HAART and 4 weeks after treatment interruption, when VLs were detectable in all participants (7,100, 58,000, 24,000, and 100,000 copies/ml, respectively). Subject 3 displayed a nucleotide substitution at position 108 in the protease regions on population sequencing, which would result in an amino acid change from methionine to isoleucine at position 36 (Table III). Sequences of clones obtained from the PCR product revealed that five of five clones displayed the M36I mutation. HIV protease genes in the plasma of this participant were further sequenced at 6 weeks after treatment interruption (29,000 copies/ml), but no M36I mutation were identified in any of the eight clones sequenced. No nucleotide substitutions were found in protease genes from the other three participants, or in reverse transcriptase genes from all participants.

DISCUSSION

After DC-based vaccination of the four subjects, immune responses to Nef138-wt in Subjects 1 and 2, and to Nef138-2F in Subject 1 were observed, whereas no detectable responses were obtained in other peptides. The results from Subjects 1 and 2 demonstrating limited breadth of response led us to consider the possibility that differences in avidity between HLA-A*2402 molecules and each peptide caused preferential presentation of Nef138 epitopes, as seven peptides were added to DCs in mixture. In fact, T2-A24 stabilization assay revealed that Gag epitopes displayed lower avidity to HLA-A*2402 than Nef138 and Env584 peptides. Thus, in Subjects 3 and 4, the seven peptides were incubated with DCs in separate wells and mixed together before vaccination, but no significant responses were observed to any of these peptides. One explanation for this observation is that when approximately 1×10^7 DCs were divided among seven peptides (approximately 1.4×10^6 cells/peptide), the numbers of DCs was too small to provide sufficient stimuli to CTLs in vivo. Although Yu et al. [2004] reported that 1.0×10^6 autologous DCs loaded with glioma-derived peptides could elicit systemic cytotoxicity in cancer patients, the number of DCs in the present cases might have been insufficient to elicit specific response from HIV-1-infected individuals. Another explanation is that Subjects 3 and 4 displayed lower nadir CD4 counts before starting HAART (50/μl and 2/μl, respectively) than Subjects 1 and 2 (164/μl and 216/μl, respectively). In untreated HIV-1-infected individuals, $CD4^+$ T cells are continuously destroyed during all stages of HIV-1 infection, causing not only quantitative, but also qualitative abnormalities in HIV-1-specific immunity. These abnormalities are carried over even after CD4 counts are normalized by the initiation of HAART. In fact, Lange et al. [2003] reported that responses to immunization of tetanus and diphtheria toxoids in chronically HIV-1-infected patients under HAART correlate with previous nadir CD4 counts, but not with current circulating CD4 counts. This kind of impaired immune function in HIV-1-infected individuals under HAART may also explain the limited breadth of immune response in Subjects 3 and 4.

In terms of safety, peptide-loaded DCs were well tolerated, and only mild local and general symptoms were observed during vaccine administration, with only one episode of acute retroviral syndrome after STI. Since treatment interruption sometimes causes viral mutation resulting in antiretroviral drug resistance [Schweighardt et al., 2002; Metzner et al., 2003; Tremblay et al., 2003], all participants changed from antiretroviral agents that are known to be susceptible to resistance mutations to other agents with short-half lives before STI. However, an M36I mutation in a protease region was transiently detected in Subject 3 when VL rebounded 4 weeks after treatment interruption, and disappeared 6 weeks after interruption. M36I mutation is regarded as one of the minor resistance mutations that can appear after emergence of major resistance mutations. We cannot determine the mechanism underlying this transient appearance of M36I in Subject 3, but replication of mono- or oligoclonal HIV-1 from reservoir cells may be responsible.

In this study with a small number of participants, DC-based vaccine elicited a limited breadth and strength of immune response, and treatment interruption failed to control rebound of viral proliferation. Several groups have tried similar therapeutic vaccines to interrupt antiretroviral therapy in both humans [Hejdeman et al., 2003; Bostrom et al., 2004; Harrer et al., 2005; Kinloch-de Loes et al., 2005; Tubiana et al., 2005; Wu et al., 2005] and macaques [Lisziewicz et al., 2005] using recombinant proteins or genes expressing HIV-1 proteins, and have reported various results of specific immune reaction and clinical outcomes after treatment interruption. Although the question as to which strategy for therapeutic vaccination is suitable for successful treatment interruption remains controversial, application of DCs as vaccine adjuvant appears theoretically attractive to improve deteriorated immune function in HIV-1-infected individuals. In agreement with this concept, therapeutic vaccine using DCs in cancer treatment has been shown to result in better tumor regression compared to vaccines using peptide alone, viral vectors or tumor cells [Banchereau and Palucka, 2005]. Recently, two groups reported preliminary results of DC-based therapeutic vaccine in HIV-1-infected patients using autologous HIV-1 as immunogens in untreated [Lu et al., 2004] and treated patients [Garcia et al., 2005]. Garcia et al. showed that DC-based HIV-1 vaccine in patients under HAART did not elicit specific immune responses, although the vaccine suppressed viral rebound in 4 of 12 vaccines after treatment interruption. Our result thus provides encouraging evidence that DC-based vaccines can induce specific immune response, albeit insufficient to suppress viral rebound, in patients under HAART. These early outcomes warrant further exploration to establish the therapeutic value of vaccination with DCs in HIV-1 infection.

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Competitive Study of Monoclonal Antibodies against the HIV-1 Gp41 Core Structure

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Abstract: **Monoclonal antibodies (MAbs) 50.69, 98.6, and T26 bind specifically to the core structure of the human immunodeficiency virus type 1 (HIV-1) envelope transmembrane glycoprotein (gp41). To clarify the specificity of the anti-core structure MAbs, we performed competitive assays using the MAbs to the H9 human T cell line infected with the IIIB strain of HIV-1 (H9/IIIB). Bound MAb 50.69 inhibited MAb 98.6 binding unidirectionally. The reason for the unidirectional cross competition between MAbs 50.69 and 98.6 is not clear, but these results help to define the antigenic structure of gp41 on the surface of infected cells.**

Key words: **HIV-1, Gp41, 50.69 antibody, 98.6 antibody**

The binding of envelope glycoprotein 120 (gp120) of human immunodeficiency virus type 1 (HIV-1) to cellular receptors on target cells leads to conformational changes of envelope transmembrane glycoprotein (gp41) that permit viral and cellular membrane fusion (12). Recent crystallographic studies have shown that fusion-active gp41 folds into a six-helix α-helical bundle, in which three N-terminal helices (N peptides) form an interior, parallel-coiled-coil trimer, while three C-terminal helices (C peptides) pack in the reverse direction into three hydrophobic grooves on the surface of this coiled coil (2, 9, 16). We have previously reported that the decline of anti-DP107 (aa 553–590) (α-helical N-peptide) antibody is associated with the clinical progression in HIV-1-infected individuals, suggesting that antibodies against the structure may have a protective role (7). To define the epitopes within these regions to which infected humans respond during the course of infection, the specificity of human MAbs to these regions was studied. Using 10 human MAbs identified initially by their reactivity to whole gp41 in HIV-1 virion lysates, Xu et al. previously reported two immunodominant regions of gp41 that define the epitopes within these regions to which infected humans respond during the course of infection (5, 17). The first region of gp41 is in the vicinity of the cysteines between amino acids 598 and 604 (cluster I). The second immunogenic region position is between 644 and 663 (cluster II). Titration of sera from HIV-1-infected patients showed that there was approximately 100-fold more efficient antibody binding to cluster I than to cluster II in patients' sera, confirming the immunodominance of cluster I. Subsequent studies have disclosed that human MAbs against gp41 could recognize the gp41 core structure (14). We called these MAbs anti-core structure MAbs, because the exact antigenic structure of gp41 clarified so far is only the core structure and the native and fusion-active structure of all gp41 molecules has not been clarified yet. MAb 50.69, which is a cluster I MAb, reacts to a N51 (aa 540–590) and C43 (aa 624–666) peptide mixture (N51/C43) (6), but neither to N36 (aa 546–581)/C34 (aa 628–661) nor a single C43 peptide (17). MAb 98.6, which is a cluster II MAb, reacts to N51/C43, N36/C34, and a C43 peptide (14). Both N51/C43 and N36/C34 are known to form a six-helical bundle (2). MAb T26 was reported to bind to the six-helix bundle N36/C34 (1, 3, 4). These studies

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry; gp41, envelope transmembrane glycoprotein 41; gp120, envelope glycoprotein 120; gp160, envelope glycoprotein 160; HIV-1, human immunodeficiency virus type 1; H9/IIIB, the H9 human T cell line infected with the IIIB strain of HIV-1; MAbs, monoclonal antibodies; MFI, mean fluorescence intensity; PE-avidin, phycoerythrin-labeled avidin.

suggest that MAb T26 is specific for the core structure because MAb T26 was reported to bind only to the oligomeric form in immunoprecipitation assays. Therefore, these MAbs are expected to bind to different epitopes. These MAbs must bind to the infected cell surface gp41 in the human body, although it is not known whether these MAbs interfere with the bindings of other MAbs. To clarify this issue we did competitive assays among human MAbs 50.69, 98.6 and T26.

One half million H9/IIIB cells, a human T cell line infected with the IIIB strain of HIV-1, were pre-incubated with saturated concentrations of MAbs 50.69, 98.6 or human IgG (Calbiochem, La Jolla, Calif., U.S.A.) for 1 hr at 4 C, followed by washing. The effects of these MAbs on the binding of biotinylated MAbs (B-50.69, B-98.6 and B-T26) to H9/IIIB cells were studied. Biotinylated MAbs 50.69 and 98.6 were provided by Dr. Miroslaw K. Gorny. MAb T26 IgG was purified from culture supernatants of hybridoma cells obtained from Dr. P.L. Earl and biotinylated. Because MAb T26 bound little at 4 C, we did not use MAb T26 as a competing MAb. B-50.69 (2 μg/ml), B-98.6 (2 μg/ml) or B-T26 (32 μg/ml) at saturated concentrations were added to the cells and incubated for 30 min at 4 C for MAb B-50.69 or MAb B-98.6, and for 15 min at 37 C for B-T26. After washing the cells twice, 4 μl phycoerythrin-labeled avidin (PE-avidin) (Serotec, Ltd., Kidlington, Oxford, U.K.) was added and the cells were incubated for 30 min at 4 C followed by fixation. PE-avidin labeled biotinylated MAb was detected by flow cytometry (FACS).

Since we could not detect efficient MAb T26 binding to H9/IIIB at 4 C, we tried to detect the temperature dependency of the MAb T26 binding. MAb T26 bound H9/IIIB and yielded a low mean fluorescence intensity (MFI) at 4 C, and a high MFI at 37 C (Fig. 1). The saturating concentrations were 64 μg/ml at 4 C and 32 μg/ml at 37 C and the saturated MFI at 4 C was 11.5 and significantly lower than the saturated MFI 18.8 at 37 C ($P<0.0167$).

We evaluated the MAb T26 binding at 37 C, and those of MAbs 50.69 and 98.6 at 4 C, and calculated the % inhibition (Fig. 2). MAb 50.69 pre-incubation blocked its own binding as a positive control. MAb 98.6 did not block MAb B-50.69 binding ($P\geqq0.05$). MAb B-98.6 was also examined in a similar condition. MAb 98.6 pre-incubation blocked MAb B-98.6 binding. The MAb 50.69 pre-incubation blocked MAb B-98.6 binding ($P<0.0167$), though the former MAb inhibited the binding more efficiently than the latter did. We were able to observe that MAbs 50.69 and 98.6 bound at 4 C and avoided non-specific binding, although the condition was not physiological (13). We also confirmed that MAb B-T26 binding at 37 C was blocked by bound MAb T26. The MAb 50.69 pre-incubation did not show a significant blocking effect for MAb B-T26 ($P\geqq0.05$). However, pre-incubation of MAb 98.6 blocked its binding very efficiently ($P<0.0167$).

MAb T26 was identified as an anti-gp41 antibody that binds only to oligomers, particularly to trimers, but not to monomers (4). Both MAbs 98.6 and T26 bind to a mixture of N36/C34, but their precise epitopes must be different from each other because MAb 98.6 also binds to monomeric gp41. MAb 98.6 pre-incubation inhibited MAb B-T26 binding to H9/IIIB, indicating the epitopes of MAbs 98.6 and T26 may partially overlap.

On the other hand, MAb 50.69 did not compete with MAb B-T26 at all, indicating that the epitopes of the two MAbs are distant. These data accord with the findings that MAb 50.69 does not bind to a mixture of N36/C34 but MAb T26 does.

MAb 98.6 binds to a unique epitope shared with a mixture of N51/C43, N36/C34 and C43. But MAb 50.69 binds specifically to a mixture of N51/C43 but not C43. It is worthwhile to note that MAb 50.69 pre-incubation inhibited MAb 98.6 binding but the interference was not reciprocal. As shown previously, MAbs 50.69 and 98.6 did not compete in an enzyme-linked immunosorbent assay (ELISA) using viral lysate or recombinant proteins derived from gp41 (17). Earl et al. also performed a competitive assay for anti-gp41 MAbs in ELISA. They also showed that the majority of the anti-gp41 MAbs are conformation dependent and most of determinant I as cluster I MAbs do not compete with determinant II as cluster II MAbs (3). MAb 50.69 was classified as a cluster I antibody, and MAb 98.6 was classified as a cluster II antibody. However, in the present study we performed competitive assays using an infected live cell line and FACS. The difference in the method used may explain the conflicting results. The epitopes of MAbs 50.69 and 98.6, which are expressed on the infected cell surface, possibly have a different conformation from the gp41 peptides previously used in ELISA, while it is known that HIV-1 envelope proteins form oligomers dominantly in viral lysate (6). The envelope proteins on the infected cell surface are heterogeneous with native gp120-gp41 complex, residual gp41 after gp120 shedding, and uncleaved envelope glycoprotein 160 (gp160) precursor, although gp160 derived from viral lysate forms a trimer (10). Atomic force microscopy investigation revealed that monomeric gp120 is dominant on H9/IIIB (8). The unidirectional competition of MAbs 50.69 and 98.6 may be explained by the positional relationship among epitopes on

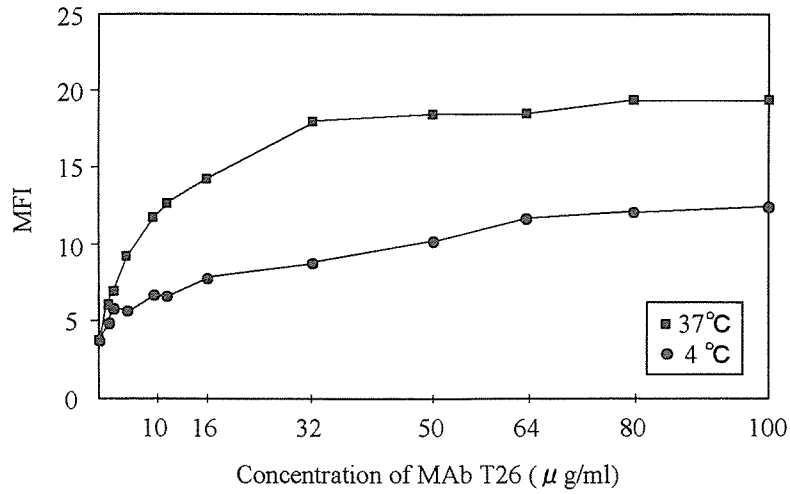

Fig. 1. The temperature dependency of MAb T26 binding. MAb T26 binding to H9/IIIB at 37 C was higher than at 4 C.

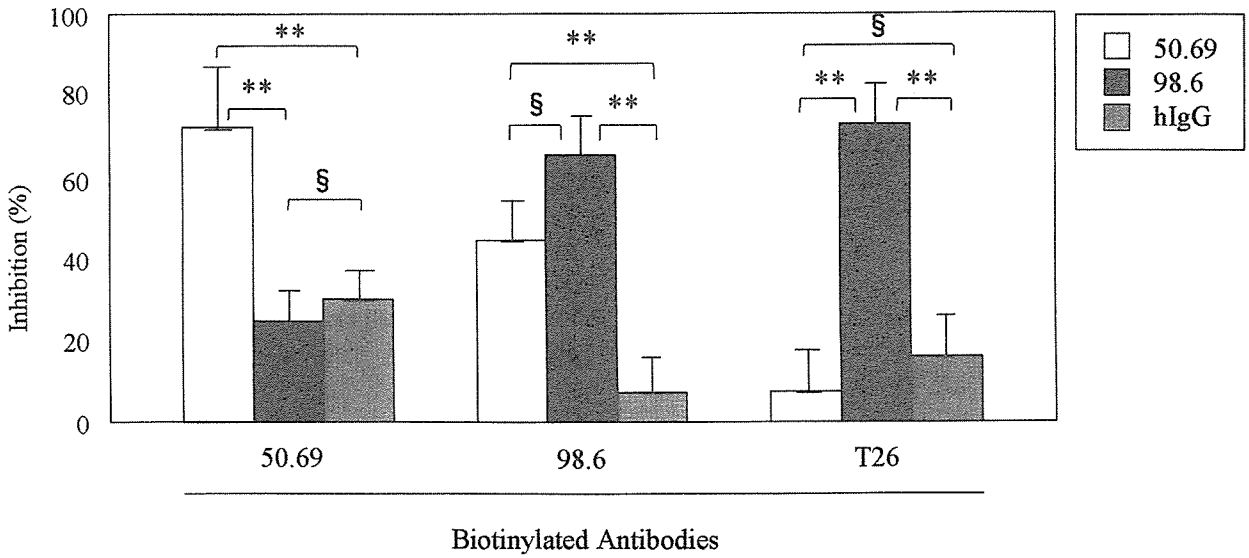

Fig. 2. The decreased binding of biotinylated MAb 50.69, 98.6, or T26 to H9/IIIB cells after preincubation with MAb 50.69 (open bars), 98.6 (filled bars), or hIgG (gray bars). The negative control was calculated as the MFI without preincubation MAb and with biotinylated MAb. The positive control was calculated as the MFI with biotinylated MAb blocked by the same preincubation MAb, the concentration of which was saturated. % inhibition was calculated as [1−(biotinylated MAb MFI−positive control MFI)/(negative control MFI−positive control MFI)]×100 (%). Data are expressed as mean±S.D. Each bar (±S.D.) represents the mean of triplicate determinations (**$P<0.0167$) ($^{\S}P\geqq 0.05$). To determine statistically significant differences among the three groups, differences were considered to be statistically significant when $P<0.0167$ by the Bonferroni/Dunn test. Data were analyzed using CellQuest software (Becton Dickinson Biosciences).

monomeric gp41. The size of the epitopes, the induced conformational changes upon binding MAb 50.69, and differences in the affinities of the MAbs also might be responsible for our results.

In this study, we analyzed the binding properties of anti-core structure gp41 human MAbs using infected cells. These analyses will contribute to understanding the structure of gp120-gp41 on the infected cell surface and the complex interactions of humoral antibodies against HIV-1. The unidirectional competition, which has not been able to be observed using gp41-derived peptides so far, suggests that the immune-response against the gp41 core structure varies much among patients and some of them are possibly dominant for certain exclusive epitopes, because patient-derived MAbs are considered to recognize the functional gp41 in the human body. We previously did competition assays between patients' sera and anti-core structure

MAbs, because patients' sera may have a diverse competition with anti-core structure MAbs (15), and found that the patients' sera competed with MAb 50.69 more than MAb 98.6 (unpublished data), suggesting that the antibodies that compete with MAb 50.69 are greater in number than those that compete with MAb 98.6 *in vivo*.

According to the above results the antibodies that bind to the epitope of MAb 50.69 are expected to compete with MAb 98.6. The lower competition of MAb 98.6 with sera suggests that some antibodies that recognize the overlapping epitope of MAb 50.69 do not compete with MAb 98.6.

Our results also indicate that there are few antibodies that have the epitope of MAb 98.6 *in vivo*, and suggest that the antibodies with this epitope may hardly ever be induced, especially among cluster II antibodies. This is assumed to be partly due to the fact that cluster II is a more variable domain than cluster I (11).

The anti-gp41 antibodies in one patient's body are assumed to compose a population including such exclusivity and heterogeneity. Our data not only contribute to analyses of the functional epitopes which are able to induce anti-gp41 antibodies *in vivo*, but also to establishing a method to evaluate the complex anti-gp41 immunity.

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YMDD mutations and genotypes of HBV in Northern China

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