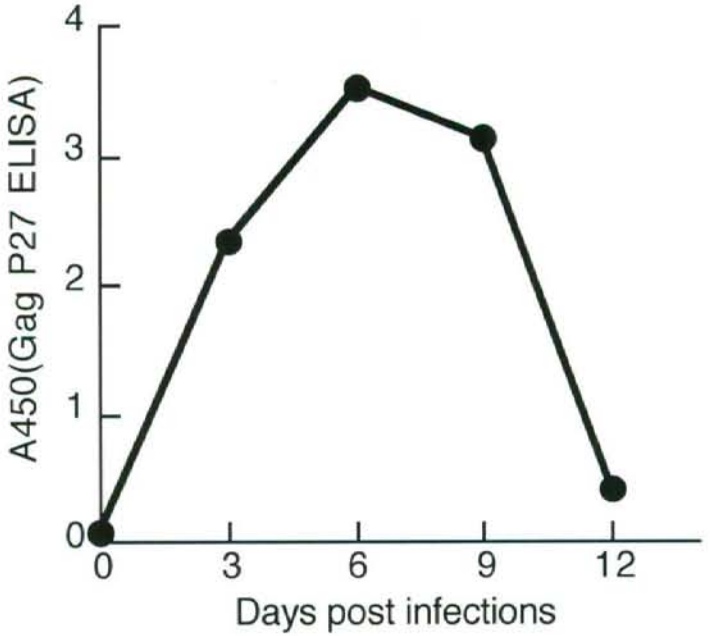


Fig.2 Optical density of SIVmac239 Gag P27 at 450 nm with ELISA.
Culture medium was corrected as the virus preparation stock at Day 6.

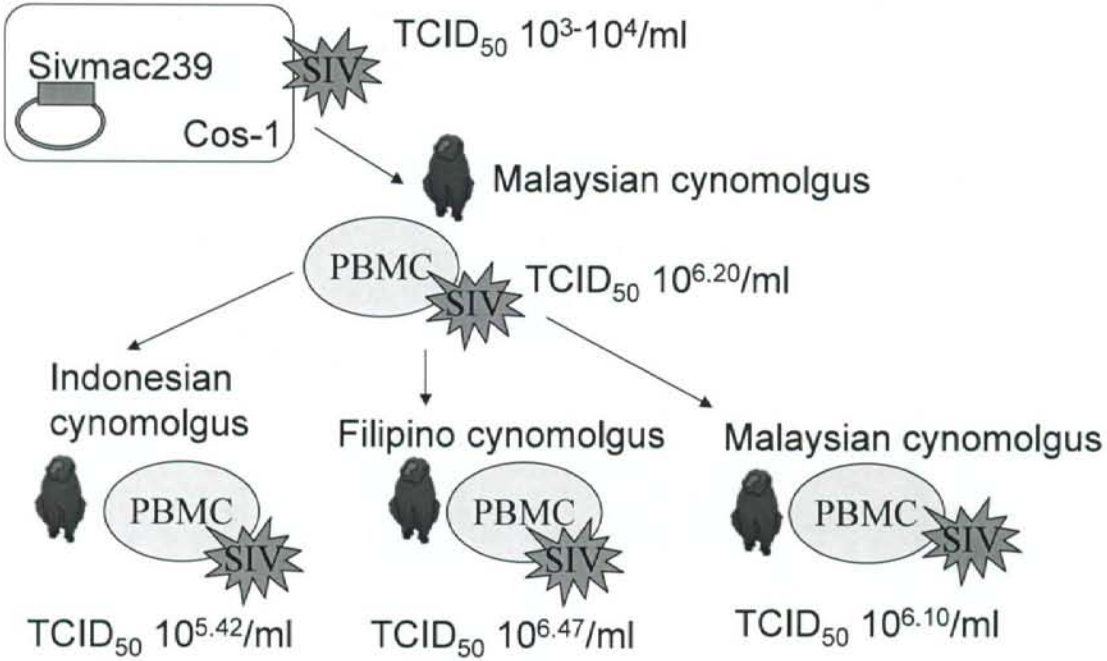


Fig.3 Preparation of SIVmac239.

厚生労働科学研究費補助金（政策創薬総合研究事業）
分担研究報告書

研究課題：エイズウイルスのサブタイプ間を越えて感染を制御する宿主応答の同定
分担研究者：駒野　淳（国立感染症研究所　エイズ研究センター　第３室　主任研究官）
分担研究課題：感染防御・制御と関連する宿主遺伝子の解析価

研究要旨
HIV 感染症を克服するためには、抗 HIV 薬開発だけでなく免疫療法や感染予防ワクチンの開発が必要である。弱毒ウイルスの感染制御ならびに弱毒ウイルスによるワクチン効果誘導メカニズムを同定することによりワクチン開発の基礎が築けると期待される。本研究では弱毒 SIV としての deglycosilation SIV mutant によって誘導される優れたワクチン効果の原因となる実行因子の同定を機能的な方法にて試みる。本年度はタンパク質発現による表現型スクリーニングによるヒトエイズウイルス複製阻害因子の系を樹立した。heterologous challenge-resistant サル由来のリンパ球組織等に由来する cDNA library から同様の手法によりサルエイズウイルス耐性に直接関与する実行因子を表現型スクリーニングにて同定するための実験系に応用可能である。

A. 研究目的

HIV 感染症を克服するためには、抗 HIV 薬開発だけでなく免疫療法や感染予防ワクチンの開発が必要である。そのためにはウイルス感染に対する免疫をより根本的に理解することが重要である。これまでのエイズウイルスに対する宿主免疫の研究は主に獲得免疫、特に中和抗体とウイルス感染細胞特異的細胞傷害活性を持つCTL を中心に行われてきた。ところが予防ワクチンにて最も有望であると考えられていた CTL 誘導型の DNA プライム-ウイルスベクターブーストワクチンがヒトでほとんど効果が見られないことが最近判明した。ワクチン開発を成功させるためには CTL 以外でウイルスを制御するメカニズムを発見することが求められる。

弱毒 SIV は病原性 SIV の感染に対して非常に強くかつ最も効果的な感染防御能を示すことがサルエイズ発症モデルによって明らかにされている。現在 HIV ワクチンとして弱毒生ワクチンを投与することは安全性の面から高いハードルがあるが、弱毒ウイルスの感染制御ならびに弱毒ウイルスによるワクチン効果誘導メカニズムを解析することは、現在最も注目されている重要な研究課題である。

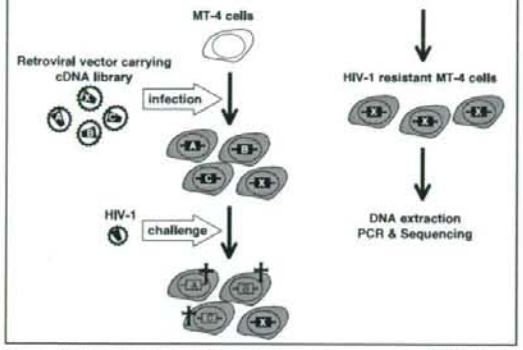
我々は弱毒 SIV としての deglycosilation SIV mutant によって誘導される抗SIV 反応の本態を解析するため多様なアプローチを試みている。抗 SIV 反応の獲得についていくつかの仮説が提唱されているが、中でもウイルス耐性遺伝子の発現が誘導されている可能性も考えられる。サル個体からウイルス耐性遺伝子を同定するためには RNA やタンパク質発現レベルでの発現上昇を足がかりにする方法も有用であるが、実際に遺伝子発現増強がウイルス感染抵抗性を与えるかどうかを検証することができれば直接的である。機能的なスクリーニングでウイルス感染抵抗性遺伝子を同定する事を目的として本年度は functional screening system の構築を行った。

B. 研究方法

ヒト T 細胞株 MT-4 細胞に HIV 感染抵抗性を有するウサギ RK13 細胞由来の cDNA ライブラリーを MLV ベクターにて導入し、GFP 発現を指標に cDNA を安定に発現する細胞を選択して HIV-1(HXB2 株)

を感染させた後ウイルス感染耐性となって生存

図1. Screening a cDNA library for "HIV-resistant" genes



してくる細胞を選択し、それらからDNAを抽出し、PCRによってMLVベクターにより導入されたcDNAを増幅し核酸配列を決定することによりウイルス感染抵抗性遺伝子候補を同定する（図1）。この候補遺伝子を再びMLVベクターにてMT-4細胞に導入し、HIV-1を感染させ、ウイルス耐性遺伝子であるかを評価する。詳細はUrano E et al., FEBS Let 2008に記載されている。

（倫理面への配慮）

特記すべきことなし。

C. 研究結果

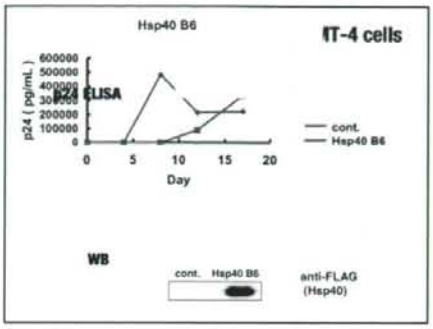
RK13細胞cDNAから独立の42個の候補遺伝子がえられた（表1）。全ての遺伝子が耐性を与えるのではなく、"background"として同定されたcDNAもあることが予想された。回収効率が高かったものに注目し、中からHSP40遺伝子を選択肢ヒトT細胞に導入してHIV-1複製を評価したところ、ウイルス複製抵抗性を与える活性を有する事が判明した（図2）。同様の方法でえられたBrd4 CTD

表1. Result of a cDNA library screening from RK13 cDNA

42 independent cDNA

Category	# ind clones
transcription	13
translation	6
trafficking	2
metabolism	2
DNA repair	3
signal transduction	3
extracellular matrix	1
glycosylation	1
cytoskeleton	1
cell cycle	1
chaperone	6
degradation	2
unknown	1

も HIV-1 複製抵抗性がある事も明らかとなった（Urano E et al., FEBS Let 2008）。この他複数の候補遺伝子について解析を並行して行っている。以上の結果より本法がエイズウイルス抵抗性遺伝子を同定するための優れた機能的スクリーニングであることが示された。



D. 考察

本実験系により異種の動物細胞からヒトT細胞を使用してHIV-1耐性遺伝子を機能的なスクリーニング方法にて選択し同定することが可能である事が証明された。本法を応用しCCR5を発現した細胞にて行えば、その延長線上にSIVに対する耐性遺伝子同定が可能である事が期待される。さらに使用するcDNAに弱毒SIVとしてのdeglycosilation SIV mutantを感染させたサル由来のリンパ組織からえられたRNAを使用する事により、将来SIVワクチンにより誘導されたウイルス耐性メカニズムの実行因子を同定することが可能である。今後はCCR5を発現した細胞とCCR5指向性のウイルスを使用して本コンセプトを実証すべく研究を展開したい。

E. 結論

タンパク質発現による表現型スクリーニングによるHIV-1複製阻害因子の系を樹立した。heterologous challenge-resistantサル由来のリンパ球組織等に由来するcDNA libraryから同様の手法によりサルエイズウイルス耐性に直接関与する実行因子を表現型スクリーニングにて同定するための実験系に応用可能である。

F. 健康危険情報

特記すべきことなし。

G. 研究発表

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H. 知的所有権の出願・取得状況 (予定を含む)

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（創薬基盤推進研究事業）
分担研究報告書

多様なエイズウイルス株の感染を制御する宿主応答：病理組織学的検索

分担研究者　中村　紳一朗　滋賀医科大学准教授

研究要旨　エイズウイルスの宿主応答を観察するためには、病理組織学的な評価が必須である。本年度はSimian Immunodeficiency Virus (SIV)を実験的に感染させたアカゲザルのパラフィン切片を用いて、免疫組織化学的にSIV抗原の局在、各種反応細胞の挙動を調べるための予備的検索を行った。数種の抗SIV抗体のうち、Nef抗原に対するモノクローナル抗体が良好な反応性を示し、免疫担当細胞に対する抗体にも良好な反応を示した。さらに免疫二重染色はSIVがモノクローナル、免疫担当細胞がポリクローナル抗体の場合は可能となった。次年度はSIVがポリクローナル、免疫担当細胞がモノクローナル抗体の染色系などを立ち上げ、ウイルス株の違いによる宿主応答を病理組織学的に検討する予定である。

A.研究目的

アカゲザルを用いたエイズ・サロゲートモデルは、エイズの基礎研究において重要な位置を占めている。近年ではウイルスの様々な機能ならびにその発現に関わる遺伝子を欠損させることによって、ウイルスの動態を詳細に観察することができるようになってきた。異なる株間で異なって表現される病態が、すなわち株間で有無を示す遺伝子のフェノタイプということになる。この病態の違いを病理組織学的に抽出するためには、ウイルス抗原ならびに免疫担当細胞を明確に検出することが必要である。本年度はすでにSIVを実験的に感染させたリンパ系臓器（脾臓・リンパ節）と小腸のパラフィン切片を用いて、それらを免疫組織化学的染色および免疫二重染色によって検出する系の確立を目的とした。

B.研究方法

（材料）SIV239株を接種後1年8ヶ月のアカゲザル（♂；3歳、動物1）、非接種のアカゲザル2例（♂；3歳と2歳、動物2と3）の脾臓、鼠径リンパ節、小腸のパラホルムアルデヒド固定、パラフィン包埋ブロックを約4μmに薄切した病理組織標本を材料とした。

（免疫染色）SIVに対する一次抗体として、抗SIV Nef-F抗体、抗SIV Nef-Q抗体、抗SIV gag p28抗体、抗SIV Env抗体（以上、マウス・モノクローナル抗体）、抗SIV gag抗体（ウサギ・ポリクローナル抗体）を用いた。免疫担当細胞に対する一次抗体として、抗CD3抗体、抗CD4抗体、抗CD8抗体、抗CD20抗体、抗CD45RA抗体、抗CD59抗体、抗CD68抗体、抗Fascin抗体

（以上、マウス・モノクローナル抗体）、抗CD3抗体（ポリクローナル抗体）を用いた。これらの抗体で酵素抗体法を行った。

（免疫二重染色）上記の各種、抗SIVモノクローナル抗体と抗CD3ポリクローナル抗体の組み合わせをAlexa488-抗マウスIgG抗体とAlexa598-抗ウサギIgG抗体で染色、抗SIVポリクローナル抗体と抗CD3モノクローナル抗体の組み合わせをAlexa488-抗ウサギIgG抗体とAlexa598-抗マウスIgG抗体で染色した。

（倫理面への配慮）

実験実施機関で承認された計画書に則って適切に行われた実験の、実験後材料を用いた実験で、特に倫理面で問題になることは無い。

C.研究結果

SIVに対するモノクローナル抗体の中では、抗SIV Nef-Q抗体、抗SIV gag p28抗体、抗SIV Env抗体がSIVを接種された動物1の脾臓、リンパ節の傍濾胞領域の円形ないしは星形細胞が陽性、抗SIV Nef-F抗体は非特異反応が強く陰性だった。抗SIV gagポリクローナル抗体も非特異が高く陰性だった。一方、抗SIV gag p28抗体、抗SIV Env抗体は動物2および3に対しても同様の陽性像を認めた。抗SIV Nef-Fおよび抗SIV Nef-Qモノクローナル抗体と抗SIV gagポリクローナル抗体は動物2および3に対して陰性だった。従って、抗SIV Nef-Q抗体のみがSIVに対して適切な反応を示した。

抗CD3抗体はT細胞、抗CD4抗体は約半数のT細胞、抗CD8抗体は辺縁帯の少数のT細胞、抗CD20抗体はB細胞、抗CD45RA抗体は少数の傍濾胞領域のBおよびT細胞、抗CD59抗体も傍濾胞領域の少数のBおよびT細胞、抗CD68抗体はマクロファージ系細胞、抗Fascin抗体は樹状細胞を染色した。

免疫二重染色では抗SIVモノクローナル抗体と抗CD3ポリクローナル抗体の組み合わせで両者に適切な陽性像を認めたが、抗SIVポリクローナル抗体と抗CD3モノクローナル抗体の組み合わせは抗SIVポリクローナル抗体に陽性像が見られず抗CD3モノクローナル抗体の陽性像のみが確認された。

D.考察

抗SIV Nef-Qモノクローナル抗体はSIV接種動物に陽性、非接種動物に陰性と、適切な染色性を示した。同モノクローナル抗体を基本としてSIVとポリクローナル抗体で検出できる免疫担当細胞との挙動を追うことは可能となった。しかし免疫担当細胞に対する抗体のほとんどがモノクローナル抗体なので、やはりSIVを特異的に染色可能なポリクローナル抗体が必要である。また抗SIV Nef-Q抗体のグロブリン・サブクラスがIgG1なので、それ以外のグロブリン・サブクラスを持つ抗体に対してはサブクラスを識別できる二次抗体を用いた染色を考える。さらにIn situ hybridizationとの二重染色も視野に入れ、様々な方法でSIVと免疫担当細胞の挙動を追える体制を整えておく必要がある。これらを実現することで明確なこれらをウイルス株の違いによる宿主応答を病理組織学的に解明できる。

E.結論

抗SIV Nef-Qモノクローナル抗体が良好な反応性を示し、種々の免疫担当細胞に対する抗体にも良好な反応を示した。さらに免疫二重染色はSIVがモノクローナル、免疫担当細胞がポリクローナル抗体の場合は可能となった。

G.研究発表

1．論文発表

なし。

2．学会発表

なし。

H.知的財産権の出願・登録状況

なし。

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

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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雑誌

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IV. 研究成果の刊行物・別刷

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Impact of glycosylation on antigenicity of simian immunodeficiency virus SIV239: induction of rapid V1/V2-specific non-neutralizing antibody and delayed neutralizing antibody following infection with an attenuated deglycosylated mutant

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Infection of rhesus macaques with a deglycosylation mutant, Δ5G, derived from SIV239, a pathogenic clone of simian immunodeficiency virus (SIV), led to robust acute-phase viral replication followed by a chronic phase with undetectable viral load. This study examined whether humoral responses in Δ5G-infected animals played any role in the control of infection. Neutralizing antibodies (nAbs) were elicited more efficiently in Δ5G-infected animals than in SIV239-infected animals. However, functional nAb measured by 90 % neutralization was prominent in only two of the five Δ5G-infected animals, and only at 8 weeks post-infection (p.i.), when viral loads were already below 10^4 copies ml^{-1}. These results suggest a minimal role for nAbs in the control of the primary infection. In contrast, whilst Ab responses to epitopes localized to the variable loops V1/V2 were detected in all Δ5G-infected animals at 3 weeks p.i., this response was associated with a concomitant reduction in Ab responses to epitopes in gp41 compared with those in SIV239-infected animals. These results suggest that the altered surface glycosylation and/or conformation of viral spikes induce a humoral response against SIV that is distinct from the response induced by SIV239. More interestingly, whereas V1/V2-specific Abs were induced in all animals, these Abs were associated with vigorous Δ5G-specific virion capture ability in only two Δ5G-infected animals that exhibited a functional nAb response. Thus, whereas the deglycosylation mutant infection elicited early virion capture and subsequent nAbs, the responses differed among animals, suggesting the existence of host factors that may influence the functional humoral responses against human immunodeficiency virus/SIV.

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INTRODUCTION

The precise role of antibody (Ab) responses in the containment of human immunodeficiency virus (HIV) remains a subject of intense study and debate. Besides the classical direct virus neutralization properties, antibodies are also capable of blocking infection via other pathways such as antibody-dependent complement-mediated inactivation of virus (Aasa-Chapman *et al.*, 2005) and antibody-dependent cellular lysis (Ahmad & Menezes, 1996; Forthal *et al.*, 2001). Acquiring an understanding of these various mechanisms for their exploitation in the

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development of candidate vaccines has been a major challenge.

The envelope protein (Env) of HIV/simian immunodeficiency virus (SIV) comprises an exterior protein (gp120) and a transmembrane (TM) protein (gp41), and trimers of the gp120/gp41 complexes form viral spikes that promote binding to receptors and co-receptors on the cell membrane for entry into the target cells (Wyatt & Sodroski, 1998). The major viral receptors of HIV/SIV include CD4 and a variety of co-receptors such as CCR5 or CXCR4. One desirable target epitope for neutralizing antibody (nAb) that shows relative conservation across clades is the binding site for the co-receptor (Burton *et al.*, 2004; Zolla-Pazner, 2004); however, this site is conformationally cryptic within the viral spike up until immediately after binding of the viral spike to CD4, providing an effective shielding mechanism to the virus. Another distinct feature of HIV/SIV Env is the extensive glycosylation that also effectively prevents access to antibodies directed at the epitopes (Chen *et al.*, 2005; Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). The gp120 protein possesses 18–33 Asn–X–Ser/Thr sequences, signals for the attachment of *N*-linked carbohydrate side chains (Leonard *et al.*, 1990; Ohgimoto *et al.*, 1998; Regier & Desrosiers, 1990; Zhang *et al.*, 2004). As the carbohydrate moiety is generally weakly immunogenic and is recognized to a large extent as self by the host immune system, the massive glycans on the surface of viral spikes constitute an immunologically silent facade (Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). As a result, mature viral spikes are protected from nAb and other host immune responses by a massive carbohydrate 'glycan shield' (Chen *et al.*, 2005; Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). In fact, a prominent role of carbohydrates of HIV/SIV in evasion from immune surveillance has been reported previously as follows. Variants of SIV that have evolved to acquire additional glycans in the variable regions of Env have increased neutralization resistance compared with the parental virus (Chackerian *et al.*, 1997; Cheng-Mayer *et al.*, 1999). Similarly, the appearance of neutralization escape mutants has been associated with altered glycosylation in HIV-1 evolved during the course of infection (Wei *et al.*, 2003). Conversely, infection with SIV239 mutants with deglycosylated Env (lacking *N*-linked glycosylation sites) in the variable loops V1/V2 of gp120 elicited markedly increased titres of nAb (Reitter *et al.*, 1998). We have reported that a deglycosylation mutant, Δ5G, lacking *N*-linked glycosylation sites at aa 79, 146, 171, 460 and 479 in gp120 of SIV239 displayed an attenuated phenotype when used to infect rhesus macaques (Mori *et al.*, 2001; Ohgimoto *et al.*, 1998). In addition, animals infected with Δ5G exhibited almost sterile protection against rechallenge with SIV239 (Mori *et al.*, 2001).

Thus, we suggest that studies aimed at identifying the mechanisms underlying the early and potent immune control of deglycosylated SIV may provide knowledge for the formulation of effective HIV/SIV vaccines. Studies performed herein were therefore directed at attempts to define more precisely the early humoral responses (both virus-specific nAb and non-nAb) generated after infection with Δ5G in rhesus macaques and to compare these responses with those observed in macaques inoculated with wild-type SIV239, with the rationale that results from such studies may help to identify their potential contribution towards viral control of primary infection.

METHODS

Viruses. The molecular pathogenic clone of SIV239 (Regier & Desrosiers, 1990) and its derived deglycosylated mutant, Δ5G, were used in this study. Δ5G was derived by mutagenesis of an SIV239 infectious DNA clone so that the asparagine residues for *N*-glycosylation at aa 79, 146, 171, 460 and 479 in gp120 were converted to glutamine residues (Fig. 1a) (Ohgimoto *et al.*, 1998). Viral stocks of SIV239 and Δ5G were prepared as reported previously (Mori *et al.*, 2001).

Peptides. A series of 72 consecutive 25 mer peptides overlapping by 13 aa were synthesized based on the entire SIV239 Env sequence (Env-1–72). These peptides were synthesized by the Microchemical Facility, Emory University School of Medicine, Atlanta, USA. Another set of 15 mer peptides overlapping by 11 aa around the V1/V2 region in gp120 (V1V2-1–12) was synthesized by Sigma-Aldrich Japan based on the Δ5G sequence (see Fig. 5b). All peptides were dissolved in DMSO diluted in PBS.

Animal infection. Juvenile rhesus macaques originating from Myanmar (Burma) (Mm12, Mm13, Mm20, Mm23 and Mm26) or from Laos (Mm07, Mm22 and Mm25) were used following the results of screening for SIV, simian T-cell lymphotropic virus, B virus and type D retrovirus infection, which were all negative prior to inception of the study. All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare as outlined by the National Institute of Infectious Diseases and National Institute of Biomedical Innovation. Animals were infected intravenously with Δ5G or SIV239 as described previously (Mori *et al.*, 2001).

Plasma viral load measurements. SIV infection was monitored by measuring the plasma viral RNA load using a highly sensitive quantitative real-time RT-PCR. Viral RNA was isolated from plasma samples from infected animals using a commercial viral RNA isolation kit (Roche Diagnostics). SIV *gag* RNA was amplified and quantified using a method originally developed by Hofmann-Lehmann *et al.* (2000) using a TaqMan EZ RT-PCR kit (Applied Biosystems). The detection sensitivity of plasma viral RNA by this method was 100 viral RNA copies per ml plasma (given as copies ml^{-1}).

Neutralization assay. SIV neutralization was tested according to a protocol using CEMx174/SIVLTR-SEAP cells, originally described by Means *et al.* (1997). To measure low levels of nAb, IgG was purified from plasma as described below and concentrated virus stocks were used.

Anti-gp120 Ab ELISA and anti-Env peptide ELISA. Recombinant SIV239 gp120 and Δ5G gp120 were expressed utilizing a Sendai virus vector as described previously (Mori *et al.*, 2005; Yu *et al.*, 1997). Culture supernatant containing approximately 2 μg secreted SIV gp120 ml^{-1} was diluted with an equal amount of PBS, dispensed into each well of an ELISA plate and allowed to incubate at 4 °C overnight.

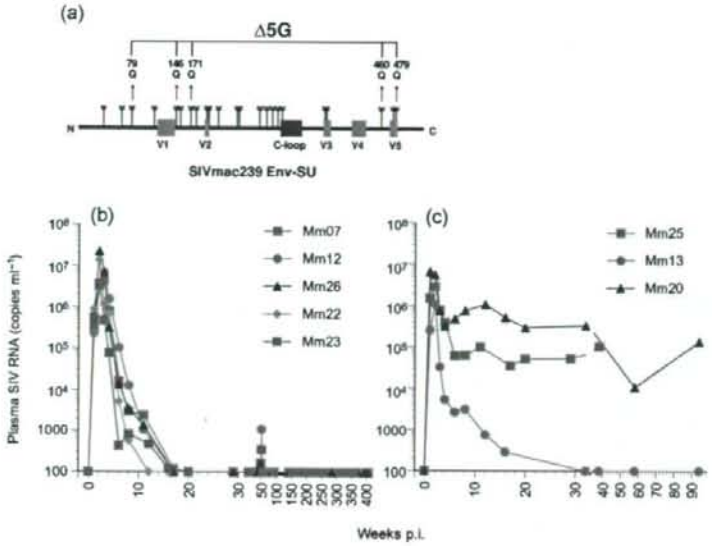

Fig. 1. Plasma SIV RNA loads in animals infected with Δ5G or SIV239. (a) *N*-Glycosylation sites in SIV239 gp120 and deglycosylation sites in Δ5G gp120. The locations of 23 *N*-glycosylation sites in SIV239 gp120, variable regions (V1–V5) and cysteine loops (C-loop) are shown. Δ5G was deglycosylated by N→Q substitutions at aa 79, 146, 171, 460 and 479 in Env (Ohgimoto *et al.*, 1998). (b, c) Plasma viral load in Δ5G-infected (b) and SIV239-infected (c) animals was measured in plasma samples using sensitive real-time RT-PCR to indicate when viral loads declined below 100 copies ml^{-1}. Three Δ5G-infected animals (Mm07, Mm12 and Mm26) were challenged with SIV239 at 48 weeks p.i.; thus, slightly increased viral loads were detected in those animals during weeks 49–51 p.i. (Mori *et al.*, 2001).

For the peptide ELISA, each peptide was diluted to 0.5 μM with 50 mM carbonate buffer (pH 9.5) and captured on Nunc Immobilizer amino plates (Nalge Nunc) at 4 °C overnight. A 1:100 dilution (150 μl) of the plasma sample to be tested was dispensed into antigen-immobilized plates and incubated at 37 °C for 1 h. Ab responses were detected using peroxidase-conjugated goat anti-monkey IgG and *o*-phenylenediamine. Absorbance was measured at 490 nm.

Removal of linear V1/V2 epitope-specific Abs from IgG fractions. A mixture of the peptides (V1V2-9, -10 and -11; see Fig. 5b) was conjugated to a HiTrap NHS-activated HP column (GE Healthcare). IgGs from plasma samples were fractionated using a mAb trap kit (GE Healthcare) and applied to the peptide-conjugated column. The flow-through fractions devoid of anti-V1V2-9, -10 and -11 peptide-specific Abs were collected. The concentration of IgG was determined using a protein assay kit (Bio-Rad).

Virion capture assay. The virion capture assay was modified using a method reported by Nyambi *et al.* (1998). ELISA plates were coated with the IgG samples described above at a concentration of 20 μg ml^{-1} in 50 mM carbonate buffer (pH 9.5) and incubated at 4 °C for 48 h. The plates were washed three times with PBS and blocked with 3 % BSA in PBS at 37 °C for 1 h. The plates were then washed three times with serum-free RPMI 1640. Δ5G or SIV239 virion solutions with a $p27^{gag}$ concentration of 15, 7.5 and 3.75 ng in 10 % fetal bovine serum/RPMI 1640 were added to each well of the IgG-coated plate and incubated at 37 °C for 3 h. The wells were washed five times with serum-free RPMI 1640 to remove unbound virus. The virus bound to IgG was lysed using MagNA Pure LC Lysis/Binding Buffer (Roche Diagnostics). The viral lysates were subjected to viral RNA purification using a MagNA Pure Compact nucleic acid purification kit (Roche Diagnostics). The copy number of the isolated SIV RNA was determined by real-time RT-PCR for SIV239 as described above.

Statistical analysis. Correlation analysis was done using Spearman's non-parametric rank test and the Mann–Whitney test using GraphPad Prism 4.0 software. Correlations were considered to be statistically significant for values of $P<0.05$.

RESULTS

Plasma viral loads of a quintuple deglycosylated SIV239 mutant in rhesus macaques

Eight rhesus macaques were infected intravenously with Δ5G ($n=5$) or SIV239 ($n=3$) (Mori *et al.*, 2001). Plasma viral RNA loads were assayed for up to 400 weeks p.i. and the data obtained in the Δ5G-infected (Fig. 1b) or SIV239-infected (Fig. 1c) animals were plotted. Both Δ5G and SIV239 replicated with similar kinetics during the early phase of primary infection for up to 4 weeks p.i. However, subsequent to this acute infection phase, virus replication was markedly different in the two groups of monkeys: SIV239-infected animals exhibited viral load set points around 10^5 copies ml^{-1} in two of three animals, with one animal (Mm13) having an undetectable viral load (<100 copies ml^{-1}) by 30 weeks p.i. (Fig. 1c). In contrast, the Δ5G-infected animals showed uniformly controlled viraemia reaching undetectable levels by 12–16 weeks p.i. and maintained this control for more than 6 years p.i. (Fig. 1b).

nAb response in Δ5G-infected animals

Although failure to detect a nAb response is characteristic of SIV239-infected rhesus macaques (Johnson *et al.*, 2003; Means *et al.*, 1997), the rapid control of viraemia in Δ5G-infected animals prompted us to determine whether nAb played a role in this control of viraemia. We hypothesized that the deglycosylation might lead to the elicitation of a markedly more vigorous nAb response than infection with SIV239. To maximize the detection sensitivity of weak nAb responses at early time points p.i., an assay that measures neutralization titres based on 50 % inhibition of virus replication (IC_{50}) in $CD4^+$ T-cell lines was initially used.

Consistent with the reported results in SIV239-infected animals, no appreciable nAb titre was detected in two animals (Mm13 and Mm25), despite the fact that viral load in Mm13 was distinctively decreased by 30 weeks p.i. However, we observed a rare animal (Mm20) that elicited a robust nAb response against SIV239 and a relatively delayed nAb response against Δ5G, despite the maintenance of a high viral load (Fig. 2a). These results indicated the lack of correlation of nAb response with viral load in SIV239-infected animals. In contrast, nAb was detected in two Δ5G-infected animals (Mm07 and Mm22) starting at 8 weeks p.i. and in two additional animals (Mm12 and Mm23) at 12 weeks p.i. (Fig. 2b, left panel). These titres peaked at either 12 or 18 weeks p.i., and the peak was followed by a decrease in titre that varied among animals. Mm12 and Mm23, which exhibited nAb induction at 12 weeks p.i., had essentially low titres, whilst Mm07 and Mm22, which exhibited nAb induction at an earlier time point, maintained vigorous nAb titres of >1 : 100. Of note, plasma from Mm26 did not contain detectable levels of nAb at any time p.i. In contrast, nAb against SIV239 was not induced in any of the Δ5G-infected animals (Fig. 2b, right panel). As low-level nAb may play a role in control of virus replication, purified IgG from the plasma samples was used to measure neutralizing activity. However, the results from the purified IgG corresponding to the plasma at a 1 : 3 dilution did not change the kinetics of nAb response in Δ5G-infected animals (data not shown).

In experiments where the passive administration of monoclonal HIV nAb successfully prevented the infection of macaques with simian–human immunodeficiency virus, the results unequivocally indicated that high titres of nAb were needed to achieve such protection (Nishimura *et al.*, 2002). In consideration of these results, data were recalculated based on a cut-off value of 90 % inhibition of virus replication (IC_{90}) in $CD4^+$ T-cell lines. As a result, nAb responses were detected in only two of the animals, Mm07 and Mm22, but with titres of 1 : 100 and 1 : 500, respectively (Fig. 2b, middle panel). Next, we examined the correlation between viral load and nAb titre at 8 and 12 weeks p.i. and found that the correlation was not statistically significant (Fig. 2c).

Anti-gp120 Ab response in Δ5G-infected animals

Next, we measured binding Ab responses against gp120. When the plasma samples were assayed for levels of Ab that bound to SIV239 gp120 or Δ5G gp120, essentially identical values were obtained. Fig. 3 shows the data obtained using SIV239 gp120. Remarkably, anti-gp120 responses during the early period p.i. between the two groups of monkeys were distinct. Whereas anti-gp120-specific Ab responses

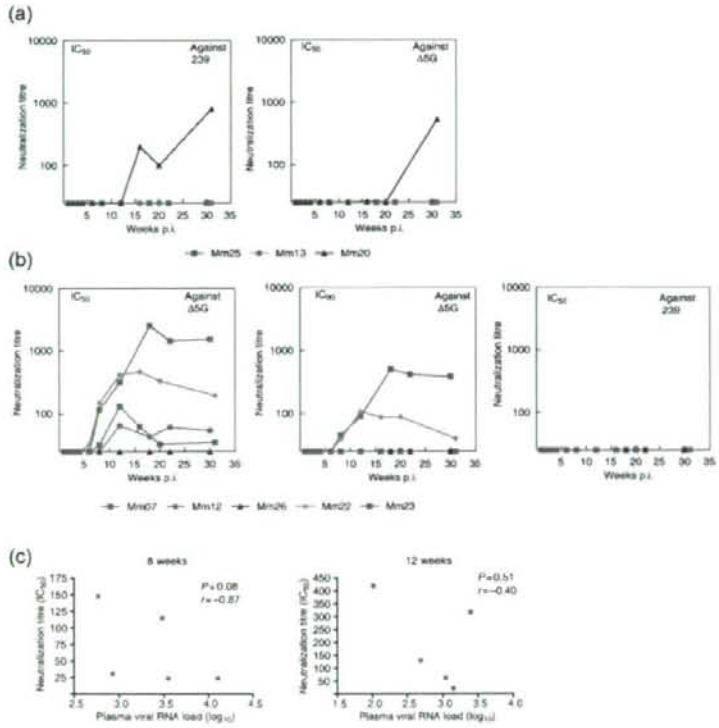

Fig. 2. nAb titres in SIV-infected animals. (a) nAb titres in SIV239-infected animals are indicated as the plasma dilution yielding 50 % inhibition (IC_{50}) of SIV239 infection (left) or Δ5G infection (right) in CEMx174/SIVLTR-SEAP cells. (b) nAb titres in Δ5G-infected animals are indicated as the plasma dilution that yielded 50 % inhibition (IC_{50}, left) and 90 % inhibition (IC_{90}, middle) of Δ5G infection or 50 % inhibition of SIV239 infection (right) in CEMx174/SIVLTR-SEAP cells. (c) Correlation between IC_{50} nAb titres and plasma viral RNA load at 8 and 12 weeks p.i. in Δ5G-infected animals.

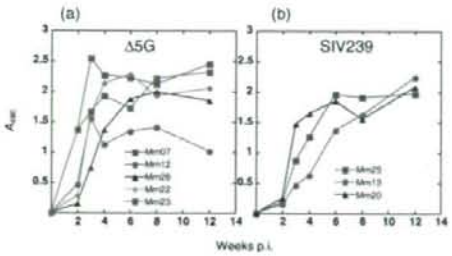

Fig. 3. Anti-gp120 Ab responses. Anti-gp120 Ab responses in Δ5G-infected (a) and SIV239-infected (b) animals were indicated as A_{490} using plasma diluted 1 : 100 in an ELISA.

peaked at 3–4 weeks p.i. in Δ5G-infected animals (Fig. 3a), those in SIV239-infected animals remained generally lower and required longer periods of time to reach their peak (Fig. 3b). Of note, whilst anti-gp120 Ab responses did not correlate well with nAb titres in the chronic phase in Δ5G-infected animals, the hierarchy detected in nAb titres (Mm07, Mm22, Mm23, Mm12 and Mm26, in descending order) was similar to that observed for gp120-binding antibodies at 2 weeks p.i. (Fig. 3a).

Ab responses to linear epitopes in gp120 and gp41 in Δ5G-infected animals differ from those detected in SIV239-infected animals

Next, we examined Ab-binding responses to linear epitopes in plasma samples from infected animals at 8 weeks p.i., as both nAb and anti-gp120-binding Ab were detected at this time point (Figs 2 and 3). We used 72 overlapping peptides encompassing the entire Env sequence of SIV239 for the detection of epitope-specific Ab in plasma samples from Δ5G-infected or SIV239-infected animals. As shown in Fig. 4 and Table 1, the plasma samples reacted with the peptides in six regions: two in gp120 and four in gp41. The regions in gp120 resided in the vicinity of V1/V2, designated region 1 (aa 109–193), and at the C terminus, designated region 2 (aa 493–529). Of note, only linear region 1 was directly affected by selected deglycosylation (aa 146 and 171). The regions in gp41 were located in the ectodomain for region 3 (aa 589–625) and region 4 (aa 660–685), and in the cytoplasmic domain for region 5 (aa 721–757) and region 6 (aa 841–879).

Although Ab responses to most of the peptides recognized in the plasma samples from Δ5G-infected animals were similar to those in SIV239-infected animals, a few peptides were recognized by Abs only in samples from Δ5G-infected animals, and Ab reactivity to some peptides was significantly different between the two groups (Fig. 4b, c and Table 1). Firstly, in region 1, whereas five peptides (Env-10, -12, -13, -14 and -15) were recognized by Abs from Δ5G-infected animals, only three peptides (Env-10, -12 and -13) reacted with Abs from SIV239-infected animals (Fig. 4b and c). Peptide Env-10 was detected by Abs from four Δ5G-infected animals, but from only one of the SIV239-infected animals. Similarly, peptides Env-12 and -13 were detected by Abs from five Δ5G-infected animals and two SIV239-infected animals. In contrast, peptides Env-14 and -15 were detected by Abs from Δ5G-infected animals but not SIV239-infected animals. The specificity of Δ5G infection in the reactivity of peptide Env-14 was statistically significant (P=0.0149) (Table 1). Secondly, the reactivity of Ab from Δ5G-infected animals with the peptides in regions 2, 3 and 4 was lower than that recorded with Ab from SIV239-infected animals (Fig. 4b and c). As shown in Table 1, the reduction in Ab reactivity from Δ5G-infected animals to peptide Env-51 (region 3) and peptide Env-56 (region 4) was significant (P=0.014 and 0.0053, respectively); however, the reduction in Ab response in region 2 was not significant. In addition, there were no significant differences in the Ab responses to the peptides in regions 5 and 6 between Δ5G-infected and SIV239-infected monkeys (Fig. 4b, c and Table 1).

A Δ5G-specific linear epitope resides in the region containing the third deglycosylation site (aa 171) between V1 and V2

As region 1 also contained the site of two mutations introduced to limit glycosylation in the Δ5G mutant, we focused additional studies on this region. To identify the Δ5G-specific epitope(s) in region 1, peptide ELISA was performed with 12 newly synthesized shorter peptides based on the Δ5G sequence spanning the V1/V2 region (Fig. 5). Ab reactivity to peptide Env-14 was mapped to peptides V1V2-9–11 (Fig. 5a). Thus, three linear epitopes (encompassed in peptides Env-10, V1V2-3 and V1V2-9–11) were identified within the V1/V2 region (Figs 4 and 5). Whilst two epitopes contained in peptides Env-10 and V1V2-3 were recognized by Ab from both SIV239- and Δ5G-infected animals, the epitope(s) corresponding to peptides V1V2-9–11 was specific to Δ5G infection (Fig. 5a). As the latter contained the third deglycosylation mutation (Figs 1 and 5b, aa 171), Δ5G specificity was probably secondary to the removal of *N*-glycan at this site in SIV239 gp120 (Fig. 5).

Δ5G-specific Ab responses to linear epitopes in Env elicited immediately following primary infection

In an effort to define the potential relevance of the linear epitope-specific Ab responses in the reduction of acute virus replication in Δ5G-infected animals, we examined the kinetics of Ab reactivity to 12 peptides: Env-10, V1V2-3 and V1V2-9, -10 and -11 for epitopes in region 1; Env-42 and -43 for epitopes in region 2; Env-50 and -51 for epitopes in region 3; Env-56 for epitopes in region 4; and Env-61 and -62 for epitopes in region 5 (Fig. 6). Whilst the induction kinetics of Ab to most peptides were variable in

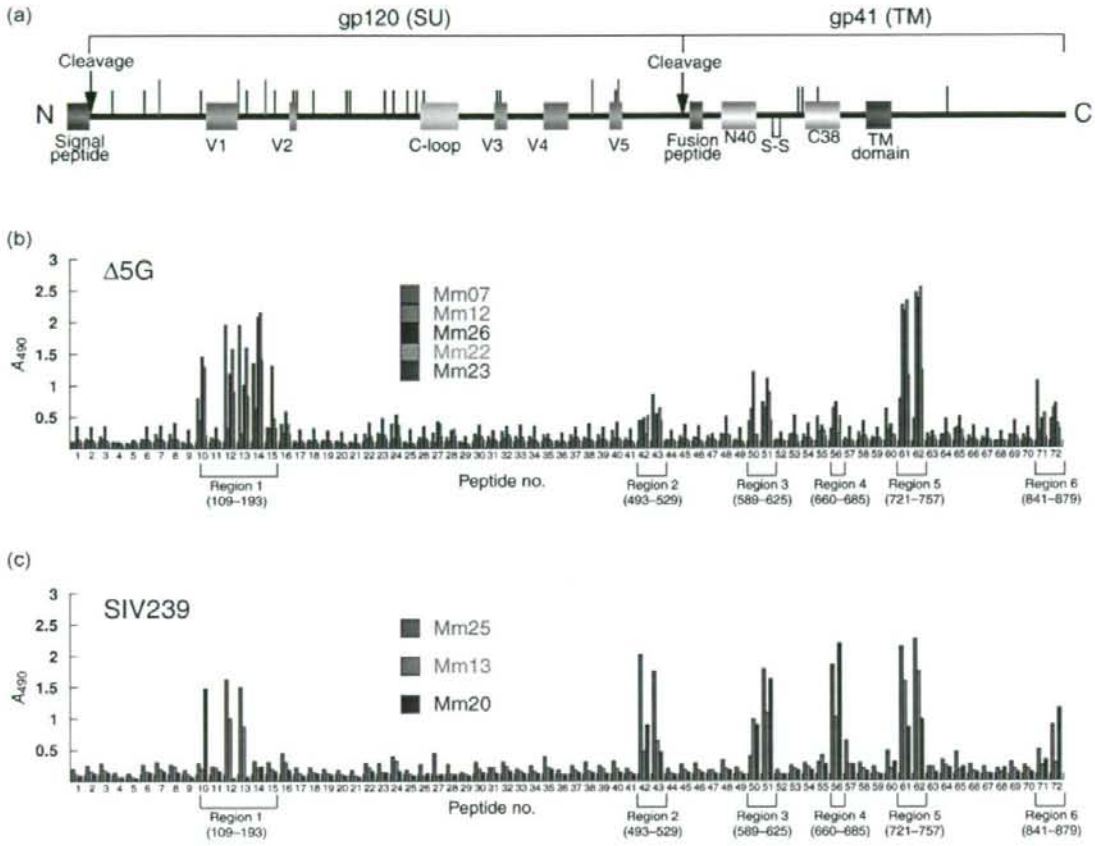

Fig. 4. Ab reactivity to synthetic overlapping peptides spanning the entire Env protein. (a) Diagram of SIV239 Env with the locations of the signal peptide (violet box), variable regions (pink boxes), cysteine loop (yellow box), fusion peptide (green box), N-terminal (N40) and C-terminal (C38) heptad repeats (light-blue boxes), membrane-spanning domain (blue box) and *N*-glycosylation sites (vertical bars) (Burns & Desrosiers, 1991; Choi *et al.*, 1994; Liu *et al.*, 2002). Red vertical bars indicate deglycosylation sites (aa 79, 146, 171, 460 and 479) in Δ5G. S-S indicates the indispensable disulfide bond for hairpin loop formation of the TM protein. (b, c) Plasma samples collected from animals infected with Δ5G (b) and SIV239 (c) at 8 weeks p.i. were used to examine Ab reactivity to 72 peptides (25 mers) overlapping by 13 residues each and spanning the entire Env protein. Reactivity was shown by A_{490}.

plasma from both groups of animals, Ab to V1V2-9, -10 and -11 was specific for Δ5G-infected animals, with rapid induction following primary infection. Ab responses to Env-61 and -62 were also induced rapidly in animals from the two groups; however, it has already been confirmed by SIV and HIV studies that a linear epitope covered by these peptides is the immunodominant epitope with no association with virus control (Eberle *et al.*, 1997; Kent *et al.*, 1992). In contrast to Ab responses to V1/V2 peptides, whilst Ab to peptides Env-51 and -56 in the gp41 ectodomain were detected in SIV239-infected animals, these reactions were low until at least 12 weeks p.i. in Δ5G-infected animals.

Properties of Ab against Δ5G-specific linear epitope

Although Ab reactivity to peptide V1V2-9, -10 and -11 was elicited specifically in Δ5G-infected animals, these Abs were non-nAbs, as these binding Abs were detected in all Δ5G-infected animals, including a nAb-undetectable monkey (Mm26), and before nAb was detected. In addition, we attempted to inhibit neutralization by the addition of excess concentrations of V1V2-9, -10 and -11 to the neutralization assay performed with plasma from Δ5G-infected animals collected at 8 and 12 weeks p.i. The reduction of nAb by the addition of an excess amount of