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ORIGINAL

Generation and characterization of APOBEC3G-positive 293T cells for HIV-1 Vif study

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Abstract : We have established a number of 293T cell lines that express a human anti HIV-1 factor APOBEC3G. Out of seven cell clones examined, four were readily demonstrated to express APOBEC3G by immunoblotting analysis. In particular, two clones (A3G-C1 and -C4) were found to produce a much higher level of functional APOBEC3G relative to that by pooled cell clones. The transfection efficiency of all these cell clones were similar to that of the parental cells, producing a comparable level of virions upon transfection of wild type and *vif*-minus proviral DNA clones. Furthermore, the expression level of APOBEC3G in the best cell line (A3G-C1) was far much higher than those of an APOBEC3G-positive lymphocyte cell line and peripheral blood mononuclear cells. We finally monitored the incorporation of APOBEC3G into virions produced in A3G-C1. APOBEC3G was easily detected in progeny viral particles upon transfection of *vif*-minus proviral clone but not of wild type. These results indicated that our new A3G-C1 cell line is eminently useful for various studies on the interaction of human APOBEC3G and HIV-1 Vif. J. Med. Invest. 54 : 154-158, February, 2007

Keywords **:** *HIV-1, Vif, APOBEC3G, 293T*

INTRODUCTION

The apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is a potent anti-HIV-1 cellular factor that modifies minus strand viral DNA during reverse transcription, resulting in either its degradation or its integration into host chromosomal DNA as a hypermutated provirus (1-3). The deleterious effect of APOBEC3G on HIV-1 comes from its packaging into progeny virions at the stage of assembly in the viral replication cycle (4, 5). HIV-1 Vif impedes the encapsidation of APOBEC3G into nascent virions (6-9). In the APOBEC3G-expressing cells such as a lymphocyte cell line named H9 and peripheral blood mononuclear cells (PBMCs), HIV-1 without functional *vif* gene is unable to grow (10, 11), and therefore, these cells are called non-permissive. Natural target cells of HIV-1 in infected individuals are thought to be all non-permissive.

The non-permissive target cells, which have been routinely used for biological and/or biochemical studies on the interaction of Vif and APOBEC3G, are of lymphocyte cell lineage. It has been well established that transfection of lymphocytic cell lines and primary lymphocytes with test DNAs are very inefficient, and that reproducible results are difficult to obtain. The APOBEC3G-positive cell lines with high transfection efficiency were obviously required

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for molecular virological analyses of Vif and its target APOBEC3G. To the best of our knowledge, however, there has been only one reported monolayer cell line which steadily expresses APOBEC3G and is very susceptible to transfection (8). In this study, we have established stable APOBEC3G-positive 293T cell lines, which are very sensitive to and suitable for transfection analysis. We show here clearly that the cell line designated 293T/A3G-C1 produced a much higher level of APOBEC3G functional against HIV-1 than that by natural target cells.

MATERIALS AND METHODS

Cells

A monolayer cell line 293T (12) was maintained in Eagles's minimal essential medium containing 10% heat-inactivated fetal bovine serum. A lymphocytic cell line H9 (13) was maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. Human PBMCs were prepared essentially as previously described (14).

Transfection

293T cells were transfected by the calcium-phosphate co-precipitation method as previously reported (15).

Western immunoblotting

Immunoblotting analysis using anti-Myc (Invitrogen), anti-APOBEC3G (NIH AIDS Research and Reference Reagent Program, Catalog no. 9906), anti-β-actin (Sigma), or anti-HIV-1 Gag p24 (NIH AIDS Research and Reference Reagent Program, Catalog no. 6521) was done essentially as previously described (16).

Reverse transcriptase (RT) assay

RT assay using ^{32}P-dTTP has been previously described (17).

MAGI assay

Infectivity of virions produced in various 293T cell lines transfected with proviral DNA clones was determined in MAGI cells as previously described (18).

Immunofluorescence (IF) assay

IF assay has been carried out essentially as previously described (19). In this study, cells for IF assay were fixed with ice-cold methanol for 10 min. First antibody used for this assay was anti-APOBEC3G (no. 9906).

DNA constructs

An infectious DNA clone of HIV-1 designated pNL432 (15) and its *vif*-minus mutant designated pNL-Nd (11) has been previously described. An expression vector of human APOBEC3G designated pcDNA-APO3G (9) and a selection vector designated pTK-Hyg (Clontech) were used for the establishment of APOBEC3G-positive 293T cell lines.

RESULTS AND DISCUSSION

Most target cells routinely used for HIV-1 infection are of lymphoid cell lineage, and it is generally difficult to introduce molecular clones into them by transfection. We were interested in analyzing, by transfection experiments, the interaction of a cellular innate anti-retroviral factor APOBEC3G and HIV-1 Vif, which counteract the activity of APOBEC3G. Recent availability of the expression vector of APOBEC3G (pcDNA-APO3G) (9) prompted us to generate monolayer cell lines producing APOBEC3G. We selected 293T cell line (12) for this purpose, because its transfection efficiency is exceptionally high and it produces a huge amount of progeny virions upon transfection of HIV-1 proviral clones.

In order to establish stable cell lines expressing APOBEC3G, 293T cells were co-transfected by the calcium-phosphate co-precipitation method (15) with the pcDNA-APO3G (or pUC19 as control) and pTK-Hyg at the ratio of approximately 20 : 1, and cultured in the presence of hygromycin (200μg/ml) for selection. Of numerous cell clones thus obtained, seven were examined for the expression of APOBEC3G by Western immunoblotting analysis (Fig. 1A) as previously described (16). To see the average expression level, dozens of cell clones were pooled and cultured, and checked also for the APOBEC3G. Quite unexpectedly, only 4/7 were apparently APOBEC3G-positive. Among the four clones, two (A3G-C1 and A3G-C4) showed a higher expression level than that of the other clones and the pooled sample. The transfection efficiency of these cells were then compared. The cells were transfected by proviral clones as above, and RT production into the culture supernatants on day 2 post-transfection was determined as previously described (17). As shown in Fig.1B, no significant difference in the results was noted. The observed transfection efficiency for these cells was similar to that of parental 293T cells (data not shown).

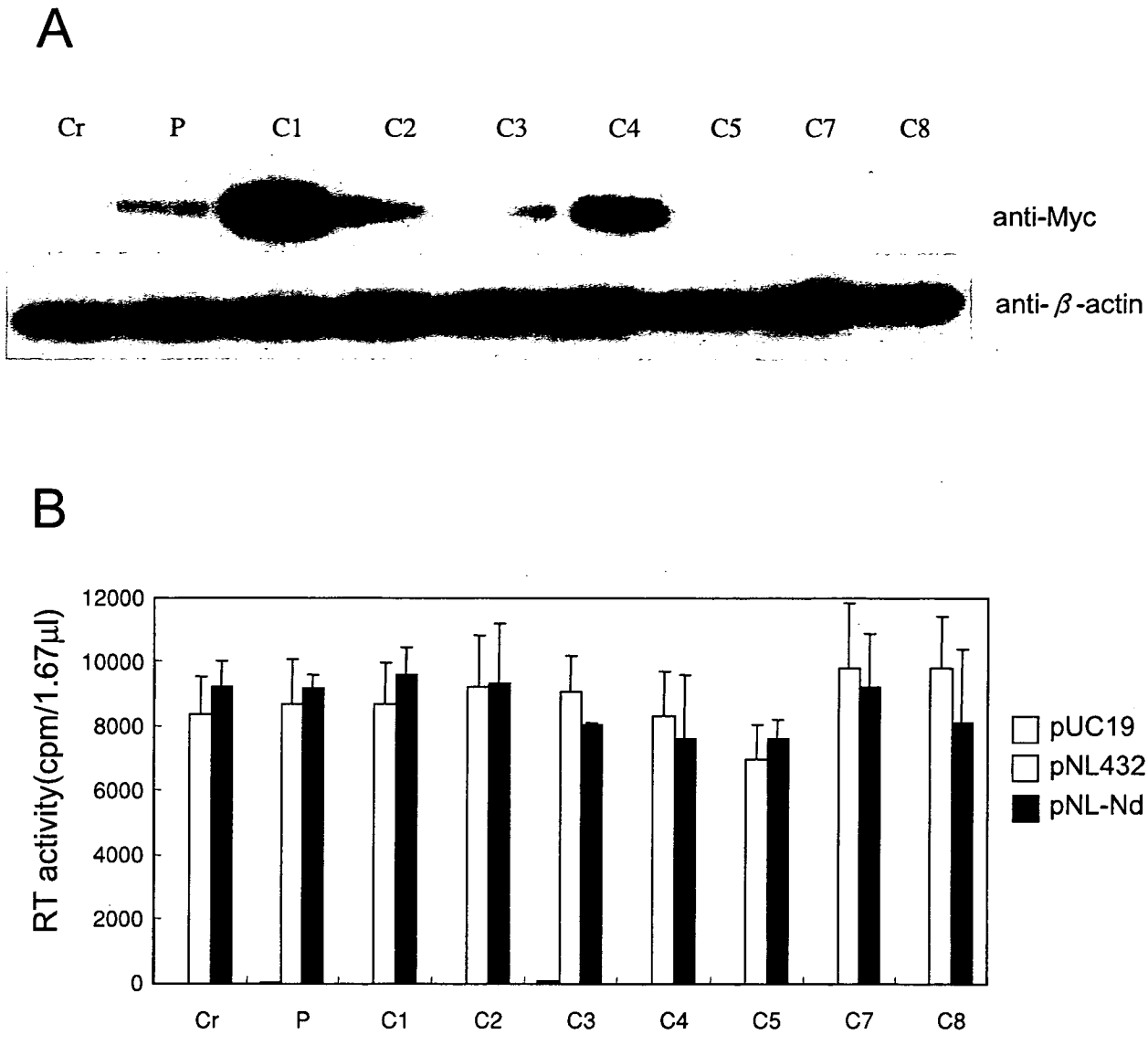

Fig. 1 : Characterization of various 293T clones established in this study. (A) Expressin levels of APOBEC3G in the cell lines established by co-transfection of pcDNA-APO3G containing Myc epitope sequence and pTK-Hyg (A3G-C1 to A3G-C8). Lysates were prepared from the cell lines, and analyzed by immunoblotting using antibodies indicated. Cr, the cell line was obtained by co-transfection of pUC19 and pTK-Hyg ; P, pooled A3G cell clones. (B) RT production in various cell lines upon transfection of proviral clones. Each cell clone indicated was transfected with pNL432 or pNL-Nd, and two days later, RT activity in the culture fluids was determined. Cr and P, the same as above.

To determine whether the A3G-C1 and A3G-C4 are expressing functionally active APOBEC3G, i.e., have the ability to suppress the replication of HIV-1 without the *vif*, the infectivity of progeny virions produced from transfected A3G-C1 and A3G-C4 was assessed in MAGI cells which are routinely used as indicator cells for HIV-1 infection (18). As is clear in Fig. 2, both cell lines consistently expressed biologically active APOBEC3G like naturally occurring non-permissive cells. In total, based on the results in Figs. 1 and 2, we concluded that A3G-C1 is the best clone here.

Next, we comparatively analyzed the expression of APOBEC3G in lymphocytic H9 cells, natural target PBMCs and newly established A3G-C1 cells. These

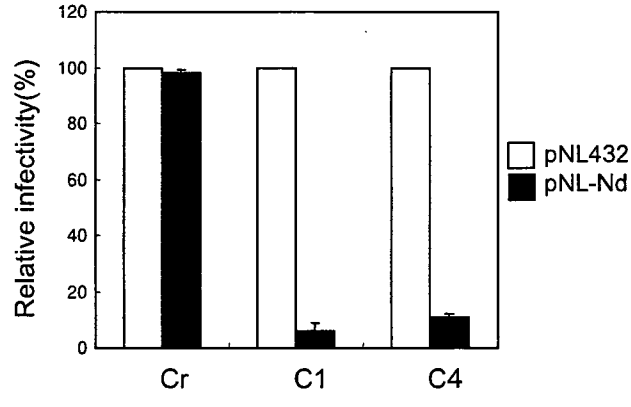

Fig. 2 : Viral infectivity of virions produced in various 293T cell lines. Virus samples were prepared from cells (A3G-C1, A3 G-C4 and Cr) transfected with pNL432 or pNL-Nd, and their infectivity was determined in the MAGI indicator cells (18). Cr, the same as in Fig.1.

cells were examined by indirect IF (19) and Western immunoblotting (16) analyses. Fig. 3A shows the results of the IF assay. Although the intensity of IF in A3G-C1 cells was much stronger than that of H9 and PBMC, APOBEC3G predominantly localized in the cytoplasm of the three cell types as judged by IF. It was also noted that some cell populations in A3G-C1 was producing a particularly high level of APOBEC3G as judged by IF. The biological meaning of this observation is not presently known. To quantitatively compare the expression level of APOBEC3G in various cell types, lysates were prepared from them and subjected to immunoblotting analysis as shown in Fig. 3B. In good agreement with the results in Fig. 3A, A3G-C1 expresses far much higher level of APOBEC3G than that of the others.

We finally monitored the incorporation of APOBEC3G from A3G-C1 cells into viral particles in the absence of Vif. A3G-C1 and control 293T cells were transfected with pNL432 or pNL-Nd, and progeny virions produced on day 2 post-transfection were collected and concentrated by ultracentrifugation as previously described (20). As shown in Fig. 4, quite expectedly, only the virions released from A3G-C1 cells transfected with the *vif*-minus mutant clone contained the APOBEC3G. In addition, we noticed no abnormal Gag profile in the mutant virions produced from A3G-C1 cells, suggesting that the Vif may not affect the virion morphology of HIV-1.

In conclusion in this report, the A3G-C1 cell line, a newly established 293T subline, was demonstrated to be very suitable for biological and molecular biological studies on HIV-1 Vif. It expressed a high level of APOBEC3G (Figs. 1 and 3). The APOBEC3G expressed in it was biologically functional (Figs. 2 and 4). Furthermore, it was very sensitive to transfection and produced a high level of HIV-1 virions (Fig.1). By using A3G-C1 cells, we would be able to analyze the molecular basis for interaction of HIV-1 Vif and APOBEC3G more easily and definitely.

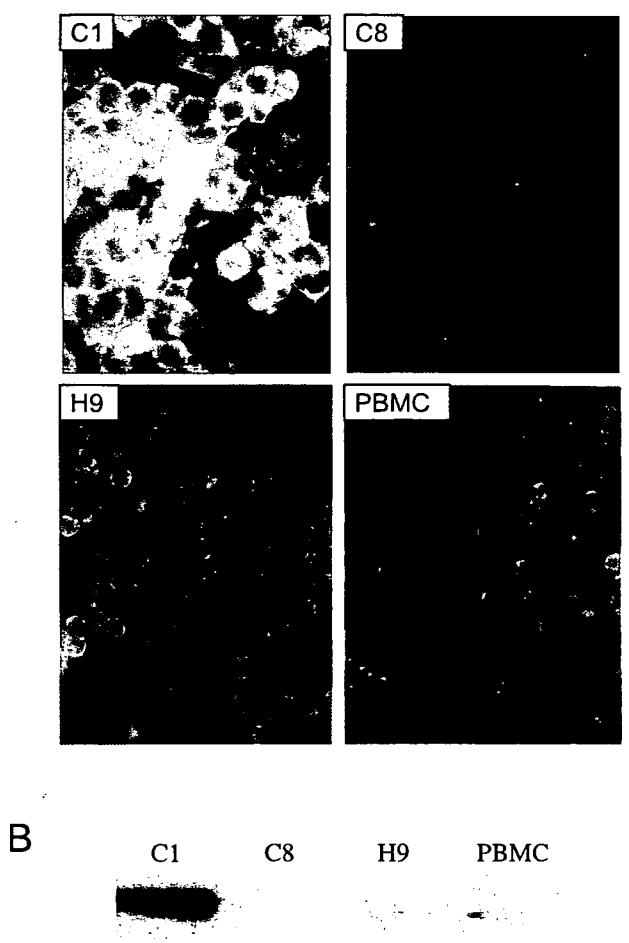

Fig. 3 : Expression of APOBEC3G in 293T/A3G-C1, 293T/A3G-C8, H9 and PBMC cultures. Expression of APOBEC3G in various cell types was monitored by indirect IF (A) and immunoblotting (B) assays. A3G-C8 was used as a negative control (see Fig.1). (A) For detection of APOBEC3G, anti-APOBEC3G (no. 9906) and FITC-conjugated anti-rabbit IgG antibodies were used as 1st and 2nd antibodies, respectively. (B) For detection of APOBEC3G, anti-APOBEC3G antibody (no. 9906) was used.

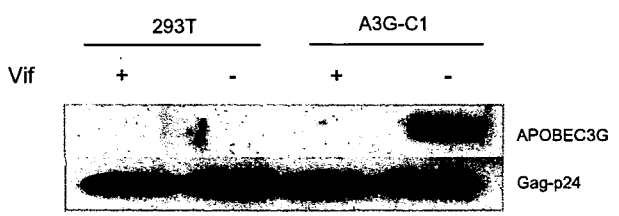

Fig. 4 : Incorporation of APOBEC3G into HIV-1 virions produced in the absence of Vif. Virus particles were prepared from 293T cells and A3G-C1 transfected with pNL432 (+) or pNL-Nd (-) as previously described (20), and their lysates were made for immunoblotting analysis. For detection of APOBEC3G and viral p24, anti-APOBEC3G (no. 9906) and anti-Gag p24 (no. 6521) antibodies, respectively, were used.

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Human Immunodeficiency Virus Type 1 Derivative with 7% Simian Immunodeficiency Virus Genetic Content Is Able To Establish Infections in Pig-Tailed Macaques[▽]

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A human immunodeficiency virus type 1 (HIV-1) derivative ($HIV_{NL\text{-}DT5R}$) containing sequences encoding a 7-amino-acid segment of CA and the entire *vif* gene from simian immunodeficiency virus (SIV) was previously shown to establish spreading infections in cultured macaque peripheral blood mononuclear cells. To assess its replicative and disease-inducing properties in vivo, $HIV_{NL\text{-}DT5R}$ was inoculated into pig-tailed macaques. $HIV_{NL\text{-}DT5R}$ generated plasma viremia in all five of the monkeys and elicited humoral responses against all of the HIV-1 structural proteins but did not cause $CD4^+$ T-lymphocyte depletion or clinical disease. Additional adaptation will be required to optimize infectivity in vivo.

Because the host range of human immunodeficiency virus type 1 (HIV-1) is restricted to chimpanzees and humans, alternative systems such as the simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV)/macaque models have been developed and used extensively for vaccine and pathogenesis studies. However, both of these HIV-1 surrogates have shortcomings that diminish their usefulness as substitutes for HIV-1 in vivo. For example, although SIV has a genomic organization very similar to that of HIV-1, it elicits distinctive cellular and humoral immune responses that are SIV specific and exhibits sensitivities to antiretroviral drugs that are not observed for HIV-1 (26). SHIVs, which contain the HIV-1 *tat*, *rev*, *vpu*, and *env* genes inserted into the SIV genetic background, have been utilized in vaccine experiments to evaluate cellular immune responses directed against SIV Gag and humoral responses directed against the HIV-1 envelope glycoprotein (1, 2, 17, 18). The absence of the other HIV-1 genes in SHIV genomes precludes an evaluation of these virus-encoded proteins during progeny virus production or as antiviral targets in vivo.

We recently reported the construction and characterization of an HIV-1 derivative, designated $HIV\text{-}1_{NL\text{-}DT5R}$, which contains a 21-nucleotide SIV Gag CA element and the entire SIV *vif* gene inserted into the genetic background of $HIV\text{-}1_{NL4\text{-}3}$ (12). $HIV\text{-}1_{NL\text{-}DT5R}$ was able to establish spreading infections in a cynomolgus monkey T-cell line and CD8-depleted peripheral blood mononuclear cells (PBMC) from pig-tailed macaques and rhesus monkeys. Those experiments indicated that the presence of a total of 666 SIV nucleotides (6.7%) at these two specific locations within the full-length 9,894-nucleotide HIV-1 genome was sufficient to counteract innate restriction factors residing in simian cells, such as APOBEC3 and TRIM5α family members, which otherwise block HIV-1 replication (23, 24). Another recently described HIV-1 derivative (stHIV-1), which contains the entire SIV CA and Vif coding sequences, exhibited similar replication properties in macaque PBMC (6).

To ascertain whether the observed infectivity of $HIV\text{-}1_{NL\text{-}DT5R}$ for cultured macaque PBMC could be extended to virus-inoculated monkeys, an animal challenge stock was first prepared from $CD8^+$ T-cell-depleted pig-tailed macaque PBMC, infected with supernatant from 293T cells transfected with pNL-DT5R DNA (12). Virus released into the culture medium on days 8 and 9 postinfection (p.i.) was pooled, and the infectivity of the resulting $HIV\text{-}1_{NL\text{-}DT5R}$ stock was determined to be 1.9×10^5 50% tissue culture infective doses ($TCID_{50}$)/ml, as measured in human T-lymphoid MT4 cells (5). Four pig-tailed macaques were inoculated intravenously with 1.9×10^6 $TCID_{50}$ of virus. Animals were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (17a) and were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Two animals (A3P027 and A4P004) were treated with anti-human CD8 monoclonal antibody (MAb) cM-T807 on days 1 (10 mg/kg of body weight, subcutaneously), 4, and 7 (5 mg/kg, intravenously each day) p.i. to suppress the induction of early antiviral cellular immunity (21). Two monkeys (A3P017 and A3P023) were not treated with cM-T807. Virus replication was determined by measuring the levels of plasma $HIV\text{-}1_{NL\text{-}DT5R}$ RNA using real-time PCR with the following primers/probes specific for the $HIV\text{-}1_{NL4\text{-}3}$ *pol* gene: PNLPOL1 forward primer (GCAGTTCATGTAGCCAGTGGATAT at 4455 to 4478), PNLPOL1 reverse primer (TGGTGAAATTGCTGCCATTG at 4596 to 4577), and PNLPOL1 probe (CAGAGACAGGGCAA

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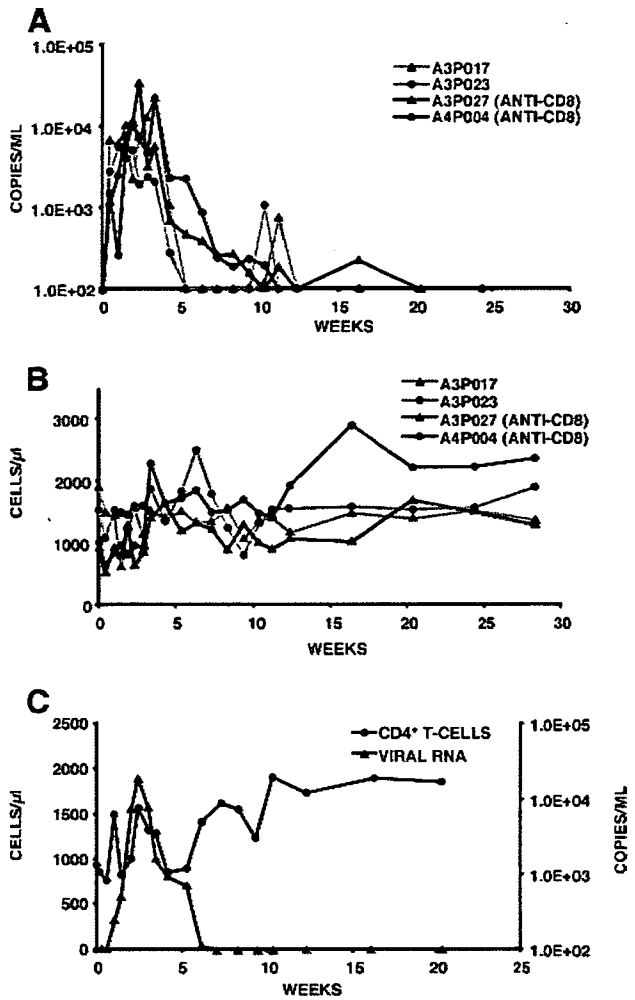

FIG. 1. Profiles of plasma viral RNA loads (A) and circulating $CD4^+$ T lymphocytes (B) in four pig-tailed macaques inoculated intravenously with 1.9×10^6 $TCID_{50}$ of $HIV\text{-}1_{NL\text{-}DT5R}$ and those of a single animal recipient of whole blood and lymph node cells prepared from the former four animals (C). The detection limit of our reverse transcription-PCR assay is 200 RNA copies/ml, and undetectable values are plotted as 100 RNA copies/ml of plasma.

GAAACAGCATACTTCC at 4501 to 4530) as previously described (3). The number of circulating $CD4^+$ T cells was monitored as a marker for virus-induced pathogenesis as described previously (3).

$HIV\text{-}1_{NL\text{-}DT5R}$ productive infections were established in all four animals with peak plasma viral loads ranging from 5.6×10^3 to 3.5×10^4 RNA copies/ml (Fig. 1A). No substantial difference was observed in the levels of peak viremia in the untreated and anti-CD8 MAb-treated monkeys. Plasma viral loads declined rapidly in the two untreated macaques and became undetectable by week 5 p.i. Viremia in the two animals treated with the cM-T807 MAb was maintained until weeks 10 to 11 at which point it fell below the limits of detection (200 viral RNA copies/ml). The prolonged viremia in the anti-CD8 MAb-treated macaques did not appear to reflect protracted suppression of $CD8^+$ T lymphocytes, since they returned to preinfection levels by week 2 postinoculation (data not shown). Although all four $HIV\text{-}1_{NL\text{-}DT5R}$-infected monkeys experienced modest declines in the numbers of circulating $CD4^+$ T lymphocytes during the initial weeks of the acute infection, presumably due to trafficking of T cells into lymphoid tissues, their levels rapidly returned to preinoculation values by week 5 p.i. (Fig. 1B). No evidence of clinical disease was observed in any of the virus-inoculated macaques during the first 6 months of their $HIV\text{-}1_{NL\text{-}DT5R}$ infections.

Extensive passaging of primate lentiviruses with impaired infectivities, both in vitro or in vivo, has resulted in the acquisition of genetic changes conferring augmented replicative properties (4, 8, 11, 13, 19, 22, 25). As an initial step in such a process, a starting virus inoculum was prepared by collecting lymph node and peripheral blood samples from each of the four $HIV\text{-}1_{NL\text{-}DT5R}$-infected monkeys at week 5 p.i. Lymph node cells (7.5×10^7 cells) were suspended in 20 ml of pooled whole blood, and the mixture was inoculated intravenously into another pig-tailed macaque (A3P024). This animal was treated with the anti-CD8 MAb on days 1, 4, and 7 at the same doses and routes as two of the monkeys in the initial infection. The plasma viral RNA levels in the recipient macaque peaked (1.9×10^4 RNA copies/ml) at week 2.4 p.i. and then rapidly declined, becoming undetectable at week 6 p.i. (Fig. 1C). The numbers of circulating $CD4^+$ T lymphocytes did not change appreciably during the initial 20 weeks of infection, and macaque A3P024 has thus far remained asymptomatic.

The infected monkeys responded to $HIV\text{-}1_{NL\text{-}DT5R}$ challenge by producing virus-specific antibodies as measured by immunoblotting of plasma samples collected during the initial 24 weeks of infection (Fig. 2). Commercially available diagnostic HIV-1 Western blotting strips (Cambridge Biotech HIV-1 Western blot kit; Maxim Biomedical Inc., Rockville, MD) were employed, and a plasma sample from an HIV-1-infected individual served as a positive control. The use of a different production lot of blotting strips resulted in the variability observed with samples from monkey A3P017 (Fig. 2). All the $HIV_{NL\text{-}DT5R}$-infected animals, regardless of anti-CD8 treatment, produced antibodies directed against HIV-1-encoded p17, p24, gp41, gp120, and gp160 (anti-gp160 as early as week 2 p.i. in macaque A4P004), and four of the five animals (A3P017, A3P027, A4P004, and A3P024) generated antibody against the HIV-1 p66 reverse transcriptase. In all five animals, the band intensities for each viral protein were maintained or increased over time, suggesting sustained virus replication, even after plasma viral RNA loads fell below the level of detection (Fig. 1). The weaker reactivity of plasma samples from monkey A3P023 was consistent with the lower values obtained with a commercially available enzyme-linked immunosorbent assay kit (Vironostika HIV-1 Microelisa system; bioMerieux Inc., Durham, NC) (data not shown).

To ascertain whether $HIV_{NL\text{-}DT5R}$ had established persistent infections in the animals, PBMC-associated viral DNA levels were measured at 45 weeks p.i. for pig-tailed macaques A3P017, A3P023, A3P027, and A4P004, and at 38 weeks p.i. for A3P024, since proviral DNA in PBMC can be detected even after plasma viral RNA loads fall below the limits of detection in animals effectively controlling virus replication (18). The same primer/probe pair and amplification conditions used to measure plasma viral RNA were employed for the detection of proviral DNA. Low levels of PBMC-associated viral DNA were detected in samples from all five animals (0.36

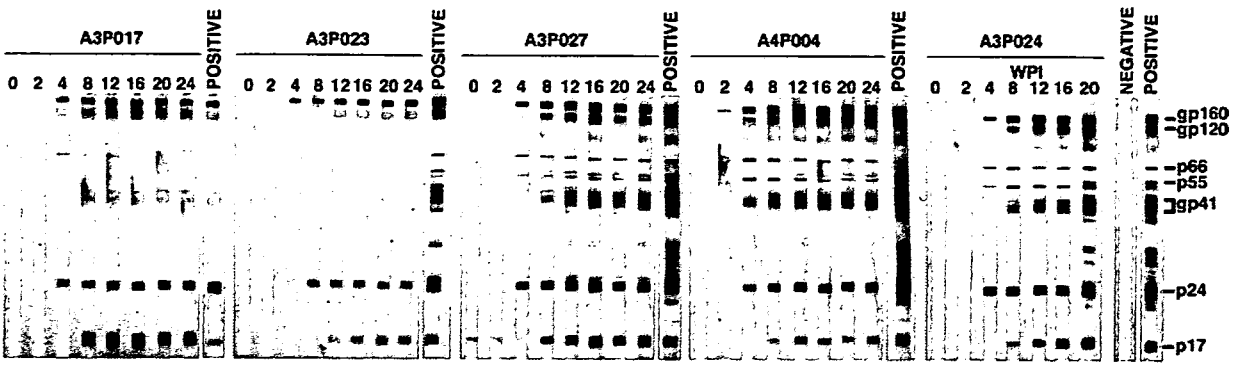

FIG. 2. Profiles of anti-HIV-1 antibody responses in pig-tailed macaques infected with HIV-$1_{NL\text{-}DT5R}$. Commercially available diagnostic HIV-1 Western blotting strips (Cambridge Biotech Western blot kit) were employed using a 1:68 dilution of pig-tailed macaque plasma samples. Normal human plasma was used as a negative control. Plasma from an HIV-1-infected individual was used as a positive control. The positions of HIV-1 proteins are indicated to the right of the blots. WPI, weeks postinfection.

copy/10^5 cells for A3P017, 0.14 copy for A3P023, 0.76 copy for A3P027, 0.70 copy for A4P004, and 0.21 copy for A3P024; the detection limit for this assay is 0.1 copy/10^5 cells). The proviral DNA loads measured in two of the anti-CD8-treated animals (A3P027 and A4P004) were somewhat higher than those in the untreated macaques (A3P017 and A3P023) and correlated with prolonged detection of plasma viremia in these monkeys. Taken together with the steady/increasing antibody responses and the presence of proviral DNA, these results indicate that $HIV_{NL\text{-}DT5R}$ is able to establish low-level persistent infections in pig-tailed macaques.

The results described in this report reinforce conclusions initially observed in transfection and single-cycle infectivity assays, which demonstrated that blocks to virus replication imposed by macaque TRIM5α and APOBEC3 cytidine deaminases markedly inhibited HIV-1 replication in simian cells (16, 24). We also reported that the restriction to the establishment of HIV-1 spreading infections in cultured monkey PBMC could be counteracted by HIV-$1_{NL\text{-}DT5R}$, which carries a short SIV Gag element and the entire SIV *vif* gene, but not by wild-type HIV-1 (12). The results from the present study now extend the previous observations to the organismal level and show that HIV-$1_{NL\text{-}DT5R}$ can replicate in vivo.

Although HIV-$1_{NL\text{-}DT5R}$ was able to establish infections in pig-tailed macaques, its replicative properties are reminiscent of first-generation SHIVs (10, 14, 15, 20). The latter generated modest levels of peak viremia during acute infections of macaque monkeys that were rapidly and durably suppressed and failed to induce any disease. Serial passaging of these SHIVs or the administration of $CD8^+$-T-cell-depleting MAbs during the primary virus infection resulted in the emergence of pathogenic virus, which replicated to high titers in infected animals and induced the systemic depletion of $CD4^+$ T lymphocytes and development of immunodeficiency (9, 11, 19). In this regard, it has also been reported that passaging of minimally cytopathic SIV_{MneCl8} (13) or even the pathogenic SIV_{B670} (8) gave rise to SIV variants exhibiting more robust replication phenotypes and augmented pathogenic properties. One could envisage using a similar approach for generating more fit HIV-$1_{NL\text{-}DT5R}$ variants for infections of macaque monkeys, including some carrying CCR5-utilizing *env* genes. These new derivatives could be used for analyses of cell-mediated immune responses directed against Gag and Pol proteins or to assess patterns of antiviral drug resistance against HIV-1-encoded proteins, not presently possible with SIV or currently available SHIVs.

Although it might be considered a step backward, one could argue that the direct substitution of additional SIV-specific sequences into the genetic backbone of HIV-$1_{NL\text{-}DT5R}$ might markedly improve its infectivity in monkeys. For example, the SIV long terminal repeat contains one (not two) NF-κB binding site, four (not three) SP1 binding sites, and unique PuB2, SF1-3, and peri-xB binding sites relative to the HIV-1 long terminal repeat (7). Similarly, the significantly larger SIV *nef* gene and the presence of both *vpr* and *vpx* genes in the SIV genome (rather than the single *vpr* gene in HIV-1) would suggest that the acquisition of nonhuman primate species-specific *cis*-acting elements and coding sequences may optimize virus infectivity in vivo. Both direct replacement and serial passaging strategies are being used to obtain HIV-$1_{NL\text{-}DT5R}$ variants with improved replicative potential in monkeys.

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VIII ウイルス病原性発現の分子機構

HIV-1の病原性とアクセサリー遺伝子

足立昭夫・鎌田和弥・八町和樹・山下知輝・内山恒夫・野間口雅子

エイズの原因ウイルスであるヒト免疫不全ウイルス1型（HIV-1）には他のレトロウイルスには存在しないアクセサリー遺伝子と総称される4種類の遺伝子がある．ウイルス発見当初からこれらの存在は認識されていたが，そのアクセサリー性のゆえにウイルス学的機能は謎であった．近年になり，アクセサリー遺伝子にコードされるウイルス蛋白質の重要な働きが明らかになってきた．アクセサリー蛋白質は宿主の防御機構を抑制・制御することなどによりウイルスの複製を最適化し，その存続・伝播・病原性発現に大きな役割を果たしている．

▶▶KEY WORDS：HIV-1　アクセサリー遺伝子　宿主域　エイズ

はじめに

後天性免疫不全症候群（エイズ）の原因ウイルスであるヒト免疫不全ウイルス1型（HIV-1）は，主としてCD4陽性Tリンパ球およびマクロファージに感染し，長い潜伏期を経たのち最終的に宿主の免疫系を破壊してエイズを発症させる．HIV-1は宿主域が非常に狭く，感染するのはヒトとチンパンジーのみであり，しかもチンパンジーはエイズにならないため，ヒトのみが発症を伴うHIV-1の自然宿主である．小動物を用いた人工的な感染モデルシステムも種々提案されているが，ヒトがHIV-1感染を受けてからエイズ発症に至るまでの経過を反映する実際的なモデルはないのが現状である．一方，HIV-1に近縁のアカゲザル免疫不全ウイルス（SIVmac）[1]やHIV-1とSIVmac間のキメラウイルスSHIV[2]もサル個体感染実験に用いられてきた．ウイルス病原性やワクチン開発研究に大きな成果があげられてきたが，SIVmacおよびSHIVはHIV-1とはウイルス学的に明らかに異なる性格ももち，遺伝子のホモロジーや遺伝子構成の違いも考慮すると，ただちに良好なHIV-1/エイズモデルとは言いがたい[3]．したがって，HIV-1の個体内複製機構・病原性発現機構の研究，とくに実験的実証的研究は困難を極めている．

HIV-1はレトロウイルスに属しているが，すべてのレトロウイルスに共通してみられる構造遺伝子*gag*，*pol*および*env*のほかにHIV-1などの霊長類免疫不全ウイルスに特異的な調節遺伝子*tat*および*rev*，さらにはアクセサリー遺伝子*vif*，*vpr*，*vpu*および*nef*が存在する（図1）．構造遺伝子および調節遺伝子は，それぞれ感染性成熟ウイルス粒子の形成およびウイルス遺伝子発現に関与し，どのような細胞種でもウイルス複製・増殖に必須である[3]．アクセサリー遺伝子には，ある特定の細胞でウイルス複製に必須である*vif*と，多くの研究者が検討した限りどのような細胞種でも非必須である*vpr*，*vpu*および*nef*とがある[3]．後者の事実は，ウイルス感染個体内におけるアクセサリー遺伝子の特別かつ重要な働きを示唆している．

上記の宿主域やアクセサリー遺伝子の存在で明らかなように，HIV-1はレトロウイルスのなかでも非常に特殊化されている．しかし，その細胞内の複製過程は，基本的にはレトロウイルスと同じである（図2）．本稿では，HIV-1の遺伝子構造，これらの遺伝子群がコードする蛋白質の機能および細胞内ウイルス複製機構について簡単に述べたのち，アクセサリー蛋白質の生物学に焦点を絞って，HIV-1の宿主域や病原性との関係について整理してまとめてみたい．

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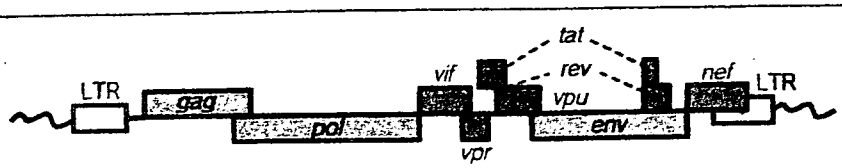
Functional roles of HIV-1 accessory genes for its pathogenicity



ウイルス蛋白質		機能
構造蛋白質		
Gag	マトリックス(matrix：MA)	ウイルス粒子形成・放出，脱殻・逆転写
	キャプシド(capsid：CA)	ウイルス粒子形成・放出，脱殻・逆転写
	ヌクレオキャプシド(nucleocapsid：NC)	脱殻・逆転写
	p6	ウイルス粒子放出，Vprのウイルス粒子へのターゲティング
Pol	プロテアーゼ(protease：PR)	Gag前駆体やGag-Pol前駆体の開裂による成熟ウイルス粒子(感染性ウイルス粒子)の生成
	逆転写酵素(reverse transcriptase．RT)	ウイルスDNA合成(含RNase H活性)
	インテグラーゼ(integrase．IN)	ウイルスDNAの細胞染色体DNAへの組込み
Env	gp120	標的細胞受容体への結合
	gp41	標的細胞への侵入
調節蛋白質		
Tat		転写の増強
Rev		構造およびアクセサリー蛋白質(Nefを除く)の発現増強
アクセサリー蛋白質		
Vif		抗ウイルス細胞因子APOBEC3G/Fの不活化
Vpr		細胞周期の停止
Vpu		ウイルス粒子の放出促進
Nef		宿主の免疫抑制

図1 HIV-1のゲノム構造とウイルス蛋白質
HIV-1のゲノムとそれにコードされるウイルス蛋白質を示す．ウイルス蛋白質は構造蛋白質，調節蛋白質およびアクセサリー蛋白質に分類し，主要な機能(本文参照)をまとめた．

I. HIV-1のウイルス学

1. HIV-1の遺伝子構造と遺伝子産物

図1に示してあるように，HIV-1ゲノムにはレトロウイルスのなかで最多の9種類の遺伝子がある．HIVには2型（HIV-2）があり，非常に限られた一部の地域（ギニアビザウ共和国など）に感染者がいるが，これにも9種類の遺伝子が存在する．しかし，遺伝子の構成は異なっており，HIV-1にある*vpu*を欠き，HIV-1にない*vpx*をもつ[3]．HIV-2はSIVmacと同じウイルスであり，近年，共通の祖先から進化してきたと推測されている[4]．HIV-2は疫学データなどにより，ヒトのエイズウイルスとしては伝播力に乏しく，かつ病原性が弱いと考えられ，世界中に蔓延し猛威をふるっているHIV-1に比べ，社会的・医学的重要性は低い．

HIV-1ゲノムからは最終的に15種類ものウイルス蛋白質が産生される（図1）．Gag，PolおよびEnv蛋白質はすべてのレトロウイルスに共通に存在し，その機能も一部を除き同じである．Gagは前駆体として合成され，ウイルス複製の最終段階（図2）で自身のプロテアーゼによりプロセシングを受け，マトリックス（MA），キャプシド（CA），ヌクレオキャプシド（NC）およびp6となる（図1）．これらはウイルス粒子の主要構成蛋白質としてさまざまな役割を担っている（図1，図2）．PolはGag-Pol前駆体として合成され，上記と同様にプロセシングされ，レトロウイルスに特徴的な酵素活性（逆転写酵素およびインテグラーゼ）を担う（図1，図2）．これに対し，Envの前駆体は細胞のプロテアーゼにより成熟型となり，標的細胞への感染を司る．HIV-1特異的蛋白質のTatおよびRevは非構造蛋白質であって，その機能はウイルス遺伝子の発現調節である（図1，図2）．ヒトT細胞白血病ウイルス1型にも同様の機能をもつウイルス蛋白質（TaxおよびRex）がある[5]．これらの働きによりウイルス複製は精妙に調節されている（図2）．すでに述べたが，アクセサリー蛋白質と総称される蛋白質群はこれらの構造・調節蛋白質と異なり，すべての細胞種で機能するわけではない．詳細は後述するが，その働きには細胞特異性がある．アクセサリー蛋白質のうち，Vprは明らかに構造

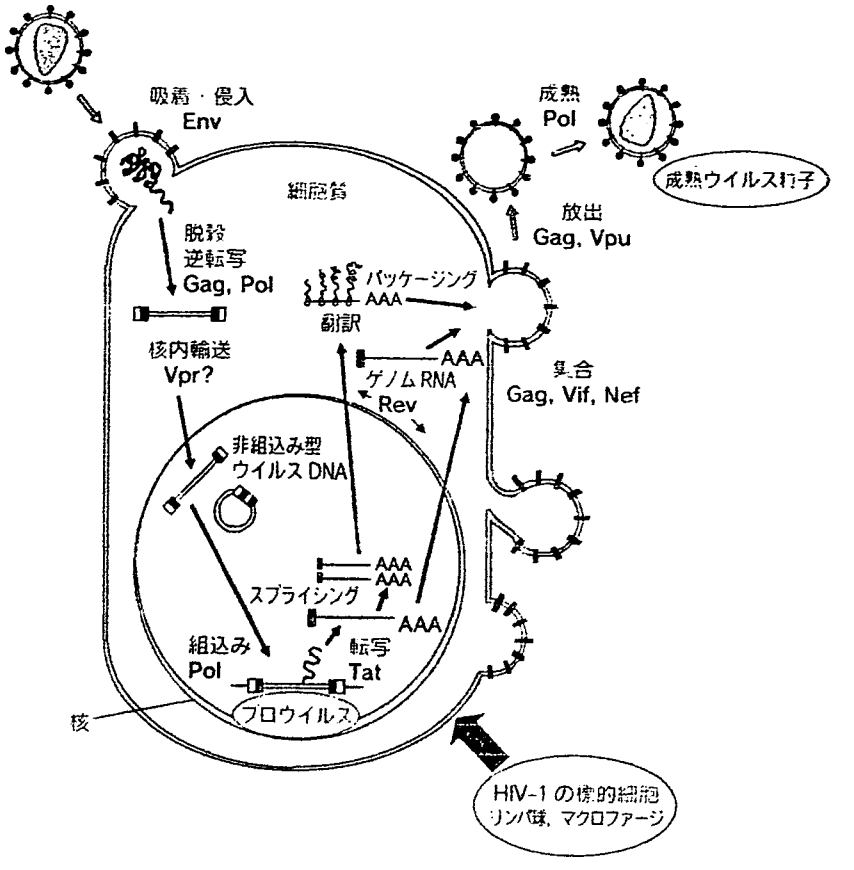


図2 HIV-1の細胞内複製機構
標的細胞中のHIV-1複製過程を模式的に示す 各複製段階で機能するウイルス蛋白質も図中に記載した

蛋白質であり，Vpuは非構造蛋白質であるが，VifとNefについては議論があり明確な結論が出ていない．このように，アクセサリー蛋白質の機能・構造を含めた全貌はいまだ未解明である．

2. HIV-1の細胞内複製

HIV-1の標的細胞における複製機序を図2に示した．成熟ウイルス粒子の細胞への吸着・侵入に始まり，RNAゲノムの逆転写，プロウイルスの生成，ウイルス遺伝子発現を経て，感染性の子孫成熟ウイルス粒子の産生に至るのがレトロウイルスの生活環である．複製前期のゲノム逆転写とその細胞DNAへの組込みが，レトロウイルスの特異的複製過程である．HIV-1特異蛋白質でこれらの過程にかかわると明確に示されているものはない．Vprがマクロファージでの HIV-1 複製に必要あるいは必須である（ウイルスゲノムの核移行）との報告はあるものの，コンセンサスとなっていない．筆者らの研究室でも，多数のヒトからマクロファージを種々の方法で調製し，Vpr欠損ウイルスの増殖能を検討したが，正常にVprを発現するウイルスとの差は顕著ではなかった（20～50％程度）．一方，HIV-1特異蛋白質（調節蛋白質およびアクセサリー蛋白質）はプロウイルスゲノムの転写過程以降（複製後期過程）に明確に関与しており，ウイルス複製を正に制御している（図2）．Tatによる転写の増強，Revによる構造およびアクセサリー蛋白質の発現増強，VifやNef蛋白質のウイルス粒子修飾，Vpuのウイルス粒子放出促進がそれである．ただし，調節蛋白質TatおよびRev，また，アクセサリー蛋白質Vifを除き，他のアクセサリー蛋白質の細胞レベルでのウイルス複製に及ぼす効果は大きくない．この点は十分注意しておく必要があると思われる．

II. HIV-1のアクセサリー蛋白質

アクセサリー蛋白質はHIV-1などの霊長類免疫不全ウイルス群（レンチウイルス）に特異的に存在し，このウイルス群の生物学的特性を担っている．これらのウイルス蛋白質の機能と作用機構の詳細は最近の総説[6~9]に譲

表1　HIV-1 アクセサリー蛋白質に関するおもな知見

蛋白質	主要な細胞・ウイルス学的知見
Vif	(1) 末梢血由来リンパ球およびマクロファージでのウイルス複製に必須 (2) ウイルス粒子を修飾することで初期過程の複製効率を制御(ウイルス産生細胞依存的) (3) ウイルス RNA に結合 (4) ウイルス DNA 合成に関与 (5) Gag 前駆体のプロセシング・アセンブリに関与 (6) 核蛋白質コアのパッキングに関与 (7) 内在性 HIV-1 抑制因子の効果を阻害
Vpr	(1) ウイルス粒子内に多量に存在 (2) HIV 遺伝子発現のトランスアクチベーター (3) マクロファージで組込み前駆体の核移行シグナルとして働く (4) 細胞分化を誘導 (5) 細胞周期を停止させる (6) アポトーシスの制御 (7) 感染個体内での高レベルのウイルス量の維持に必要 (8) エイズ発症に関与
Vpu	(1) HIV-1 にのみ存在 (2) 細胞依存的にウイルス粒子放出効率を増強 (3) CD4 を破壊 (4) アポトーシスの制御
Nef	(1) 非抗原刺激末梢血単核細胞でのウイルス複製に重要 (2) 細胞膜結合性 (3) ウイルス粒子を修飾することで初期過程の複製効率を制御 (4) ウイルスの細胞への侵入過程に関与(ウイルス産生細胞依存的) (5) ウイルス DNA 合成に関与 (6) CD4 の発現を抑制 (7) MHC-I の発現を抑制 (8) T 細胞におけるシグナル伝達系に影響 (9) 感染個体内での高レベルのウイルス量の維持に必要 (10) エイズ発症に重要

り，本稿では，そのウイルス学的意義に焦点を絞って解説したい（表1）．培養細胞株中でのウイルス複製・増殖に必須でないためアクセサリーと称されるが，HIV-1 のアクセサリー蛋白質は前出のように2つのカテゴリーに分けられる．Vif は自然宿主細胞である初代リンパ球やマクロファージではウイルス複製に必須で，その意味ではアクセサリー蛋白質ではない．2つ目のカテゴリーには Vpr，Vpu および Nef が入る．これらがウイルス複製に必須である培養細胞系は初代細胞を含めて知られていない．細胞の種類，細胞の調製法，ウイルスアッセイの方法などによっては，これらを欠損するウイルス変異体の複製・増殖は有意に低下するがウイルス学的には大きいものではない．したがって，Vpr，Vpu および Nef の真のウイルス学的機能解明が今後のアクセサリー蛋白質研究の中心課題の1つである．

1. Vif

Vif が自然宿主細胞でのウイルス複製に必須であることは早くから知られており，さまざまな現象が報告されていた（表1）．しかし，これらを統一的に理解できるようになったのは最近のことである[10]．Vif はユビキチン・プロテアソーム系により自然宿主細胞に存在する抗ウイルス因子APOBEC3Gを分解し，ウイルス粒子中への取り込みを阻害する．

2. Vpr

Vpr はその多機能性（表1）が注目されているが，既述のとおり，そのウイルス複製サイクルにおける役割は定かではない．筆者らは，Vpr が直接ウイルス複製にかかわるというより，むしろ，Vpr が標的細胞に及ぼす効果・影響が重要ではないかと考えている．

3. Vpu

Vpu が2つの活性，ウイルス粒子放出の促進および CD4 の破壊を示すことは明らかだが（表1），細胞レベルでウイルス複製に大きく影響することはない．霊長類免疫不全ウイルスのなかで HIV-1 にしか存在しないわけであるから，その働きの全容解明が待たれる．

4. Nef

Nef も Vpr と同様に多機能性が指摘されているが，報告されている知見の全体を眺めてみると，宿主の免疫との関係が浮かび上がってくる（表1）．SIVmac によりアカゲザルに惹起されるエイズと Nef の関係は早くから報告されていた[11]．

III. HIV-1 の宿主域と病原性

HIV-1 の宿主域がほぼヒトに限られるため，個体レベルの実験的研究が困難であることはすでに述べた．狭い宿主域は HIV-1 の最も顕著で重要な生物学的特性の1つ

であり，これに関するウイルス/細胞側決定因子と分子機構については精力的かつ広範な研究が展開されてきた[12,13]．ごく最近になり，筆者のグループなどによりサル指向性 HIV-1 の構築がなされるなど大きな進展があった[13,14]．ここでは，これに関連する事柄についてまとめる．

1．宿主域の決定因子

宿主域が異なる類似のウイルスがあった場合，両者間でキメラウイルスを構築してそれらの性格を調べれば容易にウイルス側要因が決められる．筆者のグループなどは，HIV-1 と SIVmac 間でさまざまなキメラウイルスを構築し，サル細胞およびサル個体でのウイルス増殖能を検討した[2,12~19]．その結果，HIV-1 の種指向性のウイルス側決定因子は Gag-CA にあるシクロフィリン結合ループと Vif とであることが明らかになった．とくに，図 3 に示した筆者らの構築した HIV-1 キメラウイルス NL-DT5R[13] は，ゲノムの約 93％が HIV-1 由来で，サル細胞だけでなくサル個体にも感染する（未発表データ）．NL-DT5R は試験管内で作製したウイルスクローンをカニクイザル細胞に馴化させたものであるが[13]，SIVmac239 と比較すると，

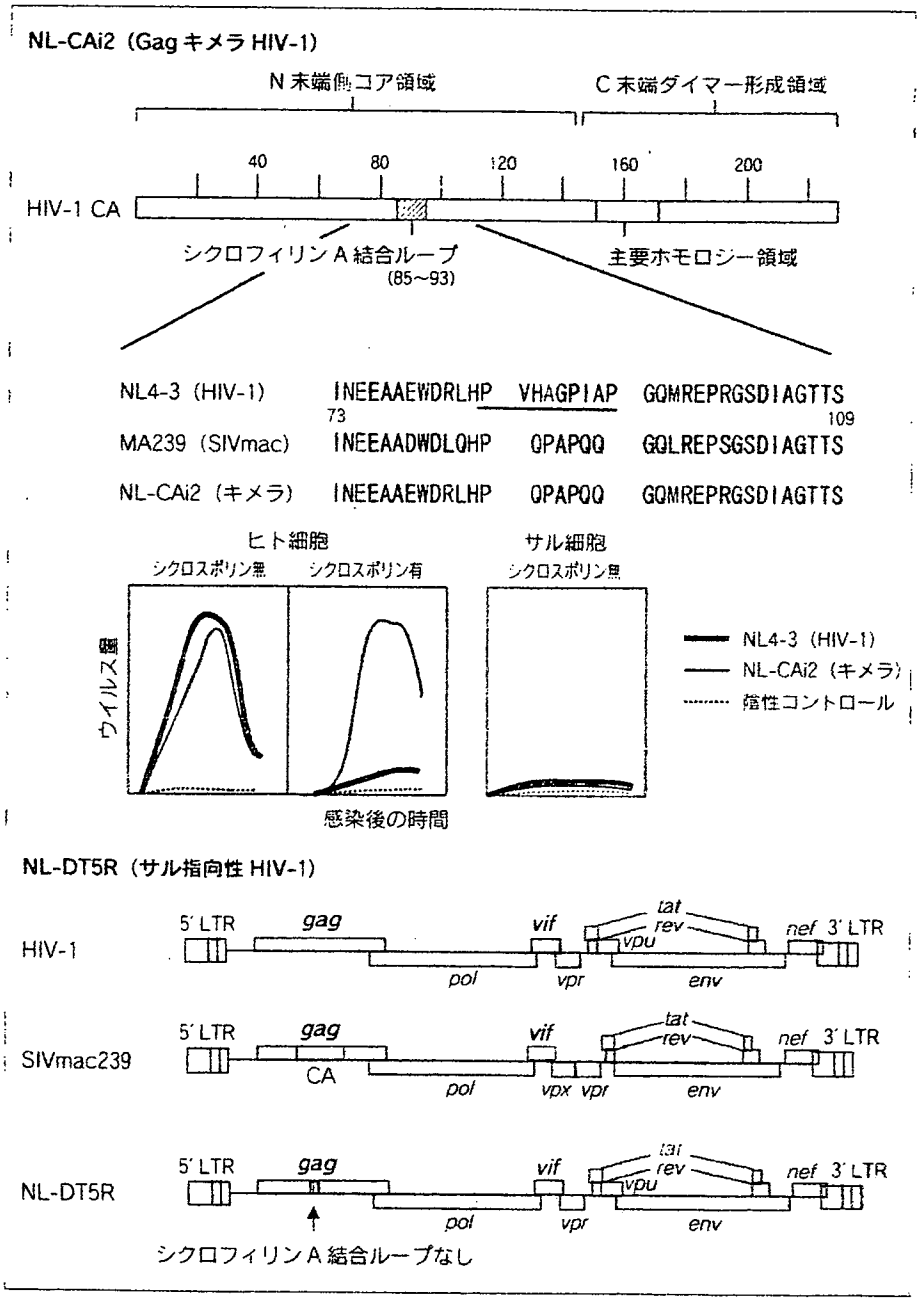


図 3　サル指向性 HIV-1 の構造
上段に SIVmac の性質を一部獲得した（シクロスポリン抵抗性）HIV-1 Gag キメラウイルス NL-CAi2[18] を，下段にサル指向性 HIV-1 キメラウイルス NL-DT5R[13] のゲノム構造を示す．NL-CAi2 はシクロフィリン A 結合ループ対応領域のみが SIVmac239 由来，NL-DT5R はこれに加えて Vif 全体も SIVmac239 のものに置換してある

サル細胞およびサル個体で増殖効率が悪い．筆者らは，この新ウイルスの抗ウイルス細胞因子からの回避が不十分であるとは必ずしも考えておらず，他の要因，すなわち，人工ゲノム作製による一定程度の欠損，Env の不適合あるいは転写能などが問題ではないかと予想している．現在，この仮説に基づいてさらなるウイルスゲノムの改良に取り組んでいる．

これらウイルス側因子と相互作用する細胞因子（TRIM5α と APOBEC3G など）についても大きく研究が発展している[10,20]．図4はこれらの作用機構について模式的にまとめたものである．HIV-1 の Gag-CA には TRIM 5α とシクロフィリン A がともに結合するが，この2つの細胞因子の関係はいまだ不明である．HIV-1 はサル細胞に存在するこれらの抗ウイルス因子を不活化・中和できないため，サル細胞では増殖不能となっている．

2. 病原性にかかわる HIV-1 蛋白質

HIV-1 の病原性に関する実験研究はいまだ不可能であるので，表2に SIVmac239 の変異体解析による病原性研究の成績をまとめた．この実験では，ウイルス複製に必須である Gag，Pol，Env，Tat および Rev については，当然，エイズ発症に必須であるという結論になる．興味深いのはアクセサリー蛋白質に関する結果である．Vif を欠損したウイルスは細胞および個体内でほとんど増殖しないため，上の5種のウイルス蛋白質欠損体と同じ成績となる．一方，Vpx あるいは Vpr 欠損ウイルスは個体内でもかなりよく増殖し，感染個体にエイズを発症させる．ただし，発症頻度や発症までの期間は正常ウイルスとは異なる．Nef 欠損および Vpx/Vpr 二重欠損ウイルスは，ある程度増殖するものの，エイズを発症させることはない．これらの成績は，個体内でのウイルス複製量と病原性に正の相関があることを示している．しかし，Nef や Vpx/Vpr の場合は必ずしもそうとは言い切れない．また，Vpu は SIVmac に存在しないため解析の対象外となっている．したがって，ヒトのエイズ発症において HIV-1 の Vpr，Vpu あるいは Nef の果たす役割を知るためには，上記の新しい HIV-1 キメラウイルスなどを用いたサル感染実験が必要であると考えられる．

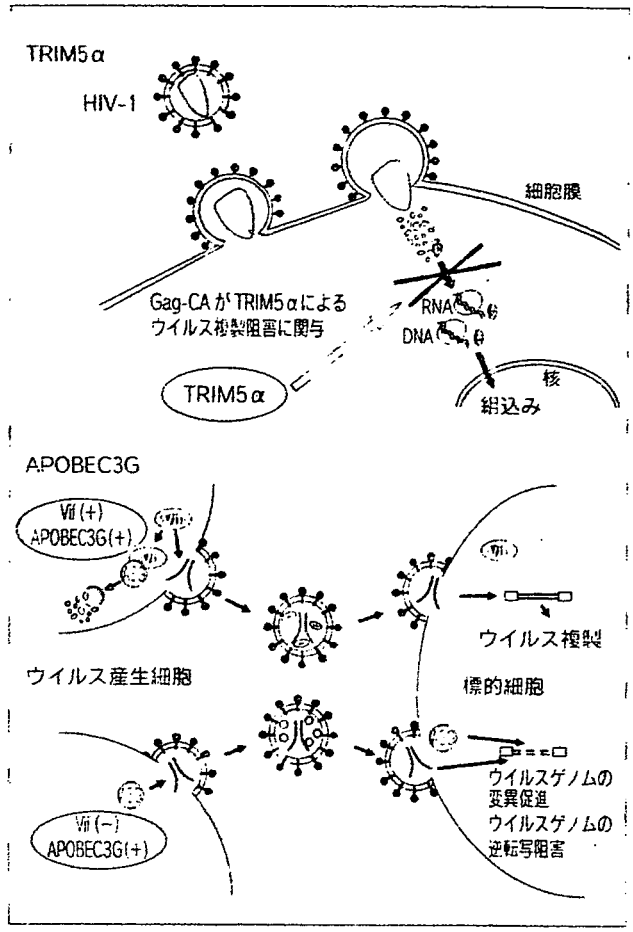


図4　抗 HIV-1 細胞因子 TRIM5α と APOBEC3G の作用機序
2つの代表的抗 HIV-1 細胞因子の作用機構を模式的に示す　HIV-1 はサル細胞に存在するこれらの抗ウイルス因子を中和できない．

表2　SIVmac239 の病原性

変異体	ウイルス増殖		エイズ発症
	培養細胞内	個体内	
SIVmac239(wt)	1.0	1.0	+
SIVmac239ΔVpx	0.01>	0.1	+
SIVmac239ΔVpr	~1.0	0.5	+
SIVmac239ΔVif	0.01>	0.00005	−
SIVmac239ΔNef	~1.0	0.01	−
SIVmac239ΔVpxΔVpr	0.05	0.01	−

おわりに

HIV-1 の病原性とアクセサリー遺伝子というタイトルで本稿をまとめたが，確定的なことはいえないというのが結論である．エイズはウイルス病であるから，当然，感染個体内で一定程度のウイルス増殖は必要である．アクセサリー遺伝子にコードされるアクセサリー蛋白質の機能・作用機序の理解は近年急速に進んだ．現在，アクセサリー蛋白質は宿主の免疫系を制御する Vif および Nef と，ウイルスの複製を最適化する Vpr および Vpu の2つのカテゴリーに分けられると考えるのが妥当ではないだ

ろうか．これを明確に証明するためには，HIV-1キメラウイルスとアカゲザルなどの霊長類を用いた動物実験が最も適当であると考える．この系が構築されれば，ヒトエイズ発症機構の解明にとどまらず，化学療法剤やワクチンの開発研究にも一石を投ずることになるであろう．

文　献

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Long-Term Control of Simian Immunodeficiency Virus Replication with Central Memory CD4$^+$ T-Cell Preservation after Nonsterile Protection by a Cytotoxic T-Lymphocyte-Based Vaccine$^\nabla$

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Induction of virus-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) responses is a promising strategy for AIDS vaccine development. However, it has remained unclear if or how long-term viral containment and disease control are attainable by CTL-based nonsterile protection. Here, we present three rhesus macaques that successfully maintained Env-independent vaccine-based control of simian immunodeficiency virus (SIV) mac239 replication without disease progression for more than 3 years. SIV-specific neutralizing antibody induction was inefficient in these controllers. Vaccine-induced Gag-specific CTLs were crucial for the chronic as well as the primary viral control in one of them, whereas those Gag-specific CTL responses became undetectable and CTLs specific for SIV antigens other than Gag, instead, became predominant in the chronic phase in the other two controllers. A transient CD8$^+$ cell depletion experiment 3 years postinfection resulted in transient reappearance of plasma viremia in these two animals, suggesting involvement of the SIV non-Gag-specific CTLs in the chronic SIV control. This sustained, neutralizing antibody-independent viral control was accompanied with preservation of central memory CD4$^+$ T cells in the chronic phase. Our results suggest that prophylactic CTL vaccine-based nonsterile protection can result in long-term viral containment by adapted CTL responses for AIDS prevention.

Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections induce acute, massive depletion of CCR5$^+$ CD4$^+$ effector memory T cells from mucosal effector sites. This is followed by chronic immune activation with gradual immune disruption leading to AIDS (7, 15, 20, 25, 26, 33, 34). Acute depletion has an impact on disease course but does not dictate everything that happens in the chronic phase (7, 26). It has also been suggested that persistent viral replication-associated chronic immune activation may be critical for AIDS progression.

Virus-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) responses are crucial for control of HIV and SIV replication (3, 8, 12, 18, 24, 29). Several vaccine regimens eliciting virus-specific CTL responses have been developed and evaluated in macaque AIDS models (6, 21). Some of them have shown protective efficacies leading to viremia control in a model of X4-tropic simian-human immunodeficiency virus (SHIV) infections (1, 16, 22, 23, 28, 31). However, assessment of the ability of vaccines to ameliorate disease progression requires analysis in macaque models of R5-tropic SIV infection (5). Although most CTL-based vaccine trials using rigorous SIV challenges in Indian rhesus macaques have failed, some of them have shown amelioration of acute memory CD4$^+$ T-cell depletion in the vaccinated animals with reduction in viral loads out to a year postinfection (4, 13, 19, 35). These findings have suggested that there may be a clinical benefit conferred by CTL-based AIDS vaccines. Unfortunately, it is still unclear as to how nonsterile protection conferred by prophylactic CTL-based vaccines can result in long-term viral containment and disease control.

We have previously developed a CTL-eliciting AIDS vaccine regimen using a DNA-prime/Gag-expressing Sendai virus (SeV-Gag) vector-boost (16, 32). Our regimen does not utilize Env immunogen that may induce neutralizing antibodies, although this antigen has been used in most of the vaccines except for a few cases (16, 31, 35). We have evaluated efficacy of this Env-independent vaccine against SIVmac239 challenge in Burmese rhesus macaques and found neutralizing antibody-independent, CTL-based control of primary SIV replication in five of eight vaccinees (17). In the present study, we have followed these macaques to examine if long-term viral containment without disease progression is possible by prophylactic CTL-based AIDS vaccines.

MATERIALS AND METHODS

Animal experiments. Twelve Burmese rhesus macaques (*Macaca mulatta*) used in our previous SIVmac239 challenge experiment (17) were followed in the

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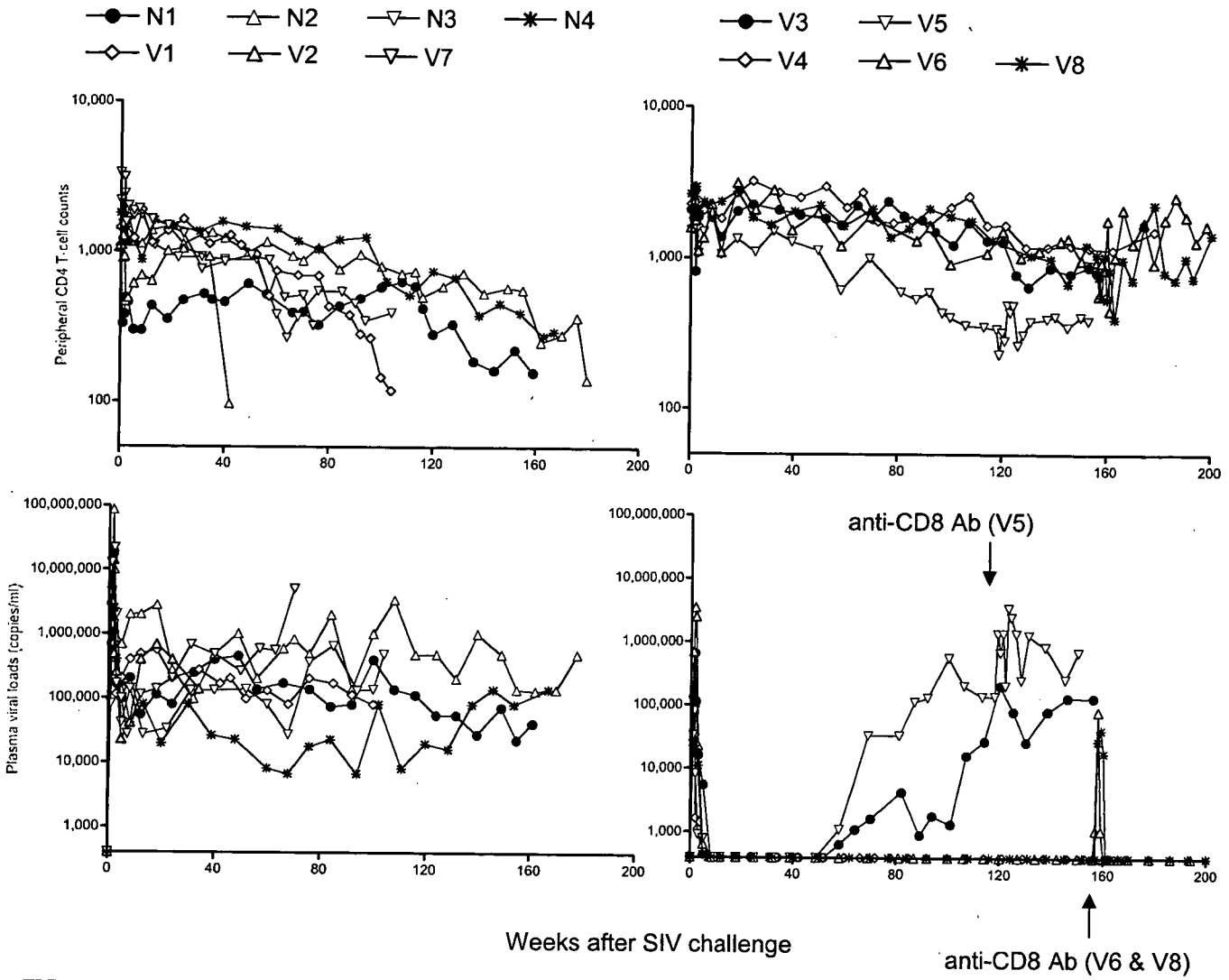

FIG. 1. Follow-up of the macaques after SIVmac239 challenge. Upper panels, peripheral $CD4^+$ T-cell counts (cells/μl); lower panels, plasma viral loads (viral RNA copies/ml plasma); left panels, the seven noncontrollers; right panels, the five controllers. All seven noncontrollers developed AIDS and were euthanized during the observation period (Table 1). Macaques V5, V6, and V8 received anti-CD8 antibody treatment starting from week 118, week 156, and week 156, respectively.

present study. These macaques were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases and the National Institute of Biomedical Innovation. Four of them were naive, whereas the other eight macaques received a DNA vaccine followed by a single boost with SeV-Gag before an intravenous SIVmac239 challenge. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA (30) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-$HIV\text{-}1_{DH12}$ chimeric Vpr, and $HIV\text{-}1_{DH12}$ Tat and Rev as described previously (17). At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally received a single boost with 1×10^8 cell infectious units of replication-competent SeV-Gag (V1, V2, V3, and V4) or 6×10^9 cell infectious units of F-deleted replication-defective F(-)SeV-Gag (9, 14, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (11).

For $CD8^+$ cell depletion, animals received a single intramuscular inoculation of 10 mg/kg of body weight of monoclonal anti-CD8 antibody (cM-T807) provided by Centocor (Malvern, PA) followed by three intravenous inoculations of 5 mg/kg cM-T807 on days 3, 7, and 10 after the first inoculation. The anti-CD8 antibody administration started at week 118 in macaque V5 and at week 156 in macaques V6 and V8. $CD8^+$ T-cell depletion in peripheral blood was confirmed by immunostaining using fluorescein isothiocyanate-conjugated anti-human CD8 antibody (DK25; Dako, Kyoto, Japan).

All the noncontrollers were euthanized when they showed typical signs of AIDS, such as reduction in peripheral $CD4^+$ T-cell counts, loss of body weight, diarrhea, and general weakness. Autopsy revealed lymphoatrophy or post-persistent generalized lymphadenopathy conditions consistent with AIDS.

Quantitation of plasma viral loads. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Serial fivefold dilutions of RNA samples were amplified in quadruplicate by reverse transcription and nested PCR using SIV *gag*-specific primers to determine the endpoint. Plasma SIV RNA levels were calculated according to the Reed-Muench method as described previously (17). The lower limit of detection is approximately 4×10^2 copies/ml.

Measurement of virus-specific neutralizing titers. Serial twofold dilutions of heat-inactivated plasma were prepared in duplicate and mixed with 10 $TCID_{50}$ of SIVmac239. In each mixture, 5 μl of diluted plasma was incubated with 5 μl of virus. After a 45-min incubation at room temperature, each 10-μl mixture was added to 5×10^4 MT4 cells in a well of a 96-well plate. After 12 days of culture, supernatants were harvested. Progeny virus production in the supernatants was examined by enzyme-linked immunosorbent assay for detection of SIV p27 core antigen (Beckman-Coulter, Tokyo, Japan) to determine the 100% neutralizing endpoint. The lower limit of detection is a titer of 1:2.

TABLE 1. Summary of responses in macaques challenged with SIVmac239

Macaque group and no.	MHC-I haplotype[a]	VL		Status[c]	CD4 count[d] at euthanasia	Opportunistic infection at autopsy[e]
		Set point[b]	After wk 60			
Unvaccinated noncontrollers						
N1	*90-088-Ij*	$>10^4$	$>10^4$	Euthanized at wk 161	158	
N2	*90-120-Ia*	$>10^4$	$>10^4$	Euthanized at wk 180	141	PCP
N3	*90-122-Ie*	$>10^4$	$>10^4$	Euthanized at wk 104	393	
N4	*90-010-Id*	$>10^4$	$>10^4$	Euthanized at wk 167	296	CMV
Vaccinated noncontrollers						
V1	*90-088-Ij*	$>10^4$	$>10^4$	Euthanized at wk 105	119	
V2	*90-120-Ib*	$>10^4$		Euthanized at wk 42	97	PCP
V7	*90-122-Ie*	$>10^4$	$>10^4$	Euthanized at wk 77	323	
Vaccinated transient controllers						
V3	*90-120-Ia*	<400	$>10^3$	Alive >3 yr		
V5	*90-120-Ia*	<400	$>10^4$	Euthanized at wk 154*	384	
Vaccinated sustained controllers						
V4	*90-120-Ia*	<400	<400	Alive >3 yr		
V6	*90-122-Ie*	<400	<400	Alive >3 yr*		
V8	*90-010-Id*	<400	<400	Alive >3 yr*		

[a] MHC-I haplotype was determined by reference strand-mediated conformation analysis as described previously (2, 17). MHC class I haplotypes *90-120-Ia* and *90-120-Ib* are derived from breeder R-90-120, *90-122-Ie* is from R-90-122, *90-010-Id* is from R-90-010, and *90-088-Ij* is from R-90-088.
[b] Plasma viral load (VL, in RNA copies/ml plasma) around week 12.
[c] All seven noncontrollers exhibited reduction in peripheral CD4 T-cell count, loss of body weight, and general weakness and were euthanized and subjected to autopsy to be confirmed as AIDS. Macaques V5, V6, and V8 (indicated by asterisks) were administered an anti-CD8 antibody for CD8 cell depletion at weeks 118, 156, and 156, respectively.
[d] Peripheral CD4 T-cell counts.
[e] PCP, pneumocystis pneumonia; CMV, cytomegalovirus infection.

Measurement of virus-specific CTL responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (17). In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a VSV-G expression plasmid and the SIVGP1 DNA, an *env*- and *nef*-deleted SHIV molecular clone DNA. Intracellular IFN-γ staining was performed using a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting nonspecific $IFN\text{-}\gamma^+$ T-cell frequencies from those after Gag-specific or SIV-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs are considered negative.

Immunostaining of $CD4^+$ T-cell memory subsets. Frozen stocks of PBMCs were thawed and subjected to immunofluorescent staining by using fluorescein isothiocyanate-conjugated anti-human CD28, phycoerythrin-conjugated anti-human CD95, peridinin chlorophyll-conjugated anti-human CD4, and allophycocyanin-conjugated anti-human CD3 monoclonal antibodies (Becton Dickinson). Memory and central memory subsets of $CD4^+$ T cells were delineated by $CD95^+$ and $CD28^+$ $CD95^+$ phenotypes, respectively, as described previously (27).

Statistical analysis. Central memory $CD4^+$ T-cell counts just before SIV challenge (at week zero) were not significantly different between the noncontrollers ($n = 7$) and the controllers ($n = 5$) by unpaired t test. We calculated ratios of the counts at week 12 to week 0, week 70 to week 0, and week 70 to week 12 in each animal and performed an unpaired t test and nonparametric Mann-Whitney U-test between the noncontrollers and the controllers by using Prism software version 4.03 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Long-term viral containment without disease progression in the sustained controllers. We followed up on our vaccinated Burmese rhesus macaques used in the previous trial (17). These macaques were vaccinated using a DNA prime–SeV-Gag boost, and they were challenged with SIVmac239. Five of eight vaccinees controlled viral replication and had undetectable plasma viremia at week 8 postchallenge. The remaining three vaccinees (V1, V2, and V7) and all four unvaccinated macaques (N1, N2, N3, and N4) failed to control viral replication. Of the five controllers, two macaques V3 and V5 (referred to as transient controllers) exhibited viremia reappearance around week 60, but the other three, V4, V6, and V8 (referred to as sustained controllers), maintained viral control (10).

In the present follow-up study, all seven noncontrollers, including three vaccinees and four unvaccinated controls, exhibited persistent viremia and a gradual decline in peripheral $CD4^+$ T-cell counts (Fig. 1). All of them finally developed AIDS and were euthanized at week 42 to 180 postchallenge (Table 1), confirming that failure in control of SIVmac239 replication results in AIDS progression even in Burmese rhesus macaques. In contrast, all three sustained controllers maintained viral control and preserved peripheral $CD4^+$ T cells without disease progression for more than 3 years (Fig. 1).

We then examined SIVmac239-specific neutralizing antibody responses by determining the end point plasma titers for killing 10-$TCID_{50}$ virus replication on MT4 cells (Fig. 2). Our vaccine regimens did not utilize Env as an immunogen, and no neutralizing antibody responses were induced before challenge in any of the vaccinees. Even after challenge, none of the SIVmac239-challenged macaques showed detectable neutralizing antibody responses until 6 months. After that, neutralizing antibody responses became detectable in some of the noncontrollers. In contrast, no or little neutralizing antibody