

# Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus

Tatsuhiko Igarashi\*, Yasuyuki Endo\*, George Englund\*, Reza Sadjadpour\*, Tetsuro Matano\*, Charles Buckler\*, Alicia Buckler-White†, Ron Plishka†, Ted Theodore\*, Riri Shibata\*, and Malcolm Martin\*\*

\*Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and

†Division of Molecular Virology and Immunology, Georgetown University Medical Center, Washington, DC 20057

Contributed by Malcolm Martin, September 24, 1999

Although simian/human immunodeficiency virus (SHIV) strain DH12 replicates to high titers and causes immunodeficiency in pig-tailed macaques, virus loads measured in SHIV<sub>DH12</sub>-infected rhesus monkeys are consistently 100-fold lower and none of 22 inoculated animals have developed disease. We previously reported that the administration of anti-human CD8 mAb to rhesus macaques at the time of primary SHIV<sub>DH12</sub> infection resulted in marked elevations of virus loads. One of the treated animals experienced rapid and profound depletions of circulating CD4<sup>+</sup> T lymphocytes. Although the CD4<sup>+</sup> T cell number partially recovered, this monkey subsequently suffered significant weight loss and was euthanized. A tissue culture virus stock derived from this animal, designated SHIV<sub>DH12R</sub>, induced marked and rapid CD4<sup>+</sup> cell loss after i.v. inoculation of rhesus monkeys. Retrospective analyses of clinical specimens, collected during the emergence of SHIV<sub>DH12R</sub> indicated: (i) the input cloned SHIV remained the predominant virus during the first 5–7 months of infection; (ii) variants bearing only a few of the SHIV<sub>DH12R</sub> consensus changes first appeared 7 months after the administration of anti-CD8 mAb; (iii) high titers of neutralizing antibody directed against the input SHIV were detected by week 10 and persisted throughout the infection; and (iv) no neutralizing antibody against SHIV<sub>DH12R</sub> ever developed.

Because HIV-1 establishes infections only in humans and chimpanzees and induces disease only in humans, chimeric simian/human immunodeficiency viruses (SHIVs) have been constructed to examine the pathogenicity and develop vaccines pertaining to HIV-1-encoded proteins in the more tractable Asian macaque model system. We have reported that SHIV<sub>DH12</sub>, previously designated SHIV<sub>MD14YE</sub>, replicates to high titers, induces p27 antigenemia, and induces immunodeficiency in pig-tailed macaques (1). However, in SHIV<sub>DH12</sub>-infected rhesus monkeys, virus loads are consistently 100-fold lower, plasma p27 is undetectable, and none of the inoculated animals have ever developed CD4<sup>+</sup> T lymphocyte depletion or signs of disease (2).

A large body of evidence now indicates that virus-specific cytotoxic T lymphocytes (CTL) play a major role in resolving acute primate lentiviral infections. In HIV-1 infections of humans, a CTL response, demonstrable before seroconversion, is temporally linked to declining virus loads (3–5). In this regard, we previously reported that the administration of the anti-human CD8 mAb T87PT3F9 at the time of primary infection of rhesus macaques with SHIV<sub>DH12</sub> led to markedly elevated (50- to 100-fold) plasma and cell-associated viral loads compared with untreated animals; p27 antigenemia also became detectable for the first time in the CD8 mAb-treated SHIV<sub>DH12</sub>-infected rhesus monkeys (2). The antibody used in that study induced only partial and transient depletions of CD8<sup>+</sup> T lymphocytes in rhesus recipients, and the responses observed varied from animal to animal. A recent study, using considerably higher doses of a humanized mouse anti-human CD8 mAb, reported more com-

plete ablation of CD8<sup>+</sup> T cells in naive rhesus monkeys that lasted for 17–60 days (6). Nonetheless, the two monkeys receiving the T87PT3F9 anti-CD8 mAb 3 days before and 4 days after i.v. SHIV<sub>DH12</sub> inoculation both experienced severe CD4<sup>+</sup> T lymphocyte declines that lasted several weeks (2).

Although the anti-CD8<sub>mAb</sub>-treated animal (565Z) with the highest virus loads eventually resolved its primary SHIV<sub>DH12</sub> infection, it experienced a second wave of viremia and CD4<sup>+</sup> T cell depletion, became symptomatic, and subsequently was euthanized. The SHIV recovered from macaque 565Z caused rapid CD4<sup>+</sup> cell loss after its i.v. inoculation into two naive rhesus monkeys and was used to prepare a tissue culture-derived virus stock with similar properties. The availability of cells and plasma collected from macaque 565Z during the course of its infection permitted a retrospective immunologic and genetic analysis of a SHIV as it evolved from a nonpathogenic to an acutely pathogenic virus during a single passage in a rhesus monkey.

## Methods

**Virus.** The structure of SHIV<sub>DH12</sub> and the preparation of virus stocks in macaque peripheral blood mononuclear cells (PBMCs) for animal inoculation has been described (1). SHIV<sub>DH12</sub> also was prepared in MT4 cells for immunoblotting.

The SHIV<sub>DH12R</sub> stock was prepared by combining  $2 \times 10^6$  lymph node cells,  $7 \times 10^6$  splenocytes plus thymocytes, and  $9 \times 10^5$  PBMCs, collected from rhesus macaque 25112 at the time of its necropsy, with  $1.3 \times 10^6$  PBMCs from rhesus monkey RQ974 (obtained 3 weeks postinfection), resuspending the mixture in RPMI 1640 medium, supplemented with 20% of heat-inactivated FBS (HyClone) in a total volume of 10 ml, and incubating the suspension for 20 hr at 37°C. The cell mixture then was cocultivated with PBMCs from two uninfected rhesus macaques ( $40.5 \times 10^6$  and  $61.5 \times 10^6$  cells, respectively in 20 ml), previously stimulated with 25 µg/ml of Con A (Amersham Pharmacia Biotech) for 20 hr. Medium was collected daily from the coculture supernatant and samples containing the highest reverse transcriptase activities (days 5–7) were pooled, filtered through a 0.45-µm membrane, aliquoted, and stored at –70°C until use.

**Animal Experiments.** Rhesus macaques (*Macaca mulatta*) were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (7) and were housed in a biosafety level 2

Abbreviations: SIV, simian immunodeficiency virus; SHIV, simian/human immunodeficiency virus; PBMC, peripheral blood mononuclear cell.

\*To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

facility; biosafety level 3 practices were followed. Animals were anesthetized with i.m. injections of Tiletamine-HCl and Zolazepam-HCl (Telazol; Fort Dodge Laboratories, Fort Dodge, IA) during phlebotomies or SHIV inoculations. Blood samples were collected with ACD-A solution or EDTA as the anticoagulant. PBMCs or plasma samples were prepared from ACD-A anticoagulated blood and stored in liquid nitrogen or  $-70^{\circ}\text{C}$ , respectively, until use. Lymphocyte immunophenotyping and complete blood counts were performed by using EDTA anticoagulated blood samples.

The SHIV<sub>DH12</sub>-infected rhesus monkey 565Z developed immunodeficiency and was euthanized 68 weeks postinfection. At the time of its necropsy, a suspension of splenocytes ( $1.4 \times 10^6$  cells) and lymph node cells ( $7.5 \times 10^5$  cells) was combined with 10 ml of its own anticoagulated blood and 6 ml of bone marrow, and the mixture was inoculated i.v. into two rhesus monkeys (macaques RQ974 and 25112). Rhesus macaques H27 and 5981 were inoculated i.v. with  $4.1 \times 10^5$  TCID<sub>50</sub> and 656 TCID<sub>50</sub> of the SHIV<sub>DH12R</sub> tissue culture-derived stock, respectively.

**Virus Load Measurements.** Plasma viral RNA levels were determined by real-time PCR (ABI Prism 7700 Sequence Detection System, Perkin-Elmer) using reverse-transcribed viral RNA from macaque plasma samples as templates. Plasma viral RNA was extracted by using a QIAamp Viral RNA Kit (Qiagen, Valencia, CA), and reverse-transcribed with MultiScribe reverse transcriptase (TaqMan Reverse Transcription Reagents Kit, Perkin-Elmer/Roche). The cDNA was amplified (45 cycles/default settings) with Ampli Taq Gold DNA polymerase (PCR Core Reagents Kit, Perkin-Elmer/Roche) by using a pair of primers derived from the *vpr* gene (forward, nucleotides 6220–6240, and reverse, nucleotides 6358–6338) of SHIV<sub>DH12</sub>. SHIV<sub>DH12R</sub>-infected animal plasma and SHIV<sub>DH12</sub>-infected rhesus PBMC culture supernatants, which previously were prequantified by the branched DNA method (8), served as standards for the assay.

p27 antigenemia was measured by an SIV core antigen assay (Coulter), using a murine mAb to capture the p27 SIV Gag protein in plasma prepared from EDTA anticoagulated blood samples.

**Long env Gene PCR.** Five-microliter samples of Hirt fractionated (9) DNA (approximately  $10^5$  cell equivalents), prepared from previously frozen PBMC samples [cultured, after thawing, for 18 hr in RPMI 1640 containing 4 mM glutamine, penicillin (25 units/ml), streptomycin (25  $\mu\text{g}/\text{ml}$ ), and 10% FBS at a density of  $10^6$  cells/ml], were used as templates for PCR amplification of a 3,048-bp region (map positions 6144–9171) of SHIV<sub>DH12</sub> proviral DNA, which encompassed the entire HIV-1<sub>DH12</sub> Env coding region. Amplifications were performed by using the Expand Long Template PCR system (Boehringer Mannheim #1681834). Reaction mixtures consisted of 5  $\mu\text{l}$  Hirt DNA, 500  $\mu\text{M}$  each dNTP, PCR buffer #2 (containing 2.25  $\mu\text{M}$  MgCl<sub>2</sub>), 0.3  $\mu\text{M}$  each primer [6144(+), 5'-CAGTAGATCCTAGACTAGAGC-3'; 9190(-), 5'-CCACTCTCCTAAGAGTCTC-3'], and 0.75  $\mu\text{l}$  of the enzyme mixture in a final volume of 50  $\mu\text{l}$  overlaid with 30  $\mu\text{l}$  mineral oil. In cases where more than 30 cycles of amplification were required, a second round of 30 cycles of amplification was performed by using nested primers [6317(+), 5'-CACAGTCAGACTGATCAAGC-3'; 9137(-), 5'-CTGTGCGAGATCTCCAGAC-3'] under the same reaction conditions. Amplifications were performed as follows: one cycle of denaturation ( $94^{\circ}\text{C}$ , 2 min); 10 cycles of amplification ( $94^{\circ}\text{C}$ , 20 s/ $63^{\circ}\text{C}$ , 30 s/ $68^{\circ}\text{C}$ , 4 min); an additional 20 cycles of amplification and extension ( $94^{\circ}\text{C}$ , 20 s/ $63^{\circ}\text{C}$ , 30 s/ $68^{\circ}\text{C}$ , 4 min + 20 s per cycle); a final extension at  $68^{\circ}\text{C}$  for 7 min. The PCR products were gel-purified on 0.6% agarose gels, and then cloned directly

into pCR2.1 (Promega Biotec #K2030–01) for sequence analysis of individual molecular clones.

**Lymphocyte Immunophenotyping.** EDTA-treated blood samples were stained with fluorochrome-conjugated mAbs [CD3-FITC (Serotec); CD4-allophycocyanin, CD8-peridinin chlorophyll protein, and CD20-phycoerythrin, (Becton Dickinson Immunocytometry Systems)] and analyzed by flow cytometry (FACSort, Becton Dickinson) as described (1).

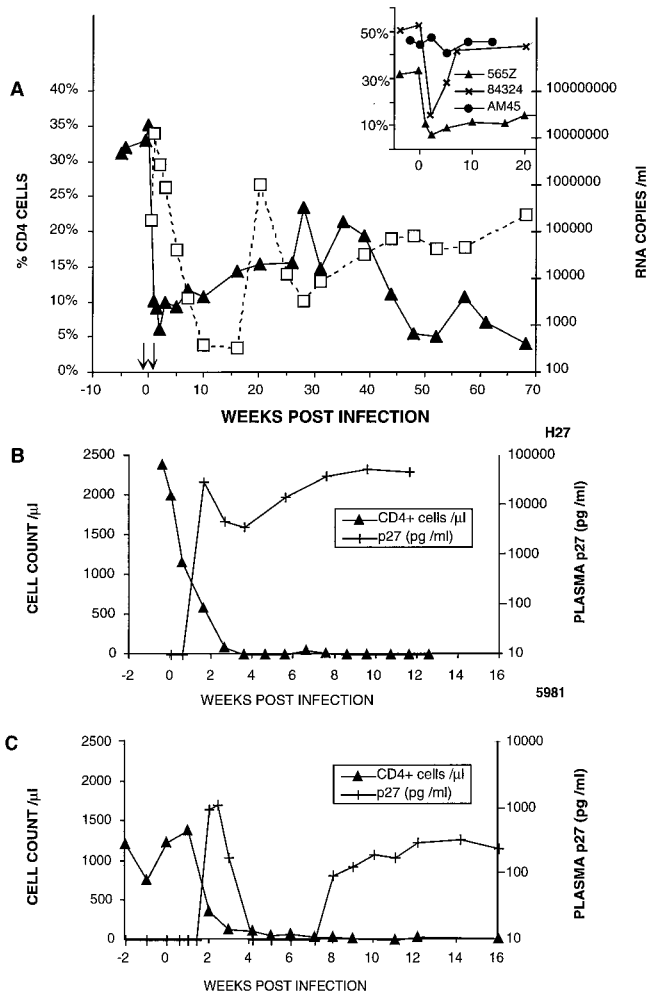
**Antibody Assays.** Limiting dilution, end-point neutralization assays (100% neutralization against 100 TCID<sub>50</sub> of SHIV<sub>DH12</sub> or SHIV<sub>DH12R</sub>) were performed as described (10). Serial 3-fold dilutions of plasma were incubated with virus, and the mixture then was used as inoculum for quadruplicate, 2-week infections of MT-4 cells. Non-neutralized culture supernatants appear as black dots on the autoradiograms of these <sup>32</sup>P reverse transcriptase (RT) assays, reflecting the presence of particle-associated RT activity in the medium. The neutralization titers were calculated by the method of Reed and Muench (11). For immunoblotting, SHIV<sub>DH12</sub> particles were concentrated by ultracentrifugation ( $100,000 \times g$  for 80 min by using an SW28 rotor in an L8–70M Ultracentrifuge, Beckman Instruments, Fullerton, CA). The pellet was resuspended in PBS and subjected to ultracentrifugation through 20% sucrose in PBS ( $200,000 \times g$  for 75 min by using an SW55 rotor in a L8–70M Ultracentrifuge, Beckman Instruments). The concentrated SHIV particles then were mixed with purified HIV-1<sub>DH12</sub> gp120, expressed from a recombinant vaccinia virus as reported (12). After incubation with SDS/PAGE sample buffer (13), the viral proteins were resolved by SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore), and incubated with serially collected plasma samples (diluted 1:100) that had been preadsorbed with sonicated lysates of *Escherichia coli* and MT4 cells, followed by incubation with sheep anti-human Ig conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Protein bands were visualized on x-ray film (Kodak Biomax MR) after incubation with chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce).

## Results and Discussion

We previously reported that the two rhesus monkeys (565Z and 84324) receiving the T87PT3F9 anti-CD8 mAb before ( $-3$  days) and immediately after ( $+4$  days) inoculation with SHIV<sub>DH12</sub> suffered significant CD4<sup>+</sup> T cell losses of several weeks duration (Fig. 1A, *Inset*). In both of these animals, the mAb-induced CD8<sup>+</sup> T cell decline (from 1,375 to 217 cells/ $\mu\text{l}$  in macaque 565Z; from 795 to 407 cells/ml in macaque 84324) was inversely related to the peak levels of plasma viremia ( $12 \times 10^6$  copies/ml in 565Z and  $0.56 \times 10^6$  copies/ml for 84324). These virus loads were 10- to 20-fold higher than macaques not receiving the anti-CD8 mAb.

In monkey 565Z, the CD4<sup>+</sup> cell number in the peripheral blood fell to 92 cells/ml 5 weeks postinfection and, as reported (2), the % CD4<sup>+</sup> T lymphocytes in lymph node biopsies declined from 49%, before infection, to 12% thereafter. The circulating CD4<sup>+</sup> cell levels in animal 565Z remained low for 2–3 months before gradually rising to approximately 60% of the preinfection level at week 35 (Fig. 1A). This partial recovery of CD4<sup>+</sup> cells accompanied the resolution of the initial plasma viremia, which fell to approximately 300 RNA copies/ $\mu\text{l}$  at week 16. However, after week 30, the virus load again increased and was accompanied by a second CD4<sup>+</sup> T lymphocyte decline, which reached 44 cells/ $\mu\text{l}$  at week 48. Monkey 565Z suffered significant weight loss by week 66, became moribund on week 68, and was euthanized at that time.

To determine whether a pathogenic variant of SHIV<sub>DH12</sub> had emerged in rhesus 565Z, a cell suspension (lymph nodes, spleen,



**Fig. 1.** (A) The dynamics of viral RNA levels and CD4<sup>+</sup> T lymphocyte counts during the SHIV<sub>DH12</sub> infection of rhesus macaque 565Z. The arrows indicate the times when anti-human CD8 mAb was administered. (B and C) SHIV<sub>DH12R</sub> induces p27 antigenemia and causes rapid CD4<sup>+</sup> T cell depletion in rhesus macaques. Blood collected at the indicated times from two infected rhesus macaques were fractionated into plasma and cells and levels of p27 and CD4<sup>+</sup> T lymphocytes, respectively, were determined as described in *Methods*.

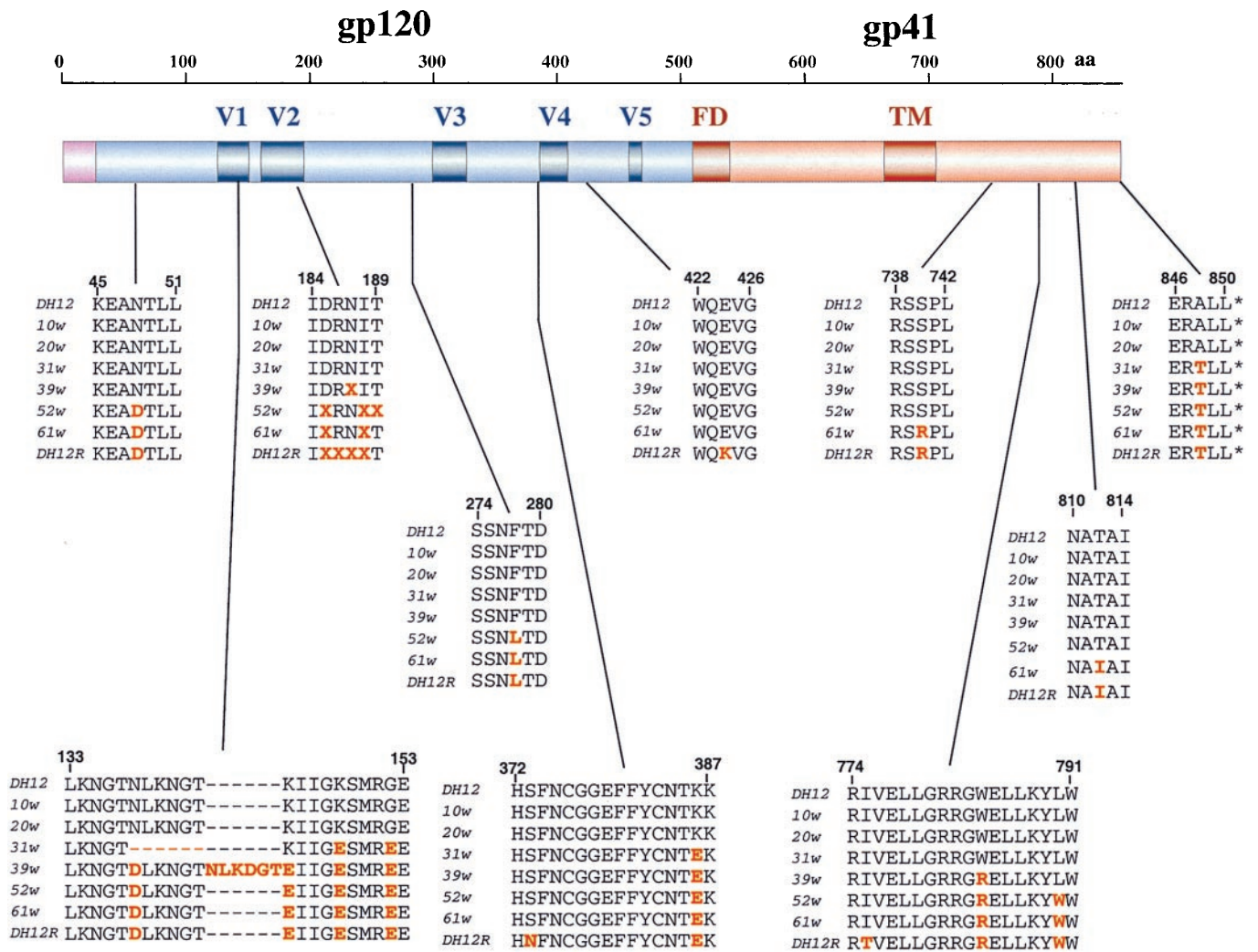
and bone marrow), prepared from tissues collected at the time of its necropsy, was mixed with anticoagulated whole blood and inoculated into two naïve rhesus monkeys (RQ974 and 25112). Both animals experienced marked and rapid CD4<sup>+</sup> T cell declines by day 10 postinfection (to 200 and 72 cells/μl, respectively), indicating that during a single passage in macaque 565Z, SHIV<sub>DH12</sub> had evolved into a highly pathogenic virus. Because we wanted to obtain a tissue culture-derived stock of a potentially highly pathogenic SHIV, a suspension of splenocytes, thymocytes, lymph node cells and PBMCs from macaque 25112 was combined with PBMCs from animal RQ974 and cocultivated with a 10-fold excess of activated PBMCs from naive rhesus monkeys. The resulting culture supernatant, designated SHIV<sub>DH12R</sub> ( $4.1 \times 10^5$  TCID<sub>50</sub>/ml), exhibited comparable *in vitro* infection kinetics in rhesus and human PBMCs as its SHIV<sub>DH12</sub> parent and directed the production of similar amounts of infectious virus progeny (approximately  $1$  to  $4 \times 10^5$  TCID<sub>50</sub>/ml). SHIV<sub>DH12R</sub>, however, induced more extensive cell killing and generated more numerous and extremely large syncytia in both PBMC types. SHIV<sub>DH12R</sub> also replicated to high levels in the continuous human MT4 T cell line.

Intravenous inoculation of two rhesus macaques (H27 and 5981) with  $4.1 \times 10^5$  TCID<sub>50</sub> or 650 TCID<sub>50</sub> of the SHIV<sub>DH12R</sub> tissue culture-derived stock, respectively, resulted in rapid and irreversible CD4<sup>+</sup> T cell depletion and high virus loads in both animals (Fig. 1 B and C). Animal H27 subsequently suffered marked weight loss (>30%), experienced respiratory distress, and was euthanized 12.6 weeks postvirus inoculation. An autopsy revealed multifocal interstitial pulmonary fibrosis associated with giant multinucleated cells. Rhesus monkey 5981 developed leukocytosis (>15,000 cells/μl) at week 9, chronic diarrhea, and weight loss and was euthanized at week 23 with a CD4<sup>+</sup> cell count of 2 cells/ml. A titration ( $4.1 \times 10^5$  TCID<sub>50</sub> to 1 TCID<sub>50</sub>) of SHIV<sub>DH12R</sub> in rhesus monkeys by the i.v. route consistently induced rapid and irreversible depletion of CD4<sup>+</sup> T lymphocytes in all of the inoculated animals (Y.E., T.I., and M.M., unpublished work). Several macaques, which received 25 TCID<sub>50</sub> or less, remain alive 1 year after SHIV<sub>DH12R</sub> infection with CD4<sup>+</sup> T cell levels in the 100 to 200 cells/μl range.

The unexpected emergence of the disease-inducing SHIV<sub>DH12R</sub> during a single *in vivo* passage of a molecularly cloned virus, rather than after multiple animal-to-animal transfers of whole blood/bone marrow as reported by others (14, 15), provided the opportunity to retrospectively follow changes introduced into the SHIV genome and to monitor the immunologic responses to both the input SHIV<sub>DH12</sub> and the emergent SHIV<sub>DH12R</sub> attending the acquisition of the pathogenic phenotype.

Alterations of HIV-1 *env* gene sequences have been previously reported to be associated with the emergence of highly pathogenic SHIVs (16, 17). Therefore, a 3,046-bp segment, which extended from the nine 3' terminal nt *vpr* to the first 36 codons of *nef*, and encompassing the entire HIV-1 Env coding region, was PCR-amplified from Hirt-fractionated DNA, prepared from the same rhesus PBMCs used to generate the SHIV<sub>DH12R</sub> stock. Nucleotide sequence analyses of 22 independent PCR clones revealed that 13 aa in gp120 and 6 aa in gp41 of SHIV<sub>DH12R</sub> had changed compared with analogous residues in the input, molecularly cloned SHIV<sub>DH12</sub> parent. These altered amino acids are indicated in the bottom row (*DH12R*) of each sequence group shown in Fig. 2. A majority of the altered gp120 amino acids mapped to the V<sub>1</sub> and V<sub>2</sub> regions whereas the gp41 changes clustered in the cytoplasmic tail. The changes in the V<sub>2</sub> region of the SHIV<sub>DH12R</sub> gp120 were quite heterogeneous, consisting of two or more amino acid substitutions and/or deletions at the indicated positions (denoted by X in Fig. 2). Some of the gp120 alterations also resulted in a loss of an N-linked glycosylation site mapping to V<sub>2</sub>. No significant change was observed in the V<sub>3</sub> region, consistent with preliminary experiments (A. Trkola and J. P. Moore, personal communication) indicating that SHIV<sub>DH12R</sub> primarily uses CCR5 and CXCR4 and is therefore indistinguishable from its dual-tropic SHIV<sub>DH12</sub> parent (18).

Analyses of independent PCR clones, generated from amplified PBMC-associated DNA collected and frozen at different times during the 68-week infection of animal 565Z, also were carried out to ascertain when and how SHIV<sub>DH12R</sub> arose. These 3-kb PCR clones were collected at weeks 10, 20, 31, 39, 52, and 61 postinfection. The deduced amino acid sequence of the SHIV Env region at these six times during the infection of rhesus monkey 565Z is indicated for each sequence grouping shown in Fig. 2. The sequence indicated at each sampling time represents the residues present in the majority of independent PCR clones analyzed. The *env* gene segments from weeks 10 and 20 were both indistinguishable from the starting SHIV<sub>DH12</sub> *env* region, harboring none of the SHIV<sub>DH12R</sub> "signature" changes. In contrast, the week 31 and 39 samples contained several residues not present in the original SHIV<sub>DH12</sub> Env glycoprotein. The V<sub>1</sub> sequence underwent drastic changes during this period, including a 6-aa in-frame deletion present at week 31. By week 39,

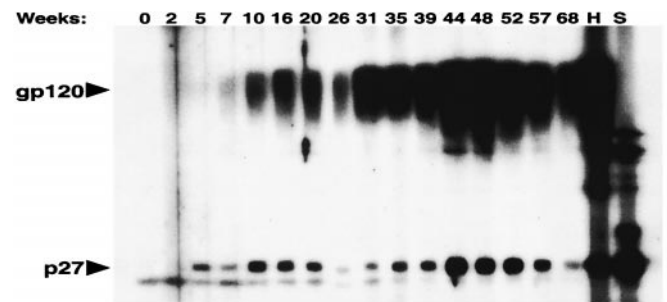


**Fig. 2.** Highly pathogenic SHIV<sub>DH12R</sub> evolves in a stepwise manner during the course of infection in rhesus macaque 565Z. The deduced amino acid sequence for the cloned SHIV<sub>DH12</sub> (DH12) and the consensus sequence from 22 independent SHIV<sub>DH12R</sub> (DH12R) PCR clones are indicated in the top and bottom lines of each grouping. Independent PCR clones also were derived from PBMC samples collected at weeks 10 (9 clones), 20 (5 clones), 31 (6 clones), 39 (14 clones), 52 (8 clones), and 61 (6 clones) postinfection as described in *Methods* and their nucleotide sequence was determined. The locations of amino acid changes in gp120 and gp41 at various times during the evolution of SHIV<sub>DH12</sub> to SHIV<sub>DH12R</sub> are indicated in red. The positions of the fusion domain (FD) and transmembrane (TM) regions of gp41 are shown.

however, the V<sub>1</sub> hexapeptide deletion had disappeared and was replaced with a 6-aa insertion, which included the nearly perfect triplication of the pentapeptide motif LKNGT, present, but unrecognized, in the original SHIV<sub>DH12</sub> gp120. Week 39 also marked the time when V<sub>2</sub> began to undergo extensive remodeling. Additional amino acid changes accumulated with time until the animal's demise at week 68. These sequence analyses clearly indicate that SHIV<sub>DH12</sub>, not SHIV<sub>DH12R</sub>, was responsible for the CD4<sup>+</sup> T cell decline and the associated high levels of viremia monitored during the first 5 weeks of the acute infection. The second, irreversible fall of CD4 and associated long-lasting viremia probably was caused by an evolving virus "swarm" from which SHIV<sub>DH12R</sub> ultimately emerged.

The kinetics of humoral immune responses during the evolution of SHIV<sub>DH12</sub> in rhesus monkey 565Z initially was examined by immunoblotting (Fig. 3). As expected, antibody levels rose in response to virus production *in vivo*. Antibody to the p27 SIV capsid protein (CA) component of SHIV<sub>DH12</sub> was first detected at week 5, and reactivity with gp120 was observed a few weeks later (Fig. 3). The initial peak of antibody production occurred between weeks 10 and 16 for anti-p27 and weeks 16 and 20 for anti-gp120 and subsequently fell over the ensuing 10 weeks. This

decline in p27 reactivity coincided with a gradual increase in the % CD4<sup>+</sup> T lymphocytes, which reached 23% on week 28 (Fig.



**Fig. 3.** Antiviral antibody responses during the course of the SHIV<sub>DH12</sub> infection of rhesus monkey 565Z. Samples of blood, collected at the indicated times, were analyzed by immunoblotting as described in *Methods*. Lanes H and S, purified IgG (0.45 μg) from a chimpanzee infected with HIV-1<sub>DH12</sub> (26) or plasma (1:200) from an SIV<sub>sm</sub>-infected rhesus macaque, respectively, were used as positive controls.

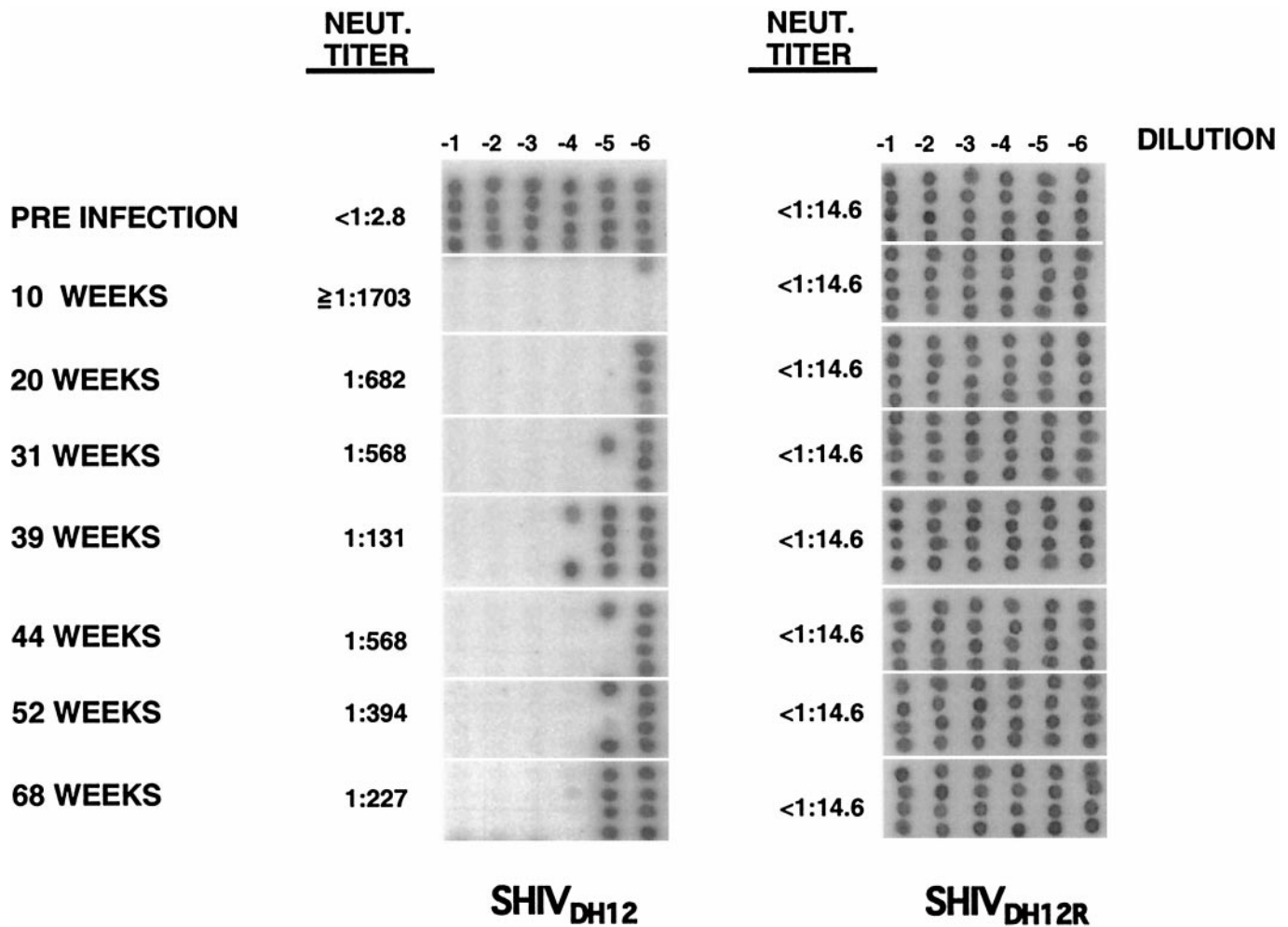


Fig. 4. Neutralizing antibody responses during the course of the SHIV<sub>DH12</sub> infection of rhesus monkey 565Z. Plasma samples prepared at the indicated times were subjected to neutralization assay as described (10).

1A). However, virus loads and the levels of both antibodies gradually increased a second time, peaking between weeks 44 and 48 postinfection. This rise in plasma viremia was accompanied by a second, unremitting loss of CD4<sup>+</sup> T cells and declining levels of both antibodies despite continuous virus production.

Neutralizing antibodies, directed against SHIV<sub>DH12</sub> or SHIV<sub>DH12R</sub>, also were examined during the course of the macaque 565Z infection, using an end-point assay that measures 100% neutralization (10, 19). High titers of antibodies, capable of neutralizing the input SHIV<sub>DH12</sub> but not SHIV<sub>DH12R</sub>, were evident 10 weeks postinfection (Fig. 4), coinciding with the emergence of binding antibody to gp120 (Fig. 3). This result indicates that the initial immune response, which peaked between weeks 10 and 20, was elicited by SHIV<sub>DH12</sub>, not SHIV<sub>DH12R</sub>. Neutralizing titers against SHIV<sub>DH12</sub> decreased somewhat after week 10 and persisted at a lower level throughout the remainder of the infection. No demonstrable neutralizing antibodies against SHIV<sub>DH12R</sub> were present in macaque 565Z at any time during the infection (Fig. 4).

Several important conclusions can be drawn from this study regarding the contribution of viral and host factors to the development of lentivirus-induced immunodeficiency. First and foremost, the pathogenic derivative SHIV<sub>DH12R</sub> emerged only in rhesus 565Z, whose CD8<sup>+</sup> T lymphocytes had been markedly depleted by mAb treatment at the time of primary viral infection. None of 16 other rhesus monkeys, inoculated with SHIV<sub>DH12</sub> but

not treated with the anti-CD8 mAb, or two other animals (2) that failed to generate high virus loads after mAb treatment, developed immunodeficiency. Our results therefore suggest the following scenario of events in animal 565Z: (i) Suppression of the initial cytotoxic T lymphocyte response, induced by the administration of the anti-CD8 mAb, interfered with the clearance of the input SHIV<sub>DH12</sub>. (ii) As a result, SHIV<sub>DH12</sub> underwent multiple cycles of replication and became widely disseminated in the infected animal during primary infection. (iii) The primary SHIV<sub>DH12</sub> infection finally was brought under control by week 12, although by that time, a pool of viral variants possibly emerged as a minor SHIV subpopulation. (iv) Variants bearing a few of the SHIV<sub>DH12R</sub> "signature" residues became predominant and could be detected by week 31, nearly 7 months after cessation of the anti-CD8 mAb treatment. (v) Stepwise evolution of the envelope sequences finally resulted in the appearance of the highly pathogenic SHIV<sub>DH12R</sub>. One might conclude, therefore, that the administration of the anti-CD8 mAb, if not the direct stimulus for the emergence of SHIV<sub>DH12R</sub>, was indirectly involved in its ultimate appearance.

Although the selective forces *in vivo* responsible for the emergence of the highly pathogenic SHIV<sub>DH12R</sub> are presently unknown, SHIV<sub>DH12R</sub> certainly can be classified as a neutralization-escape virus variant based on the assay shown in Fig. 4. Our recent study of the clearance of physical and infectious HIV particles *in vivo* demonstrated that neutralizing antibodies can

immediately inactivate autologous virus in plasma and greatly enhance its physical clearance from the blood (20). This result suggests that neutralizing antibodies are able to exert strong selective pressure, but may not, *per se*, be sufficient to generate a highly pathogenic SHIV. Other studies have reported that neutralization-resistant virus variants frequently arise during chronic SIV<sub>mac</sub>, SIV<sub>sm</sub>, and SHIV infections of Asian macaques (21–24). In the case of the HIV-1<sub>DH12</sub> isolate, the principal neutralization epitope has been mapped to the five noncontiguous variable regions (V<sub>1</sub>-V<sub>5</sub>) of gp120, using a series of HIV-1 constructs carrying chimeric gp120 molecules (Michael Cho, personal communication). Thus, the multiple changes that accumulated within the V<sub>1</sub> and V<sub>2</sub> regions of SHIV<sub>DH12R</sub> gp120 (Fig. 2) are very likely responsible for the loss of neutralization sensitivity.

Our inability to detect neutralizing antibody against SHIV<sub>DH12R</sub> at any time during the SHIV infection of macaque 565Z was not the result of the envelope of SHIV<sub>DH12R</sub> being less immunogenic. As noted above, several rhesus monkeys, inoculated with less than 100 TCID<sub>50</sub> of virus have suffered rapid and irreversible CD4<sup>+</sup> cell loss, yet have developed neutralizing antibody against SHIV<sub>DH12R</sub> (Y.E., T.I., and M.M., unpublished work). Thus the absence of neutralizing activity directed against SHIV<sub>DH12R</sub> in animal 565Z very likely reflects the inability of an immunocompromised macaque to mount a neutralizing antibody response against emerging SHIVs bearing variant envelope glycoproteins. This finding is very reminiscent of the situation with HIV-1-infected individuals in whom neutralization of autologous isolates has been difficult to demonstrate. For example,

a recent study reported that sera from long-term nonprogressors were able to neutralize contemporary autologous virus whereas only one of nine HIV-1 isolates from progressors or short-term nonprogressors was neutralized by autologous serum (25). Historically this was also the case for the original source of the HIV-1<sub>DH12</sub> isolate: autologous antibody-positive serum obtained from this AIDS patient at the time of virus isolation exhibited no neutralizing activity directed against HIV-1<sub>DH12</sub> (Ron Willey, personal communication), yet naïve chimpanzees or chimpanzees persistently infected with nonpathogenic HIV-1<sub>SF2</sub>, rapidly developed high titers of autologous neutralizing antibody after a challenge or superchallenge with HIV-1<sub>DH12</sub> (10, 26).

SHIV<sub>DH12R</sub> represents a potentially useful reagent that can be used as a challenge virus in vaccine experiments or for the study of cellular and immunologic events attending the rapid and irreversible depletion of CD4<sup>+</sup> T lymphocytes *in vivo*. Unlike some highly pathogenic SHIVs, SHIV<sub>DH12R</sub> stock can be readily prepared in either rhesus or human PBMCs and progeny virions can be titered in PBMCs or MT4 cells. Thus far, SHIV<sub>DH12R</sub> has induced marked CD4<sup>+</sup> cell loss in all i.v.-inoculated rhesus macaques (Fig. 1 B and C).

We thank Drs. Ali Azadegan, Carole Clarke, and Randy Elkins and Messrs. George Coleman, Charles Thornton, and Russ Byrum for invaluable assistance in the animal experiments; Drs. Michael Eckhaus, Georgina Miller, and David Green for pathological analyses; Dr. Michael Cho for providing vaccinia-expressed gp120; Dr. Vanessa Hirsch for providing SIV infected animal plasma; and Mr. Ron Willey for helpful suggestions.

- Shibata, R., Maldarelli, F., Siemon, C., Matano, T., Parta, M., Miller, G., Fredrickson, T. & Martin, M. A. (1997) *J. Infect. Dis.* **176**, 362–373.
- Matano, T., Shibata, R., Siemon, C., Connors, M., Lane, H. C. & Martin, M. A. (1998) *J. Virol.* **72**, 164–169.
- Carmichael, A., Jin, X., Sissons, P. & Borysiewicz, L. (1993) *J. Exp. Med.* **177**, 249–256.
- Klein, M. R., van Baalen, C. A., Holwerda, A. M., Kerkhof Garde, S. R., Bende, R. J., Keet, I. P., Eeftink-Schattenkerk, J. K., Osterhaus, A. D., Schuitemaker, H. & Miedema, F. (1995) *J. Exp. Med.* **181**, 1365–1372.
- Rinaldo, C., Huang, X. L., Fan, Z. F., Ding, M., Beltz, L., Logar, A., Panicali, D., Mazzara, G., Liebmann, J., Cottrill, M., *et al.* (1995) *J. Virol.* **69**, 5838–5842.
- Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., *et al.* (1999) *Science* **283**, 857–860.
- Committee on Care and Use of Laboratory Animals (1985) *Guide for the Care and Use of Laboratory Animals* (Natl. Inst. Health, Bethesda), DHHS Publ. No. (NIH) 85–23, revised ed.
- Dewar, R. L., Highbarger, H. C., Sarmiento, M. D., Todd, J. A., Vasudevachari, M. B., Davey, R. T., Jr., Kovacs, J. A., Salzman, N. P., Lane, H. C. & Urdea, M. S. (1994) *J. Infect. Dis.* **170**, 1172–1179.
- Hirt, B. (1967) *J. Mol. Biol.* **14**, 365–369.
- Willey, R. L., Shibata, R., Freed, E. O., Cho, M. W. & Martin, M. A. (1996) *J. Virol.* **70**, 6431–6436.
- Reed, L. J. & Muench, H. (1938) *Am. J. Hyg.* **27**, 493–497.
- Cho, M. W., Lee, M. K., Carney, M. C., Berson, J. F., Doms, R. W. & Martin, M. A. (1998) *J. Virol.* **72**, 2509–2515.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed., p. 53.
- Joag, S. V., Li, Z., Foresman, L., Stephens, E. B., Zhao, L. J., Adany, I., Pinson, D. M., McClure, H. M. & Narayan, O. (1996) *J. Virol.* **70**, 3189–3197.
- Reimann, K. A., Li, J. T., Veazey, R., Halloran, M., Park, I. W., Karlsson, G. B., Sodroski, J. & Letvin, N. L. (1996) *J. Virol.* **70**, 6922–6928.
- Karlsson, G. B., Halloran, M., Li, J., Park, I. W., Gomila, R., Reimann, K. A., Axthelm, M. K., Iliff, S. A., Letvin, N. L. & Sodroski, J. (1997) *J. Virol.* **71**, 4218–4225.
- Stephens, E. B., Mukherjee, S., Liu, Z. Q., Sheffer, D., Lamb-Wharton, R., Leung, K., Zhuge, W., Joag, S. V., Li, Z., Foresman, L., *et al.* (1998) *J. Virol.* **72**, 5207–5214.
- Zhang, Y. J., Dragic, T., Cao, Y., Kostrikis, L., Kwon, D. S., Littman, D. R., KewalRamani, V. N. & Moore, J. P. (1998) *J. Virol.* **72**, 9337–9344.
- Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., Willey, R., Cho, M. W. & Martin, M. A. (1999) *Nat. Med.* **5**, 204–210.
- Igarashi, T., Brown, C., Azadegan, A., Haigwood, N., Dimitrov, D., Martin, M. A. & Shibata, R. (1999) *Nat. Med.* **5**, 211–216.
- Burns, D. P., Collignon, C. & Desrosiers, R. C. (1993) *J. Virol.* **67**, 4104–4113.
- Rudensey, L. M., Kimata, J. T., Long, E. M., Chackerian, B. & Overbaugh, J. (1998) *J. Virol.* **72**, 209–217.
- Johnson, P. R., Hamm, T. E., Goldstein, S., Kitov, S. & Hirsch, V. M. (1991) *Virology* **185**, 217–228.
- Igarashi, T., Kuwata, T., Takehisa, J., Ibuki, K., Shibata, R., Mukai, R., Komatsu, T., Adachi, A., Ido, E. & Hayami, M. (1996) *J. Gen. Virol.* **77**, 1649–1658.
- Pilgrim, A. K., Pantaleo, G., Cohen, O. J., Fink, L. M., Zhou, J. Y., Zhou, J. T., Bolognesi, D. P., Fauci, A. S. & Montefiori, D. C. (1997) *J. Infect. Dis.* **176**, 924–932.
- Shibata, R., Siemon, C., Cho, M. W., Arthur, L. O., Nigida, S. M., Jr., Matthews, T., Sawyer, L. A., Schultz, A., Murthy, K. K., Israel, Z., *et al.* (1996) *J. Virol.* **70**, 4361–4369.