JOURNAL OF VIROLOGY, Aug. 2000, p. 6935–6945
0022-538X/00/$04.00+0
Vol. 74, No. 15

Short- and Long-Term Clinical Outcomes in Rhesus Monkeys Inoculated with a Highly Pathogenic Chimeric Simian/Human Immunodeficiency Virus

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Received 29 February 2000/Accepted 9 May 2000

A highly pathogenic simian/human immunodeficiency virus (SHIV), $SHIV_{DH12R}$, isolated from a rhesus macaque that had been treated with anti-human CD8 monoclonal antibody at the time of primary infection with the nonpathogenic, molecularly cloned $SHIV_{DH12}$, induced marked and rapid $CD4^+$ T cell loss in all rhesus macaques intravenously inoculated with 1.0 50% tissue culture infective dose ($TCID_{50}$) to 4.1×10^5 $TCID_{50}$s of virus. Animals inoculated with 650 $TCID_{50}$s of $SHIV_{DH12R}$ or more experienced irreversible $CD4^+$ T lymphocyte depletion and developed clinical disease requiring euthanasia between weeks 12 and 23 postinfection. In contrast, the $CD4^+$ T-cell numbers in four of five monkeys receiving 25 $TCID_{50}$s of $SHIV_{DH12R}$ or less stabilized at low levels, and these surviving animals produced antibodies capable of neutralizing $SHIV_{DH12R}$. In the fifth monkey, no recovery from the $CD4^+$ T cell decline occurred, and the animal had to be euthanized. Viral RNA levels, subsequent to the initial peak of infection but not at peak viremia, correlated with the virus inoculum size and the eventual clinical course. Both initial infection rate constants, *k*, and decay constants, *d*, were determined, but only the latter were statistically correlated to clinical outcome. The attenuating effects of reduced inoculum size were also observed when virus was inoculated by the mucosal route. Because the uncloned $SHIV_{DH12R}$ stock possessed the genetic properties of a lentivirus quasispecies, we were able to assess the evolution of the input virus swarm in animals surviving the acute infection by monitoring the emergence of neutralization escape viral variants.

Over the past decade, simian/human immunodeficiency virus type 1 chimeric viruses (SHIVs) have been constructed containing various amounts of human immunodeficiency virus type 1 (HIV-1) sequence and exhibiting a continuum of disease-inducing phenotypes (10, 28, 29, 34, 35). Because early studies (14, 34; our unpublished work) had indicated that SIV *gag* and *pol* sequences were required for high levels of SHIV production in cultured macaque peripheral blood mononuclear cells (PBMC), our initial strategy was to generate chimeric viruses containing as much HIV-1 genetic information as possible. The first chimeric virus we evaluated, $SHIV_{MD1}$, contained intact *tat*, *rev*, *vpu*, *env*, and *nef* genes from the dual-tropic primary $HIV\text{-}1_{DH12}$ isolate and a *vpr* gene of mixed origin (SIV_{mac239}, $HIV\text{-}1_{NL4\text{-}3}$, and $HIV\text{-}1_{DH12}$) (35). $SHIV_{MD1}$ infections were readily established in rhesus monkeys, pig-tailed macaques, and cynomolgus monkeys, but only 1 of the 21 inoculated animals developed immunodeficiency. Since our goal was to generate a pathogenic SHIV for use in vaccine experiments, a "second-generation" $SHIV_{DH12}$ (previously designated $SHIV_{MD14}$) was created in which the HIV-1 *nef* gene was replaced with the SIV_{mac239} *nef* gene. $SHIV_{DH12}$, in fact, replicated to high titers and induced disease in pig-tailed monkeys (35). However, although $SHIV_{DH12}$ infections were readily established in more than 15 rhesus monkeys, virus loads were generally low, and none of the inoculated animals suffered $CD4^+$ T cell depletions or any signs of disease.

Pathogenic SHIVs, which cause rapid $CD4^+$ T lymphocyte depletions within weeks of inoculation, have been generated as a result of serial animal-to-animal passage of whole blood and bone marrow from macaques initially infected with nonpathogenic chimeric viruses (10, 28). We recently reported the isolation of the highly pathogenic $SHIV_{DH12R}$, which arose during a single in vivo passage in a rhesus monkey treated with an anti-human CD8 monoclonal antibody at the time of its primary infection with the nonpathogenic $SHIV_{DH12}$ (8). A tissue culture-derived stock of $SHIV_{DH12R}$ induced marked and rapid $CD4^+$ T cell loss following intravenous inoculation of rhesus monkeys. Although $SHIV_{DH12R}$ retained its capacity to utilize both CCR5 and CXCR4 as coreceptors during virus entry, it could no longer be neutralized by antibodies targeting glycoprotein 120 (gp120) epitopes associated with its nonpathogenic $SHIV_{DH12}$ parent (8). The latter result was consistent with nucleotide sequence analyses of 22 independent PCR clones, amplified from the $SHIV_{DH12R}$-infected cells, which revealed changes affecting gp120 (13 amino acid) and gp41 (6 amino acid), accompanying the acquisition of increased virulence. Furthermore, the uncloned $SHIV_{DH12R}$ tissue culture-derived stock possessed the genetic properties of a lentivirus quasispecies because of the presence of additional, but common, gp120 amino acid substitutions in some of the 22 PCR clones.

We previously reported that although $SHIV_{DH12R}$ induces an extremely rapid and profound depletion of $CD4^+$ T cells in all infected rhesus monkeys, the loss of this T-cell subset did

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not appear to be irreversible in animals inoculated with small amounts of virus (8). This observation was systematically examined by performing a rigorous in vivo virus titration and various routes of inoculation. Our results show that macaques that were administered large intravenous $SHIV_{DH12R}$ inocula experienced a rapid and unremitting downhill clinical course. In contrast, rhesus monkeys receiving 25 50% tissue culture infective doses ($TCID_{50}$) or less of virus survived the primary infection with markedly reduced but stable levels of $CD4^+$ T lymphocytes and produced antibodies capable of neutralizing $SHIV_{DH12R}$. The animal-specific evolution of the $SHIV_{DH12R}$ quasispecies in surviving macaques was monitored by the emergence of neutralization escape viral variants.

MATERIALS AND METHODS

Virus. The source and preparation of the tissue culture-derived $SHIV_{DH12R}$ stock has been previously described (8). It had a titer of 1.6×10^5 $TCID_{50}$/ml measured in rhesus monkey PBMC and 4.1×10^5 $TCID_{50}$/ml measured in MT4 cells.

Animals, virus inoculation, and sample collection. The 14 rhesus macaques listed in Table 1 were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (2). They were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Phlebotomies and virus inoculations were performed with animals anesthetized with tiletamine-HCl and zolazepam-HCl (Telazol; Fort Dodge Laboratories, Fort Dodge, Iowa). EDTA-treated blood specimens were used for lymphocyte phenotyping analysis and complete blood counts; acid citrate-dextrose-A-treated samples of blood were used to prepare plasma and PBMC.

Lymphocyte phenotyping. EDTA-treated blood samples were stained with fluorochrome-conjugated monoclonal antibodies (CD3-fluorescein isothiocyanate [FITC] [Serotec, Raleigh, N.C.], CD4-allophycocyanin, CD8-peridinin chlorophyll protein, and CD20-phycoerythrin [Becton Dickinson Immunocytometry Systems, San Jose, Calif.]) and analyzed by flow cytometry (FACSort; Becton Dickinson) as previously described (8, 35).

Quantitation of plasma viral RNA levels. Plasma viral RNA levels were determined by real-time PCR (ABI Prism 7700 sequence detection system; Perkin-Elmer, Foster City, Calif.) as previously described, using reverse-transcribed viral RNA in plasma samples from $SHIV_{DH12R}$-infected macaques (8). The cDNA was amplified (45 cycles/default setting) with Ampli *Taq* Gold DNA polymerase (PCR core reagents kit; Perkin-Elmer/Roche) with primer pairs corresponding to SIV_{mac239} *gag* gene sequences (forward, nucleotides 1181 to 1208, and reverse, nucleotides 1338 to 1317) present in $SHIV_{DH12R}$. Plasma from $SHIV_{DH12R}$-infected macaques and $SHIV_{DH12}$-infected rhesus PBMC culture supernatants, previously quantitated by the branched DNA method (4), served as standards for the reverse transcription-PCR (RT-PCR) assay.

Western blot analysis. For immunoblotting, $SHIV_{DH12}$ particles were pelleted by ultracentrifugation, mixed with gp120 expressed from a recombinant vaccinia virus, and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer as previously described (8). The viral proteins were electrophoresed through 10% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore, Bedford, Mass.), and incubated with serially collected plasma samples (diluted 1:100). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-human immunoglobulin (Amersham Pharmacia Biotech, Piscataway, N.J.) and viral protein-specific bands were visualized on X-ray film using a chemiluminescent reagent (SuperSignal West Pico Chemiluminescent Substrate; Pierce).

Virus neutralization assays. The titers of virus-specific neutralizing antibody in samples of plasma were determined by a previously described limiting-dilution endpoint assay, which measures 100% neutralization against 100 $TCID_{50}$ of $SHIV_{DH12R}$, $SHIV_{DH12}$, or $SHIV_{DH12R(W30)}$ (8, 33, 40). Neutralization antibody titers were calculated by the method of Reed and Muench (27).

Mathematical analysis. The early kinetics of an in vivo virus infection can be described by the exponential function $V = V_0 exp[k(t - tc)]$, where V is the concentration of virus particles in the blood, V_0 is the virus concentration after the first cycle of virus infection at $t = tc$, tc being the time to complete a single cycle of infection, and k is the infection rate constant as previously defined for HIV-1 infections in cultured cells (5). Using this formula, one can derive an approximate relationship between the time required to reach the peak of virus infection, tp, and the input $SHIV_{DH12R}$ $TCID_{50}$:

$$tp = -(1/k)\ln(TCID_{50}) + tc + (1/k)\ln(N) \quad (1)$$

where N is the number of susceptible target cells and it is assumed that (i) the input $TCID_{50}$ equals the initial number of infected cells and (ii) the number of virus particles in the plasma is proportional to the number of virus-producing cells. The average k for the nine monkeys inoculated intravenously with $SHIV_{DH12R}$ was calculated by a linear regression analysis of the dependence of tp versus $\ln(TCID_{50})$ (see Fig. 2C) using the Scientist (MicroMath, Inc., Salt Lake City, Utah) software program. The infection rate constants for individual $SHIV_{DH12R}$-infected rhesus monkeys were calculated separately by a linear regression analysis of the dependence of $\ln(V)$ versus t, using the same software. The initial infection rate constants were also estimated using the formula:

$$k = [\ln(V_2) - \ln(V_1)]/(t_2 - t_1) \quad (2)$$

where V_2 and V_1 are the first two measurable virus concentrations at times t_2 and t_1, respectively. The same formula was used to estimate the virus decline rate constants, d, with V_1 being the peak virus concentration at time t_1 and V_2 being the virus concentration at time t_2 (equal to 2 weeks postinfection peak). Statistical analyses were performed using the Statistica software program (Statsoft, Tulsa, Okla.).

RESULTS

Titration of $SHIV_{DH12R}$ infectivity in vivo. In the initial study describing the origin of the highly pathogenic $SHIV_{DH12R}$, we reported that rhesus macaques inoculated with 4×10^5 or 6.5×10^2 $TCID_{50}$ (determined for MT4 cells) of virus became infected and experienced a rapid and irreversible decline of circulating $CD4^+$ T lymphocytes (8). To ascertain the infectivity and pathogenicity of the $SHIV_{DH12R}$ virus stock administered intravenously to rhesus monkeys, a standard limiting-dilution titration approach was used (Table 1). Three of the animals (H431, H520, and H521), inoculated with 1.0, 0.2, and 0.04 $TCID_{50}$ of $SHIV_{DH12R}$, respectively, did not become infected (no detectable PBMC-associated proviral DNA, p27 antigenemia, or virus-specific antibody responses through 24 weeks postinfection [data not shown]). Because they remained uninfected, rhesus monkeys H520 and H521 were reused to obtain a solid titration endpoint and were intravenously inoculated with 25 and 5 $TCID_{50}$ of $SHIV_{DH12R}$, respectively. Table 1 indicates that one of the two monkeys exposed to 1 $TCID_{50}$ and all animals injected with larger virus inocula became infected. In this titration, the 50% animal infectious dose of $SHIV_{DH12R}$ corresponded to approximately 1 $TCID_{50}$.

Levels of $CD4^+$ T lymphocytes in rhesus monkeys intravenously inoculated with different amounts of $SHIV_{DH12R}$. Every animal that became infected with $SHIV_{DH12R}$ administered intravenously suffered a profound and rapid depletion of circulating $CD4^+$ T cells within 2 to 3 weeks of inoculation. The $CD4^+$ T lymphocyte numbers declined irreversibly to below 50 cells/μl by weeks 3.6 to 7.6 postinfection in the four monkeys (H27, H358, 5980, and 5981) receiving the largest amounts (6.5×10^2 to 4.1×10^5 $TCID_{50}$) of virus (Fig. 1A). In addition, the monkeys which received 25 $TCID_{50}$ or less of $SHIV_{DH12R}$ also experienced rapid and marked $CD4^+$ T cell loss. However, in contrast to irreversible $CD4^+$ T cell depletions observed in animals inoculated with the larger amounts of virus, the percent $CD4^+$ cells never fell below 2%, with the exception of monkey H521 (Fig. 1B). In three of these four animals, the $CD4^+$ T-cell number remained quite low (100 to 300 cells/μl) compared to their preinoculation levels at nearly 1 year postinoculation; the number of $CD4^+$ T lymphocytes in monkey H520 was somewhat higher. One of the five rhesus monkeys (H521), which received only 5 $TCID_{50}$ of $SHIV_{DH12R}$, did experience a precipitous loss of $CD4^+$ T cells, which fell to 44 cells/μl by 12 weeks postinfection.

Plasma viral RNA levels in $SHIV_{DH12R}$-infected rhesus monkeys. All animals infected intravenously with $SHIV_{DH12R}$ rapidly developed high plasma viral RNA loads (Fig. 2). The initial peaks of viral RNA production occurred between weeks 1.6 and 4 and ranged from 7×10^6 to 2×10^8 RNA copies/ml of plasma. There was no correlation between inoculum size and peak virus loads. However, the kinetics of reaching the initial peak of plasma viremia appeared to be related to the amount of virus administered (Fig. 2C). A linear regression analysis of the dependence of the time required to reach the

TABLE 1. Profile of animals used in this study

Animal ID	Inoculum size	Route of inoculation	Infection	Clinical outcome
H27	4×10^5 $TCID_{50}$	Intravenous	+	Died at 12.6 wks PI[b]
H358	4×10^5 $TCID_{50}$	Intravenous	+	Died at 13.0 wks PI
5980	16,400 $TCID_{50}$	Intravenous	+	Died at 21.3 wks PI
5981	656 $TCID_{50}$	Intravenous	+	Died at 23.1 wks PI
6071	25 $TCID_{50}$	Intravenous	+	Healthy
H520[a]	25 $TCID_{50}$	Intravenous	+	Healthy
H521[a]	5 $TCID_{50}$	Intravenous	+	Died at 24.1 wks PI
6049	5 $TCID_{50}$	Intravenous	+	Healthy
6074	1 $TCID_{50}$	Intravenous	+	Healthy
H431	1 $TCID_{50}$	Intravenous	−	No infection established
H520[a]	0.2 $TCID_{50}$	Intravenous	−	No infection established
H521[a]	0.04 $TCID_{50}$	Intravenous	−	No infection established
H704	1×10^5 $TCID_{50}$ (3 times)	Intravaginal infusion	+	Healthy
903[a]	1×10^5 $TCID_{50}$ (3 times)	Intravaginal infusion	−	No infection established
H385	5×10^4 $TCID_{50}$	Intravaginal infusion	+	Healthy
T14	100 $TCID_{50}$	Intrarectal injection	+	Died at 12.0 wks PI
903[a]	100 $TCID_{50}$	Intrarectal injection	+	Healthy

[a] If no infection was established, this animal was reinoculated with a larger dose or via an alternative route following 24 weeks of observation.
[b] PI, postinfection.

infection peak, *tp*, versus the natural logarithm of the input inoculum size, ln($TCID_{50}$), suggested that the average infection rate constant, *k*, for the nine monkeys inoculated with $SHIV_{DH12R}$ intravenously is equal to 1.06 day^{-1} ($R = 0.81$; $P = 0.0081$; $n = 9$). Individual infection rate constants, *k*, were also determined for five of the $SHIV_{DH12R}$-infected macaques for which multiple "prepeak" plasma samples had been collected. The *k* values for these animals were 5980 (1.34; 1.27), 5981 (1.03; 1.24), 6074 (0.85; 0.95), 6049 (0.81; 0.84), and H520 (0.61; 0.98), where the numbers in parentheses denote the infection rate constants in day^{-1} calculated by using either all measurable time points, including the infection peak, or the first two measurable points, respectively (equation 2, Materials and Methods). Although a trend emerged suggesting a correlation between high initial infection rate constants and progression to fatal disease (animals 5980 and 5981, which had to be euthanized, compared to surviving and asymptomatic animals 6074, 6049, and H520), this correlation did not reach the level of statistical significance (Spearman correlation, $R = 0.87$ and $P = 0.058$; Mann-Whitney U test, $P = 0.083$ and $n = 5$). However, the effective rate decline constants, *d*, characterizing virus decay during the first two weeks after the infection peak were statistically significantly correlated to clinical outcome (Spearman correlation, $R = 0.78$ and $P = 0.01$; Mann-Whitney U test, $P = 0.027$ and $n = 9$); higher rate constants corresponded to greater survival.

By 4 to 6 weeks postinfection, the $SHIV_{DH12R}$ loads had decreased to 10^5 to 10^6 RNA copies/ml in all of the infected animals. However, the plasma viral RNA levels in monkeys inoculated with 650 $TCID_{50}$ or more of virus (Fig. 2A) subsequently rose a second time and were accompanied by clinical disease (see below). This pattern is to be contrasted with that observed for the animals inoculated with 25 $TCID_{50}$ or less of $SHIV_{DH12R}$ (Fig. 2B), in which the RNA loads continued to fall, reaching levels of 10^3 to 10^4 RNA copies/ml (macaques 6071 and 6049) or became undetectable (macaques H520 and 6074). However, PBMC-associated proviral DNA remained measurable in these latter two animals throughout the study, ranging from 820 to 6,500 copies/10^5 $CD4^+$ T cells in macaque H520 and from 22 to 120 copies/10^5 $CD4^+$ T cells in monkey 6074. As noted above, rhesus monkey H521 was the exception among the "low inoculum" group of animals. This macaque experienced a second sustained wave of high viremia (Fig. 2B) and was euthanized at week 24 postinfection.

The clinical course of $SHIV_{DH12R}$ infection in macaques correlates with inoculum size. Not unexpectedly, the pattern of $CD4^+$ T cell decline (and inoculum size) was predictive of clinical outcome. The four animals receiving 650 $TCID_{50}$ or more of virus intravenously developed disease and were euthanized between weeks 12 and 23, whereas four of the five monkeys administered 25 $TCID_{50}$ or less remained alive, with $CD4^+$ T lymphocyte counts that leveled off at the 100-to-500-cells/μl range (Table 1; Fig. 1). The two rhesus monkeys (H27 and H358) receiving the largest (4.1×10^5 $TCID_{50}$) $SHIV_{DH12R}$ inoculum both developed anorexia within 1 to 2 weeks of infection and, because of intractable diarrhea and marked weight loss, were euthanized at weeks 12 and 13, respectively. An autopsy of animal H27 revealed multifocal interstitial pulmonary fibrosis, which was associated with infiltrating multinucleated cells. Macaque 5981, originally inoculated with 650 $TCID_{50}$, experienced a somewhat slower but irreversible $CD4^+$ T lymphocyte decline (1,230 cells/μl preinfection; 142 cells/μl at week 3; 10 cells/μl at week 9) and was euthanized at week 23 because of protracted diarrhea and deteriorating clinical status. A necropsy revealed a multifocal *Candida albicans* infection of the esophagus and interstitial pneumonia with numerous giant cells. As noted earlier, animal H521 was the outlier among the rhesus monkeys receiving the small amounts of the $SHIV_{DH12R}$ inoculum. This animal developed anorexia at week 18 and diarrhea at week 23 and was euthanized at week 24 with a $CD4^+$ T cell count of 2 cells/μl of blood.

It is worth noting that the absolute levels of peak viral RNA production did not correlate with the clinical outcome (Fig. 2). Animals H521 and H358 had peak virus loads of only 7×10^6 to 8×10^6 RNA copies/ml and experienced irreversible $CD4^+$ T cell decline and death, while monkey 6049 produced 2×10^8 RNA copies/ml yet had a partial recovery of its $CD4^+$ T cell numbers and an asymptomatic clinical course.

The humoral responses of rhesus monkeys intravenously inoculated with $SHIV_{DH12R}$. Virus-specific antibodies elicited in $SHIV_{DH12R}$-infected animals were initially evaluated by im-

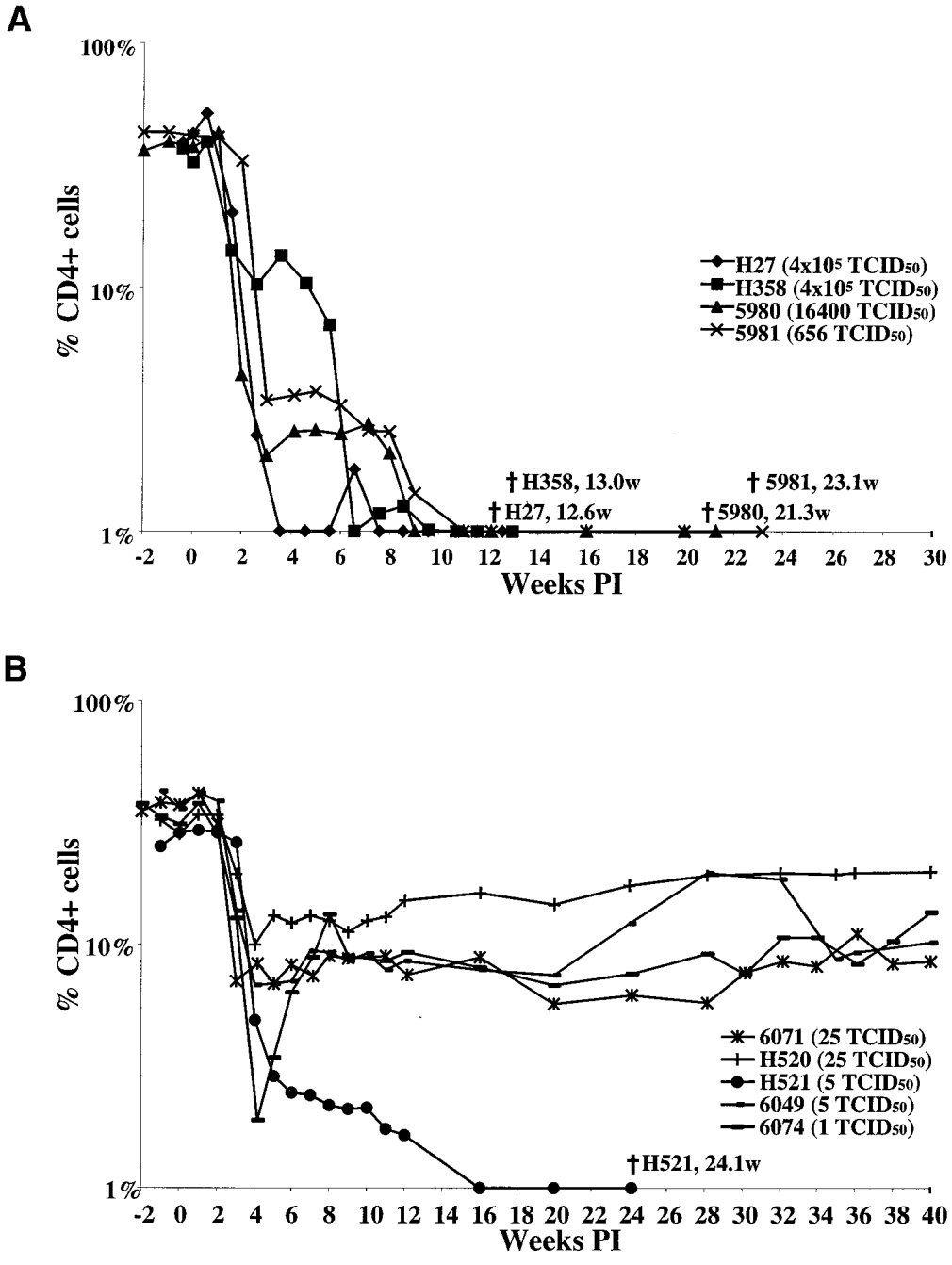


FIG. 1. $CD4^+$ T lymphocyte levels in rhesus macaques inoculated intravenously with $SHIV_{DH12R}$. Samples of blood collected at the indicated times from animals infected with 650 $TCID_{50}$ or more (A) or 25 $TCID_{50}$ or less (B) of $SHIV_{DH12R}$ were analyzed by flow cytometry as described in Materials and Methods. The time (weeks postinoculation [PI]) of death is indicated.

munoblot analysis. The four animals which received the large virus inocula and suffered irreversible $CD4^+$ T cell depletion also failed to make anti-gp120 antibodies (Fig. 3, top row). Animal H358, however, which was exposed to 4.1×10^5 $TCID_{50}$ of $SHIV_{DH12R}$, transiently produced low levels of anti-p27 (CA) antibody between weeks 2 and 8, which became undetectable at the time it was euthanized at week 13. As expected, four of the five rhesus monkeys, which had been inoculated with small amounts of virus and did not suffer a complete loss of $CD4^+$ T lymphocytes, produced high titers of anti-Gag (SIV) and -Env (HIV-1) antibodies (Fig. 3, bottom row). Animal H521, a recipient of only 5 $TCID_{50}$ of $SHIV_{DH12R}$, was the exception and, during its downhill clinical course, produced no detectable antibodies.

We previously reported that neutralization of primate lentiviruses bearing the DH12 family of envelope glycoproteins (viz. $HIV\text{-}1_{DH12}$ and $SHIV_{DH12}$) can be readily demonstrated by limiting-dilution assays utilizing reverse transcriptase production as the readout (7, 33, 40). The neutralizing activities elicited during chronic $HIV\text{-}1_{DH12}$ infections of chimpanzees

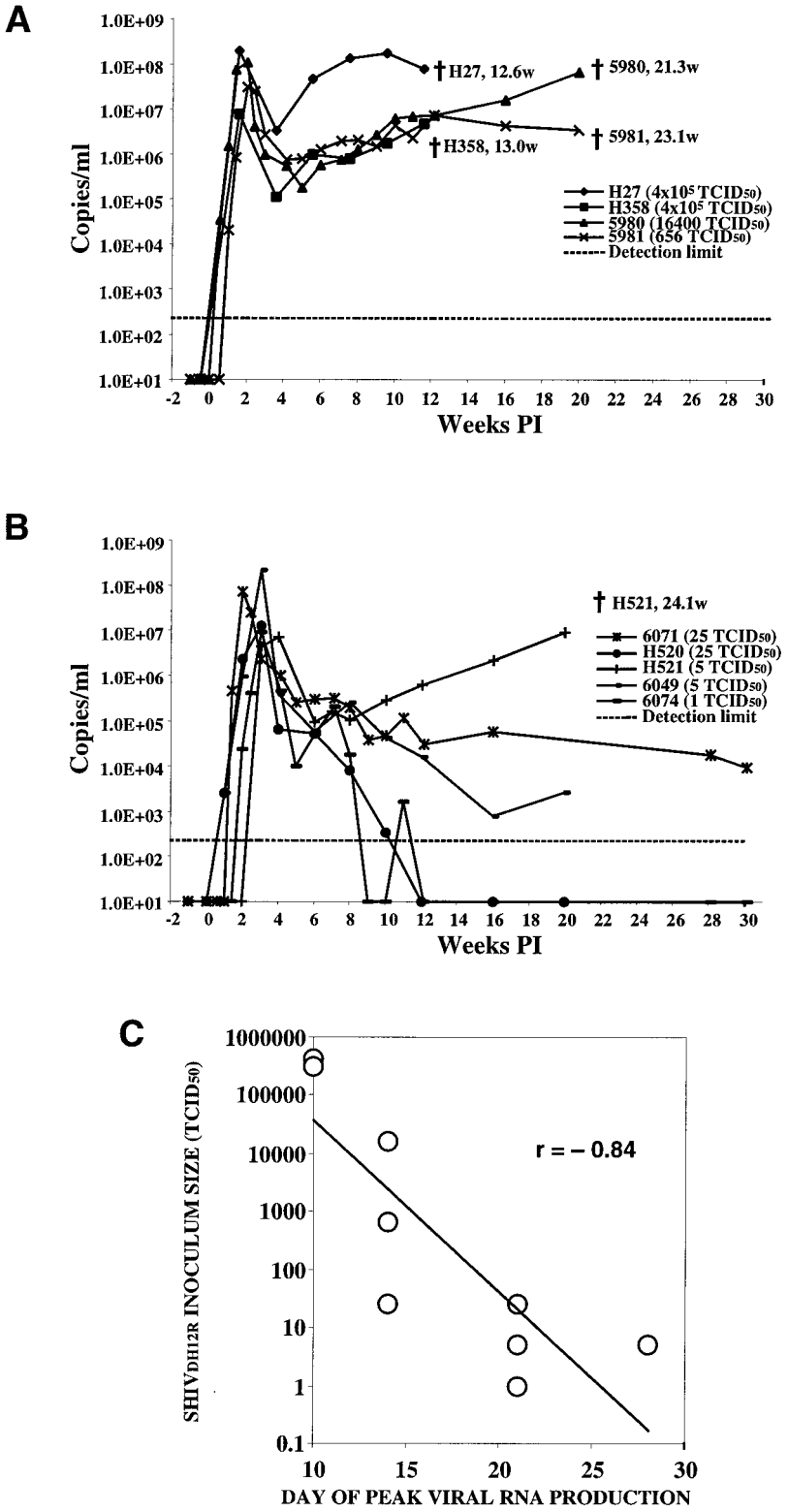


FIG. 2. Viral RNA levels in rhesus macaques inoculated intravenously with $SHIV_{DH12R}$. Samples of plasma collected at the indicated times from animals infected with 650 $TCID_{50}$ or more (A) or 25 $TCID_{50}$ or less (B) of $SHIV_{DH12R}$ were analyzed by real-time RT-PCR for viral RNA using primer pairs mapping to SIV *gag* sequences. The detection limit, approximately 200 RNA copy equivalents/ml, is indicated by the dashed line. The time (weeks postinfection [PI]) of death is indicated. (C) Relationship of the input inoculum to the time of peak virus production. The correlation coefficient is indicated.

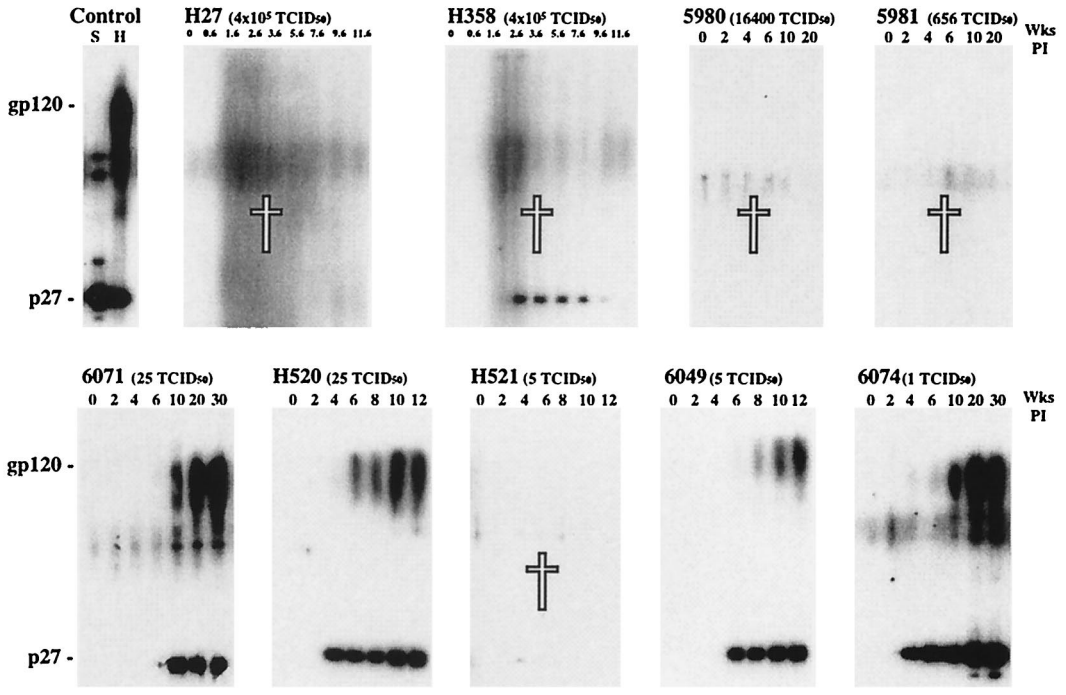


FIG. 3. Humoral immune responses in rhesus macaques inoculated intravenously with $SHIV_{DH12R}$. Plasma samples collected at the indicated times from nine monkeys were analyzed by immunoblotting as described in Materials and Methods. Plasma from a SIV_{mac239}-infected pig-tailed macaque (S) and an HIV-1_{DH12}-infected chimpanzee (H) served as positive controls. The white crosses indicate animals that died.

or $SHIV_{DH12}$ infections of macaques have been shown to be directed against the viral gp120 envelope glycoprotein (1, 36, 40). Rhesus monkeys chronically infected with the nonpathogenic $SHIV_{DH12}$ typically develop virus neutralization titers specific for $SHIV_{DH12}$ which range from 1:18 to 1:93 (7).

When plasma samples from animals inoculated with the large amounts of $SHIV_{DH12R}$ were evaluated for neutralizing antibodies, no activity was detected (Table 2). This result was consistent with the absence of anti-gp120 humoral responses, as measured by immunoblotting, in these same animals, with few or no detectable circulating $CD4^+$ T cells (Fig. 3). This result was also true for macaque H358, which had transiently produced anti-p27 but no anti-gp120 antibodies between weeks 2 and 8 postinfection (Fig. 3). In contrast, the low inoculum group of infected animals, again with the exception of monkey H521, all generated antibodies capable of neutralizing the highly pathogenic $SHIV_{DH12R}$ (Table 2). The neutralization titers measured in these monkeys fell in a range previously reported for SHIV-infected rhesus macaques with the endpoint dilution assay (7).

Infection of rhesus monkeys with $SHIV_{DH12R}$ by intravaginal infusion. In experiments modeling mucosal transmission of HIV-1 in humans, macaques have been successfully challenged with SIV and SHIVs by the vaginal and rectal routes of inoculation (6, 17, 23). To ascertain whether rhesus monkeys could be infected and develop immunodeficiency following exposure to the highly pathogenic $SHIV_{DH12R}$ through a mucosal portal of entry, 5×10^4 $TCID_{50}$ of virus was administered by a single atraumatic intravaginal infusion to three female rhesus monkeys (H704, 903, and H385). Only one (H385) of the three animals became infected; p27 antigenemia, DNA PCR, and virus-specific antibody responses (analyzed by enzyme-linked immunosorbent assay and immunoblotting) were not detectable in the other two monkeys during a 12-week observation period. Macaques H704 and 903 were subsequently rechallenged with $SHIV_{DH12R}$ by intravaginal infusion (5×10^4

TABLE 2. Titers of neutralizing antibody to $SHIV_{DH12R}$

Plasma sample taken at:	Titer for subject (inoculum size)[a]:								
	H27 (4×10^5 $TCID_{50}$)	H358 (4×10^5 $TCID_{50}$)	5980 (16,400 $TCID_{50}$)	5981 (656 $TCID_{50}$)	6071 (25 $TCID_{50}$)	H520 (25 $TCID_{50}$)	H521 (5 $TCID_{50}$)	6049 (5 $TCID_{50}$)	6074 (1 $TCID_{50}$)
Preinfection	<1.9	<1.9	<0.8	<0.8	<0.8	<3.1	<3.1	<3.1	<0.8
10 wks	<1.9	<1.9	<0.8	<0.8	<0.8	16.0	<3.1	<3.1	<0.8
20 wks	—	—	<0.8	<0.8	3.9	21.0	<1.6	7.0	11.1
30 wks			—	—	11.6	21.0	—	63.1	12.7
40 wks					7.0	43.7		75.8	8.4
50 wks					4.9	ND		ND	7.0
60 wks					7.0	ND		ND	8.4
70 wks					8.4	ND		ND	8.4

[a] ND, not done; —, animal died.

$TCID_{50}$ administered on three separate occasions over a 96-h period). Only animal H704 became infected following this rechallenge; monkey 903 remained proviral DNA negative over the ensuing 12-week observation period and did not produce virus-specific antibodies.

Although two of the three animals exposed to $SHIV_{DH12R}$ became infected as a result of intravaginal infusion, their clinical courses differed markedly from those of animals inoculated intravenously. Rhesus monkey H704 suffered a significant $CD4^+$ T lymphocyte decline beginning 3 weeks postinfection, with numbers falling to a low of 189 cells/μl of blood at week 7 (Fig. 4A). At present, the $CD4^+$ T-cell numbers in macaque H704 have risen to approximately 50% of the preinfection level. This response is similar to that observed for animals administered small $SHIV_{DH12R}$ inocula intravenously. Macaque H385, on the other hand, experienced no acute or long-term $CD4^+$ T cell decline. Both animals also exhibited patterns of viral RNA synthesis not seen in monkeys inoculated by the intravenous route (Fig. 4B). Monkey H385 produced the lowest peak viral RNA levels (1.5×10^5 copies/ml at week 4) ever measured in a $SHIV_{DH12R}$-infected macaque, with viral RNA becoming undetectable at week 6 postinfection. Although rhesus monkey H704 developed a peak viral RNA load (5.4×10^7 copies/ml) comparable to that observed in animals inoculated intravenously with $SHIV_{DH12R}$, its viremia rapidly cleared and could not be measured at 8 weeks postinfection. Results of an immunoblot analysis (Fig. 4C) using plasma from these two infected macaques were consistent with the plasma viral RNA levels. Antibodies directed against both p27 and gp120 rapidly appeared in monkey H704, whereas only a delayed anti-p27 response occurred in animal H385. Interestingly, no antibodies capable of neutralizing $SHIV_{DH12R}$ were detected in either animal (through week 70 for H385 and week 40 for H704) infected by intravaginal infusion (data not shown).

Infection of rhesus monkeys with $SHIV_{DH12R}$ by a nonintravenous parenteral route. The relative resistance of rhesus monkeys to the $SHIV_{DH12R}$ vaginal mucosal challenge is consistent with studies indicating that larger inoculum amounts are required to establish SIV infections in macaques when nonintravenous routes of inoculation are used (18, 20). It is worth noting, however, that most natural retroviral infections in nonhuman species (e.g., mice, cats, and monkeys) arise as a result of fighting (biting and scratching) or transmission through breast milk to newborn animals (9, 13, 41). Nonetheless, mucosal routes of inoculation have been used in nonhuman primates to model sexual transmission of HIV-1 in humans or, in some vaccine efficacy experiments, to reduce the immediate and systemic virus dissemination attending intravenous administration of a challenge virus. Because earlier studies have, in fact, demonstrated that lymphocytes, rather than epithelial cells, are the initial targets of SIV introduced by atraumatic mucosal routes (38), we wondered whether direct submucosal injection might retard the early steps of in vivo infections and thereby attenuate the deleterious effects of the highly pathogenic $SHIV_{DH12R}$ administered intravenously. Two rhesus monkeys were therefore inoculated with 100 $TCID_{50}$ of virus by rectal submucosal injection. One animal (T14) suffered rapid $CD4^+$ T lymphocyte depletion (49 cells/μl at 4.6 weeks postinfection) and high virus loads (1.1×10^8 RNA copies/ml at 2 weeks postinfection) and had to be euthanized at week 12 because of anorexia, lethargy, and marked weight loss (Fig. 5A and B). The second macaque inoculated by submucosal injection was the same animal (903) that failed to become infected following repeated vaginal infusions of $SHIV_{DH12R}$. Monkey 903 did indeed become infected as a result of the parenteral virus injection, experiencing a rapid but not irreversible $CD4^+$ T cell decline, which leveled off at the 200 cells/μl range (Fig. 5A). Its peak RNA load was slightly lower (2.6×10^7 copies/ml) than that measured for macaque

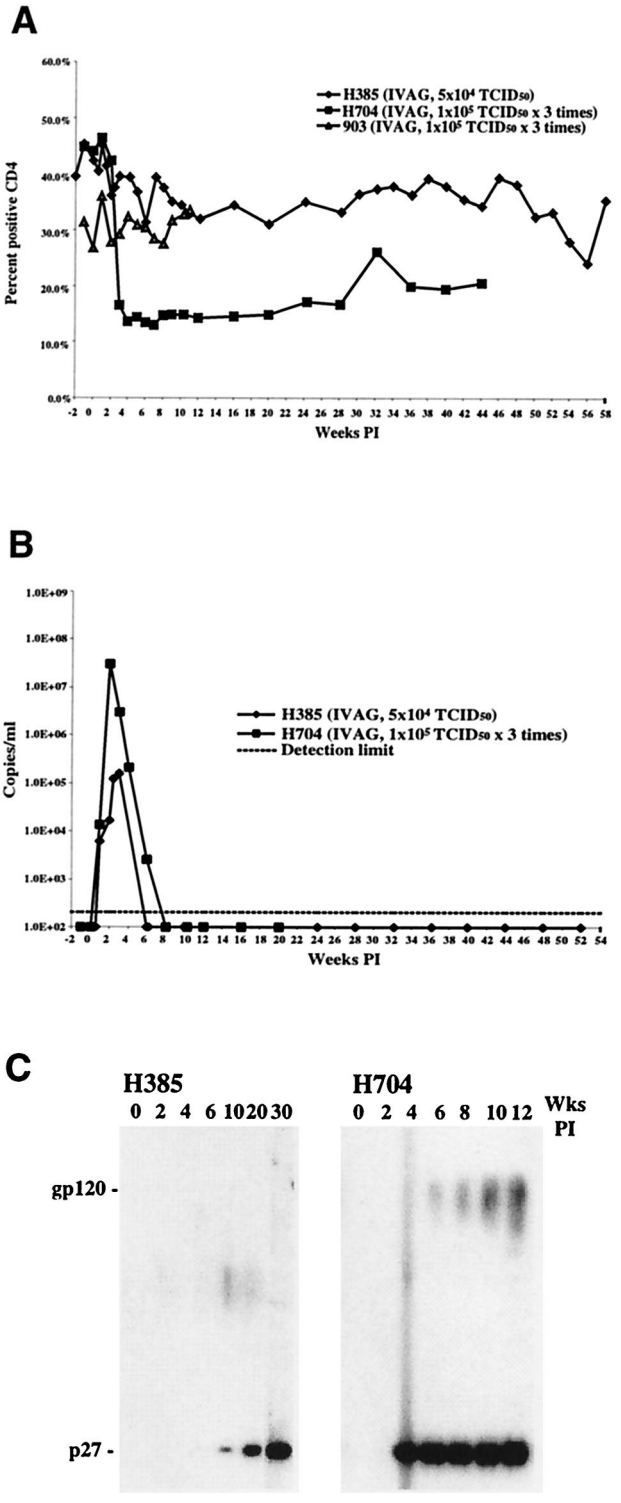


FIG. 4. The dynamics of $CD4^+$ T lymphocyte (A), viral RNA (B), and antibody (C) levels in rhesus macaques following atraumatic intravaginal infusion of $SHIV_{DH12R}$.

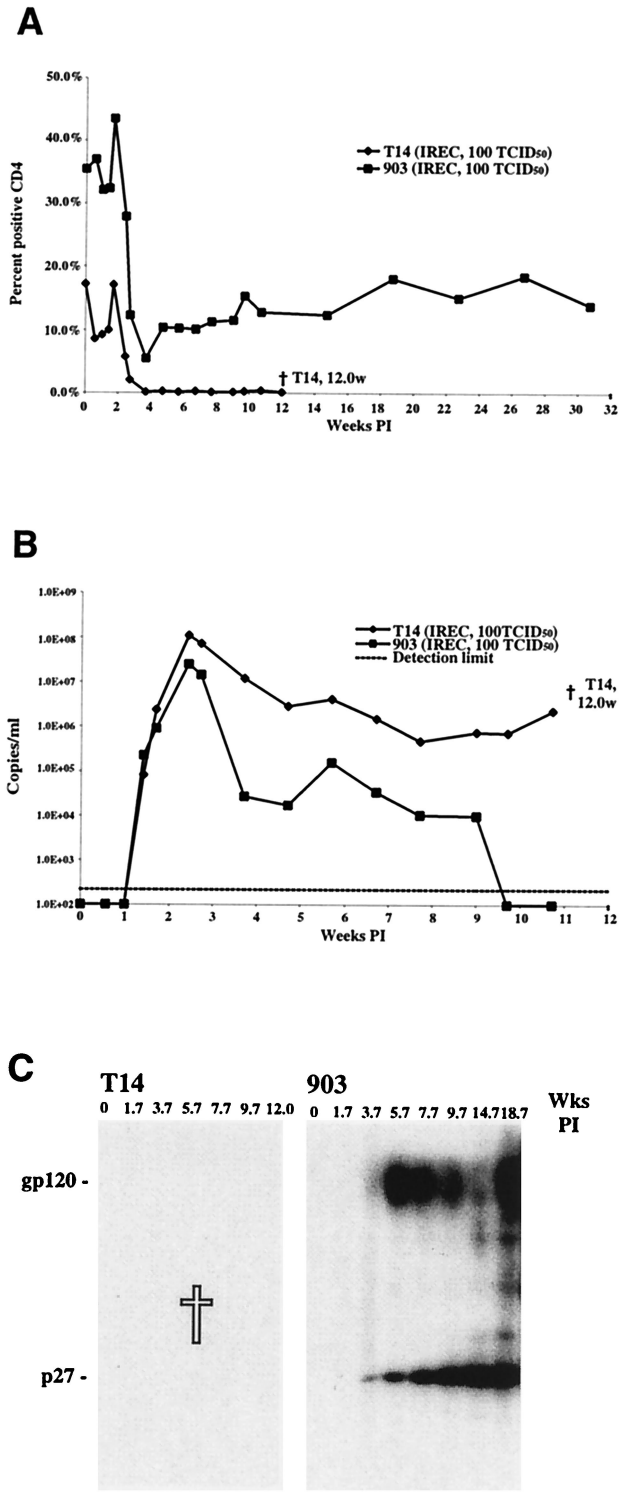


FIG. 5. The dynamics of $CD4^+$ T lymphocyte (A), viral RNA (B), and antibody (C) levels in rhesus macaques following submucosal injection of $SHIV_{DH12R}$. The time (weeks) of death is indicated.

T14 and subsequently declined to unmeasurable levels by week 10 postinfection. Results of immunoblot analyses of these two monkeys were consistent with their respective clinical courses (Fig. 5C).

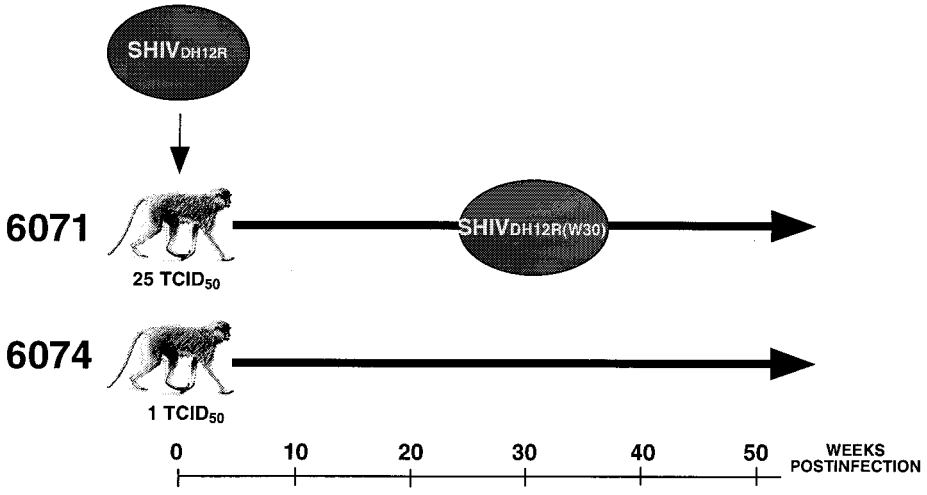
The $SHIV_{DH12R}$ quasispecies evolves in an animal-specific manner in surviving, chronically infected rhesus monkeys. We had previously molecularly characterized the $SHIV_{DH12R}$ stock by amplifying a 3-kb DNA segment encompassing the *vpu*, *env*, and *nef* genes and determining the nucleotide sequence for 22 independent PCR clones (8). Sequence analysis revealed that amino acid substitutions affecting 13 residues in gp120 and 6 residues in gp41 had accumulated during the evolution of the nonpathogenic $SHIV_{DH12}$ to the highly pathogenic $SHIV_{DH12R}$. In addition, other consistent amino acid changes were present in several but not all of the sequenced PCR clones. Thus, the $SHIV_{DH12R}$ tissue culture-derived stock resembled a typical virus quasispecies isolated from HIV-1-infected individuals. Because endpoint, 100% neutralization assays of HIV-1_{DH12}, $SHIV_{DH12}$, and $SHIV_{DH12R}$ are easy to perform (7, 8, 40), the survival of animals following $SHIV_{DH12R}$ infection provided the opportunity to assess whether the input virus quasispecies had evolved into a neutralization-resistant virus "swarm."

As indicated in Table 2, rhesus monkeys 6071 and 6074, which survived infections initiated with 25 and 1 $TCID_{50}$ of $SHIV_{DH12R}$, respectively, both generated neutralizing antibodies against the input virus. At week 30 postinfection, virus was isolated from animal 6071 by cocultivation with naïve rhesus PBMC and was designated $SHIV_{DH12R(W30)}$. Plasma samples collected at week 30 from macaques 6071 and 6074 failed to neutralize $SHIV_{DH12R(W30)}$ (Fig. 6). However, 100% neutralizing activity against $SHIV_{DH12R(W30)}$ became measurable at weeks 40 and 50 in animal 6071 but not in animal 6074. Because the plasma from monkey 6074 continued to produce neutralizing antibody directed against the common $SHIV_{DH12R}$ input even at weeks 40 and 50 postinfection (Table 2), its inability to neutralize $SHIV_{DH12R(W30)}$ is consistent with the antigenic evolution of the $SHIV_{DH12R}$ quasispecies in an animal-specific fashion.

DISCUSSION

In contrast to HIV-1 infections of humans or SIV infections of Asian macaques, in which virus-induced disease occurs in time frames of approximately 10 years and 1 year, respectively (11, 21, 31), highly pathogenic SHIVs are known to cause depletions of $CD4^+$ T lymphocytes within a few weeks of infection and death shortly thereafter (8, 10, 28). The latter clinical course was observed for $SHIV_{DH12R}$ with the interesting addition that its pathogenic effects, following intravenous virus administration, were dose dependent. The attenuating effects of reduced inoculum size were also observed when virus was inoculated by the mucosal route. The failure to establish a $SHIV_{DH12R}$ infection in one of three animals exposed multiple times to 10^5 $TCID_{50}$ of virus by intravaginal infusion and the extremely low virus loads measured in a second of these three monkeys very likely reflect the intrinsic protection afforded by mucosa, tissue, and lymph node barriers, all of which are bypassed by intravenous SHIV inoculations.

When $SHIV_{DH12R}$ was administered intravenously, marked and rapid $CD4^+$ T cell loss occurred in all animals and at all virus inoculum sizes. However, with high-input $SHIV_{DH12R}$ inocula (650 $TCID_{50}$ and greater), $CD4^+$ T lymphocyte depletion was irreversible, was associated with the absence of virus-specific humoral responses, and resulted in intractable symptoms of weakness, diarrhea, and weight loss, requiring euthanasia between weeks 12 and 23 postinfection. In contrast, $CD4^+$ T lymphocyte levels never fell below 50 cells/μl in four of the five rhesus monkeys inoculated intravenously with small (25 $TCID_{50}$ or less) $SHIV_{DH12R}$ inocula, and these animals



NEUTRALIZING ANTIBODY TITERS AGAINST $SHIV_{DH12R(W30)}$

PLASMA SAMPLES	ANIMAL 6071	ANIMAL 6074
Preinfection	<6.3	<6.3
30 weeks	<6.3	<6.3
40 weeks	**38.1**	<7.3
50 weeks	**38.1**	<7.3

FIG. 6. Evolution of the $SHIV_{DH12R}$ quasispecies in two rhesus monkeys as monitored by emergence of neutralizing antibody resistance. Macaques 6071 and 6074 were inoculated with $SHIV_{DH12R}$ and both developed $SHIV_{DH12R}$-specific neutralizing antibodies by week 20 postinfection (see Table 2). Virus was isolated from animal 6071 at 30 weeks postinfection and was designated $SHIV_{DH12R(W30)}$. Plasma samples collected from both animals at the indicated times were assayed for neutralizing activity against $SHIV_{DH12R(W30)}$.

exhibited no signs of clinical disease. Interestingly, the $CD4^+$ T cell counts in these asymptomatic macaques remained quite low compared to the preinoculation levels (Fig. 1), stabilizing at the 100- to 500-cells/μl range during a one-year period of observation. Because the accelerated and invariably fatal clinical course in monkeys exposed to large amounts of virus is determined within the first few weeks of infection and appears to be due to the seemingly synchronous loss of $CD4^+$ T cells, this SHIV system may prove useful for assessing the roles of cellular and viral determinants in disease development.

Although we observed a correlation between inoculum size and clinical outcome for rhesus macaques exposed to $SHIV_{DH12R}$ intravenously, this effect has not been reported for monkeys inoculated with pathogenic strains of SIV (3). On the other hand, there have been several reports of seronegative health care workers, newborn infants of HIV-1 positive mothers, needle-sharing drug users, and individuals who have had unprotected sexual intercourse who remain antibody negative yet mount virus-specific T cell responses (32). In some instances, integrated proviral DNA and HIV-1-specific cytotoxic T lymphocytes have been detected in such seronegative persons (12, 25). Although it is not currently understood why these individuals remain antibody negative, it is possible that they were exposed to relatively small amounts of virus and their cell-mediated immune responses controlled the initial rounds of HIV-1 replication, thereby preventing the establishment of a persistent infection.

As previously reported for SIV (19, 37), the establishment of a $SHIV_{DH12R}$ infection by a mucosal route of inoculation (viz. atraumatic intravaginal infusion) was relatively inefficient. Only two of the three animals exposed multiple times to large (5×10^4 $TCID_{50}$) $SHIV_{DH12R}$ inocula became infected, and each had a benign clinical course. One of these macaques had no significant $CD4^+$ T lymphocyte loss, and the other experienced only partial depletion of this T-cell subset. Plasma viremia in both animals became undetectable by 8 weeks postinfection, and one monkey (H385) produced anti-p27 Gag but no gp120 binding antibody. This latter result suggests that more vigorous and sustained in vivo replication is required to elicit anti-gp120 antibodies.

We have estimated the in vivo infection rate constants associated with the very initial stages of $SHIV_{DH12R}$ infections in

rhesus macaques based on an analysis of the plasma virus concentration dynamics. The average infection rate constant (1.06 day^{-1}) for the nine animals in our study inoculated by the intravenous route is lower than the rate constants recently reported for rhesus macaques inoculated with the highly pathogenic SIV_{mac251}, which ranged from 1.4 to 3.2 day^{-1} (39), for monkeys infected with SIV_{smE660} and $SIV_{smE543-3}$, which ranged from 0.9 to 2.7 day^{-1} (22), and for humans infected with HIV-1, which ranged from 1.4 to 3.5 day^{-1} (16). It had been previously reported that plasma SIV RNA levels measured on day 7 postinfection correlated with levels measured during the postacute phase of infection, suggesting that host factors could exert their effect prior to full development of specific immune responses and be of critical importance for the subsequent clinical course (15). However, a similar study assessing early events during SIV infections of rhesus monkeys found no correlation between the initial virus infection rate constants and clinical outcomes (39). Our results suggest that the very initial kinetics of primate lentivirus infections in vivo may be predictive of the subsequent virus replication patterns and clinical outcomes. However, experiments with more monkeys are needed before any definite conclusions can be reached in this regard. We also estimated the initial rates of $SHIV_{DH12R}$ decline following the peak of infection and found that they are significant predictors of clinical outcome, in agreement with the conclusions reached in a study of pathogenic SIV infections (39).

The homeostatic mechanisms underlying the markedly depressed but stable $CD4^+$ T cell levels associated with the low to undetectable virus loads, as measured by plasma RT-PCR, in rhesus monkeys recovering from $SHIV_{DH12R}$ infections is not presently understood. It is quite possible that these chronically infected animals may survive indefinitely. The presence of readily measurable neutralizing antibodies in many surviving monkeys suggests that their immune systems are capable of successfully controlling a continuous virus infection. On the other hand, because the $SHIV_{DH12R}$ stock is a heterogeneous quasispecies (8), not molecularly cloned virus, it could be formally argued that the survival rate of macaques receiving the smallest virus inocula intravenously simply reflects exposure to relatively small amounts of a highly pathogenic subpopulation present in the administered virus swarm. Resolution of and recovery from the primary $SHIV_{DH12R}$ infection in these animals would therefore depend on eliminating this component of the inoculum. If nonpathogenic variants in the $SHIV_{DH12R}$ inoculum have, in fact, been selected in vivo, one might expect the $CD4^+$ T cell counts to have increased in the surviving monkeys, an outcome that was not observed. An alternative explanation for persistently low levels of $CD4^+$ T lymphocytes is that even though a highly cytopathic virus component in the challenge stock was eliminated in vivo, $CD4^+$ T-cell subsets, possibly lost irreversibly during the primary infection, can never be or are very slowly being replenished (26, 30). These questions could be partially resolved by isolating virus from animals chronically infected with $SHIV_{DH12R}$ and inoculating naïve monkeys with the recovered virus. If, in fact, the recovered SHIV fails to induce disease following intravenous inoculation, one might conclude that the asymptomatic clinical course for the surviving macaques is due to selection in vivo of a relatively nonpathogenic virus. Whether or not this system models the disease-free period experienced by HIV-1-infected individuals following the resolution of their primary virus infections remains to be determined.

As noted earlier, gp120 epitopes associated with the DH12 family of primate lentiviruses appear to be the sole targets of neutralizing antibodies elicited in chronically infected nonhuman primate species (M. W. Cho, unpublished data). This conclusion is also consistent with results presented in this report showing that the two animals (H358 and H385) generating anti-p27 but no detectable anti-gp120 antibodies (Fig. 3 and 4C) failed to neutralize $SHIV_{DH12R}$ (Table 2 and Results). The capacity of surviving $SHIV_{DH12R}$-infected macaques to produce neutralizing antibodies has been exploited to monitor the evolution of a primate lentivirus quasispecies during long-term passage in vivo. As is the case for HIV-1-infected persons (24), a chronically $SHIV_{DH12R}$-infected rhesus monkey (6071) was unable to neutralize an autologous contemporary virus isolate ($SHIV_{DH12R(W30)}$), although neutralizing antibody against the originally inoculated $SHIV_{DH12R}$ continued to be produced (Table 2 and Fig. 6). This same animal subsequently generated neutralizing antibody against $SHIV_{DH12R(W30)}$. Not unexpectedly, the initial virus quasispecies evolved differently in a second $SHIV_{DH12R}$-inoculated macaque, which never made neutralizing antibodies against $SHIV_{DH12R(W30)}$. This result raises interesting possibilities relevant to vaccine development that could be examined with the $SHIV_{DH12R}$ system described. For example, it could be argued that virus quasispecies escape merely reflects the de novo emergence of novel gp120 genotypes, a consequence of in vivo selection and the error-prone reverse transcription reaction. Alternatively, it is possible that the $SHIV_{DH12R}$ quasispecies undergoes antigenic selection but not genotypic change by expansions and contractions of its constituent viral subpopulations, thereby altering the capacity of an earlier humoral response to control a contemporary virus swarm. Irrespective of these explanations, the persistence of neutralizing antibody directed against the initial $SHIV_{DH12R}$ quasispecies is most consistent with the continued presence and active replication of the original SHIV swarm. Some of these possibilities can be examined by biological and molecular characterizations of virus subsequently recovered from animals initially exposed to the same $SHIV_{DH12R}$ quasispecies.

ACKNOWLEDGMENTS

We are indebted to Carol Clarke, Charles Thornton, and Russ Byrum for their diligence and assistance in the care and maintenance of our animals. We are also grateful to Vanessa Hirsch for providing SIV-infected macaque plasma, Michael Cho for supplying gp120 expressed from recombinant vaccinia virus-infected cells, and Michael Eckhaus, Georgina Miller, and David Green for pathological analyses.

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