JOURNAL OF VIROLOGY, Mar. 2000, p. 2584–2593 Vol. 74, No. 6
0022-538X/00/$04.00+0

Containment of Simian Immunodeficiency Virus Infection: Cellular Immune Responses and Protection from Rechallenge following Transient Postinoculation Antiretroviral Treatment

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Received 20 August 1999/Accepted 23 December 1999

To better understand the viral and host factors involved in the establishment of persistent productive infection by primate lentiviruses, we varied the time of initiation and duration of postinoculation antiretroviral treatment with tenofovir {9-[2-(*R*)-(phosphonomethoxy)propyl]adenine}while performing intensive virologic and immunologic monitoring in rhesus macaques, inoculated intravenously with simian immunodeficiency virus SIVsmE660. Postinoculation treatment did not block the initial infection, but we identified treatment regimens that prevented the establishment of persistent productive infection, as judged by the absence of measurable plasma viremia following drug discontinuation. While immune responses were heterogeneous, animals in which treatment resulted in prevention of persistent productive infection showed a higher frequency and higher levels of SIV-specific lymphocyte proliferative responses during the treatment period compared to control animals, despite the absence of either detectable plasma viremia or seroconversion. Animals protected from the initial establishment of persistent productive infection were also relatively or completely protected from subsequent homologous rechallenge. Even postinoculation treatment regimens that did not prevent establishment of persistent infection resulted in downmodulation of the level of plasma viremia following treatment cessation, compared to the viremia seen in untreated control animals, animals treated with regimens known to be ineffective, or the cumulative experience with the natural history of plasma viremia following infection with SIVsmE660. The results suggest that the host may be able to effectively control SIV infection if the initial exposure occurs under favorable conditions of low viral burden and in the absence of ongoing high level cytopathic infection of responding cells. These findings may be particularly important in relation to prospects for control of primate lentiviruses in the settings of both prophylactic and therapeutic vaccination for prevention of AIDS.

The early stage of primary infection with the primate lentiviruses (human immunodeficiency virus (HIV) and simian immunodeficiency virus [SIV]) is an extremely dynamic period during which important aspects of the virus-host relationship are established, with far-reaching ramifications for the subsequent course of infection (18, 19, 30, 39, 51). It is a striking feature of infection with the primate lentiviruses that clearance of the initial infection is distinctly unusual. Recently it has been postulated that a major factor contributing to the failure of host containment of HIV infection is the early and continuing loss of T-cell help for development and maintenance of effective cytotoxic T-lymphocyte (CTL) responses, as a consequence of the impact of HIV infection on the $CD4^+$ helper T-cell compartment (23, 31–33, 50, 55).

Transient postinoculation antiretroviral treatment has been shown to be capable of preventing the establishment of productive persistent SIV infection (43, 45, 47). Kinetic studies have shown that both the delay between inoculation and the initiation of treatment and the duration of the treatment period can critically influence the effectiveness of postinoculation treatment in preventing the establishment of persistent infection (43, 45, 48, 49). However, most of these SIV studies have been designed as empirical animal model studies of postexposure prophylaxis regimens, and the underlying virological and host mechanisms responsible for the protective effect have not been defined. In particular, the potential contribution of immune mechanisms has not been addressed, nor has the possibility that animals protected from establishment of persistent infection by postinoculation antiretroviral treatment may be protected from subsequent rechallenge, although these issues have been explored in murine retroviral models (20, 35–38). Similarly, previous studies have suggested but not definitively demonstrated the ability of postinoculation treatment to produce a beneficial downmodulation of viral replication, following withdrawal of drug treatment, even if the establishment of persistent infection is not prevented (43, 48, 52).

Postinoculation antiretroviral treatment which prevents establishment of persistent infection, or allows persistent infection but results in sufficient sustained downmodulation of viral replication to diminish or eliminate pathogenesis and improve clinical course, might be a useful alternative model in which to study virus-host interactions of the type that might characterize

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the response of an effectively vaccinated host. Observations in this system might be expected to complement findings obtained in more traditional empirical immunization-challenge vaccine studies. In addition, this model could provide insights into the pathogenetic mechanisms that are responsible for the failure of most hosts to effectively contain primate lentivirus infection, clarifying this process. Therefore, building on previous results, we designed a study of postinoculation antiretroviral treatment, using regimens of different anticipated effectiveness, based on different delays between inoculation and initiation of treatment and different durations of treatment. The study design included intensive virological and immunological monitoring, including evaluation of SIV-specific proliferative responses as a cellular immune parameter potentially reflective of the type of responses whose loss in early infection has been implicated as a key defect in HIV-infected patients (23, 31–33, 50).

We identified postinoculation treatment regimens that effectively prevented the establishment of persistent productive SIV infection. In protected animals, we observed the development of anti-SIV proliferative responses during the treatment period, despite the absence of detectable plasma viremia or seroconversion, implying cellular immunological sensitization from very low level or anatomically restricted viral replication despite apparently effective antiretroviral therapy. Animals protected against establishment of persistent infection were also partially or completely protected against rechallenge with homologous pathogenic virus.

Even for animals treated with suboptimal drug regimens, in which the establishment of persistent infection was not prevented, the level of viral replication and pattern of infection were modulated by early postinoculation treatment, relative to untreated controls, animals treated with known ineffective regimens, or the cumulative experience with the natural history of plasma viremia in SIVsmE660-infected rhesus macaques. While measurable levels of plasma virus were detected after drug discontinuation, for many of these animals plasma viremia was self-limited, showing repeated blunted peaks and subsequent spontaneous declines, often below the threshold for detection. The results imply that immunological control of primate lentiviral infection is possible, under conditions where the initial immunological sensitization does not occur in the setting of a large viral burden and extensive cytopathic infection of $CD4^+$ helper T cells.

MATERIALS AND METHODS

Animals and animal procedures. The 18 female rhesus macaques (*Macaca mulatta*) used for this study ranged in age from 3 to 5 years and in weight from 3 to 5 kg at the time of study initiation. All animal housing and care provided and research performed were in conformance with *Guide for the Care and Use of Laboratory Animals* (9a). The National Cancer Institute Animal Care and Use Program is fully accredited by AAALAC [Association for Assessment and Accreditation of Laboratory Animal Care] International.

Blood samples were obtained from the femoral veins alternating between the left and right, under anesthesia with Telazol given intramuscularly at 3 mg/kg of body weight (combination of Tiletamine HCl and Zolazepam HCl; 100 mg/ml; Robbins). For sequential lymph node biopsies (left axillary, right axillary, and left inguinal), anesthesia was induced with Telazol (6 mg/kg intramuscularly) and maintained with 1.5 to 2% isoflurane administered via endotracheal tube.

Viruses. Animals were inoculated with SIVsmE660 (20 50% macaque infective doses [MID_{50}] intravenously [i.v.]) (15) propagated in peripheral blood mononuclear cells (PBMC) from pigtailed macaques (*Macaca nemestrina*). The virus stock had a titer of $10^{4.3}$ MID_{50}, based on in vivo titration in pigtailed macaques, and a similar titer in rhesus macaques (V. M. Hirsch, unpublished data). Conformationally intact whole SIV virions inactivated by treatment with 2,2′-dithiodipyridine to achieve covalent modification of the nucleocapsid protein $p8^{NC}$ were prepared from the clonal producer cell line SIVmne E11S (3) as described elsewhere (34) and were used as the stimulating antigen for SIV antigen-specific proliferation assays. Untreated lysed SIVmneE11S-derived virions were used as the antigen for Western blot analysis (see below).

TABLE 1. Experimental groups and tenofovir treatment regimens

Group	Tenofovir treatment		Animals[a]
	Start (h p.i.)	Duration (days)	
I.1	None		Rh 058, Rh 128
I.2	None		Rh 106, Rh 294
II	24	3	Rh 068, Rh 138
III	24	28	**Rh 009**, **Rh 120**, **Rh 300**, Rh 313
IV	72	28	Rh 125, Rh 155, Rh 184, Rh 225
V	72	63	Rh 027, Rh 056, Rh 092, Rh 110

[a] Boldface denotes animals in rechallenged 6 weeks following drug discontinuation.

Tenofovir treatment and experimental groups. Tenofovir {9-[2-(*R*)-(phosphonomethoxy)propyl]adenine} (10, 42–48) was generously provided by Gilead Sciences, Inc. (Foster City, Calif.). The drug (30 mg/kg) was administered once daily through the treatment period by subcutaneous injection at rotating sites. Previous studies have demonstrated that due to the pharmacokinetic properties of tenofovir and intracellular accumulation of the compound, this regimen is sufficient to maintain effective drug levels.

Experimental groups and tenofovir treatment regimens were as follows: group I.1, untreated controls ($n = 2$); group I.2, untreated controls ($n = 2$); group II, tenofovir started 24 h postinfection p.i. and continued for 3 days ($n = 2$); group III, tenofovir started 24 h p.i. and continued for 28 days ($n = 4$); group IV, tenofovir started 72 h p.i. and continued for 28 days ($n = 4$); group V, tenofovir started 72 h p.i. and continued for 63 days ($n = 4$) (Table 1).

Specimens and specimen preparation. Cell-free plasma was separated by centrifugation, and PBMC were prepared by density centrifugation from EDTA-anticoagulated whole blood. Lymph node mononuclear cells (LNMC) were prepared from fresh lymph node specimens by mechanical disruption using wire mesh screens. Samples were either tested fresh, viably cryopreserved, or stored at −70°C until use, depending on the analysis performed.

Virological evaluation. (i) Plasma SIV RNA. Levels of virion-associated SIV RNA in plasma were determined using a real-time reverse transcription-PCR procedure essentially as described elsewhere (40). The nominal threshold sensitivity of the assay is 300 copy eq/ml of plasma, while the interassay variation is ≤25% (coefficient of variation).

(ii) PBMC and LNMC SIV DNA. Levels of cell-associated SIV DNA were determined for PBMC and LNMC by real-time PCR essentially as described elsewhere (34). Total DNA was isolated using a commercial kit (PureGene; Gentra Systems, Minneapolis, Minn.) according to the manufacturer's recommendations. SIV *gag* DNA sequences were quantitated by real-time PCR using an assay with a lower limit of quantitation of 10 copy eq/reaction, and results were normalized relative to the copy number determined in the same extracted sample for porphobilinogen deaminase, a single-copy genomic sequence (34). Results were expressed as SIV copy eq/10^5 diploid genome eq.

(iii) Virus isolation. To evaluate the presence of infectious virus in PBMC or LNMC samples, mononuclear cells were isolated by density centrifugation from whole blood or a single-cell suspension of lymph node tissue, respectively. PBMC (10^6) were stimulated with phytohemagglutinin-P (1:50 dilution; Gibco) for 3 days and then cocultivated with 10^6 cells of the highly SIV susceptible indicator cell line AA2, clone 5 (8). Cells were cultured in quadruplicate for 8 weeks in complete medium (RPMI 1640 medium supplemented with 10% [vol/vol] heat-inactivated human AB serum, 2 mM L-glutamine, and antibiotics) supplemented with recombinant human interleukin-2 (50 U/ml; PeproTech, Rocky Hill, N.J.), with weekly medium changes. Supernatants were collected at the time of medium changes and tested for the presence of SIV $p27^{gag}$ as an index of productive viral infection, using a capture immunoassay (AIDS Vaccine Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md.).

Immunological evaluation. (i) Cellular responses. PBMC isolated by density centrifugation from EDTA-anticoagulated whole blood were seeded in round-bottom 96-well culture plates in RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 mM L-glutamine, and antibiotics (complete medium), with or without inactivated SIV virions (300 ng of SIV $p27^{gag}$ eq/ml, final concentration), with triplicate wells for each sample. Negative controls included wells seeded without antigen and wells seeded with microvesicles, prepared as described previously (5) from uninfected cultures of the same cells used to propagate the virus used as antigen. The use of microvesicles serves as a control to ensure that responses observed are directed against viral determinants rather than xenodeterminants present in the purified virus preparation and derived from the human cells used to propagate the virus used as antigen in the proliferation assay (5). Stimulation with phytohemagglutinin-P (1:50 dilution; Gibco) served as a positive control for the ability of PBMC to respond to proliferative stimuli. After 5 days of culture, plates were pulsed with [^{3}H]thymidine (1 μCi/well; 6.4 Ci/mmol; New England Nuclear) and harvested the following day. Corrected stimulation indices (SIs) were calculated as the mean value

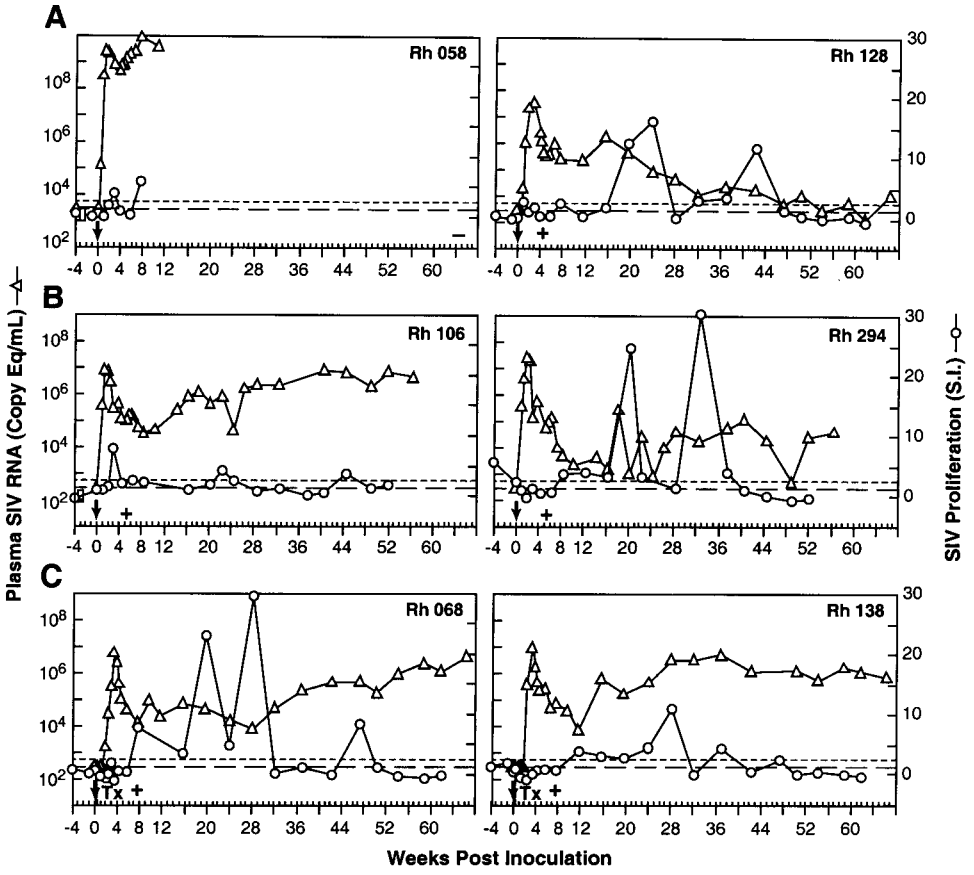

FIG. 1. Plasma viremia and SIV-specific proliferative responses for control groups. (A) Group I.1 (untreated control animals challenged at time of challenge of groups II and III). Incomplete data set for animal Rh 058 is due to death. (B) Group I.2 (untreated control animals challenged at time of challenge of groups IV and V). (C) Group II (tenofovir treatment starting at 24 h p.i. and continued for 3 days). Long-dashed line indicates threshold sensitivity of SIV RNA assay (300 copy eq/ml); short-dashed line indicates background for SIV proliferation assay (SI = 2.5). Solid arrow indicates SIV inoculation; + symbol along *x* axis indicates time of seroconversion (reactivity with ≥2 viral proteins by Western blotting); — at far right of *x* axis indicates that animal did not seroconvert during the duration of follow-up. Shaded box along *x* axis (Tx) indicates treatment duration (panel C, group II only).

for stimulated wells minus the mean value for medium control wells, divided by the mean value for the medium control wells. In testing of 102 independent samples from 62 different SIV-naive macaques (*M. mulatta*), the measured proliferative responses averaged 0.80 + 1.60 (standard deviation), with only three measurements exceeding a value of 2.5. Based on this testing, SIs of greater than 2.5 are considered positive (J. L. Rossio, unpublished data).

(ii) Antibody responses. Humoral responses were evaluated by immunoblotting. Following separation of proteins of lysed, purified SIVmneE11S virions on nondenaturing sodium dodecyl sulfate-polyacrylamide (5 to 20%) gels, proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore), and assay strips were prepared. Sera were tested at 1:500, and bound antibodies were detected using an alkaline phosphatase-conjugated secondary antibody and a chemiluminiscent procedure essentially as recommended by the manufacturer (Amersham, Arlington Heights, Ill.).

RESULTS

Infection of rhesus macaques with SIVsmE660 typically results in a defined range of viral replication patterns with associated ranges of pathogenesis and disease course (18, 19). In combination with the results from animals in the control groups in the present study, this provides a frame of reference for evaluation of results in animals receiving tenofovir according to different postinoculation treatment regimens.

Control groups. Virological and immunological parameters were followed for untreated control animals (groups I.1 and I.2), animals treated with a regimen not expected to significantly affect the course of infection (group II), and animals treated for various durations, beginning at various intervals p.i. (groups III, IV, and V). As shown in Fig. 1A and B, infection of the four untreated control animals in Groups I.1 and I.2 resulted in an exponential increase in plasma viral load following inoculation, peaking approximately 2 weeks p.i., with variable decreases subsequently. One of the two animals in group I.1 (Rh 058) showed a pattern with very high peak viral load levels, negligible downregulation of viral replication, and then a subsequent increase, characteristic of a "rapid progressor" animal (18, 19). The second animal in group I.1 (Rh 128) showed moderate downregulation of plasma viral RNA after the peak was reached and stabilization in the range of approximately 10^5 copy eq/ml in the postacute period (weeks 6 to 16 p.i.), with a further subsequent decrease to levels that are unusual in SIVsmE660-infected animals (18, 19). In group I.2, animal Rh 106 showed a more typical pattern of peak plasma viremia, with some postpeak decrease and eventual stabilization in the range of approximately 10^6 copy eq/ml (Fig. 1B). Animal Rh 294 showed marked reductions from the levels of virus seen at peak plasma viremia, with postacute levels both lower and more variable than is typical for SIVsmE660-infected animals (18, 19) (Fig. 1B).

Based on past studies (43), the treatment regimen for group II (tenofovir for 3 days, beginning 24 h p.i.) was not expected to prevent persistent infection; indeed, it was included in the experimental design primarily to demonstrate that any protective effects seen with longer treatment courses begun at 24 h

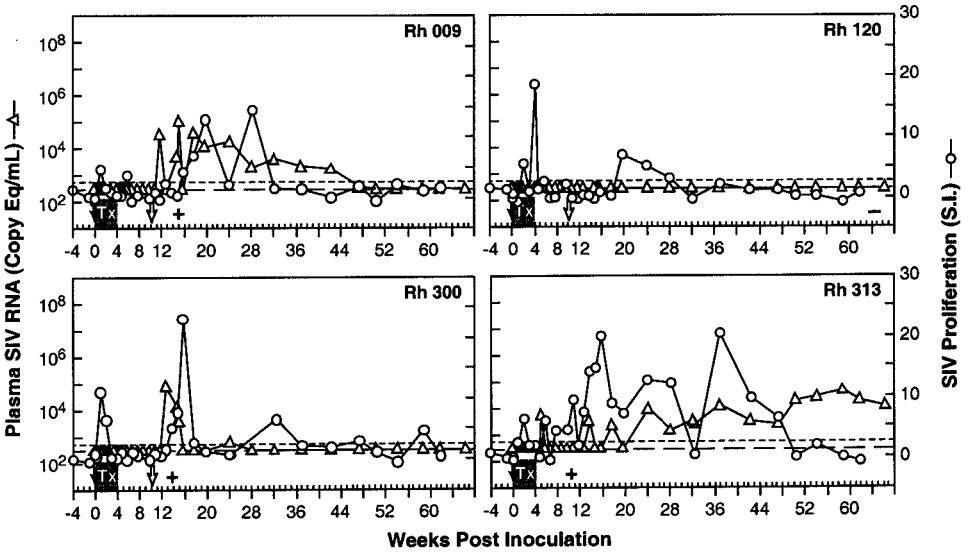

FIG. 2. Plasma viremia and SIV-specific proliferative responses for group III, tenofovir treatment starting at 24 h p.i. and continued for 28 days. Arrows indicate SIV inoculation. Open arrows indicate time of rechallenge for animals Rh 009, Rh 120, and Rh 300. All graphing conventions are as for Fig. 1.

p.i. were not simply due to blockade of the initial round of infection but required a longer duration of treatment beginning at 24 h p.i. As shown in Fig. 1C, both animals in group II (Rh 068 and Rh 138) showed plasma viremia patterns that were essentially typical of untreated animals (18, 19), with perhaps a few-day delay to peak viremia as a consequence of the brief treatment (Fig. 1C).

For the animals in groups I.1, I.2, and II, above-background SIV-specific proliferative responses were limited in frequency and magnitude during the acute phase of infection through the first 8 weeks p.i.; all animals but one seroconverted for reactivity with SIV antigens between 4 and 8 weeks p.i. The exception was animal Rh 058, which never seroconverted, as is characteristic of animals that show a rapid progressor phenotype (18, 19) (Fig. 1).

Tenofovir treatment for 28 days. (i) Beginning 24 h p.i. The above profiles of viral replication and SIV-specific immune responses, and cumulative experience with SIVsmE660 infection of rhesus macaques, provide a context for evaluating the results obtained with the different tenofovir treatment regimens that are the crux of the present study. In striking contrast to the animals in control groups I.1, I.2, and II, for the animals in group III (Rh 009, Rh 120, Rh 300, and Rh 313), no measurable plasma viremia was apparent, either during the 28 days of tenofovir treatment or, for three of four animals, during a 6-week period following discontinuation of treatment (Fig. 2). However, above-background SIV-specific proliferative responses were readily measurable during the treatment period, despite the absence of sufficient viral replication to register as measurable plasma viremia and despite the lack of seroconversion. Viral DNA was not detectable in PBMC or LNMC on the last day of tenofovir treatment or, with one exception, 1 week after cessation of tenofovir treatment; virus was not isolated from the lymph node samples from these animals, despite the ability to readily detect viral DNA and isolate virus from samples from the untreated control animals (Table 2).

Six weeks following drug discontinuation, the three animals that had shown no measurable plasma viremia during the treatment period or during the 6 weeks after treatment was stopped were rechallenged i.v. with 20 MID_{50} of SIVsmE660. As shown in Fig. 2, one of these animals, Rh 120, appeared to be completely protected following this homologous rechallenge, with no evidence of viremia during the follow-up period, although interestingly, there was a transient increase in anti-SIV proliferative responses in the postrechallenge period. Rh 120 has remained seronegative, virus isolation negative, and PBMC DNA PCR negative throughout more than 1 year of follow-up. Animals Rh 009 and Rh 300 showed relative protection following the rechallenge. Measurable plasma viremia was present following rechallenge (Fig. 2); however, in striking contrast to the primary viremia seen in the animals in control groups I.1, I.2, and II (Fig. 1), the initial viremia seen following rechallenge in animals Rh 009 and Rh 300 was self-limited, with multiple blunted peaks and spontaneous intervening declines eventually decreasing to below the threshold for measurement and remaining below this threshold for the duration of the follow-up period. Following rechallenge, both Rh 009 and Rh 300 seroconverted and showed multiple peaks of anti-SIV proliferative responses with intervening declines (Fig. 2). After the rechallenge, SIV DNA was transiently detectable in PBMC from Rh 009 but not from Rh 300.

One animal from group III (Rh 313) was not rechallenged (Fig. 1C). During the 6 weeks following cessation of tenofovir treatment, this animal showed measurable plasma viremia (5,100 copy eq/ml) on a single time point following discontinuation of tenofovir, without seroconversion, and was not rechallenged on this basis. Of note, Rh 313 was the only animal in which we were able to detect measurable amounts of SIV DNA in the lymph node sample obtained 1 week after stopping tenofovir treatment (Table 2). Subsequently, Rh 313 showed multiple blunted peaks of reemergent plasma viremia with intervening decreases, initially to below the threshold for detection, before stabilizing in the range of 10^4 copy eq/ml toward the end of the follow-up period. Rh 313 also showed multiple peaks and intervening declines in anti-SIV proliferative responses of the follow-up period, and this animal seroconverted following the emergence of measurable levels of plasma viremia after discontinuation of tenofovir treatment (Fig. 2).

(ii) Beginning 72 h p.i. Delaying initiation of tenofovir treatment to 72 h p.i. resulted in measurable levels of plasma viremia during the initial part of the treatment period in two of the

TABLE 2. PBMC and LNMC SIV DNA PCR and virus isolation[a]

Group	Animal	Last day on tenofovir treatment, or day 28 for untreated controls			1 or 4 wk[d] after stopping tenofovir treatment, or day 28 for untreated controls		
		DNA PCR[b]		Virus isolation from LNMC (ng/ml)[c]	DNA PCR		Virus isolation from LNMC (ng/ml)
		PBMC	LNMC		PBMC	LNMC	
I.1	Rh 058	1,600	2,500	+ (46.4)**	ND	ND	ND
	Rh 128	260	160	+ (16.5)	ND	ND	ND
I.2	Rh 106	ND	860	+ (10.5)	ND	ND	ND
	Rh 294	ND	720	+ (25.0)	ND	ND	ND
II	Rh 068	ND	<3	ND	ND	910	ND
	Rh 138	<3	<3	ND	ND	1,500	ND
III	Rh 009	<7	<5	Neg	ND	<18	Neg
	Rh 120	<7	<14	Neg	ND	<12	Neg
	Rh 300	<4	<3	Neg	ND	<6	Neg
	Rh 313	<4	<3	Neg	ND	4	Neg
IV	Rh 125	<13	<170	Neg	<11	ND	Neg
	Rh 155	<8	<8	Neg	<3	<3	Neg
	Rh 184	<7	<87	Neg	<8	<8	Neg
	Rh 225	<13	<3	Neg	<5	<15	Neg
V	Rh 027	<8	<4	Neg	<8	<5	Neg
	Rh 056	<5	<8	Neg	17	19	Neg
	Rh 092	<9	<3	Neg	<7	<5	Neg
	Rh 110	<2	<4	Neg	<2	<7	Neg

[a] ND, not determined; Neg, negative.
[b] SIV *gag* copy eq/10^5 diploid genome of total DNA, based on quantitation of porphobilinogen deaminase (34).
[c] Supernatant p27 content on day 28 of culture.
[d] Four weeks after stopping tenofovir treatment for group II.

four animals in group IV (Rh 125 and Rh 225) (Fig. 3). This viremia resolved over the treatment period, and no plasma virus was detectable in either animal prior to drug discontinuation (Fig. 3). The other two animals in group IV (Rh 155 and Rh 184) showed no measurable viremia during the treatment period. Variable levels of SIV-specific proliferative responses were observed, in some instances greater than the peak proliferative responses seen in the treated animals in group III. All four animals in group IV showed emergent plasma viremia after treatment was stopped, but the magnitude of the peak viremia was decreased relative to the peak acute viremia in untreated control animals, and the delay from drug discontinuation to the appearance of measurable levels of plasma virus was greater than the interval from inoculation to the onset of measurable plasma viremia in the untreated control animals (compare Fig. 3 to Fig. 1). For two of the four animals in group

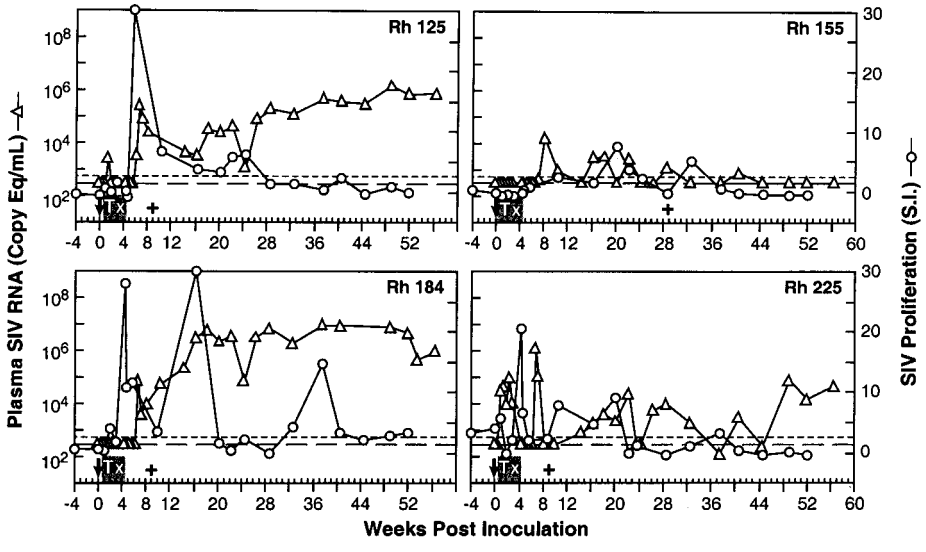

FIG. 3. Plasma viremia and SIV-specific proliferative responses for group IV, 28 days of tenofovir treatment starting at 72 h p.i. Graphing conventions are as for Fig. 1.

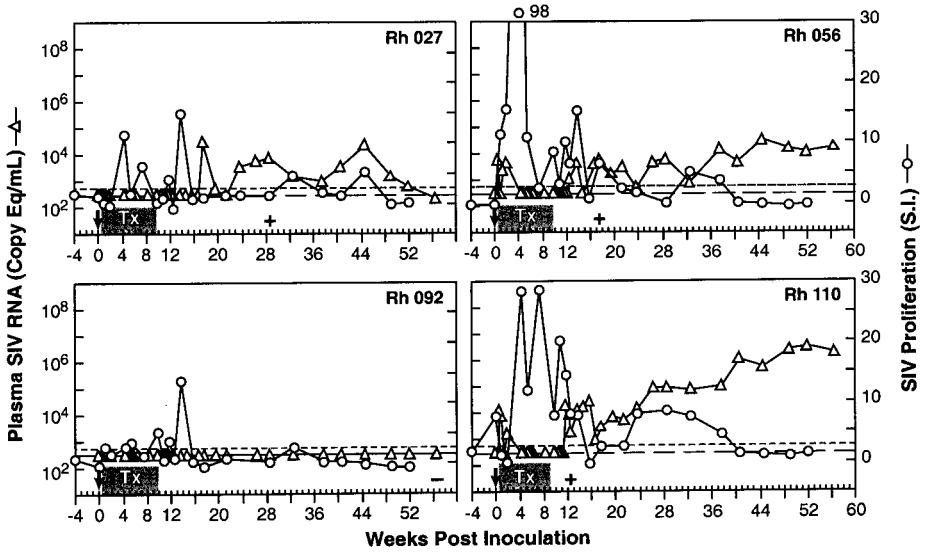

FIG. 4. Plasma viremia and SIV-specific proliferative responses for group IV, 63 days of tenofovir treatment starting at 72 h p.i. Graphing conventions are as for Fig. 1.

IV (Rh 125 and Rh 184), plasma viral load levels at the end of the follow-up period were in the range expected for untreated animals. All four animals seroconverted. Variable anti-SIV proliferative responses were observed (Fig. 3).

Tenofovir treatment for 63 days beginning 72 h p.i. Extension of the duration of treatment appeared to compensate in part for delaying the initiation of treatment to 72 h p.i. (Fig. 4). Although two of the four animals in group V (Rh 056 and Rh 110) showed measurable plasma viremia during the initial portion of the treatment period, this decreased to below the threshold of detection well before the discontinuation of treatment. One animal (Rh 092) showed no measurable viremia during treatment, or over more than 1 year of follow-up after discontinuation of tenofovir administration, although interestingly this animal showed transient boosting of the anti-SIV proliferative response following cessation of tenofovir treatment, despite the absence of measurable viremia or seroconversion (Fig. 4). The three remaining animals showed measurable levels of plasma virus and seroconversion after drug discontinuation, but both the extended delay to the appearance of measurable virus and the lower peak values seen for plasma viremia following drug cessation suggested better control of the emerging virus with extended treatment (compare Fig. 3 and 4). The mean peak posttreatment plasma level over the first 8 weeks after treatment cessation was significantly lower for the group that received 63 days treatment than for the group that was treated for 28 days ($p = 0.0286$; exact Wilcoxon rank sum test on log transformed data), while trends for longer times to first measurable plasma viral RNA level and to peak off-treatment level after treatment was stopped clearly favored the 63-day treatment group but did not reach statistical significance. All three animals with measurable posttreatment viremia (Rh 027, Rh 056, and Rh 110) showed multiple peaks and intervening declines in plasma viral load, with levels rising into the range typical of untreated animals toward the end of the follow-up period in one animal (Rh 110), suggesting a change in the virus-host balance. Variable levels of anti-SIV lymphoproliferative responses were observed through the follow-up period, although above background responses were not seen at every time point (Fig. 4).

DISCUSSION

We identified postinoculation treatment regimens that prevented the establishment of productive persistent infection following i.v. administration of a known infectious SIV inoculum, as judged by the absence of measurable plasma viremia following drug discontinuation. This effect was not due simply to blockade of the initial infection, as animals that were started on treatment at the same time but in whom treatment was continued for a much shorter interval showed a plasma viremia pattern typical of untreated control animals. Cells from all animals were readily susceptible to SIV infection vitro (data not shown), making it unlikely that the results reflect any inherent resistance to infection by some animals. Of note, the protective response observed with optimal treatment regimens was observed in animals that showed readily measurable anti-SIV lymphocyte proliferative responses during the treatment period despite the absence of detectable plasma viremia or seroconversion. In comparison to animals that showed no evidence of emergent plasma viremia upon discontinuation of treatment (Rh 009, Rh 092, Rh 120, and Rh 300), above-background anti-SIV proliferative responses were less frequent and of lesser magnitude through the initial 4 weeks of infection in untreated (groups I.1 and I.2) and ineffectively treated (group II) animals that experienced high levels of plasma viremia during primary infection ($P = 0.01$ and $P = 0.04$ for frequency and magnitude, respectively; Fisher's exact test for protected animals versus pooled groups I.1, I.2, and II). Treated animals that did not show emergence of plasma viremia upon discontinuation of drug treatment after the initial inoculation were completely (Rh 120) or partially protected (Rh 009 and Rh 300) from homologous rechallenge, as judged by plasma viremia following the rechallenge. The partial protection from rechallenge was characterized by a delayed and blunted primary viremia, which spontaneously resolved, leading to sustained control of viral replication. While alterations in the replicative properties of the virus or permissiveness of host cells due to nonimmune mechanisms could theoretically contribute to these observations, preliminary studies do not support these explanations. Viral interference-based explanations for the apparent protection observed here seem unlikely

for many of the same reasons that such explanations seem unlikely to explain protective effects of live attenuated SIV vaccines (22).

Delaying the initiation of treatment lessened its effectiveness, while extending the duration of treatment partially compensated for its delayed initiation. However, even in those animals that developed persistent productive infection following short-term treatment with suboptimal regimens of postinoculation treatment, for most we observed a sustained downmodulation of viral replication relative to the typical pattern of plasma viremia in SIVsmE660-infected animals (18, 19) (Fig. 1).

Evaluation of this study is complicated by the fact that two untreated control animals (Rh 128 and Rh 294) showed atypically low postacute levels of plasma viremia. However, even allowing for this confounding effect, which likely reflects an extreme in the spectrum of response of different individual macaques to infection with SIVsmE660, in aggregate, the data strongly suggest that transient postinoculation treatment can produce a substantial and sustained modulation of viral replication patterns, with effects persisting long after treatment discontinuation. This limitation of viral replication was also associated with sparing from the depletion of $CD4^+$ T cells typical of infection with SIVsmE660 (data not shown).

The frequent detection of SIV-specific proliferative responses during treatment, despite the absence of measurable plasma viremia, suggests that there was ongoing viral replication that was at very low levels or anatomically contained. Under such circumstances, the initial immunological sensitization in these animals may be quite different than that which occurs during the typical course of untreated infection, with disseminated infection and high-level viremia with cytopathic virus. The emergence of apparently self-limited cycles of blunted peaks of viremia following discontinuation of treatment in some animals is strongly suggestive of an ongoing dynamic interaction between virus and host, with active host control of the infection. Periodic peaks of anti-SIV proliferative responses may be one reflection of host responses attempting to control the infection, although there was not a simple absolute relationship between the presence or magnitude of such responses and viral load levels or sustained, effective control of plasma viremia.

In one recently proposed mathematical model of the pathogenesis of primate lentiviral infection, successful containment of the infection requires the development of an effective CTL memory response, which in turn is dependent on the presence of adequate T-cell help from $CD4^+$ cells (53). Absent such help, effector CTLs may be generated, but they are unable to contain the infection (6, 7, 23–25, 31–33, 41, 42, 55). According to the model, establishment of persistent SIV infection and disease progression are due to the failure to generate a CTL-memory response, which in turn is the result of $CD4^+$ T-cell impairment by the virus. Treatment during primary infection minimizes the degree of virus-induced helper T-cell impairment. Postinoculation treatment helps to ensure that the initial immunological sensitization occurs in a setting that minimizes ongoing infection of responding $CD4^+$ T cells and possible indirect SIV-related immunopathological effects. The resulting preservation of sufficient capacity for effective T cell help allows expansion of the CTL precursor population and the establishment of CTL memory, permitting long-term immunological control of the infection after withdrawal of therapy, when the virus attempts to reemerge from pharmacological suppression in the setting of a preexisting effective immune response. A contribution to viral control from noncytolytic viral suppressive activity of $CD8^+$ cells (26) upon treatment cessation is also accommodated in the model (54).

According to such models, postinoculation treatment should allow the development during primary infection of effective anti-SIV responses by $CD4^+$ cells, and our measurements of SIV-specific proliferative responses are broadly consistent with this notion. The in vivo functional significance of antigen-specific $CD4^+$ T-cell proliferative responses measured in vitro is unclear. However, $CD4^+$ T-cell responses that are indirectly measured by assessment of in vitro antigen-specific proliferation may contribute to containment of infection by several mechanisms, including importantly the provision of T-cell help for the development of effective CTL function and memory (6, 7, 23–25, 31–33, 41, 42, 55), as well as the elaboration of cytokines that may have antiviral effects. Although the present study did not include any direct measurements of $CD8^+$ T-cell function, this postulated involvement of both $CD4^+$ and $CD8^+$ T-cell compartments is also consistent with work in murine retrovirus systems in which transient postinoculation treatment with antiretroviral drugs modulated both viremia and disease course and conferred protection from subsequent homologous rechallenge (35–38). Like the resistance engendered by attenuated murine retrovirus vaccines (12), this resistance to challenge could be adoptively transferred but required transfer of both $CD4^+$ and $CD8^+$ cells (20). Direct parallel measurements of the functional activity of $CD4^+$ T cells and both cytolytic and noncytolytic activities of $CD8^+$ T cells will be an important component of future SIV studies of postinoculation antiretroviral therapy.

Postinoculation tenofovir treatment did not prevent SIV initial infection but did suppress viral replication below the threshold of detection, as measured by reverse transcription-PCR for virion-associated viral RNA in plasma. However, the observation of SIV-specific proliferative responses during the treatment period in the absence of measurable plasma viremia strongly suggests that suppression of viral replication was incomplete, with a level of replication that was quantitatively too low or anatomically not in sufficient continuity with the plasma compartment to yield a measurable level of plasma viremia. This extent of viral replication did, however, appear to be able to induce protective host responses. Indeed, this study may be considered as a model in which sensitization by the effective equivalent of an attenuated virus vaccine is achieved by pharmacological means rather than the use of a mutant virus (11, 21). An important aspect of future studies will be efforts to further clarify the ways in which host responses induced by postinoculation drug treatment are similar to or different from those induced by more conventional vaccination strategies. As many vaccine candidates seem to provide less protection than observed here, despite more readily measurable immune responses, this may provide insight into the nature of protective responses and lead to the design and development of more effective vaccine candidates.

Recent attention has focused on CD4 cell proliferative responses as a measure of antiviral immunity in HIV-1-infected subjects. While it would be ill advised to focus excessively on a single parameter in what is clearly a complex process involving multiple interdependent factors, consideration of virus-specific proliferative responses, followed here as one measurable parameter of host response, may provide some insight into pathogenesis. Rosenberg et al. (32) found a negative correlation between proliferative responses and virus load, i.e., long-term nonprogressors were characterized by high proliferative responses and low virus load, while more typical patients had low or unmeasurable proliferative responses and higher viral loads.

In this study, we observed a decline of proliferative re-

sponses over time after the discontinuation of therapy. However, this does not necessarily always indicate a loss of immunological control. Indeed, comparing averaged CD4 proliferative responses and virus load among different animals following withdrawal of treatment reveals a one-humped dependence of proliferative responses on virus load (D. Wodarz, K. Page, R. A. Arnaout, A. R. Thomsen, J. D. Lifson, and M. A. Nowak, submitted for publication), indicating that it is the relative relationship between response and viral load rather than the absolute magnitude of the response that is of critical importance. This is in accordance with predictions by a mathematical model in which loss of virus control is associated with low proliferative responses and high virus load because the degree of virus-induced immune impairment dominates over the amount of antigenic stimulation of the immune response (Wodarz et al., submitted). An increase in the strength of the immune response relative to the degree of immune impairment results in lower virus load and higher proliferative responses, as previously observed (32). However, if the strength of the immune response is further increased relative to the degree of immune impairment, virus load is reduced even more, and this reduction in virus load may be associated with a decrease in CD4 cell proliferative responses. This is because a strong response suppresses viral replication to very low levels, removing the amount of antigenic stimulus required to maintain high numbers of specific $CD4^+$ T cells. Declines in antiviral immune responses in HIV-infected patients over the course of sustained, effective antiretroviral therapy (24) are broadly consistent with this idea.

Our experimental data, in conjunction with mathematical modeling, are broadly consistent with the idea that loss of control of viral replication may be associated with high virus load and low to absent proliferative responses, while efficient immunological control of the infection is characterized by low virus load and relatively low but measurable proliferative responses. Less optimal immunological control can result in relatively higher proliferative responses along with intermediate virus load. With sustained effective control, responses may diminish as antigenic drive declines. Over time, with only partially effective control, cumulative helper T-cell impairment may lead to declining responses and increased viral load.

The interpretation that we propose is predicted on drug suppression of viral replication during primary infection facilitating effective immunological sensitization and sustained host control of the infection after drug withdrawal. However, it is formally possible that after extended tenofovir administration, tissue stores of drug may accumulate and exert effects for some time after discontinuation of drug administration. The terminal half-life for tenofovir and its metabolites in PBMC following administration to macaques is 30 to 50 h (N. Bischofberger, unpublished results). Given this, in our view neither the observed protection from rechallenge 6 weeks after stopping treatment nor effects of the prolonged duration following drug discontinuation that we observed are readily attributable to residual tissue accumulations of drug. The design of this study does not allow us to address the potential contribution to the effects observed of possible immunomodulatory properties of tenofovir, as reported in murine studies (56, 57).

Conceptually, these results provide insight into basic mechanisms of the pathogenesis of the primate lentiviruses, in particular, the impact of the initial immunological sensitization during primary infection occurring in the setting of large amounts of antigen, ongoing cytopathic infection of activated $CD4^+$ T cells, including those responding to the virus, and possible indirect mechanisms of immunopathogenesis dependent on large amounts of virus or viral proteins. While it may take months or years for the scenario to play out to a clinical endpoint, it appears that this early insult can be profound, leaving an outmatched immune system to a prolonged but ultimately futile struggle against an implacable adversary that has built an insurmountable early lead in a game the host cannot win. In contrast, it appears that when sensitization occurs under conditions of limited viral replication with reduced amounts of antigen and limited infection of $CD4^+$ T cells, a majority of animals may be capable of containment of the infection, at least in the near term.

On a practical level, these findings provide some encouragement with regard to the prospects for achieving immunological control of primate lentiviral infection, with potential direct relevance to the development of effective prophylactic vaccines for the prevention of HIV infection and AIDS. The results underscore the very real possibilities of preventing establishment of initial infection, and of achieving sustained control of infection, even if the establishment of persistent infection is not prevented. Indeed the results are consistent with earlier reports of HIV-specific interleukin-2 production by T cells from HIV-1-exposed but uninfected health care workers (9). It is also important to note, however, that the present model does not directly address or predict the elimination of latently infected cells (14) but rather deals with sustained control of the infection.

Finally, these results also suggest the potential feasibility of therapeutic vaccination of chronically infected subjects during periods of sustained pharmacological suppression of viral replication, with the objective of enhancing the host's capacity for immunological control of the infection to the extent that antiviral drugs can be discontinued (29). The urgency of this objective is enhanced by recent data indicating that current treatment regimens are very unlikely to eradicate HIV infection in practical time frames (14) and mounting experience with the difficulty of long-term compliance with complex combination antiretroviral medication regimens, due to toxicity, drug interactions, the emergence of drug resistant virus, and other factors (reviewed in references 16 and 17). Critical issues involving the effectiveness of this approach with regard to the timing of the initiation of therapy relative to the time of initial infection, and optimal immunization regimens, including the use of exogenous viral antigens, will need to be addressed in additional studies. However, our results offer hope that it may be possible to achieve sustained immunological control, provided that adequate immune function is still present or can be reconstituted in the setting of effective suppression of viral replication.

ACKNOWLEDGMENTS

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract NO1-CO-56000.

We thank Carolyn Whistler for assistance with preparation of figures, Tricia Grove for assistance with preparation of the manuscript, Gordon Heissler for assistance with animal care, Julian Bess and Mike Grimes for preparation of inactivated virus, Greg Alvord for help with statistical analyses, and Larry Arthur, Lou Henderson, and Mark Esser for helpful discussions and thoughtful review of the manuscript.

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