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Resistance of Previously Infected Chimpanzees to Successive Challenges with a Heterologous Intraclade B Strain of Human Immunodeficiency Virus Type 1

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To test whether the protective effects of attenuated simian immunodeficiency virus vaccines in macaques were applicable to the human immunodeficiency virus type 1 (HIV-1)–chimpanzee system, two groups of animals, previously infected with HIV-1_{IIIB} or HIV-1_{SF2}, were each challenged with a heterologous clade B virus, HIV-1_{DH12}. Following challenge, the parameters measured included virus isolation (from plasma, peripheral blood mononuclear cells, and lymph node tissue); quantitative DNA PCR using primers capable of distinguishing HIV-1_{IIIB}, HIV-1_{SF2}, and HIV-1_{DH12} from one another; and serologic assays to monitor changes in binding and neutralizing antibodies. In contrast to an HIV-1-naive chimpanzee that rapidly became infected following the inoculation of HIV-1_{DH12}, the two chimpanzees previously infected with HIV-1_{IIIB} resisted repeated and escalating inoculations of HIV-1_{DH12}, as monitored by virus isolation and PCR. The two animals previously infected with HIV-1_{SF2} became infected with HIV-1_{DH12}, but in contrast to the case with the HIV-1-naive chimpanzee, no cell-free viral RNA was detected in the plasma by the branched DNA procedure and levels of peripheral blood mononuclear cell-associated viral DNA were reduced 35- to 50-fold.

Since the discovery of the etiologic agent of AIDS nearly 13 years ago, significant progress has been made in determining the organization of the human immunodeficiency virus type 1 (HIV-1) genome, elucidating the structure and function of numerous viral structural and regulatory proteins, developing sensitive serological techniques to monitor and ensure the safety of human blood and blood products, devising reliable methods for isolating virus and quantitating cell-free virus loads in infected individuals, and bringing to the clinic several antiviral agents that target virus-encoded enzymes such as reverse transcriptase (RT) and protease. During this same period, however, progress in developing effective HIV-1 vaccines has been frustratingly slow. Although some protective responses have been reported (5, 7, 11, 13, 16, 17, 31), many studies evaluating passive immunization and subunit and/or whole inactivated HIV-1 vaccines in chimpanzees have failed to demonstrate efficacy against a subsequent virus challenge (3, 6, 12, 21, 30). A major impediment to the development of effective HIV-1 vaccines has been the lack of a nonhuman HIV-1 disease model which might provide insights about correlates of protective immunity. Rather than using resistance to disease development as the principal determinant of efficacy, HIV-1 vaccine studies with chimpanzees have had to rely on sterilizing immunity as the criterion of protection.

In lieu of an HIV-1 disease model, the simian immunodeficiency virus (SIV)-Asian macaque system has been increasingly utilized to evaluate different types of potentially useful vaccines. Most prominent among these have been live attenuated virus vaccine candidates, which have received considerable attention over the last 5 years. Several groups have reported that monkeys persistently infected with nonpathogenic variants of SIV or HIV-2 exhibited resistance against a subsequent challenge with disease-causing SIV strains. One frequently cited study described a genetically engineered SIV_{mac239} derivative, lacking a functional *nef* gene, which replicated to low levels in vivo, failed to cause disease, and conferred resistance to a subsequent challenge with its pathogenic parent, SIV_{mac239} (9). Other reports have described attenuated SIV or HIV-2 vaccines that protect against virus infection (1, 8, 20, 25) or merely delay the onset of immunodeficiency (24, 32).

Because attenuated derivatives of SIV currently represent the most promising prophylactic vaccine approach against lentivirus infections, we conducted a "proof of concept" study and examined whether chimpanzees persistently infected with one strain of HIV-1 could resist a challenge with a second HIV-1 strain. We employed virus isolation procedures, quantitative PCR techniques with primers capable of distinguishing indigenous from challenge HIV-1 strains, and serologic assays monitoring changes in HIV-1 antibody levels to assess the susceptibility of two groups of previously infected chimpanzees to a challenge with an HIV-1 strain (HIV-1_{DH12}) recently isolated from an AIDS patient (35). One group of two animals, chronically infected with HIV-1_{IIIB} (29), resisted repeated and escalating challenges of HIV-1_{DH12} over a two-year observation period. DNA PCR consistently amplified HIV-1_{IIIB} DNA but

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never HIV-1_{DH12} DNA in these superchallenged chimpanzees. The second group of two animals, previously infected with the HIV-1_{SF2} strain (22), were partially protected from the HIV-1_{DH12} challenge; both chimpanzees became infected, but in contrast to the case with an inoculated control animal, no cell-free viral RNA was detected in the plasma by RT PCR and the levels of PBMC-associated HIV-1_{DH12} DNA were reduced 35- to 50-fold.

MATERIALS AND METHODS

Animals. HIV-1-infected chimpanzees were housed in a biosafety level 2, AAALAC-certified facility, and biosafety level 3 practices were followed. The animals' general health and physical status were monitored at regular intervals according to accepted guidelines. Physical examinations were performed by veterinarians at times of virus inoculation, phlebotomy, or lymph node biopsy using ketamine anesthesia.

One chimpanzee (1206), shown to be negative for HIV-1 antibody (by ELISA) and PBMC-associated HIV-1 DNA (by PCR) and serving as an unimmunized control, was inoculated with 50 50% tissue culture infective doses ($TCID_{50}$) of HIV-1_{DH12} (35). Two chimpanzees (X66 and X277) had been previously inoculated with 250 and 50 $TCID_{50}$ of HIV-1_{SF2}, respectively, 22 months prior to challenge with HIV-1_{DH12} (27). Two other chimpanzees (888 and 923) had been inoculated with 40 and 400 $TCID_{50}$ of HIV-1_{IIIB} 51 and 101 months prior to challenge with HIV-1_{DH12}, respectively (2, 3). All animals became infected following this initial HIV-1 inoculation as determined by virus isolation and ELISA antibody.

HIV-1 stocks for inoculation of chimpanzees. The HIV-1_{IIIB} and HIV-1_{SF2} inocula were prepared and titered in H9 human T cells and human PBMC, respectively (3, 27). The HIV-1_{DH12} challenge stock was prepared and titered in chimpanzee PBMC, as previously described (35). The cell-free HIV-1_{DH12} inocula were thawed, diluted in serum-free RPMI 1640 medium, and maintained on ice for up to 1 h until used for intravenous inoculation. For whole-blood transmission of HIV-1, 10 ml of heparinized blood was collected from animal 1206 (17 weeks after inoculation with HIV-1_{DH12}) and immediately transfused into chimpanzee 888.

Virus isolation from blood of the infected chimpanzee. PBMC were isolated from heparinized whole chimpanzee blood by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. Fifteen million PBMC were depleted of CD8 cells by positive selection using a CD8 antibody-coated flask (Applied Immune Science) and were then cocultivated with 3×10^6 human PBMC from a HIV-1-seronegative donor. Cocultures were stimulated with 1 μg of phytohemagglutinin (Wellcome) per ml for three days and maintained in RPMI 1640 (Whittaker) medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Gibco-BRL), 20 U of recombinant human interleukin-2 (Boehringer Mannheim) per ml, 50 μg of gentamicin (Whittaker) per ml, and penicillin (50 U/ml)-streptomycin (50 μg/ml) (Gibco-BRL). Virus production was monitored by assaying RT activity (37) in the supernatants of cocultures maintained for up to 4 weeks. Stimulated human PBMC were added to the cocultures on day 14 and day 22.

HIV-1 antibody assays. HIV-1 antibody in chimpanzee plasma was measured by ELISA using a commercial HIV-1 microELISA kit (Organon Teknika) which utilizes whole HIV-1 (purified virions) as the source of antigens.

Antibody neutralization assays were conducted by incubating serial fourfold dilutions of chimpanzee sera with serial fourfold dilutions of virus (approximately 200 to 0.05 $TCID_{50}$) for 1 h; the mixture was then added to MT4 (19) cells (for 7 days) or to human PBMC (for 10 days) as previously described (4, 18). Supernatant medium was assayed for RT activity as a measure of progeny virus production. The surviving virus titer ($TCID_{50}$) was determined by using the Reed-Muench formula (33). The neutralization titers were determined from multiplicity curves in which the fraction of virus surviving neutralization (V_n/V_o, where V_n is the surviving virus titer and V_o is the titer of virus added) was plotted against the dilution of chimpanzee serum. Neutralization titers were defined as the reciprocal of the serum dilution yielding a 10-fold reduction of the infectivity titer (i.e., $V_n/V_o = 0.1$). Stocks of HIV-1_{LAI}, HIV-1_{MN}, HIV-1_{SF2}, and HIV-1_{RF} were prepared in CEM cells, and the neutralization assays were performed in MT4 cells. When stocks of HIV-1_{DH12} were prepared in human PBMC, neutralization assays were conducted in human PBMC, activated for 2 days with antibodies to CD3 (Orthoclone [Ortho]) and CD28 (L293 [catalog no. 348040; Becton Dickinson]), each at 0.1 μg/ml; similarly, MT-4-derived HIV-1_{DH12} was employed in assays carried out in MT-4 cells. The HIV-1_{DH12} neutralization titers measured in MT4 cells were usually twofold higher than those measured in human PBMC.

Detection and quantitation of HIV nucleic acids in infected chimpanzees. HIV-1 RNA was detected in the plasma by the branched DNA procedure using the HIV-1 Quantiplex RNA Assay Kit (Chiron), as previously described (10).

The amount of HIV-1 DNA present in chimpanzee PBMC was determined by DNA PCR as previously described (35). Chimpanzee PBMC (2×10^6) were lysed in 200 μl of PCR lysis buffer (100 mM KCl, 10 mM Tris-Cl [pH 8], 2.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% Nonidet P-40, 150 μg of proteinase K per ml) and incubated at 65°C for 3 h and then at 100°C for 15 min. Fivefold dilutions of the PBMC lysate (equivalent to 1×10^5, 2×10^4, 4,000, 800, and 160 cells) were subjected to 30 cycles of DNA PCR in quadruplicate and then amplified by 30 cycles of nested PCR. Under these conditions, any amplifiable molecule would be visualized in an ethidium bromide-stained agarose gel. The distribution of amplifiable molecules in each PCR should follow the Poisson equation, $P = 1 - e^{\wedge}(-m)$, where P is the positive fraction and m is the average copy number. Since when $m = 1$, P will equal 0.6321, the dilution of PBMC (starting at 10^5 cells) at which 63.21% of the samples are PCR positive would correspond to a DNA copy number of 1 per sample. The percent positive values in the PCR analyses were calculated as described by Reed and Muench (33) for determining $TCID_{50}$.

All of the primers used for PCR map to the gp120 coding region of the HIV-1 *env* gene. For the first cycle of PCR, the CTAAAGCCATGTGTAAAATTA ACCCCACTC (nucleotide positions 6575 to 6604 of HIV-1_{SF2} [GenBank accession number K02007]) and TATAGAATTCACTTCTCCAATTGTCCCT CAT (nucleotide positions 7649 to 7679 of HIV-1_{SF2}) primer pair, which can amplify all three HIV-1 strains (HIV-1_{DH12}, HIV-1_{IIIB}, and HIV-1_{SF2}), was used (35). The second amplification utilized the following primer pairs specific for one or more of the three isolates: TTGAAGAATGGTACTAATTTGAAGAATGG (HIV-1_{DH12} specific; located in the V1 region) and CCTTCAGTACCATTCCA AGTACTAT (HIV-1_{DH12} specific; located in the V4 region), which can amplify 797 bp of DNA from HIV-1_{DH12} (35); GTATGAATTCAACTGCTGTTAAATGG CAGT (nonspecific; nucleotide positions 6580 to 6609 of HIV-$1_{Bru/LAI/IIIB}$ [GenBank accession number K02013]) and CTCGGACTCATTGTTGCTATTA (HIV-1_{IIIB} specific; nucleotide positions 7194 to 7215 of HIV-$1_{Bru/LAI/IIIB}$), which can amplify 636 bp of DNA from HIV-1_{IIIB}; CAATAGATAATGCTAGTACTAC TACCAAC (HIV-1_{SF2} specific; nucleotide positions 6774 to 6802 of HIV-1_{SF2}) and GAACAACTAATTTGTCCTCCAATGG (HIV-1_{SF2} specific; nucleotide positions 7536 to 7560), which can amplify 787 bp of DNA from HIV-1_{SF2}, for HIV-1_{SF2}; and ACAGTACAATGTACACATGGAAT (nonspecific; nucleotide positions 6971 to 6993 of HIV-1_{SF2}) and CTGCCACATGTTTATAAATTGTTTTATTCTGCA (nonspecific; nucleotide positions 7475 to 7507 of HIV-1_{SF2}), which can amplify 537 bp of DNA from HIV-1_{SF2} and HIV-1_{DH12} and 555 bp from HIV-1_{IIIB}.

Ampli-Taq DNA polymerase (Perkin-Elmer) was used for all PCRs, which were performed as follows: an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 120 s; and a final extension at 72°C for 7 min.

In vitro infection of partially purified chimpanzee lymphocyte subsets. Chimpanzee PBMC, preserved in cryoprotective medium (Whittaker) supplemented with 50% fetal bovine serum (HyClone) in liquid nitrogen, were thawed and stimulated with phytohemagglutinin for 3 days prior to their use in tissue culture infections. Stimulated PBMC were depleted of CD8-positive cells by using CD8 antibody-conjugated magnetic beads (Miltenyi Biotec, Inc., Sunnyvale, Calif.) and then used for HIV-1_{DH12} infectivity studies. When necessary, the CD8-positive fraction was added back to the cultures prior to virus infection.

RESULTS

Virologic and serologic status of chimpanzees. Since the purpose of this study was to ascertain whether the protective effects of attenuated SIV vaccines could be duplicated in the HIV-1 system, the resistance of two groups of previously infected chimpanzees to a challenge with a heterologous clade B strain of HIV-1 was examined. The challenge virus strain employed, HIV-1_{DH12}, is a recently described (35) primary isolate from an AIDS patient that exhibited tropism for chimpanzee PBMC. This isolate is unique in its ability to infect human and chimpanzee monocyte-derived macrophages, to induce syncytia in human and chimpanzee PBMC, and to replicate in some human T-cell lines, such as MT4 (19). HIV-1_{DH12} is also capable of establishing in vivo infections, frequently associated with lymphadenopathy and rash (reference 35 and unpublished results), in naive chimpanzees.

The virologic and serologic status of the four HIV-1-infected chimpanzees and the single naive animal (1206; also described in reference 35), prior to challenge with HIV-1_{DH12}, is presented in Table 1. Animals 888 and 923 had been inoculated with 40 and 400 $TCID_{50}$ of HIV-1_{IIIB} 51 and 101 months prior to exposure to HIV-1_{DH12}, respectively, and had become infected on the basis of two criteria: virus isolation from PBMC and development of HIV-1-specific antibodies (2, 3). The HIV-1_{IIIB}-infected chimpanzees had high levels of ELISA antibody and substantial amounts of neutralizing antibody directed against HIV-1_{IIIB}, HIV-1_{SF2}, and HIV-1_{MN} prior to challenge with HIV-1_{DH12}. These two animals also had small but detectable numbers of virus-infected PBMC, as monitored

TABLE 1. Status of the chimpanzees prior to infection with HIV-1_{DH12}

Animal	No. of months after the primary HIV-1 infection[a]	Virus copy no.[b]	ELISA antibody titer[c]	Neutralizing antibody titer for strain[d]:				%CD3CD4[e]
				IIIB	SF2	MN	DH12	
888	51 (HIV-1_{IIIB})	24.6	1:46,875	1:2,000	1:300	1:200	<1:20	30
923	101 (HIV-1_{IIIB})	11.3	1:46,875	1:350	1:500	1:350	<1:20	24
X66	22 (HIV-1_{SF2})	<3.0	1:9,375	ND[f]	1:100	ND	<1:20	33
X277	22 (HIV-1_{SF2})	<3.0	1:375	ND	1:300	ND	<1:20	25
1206	—[g]	<3.0	<1:75	ND	ND	<1:20	<1:20	46

[a] The preexisting HIV-1 strains in the chimpanzees are shown in parentheses.
[b] Virus copy numbers per 10^6 PBMC were determined by DNA PCR (35).
[c] The highest dilutions of plasma which were positive for ELISA antibody are shown.
[d] The highest dilutions of serum which gave 90% neutralization of the indicated virus strains are shown.
[e] Percentages of CD3-CD4 double-positive cells (%CD3CD4) in blood (gated for lymphocytes) were determined by flow cytometry.
[f] ND, not done.
[g] —, naive animal.

by DNA PCR. HIV-1 was recovered from chimpanzee 888 by cocultivation during the 3-month period (1 of 3 attempts) prior to challenge with HIV-1_{DH12} but was never isolated from chimpanzee 923 during a 7-month prechallenge interval (10 attempts). Chimpanzee 923 was reinoculated with 2,000 $TCID_{50}$ of HIV-1_{IIIB} 20 weeks prior to exposure to HIV-1_{DH12} because it had been consistently virus isolation negative; no subsequent increase of the HIV-1_{IIIB} load was observed prior to the HIV-1_{DH12} challenge (data not shown).

Chimpanzees X66 and X277 had been inoculated with 250 and 50 $TCID_{50}$ of HIV-1_{SF2}, respectively, 22 months prior to challenge with HIV-1_{DH12} and had become infected as monitored by virus isolation and the appearance of HIV-1 ELISA antibody (27). Both animals had become virus isolation negative and were inoculated with 10,000 $TCID_{50}$ of HIV-1_{SF2} 8 months prior to challenge with HIV-1_{DH12}. Repeated attempts to isolate virus were unsuccessful, although a marked increase in antibody titers was observed (26). As shown in Table 1, the two animals previously infected with HIV-1_{SF2} had somewhat lower levels of ELISA and neutralizing antibodies than did the HIV-1_{IIIB}-infected chimpanzees and no proviral DNA detectable by DNA PCR (the limit of detection is three copies per 10^6 cells in our assays). Virus isolation from the PBMC of chimpanzees X66 and X277 had been negative for 18 months (seven attempts) and 19 months (nine attempts), respectively, prior to challenge with HIV-1_{DH12}.

None of the five animals had neutralizing antibodies directed against HIV-1_{DH12}.

The first exposure of chronically infected chimpanzees to HIV-1_{DH12}. A cell-free stock of HIV-1_{DH12}, prepared and titered (1.2×10^4 $TCID_{50}$/ml) in chimpanzee PBMC, was used as the inoculum. Because chimpanzees inoculated with HIV-1 do not develop disease, several other parameters, which directly or indirectly are a measure of virus levels in vivo, were monitored to ascertain whether previous exposure to live, infectious virus elicited resistance to a subsequent virus challenge. These included (i) virus isolation from plasma, PBMC, or lymph node tissue; (ii) quantitation of viral RNA in the plasma by the branched DNA amplification technique; (iii) quantitation of proviral DNA in PBMC and lymph node specimens by limiting cell dilution-DNA PCR; and (iv) quantitative ELISA and neutralization antibody determinations. Individual primer pairs that could specifically amplify (and distinguish) the V3 coding regions of HIV-1_{IIIB}, HIV-1_{SF2}, and HIV-1_{DH12} *env* genes in PCRs (Fig. 1) were used.

In their first exposure to HIV-1_{DH12}, the two HIV-1_{SF2}-infected chimpanzees received 120 $TCID_{50}$ of HIV-1_{DH12}, one HIV-1_{IIIB}-infected chimpanzee (888) was inoculated with 170 $TCID_{50}$ of HIV-1_{DH12}, and the HIV-1-naive control chimpanzee (1206) was exposed to 50 $TCID_{50}$ of HIV-1_{DH12} (Table 2). A plasma viremia which fell to background levels by week 6 was demonstrable for the control chimpanzee 2 weeks following inoculation by direct virus isolation from 1 ml of plasma (data not shown) and by the branched DNA signal amplification procedure (140,000 copies of viral RNA per ml) (Fig. 2A). In contrast, no HIV-1 RNA was detected in the plasma of the two HIV-1_{SF2}-infected chimpanzees or the single HIV-1_{IIIB}-infected chimpanzee subsequent to challenge with HIV-1_{DH12}. HIV-1_{DH12}-infected PBMC were readily detectable by DNA PCR within 1 week of infection of the naive animal (1206), peaking at a level of approximately 400 DNA copies per 10^6 cells at 3 weeks postinoculation (Fig. 2B). HIV-1_{DH12}-infected PBMC were also present in the two HIV-1_{SF2}-infected chimpanzees (X66 and X277) following challenge with HIV-1_{DH12},

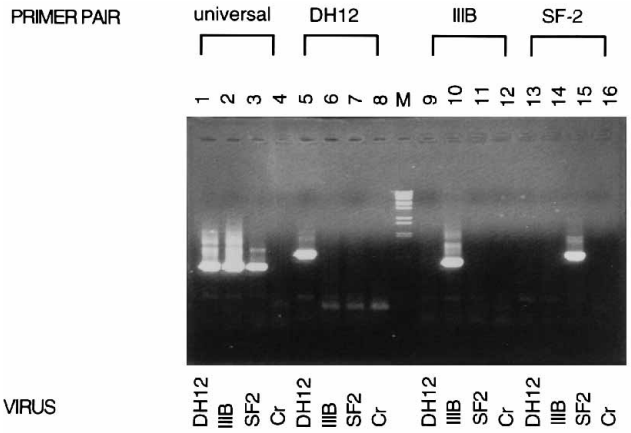

FIG. 1. Specific detection of viral DNA corresponding to three different HIV-1 strains in chimpanzee PBMC by nested PCR. Total cellular DNA was prepared from PBMC of a control, uninfected animal (Cr) or of chimpanzees infected with HIV-1_{IIIB}, HIV-1_{SF2}, or HIV-1_{DH12} and amplified for 30 cycles with an *env* gene primer pair capable of amplifying DNA from all three strains (see Materials and Methods). Approximately 5% of the PCR mixture was amplified for an additional 30 cycles with primer pairs specific for each HIV-1 strain or a primer pair (universal) able to amplify DNA from all three strains. The PCR products were electrophoresed through a 1% agarose gel and stained with ethidium bromide. The expected sizes (in base pairs) of the amplified DNAs were as follows: (i) universal primer pair (HIV-1_{DH12} DNA), 537; (ii) universal primer pair (HIV-1_{IIIB} DNA), 555; (iii) universal primer pair (HIV-1_{SF2} DNA), 537; (iv) HIV-1_{DH12}-specific primer pair, 797; (v) HIV-1_{IIIB}-specific primer pair, 636; and (vi) HIV-1_{SF2}-specific primer pair, 787. The size markers used (0.7, 1.3, 1.4, 1.9, 2.3, 3.7, 4.3, 4.8, 5.7, 6.4, 7.2, 8.5, and 14.1 kb) were electrophoresed in lane M.

TABLE 2. Chimpanzee superchallenge experiments

HIV-1_{DH12} challenge	Animal	Infection status	Size of HIV-1_{DH12} inoculum[a]	Outcome
First stage	X66	HIV-1_{SF2} infected	120	No plasma viremia; reduced PBMC viremia
	X277	HIV-1_{SF2} infected	120	No plasma viremia; reduced PBMC viremia
	888	HIV-1_{IIIB} infected	170	Protected[b]
	1206	Naive	50	Infected
Second stage[c]	888	HIV-1_{IIIB} infected	1,700	Protected[b]
	923[d]	HIV-1_{IIIB} infected	1,700	Protected[e]
Third stage[f]	888	HIV-1_{IIIB} infected	10 ml of blood (containing approx 300 infected cells) from chimpanzee 1206	Protected[b]
	923	HIV-1_{IIIB} infected	17,000	Protected[b]

[a] Sizes are expressed in $TCID_{50}$ except for the third-stage challenge of animal 888.
[b] No HIV-1_{DH12} detected by virus isolation, DNA PCR, branched DNA signal amplification, or elevated ELISA antibody levels.
[c] Twenty weeks after the first-stage challenge.
[d] This animal had not previously received HIV-1_{DH12}.
[e] No HIV-1_{DH12} detected by virus isolation, DNA PCR, or branched DNA signal amplification.
[f] Seventeen weeks after the second-stage challenge.

but at a 35- to 50-fold-lower level. In contrast, HIV-1_{DH12}-infected PBMC were not detected in the animal previously infected with HIV-1_{IIIB} (888), although low levels of PBMC infected with the indigenous HIV-1_{IIIB} were evident (Fig. 2C).

Increasing ELISA antibody levels, a sensitive indicator of active HIV-1 replication in chimpanzees, were observed in both of the superchallenged chimpanzees previously infected with HIV-1_{SF2} but not in the animal previously infected with HIV-1_{IIIB} (Fig. 3). Taken together, these results indicate that chimpanzee 888, chronically infected with HIV-1_{IIIB}, resisted an intravenous challenge of 170 $TCID_{50}$ of HIV-1_{DH12} on the basis of no detectable cell-free (branched DNA assay) or cell-associated (DNA PCR) virus and the absence of HIV-1-specific ELISA antibody elevations during a 20-week observation period. The two animals previously infected with HIV-1_{SF2} were partially protected from an HIV-1_{DH12} superchallenge: compared with the HIV-1-naive animal, no plasma viremia was detected, and the HIV-1_{DH12} proviral DNA levels were markedly lower.

Second-stage HIV-1_{DH12} challenge. Because the single HIV-1_{IIIB}-infected chimpanzee (888) appeared to resist an HIV-1_{DH12} challenge, it and a second animal (923), also persistently infected with HIV-1_{IIIB} but not previously exposed to HIV-1_{DH12}, were inoculated intravenously with 1,700 $TCID_{50}$ of HIV-1_{DH12} (Table 2). During a 17-week observation period, HIV-1_{DH12} was not detected in either animal by direct virus isolation, branched DNA amplification of plasma samples, or limiting dilution DNA PCR of PBMC (Fig. 4 and 5).

Third-stage HIV-1_{DH12} challenge. In a third experiment, the two chimpanzees previously infected with HIV-1_{IIIB} were challenged with even larger amounts of HIV-1_{DH12} (Table 2). One animal (923) was inoculated with 17,000 $TCID_{50}$ of cell-free virus, and the other (888) was transfused with 10 ml of fresh whole blood, collected on week 17, from the naive chimpanzee (1206) that rapidly became infected with HIV-1_{DH12} (Fig. 2). Quantitative DNA PCR conducted with PBMC purified from the transmission blood sample indicated that chimpanzee 888 received approximately 300 cells containing HIV-1_{DH12} DNA and 2.8×10^5 copies of virion-associated HIV-1 RNA.

As was observed in the two previous challenges, no viral RNA was detected in plasma samples and no HIV-1_{DH12} proviral DNA was detected in numerous PBMC specimens col-

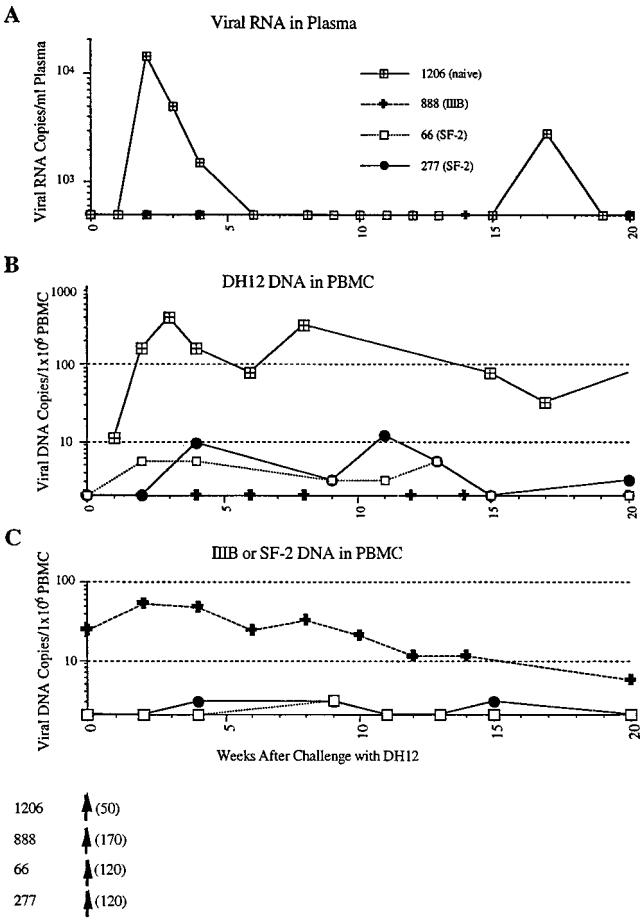

FIG. 2. Detection of HIV-1 RNA and DNA in chimpanzee plasma and PBMC following challenge with HIV-1_{DH12}. (A) Viral RNA in the plasma of the indicated chimpanzees was detected by the branched DNA amplification technique (9). The limit of RNA detection was 15×10^3 copies per ml of plasma. (B and C) HIV-1_{DH12}-specific DNA and HIV-1_{IIIB}- or HIV-1_{SF2}-specific DNA, respectively, in chimpanzee PBMC were measured by nested PCR using specific primer pairs able to distinguish each of the three isolates as described in Materials and Methods. Challenge doses (in $TCID_{50}$) of HIV-1_{DH12} are indicated at the bottom.

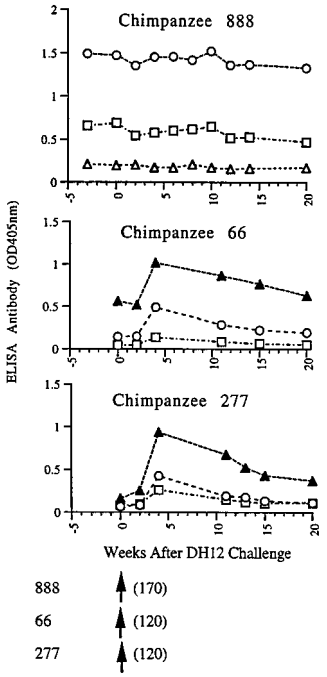

FIG. 3. Anti-HIV-1 ELISA antibody responses in previously HIV-1-infected chimpanzees following a challenge with HIV-1_{DH12}. In each case, ELISAs with whole inactivated HIV-1 were performed on fivefold serial dilutions of chimpanzee plasma. The optical densities at 405 nm (OD405nm) obtained with three different dilutions of each plasma sample are shown. Plasma dilutions were as follows: 1:1,875 (▲), 1:9,375 (○), 1:46,875 (□), and 1:234,375 (△). Challenge doses (in $TCID_{50}$) of HIV-1_{DH12} are indicated at the bottom.

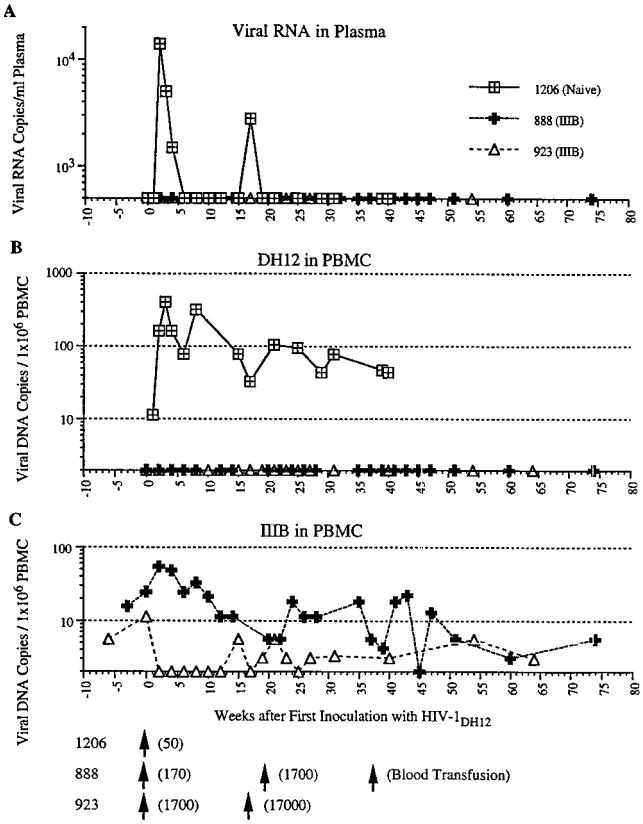

FIG. 4. Levels of HIV-1 RNA and DNA in chimpanzees previously infected with HIV-1_{IIIB} and repeatedly challenged with HIV-1_{DH12}. Chimpanzees 923 and 888 were inoculated twice and three times, respectively, with increasing amounts of the HIV-1_{DH12} inoculum. The challenge doses (in $TCID_{50}$) of HIV-1_{DH12} are indicated at the bottom except for the third challenge of chimpanzee 888, which consisted of whole blood from animal 1206 (collected at week 17). (A) Viral RNA in the plasma was measured by the branched DNA amplification procedure. (B and C) PBMC-associated HIV-1_{DH12}-specific and HIV-1_{IIIB}-specific DNAs, respectively, were measured by nested DNA PCR.

lected from either chimpanzee (Fig. 4A and B). DNA PCR analyses revealed the presence of low levels of the indigenous HIV-1_{IIIB} DNA during the 74 weeks of the experiment, with little if any change attending the HIV-1_{DH12} challenges (Fig. 4C). Similarly, the virus periodically isolated by cocultivation from the two HIV-1_{IIIB}-infected animals was invariably HIV-1_{IIIB}, and not HIV-1_{DH12}, as determined by DNA PCR (Fig. 5). In contrast, the virus recovered from the inoculated naive animal and one of the superchallenged chimpanzees previously infected with HIV-1_{SF2} was shown to be HIV-1_{DH12} (Fig. 5).

The HIV-1_{IIIB}-superchallenged animals developed neutralizing antibodies to HIV-1_{DH12}. Although HIV-1_{DH12} was never isolated and HIV-1_{DH12} RNA and DNA were never detected in the two HIV-1_{IIIB}-infected animals, a slight elevation of the ELISA antibody levels was observed in animal 923 following challenge with 1,700 $TCID_{50}$ of HIV-1_{DH12} (Fig. 6A). Similarly, the level of HIV-1 ELISA antibody in chimpanzee 888 may have increased after the challenges with 1,700 $TCID_{50}$ of cell-free HIV-1_{DH12} and 10 ml of whole blood from animal 1206 at weeks 20 and 37, respectively. Although these increases were quite modest compared with those observed in the two chimpanzees previously infected with HIV-1_{SF2} (Fig. 3), this result raises the possibility that low-level in vivo HIV-1_{DH12} infections might have occurred in the HIV-1_{IIIB}-infected chimpanzees.

Since our ELISA assay does not distinguish antibodies directed against HIV-1_{DH12} from those reacting with HIV-1_{IIIB} or HIV-1_{SF2}, neutralization assays were performed. At the time of the HIV-1_{DH12} challenge, the two chimpanzees previously infected with HIV-1_{IIIB} were producing antibodies capable of neutralizing HIV-1_{IIIB}, HIV-1_{SF2}, and HIV-1_{MN} (Table 1). Neither animal, however, had neutralizing antibodies directed against HIV-1_{DH12}. The naive chimpanzee (1206) rapidly developed HIV-1_{DH12}-neutralizing antibody, which was detectable within 7 weeks of inoculation with HIV-1_{DH12} and reached titers of greater than 1:10,000 within 4 months of infection (Fig. 6B). The two animals previously infected with HIV-1_{SF2}, as expected, developed neutralizing antibody against HIV-1_{DH12} within 9 weeks of challenge (Fig. 6B), a result consistent with the presence of HIV-1_{DH12} DNA in PBMC samples analyzed at that time (Fig. 2B). Quite unexpectedly, however, neutralizing antibody directed against HIV-1_{DH12} was detected in the HIV-1_{IIIB}-infected chimpanzee 923 following inoculation of 1,700 $TCID_{50}$ of virus and levels of this antibody continued to rise following the challenge with 17,000 $TCID_{50}$. This neutralization activity appeared in a time frame similar to that observed for the control and the two chimpanzees previously infected with HIV-1_{SF2}. In contrast to this result, HIV-1_{DH12}-neutralizing antibody was not detected in the second HIV-1_{IIIB}-infected chimpanzee (888) until several weeks after the third HIV-1_{DH12} challenge (10 ml of blood from chimpanzee 1206). Sixteen months following the third HIV-1_{DH12} challenge, the neutralization titer against HIV-1_{DH12} had declined to 1:26 compared with a 1:6,400 neutralization titer against HIV-1_{IIIB} (data not shown). It is worth noting that this fall in HIV-1_{DH12}-specific neutralization occurred against a background of relatively constant ELISA an-

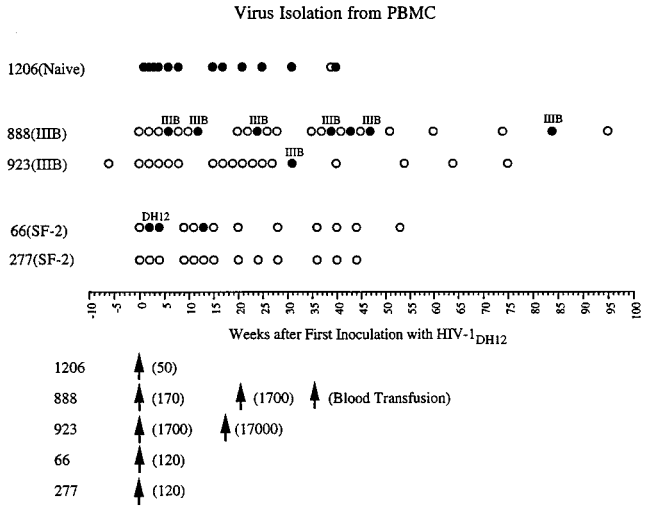

FIG. 5. Virus isolation from five chimpanzees inoculated with HIV-1_{DH12}. The challenge doses (in $TCID_{50}$) of HIV-1_{DH12} are indicated at the bottom except for the third challenge of chimpanzee 888, which consisted of 10 ml of whole blood from animal 1206 (collected at week 17). PBMC (2×10^5) from each animal were depleted of CD8 cells as described in Materials and Methods, mixed with 3×10^6 human PBMC from a seronegative donor, stimulated with phytohemagglutinin, and monitored for progeny virus production during a 4-week observation period. Filled and open circles represent positive and negative virus isolations, respectively. Viruses isolated from animals 888, 923, and X66 were identified as HIV-1_{IIIB} or HIV-1_{SF2} by specific PCR using strain-specific primer pairs.

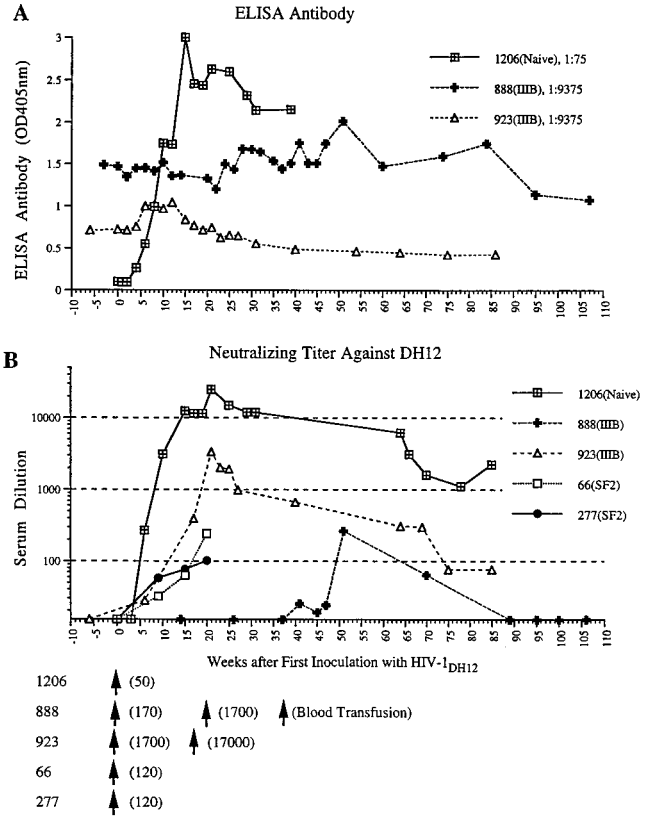

FIG. 6. Antibody responses of previously HIV-1-infected chimpanzees following successive challenges with HIV-1_{DH12}. The HIV-1_{DH12} challenge doses (in $TCID_{50}$) are indicated at the bottom except for the third challenge of chimpanzee 888, which consisted of 10 ml of whole blood from animal 1206 (collected at week 17). (A) ELISAs with inactivated HIV-1 (Organon Teknika) were performed on plasma samples from a control chimpanzee (1206) and two chimpanzees previously infected with HIV-1_{IIIB} (888 and 923), diluted 1:75, 1:9,375, and 1:9,375, respectively. OD405nm, optical density at 405 nm. (B) HIV-1_{DH12} neutralization assays were performed on the indicated naive chimpanzee, two previously HIV-1_{IIIB}-infected chimpanzees, and two previously HIV-1_{SF2}-infected chimpanzees. The dilution of plasma which gave 90% neutralization is shown on a log scale at various times subsequent to the HIV-1_{DH12} challenge.

tibody levels (Fig. 6A). The HIV-1_{DH12}-neutralizing antibody titers in chimpanzee 923 also fell over time but remained above (1:77) background levels (>1:20) nearly 70 weeks following the inoculation of 17,000 $TCID_{50}$ of HIV-1_{DH12}.

The presence of neutralizing antibody directed against HIV-1_{DH12} raised the possibility that an HIV-1_{DH12} infection of the two chronically HIV-1_{IIIB}-infected animals had in fact occurred even though HIV-1_{DH12} RNA and DNA were undetectable in the samples of plasma or PBMC analyzed. Because earlier reports had indicated that significantly higher levels of virus are present in the lymph nodes of HIV-1-seropositive individuals than are present in PBMC simultaneously collected (28, 34), inguinal lymph nodes from chimpanzees 888 and 923 were biopsied on weeks 51 (14 weeks after the blood transfusion) and 40 (23 weeks after challenge with 17,000 $TCID_{50}$ of HIV-1_{DH12}), respectively, and examined by PCR for the presence of HIV-1_{DH12} DNA. Although HIV-1_{IIIB} DNA was readily detected in both biopsy specimens, no HIV-1_{DH12} DNA could be amplified from 400,000 lymphocytes analyzed for either sample.

These results suggest that chimpanzee 923 became infected following inoculation with 1,700 $TCID_{50}$ of HIV-1_{DH12}, although cell-associated and cell-free virus and viral nucleic acids were undetectable by the methods employed. After the second HIV-1_{DH12} challenge (17,000 $TCID_{50}$), a rise in neutralizing antibody titer occurred but the level of ELISA antibody did not change appreciably (Fig. 6). As judged by the time of emergence of HIV-1_{DH12}-neutralizing antibody, chimpanzee 888 resisted the 170- and 1,700-$TCID_{50}$ virus challenges but possibly not the subsequent transfusion of whole blood from the HIV-1_{DH12}-infected animal.

Measurable differences and similarities between animals previously infected with HIV-1_{IIIB} and those previously infected with HIV-1_{SF2}. Although both groups of chimpanzees had been chronically infected with HIV-1, the animals previously infected with HIV-1_{IIIB} exhibited significantly greater resistance to the HIV-1_{DH12} superchallenge than did the two HIV-1_{SF2}-infected animals, as summarized in Table 3. The data presented in Table 1 clearly indicate that at the time of challenge, the two HIV-1_{IIIB}-infected chimpanzees had higher levels of cell-associated viral DNA than did the HIV-1_{SF2}-infected animals, from which no HIV-1 DNA could be amplified. The lower HIV-1 loads in the HIV-1_{SF2}-infected animals are also reflected in the lower HIV-1 ELISA antibody titers measured at the time of HIV-1_{DH12} inoculation.

It should be noted that none of the animals was producing neutralizing antibody directed against HIV-1_{DH12} at the time of challenge.

Because it could be argued that the different responses of the two groups of chimpanzees to HIV-1_{DH12} inoculation might have been due to different intrinsic susceptibilities of their PBMC to virus infection, independent tissue culture infections using PBMC subsets from each animal were carried out. When CD8-depleted PBMC were infected with HIV-1_{DH12}, no significant difference in the amount of progeny virions produced was observed (Fig. 7). To compare the possible suppressive effects of CD8 cells (36) from the two groups of infected chimpanzees, autologous CD8 cells were added back

TABLE 3. Summary of chimpanzee superchallenge experiments

Parameter indicative of HIV-1_{DH12} infection	Result for the following chimpanzee[a]:				
	1206[b]	HIV-1_{SF2} infected		HIV-1_{IIIB} infected	
		66	277	923	888
Plasma viremia (virus isolation)	+	−	−	−	−
Plasma RNA	+	−	−	−	−
PBMC-associated virus	+	+	−	−	−
PBMC-associated viral DNA	+	+	+	−	−
Lymph node viral DNA	+	ND[c]	+	−	−
ELISA Ab level elevation[d]	+	+	+	+	+/−
Neutralizing Ab	+	+	+	+	+[e]

[a] +, present; −, absent; +/−, see text.
[b] Naive animal.
[c] ND, not determined.
[d] Ab, antibody.
[e] Present but at a low level.

to the CD4 cell-enriched samples and then infected with HIV-1_{DH12}. Virus production was markedly reduced in the presence of CD8 cells, but no significant difference between the naive animal and the persistently infected chimpanzees was observed.

DISCUSSION

Our results clearly indicate that the protective effects of live attenuated derivatives of SIV in macaques against a subsequent virus challenge are also applicable to the HIV-1–chimpanzee system. As has been previously observed in the SIV-macaque model and as shown for HIV-1 in Table 3, chronically infected chimpanzees exhibited a continuum of resistance to a subsequent challenge. Although the animals previously infected with HIV-1_{SF2} did, in fact, become infected with HIV-1_{DH12}, no plasma viral RNA was detected in either of the two chimpanzees, PBMC-associated HIV-1_{DH12} DNA levels were 35- to 50-fold lower than those in the naive animal, HIV-1_{DH12} was isolated from the PBMC of only one of the two chimpanzees, and the titer of neutralizing antibody directed against HIV-1_{DH12} was at least 100 times lower than that in the HIV-1-naive animal. In this proof of concept study, the two previously HIV-1_{IIIB}-infected chimpanzees displayed the greatest resistance to a heterologous virus challenge: progeny HIV-1_{DH12} particles, HIV-1_{DH12} RNA, and HIV-1_{DH12} DNA were never detected in plasma, PBMC, or lymph node tissue over nearly 2 years of observation. One of the animals (923) very likely became infected following exposure to 1,700 $TCID_{50}$ on the basis of the development of HIV-1_{DH12}-specific neutralizing antibody and a modest elevation of ELISA antibody levels. Whether an HIV-1_{DH12} infection actually occurred in the second HIV-1_{IIIB}-infected chimpanzee (888) is still open to question. Neutralizing antibody directed against HIV-1_{DH12} did not appear until after the third virus challenge, nearly 1 year following the initial exposure to HIV-1_{DH12}, and the low neutralization titers detected have since fallen to background levels.

The gradient of resistance of chimpanzees to infection by a second HIV-1 strain (Table 3) is similar to the results obtained with macaques previously exposed to SIV or HIV-2 and subsequently challenged. In one of the earliest studies of this type, three cynomolgus monkeys, inoculated with HIV-$2_{SBL-K135}$ 168 days prior to challenge with SIV_{SMM-3}, were virus isolation positive for only up to 41 days following challenge and exhibited very modest elevations of ELISA antibody levels (32). This relatively benign outcome contrasts with that observed for the four unvaccinated control animals, simultaneously challenged with SIV_{SMM-3}, all of which became persistently virus isolation positive, suffered $CD4^+$ T-lymphocyte depletion, and developed a variety of clinical symptoms including the death of two of the monkeys. In another study, three rhesus macaques exposed to the nonpathogenic $SIV_{MAC-1A11}$ strain for 7 months and then challenged with 100 to 1,000 animal infectious doses of SIV_{mac251} exhibited a delay in onset of clinical disease (one monkey remained completely asymptomatic) compared with four unvaccinated animals, all of which died (24). In a follow-up experiment in which seven macaques were also vaccinated with live $SIV_{MAC-1A11}$ but were challenged intravenously with only 1 animal infectious dose, none became infected (25). More recently, this same group of researchers constructed a series of SIV chimeras that exhibited a hierarchy of virus attenuation (23). When each was independently evaluated as a potential live virus vaccine, resistance to challenge with a pathogenic strain of SIV was inversely correlated with the degree of attenuation. Unfortunately, one of four animals immunized with the least-attenuated (and most protective) of the SIV constructs developed immunodeficiency and died prior to challenge with the pathogenic SIV_{mac251}. The inverse relationship between virus attenuation and subsequent resistance to challenge observed in the SIV system is reminiscent of the results obtained with the chronically HIV-1_{IIIB}- and HIV-1_{SF2}-infected chimpanzees evaluated in the present study. Higher viral DNA loads and ELISA antibody levels (in the HIV-1_{IIIB}-infected animals) prior to challenge with HIV-1_{DH12} corre-

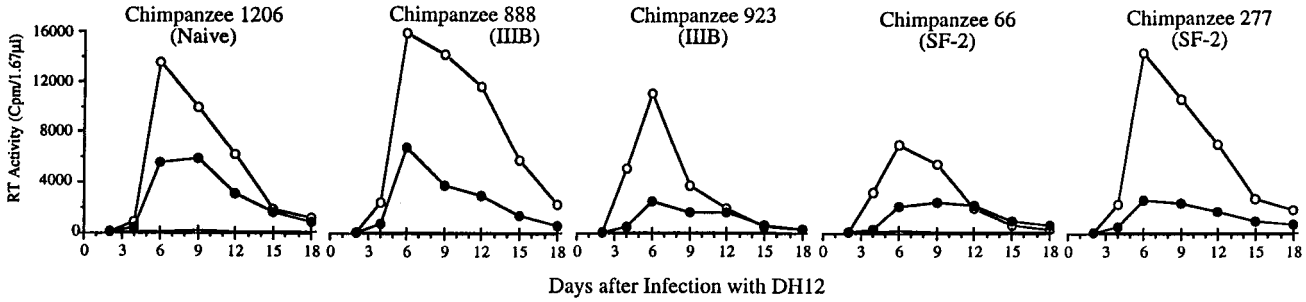

FIG. 7. Susceptibility of chimpanzee PBMC to HIV-1_{DH12} infection in vitro. Frozen PBMC, prepared from the five chimpanzees used in this study prior to inoculation with HIV-1_{DH12}, were thawed, stimulated with phytohemagglutinin for 3 days, and depleted of CD8-positive cells by using CD8 antibody-conjugated magnetic beads. CD8-depleted PBMC (10^5; open circles) or a mixture of 10^5 CD8-depleted PBMC and 10^5 autologous CD8-positive lymphocytes purified with magnetic beads (filled circles) was infected with approximately 2,000 $TCID_{50}$ of HIV-1_{DH12}. Virus replication was monitored by a ^{32}P-RT assay (37). Although chimpanzees 888, 923, X66, and X277 had been chronically infected with HIV-1_{IIIB} or HIV-1_{SF2}, mock-infected PBMC from these animals failed to release detectable progeny virus under these experimental conditions because of the extremely small number of circulating virus-infected cells. +, mock infection (CD8-depleted PBMC only).

lated with greater resistance (Tables 1 and 3). In the context of functioning as a live virus vaccine, HIV-1_{SF2} produces low levels of HIV-1 in infected chimpanzees and could be viewed as being more attenuated than HIV-1_{IIIB}.

In contrast to most studies of attenuated SIV vaccines, the indigenous and challenge HIV-1 strains that we evaluated represented heterologous pairs of viral isolates with respect to the amino acid composition of their envelope glycoproteins. For example, the gp120 of the HIV-1_{DH12} challenge virus has only 80 and 76% amino acid sequence homology with the gp120s of HIV-1_{IIIB} and HIV-1_{SF2}, respectively. Nonetheless, the results obtained demonstrate that resistance to an HIV-1 clade B heterologous challenge can be achieved. Another unexpected finding was that the resistance observed, particularly for the two animals previously infected with HIV-1_{IIIB}, was effective against extremely high titers of the HIV-1_{DH12} inoculum. An earlier titration of the HIV-1_{DH12} stock revealed that chimpanzees became infected following inoculation with 50, 30, and 16 $TCID_{50}$ of virus but not after exposure to 6 or 1 $TCID_{50}$ of virus (data not shown). Thus, the chimpanzee infectious dose for the HIV-1_{DH12} inoculum used in this study is between 6 and 16 $TCID_{50}$. The two chronically HIV-1_{SF2}-infected animals mounted a very effective, albeit incomplete, protective response against a challenge of 10 times the chimpanzee infectious dose. One of the HIV-1_{IIIB}-infected chimpanzees (888) resisted a challenge of at least 100 times the chimpanzee infectious dose of HIV-1_{DH12} as monitored by the failure to isolate virus or to detect viral RNA and/or DNA in plasma, PBMC, and lymph node specimens even though indigenous HIV-1_{IIIB} DNA could be readily amplified from PBMC.

There are at least two other reports in the literature describing the challenge of previously infected chimpanzees with a second HIV-1 isolate. In a study performed in 1987, individual animals, previously infected with HIV-1_{LAV-1} and HIV-1_{ARV-2} for 6 and 16 months, respectively, were reciprocally challenged with a very large inoculum (approximately 10^4 $TCID_{50}$) of heterologous virus (14). Using elevations in HIV antibody titers and the number of infected PBMC coupled with Southern blot hybridization as measures of infection, the authors concluded that the persistently HIV-1_{ARV-2} (now designated HIV-1_{SF2})-infected animal became infected within weeks of HIV-1_{LAV-1} challenge. The outcome of the HIV-1_{ARV-2} superchallenge of the previously HIV-1_{LAV-1}-infected chimpanzee was less clear; large numbers of HIV-1_{LAV-1}-infected PBMC were still present at the time of challenge (5 to 6 months after the initial HIV-1 infection), and no diagnostic HIV-1_{ARV-2} band was visualized on the Southern blot. A second study demonstrated that two seropositive chimpanzees, previously inoculated with unpassaged HIV-1 isolates or a suspension of a brain specimen from an AIDS patient, remained virus isolation negative following challenge with 40 chimpanzee infectious doses of HIV-1_{IIIB} (15). The interpretation of this second report is complicated by the three immunizations with γ-irradiated HIV that each animal received prior to HIV-1_{IIIB} challenge.

At the present time, the correlates of protective immunity needed for an effective prophylactic lentivirus vaccine are poorly understood. Similarly, the mechanism(s) underlying the resistance of a chronically SIV-infected macaque to a subsequent virus challenge is not known. In the present study, none of the four persistently HIV-1-infected chimpanzees had detectable levels of neutralizing antibodies against HIV-1_{DH12} prior to challenge. Although HIV-1_{DH12}-specific neutralizing antibodies were eventually detected in all of the challenged animals, neutralizing activity was never detected in one of the chimpanzees (888) following the inoculations of 170 and 1,700 $TCID_{50}$ of cell-free HIV-1_{DH12} and only low titers emerged subsequent to transfusion of 10 ml of whole blood from an HIV-1_{DH12}-infected animal. These results strongly suggest that neutralizing antibody, per se, was not responsible for resistance of animal 888 to successive exposures to HIV-1_{DH12}. Cytotoxic T-lymphocyte lysis assays, employing autologous chimpanzee Epstein-Barr virus-transformed B lymphocytes infected with recombinant vaccinia virus expressing the HIV-1_{DH12} envelope glycoprotein as targets and effector T cells from naive and persistently infected animals in conventional ^{51}Cr release assays, have been unrevealing thus far. Efforts are currently under way to examine HIV-1-specific cytotoxic T-lymphocyte lysis activities in chimpanzee PBMC subsequent to antigen-specific expansion.

The results presented here indicate that sterilizing immunity is not an achievable outcome in chronically HIV-1-infected chimpanzees subsequently challenged with a second, intraclade strain of virus. Nonetheless, when other parameters, such as virus isolation or HIV-1 RNA and DNA levels in plasma, PBMC, and lymph nodes, were used to monitor resistance to challenges with large HIV-1 inocula, even the very attenuated HIV-1_{SF2} strain was found to generate considerable protection. However, the ultimate question is whether the level of resistance observed in this study would be sufficient to prevent disease when translated to humans encountering HIV-1. At present, neither this question nor the safety of potential attenuated vaccines can be evaluated with the HIV-1–chimpanzee model, which thus far does not progress to disease. It may be possible to begin addressing both of these issues with newer versions of SIV-HIV chimeric viruses (our own unpublished data), containing the HIV-1 *vpr*, *tat*, *rev*, *vpu*, and *env* genes, which not only replicate to high titers in inoculated macaque monkeys but also cause a loss of CD4 cells and induce disease.

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