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Vaccine-Induced Protection of Chimpanzees against Infection by a Heterologous Human Immunodeficiency Virus Type 1

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The extraordinary genetic diversity of human immunodeficiency virus type 1 (HIV-1) is a major problem to overcome in the development of an effective vaccine. In the most reliable animal model of HIV-1 infection, chimpanzees were immunized with various combinations of HIV-1 antigens, which were derived primarily from the surface glycoprotein, gp160, of HIV-1 strains LAI and MN. The immunogens also included a live recombinant canarypox virus expressing a gp160-MN protein. In one experiment, two chimpanzees were immunized multiple times; one animal received antigens derived only from HIV-1_{LAI}, and the second animal received antigens from both HIV-1_{LAI} and HIV-1_{MN}. In another experiment, four chimpanzees were immunized in parallel a total of five times over 18 months; two animals received purified gp160 and V3-MN peptides, whereas the other two animals received the recombinant canarypox virus and gp160. At 3 months after the final booster, all immunized and naive control chimpanzees were challenged by intravenous inoculation of HIV-1_{SF2}; therefore, the study represented an intrasubtype B heterologous virus challenge. Virologic and serologic follow-up showed that the controls and the two chimpanzees immunized with the live recombinant canarypox virus became infected, whereas the other animals that were immunized with gp160 and V3-MN peptides were protected from infection. Evaluation of both cellular and humoral HIV-specific immune responses at the time of infectious HIV-1 challenge identified the following as possible correlates of protection: antibody titers to the V3 loop of MN and neutralizing antibody titers to HIV-1_{MN} or HIV-1_{LAI}, but not to HIV-1_{SF2}. The results of this study indicate that vaccine-mediated protection against intravenous infection with heterologous HIV-1 strains of the same subtype is possible with some immunogens.

The extraordinary genetic diversity of human immunodeficiency virus type 1 (HIV-1) is a major problem to overcome in the development of an effective prophylactic vaccine. One must contend not only with amino acid differences of up to 25% among HIV-1 strains of the same subtype or clade, but also with the presence of at least eight distinct, even more diverse subtypes, most of which can be found in multiple regions of the world (43). The first experimental attempts to demonstrate the protective efficacy of a candidate HIV-1 vaccine were optimized by using a homologous system in which the immunogens were derived from the same HIV-1 strain as the one subsequently used to challenge the immunized animals. Because only chimpanzees can be infected reliably with low doses of different HIV-1 strains, this animal model was used successfully to demonstrate that immunization with various antigens can induce HIV-1-specific immune responses that prevent infection with the homologous virus strain (6, 7, 22, 39). Furthermore, these initial studies showed that immunization of chimpanzees with the same antigens could prevent infection not only by cell-free HIV-1, but also by peripheral blood mononuclear cells (PBMC) taken from an unrelated HIV-1-infected chimpanzee (17).

Efforts to develop candidate HIV-1 vaccines have focused on the envelope glycoprotein (13), which contains a principal neutralization determinant (PND) localized in a variable region of gp120, termed the V3 loop (31, 32, 37, 51, 54). In HIV-1-infected individuals, three types of neutralizing antibodies have been described: (i) those that bind to continuous epitopes in the V2 or V3 region of gp120 (9, 19, 44, 58); (ii) those that recognize discontinuous epitopes that overlap the CD4-binding site (9, 28, 61, 65, 67); and (iii) those of lesser frequency that are targeted to epitopes in the transmembrane glycoprotein (11, 42). Passive administration of a monoclonal antibody that neutralizes HIV-1 by binding with high avidity to an epitope in the V3 loop protected chimpanzees from intravenous (i.v.) HIV-1 challenge even when the antibodies were administered after virus inoculation (12). This finding strengthened the notion that the V3 domain might be a useful component of a subunit vaccine. Additional support came when Neurath et al. (48) showed that immunization of chimpanzees with gp160 followed by boosting with a V3 loop peptide could elicit high titers of neutralizing antibodies directed against the PND. Similarly, macaques immunized with HIV-1 gp160 developed high neutralizing antibody titers when immunization was followed by injection of a PND peptide formulated with incomplete Freund's adjuvant (IFA) (53). Furthermore, the neutralizing antibodies that were elicited after immunization of chimpanzees with gp120 or gp160 or with sequential admin-

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istration of gp160 and PND peptides protected these animals against i.v. challenge with cell-free HIV-1 (6, 7, 22, 39) or virus-infected cells (17). In studies in which it was evaluated, protection appeared to correlate with the level of neutralizing antibodies that recognized the V3 domain (6, 7). More recently, Spear et al. (59) showed that antibodies to the V3 loop in serum from HIV-1-infected persons contribute a major proportion of effector functions, other than neutralization, that require antibodies.

Thus, these results appear to justify the incorporation of the V3 loop into an HIV-1 vaccine. Moreover, since this domain contains epitopes recognized by $CD8^+$ T cells (62), it might also be important for induction of cytotoxic T lymphocytes (CTL) (49, 60, 69). In general, live infectious viruses are more efficient at inducing CTL than protein or peptide antigens formulated in adjuvants. Vaccination of chimpanzees with a recombinant vaccinia virus expressing gp160 induced HIV-specific lymphoproliferative responses and major histocompatibility complex (MHC) class II-restricted $CD4^+$ CTL (68). This observation is consistent with immunization of human volunteers with gp160 or gp120, which resulted in low levels of HIV-1 envelope-specific cytotoxic activity, which was mediated primarily by $CD4^+$ cells (26, 50). In contrast, immunization of human volunteers with recombinant pox viruses expressing HIV-1 $gp160^{env}$ followed by boosting with a soluble recombinant gp160 induced both neutralizing antibodies and CTL responses (10, 24, 53).

Canarypox virus potentially has advantages over conventional vaccinia virus vectors, one of which is that replication of the canarypox virus in mammalian species is abortive; thus, safety issues in both normal and immunocompromised individuals are of less concern. Recombinant canarypox virus vectors have been used to elicit humoral and T cell-mediated immunity against diverse pathogens in animals (3, 63, 64) and have proven to be safe and immunogenic in humans (8, 53).

The hypervariability of amino acid sequences in *env* and the conformational variability of the V3 epitope due to noncontiguous amino acids (45, 47) remain formidable obstacles for the development of vaccines that will elicit broadly cross-reactive antibodies and CTL. HIV-1 vaccines incorporating antigens from multiple subtypes might be valid, provided that cross-protection could be demonstrated among HIV-1 strains that belong to the same subtype and/or have similar V3 amino acid sequences or three-dimensional configurations. To begin to address this problem, chimpanzees were immunized with different HIV-1 *env* protein and peptide antigens derived from the HIV-1_{MN} and HIV-1_{LAI} strains and then were challenged with HIV-1_{SF2}; that is, a heterologous challenge. Although these strains belong to HIV-1 subtype B, most monoclonal antibodies that neutralize LAI-related strains do not neutralize the other two strains and vice versa (23, 40, 56). Since the HIV-1_{SF2} challenge virus was passaged solely in human PBMC and never in T-cell lines, it more closely resembled primary isolates that are actually transmitted between individuals than the previously used HIV-$1_{LAI(IIIB)}$ stock.

MATERIALS AND METHODS

Animals. Adult male chimpanzees (*Pan troglodytes*), previously used in hepatitis experiments, were housed at the Laboratory for Experimental Medicine and Surgery in Primates, New York University, in biosafety level 2 facilities in accordance with institutional guidelines and standard practices for the humane care and use of chimpanzees in biomedical research. Before all procedures, chimpanzees were anesthetized by intramuscular (i.m.) injection of ketamine hydrochloride (10 mg/kg).

Vaccine preparations. Two soluble recombinant gp160 preparations were used, rgp160-LAI and rgp160-MN/LAI, the second of which contained gp120 sequences from the MN strain and gp41 sequences from the LAI strain. The HIV-1 rgp160 Env proteins were purified from the supernatants of BHK-21 cell cultures infected with recombinant vaccinia virus VVTG1163 (33, 34) or VVTG5156, which expressed uncleaved forms of either gp160-LAI or gp160-MN/LAI, respectively; both rgp160 proteins had been engineered to delete their membrane-spanning domains (22, 33, 34). To generate gp160-MN/LAI, unique restriction cleavage sites were created by site-directed mutagenesis at positions 5822 and 7272 in the gp120 moiety of the HIV-1_{LAI} *env* gene; the fragment was then excised and replaced by the corresponding gp120 portion from the HIV-1_{MN} *env* gene, obtained by PCR amplification. The hybrid $gp160^{env}$ gene was inserted into a vaccinia virus vector, and the resulting recombinant vaccinia virus, VVTG5156, was used to infect BHK-21 cells. The rgp160 protein was purified from culture supernatants by ion-exchange chromatography, lentil-lectin affinity chromatography, and gel filtration. The final preparation of rgp160-MN/LAI was 90% pure, with small amounts of cleavage fragments and traces of actin and bovine α_2-macroglobulin. The vaccine dose contained 176 μg of rgp160 in 0.45 ml of phosphate-buffered saline (PBS) and was stored at −80°C until used. The preparation of rgp160-LAI was 50% pure; each dose consisted of 150 to 200 μg of rgp160 in 0.5 to 1 ml of PBS.

The V3-LAI peptide (NeoSystem, Strasbourg, France) and the V3-MN peptide (NovaBiochem, Laufelfingen, Switzerland) were 33-amino-acid linear peptides without terminal cysteines that reproduced the hypervariable V3 loop sequences of the HIV-1_{LAI} and HIV-1_{MN} strains, respectively. Both peptides were obtained by chemical synthesis in solid phase and purified to >90% by inverse-phase and ion-exchange chromatography. The vaccine dose contained 200 μg of lyophilized peptide that was stored at −20°C until used. Syntex adjuvant formulation SAF-1 (1) or Montanide ISA 51 (Seppic, Paris, France) was added as an adjuvant. Montanide ISA 51 (0.5 ml) is an IFA composed of 1 part mannide mono-oleate (Montanide 80 or Arlacel A) to 9 parts mineral oil (Drakeaol 6VR).

Soluble recombinant proteins $p27^{nef}$ and $p18^{gag}$ were purified from *Escherichia coli* (25, 36). The $p27^{nef}$-V3 conjugate was prepared by chemical N-terminal tyrosine linkage, as described before (21). The ALVAC canarypox virus vector was developed at ViroGenetics (Albany, N.Y.). Plasmid pC5HIVMNE, obtained from Marvin Reitz (National Cancer Institute, Bethesda, Md.), was inserted into a subgenomic fragment of canarypox virus DNA. The gp160-MN gene was placed under the transcriptional control of the H6 vaccinia virus promoter and then recombined into a canarypox virus. The resulting ALVAC-HIV vCP125 recombinant was amplified in specific-pathogen-free primary chicken embryo fibroblasts, lyophilized, and stored at 4°C until used. For immunizations, $10^{6.1}$ 50% tissue culture infectious doses ($TCID_{50}$) of the canarypox virus vectors in a volume of 1 ml of distilled water were injected by the i.m. route. Recombinant vaccinia viruses expressing various HIV-1 antigens were used at a dose of 10^8 PFU and were also administered by the i.m. route.

Experimental design. The studies reported here consisted of two experiments. The large gaps in time between some series of immunizations occurred because of difficulties encountered in the generation and in vivo titration in chimpanzees of a defined heterologous HIV-1 challenge stock. In the first experiment, two chimpanzees that had been immunized initially by separate protocols with a variety of HIV-1_{LAI} antigens, including gp160, were repeatedly boosted (Table 1). During the final 34 months, one animal (C-505) received rgp160-LAI and a V3-LAI peptide, whereas the other animal (C-529) received rgp160-MN/LAI and a V3-MN peptide. The two animals were then immunized in parallel during the last 18 months before challenge. In the second experiment, two groups of two chimpanzees were immunized with different immunogens in parallel by a simplified regimen of five i.m. inoculations (Table 1). One group (C-323 and C-483) received two inoculations of rgp160-MN/LAI at time 0 and 1 month, followed by two inoculations of V3-MN peptides at 3 and 6 months; the other group (C-477 and C-641) was immunized with two inoculations of ALVAC-HIV vCP125 at time 0 and 1 month, followed by rgp160-MN/LAI at 3 and 6 months. After a 9-month rest, both groups were given a final booster inoculation that consisted of their two respective immunogens combined. All of the immunized and two naive control chimpanzees were challenged 11 weeks after the last boost by the i.v. route, using 1 ml of a 1:5 dilution of an aliquot of HIV-1_{SF2} (code 301485). This challenge virus stock, generated by nine sequential passages of the original HIV-1_{SF2} isolate by J. A. Levy on human PBMC (38), was obtained through the National Institutes of Health from Alan Schultz, National Institute for Allergy and Infectious Diseases. In our hands, the titer of this SF2 stock by end-point titrations on phytohemagglutinin-stimulated chimpanzee PBMC was 3.2×10^2 $TCID_{50}$; thus, the challenge dose was 64 $TCID_{50}$. Titration of the stock on human PBMC from different donors resulted in titers at least 100- to 1,000-fold higher, indicating more restricted infectivity of HIV-1_{SF2} for chimpanzee than for human cells. The ability of the HIV-1_{SF2} challenge virus to establish infection by i.v. inoculation was confirmed initially in one naive animal that received a slightly higher inoculum (C-623; 1 ml of a 1:4 dilution, i.e., 80 $TCID_{50}$). Furthermore, based on the p24 antigen concentration of this stock (292 ng/ml) and the estimated number of molecules of p24 per HIV-1 virion, the number of SF2 virions injected was approximately 8×10^8.

Isolation and quantitation of virus. Blood samples were obtained from chimpanzees on the day of inoculation of cell-free HIV-1_{SF2} and then every other week for 8 weeks and at monthly intervals thereafter. The presence of virus in PBMC was monitored by cocultivation of each animal's PBMC with phytohemagglutinin-stimulated human indicator PBMC and periodic assays of culture

TABLE 1. Chimpanzee immunization schedules

Chimpanzee(s)	Immunogen(s)	Adjuvant[a]	Immunization (months preinoculation)[b]
C-505	gp160-LAI, p18^{gag}, p27^{nef}	SAF-1	−66, −65, −64, −59, −58, −51
	V3-LAI peptide	SAF-1	−34, −33, −32
	gp160-LAI, V3-LAI peptide	IFA	−26, −25, −18, −3
C-529	VVgp160-Eli, VVp18^{gag}	None	−65, −64, −63
	gp160-LAI, p27^{nef}-V3 conjugate	SAF-1	−44, −43, −38
	gp160-MN/LAI	SAF-1	−32, −31, −30
	V3-MN peptide	SAF-1	−23, −22, −21
	gp160-MN/LAI, V3-MN peptide	IFA	−18, −3
C-323 + C-483	gp160-MN/LAI	IFA	−18, −17, −3
	V3-MN peptide	IFA	−15, −12, −3
C-477 + C-641	ALVAC-HIV vCP125	None	−18, −17, −3
	gp160-MN/LAI	IFA	−15, −12, −3

[a] The adjuvants were either SAF-1 (Syntex adjuvant formulation [1]) or IFA (Montanide ISA 51), as described in Materials and Methods.
[b] Time of immunization before inoculation of cell-free HIV-1.

supernatants for reverse transcriptase (RT) activity (16) or p24^{gag} antigen (29). Freshly stimulated human PBMC were added every 10 days. Cultures enriched for CD4$^+$ lymphocytes were obtained from chimpanzee PBMC by removal of CD8$^+$ cells with magnetic beads coated with anti-CD8 monoclonal antibodies (Dynabeads; Dynal). Single-cell suspensions of lymph node biopsy tissues and cells from bone marrow aspirates were also evaluated for the presence of virus by cocultivation with human PBMC. To quantitate numbers of infectious cells, serial fivefold dilutions of cell suspensions were cocultured in duplicate wells of 12-well plates with 2×10^6 human PBMC; the culture supernatants were monitored for RT activity for 6 weeks (16).

PCR and DNA sequence analysis. Specimens of chimpanzee PBMC, bone marrow cells, lymph node biopsy tissues, and cocultures of chimpanzee and human PBMC were tested independently in two laboratories for proviral DNA by nested PCR with approximately 1 μg or 10^5 cell equivalents of DNA and primers derived from conserved HIV-1 *gag* sequences. At the University of Alabama at Birmingham, the *gag* gene sense and antisense primers for the first rounds of PCR amplification, followed by the nested set used in the second round PCR, were: P17/1, 5′-TCTCGACGCAGGACTCGGCTTGCT-3′; P17/2, 5′-AAGTTCTAGGTGATATGGCCTGAT-3′; P17/3, 5′-AATTTTGACTAGC GGATCCTAGAA-3′; and P17/4, 5′-GTTCTGCAGTATAGGGTAATTTT-3′. These primers amplify a 572-bp region in p18^{gag}. The *gag* primers used in the second laboratory (Duke University) were those described by Simmonds et al. (57). The conditions for the PCR assays were those previously described by Simmonds et al. (57) and included appropriate HIV-1-positive and -negative control DNA samples. To amplify a 720-bp *env* region spanning the V3 loop, the external and internal nested primer pairs, respectively, were: 587S, 5′-ACAG-TACAATGTACACATGGAA-3′; 588, 5′-CCACTCTTCTCTTTGCCTTGGT GGGT-3′; 589, 5′-TCAACTCAACTGCTGTTAAATGGCAGT-3′; and 590, 5′-TTGTTAATAGCAGCCCTGTAATATTTGATG-3′.

To confirm that the virus recovered from the chimpanzees was HIV-1$_{SF2}$, the nested PCR products were purified, cloned with a TA cloning kit (Invitrogen, San Diego, Calif.), and sequenced by the dideoxy chain termination method (Sequenase 2 kit; U.S. Biochemicals, Cleveland, Ohio).

Serology. Serum samples were tested for total HIV-1 antibodies with two commercially available enzyme immunoassay (EIA) kits according to the manufacturer's directions (Diagnostics Pasteur, Marnes la Coquette, France; Sanofi, Seattle, Wash.). Immunoblot assays were also done with a commercially available kit (Diagnostics Pasteur) at a serum dilution of 1:100. Antibodies to rgp160-MN/LAI or linear V3 peptides were detected by enzyme-linked immunosorbent assay (ELISA) in microtiter plates coated with rgp160 (130 ng per well) or V3 peptide (100 ng per well) in carbonate buffer (pH 9.8). PBS containing 0.05% Tween 20 (washing buffer) and 5% powdered milk was used for blocking nonspecific binding and for dilution of sera. The plates were incubated sequentially with serial dilutions of chimpanzee serum samples (1:50 to 1:1250) or purified human immunoglobulins from HIV-seropositive individuals (as standards) for 90 min at 37°C and then with peroxidase-conjugated goat anti-human immunoglobulin (diluted 1:10,000) for 90 min at 37°C. *O*-Phenylenediamine dihydrochloride was used as a substrate; after 30 min of incubation, the plates were read at 490 to 650 nm. Endpoint dilution titers, calculated from binding curves, were used as measures of the magnitude of antibody binding to rgp160 or V3 peptide; antibody binding titers are reported as the $\log_{10}$ of the endpoint dilution titers.

Neutralization assays. Two different neutralization assays were used. One assay evaluated inhibition of syncytium formation in CEM-SS cells (46); the assay utilized HIV-1$_{LAI(IIIB)}$ or HIV-1$_{MN}$ stocks prepared in continuous T-cell lines and was performed as described before (46). The neutralizing antibody titer was defined as the last dilution of serum that reduced the number of syncytia by 90%. The second neutralization assay measured reduction of infectious virus titers as estimated by endpoint dilution (5). Briefly, following incubation for 30 min at room temperature of different dilutions of sera with six concentrations (0.1 to 100 TCID$_{50}$) of HIV-1, the virus-antibody mixtures were added to AA5 cells (5) in 96-well microtiter plates in duplicate. After 6 to 8 days, depending on the virus isolate, culture supernatants were tested for RT activity as a measure of infectivity. After determining the TCID$_{50}$, the surviving fraction of virus was plotted for each serum dilution. The neutralizing titer was interpolated as the reciprocal of the serum dilution that resulted in 90% reduction of the virus titer compared with titers obtained with normal chimpanzee serum. A variation of the latter assay was used to evaluate neutralization of HIV-1$_{SF2}$ passaged in human PBMC. Instead of AA5 cells, cryopreserved PBMC from 10 human seronegative donors were infected with the virus-serum mixtures after stimulation for 2 days with anti-CD3 (0.1 mg/ml) and anti-CD28 (0.1 mg/ml) antibodies. Supernatants were tested on day 12 for RT activity, and neutralization titers were calculated as above.

RESULTS

Susceptibility of chimpanzees to HIV-1$_{SF2}$ challenge stock. Before the immunized chimpanzees were challenged with virus, the infectivity for chimpanzees of the HIV-1$_{SF2}$ challenge stock was evaluated. A naive chimpanzee (C-623) was inoculated i.v. with 1 ml of a 1:4 dilution (approximately 80 TCID$_{50}$ in chimpanzee PBMC) of the stock. Using standard coculture conditions (16, 17), virus was recovered from PBMC of this animal at weeks 2 and 4 after inoculation, but not thereafter (Table 2). Single-cell suspensions of lymph node biopsy tissues and lymphocytes from bone marrow aspirates were also evaluated for the presence of virus by cocultivation with human PBMC; all cultures were negative at 6 weeks after infection and at later times. C-623 seroconverted at 8 weeks, and HIV-1-specific antibody titers persisted, albeit at lower levels than those of HIV-1$_{LAI}$-infected chimpanzees, indicating that the animal was persistently infected (Fig. 1).

Protection of immunized chimpanzees from infection. To determine whether chimpanzees could be protected from infection with HIV-1$_{SF2}$ after immunization with antigens derived from HIV-1$_{LAI}$ or HIV-1$_{MN}$, two experiments were performed. The first experiment will be described briefly because it extended over more than 5 years and utilized a diverse number of immunogens. (The extraordinary length of the study resulted primarily from a series of unforeseen delays in producing a heterologous HIV-1 challenge stock titrated in chimpanzees.) Two chimpanzees that had initially received a variety of HIV-1 antigens were repeatedly boosted with gp160-

TABLE 2. Recovery of HIV-1_{SF2} from PBMC of naive and immunized chimpanzees[a]

Expt	Chimpanzee	Status	Origin of immunogen	HIV-1_{SF2} recovered at wk postchallenge:						
				0	2	4	6	8	12	16
1	C-623	Naive		−	+	+	−	−	−	−
2	C-511	Naive		−	+	+	+	−	−	−
	C-505	Immunized	LAI	−	−	−	−	−	−	−
	C-529	Immunized	LAI + MN	−	−	−	−	−	−	−
3	C-441	Naive		−	+	+	+	−	−	−
	C-323	Immunized	MN	−	−	−	−	−	−	−
	C-483	Immunized	MN	−	−	−	−	−	−	−
	C-477	Immunized	MN	−	−	+	−	−	−	−
	C-641	Immunized	MN	−	+	−	−	−	−	−

[a] The animals were inoculated i.v. with 1 ml of a 1:4 (C-623) or 1:5 (all other chimpanzees) dilution of the HIV-1_{SF2} virus stock (38). Chimpanzee PBMC were cocultivated with an equal number of human PBMC (~10^7) from HIV-seronegative human donors.

LAI and a V3-LAI peptide (C-505) or with gp160-MN/LAI and a V3-MN peptide (C-529) (Table 1). Neutralizing antibodies were induced in both animals, but C-529 developed much higher titers and had neutralizing antibodies targeted to both HIV-1_{MN} and HIV-$1_{LAI(IIIB)}$ (Table 3). In addition, injection of the MN antigens induced a strong anamnestic antibody response to the HIV-1_{LAI} antigens, which is consistent with previous results obtained after vaccination of human volunteers with HIV-1 gp120 (35).

The two immunized chimpanzees and an unimmunized control animal, C-511, were challenged i.v. with 64 $TCID_{50}$ of the HIV-1_{SF2} stock. Virus was readily isolated by cocultivation of C-511's PBMC at weeks 2, 4, and 6 after inoculation but not later, consistent with virus recovery from C-623. In contrast, virus was not isolated from C-505's or C-529's PBMC at any time (Table 2) during a 1-year follow-up. Single-cell suspensions of lymph node biopsy tissues and lymphocytes from bone marrow aspirates were also evaluated for the presence of virus by cocultivation with human PBMC; all cultures were negative. As confirmatory evidence for protection, nested PCR assays were performed on DNA from multiple PBMC samples (weeks 2 to 28) and on cells from lymph node (24 and 52 weeks) and bone marrow (36 weeks) biopsies collected at various times after challenge. PBMC from the control C-511 and an HIV-1_{LAI}-infected chimpanzee were tested in parallel. All

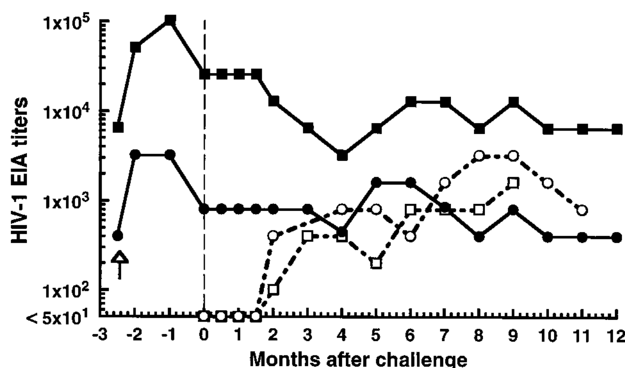

FIG. 1. Serum anti-HIV-1 antibody titers in chimpanzees. Responses following challenge with HIV-1_{SF2} of naive chimpanzees C-511 (□) and C-623 (○) and chimpanzees immunized with antigens derived from HIV-1_{LAI}, C-505 (●), or HIV-1_{LAI} and HIV-1_{MN}, C-529 (■). Titers are defined as the reciprocal of the last dilution of serum to give a positive reading by EIA. Arrow, time of last booster immunization; vertical line, time chimpanzees were inoculated with infectious HIV-1_{SF2}.

PCR assays performed on cells from the immunized chimpanzees were negative. All of the samples from C-511 and from the HIV-1_{LAI}-infected chimpanzee were positive with one exception; attempts to amplify proviral DNA from PBMC of C-511 at 6 weeks after challenge with both *gag* and *env* nested primers were negative even though PBMC from 13 and 24 weeks were positive. This result could be an artifact of the low virus burdens in HIV-1_{SF2}-infected chimpanzees (see below) and the relative numbers of cells used for coculture and each PCR (10^7 versus 10^5, respectively).

After virus challenge, no anamnestic antibody responses were detected by EIA in sera from the two immunized animals (Fig. 1); their antibody titers remained remarkably stable during the 12 months of follow-up. Similar observations were made previously in chimpanzees that were protected from infection after challenge with homologous HIV-$1_{LAI(IIIB)}$ following hyperimmunization with gp160 and V3 peptides (17, 22). The two- and fourfold decreases in C-505's and C-529's EIA titers after SF2 challenge were not significantly different from the eightfold decrease in EIA titers in both animals during the 15-month interval between their last two booster immunizations (Fig. 2). To ensure that the persistent antibody responses were directed only to the immunizing antigens, serum samples from the animals were analyzed by immunoblot (Fig. 3). Antibodies in serum samples from both animals recognized *env*-encoded antigens, as expected, but surprisingly, antibodies to $p18^{gag}$ were present before live-virus challenge. This finding is of interest because more than 5 years had elapsed since they had been vaccinated with a recombinant vaccinia virus expressing the $p18^{gag}$ antigen from HIV-1_{LAI} (Table 1). In C-505, anti-Gag antibodies appeared to increase severalfold, reaching a maximum 24 weeks after challenge and then decreasing in parallel with Env-specific antibodies. However, antibodies to $p24^{gag}$ were never detected in either animal, which contrasted with antibody reactivity in serum samples from the control C-511 (Fig. 3). As further evidence that the immunized animals were protected from infection with HIV-1_{SF2}, the HIV-1_{LAI} and HIV-1_{MN} neutralizing antibody titers in both C-505 and C-529 progressively declined (Table 3).

Verification of genotype of challenge virus stock. Although there was no reason to suggest otherwise, to verify that the challenge virus and the virus recovered from the control chimpanzees were HIV-1_{SF2}, DNA was extracted from PBMC obtained from C-511 at 4 weeks after challenge. After PCR amplification, four clones each from *gag* and *env* were selected for nucleotide sequence analysis. The results confirmed that the challenge inoculum was HIV-1_{SF2}: a combined total of 12

TABLE 3. Serum HIV-1 neutralizing antibody titers in immunized and challenged chimpanzees[a]

Time (wk)	Titer							
	Syncytium inhibition assay				Infectivity assay			
	C-505		C-529		C-505		C-529	
	LAI	MN	LAI	MN	LAI	MN	LAI	MN
−11	<32	<8	512	256	<10	<10	640	550
−7	256	<8	2,048	4,096	170	<10	4,100	4,440
−3	128	<8	2,048	8,192	155	<10	2,550	3,470
0	32	<8	1,024	4,096	208	<10	1,910	5,350
2	>32	<8	1,024	4,096	40	<10	1,600	4,800
4	32	<8	960	3,200	80	<10	570	4,800
8	<32	<8	512	256	74	<10	1,650	2,940
16	<8	<8	128	256	18	ND[b]	173	ND

[a] Chimpanzees C-505 and C-529 received their last booster injections of gp160 and V3 peptides (LAI and MN, respectively) at 11 weeks before challenge with HIV-1_{SF2} (week 0). Neutralization titers for HIV-1 strains LAI and MN were determined by a syncytium inhibition assay (46) and a neutralization-of-infectivity assay (5); for both assays, titers are the last dilution of serum that resulted in 90% inhibition.

[b] ND, not done.

point mutations were found in the four clones of the 720-bp *env* gene fragment, but only 3 point mutations were observed in the clones of the 572-bp *gag* gene fragment. Thus, in the regions sequenced, there appeared to be at least threefold greater diversity in *env* (12 of 2,880 = 0.0042) than in *gag* (3 of 2,288 = 0.0013), which is consistent with HIV-1 variation.

Comparison of immunization with gp160 and V3 peptide versus ALVAC-gp160 and gp160. To obtain additional evidence that intrasubtype heterologous protection could be achieved, a simplified vaccination protocol that compared two different combinations of immunogens was designed. Two chimpanzees (C-323 and C-483) were immunized by two inoculations of rgp160-MN/LAI 1 month apart, followed by two inoculations of V3-MN peptides at 3 and 6 months; 9 months later, the two immunogens were combined for a final booster inoculation. In parallel, two other animals (C-477 and C-641) were immunized on the same schedule as above, first with ALVAC-HIV vCP125, then with rgp160-MN/LAI, and finally with a combination of the two immunogens (Table 1). Three months after this last boost, these four immunized animals and a naive control chimpanzee, C-441, were challenged i.v. with 64 $TCID_{50}$ of HIV-1_{SF2}. As in the first heterologous challenge experiment, virus was isolated from PBMC of the control chimpanzee at weeks 2, 4, and 6 after HIV-1_{SF2} inoculation (Table 2). Virus was also isolated one time only from PBMC of the two ALVAC-rgp160-immunized chimpanzees—at 2 weeks from C-641 and at 4 weeks from C-477. However, there was no evidence of virus in whole or $CD4^+$ cell-enriched PBMC from either of the two animals immunized with the rgp160-V3 combination during 9 months of follow-up. Attempts to isolate virus from single-cell suspensions of lymph node biopsy tissue or bone marrow aspirates at 6, 12, 16, and 24 weeks after challenge were negative for all animals, including the control C-441. Similarly, nested PCR assays performed on DNA samples from PBMC and lymph node and bone marrow cells from the immunized animals were also negative. In contrast, all nested PCR assays performed on PBMC from C-441 through 28 weeks of follow-up were positive; however, PCR results on C-441's bone marrow sample were negative, which is consistent with low virus burdens.

After virus challenge, no anamnestic antibody responses to HIV-1 were detected by EIA in serum from the two virus-negative animals (Fig. 4A); their antibody titers declined over 7 months (at which time they were used in an unrelated experiment), in a manner similar to that observed in other immunized but unchallenged chimpanzees. In contrast, although

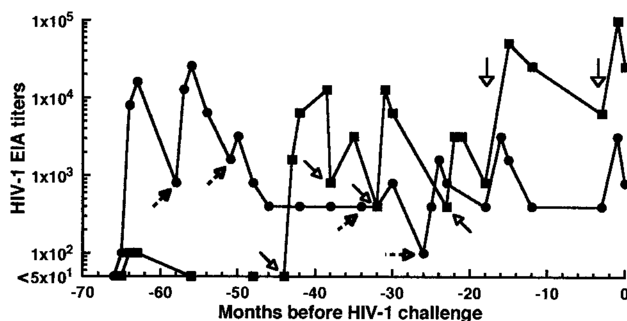

FIG. 2. Serum anti-HIV-1 antibody titers in chimpanzees C-505 (●) and C-529 (■) during immunization. C-505 and C-529 were first immunized at −66 and −65 months, respectively. Small dashed arrows for C-505 and solid arrows for C-529 indicate the times at which the first in each series of immunizations (see Table 1) was begun. The two vertical arrows indicate the times at which the two chimpanzees were immunized in parallel before virus challenge.

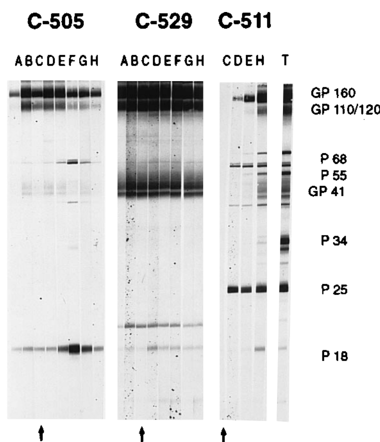

FIG. 3. Immunoblot analysis of serum samples from chimpanzees. Lanes A to H represent serum samples collected at weeks −11, −3, 0, 8, 17, 23, 31, and 39, respectively, relative to the time at which the immunized chimpanzees were inoculated with HIV-1_{SF2}, indicated by arrows. For the control chimpanzee C-511, the lanes show antibodies present in serum collected at weeks 0, 8, 17, and 39. Lane T, manufacturer's (Diagnostics Pasteur, New LAV Blot I) positive control serum. The positions of HIV-1 proteins are indicated.

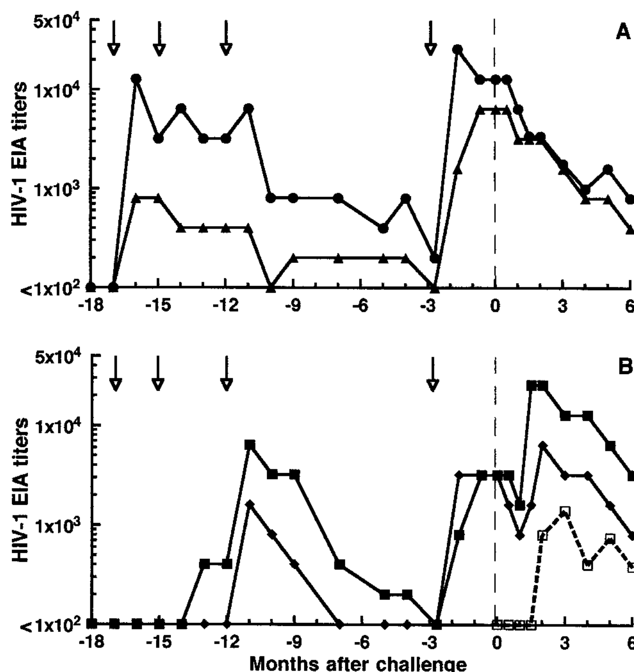

FIG. 4. Serum anti-HIV-1 antibody titers in chimpanzees. (A) Responses of chimpanzees immunized with rgp160-MN/LAI and V3-MN, C-323 (▲) and C-483 (●). (B) Responses of naive chimpanzee, C-441 (□), and chimpanzees immunized with ALVAC HIV vCP125 and rgp160-MN/LAI, C-477 (■) and C-641 (◆), respectively. Titers and dotted line are as defined in the legend to Fig. 1. Initial immunization was at −18 months; subsequent immunizations are indicated by the arrows.

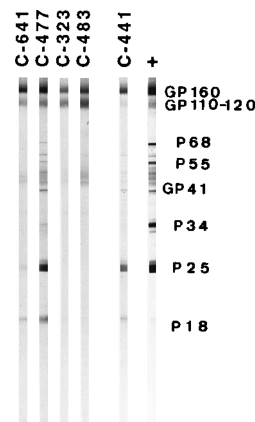

FIG. 5. Immunoblot analysis of serum samples from chimpanzees (experiment 2) at 28 weeks after inoculation with HIV-1_{SF2}. Lane +, positive serum control. The positions of HIV-1 proteins are indicated.

the two chimpanzees from which virus was recovered on only one occasion each exhibited increases in anti-HIV-1 antibodies, these increases paralleled seroconversion of the control chimpanzee (Fig. 4B). This delay indicated that the amount of viral antigen presented to the immune systems of these animals was minimal, consistent with our subsequent failure to detect virus in lymph node or bone marrow tissues. The EIA results were confirmed by immunoblot analysis (Fig. 5). The virus-negative chimpanzees (C-323 and C-483) never developed antibodies to Gag antigens, whereas antibodies to both $p24^{gag}$ and $p18^{gag}$ antigens were detected in sera from C-477 and C-641 within a few months after challenge. The respective intensities of this reactivity reflected the maximum anti-HIV-1 serum antibody titers that were attained by the two chimpanzees.

That the ALVAC/rgp160-immunized animals became infected was also apparent from the evolution of their neutralizing antibody titers (Table 4). Serum samples from both C-477 and C-641 had increases in anti-HIV-1_{MN} neutralizing antibody titers, whereas comparable neutralizing activity declined in sera from the two chimpanzees immunized and challenged in parallel. C-477 but not C-641 also developed neutralizing antibodies against HIV-1_{SF2}, consistent with the apparent lower level of infection in the latter animal.

A similar pattern was apparent in the evolution of antibodies recognizing the V3 loop, as measured by ELISA (Fig. 6). At the time of virus challenge, the titers in serum from the rgp160/V3-immunized animals were from 10- to 15-fold higher than those of the ALVAC/rgp160-immunized animals, but after challenge, the titers of the latter increased 4- to 8-fold, whereas those of the former decreased or remained constant.

Quantitation of HIV-1 virus burdens. Until now, all previous studies of immune-mediated protection of chimpanzees from infection with HIV-1, as well as the majority of studies to evaluate HIV-1 pathogenesis in chimpanzees, have used viruses derived from the LAI strain. Since there is substantial information regarding virus burden during both the acute and long-term asymptomatic phases of infection with HIV-1_{LAI} and HIV-$1_{LAI(IIIB)}$, we evaluated indicators of virus burdens in the three naive chimpanzees infected with HIV-1_{SF2}. These indicators included the frequencies of virus isolation from PBMC by routine cocultivation of 10^7 PBMC with normal human cells and quantitation of the minimum number of infected cells in single-cell suspensions of lymphocytes obtained by lymph node biopsies. Virus was not isolated from any chimpanzee's PBMC obtained more than 6 weeks after inoculation of HIV-1_{SF2}, suggesting very low virus burdens. In contrast, after infection with HIV-1_{LAI} or HIV-$1_{LAI(IIIB)}$, virus can be recovered consistently from most animals' PBMC for months and even years (2, 6, 7, 15–18, 22). In fact, isolation of HIV-1 from some chimpanzees infected with HIV-1_{LAI} for 6 or more years has been successful on more than 95% of attempts (14, 15).

Quantitation of virus burdens in single-cell suspensions of lymph node tissues from HIV-1_{SF2}- and HIV-1_{LAI}-infected chimpanzees also was consistent with the former animals' having significantly lower virus burdens. In five attempts to isolate

TABLE 4. Serum neutralizing antibody titers to HIV-1_{MN} in immunized chimpanzees[a]

Time (wk)	Titer							
	Syncytium inhibition assay				Infectivity assay			
	C-483	C-323	C-477	C-641	C-483	C-323	C-477	C-641
−11	ND[b]	ND	ND	ND	<10	<10	<10	<10
−7	128	64	32	8	82	25	17	<10
−3	64	64	16	<4	>90	10	59	<10
0	32	>64	<16	<8	222	255	36	39
2	>16	32	<16	<8	ND	ND	ND	ND
8	8	>16	64	32	ND	ND	ND	ND
12	16	8	128	<32	72	42	537	109

[a] The animals received a last booster inoculation at −11 weeks and were challenged with HIV-1_{SF2} at week 0. Neutralization assays were as described in Table 3, footnote *a*.

[b] ND, not done.

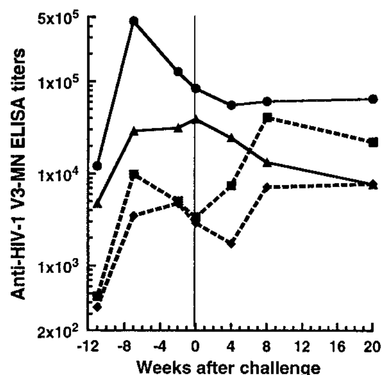

FIG. 6. Serum antibody titers to the V3-MN peptide in chimpanzees after the last booster immunization (−11 weeks) and challenge with HIV-1_{SF2} (vertical line). Animals: C-323 (▲), C-483 (●), C-477 (■), and C-641 (◆).

HIV-1 from a minimum of 10^6 lymph node cells from HIV-1_{SF2}-infected chimpanzees (biopsied 4 to 24 weeks after inoculation), virus was isolated only twice (40% success), at week 4 from C-511 and at week 6 from C-441 (Table 5). In contrast, isolation of virus from similarly prepared lymph node cells obtained from HIV-$1_{LAI(LAV\text{-}1b)}$- or HIV-$1_{LAI(IIIB)}$-infected chimpanzees (biopsied 4 to 25 weeks after infection) has been successful in 100% of seven attempts (14). The virologic results and the persistently low EIA antibody titers, as found in earlier studies (18, 41), support the conclusion that HIV-1_{SF2} replication and persistence in chimpanzees are severely restricted compared with those of strains derived from HIV-1_{LAI}.

DISCUSSION

Immunization of two chimpanzees with a variety of HIV-1 immunogens derived from HIV-1 followed by rgp160 and V3 peptides from either HIV-1_{LAI} or HIV-1_{MN} protected two of two chimpanzees from infection with HIV-1_{SF2}. Likewise, a series of five immunizations of two chimpanzees with only rgp160-MN/LAI and a V3-MN peptide provided protection against HIV-1_{SF2} in two of two animals. However, immunization of two chimpanzees with a low dose of recombinant canarypox virus expressing the gp160-MN (vCP125) followed by soluble rgp160-MN did not provide protection from the same challenge inoculum. This HIV-1_{SF2} inoculum was equivalent to a primary isolate of HIV-1, as it had been passaged exclusively in human PBMC. It therefore more closely resembled viruses transmitted during natural HIV-1 infections than do cell line-adapted viruses, such as the HIV-$1_{LAI(IIIB)}$ challenge stock used previously to test candidate HIV-1 vaccines in chimpanzees (2, 6, 7, 17, 22). The second experiment reported herein shows that immunization with envelope antigens from HIV-1_{MN} and/or HIV-1_{LAI} is sufficient to protect chimpanzees from infection with a heterologous primary isolate of HIV-1.

The reason(s) why the canarypox virus-rgp160 regimen failed to provide protection might be insufficient immunogenicity of the live recombinant virus vaccine or the use of an insufficient dose of virus for priming. Other canarypox virus–HIV-1 recombinants, such as ALVAC-HIV vCP205, which expresses rgp160-MN as well as the *gag* and *prot* genes of HIV-1, are now being tested. Despite the absence of antibodies after the primary immunization, the subsequent high levels elicited after two booster immunizations with rgp160 suggest that the gp160 expressed by recombinant canarypox virus induced memory cell populations, but that the initial T-helper-cell response was unable to stimulate efficient B-cell differentiation, leading to antibody production. This scenario is reminiscent of previous studies in chimpanzees with recombinant vaccinia virus constructs that primed the immune system to HIV-1 antigens without inducing significant antibody responses (21, 30).

Serum samples from the vaccinated chimpanzees on the day of challenge were tested for neutralization not only of the vaccine strains LAI(IIIB) and MN (Tables 3 and 4), but also of the heterologous challenge strain with HIV-1_{SF2} stocks prepared on CEM cells or human PBMC. The HIV-1_{SF2} virus grown on the CEM cell line was neutralized only by C-505's and C-529's sera at dilutions of 1:20 and 1:470, respectively. C-529, which had higher titers than the other immunized animals, was the only animal whose serum neutralized all three HIV-1 strains (LAI, MN, and SF2); it also was the only animal exposed to V3 peptides of two different HIV-1 isolates (LAI and MN), which might explain the broader specificity of its immune response. Although serum samples from C-323 and C-483 had substantial neutralizing activity against HIV-1_{MN} (Table 6), they exhibited no neutralization of HIV-1_{SF2} grown on CEM cells. Similarly, serum samples from C-477 and C-641 showed no neutralization of the SF2 strain grown on CEM cells and only weak neutralization activity against HIV-1_{MN} (Table 4). More important, none of the sera from the six chimpanzees neutralized HIV-1_{SF2} grown and tested on human PBMC, suggesting that neutralizing antibodies did not mediate protection or that the neutralization assay with PBMC lacked sensitivity.

Comparison of the humoral immune responses of the chimpanzees in the second experiment at the time of virus challenge appeared to be the most informative. Both of the rgp160/V3-immunized but neither of the ALVAC/rgp160-immunized animals were protected against infection by HIV-1_{SF2}. Although all four animals had undergone parallel immunization regimens and virus challenge, the former animals had significantly higher antibody responses to the HIV-1_{MN} V3 determinant, but not to the whole gp160; they also had substantially higher HIV-1_{MN} neutralizing antibody titers than the ALVAC/rgp160-immunized animals (Table 6). However, none of the four chimpanzees exhibited detectable neutralizing antibodies to HIV-1_{SF2}. These antibodies appeared only later in one animal, C-477, as a result of its infection.

Several points regarding the apparent protection from overt

TABLE 5. Virus burdens in lymph nodes of chimpanzees infected with HIV-1

Chimpanzee	Strain	No. of cells[a]	
		4–6 wk	16–25 wk
C-623	SF2	$>1 \times 10^6$	$>2 \times 10^6$
C-511	SF2	1×10^6	$>5 \times 10^6$
C-441	SF2	2×10^5	ND[b]
C-090[c]	LAI(IIIB)	4×10^4	ND
C-382[c]	LAI(IIIB)	ND	1×10^6
C-562[c]	LAI(IIIB)	ND	4×10^5
C-447	LAI(IIIB)	ND	1×10^5
C-655	LAI(IIIB)	ND	1×10^5
C-1196[c]	LAI(LAV-1b)	6.4×10^2	6.4×10^2

[a] The minimum number of chimpanzee lymphocytes required to isolate virus by cocultivation with normal human PBMC. A > indicates that all cocultures were negative for virus, including those with the largest number of cells tested. The time (weeks after inoculation of HIV-1) that lymph node biopsies were performed is indicated.

[b] ND, not determined.

[c] Infected by mucosal routes.

TABLE 6. Surrogate markers of protection: humoral immunity[a]

Test	Titer in serum with immunogen:			
	gp160/V3		ALVAC/gp160	
	C-483	C-323	C-477	C-641
Whole HIV-1_{LAI} (EIA)	12,800	6,400	3,200	3,200
Anti-gp160-MN (ELISA)	974,000	278,000	220,000	185,000
Anti-V3 MN (ELISA)	84,400	39,200	3,370	2,930
MN syncytium inhibition	32	64–128	<16	<8
MN neutralization of infectivity	222	255	36	39
SF2 neutralization[b]	<20	<20	<20	<20

[a] Titers were determined at time of challenge as indicated in Materials and Methods.

[b] Neutralization of HIV-1_{SF2} was evaluated with the prototypic cell line-passaged SF2 strain in AA5 cells. Similar results were obtained with the SF2 challenge virus stock and human PBMC.

infection with HIV-1_{SF2} in four of six chimpanzees deserve comment. First, the rgp160-MN/LAI protein used to immunize the animals was a noncleavable hybrid molecule composed of the gp120 moiety of HIV-1_{MN} and the gp41 anchor moiety of HIV-1_{LAI}. This hybrid gp160 binds CD4, is recognized by monoclonal antibodies to V3 that neutralize HIV-1_{MN}, and, in naive chimpanzees, induced neutralizing antibodies to HIV-1_{MN} but not to HIV-1_{LAI}, confirming that the variable antigenic epitopes in gp120 are immunodominant, as previously observed (22, 48). In the chimpanzees primed with rgp160 and V3 peptides from HIV-1_{LAI}, however, subsequent immunization with the hybrid rgp160-MN/LAI in combination with a V3-MN peptide elicited a strong anamnestic neutralizing antibody response to HIV-1_{LAI} as well as high-titered neutralizing antibodies to HIV-1_{MN}. This result suggests that successive immunizations with different antigens, rather than simultaneous immunization with mixtures of antigens, might be more effective at eliciting cross-reactive responses.

Second, in assays comparable to those that readily detected CTL activity in PBMC from vaccinated human volunteers or HIV-infected patients (63, 66), HIV-1-specific CTL activity was never detected in PBMC from the immunized chimpanzees. While CTL may have been present in lymphoid organs, such as the spleen or lymph nodes, our present results suggest that humoral immune responses mediated the apparent protection against heterologous HIV-1 infection. If CTL were responsible for protection, one would expect the ALVAC-HIV-immunized chimpanzees to be protected, not the rgp160/V3-immunized animals. In a comparable study in human volunteers, immunization with ALVAC-HIV vCP125 followed by rgp160-MN/LAI induced strong $CD8^+$ T-cell-mediated, MHC class I-restricted CTL responses in 40% of the vaccinees (52), whereas immunization with soluble rgp160-MN/LAI and V3-MN peptides elicited only weak CTL responses in a limited number of volunteers (55). All attempts to measure possible antibody-dependent cellular cytotoxicity or complement-mediated antibody cytotoxicity in sera from the immunized chimpanzees have been negative (4, 20). In the present study, our search for an immune parameter that correlated with protection identified only neutralizing antibodies targeted to the V3 loop of HIV-1_{MN} as being possibly significant (Table 6). Thus, correlates of immunity to HIV-1 remain ambiguous.

In chimpanzees C-505 and C-529, the persistence of antibodies binding to gp120 contrasts with the short-lived neutralizing antibodies, a phenomenon also observed previously in immunized chimpanzees (6, 17, 22). Irrespective of whether

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Consensus B:  CTRPNNNTRKSIHI••GPGRAFYTTGEIIGDIRQAHC
HIV-1 LAI:    ------------R-QR------V-I-K-•-NM-----
HIV-1 MN:     -----Y-K--R---••---------KN---T------
HIV-1 SF2:    ------------Y-••------H---R------K---
```

FIG. 7. Amino acid sequence alignment of the gp120 V3 loops of HIV-1 strains LAI, MN, and SF2 relative to the consensus sequence for HIV-1 subtype B (43). Identities are indicated by dashes, and dots are placed to maintain alignments.

these two immunized chimpanzees were completely protected or sustained a covert infection, it is clear that immunization prior to exposure to infectious virus can severely retard the replication and dissemination of heterologous HIV-1 and, in the case of C-323 and C-483, preclude it. Although the restricted replication of HIV-1_{SF2} in chimpanzees compared with that of HIV-1_{LAI} may have contributed to the apparent protection, the mere presence of anti-HIV-1 immunity was not sufficient to protect the chimpanzees primed with the canarypox virus vectors. It will be important, therefore, to repeat this type of heterologous challenge experiment with other HIV-1 strains more infectious for chimpanzees before one can conclude that cross-protection is possible among subtype B viruses.

The diversity of HIV-1 gp120 is considered a major problem for the development of HIV-1 vaccines, particularly for the induction of broadly cross-reactive and protective immune responses. Our results show that this problem might be less difficult than anticipated, since immunization with rgp160 and V3 peptides from HIV-1 strains MN and LAI provided protective immunity against i.v. challenge with a heterologous primary isolate of HIV-1. Although the MN, LAI, and SF2 strains of HIV-1 are classified as subtype (clade) B viruses (43), their envelope glycoprotein amino acid sequences differ by approximately 18% and those of their V3 loops differ by about 25% (44) (Fig. 7), which is typical of that expected among subtype B viruses in vaccine field trials. However, these three strains do share the GPGRAF motif at the tip of the V3 loop, which could be the cross-reactive epitope.

Although some of the immunized chimpanzees had serum antibodies with cross-neutralization activity, none of their sera neutralized the HIV-1_{SF2} challenge virus propagated on human PBMC, which might be the more relevant assay. This observation is consistent with those of others who have shown that sera from humans immunized with HIV-1 Env antigens neutralize laboratory strains grown in T-cell lines, but not clinical isolates propagated solely in PBMC (27). Since neither V3-targeted neutralizing antibodies nor CTL appeared to be responsible for the observed cross-protection, an important area of investigation is the identification of other relevant immune responses, such as those targeted to conserved gp160 epitopes. Obviously, it is of interest to understand the mechanisms involved in the vaccine-induced heterologous protection of chimpanzees.

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