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Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus

(vaccine/AIDS/neutralizing antibodies)

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ABSTRACT **Sustained high titers of neutralizing antibodies were elicited in three chimpanzees after sequential injections of different human immunodeficiency virus 1 (HIV-1) antigen preparations derived from the HIV-1 BRU strain that included whole inactivated virus or purified recombinant proteins and then synthetic peptides identical to the major HIV-1 neutralizing epitope V3. The animals were challenged i.v. with 40 chimpanzee infectious doses (equivalent to 100 tissue culture 50% infectious doses) of a stock of HIV-1 IIIB isolate. After 6 mo of follow-up, all three animals appeared uninfected by serologic and virologic criteria, including polymerase chain reaction analysis and failure to isolate virus from peripheral blood lymphocytes, bone marrow, and lymph node tissue. Of two chimpanzees monitored for 1 yr, virus was isolated initially from one animal at 32 weeks, but the second chimpanzee was virus negative by all assays through 12 mo; the third animal has remained virus negative through 9 mo of follow-up. These results indicate that it is possible to elicit protection against, or significantly delay infection of, HIV-1 by immunization, thus laying the foundation for development of an HIV-1 vaccine.**

The development of a human immunodeficiency virus (HIV) vaccine has proven to be more difficult than anticipated (1, 2). Initial attempts at immunization of chimpanzees with subunit vaccines, inactivated HIV, and/or recombinant vaccinia virus failed to prevent the establishment of persistent infection after i.v. challenge with homologous, cell-free HIV (3–6). Recently, however, protection of chimpanzees against HIV infection was achieved by immunization with the envelope glycoprotein gp120 but not with gp160 (7). In addition, vaccines comprised of whole, inactivated simian immunodeficiency virus prevented establishment of virus infection in macaques challenged with infectious virus (8, 9) and, in a similar study, horses were protected against an equine infectious anemia virus challenge (R. C. Montelaro, personal communication). These studies indicate that protection against lentivirus infections can be achieved by vaccination. Moreover, the fact that protection of a naive chimpanzee against HIV infection was observed after preincubation of the challenge inoculum with serum from an HIV-infected chimpanzee (10) suggested that neutralizing antibodies could play an important role in protection.

In attempts to elicit high neutralizing antibody titers and because of the limited availability of chimpanzees, different protocols were used to immunize chimpanzees successively with a variety of antigenic preparations derived from the lymphadenopathy-associated virus type 1 BRU strain (LAV-1_{BRU}) of HIV-1. When sustained titers of neutralizing antibodies were obtained, the chimpanzees were challenged with infectious cell-free HIV and monitored regularly to determine whether infection had occurred.

MATERIALS AND METHODS

Animals. Adult male chimpanzees (*Pan troglodytes*) that had been used previously in hepatitis A, B, and non-A non-B experiments were maintained at the Laboratory for Experimental Medicine and Surgery in Primates, in biosafety level 3 facilities. All experimental procedures were done according to institutional guidelines for containment of infectious diseases and for humane care of primates (11).

Immunogens. Sucrose gradient-purified whole HIV was inactivated by incubation with 0.025% β-propiolactone, followed by 0.025% formalin (5). Recombinant gp160env was purified from the culture medium of BHK21 cells infected with VV-1163, a recombinant vaccinia virus expressing the gp160 *env* gene modified by site-directed mutagenesis to destroy the gp120/41 cleavage site and to remove the anchor domain of gp41 (12, 13). Where indicated, the antigen was mixed with recombinant p18gag, p27nef, and p23vif antigens that were purified from *Escherichia coli* pTG2153, pTG1166, and pTG1149, respectively, as described (14, 15). Before each immunization, inactivated whole HIV (250 μg of viral protein) or the purified recombinant proteins (125–150 μg each per dose) were mixed with the Syntex adjuvant formulation 1 (SAF-1) (16), and 2 ml of the mixtures was injected i.m.

An aliquot (19.8 mg) of a 25-amino acid peptide, with the single-letter code sequence Y-NTRKSIRIQRGPGRAFVT-IGKIGN (17–19), was treated first with citraconic acid and then coupled to 19.3 mg of keyhole limpet hemocyanin (KLH) by N-terminal tyrosyl linkage using bisdiazobenzidine (pH 9.0). After formulation with SAF-1, immunizations with

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Abbreviations: HIV, human immunodeficiency virus; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; EIA, enzyme immunoassay; $TCID_{50}$, tissue-culture 50% infective dose; SAF-1, Syntex adjuvant formulation 1.

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the V3 peptide–KLH conjugate (300 μg of peptide per dose) were done by the i.m. route.

Challenge Virus. The challenge inoculum was from a stock of HIV-1, strain HTLV-IIIB (obtained from L. Arthur, National Cancer Institute), which had been titrated in chimpanzees and used in other HIV vaccine challenge studies (4, 7). The infectivity titer of this HIV-1 stock is considered to be 10^4 tissue culture 50% infective dose ($TCID_{50}$) per ml and 4×10^3 infectious units per ml for chimpanzees. The chimpanzees were challenged i.v. with 1 ml of a 1:100 dilution. Aliquots of these same 1:100 dilutions were titrated in quadruplicate by 2-fold serial dilution and infection of H9 cells in 96-well microtiter plates. After 6 days, infection was scored by immunofluorescence assay. By this method, the challenge inoculum had a titer of >64 immunofluorescent focus-forming units (end-point not reached) for the first aliquot and 170 for the second.

Neutralization Assay. Neutralization activity in serum samples from immunized chimpanzees was determined by inhibition of syncytia formation in CEM-SS cells, as described (20), or inhibition of immunofluorescent foci in H9 cells.

Virus Isolation. PBMC or bone marrow cells (obtained as aspirates) from challenged chimpanzees were cultured with normal human PBMC (21). $CD4^+$-enriched lymphocytes were obtained from chimpanzee PBMC by separation with magnetic beads to which were attached monoclonal antibodies specific for the CD8 antigen (Dynabeads, Robbins Scientific, Mountain View, CA). Lymph node tissue obtained by biopsy was minced with scissors and cultured with human PBMC. All cultures were maintained and monitored for reverse transcriptase activity for 6 weeks before being discarded.

Polymerase Chain Reaction (PCR). Both single- and double-round (nested) PCR were performed. Single-round PCR was as described (22). The positive control consisted of DNA from the 8E5 cell line persistently infected with lymphadenopathy-associated virus type 1 (LAV-1). For nested PCR, the primers for the first round of PCR(23) were 5′-GCTTCTAGATAATACAGTAGCAACCCTCTATTG-3′, corresponding to a 3-base clamp sequence, an *Xba* I restriction site, and nucleotides 1025–1048 of the pHXB2 genome (27); and 5′-GTCGGCCTTAAAGGCCCTGGGGCTTGTTCCATCTATC-3′, corresponding to a 3-base clamp sequence, a *Not* I restriction site, and nucleotides 5573–5553 of the pHXB2 genome (27). From the first round, 2.5 μl of the product was reamplified with primers SK145 and SK150 (24) over a region from nucleotides 1366 to 1507 on the pHXB2 genome.

RESULTS

Immunization Regimens (Table 1). Immunization of chimpanzee C-339 with inactivated whole HIV resulted in high titers of antibodies to *gag*- and *env*-encoded proteins, as measured by enzyme immunoassay (EIA), a low neutralizing antibody response, and no detectable cell-mediated immune response. In an effort to enhance immune responses, chimpanzee C-339 was immunized with purified recombinant gp160env. After one intradermal inoculation of gp160env with bacillus Calmette–Guérin (BCG) in multiple sites on the chest, chimpanzee C-339 was given four successive i.m. injections of the same antigen formulated with SAF-1. Total EIA antibody and neutralizing antibody titers were determined periodically: both remained unchanged and decreased rapidly after the injections were discontinued (Fig. 1*A*).

In HIV-infected persons, most HIV-neutralizing antibodies are directed against the third hypervariable region of the external envelope glycoprotein, termed the V3 loop (17–19). Antibodies to epitopes within the loop abrogate virus infectivity, probably by preventing fusion of the viral envelope to the target cell membrane. Neutralizing antibodies to V3 epitopes can, in fact, be added as long as 40 to 60 min after virus binds to the cell and still prevent infection (26). Therefore, to determine whether immunization with the V3 loop would boost neutralizing antibody titers, chimpanzee C-339 was injected with an oligopeptide having the V3 sequence of $HIV\text{-}1_{BRU}$, cross-linked to KLH, and formulated with SAF-1. No change in EIA titer was observed (Fig. 1*A*), but a significant increase in neutralizing antibody titers, which were sustained for several months, was obtained after the second immunization at week 108 (Fig. 2).

Another chimpanzee, C-433, that had been primed by vaccination with recombinant vaccinia virus VV-1139 (12), was immunized repeatedly with 125–150 μg each of recombinant soluble gp160env, p18gag, p27nef, and p23vif (see Table 1 legend). The anti-HIV antibody response induced by this regimen was clearly transient, with titers rising sharply after each booster injection and then decreasing rapidly (Fig. 1*B*). The neutralizing antibody and EIA titers of chimpanzee C-433 fluctuated in parallel. Finally, chimpanzee C-433 was injected with the same V3 peptide–KLH conjugate as chimpanzee C-339, according to the same immunization protocol. Neutralizing antibody titers increased significantly after the second injection of the V3–peptide conjugate and remained high thereafter (Fig. 2); a third immunization 4 mo later (week 126) elicited no change in titers.

At the time chimpanzee C-433 first received the purified recombinant proteins (48 weeks), a third chimpanzee, C-499, received an i.m. injection of purified gp160env and p18gag formulated with SAF-1. Chimpanzee C-499 received six booster inoculations of the same antigens, followed by a series of four injections of a mixture of 21 free (unconjugated) V3 peptides (27) in SAF-1. As with chimpanzees C-339 and

Table 1. Immunization regimens of chimpanzees with various HIV-1 antigens

Animal	Recombinant VV-1139	Inactivated HIV	Recombinant antigens				V3 peptide
			gp160	gag	nef	vif	
C-433	+	−	+	+	+	+	+
C-339	−	+	+	−	−	−	+
C-499	−	−	+	+	−	−	+

For chimpanzees C-433 and C-339, times of immunizations and virus challenge were calculated from the time that chimpanzee C-433 received its first immunization with VV-1139, which is considered week 0. Chimpanzee C-433 was first immunized with a recombinant vaccinia virus, VV-1139, that expresses a noncleavable version of the $HIV\text{-}1_{BRU}$ gp160env antigen (12). VV-1139 was administered on weeks 0, 8, and 21 by scarification on the upper back with a two-pronged needle (2×10^8 plaque-forming units per inoculum). At week 27, PBMC from chimpanzee C-433 were stimulated with phytohemagglutinin, cultured in medium containing interleukin 2, and then infected with VV-1139 at a multiplicity of infection of 7. After culture for an additional 16 hr, the PBMC were fixed with 0.8% paraformaldehyde and reinjected into chimpanzee C-433 by the i.v. route (25). At weeks 48, 54, 58, 81, 86, 88, 114, and 124, chimpanzee C-433 was inoculated i.m. with mixtures of purified gp160env, p18gag, p27nef, and p23vif (125–150 μg each per dose) formulated with SAF-1. Chimpanzee C-339 was first immunized on week 33 by i.m. injection of inactivated HIV (250 μg of viral protein) mixed with SAF-1 (1 mg of threonyl muramyl dipeptide), followed by booster inoculations on weeks 37, 41, 62, and 124. Chimpanzee C-339 was then inoculated with purified gp160env only (125 μg per dose) on weeks 66, 74, 81, 85, and 87. The V3 peptide (300 μg of peptide per dose) was administered i.m. on weeks 105, 108, and 126. Chimpanzees C-339 and C-433 were challenged on week 131 with 100 $TCID_{50}$ of $HIV\text{-}1_{IIIB}$. Chimpanzee C-499 was inoculated i.m. with a mixture of gp160env, p18gag, and SAF-1 on weeks 0, 6, 10, 33, 38, 66, and 76. (Note: week 0 for chimpanzee C-499 corresponds to week 48 for chimpanzees C-433 and C-339.) A mixture of 21 free V3 peptides (100 μg each per dose) was administered i.m. with SAF-1 on weeks 79, 83, 87, and 102. Chimpanzee C-499 and chimpanzee C-087, a naive control, were challenged on week 106 with 100 $TCID_{50}$ of HIV_{IIIB}.

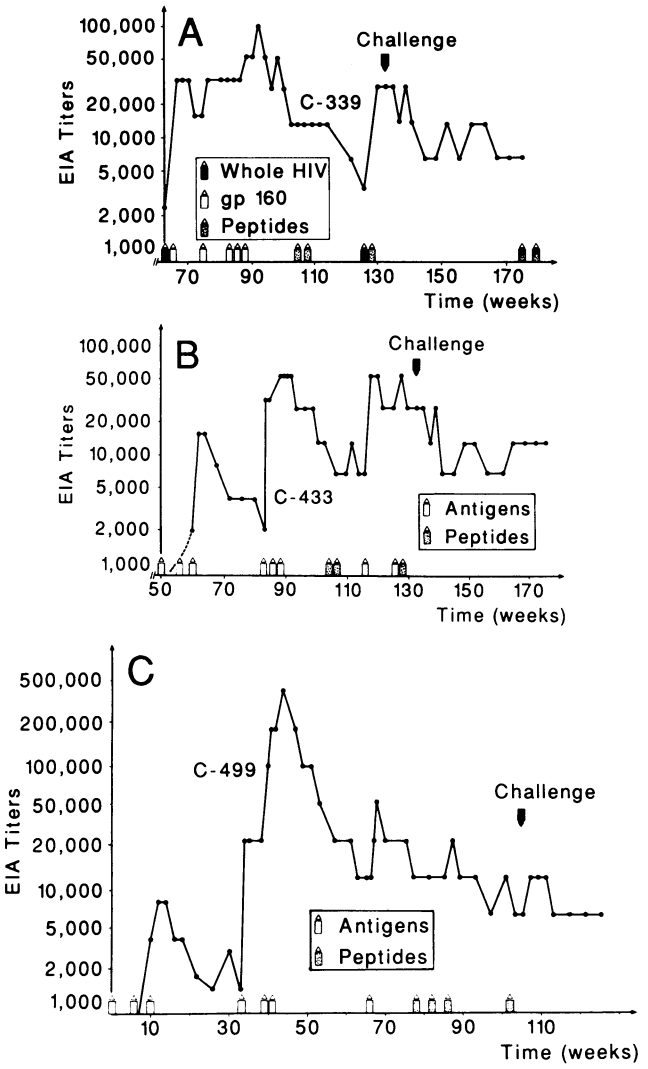

FIG. 1. Total HIV-1-specific antibody titers for chimpanzees C-339 (*A*), C-433 (*B*), and C-499 (*C*). At indicated times, chimpanzees were inoculated with various immunogens (see legend to Table 1) or challenged with HIV-1. Titers are defined as the reciprocal of the highest dilution of serum that was positive with an HIV-1 EIA kit (Genetic Systems, Seattle).

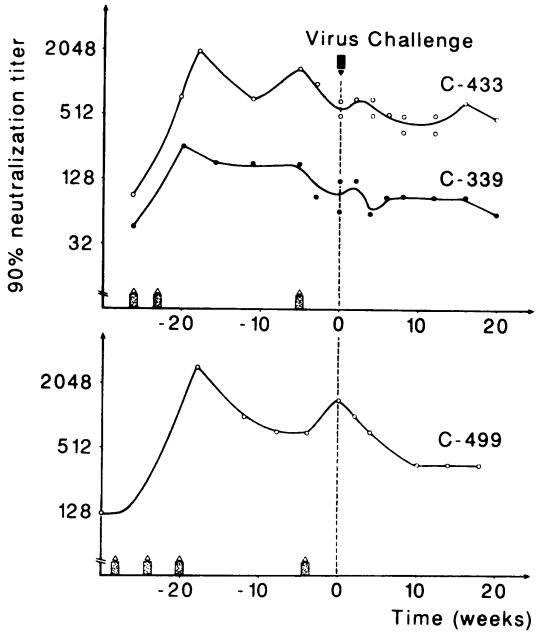

FIG. 2. Neutralizing antibody titers in sera from chimpanzees C-339, C-433, and C-499 during immunization with HIV-1 antigens. Titers are the reciprocal of the highest dilution of serum that gave 90% reduction in number of syncytia formed by CEM-SS cells (20) when compared with that obtained with control serum from a naive chimpanzee.

C-433, the EIA titers of chimpanzee C-499 declined rapidly after immunization with the purified HIV antigens, and there was no detectable effect of the V3 peptides on EIA titer. There was, however, a significant increase in neutralizing antibody titers after the V3 peptide inoculations (Fig. 2).

Challenge with Infectious HIV. Because sustained neutralizing antibody titers were achieved, chimpanzees C-433, C-339, and C-499 were challenged by i.v. inoculation of 100 $TCID_{50}$ (40 chimpanzee infectious doses) of HIV-1. At the time of challenge, 50% neutralization titers by an immunofluorescence inhibition assay were 1:2000, 1:280–350, and 1:2000, and 90% neutralization titers by a syncytia-inhibition assay (20) were 1:512–1024, 1:128, and 1:1024 for chimpanzees C-433, C-339, and C-499, respectively. Because immunization of chimpanzee C-499 was initiated at a different time from the other two animals, challenge of chimpanzee C-499 occurred 6 mo after that of chimpanzees C-339 and C-433 but was done at the same time as that of a naive control animal, chimpanzee C-087. Virus was isolated from the PBMC of chimpanzee C-087 at 2 weeks postinoculation as well as at all subsequent times, showing that 1:100 dilution of the HIV-1 stock readily infected chimpanzees under our conditions.

Attempts to Isolate HIV from Immunized and Challenged Chimpanzees. Three methods were used to assess the infection status of the animals. (*i*) Attempts to detect HIV sequences in lymphoid cells by PCR were made periodically (22–24). DNA samples obtained from PBMC of the three chimpanzees at 3 weeks and 3 and 6 mo after challenge were tested. Bands with the expected electrophoretic mobility were detected in DNA from a control HIV-infected chimpanzee but not in PBMC from the vaccinated and challenged animals or from a naive animal (data not shown). At 6 mo after challenge, nested sets of primers were used to perform PCR analyses on both PBMC and lymph node tissue of the challenged and control chimpanzees (23). This technique is more sensitive than standard PCR, and in these experiments (repeated at least seven times on all samples), approximately one molecule of viral DNA was found to produce a strong signal when present in 1.5×10^5 cell-equivalents of DNA. All PBMC and lymph node samples were consistently negative, except those from a previously infected chimpanzee, which were always positive (Fig. 3). Thus, at 6 mo after challenge, viral DNA was not present in PBMC and lymph node tissues at a frequency greater than one copy per 10^6 cells.

(*ii*) At weeks 2, 4, 6, and 8, and at monthly intervals thereafter, attempts were made to isolate virus from PBMC by cocultivation of the chimpanzees' PBMC with lymphocytes obtained from normal humans (21). Because $CD8^+$ cells have been shown to suppress virus replication not only in HIV-infected humans (29, 30) and chimpanzees (P.N.F., unpublished data) but also in simian immunodeficiency virus-infected macaques (30), in some experiments chimpanzee PBMC were depleted of $CD8^+$ lymphocytes before cultures were established. In contrast to virus recovery from the control animal, chimpanzee C-087, virus was not recovered from either total PBMC or $CD4^+$-enriched cells from chimpanzees C-339, C-433, or C-499 at any time during the first 6-mo follow-up. At 6 mo postinoculation, inguinal lymph node biopsies were performed on all animals as well as on uninfected and HIV-infected control chimpanzees. Upon

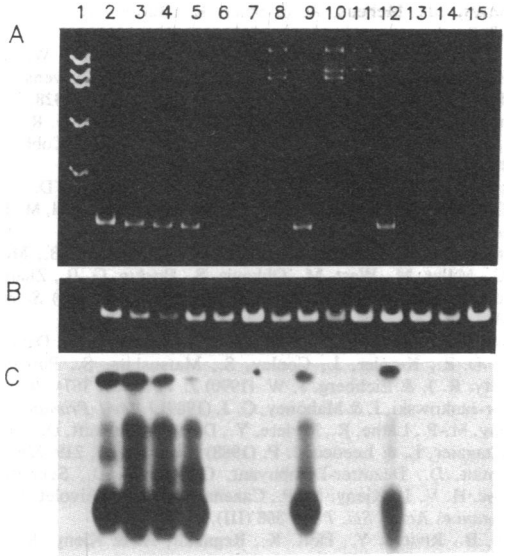

FIG. 3. PCR analysis of DNA from PBMC and lymph node tissue obtained 6 mo after challenge of chimpanzees C-339 and C-433 with HIV-1. (*A*) Ethidium bromide-stained gel of amplified HIV sequences after two rounds of PCR with nested sets of primers. The size of the HIV-specific-amplified fragment is 141 base pairs. Lanes: 1, 0.5 μg of ϕX174 DNA cleaved with *Hae* III as M_r *t* markers; 2–7, positive controls for sensitivity, each containing 10-fold fewer molecules of pHXB2 cleaved with *Xba* I than the previous sample, starting with 3000 molecules in lane 2. Each sample was amplified in the presence of 1 μg of DNA (the amount of DNA in 1.5×10^5 cells) from uninfected control chimpanzee C-519. One negative control sample (lane 14) was identified and used as the source of uninfected chimpanzee cellular DNA; all other samples were tested blindly. Chimpanzee C-487 was HIV-1-infected and used as a positive control. Lanes: 8–11, DNA from PBMC of chimpanzees C-339, C-487, C-433, and C-519, respectively; 12–15, DNA from lymph node tissue of chimpanzees C-487, C-433, C-519, and C-339, respectively. (*B*) Ethidium bromide-stained gel of an amplified portion of the β-globin gene (28) as an internal control. (*C*) Oligonucleotide hybridization of PCR-amplified sequences. PCR reaction products shown in *A* were denatured and hybridized with ^{32}P-labeled primer SK102, which hybridized entirely within the amplified sequence; the products were examined after PAGE and autoradiography according to Kwok and Kellogg (24).

cocultivation with normal human PBMC, virus was recovered from the lymph node of the infected control but not from those of the immunized and challenged chimpanzees (data not shown). Despite the fact that all attempts to detect virus during the first 6 mo after challenge had failed, virus was isolated from chimpanzee C-433 by cocultivation of PBMC obtained at 32 weeks and thereafter and of bone marrow obtained 37 weeks after challenge.

(*iii*) The challenged animals were monitored for possible seroconversion to HIV antigens that were not included in their immunization regimens. Immunoblot analysis (Diagnostic Pasteur, Marnes la Coquette, France) showed that chimpanzees C-433 and C-499, which had been immunized with, among other antigens, p18gag but not p25gag, did not seroconvert to p25 during 7-mo follow-up; however, at 32 weeks (7½ mo postinoculation), a faint p25 band was seen on immunoblots for chimpanzee C-433, which increased in intensity with succeeding serum samples. For chimpanzee C-339, which had been immunized with whole inactivated HIV, there were no detectable increases in apparent levels of antibodies to any HIV-specific proteins. Also, using purified antigens in immunoblot assays, no antibodies to the vif or nef proteins were detected in serum from chimpanzee C-339 during 12-mo follow-up.

DISCUSSION

The results presented here, as well as those reported by Berman and colleagues (7), clearly show that it is possible to elicit a protective immune response in chimpanzees with various HIV-1 antigens. We showed that chimpanzee C-499 was protected against establishment of HIV infection, at least through 9-mo follow-up, that chimpanzee C-339 was protected for 1 yr, and that chimpanzee C-433 was protected partially, as evidenced by the 7-mo delay in appearance of virus. It is possible, however, that chimpanzee C-433 also might have been fully protected if the challenge dose had been the same as that used by others (7), which was 4-fold lower than the dose we used. Protection was demonstrated by (*i*) failure to recover virus from PBMC during monthly attempts and from lymph node tissue at 6 mo postinoculation; (*ii*) negative hybridization signals in PCR analysis of DNA from PBMC at various intervals and from lymph nodes at 6 mo postinoculation; and (*iii*) the absence of antibody responses that normally follow a primary HIV infection or that are characteristic of anamnestic responses seen in previously vaccinated and challenged animals (3–5). That chimpanzee C-433 appeared to be protected for 7 mo but actually was infected from time of challenge, despite repeatedly negative tests for virus isolation and detection by PCR, is worrisome and underscores the fact that HIV can be sequestered such that it defies detection by both virologic and serologic criteria. A similar occurrence was reported (8) for a macaque immunized with inactivated whole virus and then challenged with infectious simian immunodeficiency virus. In that study, virus was not recovered initially until 32 weeks, and an anamnestic response was not observed until 39 weeks after challenge. The observation in natural HIV infections that persons remained seronegative by conventional tests for extended times, but HIV was detected by PCR or virus isolation (31, 32), suggests that high-risk individuals, such as sexual partners of HIV-infected persons, possibly could be infected despite negative serologic, virologic, or PCR analyses.

In view of the complex regimen of immunization undergone by the three chimpanzees, it is difficult to determine which of the many antigens were instrumental in eliciting protection. Chimpanzee C-339 was immunized successively with inactivated HIV, purified gp160, and the V3 peptide–KLH conjugate. Chimpanzee C-433 was immunized first with a vaccinia virus–gp160env recombinant, then with purified env, p18gag, nef, and vif antigens, and finally with the V3 peptide–KLH conjugate. The simplest immunization regimen was that of chimpanzee C-499; it consisted of purified gp160env and p18gag followed by unconjugated V3 peptides. The antigens that were common to the three animals were gp160env, p18gag, and the V3 peptide, but their relative importance remains to be determined. Adequate protection might require multiple antigenic determinants found on more than one viral protein, and/or multiple presentations of the same antigenic determinant.

It is of interest that previously tested prototype vaccines (3–6) that did not elicit significant titers of neutralizing antibodies in chimpanzees were not effective in preventing experimental infection of the animals. The observation that sustained neutralizing antibody titers were reached in chimpanzees C-339 and C-433 after two injections of the V3 peptide–KLH conjugate and in chimpanzee C-499 after three injections of V3 peptides (Fig. 2) suggests that V3 might be seen differently by the chimpanzee immune system when presented as a peptide than when presented as part of the gp160/120env molecule. We have found by immunoaffinity chromatography that virtually all HIV-neutralizing activity in the serum of the protected chimpanzees could be adsorbed by the V3 peptide (A.P., unpublished data). The booster inoc-

ulations of the V3 peptide(s) might explain why immunization with gp160 resulted in protection of chimpanzees in our experiments but not in those of Berman *et al.* (7). In this latter study, two chimpanzees were protected after immunization with gp120, and these animals had 3- to 4-fold higher titers to the principal neutralizing determinant found in the V3 loop than the two animals not protected from infection.

The question of whether the protection seen in our experiment was due solely to neutralizing antibodies or whether other immune mechanisms were involved remains unanswered. At time of challenge, antibody-dependent cellular cytotoxic activity was detected in the serum of chimpanzee C-339, but not in that of the other two chimpanzees. HIV-specific proliferative responses to the soluble proteins p18gag, gp160env, and p27nef (33, 34) were detected in PBMC from chimpanzee C-433 both before and after virus challenge, but not in PBMC from chimpanzee C-339. Interestingly, after immunization with the V3–KLH conjugate, chimpanzee C-433 displayed a sustained, strong T-helper cell reactivity to the V3 peptide, whereas chimpanzee C-339 had only a weak response; the responses of chimpanzee C-499 are under study. Repeated attempts to detect cytotoxic T lymphocytes in PBMC of the vaccinated chimpanzees before, on the day of, and after challenge have failed. It appears, therefore, that the observed protection did not correlate with T helper cell or cytotoxic T-lymphocyte activity.

The results presented here indicate that HIV vaccines can induce protection against virus infection. Numerous problems, however, remain to be solved. The high neutralizing antibody response induced by the V3 peptide was type specific; serum from the vaccinated animals at time of challenge neutralized the more diverse HIV-1 isolates RF, SF-2, and MN only marginally (unpublished data). Therefore, it will be necessary to design a vaccine that will induce high titers of neutralizing antibodies to the many HIV variants, but the recent identification (19) of principal neutralizing determinant sequences with which a majority of sera from HIV-infected persons react may make this less formidable than previously thought. The apparent success in protecting two chimpanzees and suppression of virus for an extended period in a third animal justify further efforts to develop an HIV vaccine, with the expectation that it will provide long-lasting protective immunity in humans.

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