Superinfection of a Chimpanzee with a Second Strain of Human Immunodeficiency Virus

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Two chimpanzees, one (C-499) infected with the acquired immunodeficiency syndrome-associated retrovirus type 2 (ARV-2) strain of human immunodeficiency virus (HIV) and one (C-560) infected with the lymphadenopathy-associated virus type 1 (LAV-1) strain of HIV, were inoculated with approximately 10^4 tissue culture infective doses of the reciprocal strain. At the time of the second inoculation, both chimpanzees had high titers of HIV-specific antibodies, including antibodies that neutralized both virus strains. After inoculation of the second strain of HIV, the antibody titers in both chimpanzees increased 4- to 10-fold, and in one chimpanzee (C-499), the numbers of infectious peripheral blood mononuclear cells (PBMC) increased 1,000-fold to levels that are comparable with those observed after primary HIV infections. By restriction enzyme analysis of virus recovered from PBMC, both ARV-2 and LAV-1 were identified in C-499, thus demonstrating that superinfection had occurred.

Infection of chimpanzees by various strains of the human immunodeficiency virus (HIV) is the only established animal model system for studying infection by the etiologic agent of the acquired immunodeficiency syndrome (AIDS). We showed previously (8) that this model system is very reliable as an indicator of infection after intravenous injection of the lymphadenopathy-associated virus type 1 (LAV-1) strain of HIV. Also, after primary infection by HIV, humoral immune responses of chimpanzees (9) mirror those of persons infected with HIV (5). Thus, immunization and challenge of chimpanzees will be valuable in testing candidate vaccines for efficacy in protection against HIV infection. To date, except for two chimpanzees that developed prolonged, substantial lymphadenopathy (1, 12), there is no documented evidence of an AIDS-like disease in an HIV-infected chimpanzee. Possible explanations for lack of disease include the following. (i) HIV is nonpathogenic for chimpanzees. (ii) There may be insufficient incubation time for the disease to develop. (iii) Housing chimpanzees in isolation facilities prevents their exposure to pathogens that might contribute to progression to AIDS, i.e., a cofactor is necessary. (iv) Development of AIDS may be related to particular strains of HIV, and the strains used to date to infect chimpanzees might be less pathogenic relative to other strains. (v) Multiple exposures to different strains of HIV might be necessary to facilitate development of AIDS.

To test the latter possibility and to determine whether a chimpanzee could be infected sequentially with two different strains of HIV, we attempted to superinfect two HIV-infected chimpanzees with a second, distinct strain of the virus. When inoculated with the second strain of HIV, both chimpanzees had high titers of antibody to the virus in serum. The antibodies recognized all of the major HIV proteins on immunoblots and also cross-neutralized both strains. Because chimpanzees are the closest evolutionary relatives of humans, if superinfection did not occur, it might imply that persons infected with one strain of HIV are

refractory to infection with a second strain, as suggested by Hahn et al. (10).

The strains of HIV used were LAV-1 and AIDS-associated retrovirus type 2 (ARV-2), which differ by approximately 6% in nucleotide sequence overall and by 10% in the *env* gene (17). Therefore, these strains can be distinguished by differences in the sizes of fragments generated by some restriction enzymes; in one study (3), seven restriction enzymes were used to generate maps, and these two strains differed at 13 sites throughout the genome. Initially we tested whether the ARV-2 strain of HIV could establish infection in a chimpanzee that was persistently infected with strain LAV-1 (2), originally obtained from Luc Montagnier and Jean-Claude Chermann. Approximately 6 months after receiving 1,100 50% tissue culture infective doses of LAV-1, chimpanzee C-560 was given an intravenous injection of 10^4 tissue culture infective doses of ARV-2 (13), graciously provided by Jay Levy. At the time of superinfection with ARV-2, C-560 was persistently infected with LAV-1; virus had been isolated initially 2 weeks after LAV-1 injection and at monthly intervals thereafter (Table 1). Just before ARV-2 inoculation, C-560 had an antibody titer of 25,600, as determined by enzyme immunoassay (EIA), and a neutralizing titer to LAV-1 of 200 (Table 2). The neutralizing antibodies cross-neutralized ARV-2 with a titer of 400, showing that the two virus strains have common antigenic determinants and neutralizing epitopes.

After injecting C-560 with ARV-2, we monitored two parameters: virus-specific antibody titers, as determined by EIA, and numbers of infectious peripheral blood mononuclear cells (PBMC). The latter were determined by serial 1:5 limiting dilution and cocultivation of PBMC with human PBMC, as previously described (8). Within 3 weeks after superinfection, HIV-specific immunoglobulin G antibody titers increased fourfold, and a second immunoglobulin M response was detected (Fig. 1), which suggested that an anamnestic antibody response occurred or that ARV-2 presented different antigenic determinants to the host immune system, which elicited antibodies with new specificities. Within 6 weeks after injection of ARV-2, there was an

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TABLE 1. Virus isolation and antibody detection after infection of chimpanzees with LAV-1 or ARV-2^a

Time (mo) after infection	Chimpanzee response			
	C-560		C-499	
	Virus	Antibody	Virus	Antibody
0	—	—	—	—
1	+	+	—	—
2	+	+	—	—
3	+	+	—	+/-
4	+	+	—	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	—	+
11	+	+	—	+
12	+	+	+	+
13	+	+	—	+
14	+	+	—	+
15	+	+	+	+
16	+	+	+	+

^a Chimpanzees were housed in isolation facilities at the Yerkes Regional Primate Research Center, Emory University, as described previously (8). C-560 was superinfected with ARV-2 5.5 months after infection with LAV-1. C-499 was inoculated with ARV-2 at 0 months, when C-560 was superinfected with ARV-2; C-499 was superinfected with LAV-1 15 months later. The LAV-1 stock had been passaged sequentially in two chimpanzees before use in these experiments. Both virus stocks were generated by infection of human leukocytes stimulated with phytohemagglutinin P and had been stored at -80°C before use.

apparent increase in the number of infectious PBMC (Fig. 2, C-560); however, because the numbers of infected PBMC were relatively high at the time of superinfection, the data are inconclusive.

When C-560 was superinfected with ARV-2, chimpanzee C-499 received a primary inoculation of 10⁴ tissue culture infective doses of ARV-2 to serve as a control for the ability of ARV-2 to establish infection in chimpanzees. Approximately 3 months after ARV-2 inoculation, HIV-specific antibodies were first detected by EIA (Fig. 1) and immunoblot; virus was not isolated from PBMC until 5 months after ARV-2 inoculation. In addition, our ability to isolate ARV-2 at monthly intervals varied (Table 1), which contrasted with our experience after infection of chimpanzees with LAV-1. In the latter case, once infection was established, irrespective of route of inoculation, virus was isolated from PBMC of all LAV-1-infected animals at regular

TABLE 2. Antibody titers to HIV in chimpanzees at the time of secondary virus challenge

Chimpanzee	Virus		EIA titer ^a	Neutralizing titer ^b to:	
	Primary	Secondary		LAV-1	ARV-2
C-560	LAV	ARV	25,600	200	400
C-499	ARV	LAV	64,000	10	20
C-459	LAV	LAV	32,000	40	ND ^c

^a Titers are expressed as the highest dilution of serum that gave a positive reading with the Abbott HTLV-III EIA Kit.

^b Neutralization assays were performed as described previously (6) and are expressed as the highest dilution of serum that resulted in greater than 80% reduction in reverse transcriptase activity after incubation of virus with serum and infection of human PBMC. Neutralizing titers are averages of two or more experiments or of a single experiment done in duplicate.

^c ND, Not done.

monthly intervals for more than 2.5 years (7, 8). These data suggest that different strains of HIV may have different replication potentials in chimpanzees or, alternatively, may reflect differences in responses to HIV by individual chimpanzees. We favor the former possibility, however, because in *in vitro* experiments, ARV-2 replicates in chimpanzee and human PBMC much less efficiently than LAV-1, and C-499 subsequently was readily infected by LAV-1 (see below).

To maximize our ability to detect the superinfecting virus, we did the reciprocal of the experiment with C-560, i.e., we superinfected C-499 with LAV-1. As a control for observed changes in antibody titers and possible increases in the numbers of infected cells, we gave an LAV-1-infected chimpanzee (C-459) a second intravenous dose of LAV-1. C-499 received 10⁴ tissue culture infective doses of LAV-1, whereas C-459 received twice this amount of virus. At the time of superinfection, both C-499 and C-459 had detectable neutralizing antibodies and high levels of antibodies to HIV by EIA (Table 2). Within 3 weeks after superinfection with LAV-1, virus-specific antibody titers from C-499 had increased 10-fold, and the number of infectious PBMC increased 1,000-fold to the same level as that seen early after a primary infection (Fig. 2). There was no change in the apparent number of infected PBMC from C-459, and only a twofold increase in antibody titer was observed. The number of infectious PBMC for C-459 was less than 10/10⁷ PBMC at the time of rechallenge (comparable with the 1 infectious PBMC per 10⁷ cells identified in C-499); for 3 months after the second injection of LAV-1, the number in C-459 remained less than 10 infectious PBMC per 10⁷ cells. The

FIG. 1. HIV-specific antibody response in C-560 (○) and C-499 (●) after infection with LAV-1 and ARV-2. C-560 and C-499 were infected initially with LAV-1 and ARV-2, respectively. Immunoglobulin G titers (—) were determined with the Abbott HTLV-III EIA Kit; immunoglobulin M titers (—) were determined by EIA as described previously (8). Titers are expressed as the highest dilution of serum that gave a positive reading by EIA. The arrow-head indicates time of superinfection.

FIG. 2. Number of infectious PBMC at various times after infection and superinfection of chimpanzees with LAV-1 or ARV-2. The solid line reflects the correlation between the numbers of infectious PBMC and time after infection and was determined as described previously (8) with data compiled from an analysis of infectious PBMC from five LAV-1-infected chimpanzees. Symbols: $\blacktriangle$, infectious PBMC; ∇ , below the level of detection with 10^7 PBMC; $\blacktriangle$, virus isolated only after treatment of C-499 PBMC with iododeoxyuridine ($40 \mu\text{g/ml}$) for 24 h before cocultivation with human leukocytes stimulated with phytohemagglutinin P. The time of superinfection is indicated ($\downarrow$).

absolute number of T4^+ helper cells present among PBMC in C-459 at the time of the second LAV-1 inoculation was at normal levels, indicating that target cells to support virus replication were available; therefore, the data suggest that this apparent failure of a large dose of LAV-1 to replicate may have been prevented by the HIV-specific immune response present in C-459.

To determine whether the superinfecting viruses had established infection in the HIV-infected chimpanzees, virus isolated from PBMC of C-560 at 1, 2, 6, and 12 months after ARV-2 challenge and virus isolated from PBMC of C-560 at 3.5 weeks after LAV-1 challenge were tested by restriction enzyme analysis with high-molecular-weight genomic DNA (16) or DNA in Hirt supernatants (11). High-molecular-weight DNA from leukocytes stimulated with phytohemagglutinin and infected with virus isolated from C-499 at 6 months after ARV-2 inoculation was used as the positive ARV-2 control. DNA ($10 \mu\text{g}$) was digested with *KpnI* and subjected to electrophoresis on an 0.8% agarose gel. The DNA fragments were transferred to nitrocellulose paper and hybridized to nick-translated 1.5-kilobase (kb) *BglII* fragments (nucleotides 7197 to 8627) derived from the 3' end of the human T-cell lymphotropic virus type III *env* gene (15). Hybridization was performed at 42°C for 18 h in a buffer

containing 50% formamide, $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), $5\times$ Denhardt solution (1 mg of bovine serum albumin per ml, polyvinylpyrrolidone, and Ficoll), 20 mM NaPO_4 (pH 6.5), $100 \mu\text{g}$ of salmon sperm DNA per ml, and 10% sodium dextran sulfate as previously described (14). The filters were washed sequentially in $2\times$ SSC–0.1% sodium dodecyl sulfate (three times for 5 min each time) at room temperature and in $0.1\times$ SSC–0.1% sodium dodecyl sulfate at 65°C (two times for 30 min) and exposed to Kodak XAR-5 film for 1 or 3 days. After enzymatic digestion and hybridization, a single fragment of approximately 4,900 bases, indicative of ARV-2, was observed in samples that were derived from C-499 at 6 months after ARV-2 inoculation. In parallel experiments, we detected only LAV-1, and not the superinfecting virus ARV-2, in all samples of virus derived from C-560 (Fig. 3). After 1 day of exposure of the gel to film, only the 2.7-kb fragment indicative of LAV-1, the superinfecting virus, was detected in samples from C-499 after superinfection with LAV-1, but after 3 days of exposure, the 4.9-kb fragment associated with the presence of ARV-2 was present in DNA obtained from Hirt supernatants. Enzymatic digestion with *KpnI* and hybridization of DNA from PBMC infected only with LAV-1 yielded a 2.7-kb band. As an additional control, *HindIII* was used to digest all DNA samples, and distinct banding patterns characteristic of LAV-1 (C-560) or ARV-2 (C-499, DNA used in lanes 1) were observed (results not shown). These data demonstrate unequivocally that, despite the presence of high titers of HIV-specific antibodies, LAV-1 established infection in a chimpanzee previously infected with the ARV-2 strain of HIV. Superinfection by ARV-2 of chimpanzee C-560 was questionable because only a faint band migrating with the 4.9-kb fragments is discernible in lane 3 of Fig. 3B. The inability to detect ARV-2 in PBMC

FIG. 3. Southern blot analysis of genomic and Hirt DNA from HIV-infected cells. DNA was cleaved with *KpnI* and hybridized with a 1.5-kb probe specific to the 3' end of human T-cell lymphotropic virus type III (15). Autoradiogram developed after 1 (A) or 3 (B) days of exposure. Lane 1, Genomic DNA from lymphocytes infected with virus isolated from C-499 6 months after inoculation of ARV-2; lanes 2, 3, and 4, genomic DNA from lymphocytes infected with virus isolated from C-560 at 1, 2, and 6 months, respectively, after inoculation of ARV-2; lanes 5 and 6, Hirt and genomic DNA, respectively, from lymphocytes infected with virus isolated approximately 4 weeks after superinfection of C-499 with LAV-1. *HindIII*-cleaved lambda DNA was used as molecular weight markers, as indicated by arrowheads. DNA from uninfected lymphocytes did not show hybridization with the probe used in our experiments. Kb, Kilobases.

from C-560 may result from the inherent ability of LAV-1 to replicate more efficiently than ARV-2 in culture.

While we demonstrated that an HIV-infected chimpanzee could be superinfected with a second, distinct strain of HIV type 1, we have no evidence to date that exposure to and infection by different strains of HIV facilitate development of AIDS. Ten months have elapsed since chimpanzee C-499 was superinfected, and the animal is healthy with no hematologic or immunologic abnormalities. It is probable that more than one factor (e.g., incubation time or another pathogen) plays a role in progression to AIDS.

The fact that chimpanzees can be infected with more than one strain of HIV is at variance with the suggestion of Hahn et al. (10) that an interference mechanism may prevent simultaneous infection; these investigators were able to identify only minor variants of one major HIV strain when studying serial isolates from the same individual. If a person were infected by several strains, one of which had a replication advantage over other strains, then that one strain might overgrow others in vivo or in culture, similar to what we have observed in coinfection experiments with ARV-2 and LAV-1 (unpublished data). In addition, we used a large dose of cell-free virus given intravenously to establish superinfection, which probably does not reflect the dose, presentation of virus (i.e., cell associated), or route of infection (e.g., via mucosa) commonly occurring during person-to-person transmission.

In our studies, chimpanzee C-499 was superinfected despite the presence of high titers of HIV-specific antibodies to *gag*, *env*, and *pol* gene products and, perhaps more importantly, despite neutralizing antibodies, indicating that there were no apparent defects in their humoral immune responses. Mitogenic stimulation of PBMC from HIV-infected chimpanzees with concanavalin A, pokeweed mitogen, phytohemagglutinin, and *Staphylococcus aureus* resulted in normal responses (data not shown), suggesting that cellular immune responses also were not defective. Superinfection may have occurred despite an apparently functional immune system as a result of virus-specific immune suppression, but the fact that a secondary LAV-1 infection apparently was not established in C-459 argues against this.

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