JOURNAL OF VIROLOGY, Aug. 1990, p. 3779–3791
0022-538X/90/083779-13$02.00/0

Vol. 64, No. 8

Emergence of Viruses Resistant to Neutralization by V3-Specific Antibodies in Experimental Human Immunodeficiency Virus Type 1 IIIB Infection of Chimpanzees

P. L. NARA,[1]* L. SMIT,[2] N. DUNLOP,[1] W. HATCH,[3] M. MERGES,[1] D. WATERS,[4] J. KELLIHER,[5] R. C. GALLO,[1] P. J. FISCHINGER,[6] AND J. GOUDSMIT[2]

National Cancer Institute, Frederick Cancer Research Facility[1] and Program Resources, Inc.,[4] Frederick, Maryland 21701-1013; Human Retrovirus Laboratory, Academic Medical Center, Amsterdam, The Netherlands[2]; Albert Einstein College of Medicine, Bronx, New York 10461[3]; Food and Drug Administration, Washington, D.C. 20202[5]; and Medical University of South Carolina, Charleston, South Carolina 29425[6]

Received 21 August 1989/Accepted 30 April 1990

Emergence in two chimpanzees of human immunodeficiency virus type 1 (HIV-1) IIIB variants resistant to neutralization by the preexisting antibody is described. Viruses isolated from the HIV-1 IIIB gp120-vaccinated and -challenged animal were more resistant to neutralization by the chimpanzee's own serum than viruses isolated from the naive infected animal, indicating immune pressure as the selective mechanism. However, all reisolated viruses were 16- to 256-fold more neutralization resistant than the inoculum virus to antibodies binding to the third variable domain (V3) of the HIV-1 external envelope. Early chimpanzee serum samples that neutralized the inoculum strain but not the reisolated viruses were found to bind an HIV-1 IIIB common nonapeptide (IQRGPGRAF) derived from the gp120 isolate-specific V3 domain shown to induce isolate-specific neutralization in other animals. Amplification of the V3 coding sequence by polymerase chain reaction and subsequent sequence analysis of the neutralization-resistant variants obtained from in vivo-infected animals indicated that early resistance to neutralization by an HIV-1 IIIB monoclonal antibody (0.5 β) was conferred by changes outside the direct binding site for the selective neutralizing antibody. The reisolated neutralization-resistant isolates consisted of the lower-replication-competent virus subpopulation of the HIV-1 IIIB stock, as confirmed by biological and sequence analyses. In vitro passage of the HIV-1 IIIB stock through chimpanzee and human peripheral blood mononuclear cell cultures void of HIV-specific antibody resulted in homogenic amplification of the more-replication-competent subpopulation preexisting in the original viral stock, suggesting a role for the immune system in suppressing the more-replication-competent viruses.

The human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) are members of a subfamily of nononcogenic retroviruses that persist indefinitely in the infected host, replicate continuously, and infect cells of the lymphoreticular and nervous systems (24). During their horizontal transmission, these lentiviruses undergo progressive and accumulative antigenic drift (8, 24, 25, 29, 41, 50, 52).

Three biological general phenotypes of HIV-1 and HIV-2 have been described (1, 4, 5, 46, 53, 54): one that induces syncytia in peripheral blood mononuclear cells (PBMCs), has a high replication rate, and is transmissible to permanent cell lines of the T cell and monocyte-macrophage lineage and two (one with a high and one with a low replication rate) that do not induce syncytia and are not transmissible to permanent cell lines. The most rapid progression of acquired immune deficiency syndrome and the lowest survival rate following acquired immune deficiency syndrome diagnosis have been observed in individuals from which syncytium-inducing viruses were isolated (5, 53, 55). Also, during the acute infection and subsequent clinical progression-disease course to acquired immune deficiency syndrome, a change from synctium-inducing (rapid and high) to non-synctium-inducing (slow and low) and back to synctium-inducing viruses has been documented (5, 7, 53–55). These studies as well as others (25, 48, 55) demonstrating closely related genomic variability in single patients suggest that significant biologic in vivo variation is occurring over time. Acute infections with HIV-1 lead to a rapid plasma viremia (1, 13, 17) which generally subsides concomitantly with the appearance of a serologic response. It is postulated that the host immune surveillance plays a critical role in the ability to suppress syncytium-inducing HIV variants.

The host immune response to this biological and genomic variation exhibited a HIV-1, in particular the antibody response, is best studied in the natural infection of humans (19, 20, 53, 54) and the experimental infection of chimpanzees (2–4, 16, 18, 21–23, 35, 36, 38, 39). Shortly after primary infection, virus-neutralizing, virus-induced cell-fusion-inhibiting, and antibody-dependent cellular-cytotoxicity-mediating antibodies as well as cytotoxic lymphocytes are induced. In the majority of cases following acute HIV-1 infection, free virus and viral antigens usually disappear from the serum over time (1, 19, 20). In the acute phase of infection, virus neutralization and cell-fusion-inhibiting antibodies that block subsequent cell-to-cell transmission are type specific (16, 31, 39). Within the subsequent 1 to 2 years, a lower-titered, generally broader virus-neutralizing antibody (NA) develops, to include strains with a divergent gp120 third variable domain (V3) (23, 39). The transmembrane protein of 41 kilodaltons (gp41) and gp120 appeared to present the most important neutralizing epitopes (6, 9, 16, 26, 27, 31, 32, 44, 47, 56). Several groups have shown that the V3 within the carboxyl-terminal half of gp120 contains a major neutralization domain (16, 43, 47). The architecture of this neutralization domain is conserved by a GPGR sequence of high β-turn potential flanked by two cysteine residues (34) at amino acid

* Corresponding author.

positions 296 to 331. Because the antibody-contacting amino acids on either side of the GPGR are divergent among viral strains, antibodies binding this region only neutralize viruses sharing those contact residues. The amino acid sequence of the human and the chimpanzee antibody-binding site was shown to be nine amino acids in length, of which at least five consecutive amino acids were crucial to antibody binding (34).

Detailed studies of NA for HIV-1 have been reported, and it has been demonstrated to be kinetically biologically functional in vitro (26, 35). NA from HIV-1-infected humans and chimpanzees and gp120-immunized animals was shown to block infectivity of the virus in a post-CD4 binding fashion, mediated through this V3 immunodominant neutralization epitope (35). A biologically functional postadsorption interaction, involving this V3 domain in association with codons 128 and 267 of the viral envelope, has also been recently described (26, 57). NA to one of these regions, the 267 site, was additionally shown to inhibit infectivity of HIV-1 in a postbinding manner.

The infection of chimpanzees with HIV-1 leads to a persistent state of viral infection, as demonstrated by the ability to recover virus from their circulating PBMCs over a period of years (2, 11, 12, 14, 38, 39). Following intravenous inoculation, virus isolation occurs within 2 to 6 weeks postinoculation (PI), with subsequent seroconversion to core and envelope viral proteins within 2 weeks (3, 38, 39). Subsequent serial virus isolations occur frequently throughout the first 36 weeks (3). Over time, the animals make antibody to all the known immunoreactive viral proteins found in HIV-1-infected humans.

The objectives of the present report were to study the antigenic variation of a molecularly characterized viral inoculum of HIV-1 in chimpanzees. Due to the critical functional characteristics of the V3 envelope domain, sequential viral isolates as well as serum samples provided the means to define serologically and molecularly the process by which neutralization-resistant variants (NRVs) emerge in vivo and the role the V3 region played in the process. Also, the fortuitous chance of having a viral stock containing molecularly defined subpopulations of relatively higher- and lower-replication-competent viruses provided a unique model of the process of selection, antigenic drift, and host immune responses in a living animal. A comparison was made between virus variants yielded by in vitro passage through chimpanzee PBMCs (C-PBMCs) and human PBMCs (H-PBMCs) and by in vivo passage in a naive and gp120 antibody-positive chimpanzee. The serological, biological, and molecular characteristics of HIV-1 NRVs are described.

MATERIALS AND METHODS

Preparation of virus stocks. The HIV-1 infectious challenge stock was prepared from HIV-1 IIIB-infected H9 cells as previously described (3). Virus stocks for additional neutralization, replication, and in vitro passage studies of HIV-1 IIIB and its three infectious clones, HX10, HXB2, and HXB3, as well as RF were prepared and cryopreserved as previously described (37). The HIV-1 MN and CC were used as fresh titered viral stocks due to their temperature lability at −70°C.

Animals. To conserve the scarce supply of available chimpanzees, young adult to adult animals previously used in experiments involving human hepatitis A, B, and non-A, non-B were used for our HIV-1 gp120 vaccine trial. Many chimpanzees used in hepatitis research have received human blood products which possibly included HIV. Therefore, both animals were pretested for HIV antibodies prior to use. Housing conditions for the chimpanzees and the serological assays used to detect HIV-1 antibodies have been previously described (3). The HIV-1 IIIB gp120 and the virus challenge inoculum or protocol used for immunizations and subsequent challenges of the chimpanzees have also been previously described (3). The two animals, no. 911 (naive) and no. 1125 (IIIB gp120 vaccinated), described in this study received 40 and 400 cell culture infective doses intravenously, respectively. The animals were physically examined by staff veterinarians every 2 weeks, at which time blood samples were drawn.

Analysis of the HIV-1 IIIB parental stock for NRVs in vitro. To assure that the HIV-1 IIIB/H9 parental viral stock did not contain a minor neutralization-resistant fraction or subpopulation other than the three infectious clones HX10, HXB2, and HXB3, numerous endpoint-limiting-dilution and amplification analyses were performed on the HIV-1 IIIB stock. These were done in both CEM-SS cells and C- and H-PBMCs. All resulting cultures (n-89) were diluted to a theoretical point of a single infectious unit. There, resultant viruses were amplified in culture and serologically phenotyped with HIV-1 IIIB V3 gp120-specific NAs and found to be devoid of NRVs (data not shown). In addition, polymerase chain reaction (PCR) analysis of the HIV-1 IIIB/H9 stock was performed, and it also failed to demonstrate any nonrelated HIV-1 IIIB V3 sequences. To control for the effects of in vitro selection, spontaneous mutations or differences in glycosylation patterns in the viral envelope (which could occur as a result of using primary PBMCs during the virus isolation and subsequently influence directly or indirectly the V3 neutralization epitope), the HIV-IIIB virus stock was passaged through phytohemagglutinin-stimulated primary H- and C-PBMCs and retested for sensitivity to HIV-1 IIIB NA.

In vitro biological characterization of HIV-1 IIIB, HX10, HXB2, HXB3, and reisolated NRVs. To determine in vitro infectivity, to estimate the chimpanzee infective dose, and to perform kinetic viral-replication studies, the HIV-1 IIIB stock used in a previous minimal-infective-dose study (3) and its three molecular clones were recovered from liquid nitrogen storage and tested for infectivity in the continuous cell lines H9 and CEM-SS as well as in phytohemagglutinin-stimulated normal H- and C-PBMCs. Infectivity kinetic studies on 2-day phytohemagglutinin-stimulated C- and H-PBMCs for IIIB, HX10, HXB2, and HXB3 were done at a multiplicity of infection 0.01 as described previously (3). The primary cell passage studies were repeated two times at 400 and 40 cell culture infectious units per ml of the HIV-1 IIIB challenge stock to approximate the in vivo challenge.

Isolation and preparation of viral stocks from HIV-1-infected chimpanzees. C- and H-PBMCs were prepared by diluting heparinized blood with equal volumes of Dulbecco phosphate-buffered saline as previously described (3). A constant cell concentration of 3×10^6 to 4×10^6 cells was seeded from each animal during each virus isolation attempt into T-75 flasks, and they were incubated at 37°C in 5% CO_2. This was done to assess the biologic behavior of each reisolated virus, as well as to provide a rough measure of the kinetics and level of viral expression during the early experimental period. In a few select cases, reisolated viruses from the HIV-1 IIIB-infected chimpanzees reported here were simultaneously derived in both H- and homologous C-PBMCs. This was done in parallel during the standard cocultivation procedure described to assess the degree of

viral selection and other unknown host-specific cellular factors (i.e., glycosylation) which could influence the serotyping and neutralization assays. All reisolated viruses were harvested at 2-day intervals, cryopreserved, titered, and serotyped. NRV stocks were further analyzed for their ability to replicate and cause syncytium formation in the CEM-SS cell line. This somewhat empirical biological analysis of the isolated NRV, IIIB, and its molecular clones was done by parallel titration and subsequent comparison of the endpoint syncytial-forming units (SFUs) and p24 levels in the quantitative microtiter infectivity assay previously described (37). In addition, the NRV stocks were analyzed by terminal endpoint dilution-amplification analysis for the presence of nonsyncytial variants (37).

Serotyping-neutralization assays. Three HIV-1 IIIB gp120 V3-specific NAs were used in these studies. One monoclonal, 0.5 β (31), and two polyclonal goat antisera were raised against either the native gp120 (40) or the gp120 V3 domain of the HIV-1 IIIB strain (47). Antibodies used for serotypic neutralization analysis of HIV-1 IIIB, its three infectious clones, and the NRVs were quantitated by using the HIV-1 microtiter infectivity syncytium-forming assay previously described (37). The neutralizing titers in the text and tables were determined as means of duplicates of the virus-surviving fraction (V_n/V_o) resulting from the statistically significant reduction in virus-induced SFUs in the presence of duplicate twofold dilutions of serum (V_n) divided by the number of total SFUs added (V_o). A 90% neutralization titer was demonstrated at a $V_n/V_o = 0.1$ and was repeatable to within a two- to fourfold dilution endpoint ($P \leq 0.001$).

Seroreactivity to HIV-1 IIIB and RF gp120 V3-specific peptides. The design of the synthetic peptides for the V3 (KSIRIQRGPGRAFVTIG, HX10; IQRGPGRAF, 3B; IQRGPGRVI, 3B/RF; ITKGPGRAF, RF/3B; ITKGPGRVI, RF) has previously been described (15). The synthetic peptides were produced by using Merrifield solid-phase synthesis by the European Veterinary Laboratory (Amsterdam, The Netherlands). The presence of antibodies was determined by using a noncompetitive solid-phase immunoassay. Bound antibodies were visualized by using *ortho*-phenylenediamine as substrate. The reaction was stopped with 1 N H_2SO_4, and optical density was read at an absorbance of 450 nm. That dilution for which the logarithm corresponded to an optical density at 450 nm of (optical density maximum − optical density minimum)/2 was considered to be the midpoint titer.

PCR. The V3 region was amplified by using the oligonucleotides 5′ ksi (5′ AT AAGCTT GCAGTCTAGCAGAAGA AGA 3′) and 3′ ksi (5′ AT GAATTC TGGGTCCCCTCCTG AGGA 3′) which bracket the V3 region (the first 8 nucleotides were added to create and protect a *Hin*dIII and an *Eco*RI site) as described by Saiki and co-workers (49). The polymerase chain reaction (PCR) mixture (100 μl) contained 100 ng of DNA in 50 mM Tris hydrochloride (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 1 mM of each of four deoxyribonucleotide triphosphates, 10 pmol of oligonucleotide primer 5′ ksi and 10 pmol of oligonucleotide 3′ ksi, and 1 U of *Taq* polymerase (a gift from Perkin Elmer-Cetus, Emeryville, Calif.). The samples were overlaid with paraffin-oil, denatured for 5 min at 95°C, and amplified for 35 cycles. Each cycle consisted of a 1-min denaturation step at 95°C, a 1-min annealing step at 55°C, and a 2-min extension step at 72°C. Following 35 cycles, the samples were extended for another 10 min at 72°C and stored at −20°C. The amplified fragment was purified by preparative gel electrophoresis and phenol chloroform extraction. The amplified and purified DNA was made single stranded by performing an additional PCR for 15 cycles in the presence of only one primer. The single-stranded DNA was sequenced by using the dideoxy-chain termination method with the complementary primer.

Sequencing. The amplified fragment was purified by preparative gel electrophoresis and phenol chloroform extraction. The amplified and purified DNA was made single stranded by performing an additional PCR for 15 cycles in the presence of only one primer. The single-stranded DNA was sequenced by using the dideoxy-chain termination method with the complementary primer (51). In addition to the direct sequences, four clones of each virus isolate studied were additionally sequenced with Sequenase (United States Biochemical Corp., Cleveland, Ohio) containing amplified DNA obtained after doing a new PCR on the isolated DNA. The clones were generated by digestion of the amplified DNA with *Hin*dIII and *Eco*RI, cloning of this digested fragment with *Hin*dIII- and *Eco*RI-digested pGEM7 (Promega Biotec, Madison, Wis.), and transformation of this newly formed plasmid to HB101 (*Escherichia coli*).

RESULTS

Neutralization analysis of HIV-1 IIIB, HX10, HXB2, HXB3, and NRVs following inoculation of HIV-1 IIIB in naive and HIV-1 IIIB-gp120 immunized chimpanzees. To demonstrate how the NRVs from the two chimpanzees were evaluated in this study, virus-surviving fraction curves (V_n/V_o) were constructed and presented (Fig. 1). These figures compared the neutralization sensitivity of the parental HIV-1 IIIB virus to the isolated 6- and 16-week NRVs. The HIV-1 IIIB gp120 V3-specific polyclonal reference serum used in typing and neutralization studies was represented as the closed circles on the left side of panel 4B. This line represents the NA activity against the parental HIV-1 IIIB inoculum. Note that the 90% point of neutralization ($V_n/V_o = 0.1$) was between 256 and 512. Comparison of this curve to the virus-surviving fraction curves of the 6- and 16-week isolates from both animals demonstrates a greater than 64-fold reduction in neutralization at the same serum dilution. Intravenous inoculation with 40 cell culture infectious units per ml of the HIV-1 IIIB stock in a naive chimpanzee (no. 911) resulted in an antibody response that neutralized the parental inoculum virus at a 1:4 (open circles) titer 6 weeks PI (Fig. 1A and Table 1A). This titer as well as all others reported represented a quantitative, and therefore statistically significant, 90% reduction ($V_n/V_o = 0.1$) of approximately 150 to 200 SFUs of virus (37). This NA activity was not seen in prebleeds from these animals. Additionally, the NA was mapped by pep scan analysis during seroconversion to a nonapeptide (IQRGPGRAF) localized at the center of the V3 domain to which the sequence was common to all three HIV-1 IIIB clones present in the IIIB parental stock and occurred at 6 weeks PI in parallel to the emergence of antibodies neutralizing the inoculum virus mixture (36). This NA activity was present either simultaneously with or in the absence of seroreactivity to other viral-specific proteins, as well as the HIV-1 IIIB gp120 V3 region. Animal no. 911 is representative of three animals in which NA was detectable in the absence of other viral-specific proteins. Subsequently, the neutralization titer to the parental IIIB stock rose continuously to 256 after 28 weeks (Table 1A).

To better understand the specificity and kinetics of the NA response in the animals to the viral subpopulations contained within the HIV-1 IIIB challenge stock, we tested sequential serum samples from the animals for NA against the individ-

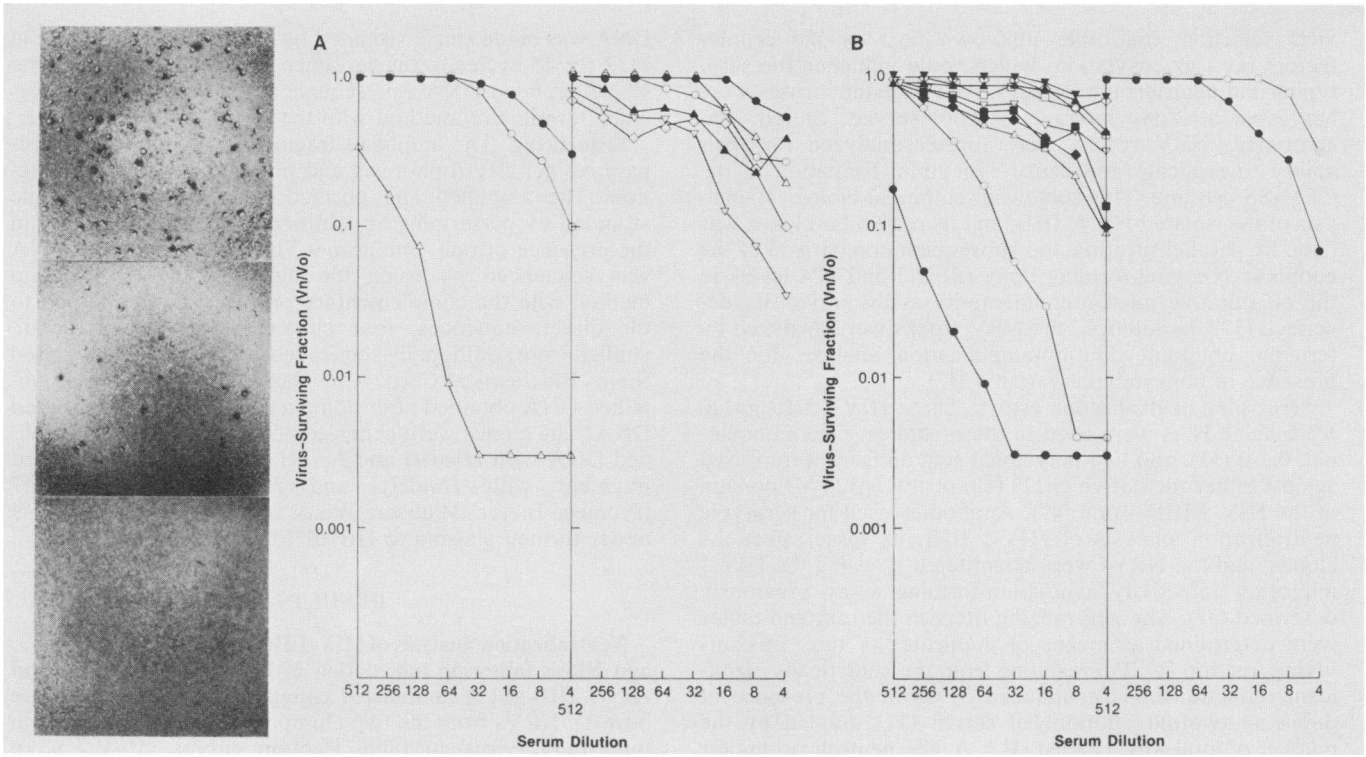

FIG. 1. Comparative serotypic neutralization analysis of the HIV-1 IIIB challenge stock and NRVs reisolated from experimentally infected chimpanzees. (A) The naive animal (no. 911) is represented by the following symbols: (left side) ●, prebleed versus IIIB; ○, 6 weeks PI versus IIIB; △, 16 weeks PI versus IIIB; (right side) ●, prebleed versus 6-week NRV; ○, prebleed versus 16-week NRV; ▽, 16 weeks PI versus 6-week NRV; △, 16 weeks PI versus 16-week NRV; ▲, IIIB gp 120-V3 NA versus 6-week NRV; ◇, IIIB gp120-V3 NA versus 16-week NRV. (B) The gp120 IIIB-vaccinated animal 1125 is represented by the following symbols: (left side) ●, IIIB gp120-V3 NA versus IIIB; ○, gp120 immune challenge versus IIIB; ▲, prebleed versus 6-week NRV; ▼, prebleed versus 16-week NRV; □, immune versus 6-week NRV; ◇, gp120 immune versus 16-week NRV; ■, 6 weeks PI versus 6-week NRV; ▽, 6 weeks PI versus 16-week NRV; △, 16 week PI versus 6-week NRV; ◆, 16 weeks PI versus 16-week NRV; (right side) ●, IIIB gp120-V3 NA versus 6-week NRV; ○, IIIB gp160-V3 NA versus 16-week NRV. The photomicrographs (top, middle, and bottom on the left) represent the actual virus-induced syncytial plaques for the various levels of neutralization (i.e., top, V_n/V_o = 1.0; middle, V_n/V_o = 0.1; and bottom, V_n/V_o = 0.01).

ual infectious molecular clones derived from the HIV-1 IIIB inoculum. As demonstrated in Table 1A, animal no. 911 NA at 6 weeks PI was capable of first recognizing HXB2 and HXB3 viral subpopulations at a titer of 1:4. Neutralization to the HX10 virus was not demonstrated until 10 to 16 weeks PI in this chimpanzee. Sera taken after 28 weeks PI neutralized the three cloned viruses to within a two- to fourfold titer. Despite the relative NA sensitivity of HX10 to the polyclonal HIV-1 IIIB V3-specific NA (Fig. 2), the NA titer in animal no. 911 for HX10 was delayed for 10 weeks PI and remained four- to sixfold less than that of the parental IIIB strain, further suggesting an absence of HX10-specific NA recognition. Also, the serum taken from animal 911 at 6 weeks, which exhibited NA for HIV-1 IIIB, did not neutralize its own virus isolated at this time (nor the HX10 molecular clone; see box in Table 1); the subsequent sera did (Table 1). The virus isolate recovered at 16 weeks was neutralized for the first time at 28 weeks, whereas the 32-week isolate was equivalently neutralized at 32 weeks, although at much lower titers (Table 1). Sequential viruses reisolated from four additional chimpanzees utilized in the previously mentioned minimal-infective-dose titration, and reported by us earlier, were all found to be NRVs (36).

Sequential serum samples taken from the HIV-1 IIIB gp120-vaccinated animal (no. 1125), which was subsequently challenged with 400 cell culture infectious doses in parallel with the same HIV-1 IIIB stock previously characterized, showed an enhanced and rapid anamnestic pattern of neutralization by 4 weeks PI (Fig. 1, panel B; Table 1). A 16- to 32-fold NA increase to the HIV-1 IIIB challenge virus (from which the gp120 vaccine was derived) and a 4- to 8-fold NA increase for the molecular infectious clones HX10, HXB2, and HXB3 between 0 and 10 weeks PI (Table 1) were detected when compared with no. 911, the unvaccinated animal (Table 1). In parallel to the presence of low-titer antibodies neutralizing the challenge virus, antibodies to the nonapeptide IQRGPGRAF were detected preinoculation in this HIV-1 IIIB gp120-vaccinated animal. These correlated with low neutralization titers for HX10, HXB2, and HXB3 at 1:8, 1:4, and 1:4, respectively. Prechallenge heterologous viruses like RF, MN, and CC were not neutralized. The NRVs obtained from this animal at 6, 16, and 32 weeks PI are depicted in Table 2. The neutralization resistance of these three isolates with sera of the same animal (Table 1) up to 16 weeks was complete, and only 28 weeks after challenge did the 6-week isolate become neutralized at low titer. Unlike the neutralization response seen in animal no. 911, neutralization of the 6-, 16-, or 32-week NRVs with homologous sera from the challenged chimpanzee (no. 1125) was delayed by an additional 18 weeks. The V3 genotypes of these NRVs are discussed in the next section and appear to have resulted again from the early selection due to variable levels of preexisting NA against the clonal subpopulations HX10, HXB2, and HXB3. Unlike the naive animal (no. 911),

TABLE 1. Serological and molecular analysis of the HIV-1 gp120 envelope region (290 to 337) from neutralization escape mutants reisolated over time from experimentally HIV-1 IIIB-infected chimpanzees

Animal no. and virus	V3 typing MAb (0.5 β) titer	Viral env[a] sequence (290–331)	Animal serum history (neutralization titers at following wk PI)							
			0	4	6	10	16	28	32	80
911 (Naive)										
Laboratory stock										
HIV-1 IIIB/H9	128[b]	Q---S(1/3)[c] T---R(1/3) T---K(1/3)	<4	<4	4	16–32	64–128	256–512	256–512	256
HX10	128	Q---S(only)	<4	<4	<4	<4	4–8	32–64	32–64	32–64
HXB_2	32–64	T---R(only)	<4	<4	4	4–8	8–16	64–128	64–128	64–128
HXB_3	32–64	T---K(only)	<4	<4	4	4–8	8–16	64–128	64–128	64–128
Virus isolate										
6 wk PI	8–16	Q---S(only)	<4	<4	<4	4–8	8–16	32	32	32–64
16 wk PI	<4	Q---R(only)	<4	<4	<4	<4	<4	4–8	16–32	16–32
32 wk PI	<4	Q---S(only)	<4	<4	<4	<4	<4	<4	4	4–8
1125 (gp120 vaccinated)										
Laboratory stock										
HIV-1 IIIB/H9	128		16–32	64–128	128–256	256–512	256–512	256–512	256–512	256
HX10	128	Q---S(only)	8	4–8	16	32	32–64	32–64	64	ND[d]
HXB_2	32–64	T---R(only)	4	16–32	32–64	64–128	64–128	128	128	ND
HXB_3	32–64	T---K(only)	4	16–32	64	64–128	64–128	64–128	128	ND
Virus isolate										
6 wk PI	<4	Q---S(3/4) Q---R(1/4)	<4	<4	<4	<4	<4	8	8–16	16–32
16 wk PI	<4	Q---S(2/4) Q---R(2/4)	ND	<4	<4	<4	<4	<4	<4	8
32 wk PI	ND	S(only) aa 325-I[e] aa 330-E	ND	<4	<4	<4	<4	ND	ND	ND

[a] The capital letters listed under this portion of the table represent the amino acid sequence at position 290 (Q or T) and 306 (S, R, or K). The dashes indicate sequence identity. The term "only" means that all four clones as well as the direct V3 sequence were homogenous with respect to the V3 genotype listed.

[b] The number listed under this portion of the table represents the reciprocal of the serum dilution giving 90% virus neutralization ($V_n/V_o = 0.1$) at a value of $P < 0.001$.

[c] The ratio listed next to the V3 sequence represents the fraction of clones identified by this sequence.

[d] ND, Not determined.

[e] The position in the V3 sequence where a different amino acid substitution occurred is indicated by the three-digit number, and the identity of the amino acid is indicated by standard single-letter alphabet nomenclature.

in the gp120-vaccinated animal (no. 1125), when the NA titers for the HX10 and HXB2 approximate each other at 10 to 16 weeks PI, the isolated NRVs consist of an equal mixture of HX10 and HXB2-like V3 sequences (Table 2; see Fig. 5A). However, by 28 to 32 weeks, when a slightly higher NA titer was detected for HXB2 and HXB3, an NRV with a V3 HX10-like population again dominated the culture. This NRV isolate, unlike the 32-week isolate from animal no. 911, was found to have acquired two new amino acid substitutions in the V3 region. These results indicate that vaccination not only failed to confer protection from the initial viral challenge pool but may have even increased the degree of the virus escape by delaying the host immune humoral selection and subsequent NA pressure. In neither of the two animals was the V3 genotype of the HXB3 clone ever detected by PCR directly or after recovery by cocultivation. Although not reported here, an additional gp120-vaccinated challenged animal (no. 1068) that failed an intravenous challenge with HIV-1 IIIB also demonstrated NRV throughout its virus reisolation protocol (data not shown). To evaluate whether the NRVs described here represented novel HIV-1 neutralization variants, we tested them against two previously published human acquired immune deficiency syndrome neutralization sera (37) and other HIV-1 IIIB-infected chimpanzee sera. In all cases, either the human or chimpanzee serum was capable of partial neutralization, between one-fourth and one-half of the NA titer for the parental HIV-1 IIIB strain (data not shown).

Serological and molecular analysis of HIV-1 IIIB resulting from in vitro passage in H- and C-PBMCs. The PBMC cultures were infected with the HIV-1-IIIB challenge stock produced in H9 cells. DNA from this producer cell line was isolated, and the V3 domain was amplified by PCR. This stock contained three distinct viral genomes, in approximately equal proportions, that corresponded in the V3 sequence to the previously described infectious clones and have been depicted in this study as HX10-S, HXB2-R, and HXB3-K (Table 1 and Fig. 3). Short-term (4 to 6 days) primary passage of this HIV-1 IIIB mixture in both H- and C-PBMCs yielded a rather homogeneous population of vi-

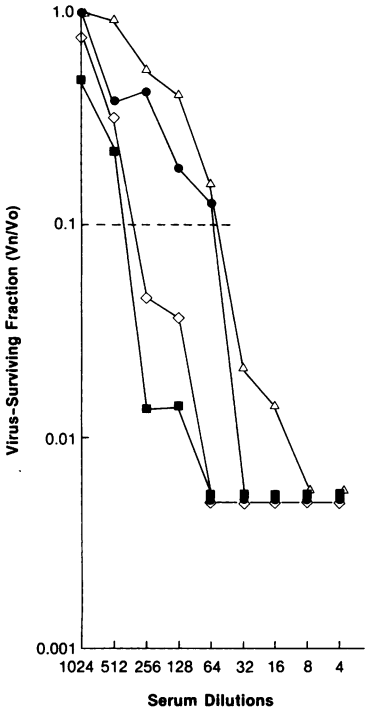

FIG. 2. Comparative phenotypic-serotypic neutralization analysis of HIV-1 IIIB and its molecular clones with polyclonal HIV-1 IIIB V3-specific NA. A 90% SFU inhibition point is demonstrated by the dashed line. The symbols are: ■, HIV-1 IIIB; ◇, HX10; ●, HXB2; and △, HXB3.

ruses with the HXB3 intra- and extra-V3 nucleic acid sequence coding for a Lys at position 306 and glutamine at position 290. All four sequenced clones as well as the sequence obtained directly from the PCR product showed this Lys V3 genotype after H- as well as C-PBMC passage (Fig. 3). The viruses passaged singly through both H-PBMCs and C-PBMCs were six- to eightfold more resistant to neutralization with the polyclonal HIV-1 III B V3 gp120-specific NA than was the parental HIV-1 IIIB stock (Fig. 4). Similar results were obtained with a polyclonal rabbit serum raised against a 24-mer peptide covering the V3-neutralizing domain (data not shown).

To establish whether this neutralization resistance could be attributed solely to nonsilent mutations amino terminal and carboxyl terminal of the amino-terminal cysteine, three viruses produced by transfection of the infectious clones HX10, HXB2, and HXB3 were used to test residual infectivity after incubation with the same polyclonal HIV-1 IIIB V3-specific NA (Fig. 2). Four clones of the PCR-amplified V3 domain of each of these three viruses were sequenced and shown to have the sequences HX10, Q through S; HXB2, T through R; HXB3, T through K as published previously (31; Fig. 4).

The virus yielded from cultures transfected with the infectious clone HX10 could be neutralized as readily as the parental HIV-1 IIIB stock. However, the viruses of the infectious clones HXB2 and HXB3 exhibited a statistically significant ($P \leq 0.001$) eightfold resistance to neutralization by HIV-1 IIIB V3-specific NAs than either HX10 or the parental HIV-1 IIIB mixture (Fig. 2). These viruses differ by only one amino acid inside and outside the V3 domain and parallel the findings by Looney and co-workers (30). Although only the coding region for the gp120 V3 domain was sequenced, these results strongly suggest that homogeneous amplification of the HXB3 virus strain occurred during primary passage in both H- and C-PBMCs. The neutralization resistance of resultant virus from the passage of HIV-1 IIIB through H- and C-PBMC (the HXB3-like V3 sequence) was identical to that seen with the molecularly cloned HXB3 (Fig. 2 and 4). These studies were repeated twice with identical results.

In vitro biology of the HIV-1 IIIB stock, its molecular clones, and reisolated NRVs. To confirm the previously described differences in the replication rates of these viruses, C- and H-PBMCs were infected by using identical multiplicities of infection of parental HIV-1 IIIB virus and its infectious molecular clones HX10, HXB2, and HXB3. As can be seen in Table 2, the HIV-1-IIIB parental stock in C- and H-PBMC required only 6 and 3 days, respectively, to reach significant levels of extracellular virus, as measured by p24 antigen capture analysis. When the IIIB molecular clones were analyzed, the fastest and most replication-competent subpopulation was HXB3. Comparatively, at the same multiplicity of infection, the HX10 virus required a 10-fold higher virus inoculum (to get a multiplicity of infection of 0.01) and twice as long in vitro in the C-PBMC to obtain an equivalent p24 level as the HXB3 clone. In

TABLE 2. Kinetics of cytopathic and replication competency of HIV-1 IIIB, molecular clones, and reisolated NRV[a]

Source	Virus strain	p24 kinetics[b]		Syncytial-forming capacity[c]	RT level[d]	
		C-PBMC	H-PBMC		C-PBMC	H-PMBC
Lab stocks	IIIB/H9	6	3	+++	21,292 (9)	337,644 (6)
	(S)HX-10	12	6	+	36,103 (15)	62,164 (9)
	(R)HXB2	9	12	++	41,880 (15)	74,057 (12)
	(K)HXB3	6	6	+++	43,375 (18)	105,686 (9)
Reisolated NRV	(S)911/6	9	6	++	38,150	105,080
	(R)911/16	9	6	+++	31,610	95,154
	(S/R)1125/6	6	3	+++	40,138	152,165
	(S/R)1125/16	6	6	+++	39,399	131,132

[a] Values for P24 and RT were obtained at a multiplicity of infection of 0.001.

[b] p24 levels were determined by the Dupont antigen capture assay. The values listed are from one representative experiment and represent the day at which the culture supernates equaled or exceeded 0.5 ng of p24 per ml.

[c] Syncytial-forming capacity is measured by the ability to form syncytia in the quantitative microtiter infectivity assay. Infectious endpoint dilutions were determined by comparing the SFUs with p24 levels. A rating of three (+++), two (++), and one (+) plus is given when the infectious endpoint as determined by the SFU and p24 levels is within a twofold (+++), two- to fourfold (++), and four- to eightfold (+) endpoint of each other.

[d] RT stands for reverse transcriptase. The values listed here are the highest values obtained over the course of the 21-day in vitro cultivation period. The number in parenthesis indicates the day postinfection it was recorded.

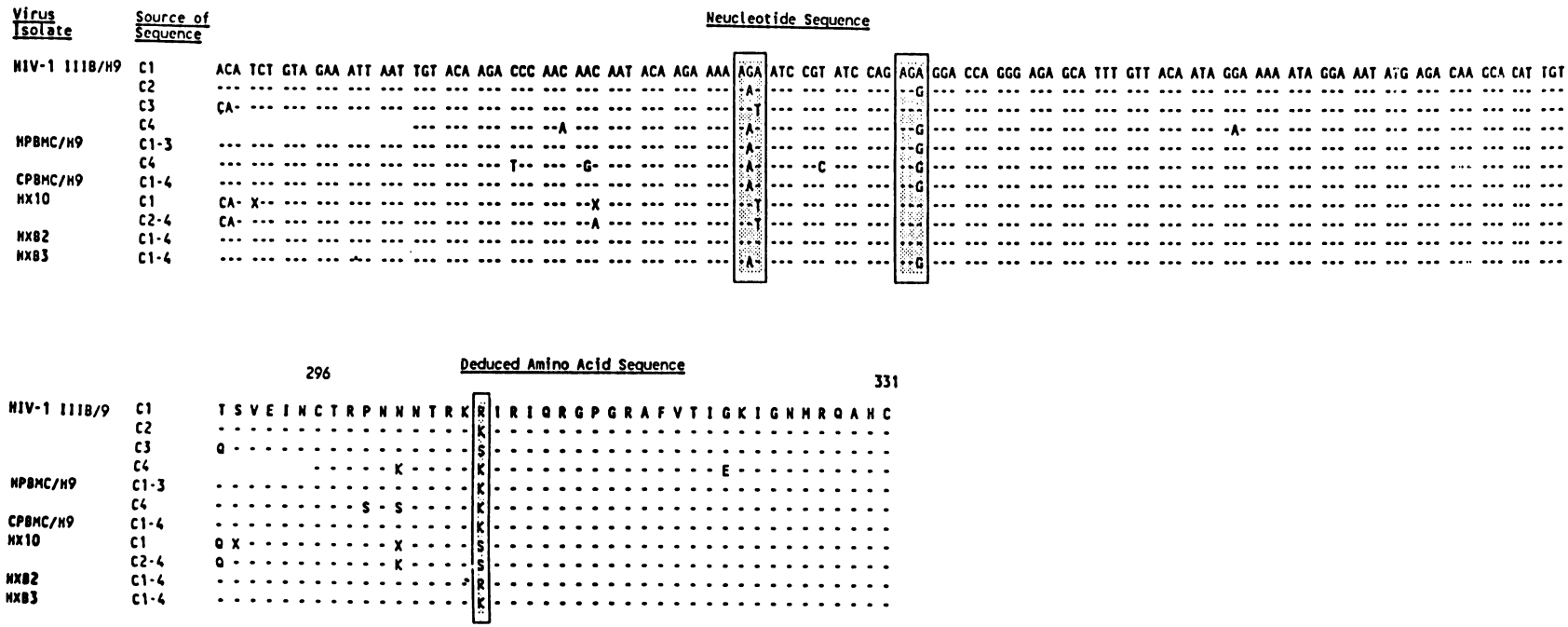

FIG. 3. Nucleic acid sequence and deduced amino acid sequence of the HIV-1 IIIB virus stock (H9/HIV-1 IIIB) used for inoculation of the cultures as well as neutralization and replication assays; the three viruses obtained after transfection of the molecular clones HX10, HXB2, and HXB3; the passaged virus through human PBMC (H-PBMC/H9); and the passaged virus through chimpanzee PBMC (C-PBMC/H9). Each of the viral sequences obtained in these studies for our molecular clones are indicated (C1 to C4). Within the sequence, X indicates that the nucleic acid and derived amino acid could not be identified.

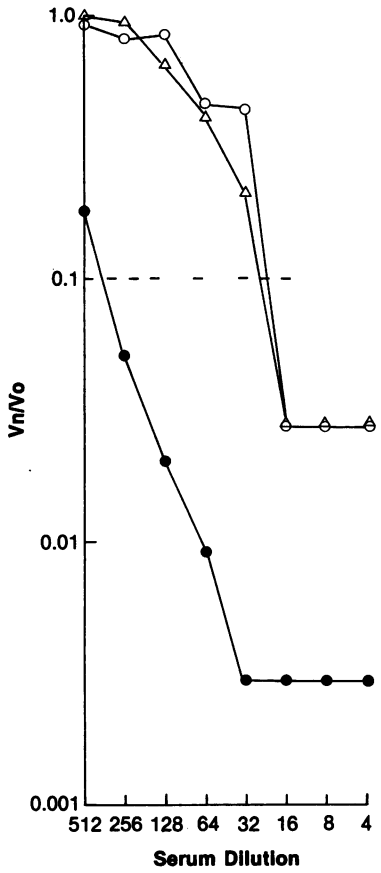

FIG. 4. Comparative phenotypic-serotypic neutralization analysis of HIV-1 IIIB passaged through primary C- and H-PBMCs. The horizontal dashed line demonstrates the 90% SFU inhibition point. The symbols are: ●, HIV-1 IIIB/H9 versus polyclonal IIIB V3-specific NA; ○, HIV-1 IIIB/H-PBMC versus IIIB V3-specific NA; and △, HIV-1 IIIB/C-PBMC versus IIIB V3-specific NA.

H-PBMC, however, it appears that HX10 replicated more efficiently, based on p24 kinetics (Table 2). NRVs from both animals obtained directly from cocultivation with H-PBMCs exhibited typical HIV-1 IIIB replication kinetics and cytopathology in vitro (Table 2). In one case, the 6-week NRV isolate from chimpanzee 911, designated 911/6(S), with the HX10 V3 genotype(s) was approximately twofold more replication competent and slightly better at quantitative endpoint syncytia induction (Table 2). Analysis of the NRV stocks for nonsyncytial variants was negative (data not shown).

V3 PCR analysis of isolated NRVs from a naive and gp120-immunized chimpanzees following inoculation with HIV-1 IIIB. The overall pattern that emerged from the serotypic neutralization analysis of virus from the naive chimpanzee (no. 911) over time suggested that as NA titers rise and recognize the most-replication-competent viruses; the relatively less-replication-competent subpopulation(s) is isolated from the animal. This is demonstrated in Fig. 5 and Table 2, where the initial V3 genotype directly sequenced from the earliest isolated (6 weeks PI) virus was the HX10-S type. PCR analysis of the V3 region of later isolates demonstrated a switch from HX10-like (S-V3 genotype) to HXB2-like (R-V3 genotype) at 16 weeks PI, with a return to the HX10-like (S-V3 genotype) occurring again at 32 weeks PI. As mentioned previously, at the various time points where a V3 genotypic shift is observed, the neutralization titers (for these two clones) approximated each other, thus apparently shifting the neutralization and reisolation threshold to another isolate. At 6 and 16 weeks PI, no heterologous viruses (i.e., RF, MN, and CC) were neutralized.

Although the V3 domains from the NRV isolates recovered at 6 and 32 weeks from animal no. 911 were homogeneous by direct and cloned sequences for the HX10 V3 amino acid coding sequence (Fig. 5), the resistance to neutralization by HIV-1 IIIB V3-specific NA was far greater than that of the original HX10 or HXB2 clones (Table 1). When additional NRVs from two other animals were simultaneously isolated in parallel by cocultivation in either H-PBMCs or their own C-PBMCs, no differences in the V3 sequence or degree of resistance to the typing serum was noted. This suggested that the isolation procedure in which phytohemagglutinin-blasted H-PBMCs were used did not alter the recoverable NRVs present in the chimpanzee's own cells (data not shown). It has to be noted, however, that the deduced amino acid sequence for the infectious clone HXB2 (T through R) differed from that of the 16-week isolate (Q through R) and that the nucleic acids for the 16-week isolate coding for Arg at position 306 were AGG and CGT, while in HX10 it was AGA (Fig. 5).

Sequencing clones derived from the PCR product of the V3 domain of the gp120 IIIB-vaccinated animal revealed a substantial degree of heterogeneity for all three NRVs (6, 16, and 32 weeks) analyzed (Fig. 5). Three molecular clones of the 6-week NRV isolate showed that the HX10 sequence, both at the nucleic acid and at amino acid levels, and one of the four clones had the unique amino acid sequence Q through R and an AGG codon instead of an AGA for the characteristic Arg at position 306, similar to that seen in the naive animal (Fig. 5; Table 1). The virus isolated at 16 weeks showed two of four clones with the nucleic acid and amino acid sequence of HX10 and two with the previously seen unique Q through R sequence. The virus isolated at 32 weeks showed all four clones with the HX10-like Q through S genotype, with additional amino acid substitutions occurring for the first time on the C-terminal portion of the V3 domain. These substitutions were all different from any of the original HIV-1 IIIB clones sequenced from the HIV-1 IIIB inoculum challenge stock.

DISCUSSION

Sequential isolates recovered during the course of an experimental HIV-1 IIIB infection in chimpanzees were far more resistant to neutralization by HIV-1 IIIB V3-specific NA (including the monoclonal antibody [MAb] 0.5 β) than the three molecular cloned viruses or the parental stock virus. This was most apparent in the case of the isolates with relatively heterogeneous genomes from the HIV-1 IIIB gp120-immunized animal.

Resistance to the HIV-1 IIIB V3-specific NA (MAb 0.5 β) was conferred by in vivo passage in both naive and gp120-immunized animals. Serum neutralization of the parental stock coincided with the appearance of antibody to a non-americ peptide (IQRGPGRAF) in a solid-phase enzyme-linked immunosorbent assay. This antibody by itself induced neutralization of the stock HIV-1 IIIB virus in animals, thus strongly indicating the chimpanzee antibody population binding to the V3 domain of the gp120 as the early immunoreactive site and the virus "prison" to escape from.

Sequencing the V3 domains of sequential isolates from

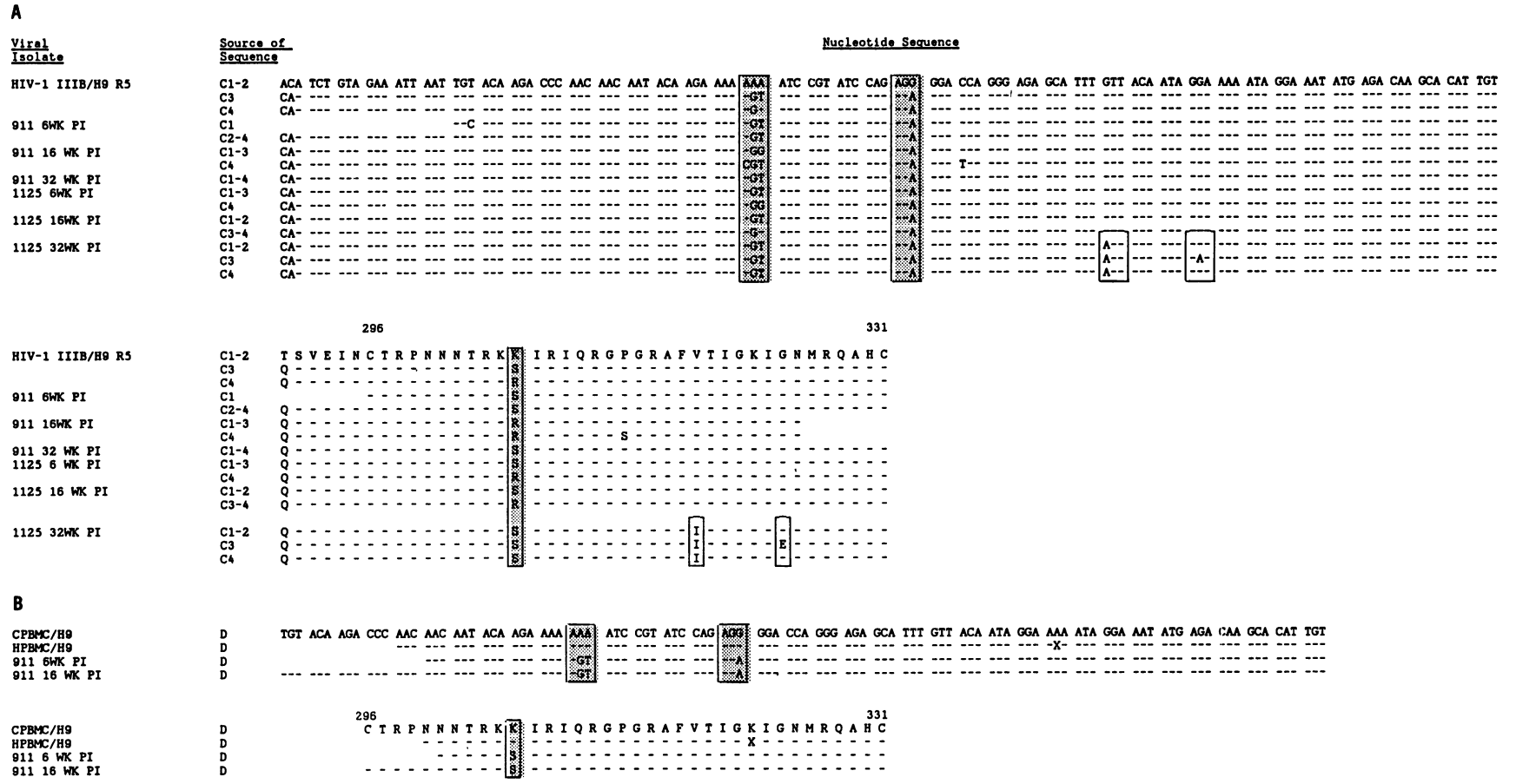

FIG. 5. (A) Nucleic acid sequence and deduced amino acid sequence from four clones(C1 to C4) of the V3 gp120 coding region of the HIV-1 IIIB stock used to inoculate the two animals and the subsequently reisolated viruses obtained at several time points after infection from the naive (911) and the preimmune animal (1125). (B) Nucleic acid and deduced amino acid sequences directly obtained (D) from PBMC DNA of the V3 gp120 coding region of the HIV-1 IIIB passaged through primary H- and C-PBMC as well as from NRVs obtained at 6 and 16 weeks PI from an experimentally HIV-1 IIIB-inoculated chimpanzee.

both the naive and the gp120-immunized animal revealed the complete disappearance of Lys-306 coding sequences, indicating that the in vivo passage selected against the HXB3-like (K-V3 genotype) virus. The selection against of HXB3 in vivo may or may not have been immune based. The data in this study, however, suggest that the earliest NA detected (6 weeks PI) was capable of neutralizing both HXB2 and HXB3, but not HX10, lending support to the notion of V3 NA-mediated selection. The fact that HXB3 was found to be the most replication-competent virus in the inoculum in vitro and that an HX10-like NRV based on the V3 genotype was isolated first lends further support to this notion. An identical independent result was observed in a recent passive in vitro neutralization experiment of chimpanzees (Emilio Emini, personal communication). The role of T-cell-mediated immunity during this early selection is currently unknown. Although cell-mediated immunity has been demonstrated in HIV-1-infected chimpanzees 3 months after HIV-1 inoculation (10) and following two vaccinations with vaccinia expressing the HIV-1 envelope, which failed to confer protection (28), its role in early viral selection remains an open issue.

The identity of the V3 amino acid sequence between the neutralization-resistant mutants generated in vivo that were highly NA resistant to HIV-1 IIIB V3-specific NA (MAb 0.5 β) and the NA-sensitive molecular clones (in particular HX10) points to changes somewhere outside the V3 domain that conferred resistance. The MAb 0.5 β used in these studies binds to a region of the V3 domain that is conserved between the three molecular clones observed in the parental virus stock, yet a degree of neutralization resistance can be detected between the three clones (30, 36). This finding also suggests that changes occurring outside the direct antibody-binding site can confer neutralization resistance in the V3 domain to the HXB2 and HXB3-like viruses. Notably, HXB2 and HXB3 share additional amino acid similarity at the carboxyl end of the 267 region and V4 not found in HX10. Such a phenomenon has previously been shown in feline leukemia virus infection (42) and HIV-1 in vitro (45), where nonsilent mutations, distant from the direct antibody-binding site, resulted in resistance to neutralization by a particular antibody.

In contrast, primary passage of an HIV-1 IIIB stock containing V3 viral subpopulations with genomes identical to the HX10, HXB2, and HXB3 molecular clones through C- and H-PBMCs yielded a homogeneous viral population with nucleic and amino acid sequences resembling HXB3. This in vitro-passaged population of viruses was partially (six- to eightfold more) resistant to neutralization by the HIV-1 IIIB V3-specific NA relative to the HIV-1 IIIB parental stock but similar in neutralization to a pure population of HXB3 virus produced by an HXB3-infected transformed human T-cell line. HXB3 was subsequently demonstrated to be the most-replication-competent virus population in vitro and rapidly outgrew the other two preexisting viruses (HX10 and HXB2) when inoculated onto C- and H-PBMCs. We concluded, therefore, that the resistance to neutralization by HIV-1 IIIB V3-specific NA after a single passage of the IIIB stock virus through PBMCs was due to the in vitro selection and generation of a homogenous population of the more neutralization-resistant and replication-competent HXB3 subpopulation of the HIV-1 IIIB inoculum. This in vitro passage phenomena has subsequently been observed by another investigator (Marc Girard, personal communication).

The NRV isolates obtained later from the HIV-1 IIIB gp120-immunized animal showed more V3 genomic heterogeneity and neutralization resistance over a longer period of time when compared to isolates and sera obtained from the serologically naive animal. When compared to the naive animal, the gp120-immunized animal had as much as a 64- to 128-fold increase in NA over a period of 22 weeks. This increased level of humoral immunity appeared to be correlated with the "apparent" increased level of neutralization resistance exhibited by these viruses during subsequent infection, selection, and replication in the host. It is interesting to note in this animal, as compared to the naive one, the appearance of additional primary amino acid changes in the V3 region. This finding of V3 amino acid substitutions occurring later in the host suggests that amino acid variability within the antibody-binding site related to isolate-specific neutralization may occur as a somewhat later event (months to years). Preference for certain amino acid residues (Ser and Arg) at position 306, irrespective of the nucleic acid coding triplet, suggested not only the nonidentity of both the NRVs in vivo and the molecular clones HX10 and HXB2 but also a functional constraint on that position. Again McKeating and co-workers have shown that an Arg-308 → Gly-308 change not only conferred escape from the selecting MAb but also yielded a noninfectious virus (33). The in vivo occurrence of antigenic drift, as shown for HIV-1 in the present study, might also be operational in the natural infection of humans, thus resulting in increasing genomic diversity during the course of infection (25, 48, 55). Not unsurprisingly, these results strongly suggest that the neutralization and serologic diversity of the principal neutralization epitope of HIV-1 may not be reflected completely by its primary amino acid sequence.

The isolation of relatively slower replicating variants from chimpanzees in the face of a measurable immune response to the more-replication-competent viruses of chimpanzees may also point to the possible association of less-replication-competent virus variants with the asymptomatic healthy phase and more-replication-competent viruses with disease in infected humans (5, 53–55). Extending the findings of this study to HIV-1-infected humans would suggest that the previously reported "rapid/high"-replication viruses seen in the acute and later stages of disease (5, 7, 54, 55) may not necessarily represent the emergence in the host over time of a more highly cytopathic and replication-competent variant but rather be due to "reactivation" of the initially controlled, more-replication-competent virus subpopulations. This reactivation may occur simply due to the associated loss of humoral and cellular immune cell function secondary to immune system dysregulation and ablation.

Prior to this report, the V3 domain of HIV-1 was thought to allow escape mutants to arise solely due to its primary amino acid hypervariability, and there was hope that a limited number of critical V3 amino acids could be identified to account for the type specificity of the NA response reported earlier (16, 30, 32, 38, 46). It now appears that the early escape provided by the NRV is probably related to sequence changes elsewhere in the envelope. These primary changes must be conveyed to the V3 domain through secondary changes in the tertiary and quaternary structure of the gp120/41 molecule. Operational mechanisms for this lack of neutralization may be that the V3 region is in an alternate molecular conformation. Due to other changes in continuous or discontinuous regions of the envelope, such as 127, 256, or changes in glycosylation patterns, the resultant stereochemistry of the epitope may shroud it from the antibody. Thus, it may be that HIV has evolved a single, extremely

interdependent, conformationally induced hypervariable linear neutralization epitope for its initial survival.

In conclusion, primary passage of HIV-1 IIIB in C- and H-PBMCs, in the absence of a selective MAb, yielded a homogenous virus strain due to amplification of the most-replication-competent variants in the stock. The antibody population neutralizing virus, through binding to the V3 domain of the virus, strongly appeared to be the selective force in vivo that suppressed and removed the relatively more-replication-competent virus subpopulations from the circulating recoverable virus pool. Thus, the initially isolated neutralization escape mutants appear to arise from the less-replication-competent virus subpopulations. The fact that virus mutants isolated from the gp120 immune animal were more resistant over time to neutralization by preexisting antibody relative to the naive animal confirmed this notion. Comparison of the nucleic acid sequences of the NRVs emerging in vivo with the molecular clones HX10, HXB2, and HXB3 suggested that changes outside the direct binding site for the selective natural antibody or a V3 binding MAb determined the ability of the mutant virus to resist neutralization. The findings presented here, implying a complex conformational nature to the V3 domain of HIV-1, will complicate peptide-derived, envelope-based vaccine approaches. Mapping these conformationally interdependent envelope sites to reveal the degree and extent that these sites affect the principal neutralization epitope should provide a better understanding of the dynamics of the viral envelope in future vaccine development.

ACKNOWLEDGMENTS

We thank Scott Putney for polyclonal antisera to peptides derived from the gp120 V3 domain, Larry Arthur and Steve Pyle for antisera to native gp120, Hiroaki Mitsuya for the 0.5 β MAb, Michael Poore and Shawn Conley for viral quantitation and technical support, and Linda Brandenburg and Erna Albers for manuscript preparation.

Research was sponsored, at least in part, by Public Health Service grant NO1-CO-74102 from the National Cancer Institute, with Program Resources, Inc.

ADDENDUM IN PROOF

Subsequent work recently published (J. Albert, B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nyström, and E. Fenyo, AIDS 4:107–112, 1990, and J. Albert, B. Abrahamsson, and E. M. Fenyo, UCLA Symp. Mol. Cell. Biol., Suppl. 14D, p. 163, 1990) has independently confirmed the findings presented here of the existence in humans of rapidly occurring neutralization escape mutants without changes in the V3 amino acid sequences.

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