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Induction of Neutralizing Antibodies to T-Cell Line-Adapted and Primary Human Immunodeficiency Virus Type 1 Isolates with a Prime-Boost Vaccine Regimen in Chimpanzees

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Five chimpanzees were immunized by administration of one or more intranasal priming doses of one to three recombinant adenoviruses containing a gp160 insert from human immunodeficiency virus type 1 (HIV-1) MN (HIV-1_{MN}) followed by one or more boosts of recombinant HIV-1_{SF2} gp120 delivered intramuscularly with MF59 adjuvant. This regimen resulted in humoral immune responses in three of five animals. Humoral responses included immunochemically active anti-HIV-1 antibodies (Abs) directed to recombinant gp120 and neutralizing Abs reactive with T-cell-line-adapted HIV-1_{MN} and HIV-1_{SF2}. In addition, neutralizing activity was detected to the two homologous primary isolates and to two of three heterologous primary isolates which, like the immunizing strains, can use CXCR4 as a coreceptor for infection. The three animals with detectable neutralizing Abs and a fourth exhibiting the best cytotoxic T-lymphocyte response were protected from a low-dose intravenous challenge with a cell-free HIV-1_{SF2} primary isolate administered 4 weeks after the last boost. Animals were rested for 46 weeks and then rechallenged, without a boost, with an eightfold-higher challenge dose of HIV-1_{SF2}. The three animals with persistent neutralizing Abs were again protected. These data show that a strong, long-lived protective Ab response can be induced with a prime-boost regimen in chimpanzees. The data suggest that in chimpanzees, the presence of neutralizing Abs correlates with protection for animals challenged intravenously with a high dose of a homologous strain of HIV-1, and they demonstrate for the first time the induction of neutralizing Abs to homologous and heterologous primary isolates.

Most viral vaccines induce immunity which limits virus replication, prevents disease, and facilitates clearance of the infection. Few, if any, induce sterilizing immunity, defined as the ability to completely prevent virus infection (19). Protection is achieved by stimulating the humoral and cellular arms of the immune response, both of which are needed to eliminate free virus and infected cells.

It has been postulated that to prevent and contain human immunodeficiency virus type 1 (HIV-1) infection, stimulation of cellular immunity is more critical than induction of antibodies (Abs) because infection is thought to occur as the result of contact with infected cells as well as with free virions. Support for this concept comes from the findings that many exposed but uninfected subjects display cellular responses in the absence of Abs (reviewed in reference 37) and that vaccine-induced immunity to certain other retroviral infections, such as feline leukemia virus, does not correlate with the presence of neutralizing Abs (NAbs) (12). Moreover, in the absence of sterilizing immunity, cellular immunity is believed to be necessary if eradication of infection is to be achieved.

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The need for Abs to protect against HIV-1 and other viruses is also well documented. Most efficacious viral vaccines in current clinical use induce NAbs, which play a crucial role in prophylaxis (33). Abs are essential for the elimination of free virus particles and thus for reduction of the magnitude of the infectious inoculum. Furthermore, effective levels of functional Abs also help to contain virus spread as virions are produced by infected cells. Indeed, an important role for Abs has been documented in studies of HIV-1 infection with humans and chimpanzees in which Abs have been shown, directly or by association, to be involved in preventing, delaying, and decreasing the extent of HIV-1 infection (8, 9, 15, 31, 36, 38).

Chimpanzees represent a particularly valuable model for the study of HIV-1 prophylaxis because they are the only nonhuman primates that can be readily infected with HIV-1 and because they can be immunized and challenged with HIV-1 under controlled conditions. Numerous studies have been conducted with either passive or active immunization and challenge with free or cell-associated virus. In most of these studies, protection has been correlated with the presence of NAbs (3, 9–11, 15) and, when sterilizing immunity was not achieved, the presence of NAbs was frequently associated with delayed onset or reduced parameters of infection (9, 15, 38).

Like sera from human recipients of various candidate HIV-1 vaccines, sera from immunized chimpanzees have been shown to display significant levels of NAbs for the immunizing HIV-1 strains, but the detection of NAbs to primary isolates has been difficult to achieve (4, 16, 38). This failure to demonstrate primary isolate NAbs could be due to the inability of a partic-

TABLE 1. Summary of chimpanzee immunization and challenge results

Chimpanzee	Reagent administered at wk:								Challenge results with indicated HIV_{SF2} dose:	
	0[a]	12[a]	24[a]	38[b]	48[b]	52[c,d]	96[a]	98[c,e]	Low (wk 52)[d]	High (wk 98)[e]
1P		$Ad5/160_{MN}$		$rgp120_{SF2}$	$rgp120_{SF2}$	HIV		HIV	Protected	Protected
$2P_A$		$Ad5/160_{MN}$	$Ad7/160_{MN}$		$rgp120_{SF2}$	HIV		HIV	Protected	Protected
$2P_B$		$Ad5/160_{MN}$	$Ad7/160_{MN}$		$rgp120_{SF2}$	HIV		HIV	Protected	Infected
$2P_{POS}$		$Ad5/160_{MN}$, $Ad7/160_{MN}$, $Ad4/160_{MN}$	$Ad5/160_{MN}$, $Ad7/160_{MN}$, $Ad4/160_{MN}$		$rgp120_{SF2}$		$Ad5/160_{MN}$, $Ad7/160_{MN}$, $Ad4/160_{MN}$	HIV		Infected
3P	$Ad5/160_{MN}$	$Ad7/160_{MN}$	$Ad4/160_{MN}$		$rgp120_{SF2}$	HIV		HIV	Protected	Protected
3C	Ad5	Ad7	Ad4		MF59	HIV		HIV	Infected	Infected
C								HIV		Infected

[a] Intranasal administration; for chimpanzee $2P_{POS}$, 10^7 PFU of each of the three recombinants was administered ($Ad5/160_{MN}$, $Ad7/160_{MN}$, $Ad4/160_{MN}$).
[b] Intramuscular administration.
[c] Intravenous administration.
[d] One milliliter of a 1:40 dilution of $HIV\text{-}1_{SF2}$ challenge stock (HIV) (28).
[e] One milliliter of a 1:5 dilution of $HIV\text{-}1_{SF2}$ challenge stock (HIV).

ular vaccine to induce such Abs, to an antigenic mismatch between the immunizing strains and the primary isolates used to detect neutralizing activity in vitro, and/or to inadequate sensitivity of the assays used to measure Ab activity. In this study, we used several different viruses and assay techniques to study NAbs in the sera of chimpanzees immunized with vaccines which had protected four of four chimpanzees against a low-dose HIV-1 challenge and, after a 1-year rest period, had protected three of the same four chimpanzees against a high-dose challenge with cell-free $HIV\text{-}1_{SF2}$ (25).

The vaccine regimen used consisted of intranasal administration of recombinant adenoviruses containing gp160 of $HIV\text{-}1_{MN}$ ($Ad/160_{MN}$; priming doses) followed by intramuscular administration of a recombinant gp120 (rgp120) subunit of $HIV\text{-}1_{SF2}$ ($rgp120_{SF2}$; boosts). Sera of immunized and control animals were tested for neutralizing activity against the cell-line-adapted immunizing strains ($HIV\text{-}1_{MN}$ and $HIV\text{-}1_{SF2}$), against primary isolates homologous to the immunizing strains (previously cultured only in peripheral blood mononuclear cells [PBMCs]), and against other, heterologous, syncytium-inducing (SI) or dualtropic primary isolates which, like the immunizing strains, can use CXCR4 as a coreceptor for infection of cells (13, 22).

The studies showed that the administration of priming doses of $Ad/160_{MN}$ rarely elicited NAb responses, whereas high titers of NAbs were induced swiftly after the administration of $Ad/160_{MN}$ and one or more boosts with rgp120 to the three chimpanzees that were protected against both the low- and the high-dose challenges. After priming doses and boosts, NAbs to the cell-line-adapted immunizing strains, to the homologous primary isolates, and to two of three heterologous primary isolates were detected. NAbs remained detectable for more than 1 year after the last boost and correlated with protection from a high-dose challenge with cell-free $HIV\text{-}1_{SF2}$ administered approximately 1 year after the last boost. As previously shown (25), CD8 cytotoxic T lymphocytes (CTL) were detected transiently in all five chimpanzees over the immunization course and apparently contributed to protection from low-dose challenge but not from high-dose challenge in one NAb-negative chimpanzee. These studies suggest that NAbs play an important role in protection from intravenous infection by low-dose and high-dose cell-free viruses in chimpanzees and demonstrate for the first time that NAbs to homologous and heterologous primary isolates can be induced with a prime-boost immunizing regimen in chimpanzees.

MATERIALS AND METHODS

Immunization and challenge of chimpanzees. As previously described (25) and as summarized in Table 1, five chimpanzees were immunized intranasally with 10^7 PFU each of recombinant adenoviruses (adenovirus type 5 [Ad5], Ad7, and/or Ad4) containing a gp160 insert derived from $HIV\text{-}1_{MN}$ in tandem with a separate copy of the *rev* gene ($Ad/160_{MN}$). The chimpanzees were immunized with $Ad/160_{MN}$ once (chimpanzee 1P), twice (chimpanzees $2P_A$, $2P_B$, and $2P_{POS}$), or three times (chimpanzee 3P). These immunizations are referred to as priming doses; they were followed by one or two intramuscular immunizing doses of 50 μg of CHO cell-derived $HIV\text{-}1_{SF2}$ gp120 delivered with MF59 adjuvant (referred to as boosts). As shown in Table 1, chimpanzees were challenged intravenously 52 and 98 weeks after the first vaccine dose with a low virus dose (1 ml of a 1:40 dilution of a PBMC-grown $HIV\text{-}1_{SF2}$ challenge stock) or with a high dose (1 ml of a 1:5 dilution of virus stock) (27). One chimpanzee ($2P_{POS}$) received only the high-dose challenge at 98 weeks, 2 weeks after a third intranasal immunization with $Ad/160_{MN}$. One control chimpanzee (3C) received three immunizations with wild-type adenovirus vectors lacking the HIV-1 insert and a subsequent injection of MF59 adjuvant. Another chimpanzee (C) which received no prior immunizations or mock injections served as a control for the high-dose challenge.

Infection after challenge was assessed by periodic monitoring of PBMCs for virus isolation, by RNA PCR of plasma, and by PCR of DNA extracted from PBMCs with conserved *gag* primer pairs as previously described (25). Sera from periodic bleeds were stored frozen prior to assay for immunochemical and neutralizing activities.

Immunochemical reactivity of serum specimens. Chimpanzee sera were tested for reactivity against $rgp120_{SF2}$ as described previously (9, 39). The enzyme-linked immunosorbent assay (ELISA) titers obtained were the reciprocals of the dilutions that resulted in an absorbance corresponding to 50% of the maximum signal in the assay. The results were the means of duplicate assays; the standard deviations were ≤10% of the values reported. Sera that showed no evidence of a reaction at a 1:100 dilution are indicated as having titers of <1:100.

Viruses. Cell-line-adapted $HIV\text{-}1_{SF2}$ was carried in HUT78 cells, and cell-line-adapted $HIV\text{-}1_{MN}$ was carried in H9 cells. Several HIV-1 primary isolates which had been carried only in PBMCs were also used. These included $HIV\text{-}1_{MN}$ and SF33 (23, 42), which had been passaged extensively in PBMCs, as well as $HIV\text{-}1_{SF2}$, BZ167 (24), and 92HT593 (from the National Institutes of Health AIDS Reference and Reagent Program), which were used as low-passage viruses in the experiments described below.

Neutralization assays. Seven different neutralization assays were used to assess activity in the sera. First, T-cell-adapted $HIV\text{-}1_{SF2}$ was tested with HUT78 as target cells according to the methods of El-Amad et al. (9) and Haigwood et al. (18). The readout for this assay was a p24 ELISA, and data were normalized by running a human serum pool as a standard in each assay. The normalized neutralization titer was defined as the reciprocal of the dilution of serum (in the serum-virus mixture) that resulted in a 50% decrease in intracellular p24 levels. HUT78 cell-grown $HIV\text{-}1_{SF2}$ was also tested with CEMx174 as target cells according to the method of Robert-Guroff (34). The readout for this assay was a reduction by 50% in the number of cells staining positively for p24, read

microscopically. Data were normalized by setting the percentage of infected cells in the presence of control serum as 100%. Tissue-culture-adapted HIV-1_{MN} was assayed with H9 as target cells in the same manner as that described above for the assay of HIV-1_{SF2} with CEMx174 cells.

Stocks of HIV-1_{SF2} and HIV-1_{MN} which had been passaged only in PBMCs (and never carried in T-cell lines) were also tested for neutralization. The primary isolate HIV-1_{SF2} stock was tested for neutralization on PBMCs stimulated with anti-CD3 and anti-CD28 as previously described (4). The readout for this neutralization assay was a 10-fold reduction (compared to control cultures with normal chimpanzee sera) in the titer of infectious virus, measured by the presence or absence of reverse transcriptase. Primary isolate HIV-1_{MN} was tested for neutralization with human phytohemagglutinin (PHA)-activated PBMCs as target cells as previously described (25, 28). Virus infectivity was assessed with a p24 ELISA, and neutralization titers were expressed as the dilutions of sera capable of reducing p24 production by 50%.

Chimpanzee sera were also tested for their capacity to neutralize heterologous primary isolates BZ167, 92HT593, and SF33. These viruses were grown in PHA-activated PBMCs as previously described (45) and frozen prior to use in neutralization assays. BZ167 and 92HT593 were tested using as target cells unstimulated PBMCs that had been exposed to each virus-serum mixture for 1.5 h (the resting cell assay [20, 45]). After replacement of the virus-serum mixture with culture medium and overnight incubation, the medium was supplemented with PHA and interleukin-2; ELISA quantitation for reduction in p24 levels was performed 6 days postinfection. Positive and negative chimpanzee serum controls were included in these experiments. Data are reported as the percent reduction in p24 levels at various serum dilutions compared to p24 levels in cultures in which virus was preincubated with sera drawn from the chimpanzees prior to immunization. Neutralization assays in which SF33 was used were performed with CEM-SS as target cells as previously described (43), with the modification that the virus-serum mixture was incubated with the cells overnight, followed by three washes with RPMI-1640 medium. A p24 ELISA readout was performed 6 days postinfection.

RESULTS

Immunization and challenge results. Chimpanzees were immunized and challenged according to the schedule shown in Table 1. The results of challenging the animals with a low dose of HIV-1_{SF2} at week 52 and with a high dose of HIV-1_{SF2} at week 98 are also shown. In both experiments, virus replication and seroconversion in the control chimpanzees challenged for the first time became evident by weeks 4 and 6 to 8, respectively (25). Four immunized animals were challenged with the low dose of virus at week 52, and all four were protected. Three of these four protected chimpanzees (1P, $2P_A$, and 3P) mounted a detectable Ab response, as measured by ELISA and/or NAbs for the immunizing strains of HIV-1 (Table 2). The fourth animal ($2P_B$) had no detectable anti-HIV-1 Abs but did display HIV-1-specific cytotoxic T-cell activity in PBMCs and lymph node cells prior to the low-dose challenge, as previously described (25). Five immunized animals were challenged with the high dose of virus at week 98, and three of the five were protected (Table 1). Sera from all three of these protected chimpanzees (1P, $2P_A$, and 3P) displayed detectable Abs by ELISA and/or neutralizing activity for the immunizing strains of HIV-1 prior to the high-dose challenge (Table 2); sera from the infected chimpanzees ($2P_B$ and $2P_{POS}$) displayed no Abs (Table 2).

Neutralization of immunizing strains. Neutralizing titers for HIV-1_{MN} and HIV-1_{SF2} were measured by different methods before and after the low-dose challenge. Neutralizing titers for HIV-1_{MN} grown in and tested on H9 cells were consistently higher than those for HIV-1_{MN} grown in and tested on PHA-stimulated PBMCs (Table 2). Neutralizing titers for HIV-1_{SF2} grown in and tested on HUT78 cells were generally higher than those detected when CEMx174 cells were used as target cells. Moreover, NAb titers measured for T-cell-line-adapted HIV-1_{SF2} were generally higher than those measured for the HIV-1_{SF2} primary isolate. Indeed, in results similar to those obtained with HIV-1-positive human sera (35, 41), chimpanzee NAbs to HIV-1_{MN} and HIV-1_{SF2} were best detected with assays based on cell lines (Table 2). The NAb titers were usually highest when cell-line-adapted HIV-1_{MN} was used for detection; however, the results with both cell-line-adapted and PBMC-grown stocks of HIV-1_{MN} and HIV-1_{SF2} demonstrated that NAbs were generated in the three of four animals protected from a low-dose challenge and in all three animals protected from a high-dose challenge. However, the levels of NAbs detected depended on several variables, including the host cell in which the virus was grown and the target cell used in the assay.

Neutralization of heterologous primary isolates. Neutralizing activity was detected against HIV-1_{MN} and HIV-1_{SF2} primary isolates which, by definition, had been carried only in PBMCs. To determine if neutralizing responses were generated against heterologous primary isolates, the sera were tested against three clade B primary isolates: SF33 (23, 42), BZ167 (1, 44, 45), and 92HT593. All of these viruses, like the immunizing strains, use CXCR4 as a coreceptor for infection and infect MT2 cells; SF33 and 92HT593 can also use CCR5 as a coreceptor (data not shown) and thus are dualtropic viruses. Sera initially tested were from the three animals which had been protected from both the low-dose and the high-dose challenges; the specimens tested were drawn within 1 month of the low-dose challenge. Neutralizing activity was assessed against SF33 grown in PBMCs and tested on CEM-SS target cells; neutralizing activity against BZ167 and 92HT593 was measured with virus grown in PHA-activated PBMCs and tested on unstimulated PBMCs in the resting cell assay. The results of duplicate experiments with SF33 and BZ167 are shown in Table 3. Activities against the two viruses were comparable in sera from chimpanzee 3P but stronger for BZ167 than for SF33 in sera from animals 1P and $2P_A$. Preimmune sera from these animals displayed no neutralizing activity against BZ167, and serum from chimpanzee 3C, immunized with the adenovirus construct without the gp160 insert, showed no activity against SF33. No consistent neutralizing activity against isolate 92HT593 was detected in multiple bleeds from chimpanzees 3P, 1P, and $2P_A$; preimmune serum from control chimpanzee 3C was also negative against 92HT593 (data not shown). These experiments demonstrate that the immunizing regimen induced neutralizing activity against two of three heterologous primary isolates as well as NAbs against the two homologous primary isolates.

To ascertain whether the presence of the primary isolate NAbs correlated with protection, we performed further experiments with primary isolate BZ167 in which the levels of NAbs and their presence over time were measured. For this, a coded panel of 85 sera was tested for neutralizing activity against BZ167 in the resting cell assay. Sera were tested at a dilution of 1:20. In some cases, sera were also tested at a dilution of 1:200. The data were plotted after the codes were broken, and the results are shown in Fig. 1. Figure 1A shows that no significant neutralization was detected in sera drawn at three time points from control chimpanzee 3C, primed with adenovirus lacking the gp160 insert and boosted with adjuvant alone. In marked contrast were the data for sera from chimpanzee 3P (Fig. 1A), primed with three doses of Ad/160_{MN} and boosted once with rgp120. NAb levels increased gradually during immunization with Ad/160_{MN}, reaching a maximum 4 weeks after administration of the rgp120 boost. Thereafter, sera from this animal, tested at a 1:20 dilution, mediated a mean level of 93% neutralization over a period of 1 year. This animal was protected from the first and second virus challenges when NAb levels were high. When sera from this animal were tested at a dilution of 1:200, the level of neutralizing activity was found to drop gradually over time (Fig. 1A).

Figure 1B shows results for sera from chimpanzee 1P, which received a single priming dose of Ad/160_{MN} and two boosts of

TABLE 2. ELISA results and NAb activity for the immunizing strains in chimpanzee sera

Chimpanzee	wk postinoculation (date of bleed, mo/day/yr)	Reciprocal ELISA titer vs $rgp120_{SF2}$	Neutralization data (reciprocal titers)[a]				Challenge results	
			50% Neutralization with[b]:			Reciprocal neutralization titer with SF2[c], anti-CD3/28PBMC, anti-CD3/28PBMC[b,d]		
			MN, H9, H9	MN[c], PHA-PBMC, PHA-PBMC	SF2, HUT78, HUT78	SF2, HUT78, CEMx174	Low dose	High dose
3C	51 (04/19/94)		<20			<20	<50	Inf	Inf
	52 (04/28/94)	<100	<20	<20	<10	<20			
	56 (05/25/94)	<100	<20		<10	<20			
	92 (01/31/95)	291	<20		80	<20			
$2P_{POS}$	46 (03/14/94)		<20					NA	Inf
	50 (04/12/94)	<100	<20			<20			
	51 (04/19/94)		<20			<20	<50		
	92 (01/31/95)		<20			<20			
$2P_B$	50 (04/12/94)	<100	<20			<20		Prot	Inf
	51 (04/19/94)		<20			<20	<50		
	52 (04/28/94)	<100	<20	<20	<10	<20			
	92 (01/31/95)	<100	<20		<10	<20			
1P	40 (02/01/94)	12,190	800			170	50	Prot	Prot
	50 (04/12/94)	9,351	360	30		70	50		
	51 (04/19/94)		430	20		120	<50		
	52 (04/28/94)	11,424	360	70	148	90	<50		
	56 (05/25/94)	7,245	240		200	70	<50		
	62 (07/05/94)	3,794	50		240	30	<50		
	76 (10/11/94)	1,745	80		62	20	<50		
	92 (01/31/95)	1,478	60		50	<20			
$2P_A$	50 (04/12/94)	10,079	820	70		90	<50	Prot	Prot
	51 (04/19/94)		660			190	237		
	52 (04/28/94)	15,274	560	30	455	80	516		
	56 (05/25/94)	8,670	200		320	80	103		
	62 (07/05/94)	4,538	120		120	40	<50		
	76 (10/11/94)	1,608	40		285	60	<50		
	92 (01/31/95)	1,014	110		60	25			
3P	14 (08/03/93)		450			140	<50	Prot	Prot
	26 (10/26/93)		470	160		30	<50		
	48 (03/29/94)	9,829	160	150		30	122		
	50 (04/12/94)	235,897	2,670	110		430	857		
	52 (04/28/94)	39,476	2,550	640	515	1,030	336		
	56 (05/25/94)	20,963	1,600	>1,620	360	240	175		
	62 (07/05/94)	12,815	570		400	100	122		
	76 (10/11/94)	6,542	450		200	120	<50		
	92 (01/31/95)	5,037	820		280	255			

[a] Low-dose challenge was at week 52; high-dose challenge was at week 98. Inf, infected; Prot, protected; NA, not applicable.
[b] The first item is the virus strain tested, the second item is the host cell used to produce the virus, and the third item is the target cell used in the neutralization assay. PHA-PBMC, PHA-activated PBMCs; anti-CD3/28 PBMC, PBMCs stimulated with anti-CD3 and anti-CD28.
[c] Passaged only in PHA-activated PBMCs.
[d] V_N/V_O, 0.1.

rgp120. The results were similar to those for chimpanzee 3P: a single priming dose did not induce significant levels of NAbs, but the boosts induced high levels of NAbs, which remained elevated for approximately 1 year. This animal was protected from the low- and high-dose challenges when NAb levels were high.

Figure 1C shows the data for sera from chimpanzee $2P_A$, which received two priming doses of $Ad/160_{MN}$ and one boost of rgp120. Again, NAb levels remained low until after the administration of the boost, which gave rise to a high neutralizing titer against primary isolate BZ167. This animal was protected at this time from a low-dose challenge. In this animal, NAb titers dropped gradually over a period of 1 year but remained detectable (compared to those in sera drawn prior to immunization). This animal was protected from a high-dose challenge in that virus isolation was negative throughout 64 weeks of follow-up. However, as reflected by the anamnestic Ab response and the presence of a low level of plasma RNA 41 weeks after the high-dose challenge (25), a transient low-level infection occurred, but further HIV-1 replication was efficiently suppressed.

The results of measurements of NAbs in the two remaining chimpanzees are shown in Fig. 1D. Chimpanzee $2P_{POS}$ was given two priming doses of $Ad/160_{MN}$ and one rgp120 boost. This animal produced no consistent pattern of NAbs for BZ167, $HIV\text{-}1_{MN}$, or $HIV\text{-}1_{SF2}$. The poor humoral response of this animal to priming with $Ad/160_{MN}$ and subsequent boosting may have been the result of high preexisting Ab titers (1:128) to all three adenovirus vectors (25). This animal was not given a first, low-dose challenge. This animal was, however, given another priming dose of $Ad/160_{MN}$ 2 weeks prior to the high-

TABLE 3. Neutralizing activity of sera from immunized chimpanzees for heterologous primary HIV-1 isolates[a]

Chimpanzee	Time of bleed	% Neutralization of the following viruses at the indicated serum dilutions:			
		SF33[b]		BZ167[c]	
		1:20	1:320	1:20	1:200
1P	At challenge[d]	56, 69	0, 0	97, 100	72, 56
$2P_A$	At challenge[d]	52, 26	23, 0	91, 89	57, 0
3P	At challenge[d]	99, 97	87, 66	93, 93	98, 49
1P	Preimmune	ND, ND	ND, ND	0, 0	22, 11
$2P_A$	Preimmune	ND, ND	ND, ND	0, 0	0, 0
3P	Preimmune	ND, ND	ND, ND	0, 0	10, 0
3C	At challenge[d]	22, 0	8, 0	ND, ND	ND, ND

[a] Results of duplicate experiments are shown throughout. ND, not done.
[b] SF33 was grown in PBMC host cells and tested on CEM-SS target cells.
[c] BZ167 was grown in PBMC host cells and tested in the resting cell assay.
[d] Sera were drawn within 1 month of the low-dose challenge.

dose challenge. This immunization failed to induce NAbs and the animal, when challenged, was infected.

Chimpanzee $2P_B$ also received two priming doses of Ad/160_{MN} and one rgp120 boost. This animal never mounted a NAb response, although, as previously described (25), it exhibited sporadic CTL activity in PBMCs over the immunization course and displayed major histocompatibility complex-restricted, HIV-1-specific CTL in lymph node cells. This animal was protected from the low-dose challenge. However, after a 1-year rest without intervening antigenic stimulation with HIV-1, the animal failed to display NAbs (Fig. 1D and Table 2), lost its cytotoxic T-cell response (25), and was infected upon challenge with the high dose of HIV-1_{SF2}.

The data in Fig. 1 and Table 2 indicated that NAb levels to both the immunizing strains and heterologous primary isolate BZ167 remained detectable in the animals protected from a high-dose challenge for the entire period between the first and second challenges. Thus, for example, chimpanzee 3P displayed 50% neutralizing titers of 1:820 and 1:255 at week 92 against cell-line-adapted HIV-1_{MN} and HIV-1_{SF2}, respectively (Table 2). This same animal at week 92 (January 1995) displayed a 50% neutralizing titer of 1:200 for BZ167 (Fig. 1A). Similar results were observed for chimpanzees 1P and $2P_A$, while no consistent pattern of neutralizing activity to any of the three viruses was detected during this same period in chimpanzees infected by the high-dose challenge (Fig. 1 and Table 2).

Finally, selected sera were titrated to determine if a dose-response relationship could be demonstrated between serum concentration and neutralizing activity for BZ167. Figure 2 shows the results of three separate experiments with sera drawn from chimpanzees 3P, 1P, and $2P_A$ at the time of the

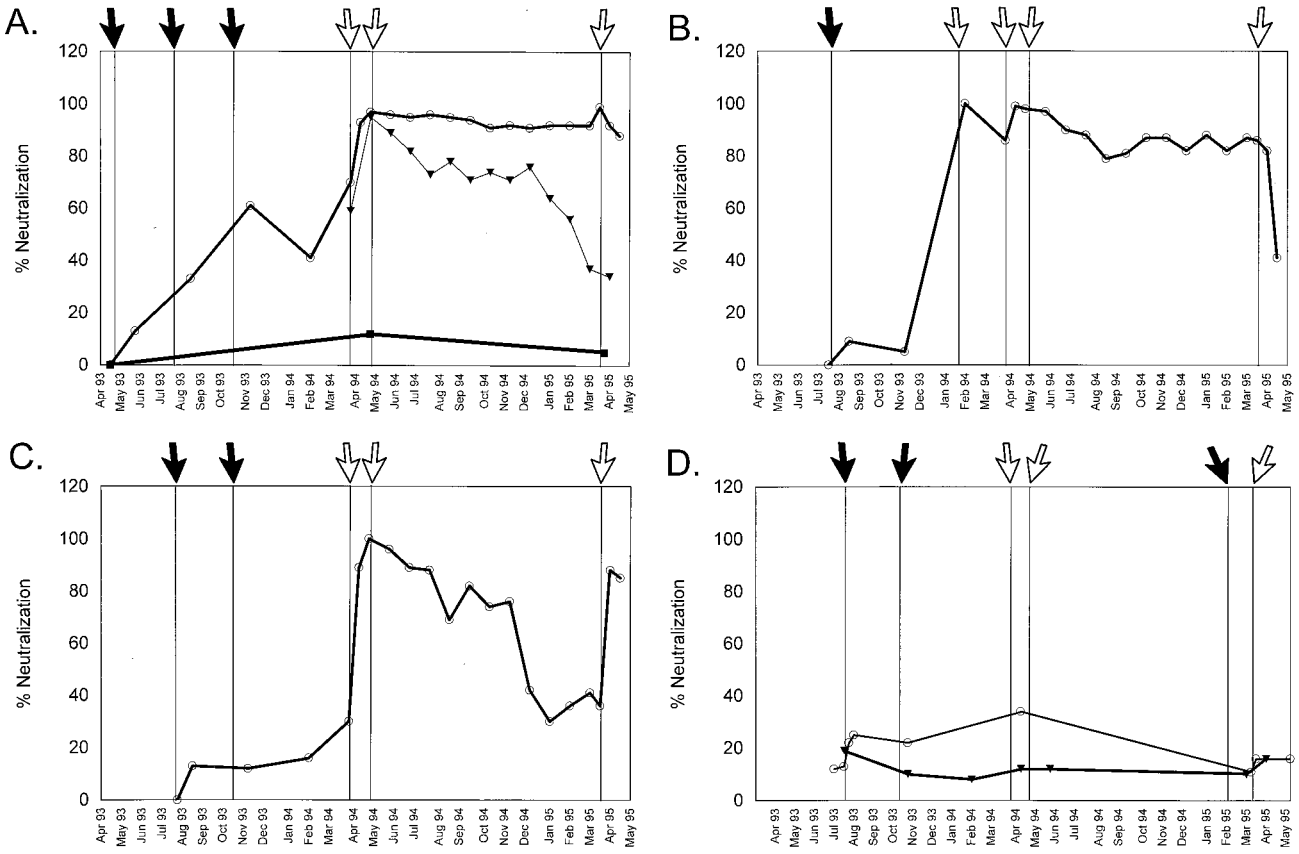

FIG. 1. Neutralizing activity for heterologous primary isolate BZ167 in the sera of immunized chimpanzees. Percent neutralization is shown on the ordinate; the date on which blood was collected is shown on the abscissa. Immunization with Ad/160_{MN} is denoted by solid arrows, and boosting with $rgp120_{SF2}$ is shown by open arrows. The gray arrows denote the times of the low-dose challenge (April 1994) and the high-dose challenge (March 1995). (A) Activity in sera from chimpanzee 3C tested at 1:20 (■) and from chimpanzee 3P tested at 1:20 (○) or 1:200 (▼). (B) Activity in serum from chimpanzee 1P tested at 1:20. (C) Activity in serum from chimpanzee $2P_A$ tested at 1:20. (D) Activity in sera from chimpanzees $2P_B$ (▼) and $2P_{POS}$ (○) tested at 1:20. Only chimpanzee $2P_B$ was given the low-dose challenge, and only chimpanzee $2P_{POS}$ was given a boost with Ad/160_{MN} before the high-dose challenge.

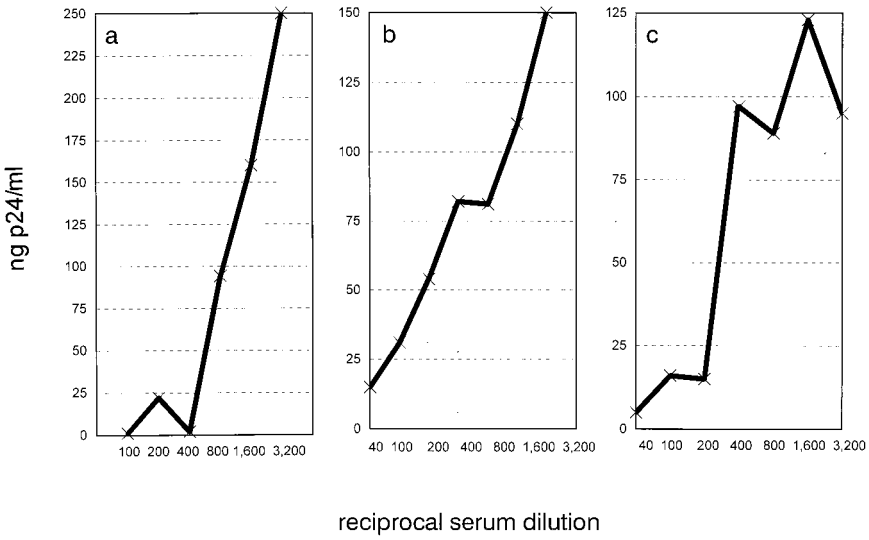

FIG. 2. Titration of sera from chimpanzees 3P (a), 1P (b), and $2P_A$ (c). Sera were tested at various dilutions for their ability to block BZ167 infection of unstimulated PBMCs, assessed on the basis of p24 production.

first challenge or within 2 months. Each serum sample showed a classic titration curve with decreasing neutralizing activity, i.e., increasing p24 production, as the serum sample was diluted.

DISCUSSION

Immunization of chimpanzees by use of a prime-boost regimen with $Ad/160_{MN}$ and $rgp120_{SF2}$ resulted in the protection of four of four chimpanzees from a low-dose challenge and three of the four from a subsequent high-dose challenge with cell-free HIV-1_{SF2} grown in PBMCs. Immunity, as measured by the presence of NAbs, was demonstrable 1 year after the last immunizing dose. Protection was correlated with the presence of NAbs to homologous cell line-adapted HIV-1 strains and to homologous and heterologous primary isolates in three of the four animals protected from the low-dose challenge and in all three animals protected from the high-dose challenge. The Ab responses in the protected animals are among the longest lived and broadest described to date for subjects immunized with HIV-1 vaccines (14, 17). As shown previously, CTL responses, elicited in all immunized chimpanzees, appeared to play a role in the protection of at least one animal against a low-dose challenge but were not sufficient to protect against a high-dose intravenous challenge in the absence of NAbs (25).

The protection of actively immunized chimpanzees against homologous and heterologous challenges with clade B viruses has been described by several groups (3–5, 9, 14–16). Immunization can also result in the delay of infection and in a decrease in the parameters of infection (9, 15, 38). Consequently, this animal model of HIV-1 infection provides one of the most convincing arguments for the feasibility of developing a protective vaccine and for the role of Abs in protection against infection. Moreover, many studies have reported a correlation between the presence of NAbs and these protective events (3, 9–11, 15). It is notable, however, that while Girard et al. (16) protected chimpanzees from challenge with PBMC-grown HIV-1_{SF2} by immunization with various combinations of immunogens from HIV-1_{MN} and HIV-1_{LAI}, protection was correlated with the presence of NAbs against HIV-1_{MN} and HIV-1_{LAI} grown in cell lines, while no NAbs to HIV-1_{SF2} grown in and tested on activated PBMCs were demonstrable. Berman et al. (4) were also able to protect chimpanzees from challenge with a heterologous HIV-1_{SF2} primary isolate by immunization with $rgp120_{MN}$; protection again was correlated with NAbs for three cell-line-adapted strains (HIV-1_{MN}, HIV-1_{SF2}, and HIV-1_{IIIB}) but not with NAbs to the challenge strain, HIV-1_{SF2}, grown in and tested on activated PBMCs. Similarly, Shibata et al. (38) protected chimpanzees from challenge with a heterologous DH12 primary isolate by prior infection with HIV-1_{IIIB} or HIV-1_{SF2}; again, however, while protection was correlated with NAbs for cell-line-adapted strains (HIV-1_{IIIB}, HIV-1_{SF2}, and HIV-1_{MN}), no NAbs were detected against DH12 grown in and tested on activated PBMCs. In contrast, in the study described here, protection was correlated with the presence of NAbs against homologous cell-line-adapted viruses (HIV-1_{MN} and HIV-1_{SF2}) and against homologous and heterologous primary isolates (HIV-1_{MN}, HIV-1_{SF2}, BZ167, and SF33) grown in PBMCs and tested in a variety of neutralization assays. As in previous studies, the level of NAbs associated with protection was dependent upon the virus strain and assay conditions used. Thus, for example, at the time of a low-dose challenge, serum from chimpanzee 1P displayed no detectable neutralizing activity against HIV-1_{SF2} in an assay in which the virus was grown in and tested on PBMCs stimulated with anti-CD3 and anti-CD28 (Table 2), but the same serum specimen from this protected animal displayed a neutralization titer of 1:360 against HIV_{MN} grown in and tested on H9 cells (Table 2) and a titer of >1:200 when tested against BZ167 in the resting cell assay (25). Thus, because the virus strain and assay conditions affect the quantitation of NAbs, it is not a simple matter to assign an absolute value for titers of NAbs which correlate with protection.

An effective vaccine will have to induce a strong immune response to a broad spectrum of HIV-1 strains. In previous studies, it was shown that the levels of Abs increase with repeated immunization and that, initially, Abs react most strongly with the immunizing strain but, upon reimmunization, the breadth of reactivity increases. Thus, Arthur et al. (2) showed that chimpanzees immunized three times with $rgp120_{LAI}$ developed increasing neutralizing responses to

HIV-1_{LAI}, and Berman et al. (4) showed that chimpanzees immunized with three or four injections of $rgp120_{MN}$ developed strong neutralizing titers for the homologous strain as well as cross-neutralizing Abs to HIV-1_{SF2} and weak but demonstrable neutralizing activity against HIV-1_{IIIB}.

The demonstration in our study of NAbs for homologous laboratory strains as well as for homologous and heterologous primary isolates extends the observation that appropriate HIV-1 immunogens can induce cross-neutralizing responses. Moreover, the finding that cross-neutralizing Abs for primary isolates BZ167 and SF33 appeared soon after boosting suggests that Abs to shared epitopes can readily be induced with a relatively short course of immunization. Both the breadth and the sustained nature of the response to the prime-boost regimen used here attest to the better quality of the immune response engendered with these reagents than with subunit rgp120 administered without previous priming of the immune system (3, 9).

The immunogens used in this study were derived from SI HIV-1 isolates which use the CXCR4 coreceptor (13, 22). The primary isolates selected for use in neutralization assays were purposefully chosen as CXCR4-utilizing isolates to maximize the immunologic and phenotypic matches between immunogens and test strains. Isolates HIV-1_{MN} and HIV-1_{SF2} were chosen because of their homology to the immunizing strains, and primary isolate BZ167 was examined because, in addition to being an SI virus, it is similar to the immunizing strains in a 630-bp region of gp120 (in the C2 and V3 to V5 regions) (1). Two dualtropic primary isolates were tested; of these, SF33, but not 92HT593, was neutralized. Thus, four of the five primary isolates tested were neutralized by sera from chimpanzees that mounted protective Ab responses. Moreover, positive results were obtained against these primary isolates in four different neutralization assay formats.

The data presented above suggest that the immunizing regimen induced NAbs to epitopes which are shared by several, but not all, primary viruses that utilize CXCR4, and the diversity of the primary isolates that were neutralized attests to the rather broad neutralizing activity induced by the immunizing regimen. Given the diversity of the V3 loops of the viruses that were neutralized (29, 45), it is improbable that all of the neutralizing activity was directed at this region of the envelope; experiments that are under way will clarify the specificity or specificities of the Abs. While HIV-1_{MN}, HIV-1_{SF2}, and SF33 were all isolated in the United States in the mid-1980s (23, 32), BZ167 was isolated in 1990 from a patient in Brazil (24). Similarly, while HIV-1_{MN} and SF33 were carried for several passages in PBMCs in vitro, the HIV-1_{SF2} and BZ167 strains used here were low-passage strains. Furthermore, the V3 loops of the four neutralized viruses were quite divergent from those of the immunizing strains (29, 45). What differentiates the virus which was not neutralized (92HT593) from the others which were neutralized is not clear at this point. (92HT593 was isolated in 1992 from a patient in Haiti, was used as a low-passage virus, and has a V3 loop which shows more similarity to those of the immunizing strains than do the V3 loops of the two heterologous primary isolates that were neutralized.)

Now that an immunizing regimen which induces NAbs for several primary isolates has been identified, the breadth of this immune response needs further study. The extent of clade B primary isolates that can be neutralized needs to be investigated further. Also to be determined is whether viruses from other clades can also be neutralized and whether any activity extends to non-SI viruses. Similarly, the benefit that would be derived from the use of multiple strains for priming and/or boosting needs to be elucidated.

Nonetheless, the detection of NAbs for four homologous and heterologous primary isolates is a first critical step in dispelling the concept that NAbs for primary isolates of HIV-1 cannot be induced by immunization. Since it is well established that polyclonal antiserum from HIV-1-infected subjects can neutralize primary isolates of HIV-1 (21, 30, 40) and that monoclonal Abs derived from the cells of HIV-1-infected subjects can neutralize primary isolates (6, 7, 21, 26), it is clear that the B-cell repertoire includes protective anti-HIV-1 NAbs. A priori there is no reason why proper immunization should not similarly induce protective NAbs for primary isolates. This study with chimpanzees and parallel studies with a different prime-boost regimen with humans (43) are the first to suggest that, in fact, immunization with various candidate HIV-1 vaccines can induce NAbs against at least a limited range of HIV-1 primary isolates.

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