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Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by immunization

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A combination AIDS vaccine approach consisting of priming with adenovirus-HIV-1_{MN} gp160 recombinants followed by boosting with HIV-1_{SF2} gp120 was evaluated in chimpanzees. Long-lasting protection, requiring only three immunizations, was achieved against a low-dose challenge with the SF2 strain of HIV-1 and a subsequent high-dose SF2 challenge administered 1 year later without an intervening boost. Notably, neutralizing antibody responses against both clinical and laboratory isolates developed in three chimpanzees and persisted until the time of high-dose challenge. The possibility that cytotoxic T-lymphocytes contribute to low-dose protection of a chimpanzee lacking neutralizing antibodies is suggested. Our results validate the live vector priming/subunit booster approach and should stimulate interest in assessing this combination vaccine approach in humans.

The chimpanzee HIV-1 model has been exploited with varying degrees of success in AIDS vaccine research[1-13]. In general, protection of chimpanzees against infection from a live HIV-1 challenge has required multiple immunizations and has been demonstrated when the viral challenge was administered soon after completion of immunization regimens and at the peak of induced immune responses. Also, the immune responses elicited by the various vaccine regimens tested have shown *in vitro* reactivity only against laboratory adapted viruses and not against clinically relevant primary HIV-1 isolates. Therefore, a vaccine approach using only a few immunizations to induce long-lasting immunity, including reactivity against clinical isolates, would represent a significant advance.

Because HIV-1 infection can result from either cell-free or cell-associated virus, an ideal vaccine should induce both humoral and cellular immunity. Thus, live, replicating recombinant vectors (vaccinia, canarypox, adenovirus) that can induce cellular immunity and prime humoral immune responses to expressed genes, together with HIV-1 subunit boosters, have been pursued[1,9-11]. Human adenoviruses (Ad) represent particularly attractive vaccine vehicles as Ad-HIV-1 recombinants should induce not only HIV-specific humoral and cellular immunity, but also mucosal immune responses because of their replication at mucosal sites in the upper respiratory tract and gut. Further, the existence of over 45 antigenically distinct Ad serotypes[14] suggests that multiple Ad could be developed as vectors if necessary, providing flexibility for sequential immunizations.

Previously, the immunogenicity of Ad-HIV-1_{LAI} gp160 recombinants followed by baculovirus-expressed HIV-1_{LAI} gp160 subunit was evaluated in chimpanzees[10,11]. Transient, elevated neutralizing antibody (NAb) levels were induced but not sustained, perhaps because the gp160 booster used was denatured. Subsequently, Ad-HIV-1_{MN} gp160 recombinants were prepared and evaluated together with a CHO-expressed HIV-1_{SF2} gp120 subunit possessing native conformation[15,16]. Both the MN and SF2 strains are antigenically more representative of the HIV-1 isolates occurring in North America than the LAI strain. The goals of this study were to investigate the number of Ad-HIV-1

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Table 1 Schedule for immunization of chimpanzees with Ad-gp160$_{MN}$ and gp120$_{SF2}$ subunit vaccines and subsequent challenge with HIV-1$_{SF2}$.

Chimpanzee	Pre-existing Ad-Antibody[a]	0w	12w	24w	38w	48w	52w	96w	98w
1P	<8,–,–	–	Ad5-gp160	–	gp120	gp120	HIV-low[d]	–	HIV-high[e]
2P$_A$	<8,32,–	–	Ad5-gp160	Ad7-gp160	–	gp120	HIV-low	–	HIV-high
2P$_B$	8,<8,–	–	Ad5-gp160	Ad7-gp160	–	gp120	HIV-low	–	HIV-high
3P	<8,<8,<8	Ad5-gp160	Ad7-gp160	Ad4-gp160	–	gp120	HIV-low	–	HIV-high
2P$_{POS}$	128,128,128	–	Ad5,7,4,-gp160[c]	Ad5,7,4-gp160	–	gp120	–	Ad5,7,4-gp160	HIV-high
3C	16,<8,<8	Ad5	Ad7	Ad4	–	MF59	HIV-low	–	HIV-high
C	nt[b]	–	–	–	–	–	–	–	HIV-high

[a]Chimpanzees were prescreened for the presence of Ad type-specific antibodies (Ad5, Ad7, Ad4) by a microneutralization assay.
[b]nt = not tested
[c]10^7 pfu of each of the 3 recombinants was administered.
[d]The low-dose HIV-1$_{SF2}$ challenge represents 1 ml of a 1:40 dilution of the challenge stock. This stock, consisting of the original HIV-1$_{SF2}$ isolate grown on human PBMC, was obtained from Dr. Alan Schultz, NIH, NIAID. Various estimates of the infectivity of this stock have been reported, however as recently estimated by *in vivo* titration, it contains at minimum, 200 chimpanzee infectivity doses per ml[17].
[e]The high-dose HIV-1$_{SF2}$ challenge represents 1 ml of a 1:5 dilution of the challenge stock.

recombinant administrations necessary to optimally prime the chimpanzees before subunit booster immunizations, to evaluate the influence of preexisting antibody to the Ad vectors on priming, to assess the protective efficacy of the combination vaccine regimen, and to determine whether vaccine protection is associated with a particular immune response.

Several significant findings have emerged from these experiments. Complete protection lasting more than 10 months in all four immunized chimpanzees against a low-dose HIV-1$_{SF2}$ challenge was observed. More importantly, three of the four chimpanzees were protected against a subsequent high-dose challenge administered nearly a year later without an intervening booster inoculation. Only three immunizations were necessary to elicit the protective immune responses. Notably, antibodies capable of

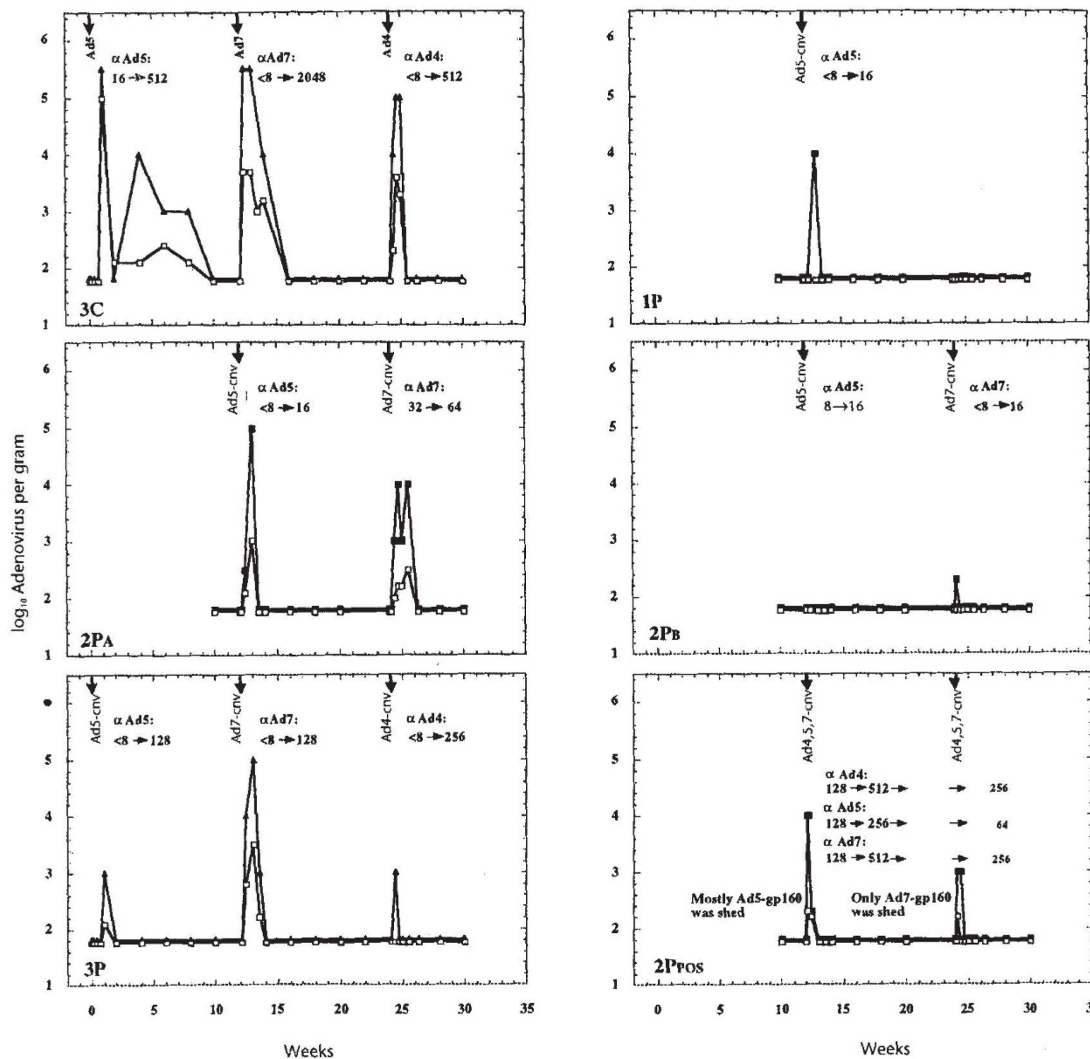

Fig. 1 Shedding of recombinant Ad in stools of individual chimpanzees and seroresponses to Ad vectors following immunization with Ad-HIV. Inverted arrows show the timing of intranasal inoculations with the indicated viruses (10^7 PFU/Ad-HIV). Anti-Ad pre-inoculation and peak post-inoculation titers, are shown.

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neutralizing both primary and laboratory-adapted HIV-1 isolates were induced and persisted over a year following the last immunization. Whereas earlier studies have concluded that NAb are necessary for vaccine protection against an intravenous challenge in the chimpanzee model, here protection against low-dose challenge was observed in a chimpanzee possessing HIV-1-specific cytotoxic T-lymphocyte (CTL) activity but lacking NAb, suggesting for the first time a possible role for CTLs in vaccine protection of chimpanzees. Protection against a high-dose challenge was observed in the chimpanzees that developed persistent NAb responses. Finally, HIV-specific immune responses were induced in chimpanzees possessing low-titered Ad antibodies, suggesting preexposure of humans to Ad may not preclude immunization with Ad-based recombinants. These observations suggest that the Ad-recombinant/subunit boosting approach merits further evaluation in humans, the natural host of Ad.

Immunization and induction of immune responses

The immunization and challenge protocol for six female chimpanzees, aged 7–21 years, and a naive female control, aged 31 years, is listed in Table 1. To investigate optimal immunization requirements, chimpanzees received one, two or three sequential primings (chimpanzees 1P, 2P or 3P, respectively) with Ad-HIV-1_{MN} recombinants. To assess the effect of preexisting immunity to Ad on priming, a chimpanzee ($2P_{POS}$) that had a high antibody level to each of the three Ad vectors was included in the study. Other animals possessed low or undetectable (8 or less) or moderate (16–32) levels of Ad antibodies (Table 1).

Intranasal administration of wild-type or recombinant Ad produced no respiratory or enteric disease. Ad replication in the upper respiratory tract (URT) was limited, but replication in the gut was extensive (Fig. 1), as previously reported[11]. Minimal or no infectious virus was detected in URT or stool samples of chimpanzees 1P, $2P_B$ and $2P_{POS}$, whereas Ad shedding was greater in samples of chimpanzees $2P_A$ and 3P. Wild-type Ad replicated longer and to a higher titer in chimpanzee 3C. Overall, Ad antibodies correlated with levels of Ad shedding. Moderate levels of Ad serum antibodies did not block Ad replication in chimpanzees $2P_A$ or 3C.

High-titered NAb to MN and SF2 laboratory isolates, associated with good replication of Ad recombinants, developed in chimpanzees $2P_A$ and 3P and in chimpanzee 1P after booster immunizations, but not in chimpanzees $2P_B$ and $2P_{POS}$ (Fig. 2, *a* and *b*; Table 2). Sporadic HIV-1- specific CTL activity was detected in peripheral blood lymphocytes (PBLs) of all immunized chimpanzees over the course of immunization. The highest levels were seen in chimpanzees $2P_B$ and $2P_{POS}$, which lacked NAb (Fig. 3*a*; Table 2), and the lowest were in chimpanzee 3P, which had the highest NAb titers. A lymph node was obtained from each chimpanzee 50 weeks after initial immunizations, and only lymph node cells from chimpanzee $2P_B$ exhibited >10% specific lysis of autologous B cells infected with a vaccinia HIV-1_{SF2} envelope construct or pulsed with HIV-1_{MN} envelope peptides (Fig. 3*b*). CTL activity in frozen PBL of chimpanzee $2P_B$ obtained 2 weeks before challenge was subsequently attributed to $CD8^+$ T-cells and was MHC-restricted (Fig. 3*c*).

Low-dose challenge

A low-dose challenge (1 ml of 1:40 dilution of HIV-1_{SF2} stock[17]) was administered intravenously 52 weeks after inoculation to control chimpanzee 3C and to the four immunized chimpanzees with either NAb or lymph node CTL activities (Table 2; Fig. 3*b*). Although chimpanzee $2P_{POS}$ exhibited CTL activity in PBLs (Fig. 3*a*), it was not challenged, as it failed to exhibit either lymph node CTL or NAb activities. Immune responses present at challenge are shown in Table 2. Chimpanzees 1P, $2P_A$ and 3P had NAb against HIV-1 laboratory isolates and an HIV-1_{MN} primary isolate passaged multiple times in PBLs. Furthermore, chimpanzees $2P_A$ and 3P had NAb activity against the HIV-1_{SF2} clinical isolate challenge stock. Notably, chimpanzees 1P, $2P_A$ and 3P had high-titered NAb against a heterologous HIV-1 clinical isolate (BZ167) as assessed on unstimulated PBLs[18]. CTL activity in PBLs was low at challenge in all animals.

Four weeks after challenge, control chimpanzee 3C revealed HIV-1 infection by virus isolation, DNA and RNA polymerase chain reaction (PCR) analyses, and seroconversion to HIV-1 p24 (Table 2). To date, all five control chimpanzees (one here and four others, ref. 19 and Murthy, K.K. *et al.* Presented at the Conference on Advances in AIDS Vaccine Development, Sixth Annual Meeting of the National Cooperative Vaccine Development Group for AIDS, October–November 1993, Alexandria, VA) have become infected within 4–8 weeks following intravenous inoculation with the same low-dose of HIV-1_{SF2}. In contrast, the four immunized chimpanzees were completely protected, showing no virological or serological evidence of HIV-1 infection over the 46-week period following challenge.

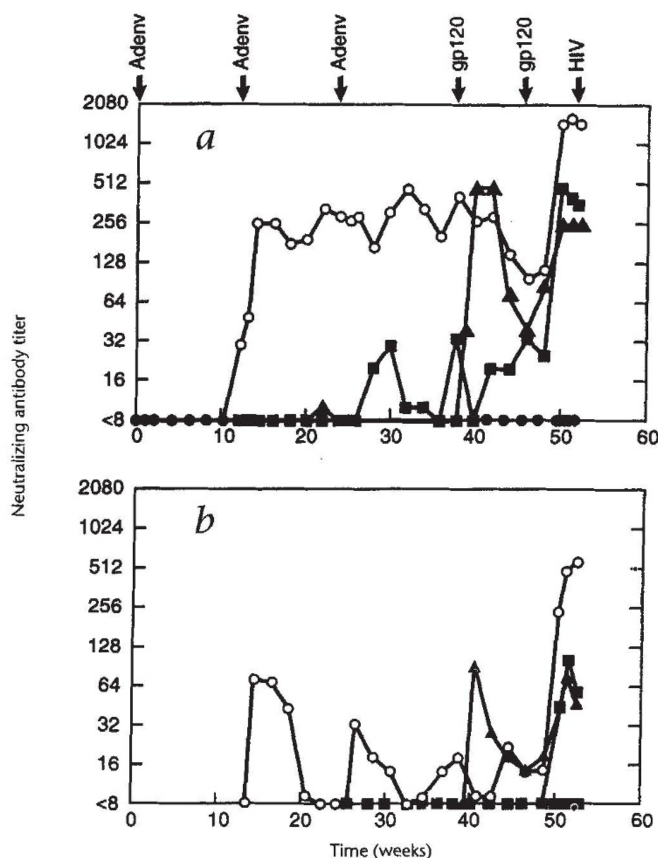

Fig. 2 Neutralizing antibody responses in immunized chimpanzees. NAb responses against cell-free HIV-1 laboratory isolates MN (*a*) and SF2 (*b*) are shown over the course of immunization. The chimpanzees are designated as follows: (solid triangle) 1P (solid square) $2P_A$ (open square) $2P_B$ (open circle) 3P (solid circle) $2P_{POS}$ (open triangle) 3C. Arrows represent the timing of all immunizations and the low-dose challenge (week 52). However, reference should be made to Table 1 for the immunization regimen of specific chimpanzees.

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High-dose challenge

To establish a measure of the duration of protective immunity and to evaluate protection against high-dose infection, 1 ml of a 1:5 dilution of the HIV-1_{SF2} challenge stock was administered intravenously 46 weeks after low-dose challenge without prior immunization to the four protected animals and chimpanzee 3C (Table 1). Chimpanzee $2P_{POS}$ was also given a high-dose challenge, but because it had never demonstrated NAb or CTL activity of lymph node cells, it received a third Ad-gp160 administration 2 weeks before challenge (Table 1). This did not elicit NAb, but successfully boosted PBL CTL activity. At the time of the boost, PBLs of chimpanzee $2P_{POS}$ exhibited no CTL activity (Fig. 4*a*). One week later, specific lysis of autologous B cells infected with a vaccinia HIV-1_{MN} envelope construct reached 17%. At high-dose challenge, this activity had declined to <10%. Two other chimpanzees, 1P and 3P, were not boosted, but retained CTL activity in PBLs of over 10% specific cell lysis at the time of high-dose challenge (Fig. 4*b*; Table 2). Further lymph node biopsies were not performed on any of the chimpanzees. Low-titer NAb to HIV-1 laboratory isolates and to clinical isolate BZ167 persisted in sera of chimpanzees 1P, $2P_A$ and 3P without a booster immunization, and were readily detected at high-dose challenge (Table 2).

Table 2 **(cont. on p. 655)** Immune status of chimpanzees at times of HIV-1SF2 low- and high-dose challenges; virological and serological evidence of infection

	NAb Titer at Challenge					
	Lab Isolates			Clinical Isolates		
	MN	SF2	SF2	MN	SF2	BZ167
Low-dose Challenge						
1P	250	50	148	40	<50	>200
$2P_A$	355	60	455	20	516	>200
$2P_B$	<10	<10	<10	<10	<50	<20
3P	1450	635	515	390	336	>200
$2P_{POS}$	<10	<10	nt	<10	<50	<20
3C	<10	<10	<10	<10	nt	nt
High-dose Challenge						
1P	35	35	nt	nt	nt	>20
$2P_A$	40	<10	nt	nt	nt	>20
$2P_B$	<10	<10	nt	nt	nt	<20
3P	105	35	nt	nt	nt	>20
$2P_{POS}$	<10	<10	nt	nt	nt	<20
C	<10	<10	nt	nt	nt	nt
3C	<10	<10	nt	nt	nt	nt

[a]Peak CTL responses in PBLs observed with any MN- or SF2-specific target cell before and at the time of low- and high-dose challenges are summarized from Figs. 3*a* and 4*b* and expressed as % cell lysis. CTL activity, also expressed as % cell lysis, in lymph node cells taken 2 weeks prior to low-dose challenge is summarized from Figure 3*b*.

Four weeks after high-dose challenge, naive control chimpanzee C revealed infection by HIV-1 isolation and RNA PCR of plasma (Table 2). Seroconversion to p24 occurred at 6 weeks. Previously protected chimpanzee $2P_B$ exhibited reduced HIV infection relative to control chimpanzee C, as demonstrated by more transient virus isolation, no early detection of HIV-1 RNA in plasma after challenge (compared with 4–8 weeks for chimpanzee C), and delayed seroconversion to p24 (Table 2). The previously infected control chimpanzee (3C) apparently resisted significant reinfection — virus was not isolated until 12 weeks after high-dose challenge, and viral RNA was not detected in plasma immediately after challenge. Thus, a low-dose infection seemed to protect against subsequent homologous high-dose challenge. Chimpanzee $2P_{POS}$, which replicated Ad-recombinants poorly (Fig. 1), became infected at a rate similar to that of control chimpanzee C.

In contrast, chimpanzees 1P, $2P_A$ and 3P remained protected. These chimpanzees exhibited no plasma viremia shortly after challenge. A low level of plasma RNA was seen in chimpanzees $2P_A$ and 3P at 41 weeks after high-dose challenge, and proviral

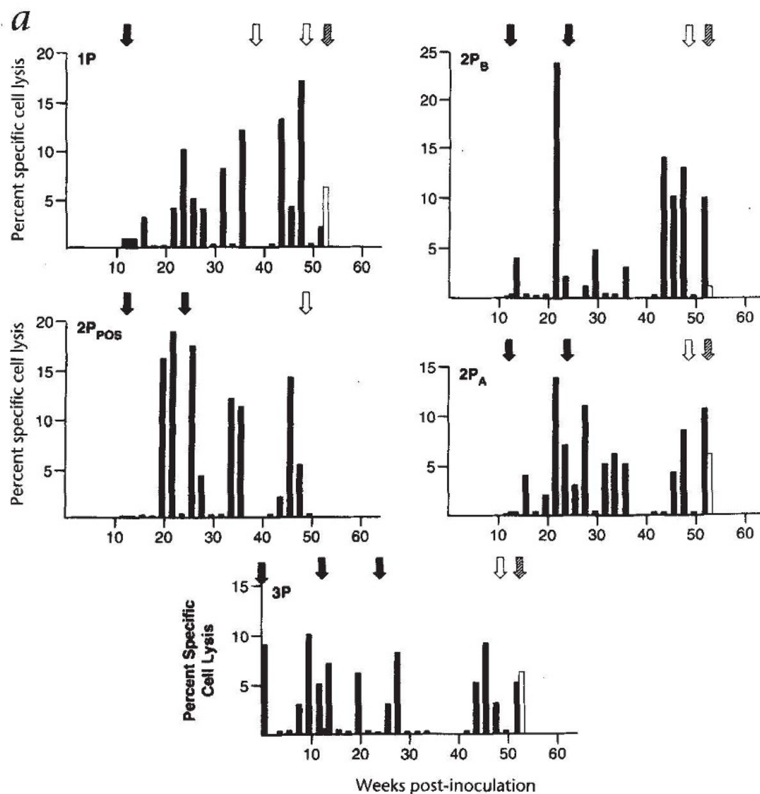

Fig. 3 **(cont. on p. 655)** Cytotoxic T lymphocyte activity in immunized chimpanzees. *a*, CTL activity in PBL of immunized chimpanzees. The peak percent cell lysis obtained with any of the HIV-1-specific target cells at a given time point is plotted for each chimpanzee. Percent cell lysis was calculated as described in Methods, and percent specific cell lysis was obtained by subtracting percent cell lysis obtained with control target cells (vsc-8-infected or pulsed with scrambled peptide). In addition, percent specific cell lysis obtained with effector cells of the control 3C chimpanzee and the appropriate HIV-1-specific target cell (ranging from 0 to 14%, mean of 2%) was also subtracted from plotted values for simplification of data presentation. The data are reported for an E:T ratio of 100:1. Solid bars reflect activity with HIV-1_{MN}-specific target cells and open bars with HIV-1_{SF2} specific target cells. The solid arrows represent immunizations with Ad- gp160 recombinants, the open arrows represent immunizations with gp120, and the hatched arrow represents the low-dose SF2 challenge.

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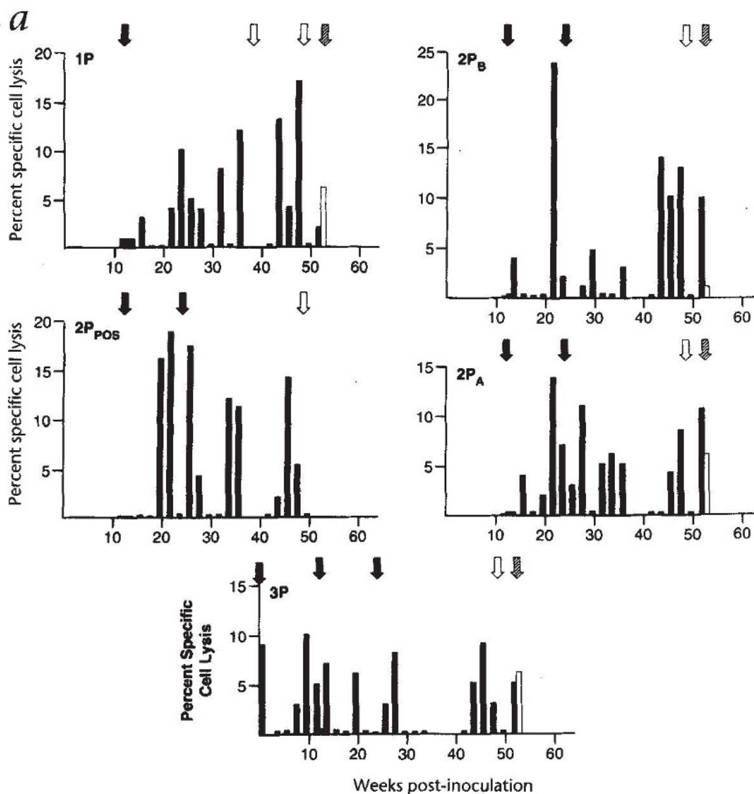

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CTL response[a]					HIV Infectivity parameter[b]			
Peak in PBL before challenge	PBL at challenge		Lymph node prior to challenge		Weeks post-challenge [Peak copies/ml]			
MN	MN	SF2	MN	SF2	Virus isolation	DNA PCR	RNA PCR	Serology
17	2	6	1	5	neg	neg	neg	neg
14	11	6	8	3	neg	neg	neg	neg
21	10	1	10	20	neg	neg	neg	neg
10	5	6	0	0	neg	neg	neg	neg
19	NA	NA	2	0	NA	NA	NA	NA
0	0	0	0	0	4–46	4–44	4–6[14800]	8–46
nt	6	14	nt	nt	neg	neg	neg	neg
nt	0	0	nt	nt	neg	45	41 [100]	neg
nt	0	0	nt	nt	4–6	45	33 [500]	10
nt	1	12	nt	nt	neg	neg	41 [100]	neg
nt	3	7	nt	nt	4–15	29	2–4 [6200]	6
nt	nt	nt	nt	nt	4–17	neg	4–8,25 [6200]	6
nt	0	0	nt	nt	12–15	37,49	33,49 [100]	NA

[b]The time to first detection of infection and the duration of a detectable signal is indicated. The peak number of RNA copies in plasma is indicated. Plasma samples on weeks 41 and 45 were shown to be contaminated with HIV-1. RNA PCR data at these time points reflect results obtained from serum samples obtained at these same times. A positive serologic response indicates the presence of antibodies to gag proteins. nt = not tested. NA = not applicable.

DNA was detected in PBMCs of chimpanzee $2P_A$ at 45 weeks after challenge (Table 2), indicating that although an initial HIV infection apparently occurred in these two chimpanzees, further HIV replication was highly suppressed. Notably, all three chimpanzees have been persistently virus isolation negative, and they have also remained seronegative for Gag antibodies out to 64 weeks after high-dose challenge.

Immune responses to human cellular antigens have been associated with protection against SIV infection in rhesus macaques[20]. Both HLA-DR and HLA class I antigens are physically associated with HIV-1, HIV-2 and SIV particles[21], and immunization of macaques with purified human HLA-DR has conferred protection against subsequent challenge with SIV propagated in human cells[22]. Because the $HIV-1_{SF2}$ challenge stock was propagated in human PBMCs, immune responses to human cellular antigens elicited by the low-dose challenge may have contributed to the subsequent protection from high-dose challenge. We examined serum sample from the challenged chimpanzees for antibodies to human HLA-DR1, HLA-DR4 and β2- microglobulin (see the Methods section). Sera obtained at the time of challenge and 2 and 4 weeks after the low- and high-dose challenges were all negative for antibodies to the three antigens (data not shown). Therefore, human class I and class II antigens present in the $HIV-1_{SF2}$ challenge stock were poorly immunogenic in the chimpanzee, either because of insufficient antigen present in the viral inocula together with the absence of an adjuvant, or because

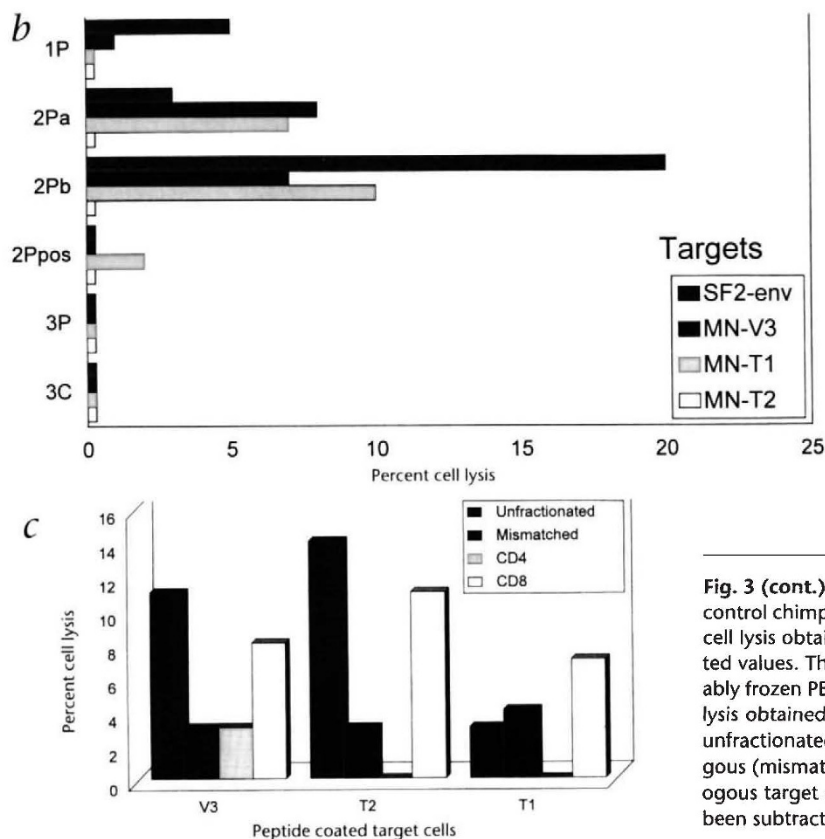

Fig. 3 (cont.) *b*, CTL activity in fresh lymph node cells of vaccinated and control chimpanzees obtained 2 weeks before low-dose challenge. Percent cell lysis obtained with control target cells has been subtracted from plotted values. The E:T was 100:1. *c*, MHC-restricted $CD8^+$ CTL activity in viably frozen PBLs of chimpanzee $2P_B$, 2 weeks before challenge. Percent cell lysis obtained with three different target antigens is plotted for killing by unfractionated effector cells of autologous (unfractionated) or nonautologous (mismatched) target cells, or by $CD4^+$ or $CD8^+$ effector cells of autologous target cells. Percent cell lysis obtained with control target cells has been subtracted. The E:T was 100:1.

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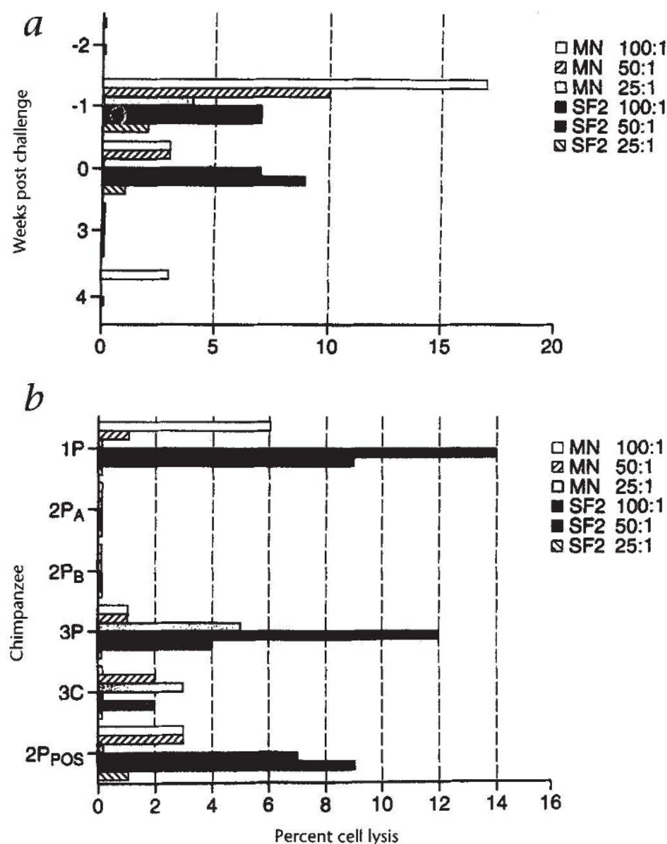

Fig. 4 Cytotoxic T lymphocyte activity in chimpanzees at time of high-dose challenge. *a*, CTL activity in PBL of chimpanzee $2P_{POS}$ at time of third boost and high-dose challenge. As shown in Table 1, a mixture of Ad-gp160 recombinants was administered at 96 weeks (indicated as –2 weeks here) and high-dose challenge at 98 weeks (0 weeks here). Percent cell lysis of autologous B cell targets infected with the vaccinia recombinant vMN462 expressing MN gp160, or with the vaccinia-SF2 envelope recombinant was determined. Percent cell lysis with the control vsc-8-infected target cells has been subtracted from plotted values. The ratio of effector cells used with either MN- or SF2-specific target cells is indicated. *b*, CTL activity in chimpanzee PBLs at the time of high-dose challenge. Percent cell lysis was assessed as in *a*.

of the homology between chimpanzee and human antigens. Thus, immunity to the major cellular antigens present on viral particles did not appear to contribute to the observed protection against high-dose challenge.

Discussion

This first demonstration in chimpanzees of protection resulting from an Ad-HIV-1/gp120 combination vaccine regimen provides several significant advances. First, persistent NAb were induced following only three immunizations: one Ad-HIV-1 recombinant immunization and two gp120 boosts, or two Ad-HIV-1 recombinant immunizations and one gp120 boost. However, multiple administrations of Ad-HIV-1 recombinants were generally not effective at priming HIV immune responses unless significant replication occurred, as shown by viral shedding. Previous work showing that induced antibody responses to gp160 have a longer half-life in humans than in chimpanzees[23] imply even more persistent responses might follow vaccination of humans. It is noteworthy that Ad-HIV-1 recombinant replication in the presence of moderate, but not high, levels of Ad antibody was observed, suggesting that preexisting immunity to Ad may not accurately predict the ability of Ad recombinants to replicate. Making firm conclusions regarding the influence of preexisting Ad antibody on virus replication will require further study. However, this observation suggests prior exposure and seroconversion to an Ad vector would not necessarily interfere with the ability of an Ad-HIV-1 recombinant to prime an HIV-1-specific immune response.

Second, high-titered NAb to clinical isolates (Table 2; S.Z.-P. *et al.*, manuscript in preparation) were elicited. These antibody reactivities were demonstrated against homologous MN and SF2 isolates never cultured on T-cell lines using activated PBLs as targets for infection, as well as against the heterologous BZ167 primary isolate, using unstimulated PBL targets. Such antibody activities have not previously been demonstrated in other approaches, but likely are crucial for further vaccine development.

Third, the resistance of chimpanzee $2P_B$ (lacking NAb but having CTL activity in PBLs and lymph node) to a low-dose challenge suggests a possible role for cell-mediated immunity in vaccine protection against intravenous challenge in the chimpanzee model. The apparent partial resistance of this chimpanzee to high-dose challenge is also noteworthy. Previously, CTL activity detected in cloned but not unfractionated effector cells of immunized chimpanzees[24] failed to confer protection against HIV challenge[1]. Although protection against high-dose challenge occurred in chimpanzees with NAbs, consistent with results of earlier studies showing the importance of NAbs (including V3-loop antibodies) in vaccine protection[4,5,8,9,25,26], our findings suggest that many mechanisms may mediate protective immunity. With regard to CTLs, Gallimore *et al.* have reported that CTLs may be involved in vaccine protection of rhesus macaques, showing that Nef-specific CTL precursor frequency was inversely correlated with virus load following intravenous challenge with a high dose of SIV (ref. 27). Although studies in both macaque and chimpanzee models therefore imply that CTLs contribute to protection from viral challenge, definitive proof must await results of adoptive transfer experiments. Other, as yet unidentified factors may also play a role.

Fourth, and most importantly, long-lasting protection extending nearly one year (50 weeks) beyond the last immunization was achieved against high-dose challenge. This represents a significantly longer period of protection than any demonstrated previously in studies using cell-free HIV challenge. The absence of antibodies in chimpanzee sera to human MHC class I and class II antigens following low-dose challenge suggests that immunity to human cellular antigens present on the challenge virus was not a major factor associated with the observed protection against the high-dose challenge.

Safety concerns will be paramount in moving Ad-HIV-1 recombinants into human trials. As illustrated in Fig. 1, the wild-type Ad vectors replicated to higher titer and were shed for longer periods of time than the Ad-HIV-1 recombinants, suggesting the recombinants may be somewhat attenuated *in vivo* with regard to replication. It is also noteworthy that intranasal administration of Ad-HIV-1 recombinants did not cause any upper respiratory tract disease in the immunized chimpanzees nor did it establish latent infection of chimpanzee PBLs (data not shown). Finally, while intranasal administration was necessary for good replication of the Ad-HIV-1 recombinants in chimpanzees, oral administration, which bypasses the lungs, may prove satisfactory in

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humans — in fact, oral administration of Ad vaccines has been carried out safely and efficaciously for years by the military[28].

Overall, our results further validate the live vector priming/subunit booster approach and should stimulate interest in assessing this combination vaccine in humans, the natural host of Ad. Whereas significant advances have been achieved using this HIV envelope-based vaccine regimen, inclusion of other HIV-1 antigens (Gag and Nef, for example) in a recombinant vectored vaccine would be expected to further enhance vaccine efficacy.

Methods

Immunization and challenge of chimpanzees. Chimpanzees were primed intranasally at 12-week intervals once (chimpanzee 1P), twice (chimpanzees $2P_A$, $2P_B$ and $2P_{POS}$), or three times (chimpanzee 3P) with 10^7 plaque-forming units (PFU) Ad-HIV-1_{MN} gp160 recombinants based in Ad5, Ad7 or Ad4 vectors (Table 1), prepared as described previously[29]. A control chimpanzee (3C) received three immunizations with a similar dose of the wild-type vectors. The immunization schedule was staggered to allow for simultaneous challenge. Twenty-four weeks (26 weeks for 1P) following the last priming immunization the chimpanzees were inoculated intramuscularly with 50 μg of HIV-1_{SF2} gp120 expressed in CHO cells, formulated in MF59 adjuvant. Chimpanzee 1P received a second gp120 booster at 48 weeks together with the other animals. Chimpanzee 3C received MF59 adjuvant alone. Chimpanzees were challenged with low- or high-dose HIV-1_{SF2} at 52 and 98 weeks after initial immunization as indicated. Chimpanzee $2P_{POS}$ received only the high-dose challenge following an additional intranasal immunization at 96 weeks with a mixture of Ad HIV-1_{MN} recombinants. Naive chimpanzee (C) served as control for the high-dose challenge.

Assessment of Ad replication. Shedding of recombinant Ad in stools was measured by plaque assay and by an Ad type-specific PCR (ref. 30). For recovery of infectious Ad, stool samples were collected and stored as 10% suspensions in DMEM containing gentamycin (200 μg/ml) and amphotericin (10 μg/ml). The suspensions, clarified by centrifugation at 5000*g*, were titrated for Ad infectivity on confluent A549 cell monolayers under 0.5% agar overlays[10]. Plaques were viewed under neutral red staining and enumerated, and the viral titers were expressed as PFU per gram of stool. Identification of recombinant Ad was accomplished by plaque hybridization using radioactive DNA oligoprobes to the HIV *env* gene as described[10]. For nested PCR analysis, 10 μl of the clarified stool supernatant was evaluated by a GeneAmp PCR reagent kit (Perkin Elmer, Norwalk, CT), using primers selected from the regions of least homology among the Ad4, 5 and 7 fiber genes. The assays, shown to be Ad serotype-specific, were sensitive to 100 DNA molecules per milliliter (corresponding to 1–10 PFU/ml). A negative and positive control (containing 1–100 Ad DNA copies) were run concurrently with the test samples. Serum antibody levels to Ad vectors were determined by a type-specific microneutralization assay[31].

HIV-1-specific immune responses. Neutralizing antibody responses against HIV-1 laboratory isolates MN and SF2 (assay 1) were assessed as previously described[32] on chimpanzee serum samples obtained periodically. H9 and CEMX174 cells were targets for infection with frozen titered stocks of HIV-1_{MN} and HIV-1_{SF2}, respectively. End-point titers are defined as the reciprocal of the serum dilution at which infectivity levels were 60% of control values after normalization to control infectivity levels. Neutralization of SF2 (assay 2)[19] used a frozen, titered stock of HIV-1_{SF2} and HUT78 cells as targets of infection. A 50% neutralization titer is reported.

Neutralizing antibodies against the MN clinical strain, representing the original HIV-1_{MN} isolate passaged only in PBLs, were assessed as follows. Cell-free viral supernatant (20 μl) containing twenty 50% tissue culture infectious doses was incubated at 37 °C for 1 h with an equal volume of threefold serially diluted and heat-inactivated serum (beginning at 1:10). Fresh human PBLs (10 μl, 10^7/ml), stimulated for 48 h with phytohemagglutinin (PHA), and 50 μl of medium were added and incubated overnight at 37 °C. After 3 washes with RPMI 1640, the cells were suspended in 40 μl of medium. Cells (20 μl) were transferred into 200 μl of complete medium containing IL-2 (2.5 ng/ml). Virus infectivity was assessed by p24 expression 5 days later using an HIV-1 p24 antigen capture kit (NCI-FCRDC, Frederick, MD). NAb titers were expressed as above for the MN laboratory isolate. The SF2 clinical isolate, also passaged only in PBLs, was assayed as previously described[33]. A 50% end-point titer is given. Neutralization of the primary isolate BZ167, grown in activated PBLs and used at low passage, was assessed on unstimulated PBLs as described[17]. Fifty percent end-point titers are given.

HIV-1-specific CTL responses were assessed on PBLs periodically over the course of immunization. Effector cells obtained from PBLs or single-cell suspensions of lymph nodes were cultured for 5 days as previously described[34] in the presence of HIV-1_{MN} envelope (2 μg/ml) and/or a mixture of synthetic peptides (1 μg/ml each), known to be CTL epitopes[35] and representing the MN amino acid sequence, including V3 (the V3 loop "p18" peptide: RIHIGPGRAFYTTKN[36]), T1 (KQIINMWQEVGKAMYA[37]), and T2 (HEDIISLWDQSLK[37]), plus IL-2 (10 U/ml). Alternatively, effector cells were cultured for 5 days in the presence of concanavalin A (conA, 5 μg/ml) and IL-2. Target cells, prepared as previously described[34], were autologous chimpanzee B lymphocytes transformed with EBV and infected with a vaccinia-SF2 envelope recombinant[38]; a vaccinia HIV-1_{MN} gp160 recombinant, vMN462 (ref. 39); or vsc-8 (ref. 40), a vaccinia β-galactosidase recombinant as control. Alternatively, target cells were pulsed with the V3, T1 or T2 peptides or a scrambled "p18" peptide as control. Following a 4- to 6-hour chromium release assay using effector:target cell ratios (E:T) of 100:1, 50:1 and 25:1 (ref. 34), percent cell lysis was expressed as [(experimental c.p.m. minus spontaneous c.p.m.)/(total c.p.m. minus spontaneous c.p.m.)] × 100. Results were discarded if spontaneous release was more than 30%. Percent cell lysis obtained with control target cells (vsc-8-infected or coated with scrambled peptide) was subtracted from reported values. The data, unless otherwise indicated, are reported for E:T ratios of 100:1. CTL effector cells were characterized by culturing viably frozen PBLs for 5 days with conA and IL-2 as described above. Portions were depleted of either $CD4^+$ or $CD8^+$ cells using Leu3a or Leu2a monoclonal antibodies and Dynabeads M-450 (Dynal, Lake Success, NY) coated with goat anti-mouse IgG, and the various cell populations were subjected to chromium release assay.

Virologic assessment of infection by low- and high-dose HIV-1_{SF2} challenge. Peripheral blood, serum, and plasma samples were monitored periodically following challenge. Virus isolation, carried out by two laboratories, is scored positive if virus was detected by either laboratory. PBMCs were depleted of $CD8^+$ cells using Dynabeads M-450 anti-CD8, and cocultured with PHA-stimulated, polybrene-treated PBLs from seronegative humans. Culture supernatants were monitored for virus expression by p24 antigen capture assay or by measurement of reverse transcriptase activity. A positive result required a positive signal on two successive assays.

HIV proviral DNA in PBLs was assessed by PCR using conserved *gag* primer pairs (Biotech Research Laboratories, Rockville, Maryland). The sensitivity of the assay was 2–4 proviral DNA copies. A sample was scored positive if duplicate PCRs were positive. For indeterminate samples, DNA was extracted from another aliquot of cells, and the PCR was again performed in duplicate. Results were reported positive or negative depending on the outcome of the majority of the PCRs. RNA in plasma was assessed by two different laboratories by the NASBA technique[41] or by quantitative RT-PCR (ref. 19). A sample was scored positive if either assay was positive.

Serologic responses were assessed using HIV western blot kits (Cambridge Biotech, Worcester, MA).

Immune response to human cellular antigens. Antibodies to human HLA-DR1, HLA-DR4 and β2-microglobulin were assessed by radioimmunoassay[22]. Serial threefold dilutions, beginning at 1:20, of sera obtained before and after HIV-1_{SF2} challenges were assayed.

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