

# Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations

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**Eight different protocols were compared for their ability to raise protection against immunodeficiency virus challenges in rhesus macaques. The most promising containment of challenge infections was achieved by intradermal DNA priming followed by recombinant fowl pox virus booster immunizations. This containment did not require neutralizing antibody and was active for a series of challenges ending with a highly virulent virus with a primary isolate envelope heterogeneous to the immunizing strain.**

With the prospect of HIV, first recognized in 1984, infecting 1% of the world's population by the year 2000, the need for the development of a vaccine for AIDS is urgent<sup>1</sup>. Challenges for an effective vaccine include preventing infection by a virus that is not easily blocked by neutralizing antibody<sup>2,3</sup>, containing a virus that preferentially kills the T-helper cells that support the amplification and differentiation of anti-viral immune responses<sup>4,5</sup>, and preventing the establishment of the reservoir of latent proviral DNA that can serve as a source of re-emergent virus<sup>6</sup>. These are difficult goals for a vaccine to achieve.

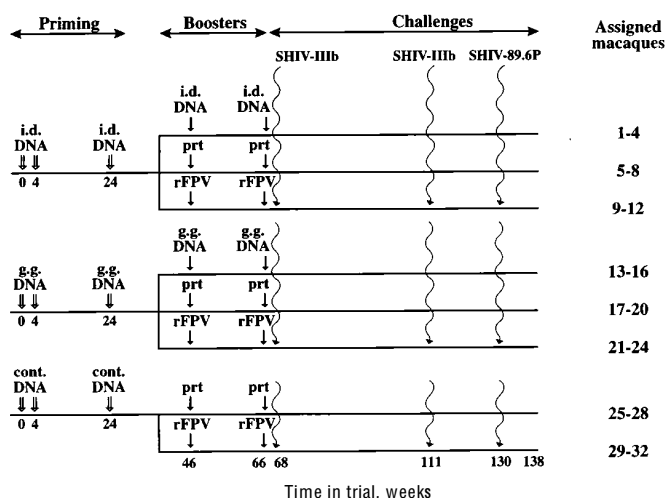
Among the AIDS vaccines that have been tested in macaque models, only live attenuated viruses with potential risks for disease have contained highly pathogenic challenges<sup>7-9</sup>. Three safer approaches for vaccination, including purified proteins, recombinant viral vectors and DNA vaccines, have held some promise in less-virulent challenge models. Purified envelope glycoproteins (Envs) have generated sterilizing immunity that correlated with the presence of neutralizing antibody. However the efficacy of this neutralizing activity has been limited by its poor persistence and its specificity for the immunizing Env, but not a spectrum of patient Envs (refs. 10-12). Live vectored vaccines, such as recombinant pox viruses expressing Env or capsid proteins (Gag) have raised both antibody and cell-mediated immune responses<sup>13-15</sup>. For live vectors, the expression of both Env and Gag has raised better containment than the expression of only Env or

Gag<sup>16</sup>. The third approach, DNA vaccines, also raises both humoral and cell-mediated immune responses<sup>17,18</sup>. DNA vaccines have contained a highly attenuated HIV-1 infection in chimpanzees (HIV-1-SF2)(ref. 19), and achieved sterilizing immunity when the DNA vaccine was followed by a protein booster and challenge was with a virus with an Env homologous to the booster Env protein<sup>20</sup>.

We undertook this study to gain experience with the use of DNA for an immunodeficiency virus vaccine and to evaluate the ability of DNA, or DNA followed by protein or recombinant pox virus booster immunizations, to raise protection. Would vaccines that were providing some success when used individually have synergy when used together? Our results show that intradermal DNA priming followed by recombinant pox virus boosters can contain challenge infections below the level of detection of our PCR assays for viral RNA. This apparently cell-mediated containment did not require detectable levels of neutralizing antibody.

## Vaccine trial and immunogens

The vaccine trial had eight groups with four rhesus macaques in each (Fig 1). Two methods of DNA delivery were evaluated: intradermal injections of DNA in saline, and gene gun bombardments of DNA-coated gold beads. A popular method of DNA delivery, intramuscular inoculation, was not used, because of overlap with other NIH sponsored trials. Groups 1-3 tested intra-

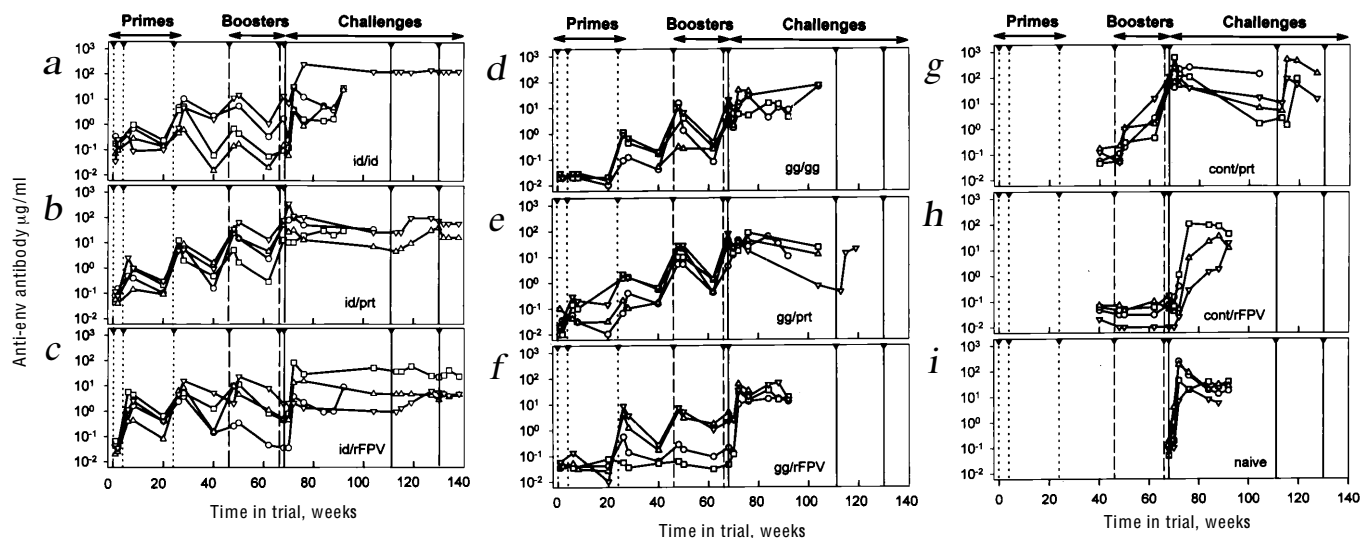


**Fig. 1** Vaccine trial. Downward arrows, timing for priming and booster immunizations and challenge infections for the eight sections of the trial. Far right, monkeys assigned to each group. i.d., intradermal; g.g., gene gun; DNA, five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox viruses; cont. DNA, vaccine vector without an insert.

dermal priming with DNA followed by booster immunizations with intradermal DNA inoculations, purified Env protein or recombinant fowl pox virus (rFPV). Groups 4–6 evaluated priming with DNA delivered by gene gun followed by booster immunizations with DNA delivered by gene gun, Env protein or rFPV. Groups 7 and 8 tested priming with control DNA (plasmid vector without a vaccine insert) followed by boosting with Env protein or rFPV. The DNA 'primers' were administered at 0, 4 and 26 weeks and the booster immunizations, at 46 and 66 weeks. Intravenous challenges were with chimeras between simian and human immunodeficiency viruses (SHIVs) that allow infection of rhesus macaques with viruses with HIV-1 Envs. The first two

challenges were with SHIV-IIIb, the virus from which the immunogens had been derived. SHIV-IIIb is a nonpathogenic virus that grows to lower titers in macaques than typical primary isolates achieve in humans<sup>21,22</sup>. The third challenge was with SHIV-89.6P, a very virulent virus with the envelope glycoprotein of a primary isolate<sup>23</sup>. In rhesus macaques, SHIV-89.6P causes the depletion of CD4<sup>+</sup> T cells within 2 weeks of infection and death from opportunistic infections within 6 months.

The DNA and rFPV immunogens were designed to express the *gag*, *pol*, *env* and *nef* genes of SHIV-IIIb. Five DNA constructs were used for each DNA inoculation: The first vaccine DNA, pRS102, encoded the Gag and polymerase proteins of SIVmac239; the second, pCMV/nef, encoded the Nef protein of SIVmac239; the third, pJW4303/HXB-2d pol, was designed to express non-infectious HIV-1-IIIb virus-like-particles; the fourth, pJW4303/HXB-2.gp140, encoded a secreted oligomeric form of the HIV-1-IIIb Env that is unstable and sheds gp120; and the fifth, pJW4303/HXB-2.gp120, encoded a secreted monomeric form of the receptor binding subunit of the HIV-1-IIIb Env (gp120). A combination of Env-expressing plasmids was used because a similar combination had proved effective at raising neutralizing antibody in another DNA vaccine trial<sup>24</sup>. For intradermal immunizations, 500 µg of each DNA were administered for a total of 2.5 mg of DNA per inoculation. For the gene gun inoculations, 4 µg of each DNA was delivered for a total of 20 µg of DNA per inoculation. Less DNA is required to raise immune responses by gene gun administration than by saline injections of DNA because of the relatively efficient transfection that results from the direct delivery of DNA into cells by DNA-coated gold bullets<sup>25,26</sup>. The protein boost consisted of 100 µg of the full-length, unmutated Env of HIV-1-IIIb (ref. 27) in incomplete Freund's adjuvant administered intramuscularly. The IIIb Env had an apparent molecular weight of 160 kDa with gp120 and gp41 covalently attached. Recombinant fowl pox was chosen for the pox virus boost because fowl pox infects but does not replicate in macaques, posing no risk of unwanted dissemination of the vector in the vaccinated monkey. Three SHIV-IIIb-expressing rFPVs



**Fig. 2** Temporal antibody responses during the immunization and challenge phases of the vaccine trial. Titers of IgG specific for Env were determined by ELISA. Each panel presents data for one group of the trial. Lower right corners, priming immunizations/booster immunizations: i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant;

rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert. Symbols indicate order in group: ○, first monkey in each group (macaques 1, 5, 9, 13, 17, 21, 25, 29, 33, 37 and 39); □, second (2, 6, 10, 14, 18, 22, 26, 30, 34, 38 and 40); △, third (7, 11, 15, 19, 23, 27, 31, 35 and 41); ▽, fourth (4, 8, 12, 16, 20, 24, 28, 32 and 36).

Table 1 Specific CTL activity generated in different groups of the trial

Trial group, macaque number		Time in trial							
		6 weeks, 2 weeks after second prime	20 weeks, 4 weeks before third prime	26 weeks, 2 weeks after third prime	40 weeks, 6 weeks before first boost	48 weeks, 2 weeks after first boost	62 weeks, 4 weeks before second boost	68 weeks, 2 weeks after second boost,	111 weeks, 43 weeks after day of first challenge
i.d./i.d.	1	-/- <sup>a</sup>	-/-	-/-	-/-	1/-		-/-	
	2	-/-	-/-	-/-	-/-	-/-		-/-	
	3	-/-	-/-	-/-	-/-	1/-/2		-/-	
	4	-/-	-/-	-/-	-/-	-/-		-/-	1/1-
i.d./prt	5	-/-	-/-	-/-	-/-	-/-		-/-	1/1-
	6	-/-	-/-	-/2/2	-/-	-/-		-/-	
	7	-/-	-/-	1/-	2/1-	-/-		-/-	
	8	-/-	-/-	-/-	-/-	-/-		-/-	-/-
i.d./rFPV	9	-/-	-/-	-/-	-/-	-/-		1/-/1	
	10	2/-	-/-	-/-	2/1/2	-/1		-/-	
	11	-/-	-/-	-/-	-/-	1/-		1/-	2/2-
	12	-/-	-/-	1/-	2/2/2	1/2/3		1/-	2/-
g.g./g.g.	13	1/-	-/-	-/-	1/-	-/-	-/-	-/-	4/3/1
	14	1/-	-/-	-/-	1/-	1/1-	-/-	-/-	
	15	-/1	-/-	-/-	-/-	1/1/1	-/-	-/-	
	16	-/-	-/-	-/1-	-/-	1/-	1/-	-/-	2/-
g.g./prt	17	-/-	2/-	-/-	1/-/1	1/-/2	-/1	-/-	
	18	-/-	-/-	-/-	-/-	1/-	-/-	-/-	2/1-
	19	-/-	-/-	-/-	-/-	-/1-	-/1	-/-	1/-/1
	20	-/-	-/-	-/-	-/-	-/1-	-/-	-/-	-/-
g.g./rFPV	21	2/-	2/-	1/-	-/-	1/-	-/2	-/-	
	22	2/5-	-/-	-/-	-/-	2/1/1	1/-	-/-	
	23	2/-	2/-	-/-	-/-	3/4-	-/-	-/-	3/1-
	24	-/2	-/2	-/-	1/-	1/-	2/-	1/-	
cont/prt	25			-/- <sup>b</sup>			-/-	-/-	-/1-
	26			-/-			-/-	-/-	-/-
	27			-/-			-/-	-/-	-/-
	28			-/-			-/-	-/-	-/-
cont/rFPV	29			-/- <sup>b</sup>			-/-	-/-	
	30			-/-			-/-	-/-	
	31			-/-			-/-	-/-	
	32			-/-			-/-	-/-	

CTL responses were measured after 2 weeks of re-stimulation. Herpes papio-immortalized B cell lines and recombinant vaccinia viruses expressing Gag, Env or Nef were used for restimulation and for targets<sup>29</sup>. Spontaneous release of target cells was <25% in all assays. Effector:target ratios for which background lysis of control vaccinia virus-expressing cells exceeded 20% were excluded from analysis. Based on examination of naive monkeys, specific lysis of greater than 5% observed at more than one effector:target ratio was interpreted as significant. Three effector to target ratios were used in assays (40:1, 20:1 and 10:1). <sup>a</sup>Scores for specific lysis for Gag/Env/Nef targets. -, <5%; 1, 5–10%; 2, 10–20%; 3, 20–30%; 4, 30–40%; 5, 40–50% specific lysis. In concurrent CTL assays on PBMCs from macaques infected with live-attenuated viruses, CTL assays consistently scored higher than 2. <sup>b</sup>Assays done at week 34. i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert.

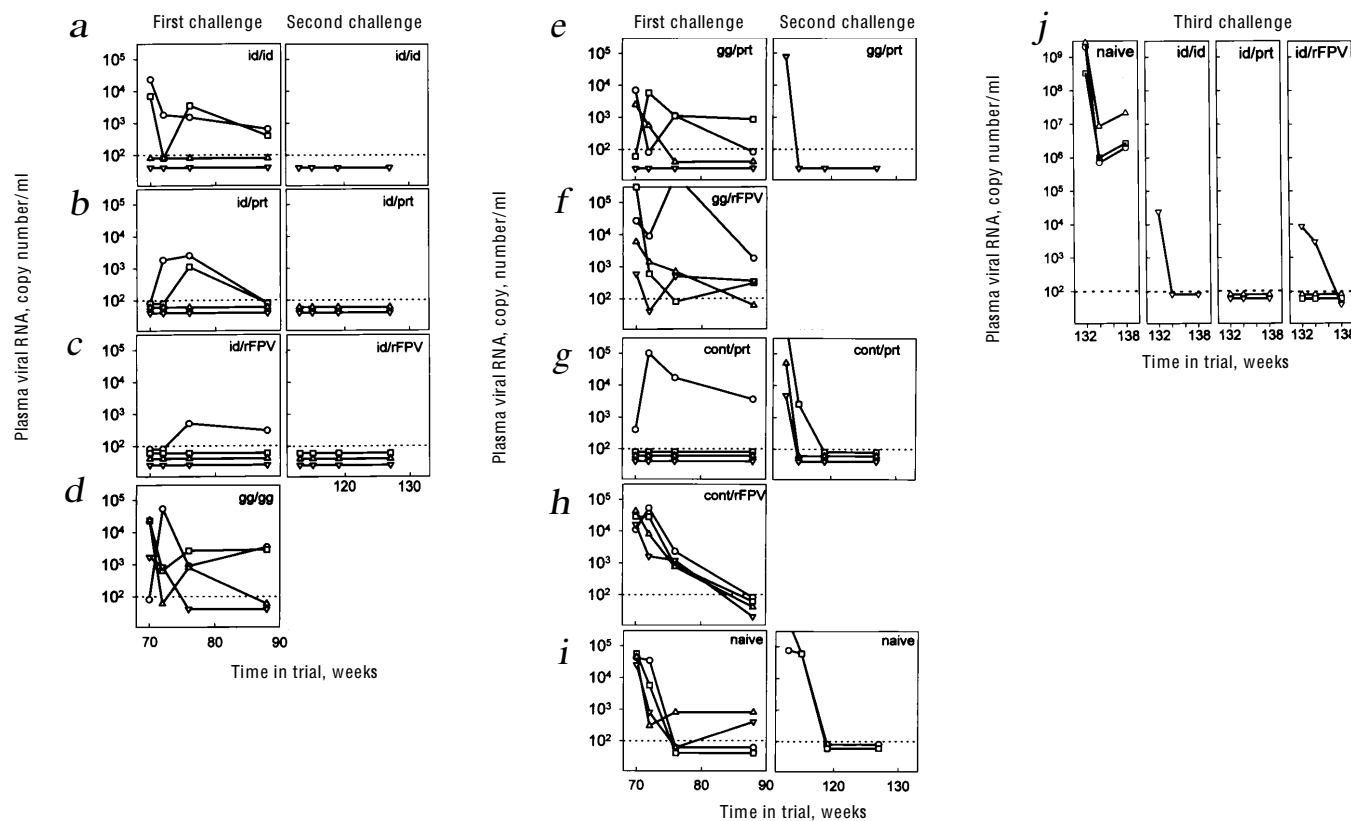
included one expressing Gag-Pol, one expressing Env, and one expressing Nef (ref. 28). Of each rFPV,  $5 \times 10^8$  PFU were administered intramuscularly, for a total dose of  $1.5 \times 10^9$  PFU.

### Antibody and CTL responses to the immunogens

The different DNA priming and booster inoculations generated distinct patterns of antibody responses (Fig. 2). During the priming phase of the immunizations, the intradermal DNA inoculations generated antibodies against Env that appeared earlier and rose to higher titers than the gene gun-generated antibody (geometric mean titers of 3.5 and 0.47  $\mu$ g per ml, respectively;  $P = 0.01$ , Mann-Whitney U test). The fourth and fifth intradermal DNA boosters failed to increase the levels of antibody above the peak primed response (Fig. 2a), whereas the gene gun DNA boosters increased specific antibody to a geometric mean titer of 6.1  $\mu$ g per ml (Fig. 2d). The protein boosters generated the highest titers of antibody achieving geometric mean titers of 43, 58 and 71  $\mu$ g per ml, or 20–30% of the level of antibody to Env pre-

sent in a pooled serum from rhesus macaques chronically infected with SHIV-IIIb. The rFPV boosters were the least successful booster immunization for antibody. For the monkeys primed with DNA delivered intradermally, the first, but not the second rFPV booster, increased the levels of falling antibody (Fig. 2c). For the monkeys primed with DNA delivered by gene gun, low level increases in falling responses were observed for each of the rFPV boosts (Fig. 2f). Undetectable levels of anti-Env IgG were raised in the group receiving control DNA plus rFPV (Fig. 2h).

The frequency and activity of specific cytolytic T lymphocytes (CTLs) were low throughout the trial (Table 1). Of the 156 assays for CTLs, only 70 were positive. In these positive assays, Gag was a target in 39 assays; Nef, in 17; and Env, in 14. Analysis of a subset of monkeys demonstrated that the CTL activity was MHC-restricted and mediated by CD8<sup>+</sup> cells (data not shown). As an index for CTL activity, scores were assigned that reflected the presence and extent of CTL lysis; these scores were summed over time for the monkeys within a group. In the four assays before



**Fig. 3** Levels of viral RNA in plasma after the first, second and third challenges. Temporal levels of plasma viral RNA were determined using real-time PCR (ref. 31). **a–i**, first and second challenges; **j**, third challenge. Upper right corners, priming immunizations/booster immunizations: i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl

pox virus vectors; cont., plasmid vector without a vaccine insert. Symbols indicate order in group: ○, first monkey in each group (macaques 1, 5, 9, 13, 17, 21, 25, 29, 33, 37 and 39); □, second (2, 6, 10, 14, 18, 22, 26, 30, 34, 38 and 40); △, third (7, 11, 15, 19, 23, 27, 31, 35 and 41); ▽, fourth (4, 8, 12, 16, 20, 24, 28, 32 and 36). Levels of viral RNA below detection were scored as 100; limit of detection, 300–1,000 copies of viral RNA per ml of plasma.

the booster immunizations, specific cytolytic activity for the monkeys primed with DNA delivered by gene gun had a cumulative score of 32, compared with 23 for the monkeys primed with DNA delivered intradermally. At 2 weeks after the first booster, groups receiving the rFPV booster had the highest scores. After the second rFPV booster immunization, the lytic scores were lower than those after the first boosters. On the day of the first challenge, only four monkeys had low levels of specific CTL activity (<10% lysis). These included three macaques in the group primed with DNA delivered intradermally and boosted with rFPV virus, and one monkey in the group primed with DNA delivered by gene gun and boosted with rFPV. No monkey receiving control DNA vaccination had a positive CTL response at any time before challenge (data not shown). Concurrent assays for cytolytic activity using peripheral blood mononuclear cells (PBMCs) from macaques infected with live attenuated viruses consistently showed intermediate to high levels of lytic activity<sup>29</sup>. Thus, the levels of vaccine-raised CTLs were lower and less consistent than those seen for live attenuated SIVs.

#### First and second challenges: SHIV-IIIb

The first two challenges were with SHIV-IIIb, a virus homologous to the immunogens. Ten macaque infectious doses of SHIV-IIIb were administered intravenously at 2 weeks after the second booster immunization, a time when peak titers of neutralizing antibody were expected to be present<sup>30</sup>. Protection was evaluated

by measuring virion-associated SIV RNA in plasma using a quantitative real-time RT-PCR (ref. 31) and by determining the frequency of PBMCs that could initiate an infection when co-cultivated with CEMx174 cells, a very susceptible cell line<sup>32</sup>. Monkeys were given subsequent challenges if plasma viral RNA could not be detected at any time after challenge and if only one co-cultivation assay showed low levels of infected cells. After the first challenge, ten monkeys met these criteria for protection and were given a second SHIV-IIIb challenge 43 weeks after the first challenge. No booster immunization was given before the second challenge to allow assessment of the long term efficacy of the different vaccine protocols against the homologous challenge.

Unexpectedly, the intradermal DNA priming provided better containment of the SHIV-IIIb challenge than the gene gun DNA priming ( $P = 0.01$ , Fisher exact test) (Fig. 3). All four of the naive control monkeys had levels of plasma viral RNA greater than  $1 \times 10^4$  copies/ml. Six of the twelve monkeys primed with DNA delivered intradermally had undetectable levels of plasma viral RNA after the first two challenges, whereas all of the monkeys primed with DNA delivered by gene gun had easily detected levels of viral RNA after the first or second challenge. The best containment occurred in the group primed with DNA delivered intradermally and boosted with rFPV (Fig. 3c). After the first challenge, three members of this group had undetectable levels of plasma viral RNA, and the fourth had very low levels of viral RNA. Three of the monkeys receiving control DNA plus Env pro-

**Table 2** Detection of infection after challenge: cumulative levels of plasma viral RNA and cumulative number of co-cultivation-positive PBM Cs

Group/ macaque		First SHIV-IIIb challenge		Second SHIV-IIIb challenge		Third SHIV89.6P challenge	
		Viral RNA, copies <sup>a</sup>	Co-cultivation-positive cells <sup>a</sup>	Viral RNA, copies	Co-cultivation-positive cells	Viral RNA, copies	Co-cultivation-positive cells
naive <sup>a</sup>		76,000	770	649,000	149	2.6 × 10 <sup>9</sup>	83,455
		61,700	743	135,000	8,751	0.3 × 10 <sup>9</sup>	ND
		45,900	177,178			2.7 × 10 <sup>9</sup>	ND
		27,200	6,615				
id/i.d	1	26,950	6578				
	2	10,800	252				
	3	<	41				
	4	<	<	<	<	24,000	27
i.d./prt	5	4,300	29				
	6	1,100	14				
	7	<	<	<	9	< <sup>d</sup>	<
i.d./rFPV	8	<	<	<	9	< <sup>d</sup>	<
	9	800	41				
	10	<	14	<	<	< <sup>d</sup>	9
g.g./g.g.	11	<	9	<	<	< <sup>d</sup>	<
	12	<	<	<	<	11,600	56
	13	58,400	<*				
	14	29,250	14*				
g.g./prt	15	24,800	243				
	16	2,550	14*				
	17	9,300	81				
	18	7,750	<*				
g.g./rFPV	19	3,050	14				
	20	<	<*	78,000	49		
	21	727,800	490				
	22	30,900	246				
	23	8,100	13				
	24	1,450	246				
cont/prt	25	121,000	14				
	26	<	14	752,600	29,426		
	27	<	<	51,000	136		
	28	<	<	4,900	27		
cont/rFPV	29	66,300	406				
	30	57,850	3,281				
	31	51,750	732				
	32	18,800	1,098				

<sup>a</sup>Naive macaques are not numbered because different macaques were used to control for the three challenges. <sup>b</sup>Sum of the titers of plasma viral RNA/ml at 2, 4, 8 and 20 weeks after the first challenge; 2, 4, 8 and 16 weeks after the second challenge; and 2, 4 and 8 weeks after the third challenge. <, below the background for detection. Backgrounds for detection, 300–1,000. <sup>c</sup>Sum of the co-cultivation-positive cells per 1 × 10<sup>6</sup> PBM Cs at 2, 4, 8 and 12 weeks after the first challenge; 2, 4 and 8 weeks after the second challenge; and 2 and 4 weeks after the third challenge. \*, monkeys for which data for 4 weeks after challenge were not available. <, no co-cultivation-positive cells detected in 1 × 10<sup>6</sup> PBM Cs. <sup>d</sup>Monkeys given the transfusion test for detection of occult virus (Table 3). ND, not done; i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert.

tein also had undetectable levels of viral RNA after the first challenge. However, all of these were infected by the second challenge (Fig. 3g). None of the monkeys receiving control DNA plus rFPV were protected against the first challenge (Fig. 3h).

Assays for co-cultivation positive cells and anamnestic antibody responses showed that monkeys that did not have detectable plasma viral RNA by PCR assay had sustained low levels of infection. After the first challenge, four of the eleven monkeys with undetectable levels of viral RNA had detectable levels of co-cultivation-positive cells and anamnestic antibody responses (Table 2 and Fig. 2). Three additional monkeys had detectable levels of anamnestic antibody responses (Fig. 2a, macaque ▽; macaques △ and ▽). After the second challenge, two of the six monkeys with undetectable levels of viral RNA had low levels of co-cultivation-positive cells and anamnestic antibody responses (Table 2 and Fig. 2), and a third had an increase in antibody levels (Fig. 2c, macaque ▽).

### Third challenge: SHIV-89.6P

The six monkeys that did not have detectable levels of plasma viral RNA after the first two challenges were challenged 19 weeks later with the highly virulent SHIV-89.6P virus. Two weeks after the SHIV-89.6P challenge, the naive control monkeys had undergone a precipitous decrease in their numbers of CD4<sup>+</sup> T cells (data not shown) and had very high levels of plasma viral RNA (Fig. 3j). All of the monkeys given the third challenge were protected against the rapid loss of CD4<sup>+</sup> cells (data not shown). Four did not have detectable levels of viral RNA by PCR assay (Fig. 3j). Three of these did not have detectable levels of co-cultivation-positive cells (Table 2). The two monkeys that did have detectable levels of viral RNA by PCR assay had titers 0.001% those of the naive control monkeys. By 8 weeks after challenge, these two monkeys had reduced their levels of viral RNA to below background, whereas levels of viral RNA of greater than 1 × 10<sup>6</sup> copies/ml persisted in the naive control monkeys.

### Neutralizing antibody

Neutralizing antibody was present at different titers in the different sections and phases of the trial (Fig. 4). On the day of the first challenge, low-to-undetectable levels of neutralizing antibody were present in the group primed with DNA delivered intradermally and boosted with rFPV. In contrast, in the group primed with control DNA and boosted with Env protein, neutralizing antibody approximated the titer of 200, which is considered a correlate of protein-raised protection against SHIV-IIIb (ref. 33). In the groups primed with vaccine DNAs and boosted with protein or DNA, titers of neutralizing antibody ranged from intermediate to low. For monkeys given the second challenge, titers of neutralizing antibody against SHIV-IIIb on the day of challenge were both higher and lower than those on the day of the first challenge for the three groups of monkeys primed with DNA delivered intradermally, but were consistently lower for the monkey primed with DNA delivered by gene gun and boosted with protein, and monkeys receiving control DNA and protein boosters. On the day of the third challenge, none of the monkeys remaining in the trial had detectable levels of neutralizing antibody for SHIV-89.6P.

### Transfusion test

At 8 weeks after the third challenge, 10 ml of whole blood from the four macaques with undetectable levels of infection (Table 2, macaques 7, 8, 10 and 11), were transfused into naive macaques to see if an occult infection could be transmitted (Table 3). Not unexpectedly, macaque 10, which had co-cultivation-positive cells at two weeks after the SHIV-89.6P challenge transmitted SHIV-89.6P. However, unexpectedly, macaque 7 that had neither detectable levels of viral RNA nor co-cultivation-positive cells for SHIV-89.6P transmitted the earlier SHIV-IIIb challenge virus. This macaque had transient low levels of co-cultivation-positive cells (9 cells per  $1 \times 10^6$  PBMCs) at 2 weeks after the second challenge but never had detectable levels of plasma virus RNA (Table 2). Macaque 8, which transiently had co-cultivation-positive cells for SHIV-IIIb after the second challenge, did not transmit SHIV-IIIb. Macaque 11, which transiently had co-cultivation-positive cells for SHIV-IIIb after the first challenge, also did not transmit SHIV-IIIb.

### Discussion

We tested eight different protocols for vaccination against immunodeficiency viruses and found one protocol that contained challenge infections in the absence of detectable levels of neutralizing antibody. This very promising protocol consisted of intradermal DNA priming followed by rFPV booster immunizations (Fig. 3 and Table 2). The presumably cellular immune responses that mediated protection were at levels close to those considered 'background' in our assays for CTLs (Table 1), and were well below the level of CTLs in concurrent assays conducted on rhesus macaques infected with live attenuated SIVs (ref. 29). This raises the possibility that the protective response might have been mediated by a nonlytic T-cell activity. Two types of T-cell-mediated anti-immunodeficiency virus responses have been reported: a cytolytic, MHC-I-restricted activity, and a nonlytic, secreted activity that suppresses HIV-1 infections<sup>34,35</sup>.

Intradermal DNA priming was more effective than gene gun DNA priming in raising protective responses ( $P = 0.01$ ). This was seen in the groups receiving DNA

and Env protein boosters as well as in the groups boosted with rFPV (Fig. 3 and Table 2). In the three groups primed with DNA delivered intradermally, six of twelve monkeys contained the first two challenges below the level that could be detected by RT-PCR assays for plasma viral RNA. In contrast, none of the monkeys primed with DNA delivered by gene gun were similarly protected. This difference in efficacy did not manifest itself in before-challenge assays for CTL or antibody (Table 1 and Figs. 2 and 4) and would not have been realized in the absence of the challenges. One manifestation of the difference in efficacy was lower levels of plasma viral RNA per co-cultivation-positive cell in the monkeys primed with DNA delivered intradermally than in the groups primed with DNA delivered by gene gun ( $P < 0.03$ , Mann-Whitney U test) (Table 2). This could reflect the possibility that intradermal but not gene gun DNA inoculations prime T-cell responses with suppressive activity for HIV-1 infections.

Assuming results can be extrapolated from murine to primate models, the difference in the efficacy of the responses primed with DNA delivered intradermally and by gene gun may be due to saline injections of DNA priming predominantly type 1 T-cell help, and gene gun deliveries of DNA priming predominantly type 2 T-cell help<sup>36</sup>. Differences in the type of a primed T-cell response are maintained during subsequent booster immunizations or challenge infections<sup>36</sup>. Type 1 and type 2 T-helper cells express different patterns of lymphokines and chemokines and are associated with distinctive CTLs (refs. 37,38). Differences in these two types of immune responses could have profound effects on the ability of cell-mediated immune responses to contain immunodeficiency virus challenges.

Different aspects of the immune response seemed to play different parts in the control of the immunodeficiency virus challenges in the different sections of the trial and for the different challenges within a section. This is consistent with multiple aspects of immune responses having the potential to contribute to protective immunity<sup>39</sup>. At the time of the first challenge, an apparently cell-mediated protection (in the absence of detectable levels of neutralizing antibody) occurred in the rFPV-boosted group of the monkeys primed with DNA delivered intradermally (Fig. 4). In the groups not boosted with rFPV, containment was more likely in monkeys that had intermediate-to-high levels of neutralizing antibody than in monkeys with low titers of neutralizing antibody ( $P < 0.04$ , Mann-Whitney U test) (Fig. 4). At the time of the second challenge, the titers of neutralizing antibody had decreased in the one monkey primed with DNA delivered by gene gun and boosted

**Table 3** Use of blood transfusions to test for the presence of infection in the protected macaques

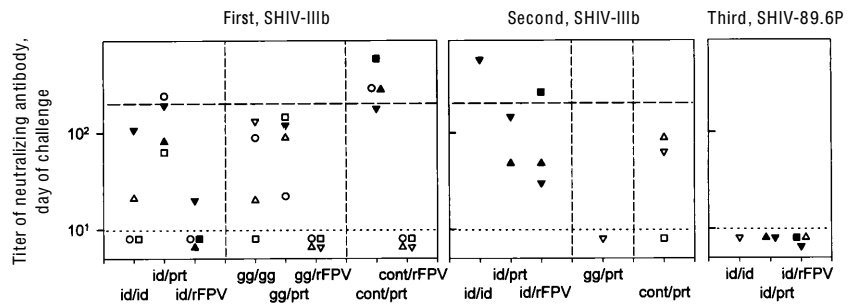
Donor macaque	History of after-challenge virus recovery for donor, (RT-PCR/co-cultivation)			Infection of recipient	Proviral V3 region of infecting virus <sup>a</sup>
	First IIIb	Second IIIb	Third 89.6P		
vaccinated					
7	-/-	-/+	-/-	yes	SHIV-IIIb
8	-/-	-/+	-/-	no	-
10	-/+	-/-	-/+	yes	SHIV89.6P
11	-/+	-/-	-/-	no	-
Control					
39			+/+	yes	SHIV-89.6P

<sup>a</sup> -, failure to detect proviral DNA in PBMCs by PCR. IIIb, SHIV-IIIb; 89.6P, SHIV-89.6P.

## ARTICLES

**Fig. 4** Titers of neutralizing antibody for the challenge virus on the days of the first, second and third challenges. Neutralizing antibody responses are the reciprocal of the serum dilution giving 50% neutralization of SHIV-IIIb (a and b) or SHIV-89.6P (c) using a cell-killing assay and neutral red staining<sup>49</sup>. Symbols indicate order in group: ○, first monkey in each group (macaques 1, 5, 9, 13, 17, 21, 25, 29, 33, 37 and 39); □, second (2, 6, 10, 14, 18, 22, 26, 30, 34, 38 and 40); △, third (7, 11, 15, 19, 23, 27, 31, 35 and 41); ▽, fourth (4, 8, 12, 16, 20, 24, 28, 32 and 36); Open symbols, infected monkeys; filled symbols, monkeys with strongly contained infections.

Horizontal dotted lines, the limit of detection for the assay; horizontal dashed lines, titer of neutralizing antibody that correlates with protection against the SHIV-IIIb challenge in SHIV-IIIb Env protein-immunized monkeys<sup>33</sup>. Below graphs, priming immunizations/booster immuniza-



tions: i.d., intradermal delivery of five vaccine DNAs; g.g., gene gun delivery of five vaccine DNAs; prt, Env protein in incomplete Freund's adjuvant; rFPV, three recombinant fowl pox virus vectors; cont., plasmid vector without a vaccine insert.

with protein and in the three monkeys primed with control DNA and boosted with protein, which had contained the first challenge. All four of these monkeys were infected by the second challenge virus. However, monkeys primed with DNA delivered intradermally that had titers of neutralizing antibody overlapping those of the monkeys primed with control DNA and boosted with protein contained the second challenge, and were presumably protected by cell-mediated responses as well as neutralizing antibody. By the third challenge, a neutralizing antibody-independent, presumably cell-mediated containment of the SHIV-89.6P challenge seemed to be occurring in monkeys from all three of the groups primed with DNA delivered intradermally (Fig. 4 and Table 2).

In the monkeys primed with DNA delivered intradermally, protective cell-mediated immune responses are likely to have been boosted by the challenge infections (much as they were boosted by the rFPV infection). These protective boosters were not observed in the groups primed with gene gun DNA or control DNA. This may reflect the live virus challenge boosting protective cell-mediated responses only in those groups in which protective cell-mediated responses had been primed. The putative booster immunizations differed from infection with SHIV-IIIb in their very low levels of virus replication. Infection of naive monkeys with SHIV-IIIb resulted in 27,000–649,000 copies of viral RNA and 149–177,000 co-cultivation-positive cells (Table 2). By contrast, the putative SHIV-IIIb boosters were accomplished with levels of infection that did not have detectable levels of plasma viral RNA and had  $\leq 14$  co-cultivation-positive cells (Table 2). These levels of infection are below the threshold of infection required for protection by live attenuated vaccines<sup>40</sup> and would not have provided containment in the absence of a vaccine-primed immune response.

The raising of protective cell-mediated responses by DNA primes and pox virus booster immunizations has precedence in studies in mice and macaques<sup>41–43</sup>. In these studies, the DNA prime was hypothesized to focus the immune response on the desired antigens, whereas the recombinant pox virus booster immunization was hypothesized to boost this response, both by the expression of higher levels of the recombinant antigen and the immunostimulatory activity of a pox virus infection. Our results contrast with those of a study in which gene gun DNA priming and rFPV booster immunizations protected three pig-tail macaques against an HIV-1 challenge<sup>43</sup>. In this highly attenuated infection (a maximum of 300 copies of viral RNA in

the naive control monkeys), gene gun DNA priming plus rFPV boosting may be able to afford protection against an immunodeficiency virus challenge.

Unlike antibody that can prevent infection, cell-mediated immune responses act after infection, and thus afford immunodeficiency virus infections the opportunity to establish proviral DNA. A transfusion test at the end of the trial showed an otherwise undetected SHIV-IIIb infection in one of four monkeys tested (Table 3). In this monkey, the last SHIV-IIIb challenge had been 27 weeks before the transfusion. This monkey had never tested positive for plasma viral RNA and had shown low levels of co-cultivation-positive cells only once, at 25 weeks before the transfusion (Tables 2 and 3). This result indicates that HIV-1 vaccines that operate after infection, despite even very low levels of infection (below those able to be detected by RT-PCR), will not prevent the long term persistence of virus and the potential for re-emergent virus<sup>44</sup>.

The ability to achieve stringent containment of serial SHIV challenges using intradermal DNA priming and recombinant pox virus boosters holds promise for the development of a vaccine capable of considerably reducing viral replication and thus stemming the transmission of AIDS. This vaccine would be effective against a diversity of isolates because of its ability to raise neutralizing antibody-independent containment of infections. If our hypotheses are correct, the boosting of responses by challenge infections should bolster the effectiveness of the vaccine in the high-risk populations, which are the chief source of transmission.

## Methods

**Vaccine trial.** For intradermal immunizations, 500  $\mu$ g of each DNA was administered in four 100- $\mu$ l inoculations in the skin of the upper back for a total of 20 injections and 2.5 mg DNA per time point. For the gene gun inoculations, 4  $\mu$ g of each DNA was bombarded at 350 psi in eight shots, each containing 0.5 mg of gold, into shaved abdominal skin, for a total of 20  $\mu$ g and 40 gene gun deliveries of DNA per inoculation. The protein boost consisted of 100  $\mu$ g of the full-length Env of HIV-1-IIIb (ref. 27) in incomplete Freund's adjuvant, administered in two 0.5-ml inoculations, one in each biceps. Of each of three rFPV expressing Gag-Pol, Env or Nef (refs. 28,45),  $5 \times 10^8$  PFU were mixed in 1 ml and delivered in two 0.5-ml injections, one to each biceps, for a total dose of  $1.5 \times 10^9$  PFU. Care of the macaques was in accordance with institutional guidelines for the care and use of experimental animals.

**Immunogens.** A vaccine plasmid called pRS102 expressed SIVmac239 Gag and Pol proteins. The vaccine insert for pRS102 comprised a Kozak sequence, the SIV239 gag-pol region (nucleotides 1309–5753) and the

Mason-Pfizer Monkey virus cytoplasmic transport element. This insert was cloned into the *HindIII* and *NheI* sites of the eukaryotic expression vector pJW4303, and expression in transiently transfected COS cells was verified. pJW4303 was provided by J.I. Mullins (University of Washington, Seattle). A vaccine plasmid called pCMV/nef comprised the *PstI*-*StuI* Nef-encoding fragment of clone BK28 inserted into pCMV5 (ref. 46), and was provided by G. Viglianti (Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts). The pJW4303/HXB-2.dpol, pJW4303/HXB-2.gp140 and pJW4303/HXB-2.gp120 vaccine plasmids have been reported<sup>30</sup>.

**Assays for ELISA antibody.** The titers of anti-Env IgG in macaque sera were determined using an ELISA with affinity-purified sheep antibody against Env (catalog number 6205; International Enzymes, Fallbrook, California) to capture the gp120 form of a IIIB Env. Gp120 was produced using recombinant vaccinia virus vPE-50 (ref. 47). A standard curve was made using pooled sera from SHIV-IIIB-infected macaques whose anti-Env IgG concentration had been determined to be about 210 µg/ml. Sera were assayed at threefold dilutions in duplicate wells. Biotinylated anti-human IgG (H and L) and horse radish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, California) were used to detect bound IgG. Standard curves were fitted and sample concentrations were interpolated as µg per ml of sera using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, California).

**Blood transfusion assay.** At 8 weeks after the third challenge, 10 ml of whole blood was transfused into naive recipients to test whether virus that could not be detected by co-cultivation or RT-PCR could be transmitted. Three naive monkeys challenged with SHIV-89.6P were used as positive controls. Transfusion recipients were tested over a 3-month period for infection by testing for 'antigenemia' and by co-cultivation of PBMCs with CEM-X174 cells at 0, 2, 4, 8 and 12 weeks after transfusion. The presence of virus was assayed using an antigen-capture ELISA for the p27 Gag protein of SIV (Coulter, Hialeah, Florida). The identity of viruses transmitted was tested by PCR amplifying and sequencing the C2V3C3 region of the proviral DNA in the PBMCs of recipient macaques<sup>48</sup>.

**Statistical analyses.** The Mann-Whitney U test was used to evaluate between-group differences in antibody titers or levels of plasma virus RNA per co-cultivation-positive cell. The Fisher exact test was used to examine differences in the frequency with which different trial groups contained viral infections. A type I error rate of 0.05 (two-tailed) was adopted as the criterion of significance for statistical inference.

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