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Neutralization of primary HIV-1 SF13 can be detected in extended incubation phase assays with sera from monkeys immunized with recombinant HIV-1 SF2 gp120

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Abstract

Phase III efficacy trials of recombinant human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins were postponed. In Phase I and II trials these candidate vaccines had failed to induce neutralizing antibodies against virus which had been isolated by co-culture with human peripheral blood mononuclear cells (PBMC). The aim of the present study was to determine assay conditions for detecting neutralization of primary HIV-1 isolates with sera from immunized individuals. We show that in two immunogenicity trials in rhesus macaques, recombinant HIV-1 SF2 gp120 induced antibodies which neutralized the primary HIV-1 SF13 isolate. Statistically significant in vitro neutralization required assays in which the incubation phase was extended. Sterile immunity was only seen with the highest level of neutralization, induced by a recombinant prime, peptide boost strategy. We recommend that neutralization assays with extended incubation phases should be used to monitor Phase III efficacy trials.

Keywords: Envelope glycoprotein; DNA vaccine; Primary HIV-1 isolates; Recombinant prime; Peptide boost; Neutralizing antibodies

1. Introduction

Recombinant human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins were shown to be safe and immunogenic for humans but Phase III efficacy trials of these candidate vaccines were nevertheless postponed [1]. These immunogens failed to induce antibodies able to neutralize isolates (primary) which had been minimally passaged in artificially activated human peripheral blood mononuclear cells (PBMC) so that they were as close as possible to the virus involved in natural transmission events [2]. There are two potential explanations for this failure: either the immunogens are not inducing the correct antibodies; or, the assays are not capable of detecting the induced antibodies. Although the first Phase III trial has now been completed this problem has not been resolved.

We [3,4] and others [5] have presented evidence indicating that there are at least two sets of epitopes associated with neutralization of primary isolates. The first set is exposed on the surface of the free virion so that the level of neutralization increases as the time for the reaction between antibody and virus (incubation phase of an in vitro assay) is extended. These epitopes are shared between isolates within a single envelope genetic subtype. Binding of antibody leads to an all or nothing loss in virus infectivity so that no replication of a neutralized virus can be detected within 14 days of culture [4]. The second set of epitopes is shared between virus isolates from different subtypes. Viruses are more readily neutralized after they have bound to their target cell so that neutralization increases with the absorption phase of an in vitro assay. However, except for the most neutralization sensitive isolates, antibody binding to these epitopes only slows down virus replication (or influences some other property associated with viral pathogenicity). In the early stage of culture, virus produces considerably less antigen following

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exposure to neutralizing antibodies, relative to control sera, but a single infectious dose of neutralized virus can still release a detectable quantity of antigen within 14 days [3].

In the present paper, we demonstrate that recombinant HIV-1 SF2 gp120 can induce antibodies able to neutralize primary isolates provided the incubation phase of the assay is extended.

2. Materials and methods

2.1. Peripheral blood mononuclear cells

Buffy coats were obtained from the Antwerp Red Cross Blood Transfusion Centre. PBMC were separated using Linfosep (Biomedics, Madrid, Spain), adjusted to 1×10^6 ml^{-1} in RPMI medium supplemented with 0.5 μg/ml of phytohaemagglutinin (PHA; Abbott Murex, Dartford, UK), 15% foetal calf serum (FCS; Bio-Whittaker, Verviers, Belgium) and 2 μg/ml polybrene (Sigma–Aldrich, Bornem, Belgium) and incubated for 48–72 h. The PHA-transformed PBMCs were then cultured in RPMI medium where the PHA was replaced by 20 U/ml of recombinant human interleukin 2 (IL2; Roche Diagnostics, Brussels, Belgium) [3,4].

2.2. Dendritic cell, T4 lymphocyte co-culture

For dentritic cell/T4 lymphocyte co-cultures, the purified leukocytes were fractionated into monocytes and lymphocytes by counter-flow elutriation using a J6-MC elutriator (Beckman Instruments, Palo Alto, California, United States of America). The monocyte fractions were pooled and further purified by mixing with sheep red blood cells and removal of rosettes over Linfosep. The monocytes were then cultured in RPMI 1640 medium with 10% freshly prepared heat-inactivated human serum and recombinant human granulocyte–monocyte colony-stimulating factor (GM-CSF; Boehringer-Mannheim, Mannheim, Germany; 20 ng/ml) and recombinant human interleukin 4 (IL4; Genzyme, Cambridge, MA, USA; 20 ng/ml) for 7 days, with two changes of medium. The lymphocyte fractions were frozen and stored in liquid nitrogen. At day 6, the lymphocytes were thawed, positively purified with CD4 magnetic beads (Dynal, Oslo, Norway) and cultured overnight. Purity of the preparations was monitored by FACS analysis. Autologous lymphocytes were added to the dendritic cells which had been pulsed with HIV and cultured without any additional lymphokines. There is no need to add recombinant interleukins since the lymphocytes transform and produce the conditions necessary for their own and the dendritic cells' survival [6].

2.3. Virus isolates

Virus stocks were prepared by infecting a 5 ml-culture of PHA-transformed PBMCs with HIV-1 obtained from the international repositories [3,4]. Twice weekly, the cells were divided and freshly transformed cells from a different donor added. This procedure was repeated to eventually generate 30 ml of viral supernatant with a high HIV-antigen content. The supernatants from these cultures were dispensed into aliquots and stored at −80 °C. HIV-1 SF162 (original donor Levy and co-workers [7]) was obtained from the Centralized Facility for AIDS Reagents, the European Programme EVA at the United Kingdom National Biological Standards and Control Laboratory. The primary isolates of HIV-1 MN (original donor Gallo [8]) and HIV-1 SF13 (original donor Levy and Shimabukuro [9]) were obtained from Zolla-Pazner et al. [8]. Small stocks of HIV-1 SF13 were also prepared in dendritic cell, T4 lymphocyte co-cultures.

2.4. Sera and plasma

Sera and plasma were obtained from rhesus macaques involved in immunization experiments undertaken at the Biomedical Primate Research Centre (BPRC), Rijswijk, the Netherlands [10,11]. The description of these trials is reduced to an outline of the immunogens, injection schedules and challenge since full details have been published elsewhere [10,11].

In a recombinant prime, peptide boost vaccination strategy, macaques were primed with Chiron's recombinant HIV-1 SF2 gp120 formulated into immune stimulating complexes (ISCOM). The injection was repeated 6 weeks later. At the same time, the macaques were also immunized with 15-mer synthetic peptides (Affiniti Research Products Ltd., Exeter, UK), representing the HIV-1 SF2 gp120 V2 and V3 neutralizing epitopes, covalently coupled to FluPR8 envelope glycoproteins in ISCOMs. The peptide-conjugate injection was repeated at week 16. Two control macaques were immunized with FluPR8-ISCOMs at weeks 0, 6 and 16. Serum was taken 2 weeks after the last injection. Each immunized macaque had sterilizing immunity to an intravenous challenge with 30 animal infectious doses (AID_{50}) SHIV SF13 at week 20 [10].

Plasma were obtained at week 38 from a second immunization study involving HIV-1 SF2 immunogens [11]. The four macaques in group 1 were injected at weeks 0, 12, 24 and 36 with plasmid DNA (8 μg per dose) which expressed HIV-1 SF2 gp120. In groups 2 and 3, macaques were immunized with Chiron's recombinant HIV-1 SF2 gp120 in MF59C-0 (50 μg immunogen per dose) or ISCOMs (30 μg per dose), respectively. Four monkeys were injected intra-muscularly with each formulation at weeks 0, 12 and 36. In the controls, group 4, two monkeys were injected with 4 μg × 8 μg DNA expressing an irrelevant antigen or 3 μg × 50 μg of an irrelevant immunogen in MF59C-0 adjuvant. All 16 monkeys were challenged intravenously at week 40 with 50 AID_{50} SHIV SF13. The outcome of challenge is summarized in Table 6.

2.5. Neutralization assay

A fivefold dilution series of virus was prepared and a 190 μl aliquot of each dilution mixed with 10 μl of serum or plasma and incubated at 37 °C. A 20 μl aliquot of this mixture was then added to cells and residual virus allowed to absorb at 37 °C before removing any unbound material by washing. The cells were then cultured at 37 °C. For ease of presentation in the subsequent text, the various assay formats are summarized as *a*/*b*/*c* ($n = x$) where *a* is the incubation phase (h), *b* the interval allowed for absorption (h), and *c* the number of days the cells are cultured starting from their first exposure to virus. The number of duplicate cultures is given by *x* [3,4,12]. For the dendritic/T4 co-cultures, the dendritic cells were exposed to the virus/antibody mixture, incubated for 2 h and then washed before the lymphocytes were added. Release of HIV antigen into culture supernatants was monitored using an 'in-house' ELISA [13].

2.6. Statistical techniques

The titre of virus was calculated within each individual experiment using the method of Reed and Muench [14]. In the virus dilution series, doses ranged between those infecting all cultures (100%) to those infecting none (0%). Wells giving an optical density of >0.3, against a background of 0.03–0.05 in the ELISA, were considered to be infected. The neutralization index was the titre, expressed in $\log_{10}$, following virus incubation with serum/plasma taken before a monkey had received any injection, divided by its titre following exposure to the serum/plasma taken from the same monkey at the end of its injection schedule. So that the statistical significance of each individual monkey's serum/plasma neutralization could be calculated, antigen release into culture supernatants was determined in $\log_{10}$ arbitrary units against a standard curve of HIV-1 SF33 cultured in HUT 78 cells. Since multiple comparisons were possible within any neutralization experiment, reductions in antigen release were compared using the Studentised range test [15].

3. Results

3.1. Short incubation phase assays fail to detect neutralization of a primary isolate

Virus produced in lymphocytes which have been activated without exposure to mitogen is not neutralized in assays with short incubation phases by sera from monkeys immunized by a recombinant prime, peptide boost strategy. No neutralization was seen in assays with 1 h incubation and a 2 h absorption phase when dendritic cell/T4 lymphocyte co-cultures were used as target cells (Table 1). There was a reduction in the release of HIV antigen into the culture supernatant when the virus inoculum was 2 $TCID_{50}$ and this reached 50% or more for all three monkeys, whether reductions were compared with cultures inoculated with virus which had been incubated without any serum, virus incubated with the monkey's own pre-immune serum or serum from the control monkey (9258) immunized with FluPR8-ISCOMs (Table 2). Serum from monkey 9251 induced a 90% reduction in antigen release, in comparison to the FluPR8 immunized monkey serum. However, the variation between duplicate cultures was such that these reductions did not reach statistical significance.

Table 1
Neutralization of primary HIV-1 SF13 by sera from vaccine-protected monkeys using dendritic/T4 co-cultures (1/2/*x*) assays

Monkey plasma	Supernatants collected after culturing cells for				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
Monkeys immunized with HIV immunogens					
9251	0.50[a]	**0.70**	0.60	0.60	**1.05**
9263	0.35	0.30	0.15	0.45	0.35
BB85	0.50	**0.70**	0.50	0.35	0.35
Control monkey immunized with Flu immunogen					
9258	0.20	0.10	−0.35	0.35	0.10

Bold indicates a neutralization index ≥0.70 (= 80% neutralization).
[a] Neutralization index: titre of virus following incubation with serum obtained from monkey before any injection, divided by its titre following incubation with sera from the same monkey 2 weeks before challenge.

3.2. Assays with extended incubation phases are required for statistically significant results

The level of neutralization may be increased and the error mean square reduced, allowing statistically significant results to be obtained with fewer duplicate cultures, if the incubation phase of the assay is extended. When the assays in Tables 1 and 2 are repeated with a 24 h incubation phase, neutralization of more than 90% (neutralization index >1.0) of virus can be demonstrated after 1 week in culture (Table 3).

After 7 days in culture there was close to 90% reductions in the release of antigen from cells inoculated with virus exposed to HIV immune serum in comparison to virus incubated with the individual monkey's own pre-immune serum (Table 4). This difference increased to 99% after 2 weeks in culture, but fell in those cultures inoculated with the highest dose (100–300 $TCID_{50}$) of virus after 4 weeks. In cultures inoculated with a lower virus dose (20–60 $TCID_{50}$), three sera (monkeys 9111, 9251 and BB85) produced close to 99.9% reductions in antigen release after 4 weeks in culture. Serum from each monkey produced results which were individually statistically significant ($P < 0.01$).

3.3. Neutralization can be demonstrated with conventional PBMC assays

Statistically significant neutralization is also detectable using mitogen-activated PBMCs as target cells and assays with extended incubation phases (Table 5). In the PBMC

Table 2
Concentration of HIV antigen released by cells into supernatant after 5 weeks of culture of primary isolate of HIV-1 SF13: neutralization assay in 1/2/35 ($n = 6$) dendritic/T4 cell co-cultures with serum from protected monkeys

Virus dose in culture	Monkeys immunized with recombinant prime, peptide boost			Flu-ISCOMs	Virus alone
	9251	9263	BB85	9258	
Pre-immune plasma					
50 $TCID_{50}$	3.16 (0.06)	3.10 (0.03)	3.09 (0.03)	3.01 (0.02)	
10 $TCID_{50}$	3.03 (0.07)	2.88 (0.08)	2.79 (0.03)	2.97 (0.04)	
2 $TCID_{50}$	2.55 (0.54)	2.81 (0.04)	2.87 (0.13)	2.64 (0.13)	
0.4 $TCID_{50}$	1.73 (0.65)	0.63 (0.63)	1.36 (0.78)	0.86 (0.59)	
0.08 $TCID_{50}$	1.25 (0.50)	0.31 (0.31)	0.00 (0.00)	1.14 (0.69)	
Plasma from 2 weeks before challenge					
50 $TCID_{50}$	3.08 (0.06)	3.18 (0.03)	3.04 (0.04)	3.22 (0.07)	2.99 (0.08)
10 $TCID_{50}$	2.99 (0.10)	3.12 (0.05)	3.01 (0.08)	**2.67** (0.49)	2.84 (0.16)
2 $TCID_{50}$	1.69 (0.61)[a]	1.85 (0.70)[b]	2.20 (0.74)[b]	2.84 (0.15	2.52 (0.20)
0.4 $TCID_{50}$	**0.61** (0.61)[a]	**0.00** (0.00)[a]	**0.00** (0.00)[a]	1.87 (0.85)	0.00 (0.00)
0.08 $TCID_{50}$	**0.57** (0.50)	0.69 (0.69)	0.53 (0.53)	**0.61** (0.64)	0.69 (0.69)

Data expressed as mean ± standard error of HIV-1 antigen released in $\log_{10}$ arbitrary units. Bold indicates 50% reduction in comparison to pre-immune serum. Underlined indicates 50% reduction in comparison to virus alone. Minimum significant range at $P < 0.05$ for 1/2/35 with a 2 $TCID_{50}$ inoculum: 1.88; no serum reaches statistical significance.

[a] 90% reduction in comparison to Flu-ISCOM serum.

[b] 50% reduction in comparison to Flu-ISCOM serum.

assays with sera from monkeys immunized with the recombinant prime, peptide boost strategy, the absorption phase was extended in accordance with the HIV Vaccine Trials Network (HVTN) and other studies claiming to demonstrate neutralizing antibodies induced by candidate vaccines [16–19].

Three monkeys (9251, 9263 and BB85) have serum which can neutralize primary HIV-1 SF13 in conventional 24/24/14 PBMC assays (Table 5). No more serum was available from the fourth monkey, 9111. Although reductions in the release of HIV antigen into culture supernatants reached statistical significance, the neutralization indices were lower than in the dendritic cell/T4 lymphocyte co-cultures for monkeys 9263 and BB85.

The recombinant prime, peptide boost immunization strategy induces antibodies which are specific for HIV-1 SF13.

Table 3
Neutralization of primary HIV-1 SF13 by sera from vaccine-protected monkeys using dendritic/T4 co-cultures (24/2/x) assays

Monkey plasma	Supernatants collected after culturing cells for			
	1 week	2 weeks	4 weeks	6 weeks
Monkeys immunized with HIV immunogens				
9111	≥**1.25**[a]	**1.60**	**1.65**	**1.75**
9251	≥**1.80**	**1.50**	**1.80**	**1.65**
9263	≥**1.60**	**1.95**	**1.75**	**1.55**
BB85	1.75	**2.10**	**2.00**	**1.90**
Control monkey immunized with Flu immunogen				
9258	0.10	0.30	−0.20	−0.10
BB70	−0.05	−0.10	−0.10	0.00

Bold indicates a neutralization index ≥0.70 (= 80% neutralization).

[a] Neutralization index: titre of virus following incubation with serum obtained from monkey before any injection, divided by its titre following incubation with sera from the same monkey 2 weeks before challenge.

Only one serum (monkey 9251) showed neutralization of a fully heterologous subtype B primary isolate, HIV-1 MN (Table 5).

3.4. Plasma from a second monkey immunization trial also neutralize primary HIV-1 SF13

To remove any potential interaction effect between extended incubation and absorption phases [4], in the assays with the second BPRC immunization trial the latter was shortened to the minimum required for virus to bind to cells. Plasma from the monkeys in group 2 (immunized with recombinant HIV-1 SF2 gp120 in MF59) and group 3 (immunized with recombinant HIV-1 SF2 gp120 in ISCOMs), which showed some protection following challenge with SHIV SF13, neutralized more than 90% (index >1.0) of primary HIV-1 SF13, except monkey X009 where 80% neutralization was observed (Table 6). Each plasma showed a statistically significant reduction in release of viral antigen into culture supernatants with at least one virus dose. Three of the monkeys (I 038, Q 062 and X 007) immunized with HIV-1 SF2 gp120 DNA showed a statistically significant reduction in antigen release of >50%, although the neutralization indices showed no difference from their equivalent controls (group 4). However, the reduction was only seen after 7 days in culture. In neither group 1 nor group 4 were macaques protected against challenge with SHIV SF13.

4. Discussion

Neutralizing antibodies should be able to prevent virus spreading through the plasma to organs remote from the

Table 4
Release of antigen into supernatants of cultures infected with primary isolate of HIV-1 SF13: neutralization assay in 24/2/x ($n = 6$) dendritic/T4 cell co-cultures with serum from protected monkeys

After week 1: serum	Dilution (concentration) of virus in neutralization assay			
	1 in 2 dilution (= 100 $TCID_{50}$)		1 in 10 dilution (= 20 $TCID_{50}$)	
	Pre-immune	Immune	Pre-immune	Immune
9111	1.87 (0.14)	**0.53 (0.11)**[a]	1.20 (0.19)	**<0.30 (0.00)**[b]
9251	2.19 (0.04)	**0.47 (0.12)**[a]	1.71 (0.15)	**0.33 (0.03)**[a]
9263	1.63 (0.06)	**0.41 (0.10)**[a]	1.45 (0.05)	**0.38 (0.07)**[a]
BB85	2.26 (0.11)	**0.93 (0.13)**[a]	1.91 (0.11)	**<0.30 (0.00)**[a]
9258	2.11 (0.07)	2.03 (0.23)	1.58 (0.11)	1.57 (0.13)
BB70	2.03 (0.13)	2.29 (0.11)	1.56 (0.08)	1.65 (0.12)
95% (99%) minimum significant range	0.58 (0.68)		0.49 (0.57)	
After week 2: serum	1 in 2 dilution (= 100 $TCID_{50}$)		1 in 10 dilution (= 20 $TCID_{50}$)	
	Pre-immune	Immune	Pre-immune	Immune
9111	3.13 (0.14)	**1.52 (0.84)**[a]	2.06 (0.17)	**0.34 (0.03)**[a]
9251	3.56 (0.13)	**0.82 (0.29)**[c]	2.92 (0.13)	**0.31 (0.25)**[c]
9263	3.15 (0.13)	**0.85 (0.22)**[c]	2.80 (0.06)	**0.93 (0.38)**[a]
BB85	3.41 (0.10)	**1.56 (0.21)**[a]	2.52 (0.12)	**<0.30 (0.00)**[c]
9258	3.58 (0.11)	2.90 (0.28)[b]	2.71 (0.11)	2.63 (0.18)
BB70	3.12 (0.11)	3.33 (0.19)	2.47 (0.11)	2.60 (0.10)
95% (99%) minimum significant range	0.98 (1.14)		0.73 (0.85)	
After week 4: serum	1 in 2 dilution (= 300 $TCID_{50}$)		1 in 10 dilution (= 60 $TCID_{50}$)	
	Pre-immune	Immune	Pre-immune	Immune
9111	4.10 (0.11)	3.30 (0.24)[b]	4.02 (0.05)	**0.32 (0.17)**[c]
9251	4.06 (0.03)	**2.03 (0.41)**[c]	3.88 (0.21)	**1.07 (0.64)**[c]
9263	4.32 (0.04)	**2.39 (0.59)**[a]	4.14 (0.10)	**2.06 (0.75)**[c]
BB85	4.04 (0.10)	**3.10 (0.46)**[b]	4.20 (0.07)	**0.48 (0.33)**[c]
9258	4.01 (0.04)	4.02 (0.05)	3.91 (0.08)	4.22 (0.06)
BB70	4.09 (0.04)	3.99 (0.12)	4.15 (0.07)	4.41 (0.06)
95% (99%) minimum significant range	1.12 (1.30)		1.36 (1.59)	

Data expressed as mean ± standard error in $\log_{10}$ arbitrary units. Bold indicates 99% significance (Studentised range test used to calculate minimum significant ranges).

[a] 90% reduction in antigen release relative to pre-immune serum.

[b] 50% reduction in antigen release relative to pre-immune serum.

[c] 99% reduction in antigen release relative to pre-immune serum.

Table 5
Neutralization indices of primary HIV-1 isolates by sera from vaccine-protected monkeys using conventional PBMC (24/24/x) assays

Plasma	Primary virus exposed to antibodies					
	SF13		SF162		MN	
	$x = 7$ days	$x = 14$ days	$x = 7$ days	$x = 14$ days	$x = 7$ days	$x = 14$ days
Monkeys immunized with HIV immunogens						
9111	N.D.	N.D.	N.D.	N.D.	0.22	0.00
9251	**1.55**[a]	**1.40**[a]	0.40	0.65	**0.95**[a]	**1.30**[a]
9263	0.60[a]	**0.75**[a]	0.35	0.45	−0.25	−0.35
BB85	**1.05**[a]	**0.70**[a]	N.D.	N.D.	0.15	0.20
Monkeys immunized with Flu immunogens						
9258	0.20	0.10	N.D.	N.D.	0.40	0.10
BB 70	0.15	0.30	0.20	0.10	N.D.	N.D.

Data expressed as neutralization indices: titre of virus following incubation with serum obtained from monkey before any injection, divided by its titre following incubation with sera from the same monkey 2 weeks before challenge. N.D.: not done—no more serum is available to complete this table. Bold indicates a neutralization index ≥0.70 (≥80% reduction in virus titre).

[a] Indicates that a statistically significant reduction in antigen release was seen with at least one virus dose.

Table 6
Neutralization of primary HIV-1 SF13 in 24/1/x ($n = 6$) PBMC assays by plasma from HIV-1 SF2 immunized monkeys challenged with SHIV SF13

Group	Immunogen	Replicate	Monkey	Neutralization indices (days in culture)		Log_{10} antigen reduction				Result of challenge
				Day 7	Day 14	Day 7		Day 14		
						1 in 2[a]	1 in 10[a]	1 in 2[a]	1 in 10[a]	
1	gp120/DNA	1	I 038	−0.05	−0.25	**0.43**	0.15	0.06	−0.13	Infected
		2	Q 062	0.30	**0.75**	**0.34**	−0.01	0.26	−0.18	Infected
		3	I 044	0.15	0.50	0.17	−0.12	−0.12	−0.05	Infected
		3[b]		−0.45	−0.60	−0.58	−0.77	−0.64	−0.35	
		4	X 007	**0.70**	0.60	**0.32**	−0.16	0.02	0.13	Infected
2	rgp120/MF59	1	I 046	**1.25**	**1.30**	**0.57**	0.82	0.40	0.61	Transient infection
		2	T 118	**1.35**	**1.30**	**2.21**	**1.55**	**1.91**	**1.88**	Transient infection
		3	J 041	**1.20**	**1.15**	0.14	0.63	0.14	−0.13	Transient infection
		3[b]		**1.35**	**1.40**	**0.84**	**1.33**	0.59	**2.04**	
		4	Z 064	**1.40**	**1.65**	0.48	**1.83**	−0.01	**1.95**	Transient infection
3	rgp120/ISCOM	1	T 122	**1.20**	**1.20**	0.76	**2.23**	0.05	**1.54**	Protected
		2	L 159	**1.45**	**1.45**	0.62	**1.98**	0.12	**2.12**	Transient infection
		3	X 009	**0.85**	0.60	0.01	0.40	−0.10	0.10	Protected
		3[b]		0.50	**0.75**	0.47	0.85	**0.81**	**1.82**	
		4	Q 048	**1.65**	**1.65**	**0.83**	**1.50**	**0.74**	**2.02**	Transient infection
4	DNA control	1	Q 045	0.00	−0.20	0.16	0.12	0.08	−0.01	Infected
		2	Q 054	−0.30	**0.70**	−0.06	0.06	0.04	0.03	Infected
	MF59 control	3	EP 4	**0.70**	0.60	**0.39**	−0.05	0.33	−0.24	Infected
		3[b]		0.20	0.50	0.11	−0.64	0.17	0.04	
		4	WK 2	0.25	0.45	−0.03	−0.26	−0.22	−0.30	Infected

Bold indicates a neutralization index ≥0.70 (≥80% neutralization). Similarly, reductions in antigen release into culture supernatants reaching statistical significance ($P < 0.05$, Studentised range test) are also highlighted. Minimum significant ranges were calculated for each virus dilution within each group of monkeys.

[a] Virus dilution.

[b] Four plasma, from one monkey in each treatment group, were tested in each replicate. The PBMCs used in replicate 3 may have been more sensitive to primary HIV-1 SF13 since its titre was 7450 infectious units per dose giving 3225 $TCID_{50}$ for the 1 in 2 dilution (instead of a mean of 118 $TCID_{50}$ in the other three replicates) and 745 $TCID_{50}$for the 1 in 10 dilution (instead of 24 $TCID_{50}$). Assays with these plasma were therefore repeated to give replicate 3.

initial site of infection no matter by which transmission route an individual person is exposed. Whether preventing an infectious plasma viraemia does restrict viral replication to the point of entry and result in clinical benefit with a virus which can integrate into the host's genome can only be addressed as part of a Phase III efficacy trial [20]. Hitherto, this hypothesis could not be tested since it has not been possible to demonstrate in vitro neutralization of virus which is as close as possible to that involved in natural transmission events, using vaccine-induced antibodies [2]. However, if antibodies can only slow down viral replication rates, they can not reasonably be expected to prevent an infectious viraemia [21]. In the present paper, we use the neutralization index to quantify all or nothing reductions in viral infectivity. The culture phase is extended so that a single infectious unit of virus has sufficient time to release a detectable quantity of antigen into the culture supernatant (Tables 1 and 3). We show that sera/plasma from monkeys immunized with a first generation candidate HIV-1 vaccine can neutralize a primary HIV-1 isolate. The same sera showed no neutralization in assays with a conventional format of 1 h incubation and allowing 2 h for virus to infect target cells. Statistically significant neutralization was only seen when virus and antibody were allowed to form complexes over extended incubation phases.

The results of the present study are consistent with the hypothesis that protection is determined simply by the magnitude of the immune response. As was seen with passive transfer experiments, the level of neutralizing antibodies in the plasma corresponds with protection [22–25]. Sera from monkeys immunized with the recombinant prime, peptide boost strategy have the highest neutralizing activity, reducing virus infectivity by 98.2–99.2% (neutralization indices of 1.75–2.10, Table 3) with reductions in antigen release of 99.17–99.98% (Table 4). Plasma from monkeys immunized with HIV-1 SF2 gp120 in either ISCOMs or MF59 as adjuvants neutralize 85.0–97.8% of virus (index = 0.85–1.65, Table 6) with reductions in antigen release of 85–99.4% (Table 6). The monkeys immunized with DNA have the lowest immune response: a 1 in 20 dilution of their plasma reduces the release of antigen in cultures infected with 100 $TCID_{50}$ by more than 50%, although virus infectivity is reduced by no more than is seen with plasma from control monkeys. As with humans immunized with placebos in the HVTN trials [18], neutralization close to 80% was recorded

with plasma from control monkeys in the present study, e.g. monkeys Q 054 and EP4 in Table 6.

In the immunized monkeys, the presence of neutralizing antibodies in the plasma correlates with the absence of virions in their circulation. (Although no attempt was made to detect infectious virus in the plasma of the monkeys following challenge in the present study, the absence of viral RNA indicates that the spread of virus through the circulation was prevented.) Following the recombinant prime, peptide boost strategy all four monkeys with the highest level of neutralizing antibodies had sterilizing immunity: no virus was detected by co-culture of monkey cells with PHA-transformed human PBMCs, no virion RNA could be detected in the circulation and there was no anamnestic antibody response [10]. Two of the monkeys immunized with recombinant HIV-1 SF2 gp120 alone in ISCOMs also had sterilising immunity while all four monkeys had no circulating free virions. Monkeys L 159 and Q 048 had provirus in their circulating leukocytes and with the latter these were infectious in co-culture. Monkeys I 046, T 118 and J 041, vaccinated with recombinant HIV-1 SF2 gp120 in MF59 adjuvant, each had circulating proviral DNA but no infectious virus was detected in co-culture. Only the fourth monkey in group 2, Z 064 had a free virion viraemic phase. All four monkeys which were immunized with DNA had viral RNA in their plasma and infectious provirus in their leukocytes [11].

The HIV-1 SF2 recombinant prime, peptide boost vaccination strategy induces antibodies which are highly specific. HIV-1 SF13, HIV-1 MN and HIV-1 SF162 are each very sensitive to losing their infectivity when exposed to neutralizing antibodies [4], although HIV-1 SF162 has previously been reported to resist neutralization in 1/overnight/7 PBMC neutralization assays [26]. The laboratory isolate of HIV-1 MN is neutralized [27] by antibodies raised to the HIV-1 SF2 V2 region peptide used to boost the immune response in the recombinant prime, peptide boost vaccination strategy (Tables 1–5). However, only one monkey (9251) had antibodies able to neutralize the primary isolate of this virus. No significant neutralization was seen with sera from monkeys 9251 and 9263 (Table 5) against HIV-1 SF162.

Neutralization of primary isolates can be demonstrated using antibodies induced by the first generation of HIV-1 vaccine candidates with extended incubation phase assays [12]. However, inducing these antibodies requires a strategy which is not yet licensed for human use. Neutralization of HIV-1 SF162 and HIV-1 Han2 (a virus ‘moderately’ resistant to losing its infectivity [4]) have been demonstrated in assays with extended (24 h) incubation phases using GHOST cells as targets, and plasma from human volunteers in the AIDS Vaccine Evaluation Group protocol 029 [12]. Volunteers were primed with four injections of canary pox expressing, inter alia, HIV-1 MN gp120 and then boosted with two injections of HIV-1 SF2 gp120, giving six injections all together [18] (i.e. three more than a standard human immunization schedule). The recombinant prime, peptide boost vaccination strategy represents a potential substitute for this unacceptable strategy since high levels of neutralizing antibodies were induced with a three-injection schedule. Broader neutralization may be expected by substituting mimotopes to monoclonal antibodies able to neutralize a wide range of primary HIV isolates as boosters in place of the peptides representing linear epitopes and an immunogen able to prime the host’s B-lymphocytes to the epitope’s conformation [28].

We can offer a potential solution to the perceived failure of HIV-1 vaccines to induce the correct antibodies [1]: given a sufficiently potent adjuvant, the immunogens appear to retain a conformation which can potentially induce neutralizing antibodies but it seems unlikely that short-term incubation phase assays would be able to detect such antibodies even if they were present. We, therefore, recommend that assays with extended incubation phases should be used to monitor the neutralizing antibody response of volunteers in Phase III trials [20]. One caveat should be added. Primary HIV-1 SF13 is very sensitive to neutralization, producing an all or nothing loss in its infectivity [4]. The correlation between the results from in vitro assays and in vivo vaccine-induced protection may not hold if neutralization resistant isolates which can still replicate, albeit at a reduced rate, following exposure to antibodies are introduced directly into the bloodstream. However, when these resistant viruses are transmitted heterosexually, vaccination may achieve the same effect (i.e. preventing an infectious plasma viraemia), if viral replication can be slowed down to the point where cell-mediated or innate immune responses can control the virus infection. In the present paper, we have demonstrated that antibodies can neutralize HIV in dendritic cell/T4 lymphocyte co-cultures which simulate the early stages following heterosexual transmission [29].

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