Long-term protection against SIV-induced disease in macaques vaccinated with a live attenuated HIV-2 vaccine

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The aim of this study was to test the ability of a live attenuated human immunodeficiency virus type 2 (HIV-2) vaccine to protect cynomolgus monkeys against superinfection with a pathogenic simian immunodeficiency virus (SIV_{sm}) This report is an update on our previously reported observation period of nine months. The new data here show that three of four monkeys vaccinated with live HIV-2 were protected against immunosuppression and SIV-induced disease during more than five years of follow-up. The quality of the immunity was permissive for infection, but monkeys that survived showed restricted viral replication in peripheral blood and lymph nodes. This study shows that it is possible to induce protection against a pathogenic heterologous primate lentivirus and to prevent disease in vaccinated monkeys even if infection is not prevented. These findings provide evidence that protection against AIDS can be achieved by immunization.

Human immunodeficiency virus (HIV) infection and AIDS are now pandemic and the development of a prophylactic vaccine against HIV has a very high priority. A useful vaccine against HIV and AIDS must be able to elicit broadly cross-protective responses against diverse strains of HIV. In the present study we have addressed this question by using the SIV macaque model[1]. This animal model is appropriate because the macaque species is a close phylogenetic relative of humans and because simian immunodeficiency virus (SIV) and HIV-1 infections follow similar disease courses[2].

Based on sequence homology and biological characteristics certain strains of SIV are the closest known relatives of HIV, particularly HIV-2 (ref. 3). We have previously shown that cynomolgus monkeys can be experimentally infected with SIV_{sm} (ref. 4) as well as HIV-2 (ref. 5) The clinical outcome of infections with these viruses differ. SIV_{sm} infection of cynomolgus monkeys leads to a fatal AIDS-like disease and death within 2–26 months[2]. Furthermore, SIV_{sm}-infected macaques exhibit progressive lymph node changes similar to those seen in HIV infection in humans[2]. In contrast, certain strains of HIV-2 that have been propagated in human cell lines replicate poorly *in vivo* and HIV-2-infected macaques do not develop disease[5].

It would be optimal if a vaccine against HIV would induce sterilizing immunity. However, successful live viral vaccines in current use in humans such as vaccines against yellow fever, adenovirus, polio, measles, and rubella do not prevent an initial infection but rather limit viral replication resulting in prevention or delay of disease[6]. Several SIV vaccine candidates have been tested in the SIV macaque model[1]. Vaccination of macaques with SIV envelope vaccines has been reported to have the capacity to reduce the viral load early after infection[7,8]. There are reports of vaccination delaying SIV-induced disease, which include the use of live attenuated vaccines[9], whole inactivated virus[10,11], and peptide vaccines[12].

A recent encouraging report from Daniel *et al.*[13] demonstrates that a live attenuated SIV_{mac} vaccine with a deletion in the *nef* gene protects macaques against challenge with a high dose of homologous virus. In 1990 we showed that an HIV-2 infection restricted the replication of a subsequently administered pathogenic SIV_{sm} and prevented acute early immunosuppression and death[14]. The previously published observation time of nine months has now been extended to more than five years. Here we demonstrate that the monkeys vaccinated with a live attenuated vaccine and challenged with a heterologous pathogenic SIV_{sm} were protected against SIV-induced disease.

Outcome of immunization with live attenuated HIV-2

Four cynomolgus monkeys (Table 1) were first inoculated with live HIV-2 (referred to as vaccinated animals) and then superinfected with 10–100 monkey infectious doses of pathogenic SIV_{sm} to study protective immunity. After HIV-2 inoculation (for details of the outcome of HIV-2 infection, see ref. 14), virus could be isolated from the peripheral blood mononuclear cells (PBMC) for one month, and seroconversion was observed in all four monkeys four to six weeks after inoculation with HIV-2. At the day of SIV_{sm} challenge the HIV-2-inoculated monkeys were clinically healthy with

normal CD4 counts. Attempts to isolate HIV-2 from PBMC on the day of SIV_{sm} challenge were unsuccessful, but HIV-2 DNA was detected by PCR. Before SIV_{sm} challenge, the vaccinated animals had neutralizing antibodies against HIV-2 as previously published[14]. In contrast, none of the HIV-2-infected animals developed detectable neutralizing antibody titres against SIV_{sm} before challenge (Table 2). Western blot analysis showed that sera from the HIV-2-inoculated animals contained antibodies that reacted with HIV-2 *gag*, *pol* and *env* structural proteins.

Clinical outcome of pathogenic SIV_{sm} challenge

The ability of the live attenuated HIV-2 vaccine to protect against SIV infection and disease was assessed by exposure of the four vaccinated monkeys and four naive controls to a heterologous pathogenic SIV_{sm} isolate. All four control monkeys showed declines in CD4+ lymphocytes after SIV_{sm} challenge (Fig. 1) and by two years and two months they had either died or had to be killed because of clinical signs of AIDS (Table 1). All vaccinated animals survived longer than the SIV_{sm} controls. Three of four vaccinees have not shown any disease and have maintained normal levels of CD4+ cells for more than five years after challenge (Fig. 1). Starting at about 16 months after SIV_{sm} inoculation, a decline in CD4+ T cells was observed in the vaccinee H7 and 41 months after challenge the monkey died with clinical signs of simian AIDS and low CD4 count.

Viral antigenemia and virus isolation after SIV_{sm} challenge

Two weeks after SIV_{sm} challenge, viral antigen in plasma was detectable in all four controls but in none of the four vaccinated monkeys. Virus was repeatedly isolated from PBMC of the four controls throughout the course of infection (Table 3). In contrast, virus was isolated from PBMC of the vaccinated monkeys once or twice during the first two to six weeks and thereafter only occasionally despite repeated attempts (Table 3). However, virus was isolated from lymph node cells of monkey H8 and K26 from a four- and five-year biopsy. Beginning at 16 months after SIV_{sm} inoculation, virus was repeatedly isolated from PBMC of vaccinee H7, a total of eleven positive cultures was collected before death.

Serology

On the day of challenge the vaccinated macaques had no detectable neutralizing antibodies against SIV_{sm}, but they developed strong anamnestic antibody responses postchallenge as determined by whole-virus ELISA (data not shown) and by neutralizing assay (Table 2). Superinfection induced a rapid increase in the neutralization titres to SIV_{sm} in all four macaques in the vaccine group (Table 2). This effect was not seen in sera of unvaccinated controls sampled one month after challenge.

To rule out the possibility that protection was due to anti-cellular mechanisms[15], we assessed the presence of anti-cellular antibodies. Sera from the vaccinated animals were examined by flow cytometry for their ability to bind human PBMC. Sera taken on the day of challenge showed no increase in the binding of human PBMC when compared with sera taken before the injection of HIV-2.

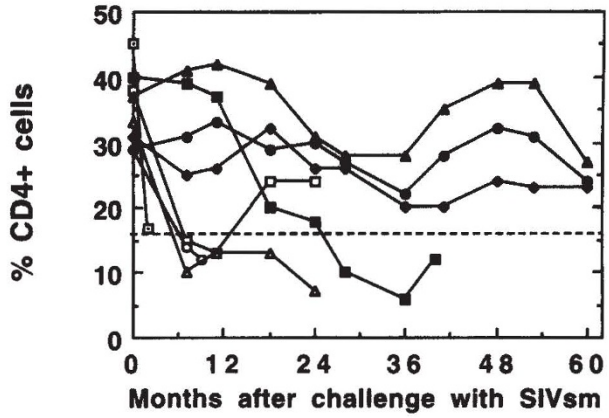

Fig. 1 Percentage of CD4+ cells in blood after SIV_{sm} superinfection of four cynomolgus monkeys vaccinated with live HIV-2 [H2 (▲), H7 (■), H8 (●), K26 (◆)] and in four control monkeys inoculated with SIV_{sm} only [H19 (⊡), H20 (□), H25 (○), H26 (△)]. Lower normal limit of percentage of CD4+ T cells in blood from a reference group of 200 healthy cynomolgus monkeys (- - - -).

Differential PCR analysis after SIV_{sm} challenge

On the day of challenge all vaccinated monkeys were positive for HIV-2 but negative for SIV by PCR (Table 3). Twelve days after SIV challenge, all four vaccinated monkeys showed a mixed PCR pattern with detection of both SIV and HIV-2. In the following samples (27 days and four months) two of four monkeys showed a mixed pattern, but in one monkey (K26) only SIV was detected. Between one and four years after challenge we were not able to amplify any viral DNA from two of four vaccinated monkeys. At five years after challenge SIV DNA was detected in all three surviving vaccinated macaques. The PBMC samples from the SIV-inoculated controls were always PCR positive for SIV only.

Histopathology and immunohistochemistry

Histopathological findings in lymph nodes of the four unvaccinated SIV-infected controls revealed hyperplasia of follicular B-cell areas at early stages of infection, whereas progressive disease was associated with follicular fragmentation, atrophy and depletion. Immunostaining (for details see ref. 14) showed SIV antigens associated with the follicular dendritic cell (FDC) network, but the

Table 1 Long-term outcome and survival of macaques vaccinated with live HIV-2 and challenged with SIV_{sm} and of naive control animals

Animal no.	Primary infection	Time from HIV-2 inoculation to SIV challenge	Months to death	Clinical signs of infection
H2	$HIV\text{-}2_{SBL\text{-}K135}$	5 months	Alive	Clinically normal
H7	$HIV\text{-}2_{SBL\text{-}K135}$	"	41	LAS
H8	$HIV\text{-}2_{SBL\text{-}K135}$	"	Alive	Clinically normal
K26	$HIV\text{-}2_{SBL\text{-}6669}$	9 months	Alive	Clinically normal
H19	None	-	2	LAS, WL, D
H20	None	-	26	LAS, S, WL, D
H25	None	-	9	LAS, S, WL
H26	None	-	25	LAS, S, WL, D

The observation time is five years postchallenge with SIV_{sm}.
LAS, lymphadenopathy syndrome; WL, weight loss; D, diarrhoea; S, splenomegaly.

amount of demonstrable antigen decreased markedly in relation to the degree of FDC destruction and follicular involution. In contrast, three of the surviving vaccinated monkeys (H2, H8 and K26) showed neither histopathological changes nor demonstrable SIV or HIV-2 antigens in lymph nodes taken at two, three, four and five years after challenge. Monkey H7 had detectable viral antigen associated with follicular hyperplasia in a two-year biopsy and a terminal sample at 41 months revealed evidence of follicular fragmentation with a low number of FDCs with associated SIV viral antigen.

Table 2 Neutralizing antibody titres against SIV_{sm} in macaques before and after SIV_{sm} challenge

Animal	*Neutralization titre* At the day of SIV challenge	One month after challenge	One year after challenge
Vaccinated			
H2	<10	160	40
H7	<10	160	>320
H8	<10	160	320
K26	<10	80	>320
Naive animals			
H19	<10	<10	ND
H20	<10	<10	40
H25	<10	<10	320[a]
H26	<10	<10	>320

ND, not done.
[a]Monkey H25 died and was sampled on day 265.

Long-term follow-up of vaccinated controls not exposed to SIV

Two monkeys inoculated with HIV-$2_{SBL\text{-}K135}$ and followed up for five years, and two monkeys inoculated with HIV-$2_{SBL\text{-}6669}$ and followed up for two years were virus isolation positive for two to four weeks after inoculation but not thereafter. All four monkeys remained healthy with normal CD4 values during the observation period and showed no histopathological lesions in lymph nodes.

Discussion

The present study shows that a live attenuated HIV-2 vaccine can induce long-term protection in cynomolgus monkeys against SIV-induced disease and death, even though there was no protection against infection. All vaccinated monkeys clearly became superinfected with SIV_{sm} but the vaccine provided sufficient immune priming to control SIV replication and protect against declining $CD4^+$ lymphocyte counts in three of four vaccinated monkeys. The fourth vaccinated monkey that ultimately developed AIDS still lived almost twice as long as the last survivor in the control group. The SIV-infected controls had abundant viral antigen in the lymph nodes, whereas successfully vaccinated animals had no demonstrable viral antigen, suggesting a greater

Table 3 Isolation of virus from PBMC (I) and discrimination between HIV-2 and SIV_{sm} by PCR analysis (P) from monkeys vaccinated with live HIV-2 and challenged with SIV_{sm} and control monkeys infected with SIV_{sm} only.

	Monkeys vaccinated with live HIV-2 and challenged with SIV								Control monkeys infected with SIV only							
Time after SIV inoculation	H2		H7		H8		K26		H19		H20		H25		H26	
	I	P	I	P	I	P	I	P	I	P	I	P	I	P	I	P
0 days	–	(H)	–	(H)	–	(H)	–	(H)	–		–		–		–	
12 "	+	(S/H)	+	(S/H)	+	(S/H)	+	(S/H)	+	(S)	+	(*)	+	(*)	+	(S)
20 "	+		–		–		–		–		+		+		+	
27 "	–	(S)	–	(S/H)	–	(S/H)	ND	(S)	ND	(S)	ND	(S)	+	(ND)	+	(S)
41 "	–		–		+		ND		ND		ND		+		+	
70 "	–		–		–		–		+		–		+		+	
3 months	–		–		–		ND				+		+		–	
4 "	–	(H)	–	(S/H)	–	(S/H)	+	(S)			+	(S)	–	(S)	+	(S)
9 "	–		–		–		–				+		+		+	
1 year	–	(–)	–	(S)	–	(ND)	–	(–)			+	(S)			+	(S)
2 "	–	(–)	+	(S)	–	(H)	–	(–)					+		+	
3 "	–	(–)	+	(S)	–	(S)	–	(–)								
4 "	–	(–)			–	(S)	–	(–)								
5 "	–	(S)			–	(S)	+	(S)								
Virus recovery[a]	3/27		12/21		4/27		6/23		2/3		11/12		7/8		13/15	

+, virus isolated; –, negative virus isolation; (H), HIV-2 DNA detected by PCR; (S), SIV DNA detected by PCR; (S/H), both SIV and HIV-2 DNA detected by PCR; (–), negative PCR for both SIV and HIV-2 DNA; ND, not done; (*), not determinable for technical reasons.
[a]Number of times virus isolation from PBMC was successful divided by the number of times virus isolation was attempted throughout the period of infection.

ability of their immune system to control viral replication *in vivo*. Histopathologic examination showed that the controls with disease exhibited characteristic follicular involution in lymph nodes[2], whereas the protected animals had preserved lymph node architecture for at least five years after SIV challenge. These findings in the protected animals are strikingly similar to histopathological, immunologic and virologic characteristics seen in human long-term survivors with nonprogressive HIV-1 infection[16,17].

To date, the most successful immunization approach against SIV has been a live attenuated vaccine[9,13]. The present study extends these findings by demonstrating induction of cross-protective immunity against disease and immunosuppression. To our knowledge this is the first published report of successful long-term prevention of disease in macaques with vaccination where the main end point is death from AIDS in all controls. The results are even more encouraging since a heterologous challenge virus was used. HIV-2 and SIV_{sm} are as similar as members of different HIV-1 subtypes[18]. Thus, our results suggest that it may be possible to induce cross-protection with a replicating HIV-1 vaccine. The finding that suppressed viral replication in the vaccinated monkeys was associated with prevention or delay of disease suggest that much can be achieved with an HIV vaccine that allows infection and limited replication, even if sterilizing immunity would be the ideal.

The major disadvantage of attenuated vaccines is the possibilty of their reversion to a virulent form and recent evidence shows that neonatal monkeys get AIDS with this type of vaccine[19]. Thus live attenuated vaccines against HIV and AIDS in humans will probably only be considered if approaches involving non-infectious agents fail. However, the use of attenuated vaccines in monkeys represents a model to study the mechanisms involved in suppression of virus replication. In the present study, none of the vaccinees had detectable neutralizing antibodies on the day of challenge against the incoming virus. However, challenge had a significant effect in boosting the neutralization response. This finding suggests that down-regulation of viral replication was associated with the ability of the host to rapidly mount a neutralizing antibody response after challenge. This induction of immunological memory following challenge is probably of critical importance. Although only humoral immune responses were characterized in the present study, it is likely that cell-mediated immune responses specific to SIV were induced and that these also may contribute to the control of the virus. Live attenuated vaccines have proved successful for many viruses, presumably because they induce $CD8^+$ cytotoxic T lymphocytes and immunological memory[20].

We conclude that an HIV vaccine can elicit long-term protection against immunosuppression and disease caused by a heterologous immunodeficiency virus, even if prevention of infection is not achieved. This raises hope that a similar long-term protection against disease caused by heterologous HIV strains can also be elicited by HIV vaccines in humans. Thus, these findings may have considerable relevance for human vaccination against HIV-1 and provide hope that an effective vaccine against AIDS can be developed.

Methods

Experimental design. Twelve cynomolgus monkeys (*Macaca fascicularis*), both males and females of various ages, were used in this study. Eight cynomolgus monkeys were vaccinated by intravenous (i.v.) inoculation with a single injection of cell-free HIV-2. Five animals (H2, H3, H4, H7 and H8) were inoculated i.v. with 1 ml of undiluted cell-free supernatant containing $HIV\text{-}2_{SBL\text{-}K135}$ (ref. 21), and the remaining three animals (K26, H5, H6) were vaccinated by i.v. inoculation of 1 ml of cell-free $HIV\text{-}2_{SBL\text{-}6669}$ (ref. 22). These eight inoculated macaques were divided into two groups. The first group (monkeys H2, H7, H8 and K26) were later challenged together with four control animals with 10–100 monkey infectious doses of pathogenic SIV_{sm} given intravenously to test whether superinfection occurs. The challenge virus was grown on fresh human cells[14]. The second group of four monkeys (H3–H6) were followed to assess the virulence of the two HIV-2 strains used for vaccination.

Determination of infection after immunization and challenge. Signs of infection were monitored by virus isolation from monkey PBMC or lymph node cells, seroconversion and by polymerase chain reaction (PCR). The techniques of virus isolation from monkey PBMC or lymph node cells and viral antigen detection in serum have been described elsewhere[23].

Two nested PCR approaches were used to distinguish between SIV and HIV-2 and were performed essentially as previously described[23,24]. Details will be presented elsewhere (L. Walther *et al.*, manuscript in preparation). In the first discriminative PCR, two separate sets of nested primers were used; one specific for SIV vif-vpx-vpr region and the other for HIV-2 LTR region. The sensitivity and specificity was 100% when 38 samples from monkeys infected with $HIV\text{-}2_{SBL\text{-}6669}$ and 43 monkeys infected with SIV_{sm} were analysed with this PCR assay. The other discriminative PCR took advantage of the fact that the HIV-2 LTR contains a conserved insertion of 40–44 base pairs as compared with SIV LTR (ref. 25).

Antibody titres were determined by whole-virus ELISA[2], by western blot[2] and by an assay for neutralizing antibody. A virus neutralizing assay was used as described previously[26] with the following modifications. Thirty 50% infectious doses (ID_{50}) of virus was used in the neutralization assay with duplicated wells in 96-well plates. All serum samples were tested against HIV-2 and SIV_{sm} at the same time.

To evaluate the rate of disease progression, clinical parameters, determination of T-lymphocyte subsets and evolution of histologic changes within lymph nodes were monitored as previously described[14].

Acknowledgements

We thank E. Norrby, H. Wigzell and E-M Fenyö for helpful cooperation, and Silvia Marquina, Kerstin Hild and Reinhold Benthin for expert technical assistance. We thank H. McClure for providing the SIV isolate and Olov Grankvist for helpful advice with PCR primers. This work was supported by grants from the Swedish Medical Research Council, Swedish Agency for Research Cooperation with Developing Countries (SAREC) and from the Swedish National Board for Industrial and Technical Development (NUTEC).

RECEIVED 27 FEBRUARY; ACCEPTED 20 JULY 1995

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