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Vaccine protection against simian immunodeficiency virus infection

(acquired immunodeficiency syndrome/immunization/animal model/*Macaca mulatta*)

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ABSTRACT **Rhesus monkeys were immunized by multiple inoculations with purified, disrupted, noninfectious simian immunodeficiency virus (SIV) in adjuvant. Immunized monkeys developed anti-SIV antibodies detectable by whole-virus ELISA and by immunoblot reactivity; these antibodies had weak neutralizing activity. One week after the last immunization, monkeys were challenged with 200–1000 animal infectious doses of uncloned, live SIV. The same strain of SIV that was used for vaccination was also used for challenge. Anamnestic antibody responses and SIV recovery from peripheral blood were used to evaluate infection following the live virus challenge; two of six vaccinated monkeys showed no evidence of infection following the live virus challenge. Transfusion of 10 ml of whole blood from these two into uninfected, naive rhesus monkeys did not result in infection of the recipients, providing further support for the lack of infection in the two previously vaccinated animals. Four of four unvaccinated control monkeys inoculated with these doses of live SIV became infected and three of these died with AIDS 118–258 days after infection. Only one of the six vaccinated monkeys has died to date. *In situ* hybridization with lymph node biopsy specimens suggested that the virus load was much higher in control macaques than in vaccinated macaques. These results indicate that vaccination with inactivated whole virus can protect macaques against challenge with live SIV. Furthermore, they provide hope that vaccine protection against human AIDS virus infection may be possible.**

Development of safe, effective vaccines against the human immunodeficiency virus (HIV) is likely to be a very difficult task. The reasons for this pessimism stem as much from the biological properties of the HIV lentivirus as from the failure of initial HIV vaccine trials in chimpanzees. HIV, like other lentiviruses, has a remarkable ability to persist and to eventually induce a chronic, debilitating disease in spite of an apparently strong host immune response to the virus. HIV-infected individuals may remain clinically well for years while maintaining easily detectable humoral and cellular immune responses, only to succumb eventually to the virus. Initial attempts to immunize chimpanzees with subunit and vaccinia recombinant vaccines have failed to prevent persistent infection following challenge by homologous, live virus (refs. 1 and 2; L.O.A., unpublished data). Vaccine strategies are complicated even further by the wide variety of strain types that exhibit little or no cross-neutralization (3).

The simian immunodeficiency viruses (SIVs) are ideal in many respects for studying AIDS vaccine strategies. SIVs are the closest known relatives of the HIVs, with extensive homology gene-for-gene along the genome (4, 5). The SIVs have a lentiviral morphology, have a propensity to replicate in lymphocytes and macrophages, use the CD4 molecule as receptor, and persist *in vivo*; these properties are all similar to those of the HIVs. Some stocks of SIV are able to induce in rhesus monkeys (*Macaca mulatta*) a disease remarkably similar to AIDS in humans. This AIDS disease has a high mortality in a time frame suitable for laboratory investigation (6–8). Rhesus monkeys are not endangered in the wild, breed well in captivity, and can be made available in large numbers for such studies.

In this report we present results of our initial attempts to use inactivated whole virus to protect macaques against SIV infection and disease induction.

METHODS

Virus and Cells. Early passages of the uncloned SIVmac251 isolate were used for this study (9). Phytohemagglutinin-stimulated normal human peripheral blood lymphocytes (PBLs), growing in medium containing interleukin 2, the human T-cell lymphoma line HuT 78, and the hybrid cell line CEMx174 were used as indicator cells for virus-recovery attempts from peripheral blood and lymph nodes of inoculated macaques. On several occasions, $CD8^+$ lymphocyte depletion was employed to assist virus recovery (10).

Animal Titration. The live SIVmac251 used for challenge in the second and third vaccine studies was titered in animals. Passage 1, PBL-grown SIVmac251 was inoculated onto HuT 78 cells and 27 days later more than 200 vials of filtered, cell-free supernatant virus in RPMI 1640 with 15% fetal bovine serum were stored in liquid nitrogen. Per milliliter, this stock contained 420,000 cpm of reverse transcriptase activity, measured as described (11). Prior to the second vaccine study, 10-fold dilutions (10^{-1}–10^{-5}) of this stock were prepared in RPMI 1640 medium without serum, and 1 ml of each dilution was inoculated intramuscularly into a rhesus macaque. All five macaques became infected. After the third vaccine study was begun, we prepared 10^{-6} and 10^{-7} dilutions of this same stock and 1 ml of each dilution was inoculated intramuscularly into a macaque. The rhesus macaque that received the 10^{-6} dilution became infected but the rhesus macaque that received the 10^{-7} dilution did not become infected. Infection was monitored by SIV recovery from peripheral blood and by antibody responses, using blood samples taken periodically following inoculation. We thus consider this stock to contain $\approx 10^6$ animal infectious doses per ml as measured by intramuscular inoculation. When tested in CEMx174 and other cultured cells, this stock was found to contain $10^{3.5}$–10^4 tissue culture infectious doses (TCID) per ml. The TCID titer was not found to change appreciably over 18 months of storage in liquid nitrogen.

Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; TCID, tissue culture infectious dose; PBL, peripheral blood lymphocyte.

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Table 1. First vaccine study

Animal number	Antibody titer* ELISA doc	ELISA 2 wpc	Neutralization doc	Neutralization 2 wpc	SIV recovery	Outcome†
			Vaccinated group			
68-85	2560	20,480	16	2560	+	Alive
205-85	1280	10,240	16	2560	+	258 dpc
206-85	2560	20,480	32	2560	+	347 dpc
274-85	1280	10,240	32	2560	+	275 dpc
			Unvaccinated group			
120-85	0	0	0	0	+	257 dpc
121-85	0	0	0	0	+	367 dpc
248-85	0	0	0	0	+	125 dpc
249-85	0	0	0	0	+	551 dpc

Vaccination. Four juvenile rhesus macaques 7–11 months of age received three inoculations of an inactivated whole-virus vaccine spaced 3 weeks apart. Four juvenile rhesus macaques 8–11 months of age receiving no vaccine served as controls. Sucrose gradient-purified SIVmac (uncloned strain 251) was treated with formalin (1:4000) at 37°C for 2 days; treated virus was not infectious in cell culture and vaccinated macaques had no recoverable virus in their peripheral blood prior to live virus challenge. The inactivated whole virus was delivered to macaques by intramuscular inoculation with the threonyl muramyl dipeptide adjuvant/vehicle mixture of Syntex (14). Each macaque received 75 μg of protein per inoculation. *Challenge.* Early-passage SIVmac251 grown on normal human PBLs was used for macaque inoculation 18 weeks after the first vaccination (12 weeks after the last vaccination). Infected cells were removed by centrifugation, the supernatant was filtered, and 2 ml of the undiluted cell-free virus was inoculated intravenously. This supernatant had a reverse transcriptase activity of 36,000 cpm/ml and contained $\approx 10^4$ $TCID_{50}$ per ml.

*Serum was collected on the day of challenge (doc) and 2 weeks postchallenge (2 wpc). Binding antibodies were determined by ELISA and neutralizing antibodies were determined by virus neutralization in the MT4 cell killing assay (see *Methods*).

†Macaques were either found dead or sacrificed when death appeared imminent on the indicated days postchallenge (dpc). Macaque 68-85 was still alive 930 dpc. Three of the four vaccinated–challenged macaques died with clinical signs and autopsy findings of AIDS 258–347 dpc. Postmortem examinations revealed that all three animals had profound thymic atrophy and lymphocytic depletion of lymphoid organs; two of the three (205-85 and 274-85) had *Pneumocystis carinii* pneumonia and colonic trichomoniasis, and one (205-85) also had adenoviral pancreatitis. Necropsy findings revealed that three out of the four unvaccinated control animals (121-85, 248-85 and 249-85) died with SIV-induced meningoencephalitis and thymic atrophy. One of these (121-85) also had *Pneumocystis carinii* pneumonia, adenoviral pancreatitis, and giant-cell lymphadenopathy. Another (249-85) had generalized *Mycobacterium avium–intracellulare* infection. The fourth animal (120-85) had marked lymphadenopathy; generalized B-cell proliferation in the lung, kidney, bone marrow, thymus, stomach, and skin; pulmonary thrombosis; and hemorrhagic enterocolitis.

Vaccine Preparation. Procedures for sucrose gradient (12) and column (13) purification of virus have been described. Formalin-inactivated (study 1) or detergent-disrupted (studies 2 and 3) SIV was mixed with the adjuvant/vehicle mixture of Syntex (14) and ≈1 ml was inoculated intramuscularly. The adjuvant/vehicle mixture contained 0.1% Tween 80, 2.5% pluronic L121, 5% squalene, and 100–200 μg of threonyl muramyl dipeptide per ml.

Antibody Measurement. Procedures for measurement of binding antibodies by whole-virus ELISA and by immunoblot and of neutralizing antibodies in the sensitive MT4 cell killing assay have been described (7, 13, 15). ELISA titers refer to the serum or plasma dilution that yielded an absorbance at 410 nm of 0.1. Sera or plasma from all animals yielded values of <0.1 at a 1:20 dilution prior to inoculation with vaccine materials or live virus.

Table 2. Second vaccine study

Animal number	Antibody titer (doc)* ELISA	Neutralization	Anamnestic response†	SIV recovery	Outcome‡
		Vaccinated group			
201-86	20,480	320	No	–	Alive
221-86	20,480	160	24–40 wpc	+	311 dpc
		Unvaccinated group			
165-86	0	0	—	+	118 dpc
188-86	0	0	—	+	258 dpc

Vaccination. Two juvenile rhesus macaques began receiving a total of five inoculations with inactivated whole-virus vaccine at ≈7 months of age; they received three inoculations spaced 3 weeks apart, and 4 months later they received two inoculations spaced 2 weeks apart. Two other juvenile rhesus macaques of similar age served as unvaccinated controls. SIVmac251 purified by Sepharose 4B column chromatography (13) was pelleted and resuspended at 1/10,000th the initial volume (≈20 mg of protein per ml) in 0.5% Triton X-100 in phosphate-buffered saline. This disrupted preparation lacked infectivity in cell culture and vaccinated macaques had no recoverable virus in their peripheral blood. The inactivated whole virus was diluted in phosphate-buffered saline and delivered to macaques by intramuscular inoculation with the threonyl muramyl dipeptide adjuvant/vehicle mixture of Syntex (14). Each macaque received ≈400 μg of protein per inoculation. *Challenge.* One week after the last immunization, each animal was inoculated intramuscularly with 1000 animal infectious doses of live SIVmac251 (see *Methods*).

*Antibody titers were determined by ELISA titration and by virus neutralization, using serum collected on the day of live virus challenge (doc).

†Antibody titers were determined by ELISA titration, using serum collected after inoculation with live SIVmac (see Fig. 2*A*); wpc, weeks postchallenge.

‡Macaques either were found dead or were sacrificed when death appeared imminent on the indicated days postchallenge (dpc). Macaque 201-86 was still alive 590 days following live virus challenge. Postmortem examination of the other vaccinated animal, 221-86, revealed that it died of overwhelming bacterial sepsis with disseminated thromboembolic complications. This was manifested by a purulent meningoencephalitis, splenic abscesses, widespread somatic and visceral hemorrhages, thromboses, and renal and splenic infarction. The source of the infection was not determined. The thymus and lymph nodes were only mildly depleted of lymphocytes. Pathologic examination revealed that one of the two unvaccinated animals, 165-86, had SIV-induced meningoencephalomyelitis, moderate lymphocytic depletion of lymph nodes, and a severe, diffuse interstitial pneumonia. The second control animal, 188-86, had profound thymic atrophy, giant-cell lymphadenopathy, fibrinous pneumonia with giant-cell infiltrates, atrophy of intestinal mucosa with giant-cell infiltrates, colonic trichomoniasis, and granulomatous peritonitis due to intestinal perforation.

Pathology. All animals that either died or were euthanized as part of these studies were subjected to complete postmortem and histopathologic examination to determine the nature and distribution of lesions as well as the cause of death.

***In Situ* Hybridization.** Procedures for *in situ* hybridization have been described (16, 17).

RESULTS

First Vaccine Study. In our initial immunization study, four rhesus monkeys received three inoculations of formalin-inactivated SIV in the adjuvant/vehicle mixture; four rhesus monkeys served as unvaccinated controls (Table 1). Vaccinated macaques developed anti-SIV antibodies as measured by whole-virus ELISA (Table 1); these antibodies had very weak but detectable neutralizing activity (Table 1) in the sensitive MT4 cell killing assay for neutralization (7). Vaccinated macaques were challenged 12 weeks after the last

immunization by intravenous inoculation of undiluted SIVmac (10^3–10^4 $TCID_{50}$) grown in human PBLs. All eight macaques in this study became infected; SIV was recovered from peripheral blood of all animals at weeks 1, 2, 4, 8, and 12 after challenge and a dramatic anamnestic antibody response was evident by 2 weeks after challenge (Table 1).

All four unvaccinated macaques died with clinical signs and autopsy findings of AIDS 125–551 days after the live virus challenge; three of the four vaccinated–challenged macaques also died with clinical signs and autopsy findings of AIDS, 258–347 days after the live virus challenge. These are summarized in Table 1. The fourth vaccinated–challenged macaque remains persistently infected and healthy 30 months after the live virus challenge.

Second Vaccine Study. We changed a number of parameters in our second vaccine study in order to enhance the likelihood of a successful vaccine protection (Table 2). Column-purified SIV rather than sucrose gradient-purified virus was used in the vaccine preparation, to better preserve the external envelope glycoprotein gp120. Previous studies have documented the underrepresentation of this protein in even the crudest of HIV and SIV preparations (12, 18). The regimen of inactivated virus administration was changed so that animals received more doses and larger amounts of viral protein per dose. Furthermore, animals were challenged 1 week after the last immunization—i.e., near the peak of immunization-induced anti-SIV antibody titer. We used intramuscular challenge with live SIV. Finally, we controlled the dose of live challenge virus, using a 10^{-3} dilution of an SIV stock stored in liquid nitrogen; this stock had been titered in animals (see *Methods*).

This vaccination regimen did result in higher anti-SIV antibody titers on the day of live virus challenge (Table 2). By immunoblot analysis, post-immunization sera recognized primarily p17 and p26 gag proteins; reactivity was also detected to the external envelope glycoprotein gp120 on immunoblots (Fig. 1). Sera from vaccinated macaques showed weak but readily detectable neutralizing activity (Table 2). The neutralization titers were correspondingly higher than in the first study (Tables 1 and 2). These neutralizing titers are still 1–2 orders of magnitude lower than those observed in macaques infected with SIV (7).

Following challenge with 1000 animal infectious doses of live SIV, SIV was recovered from the peripheral blood of both the control unvaccinated macaques, and both control animals died with AIDS, 118 and 258 days after inoculation (Table 2). SIV was not recovered from either of the vaccinated macaques during the first 28 weeks following challenge. SIV was recovered, however, from peripheral blood samples of macaque 221-86 at weeks 32, 36, and 39 and on the day of death at week 44. An anamnestic response did appear in 221-86 but it was not evident until more than 24 weeks after the live virus challenge (Fig. 2*A*). Postmortem examination of 221-86 revealed that this animal died of overwhelming bacterial sepsis with disseminated thromboembolic complications (Table 2).

Rhesus monkey 201-86 has been observed for more than 18 months after the challenge and appears to have been protected against infection. No anamnestic antibody response was detected at any time in this animal (Table 2; Fig. 1; Fig. 2*A*) and by 18 months after challenge, his antibody titer had declined to a low level. SIV has never been recovered from his peripheral blood, even with the use of $CD8^+$ lymphocyte depletion to assist virus recovery. Eighteen months after the live virus challenge, 10 ml of heparinized whole blood from 201-86 was transfused into a naive rhesus monkey; the recipient did not become infected. On at least 10 separate occasions we have infected naive recipients by transfusing whole blood or tissue extracts from SIV-infected animals. In fact, we have never failed to infect recipients by such transfusions from animals known to be infected with SIV.

Lymph node biopsies were taken from vaccinated macaques 201-86 and 221-86 and from the unvaccinated control 188-86 at 190 days after the live virus challenge. Minced lymph node cultures yielded SIV from the control 188-86 macaque but not from the vaccinated 201-86 and 221-86 macaques.

Third Vaccine Study. Four rhesus monkeys received disrupted-whole-virus vaccination very similar to that in the previous study, and comparable binding and neutralizing antibody titers were detected on the day of live virus challenge (Tables 2 and 3 and Fig. 1). Two animals served as unvaccinated controls. All animals in the third study received an intramuscular challenge with 200 animal infectious doses of the same stock of SIVmac251.

As expected, both control macaques became infected with this dose and to date one has died with AIDS, 196 days after inoculation (Table 3).

Of the four vaccinated macaques, SIV was recovered from peripheral blood samples of three (36-83, 37-83, and 444-82) on at least four separate occasions (Table 3). These same three animals also had anamnestic antibody responses (Fig.

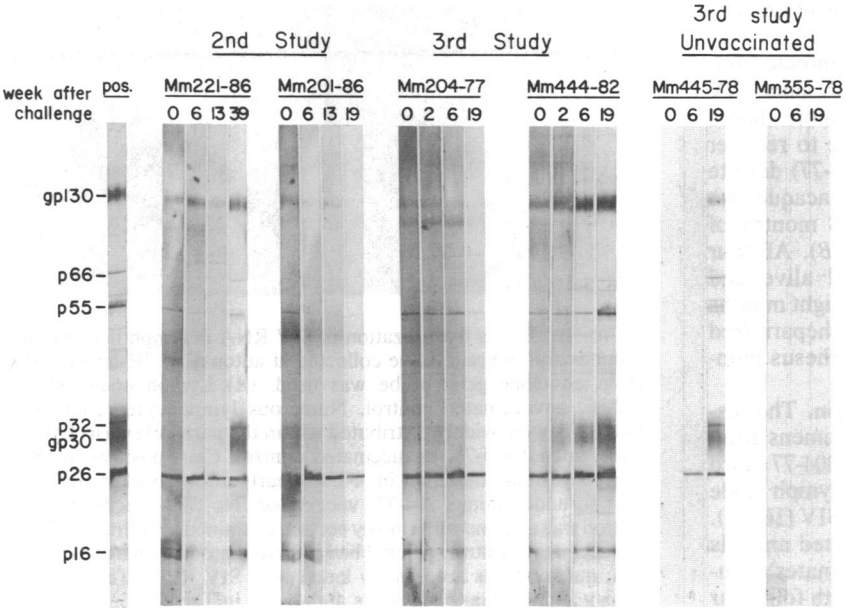

FIG. 1. Immunoblot reactivity of *M. mulatta* (Mm) sera to viral proteins. pos., Positive control.

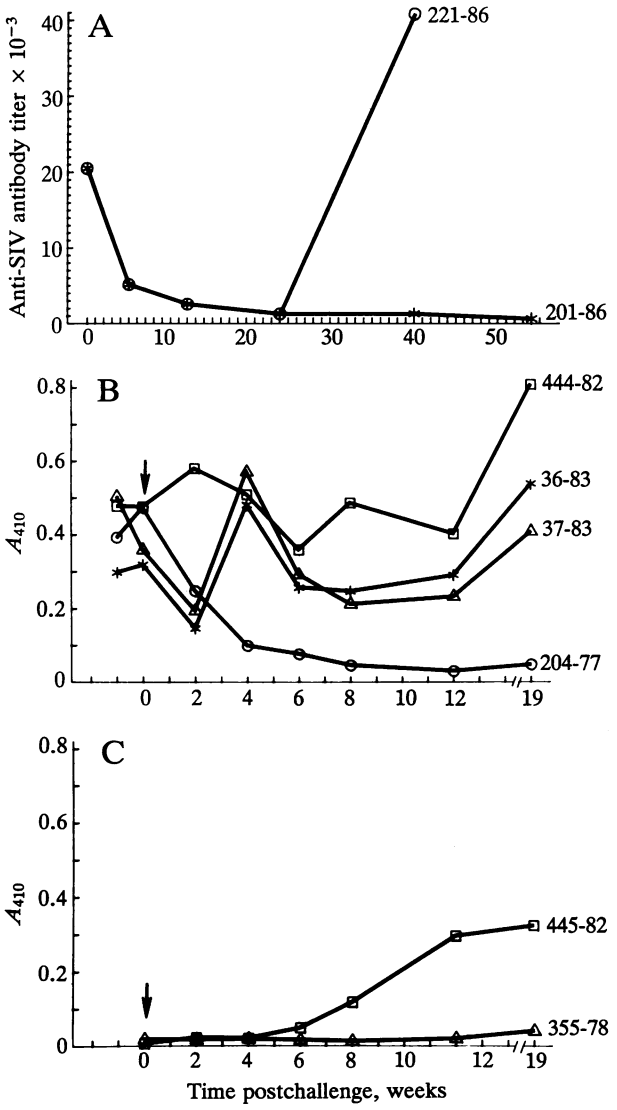

FIG. 2. Anamnestic antibody responses in the weeks following challenge with live SIV. Time zero is the day of challenge (arrow in *B* and in *C*) with live SIV. (*A*) Second vaccine study; antibody titer by ELISA. (*B*) Third vaccine study; ELISA absorbance at 410 nm at 1:2000 dilution of serum. (*C*) Unvaccinated controls in third vaccine study; ELISA absorbance at 410 nm at 1:2000 dilution of serum.

1*B*). These vaccinated macaques that became infected following challenge remained persistently infected, since SIV was recovered from peripheral blood samples more than 6 months after challenge. We have not been able to recover SIV from one of the vaccinated macaques (204-77) despite nine separate attempts to do so (Table 3). This macaque also had no detectable anamnestic response over 8 months of testing following the live SIV challenge (Fig. 2*B*). All four vaccinated macaques in this group remained alive and healthy 10 months after the live SIV challenge. Eight months after the live virus challenge, 10 ml of whole heparinized blood from 204-77 was transfused into a naive rhesus monkey; the recipient did not become infected.

Lymph Node Biopsies and *in Situ* Hybridization. The histologic appearances of lymph node biopsy specimens from animal 221-86 (second vaccine study) and animal 204-77 (third vaccine study) were consistent with inguinal lymph node morphology seen in macaques not infected with SIV (16, 19). Inguinal lymph node biopsy samples from infected animals (unvaccinated controls and nonprotected vaccinates) contained various stages of follicular hyperplasia with follicular

Table 3. Third vaccine study

Animal number	Antibody titer (doc)* ELISA	Antibody titer (doc)* Neutralization	Anamnestic response†	SIV recovery	Outcome‡
		Vaccinated group			
36-83	20,480	320	4 wpc	+	Alive
37-83	20,480	320	4 wpc	+	Alive
204-77	20,480	160	No	−	Alive
444-82	20,480	160	2 wpc	+	Alive
		Unvaccinated group			
355-78	0	0	—	+	196 dpc
445-82	0	0	—	+	Alive

Vaccination. Four rhesus macaques received a total of six inoculations with disrupted SIV as described in the legend to Table 2. The first three inoculations were spaced 3 weeks apart; 9 months later the macaques received three more inoculations over a 5-week period.
Challenge. Animals were challenged 1 week after the last immunization by intramuscular injection of 200 animal infectious doses of live SIVmac251.
*Determined by ELISA and by virus neutralization, using serum collected on the day of challenge (doc).
†Determined by ELISA (see Fig. 2*B*); wpc, weeks postchallenge.
‡Macaque 355-78 was found dead 196 days postchallenge (dpc). The others remained alive 245 dpc. Postmortem examination showed that 355-78 died with SIV-induced meningoencephalitis, diffuse interstitial giant-cell pneumonia, lymphocytic depletion of lymph nodes, and mild pyelonephritis.

fragmentation and involution consistent with changes previously described in SIV-infected macaques (16, 19). In two unvaccinated animals, follicles were depleted and the paracortical regions were either expanded (188-86) or normal (355-78).

In situ hybridization results are summarized in Fig. 3 and Table 4. Positively hybridizing cells compatible with lymphocytes and macrophages were easily identified in all unvaccinated controls. Among the previously vaccinated animals, five of the six contained no positive hybridization signal

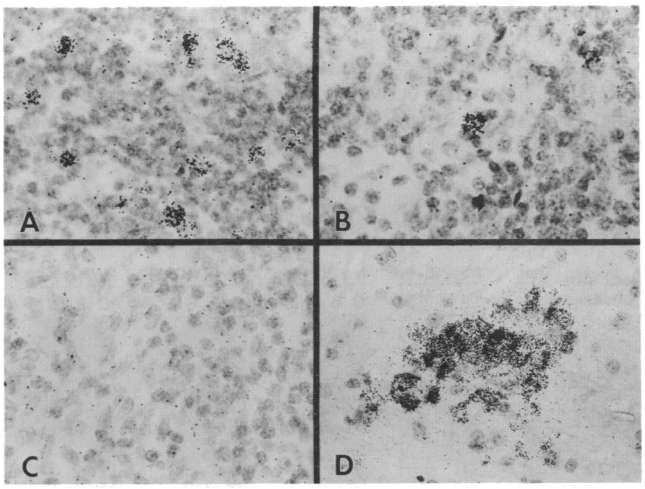

FIG. 3. *In situ* hybridization of SIV RNA in lymph node biopsy samples and in brain tissue collected at autopsy. A ^{3}H-labeled SIV DNA envelope gene probe was used. (*A*) Lymph node, animal 188-86, unvaccinated control. Numerous lymphocytes containing SIV RNA were widely distributed within the paracortex. (*B*) Lymph node, animal 355-78, unvaccinated control. Cells positive for SIV were less numerous than in *A* but clearly above background. (*C*) Lymph node, animal 204-77, vaccinated. No SIV-specific hybridization was demonstrable in any section examined. (*D*) Brain, 165-86, unvaccinated control. Animal had granulomatous meningoencephalitis; macrophages are heavily laden with SIV RNA. The times of biopsy and summary of results are shown in Table 4.

Table 4. Lymph node *in situ* hybridization

Animal number	Vaccination	Positive cells per section	Positive sections per number examined
		Second study, 190 dpc	
221-86	Yes	0	0/10
201-86*	Yes	0 (0)	0/10 (0/10)
188-86	No	>>>20	10/10
165-86†	No	>>>20	10/10
		Third study, 157 dpc	
36-83	Yes	1–5	4/6
37-83	Yes	0	0/10
204-77	Yes	0	0/10
444-82	Yes	0	0/10
355-78	No	>>20	10/10
445-82	No	1–5	7/10

Lymph node specimens were from biopsies done 190 (second study) or 157 (third study) days postchallenge (dpc), unless otherwise indicated.

*Animal 201-86 had two lymph node biopsies; results of the second biopsy (486 dpc) are indicated in parentheses.

†Lymph nodes from animal 165-86 were examined at autopsy (118 dpc).

in the sections examined; monkey 36-83 had one to five positive cells in four of six sections examined.

DISCUSSION

Two of the six vaccinated macaques in our last two studies appear to have been protected against SIV infection by live virus challenge. Furthermore, prior vaccination appeared to have a beneficial effect even in those four vaccinated–challenged macaques that became infected. By *in situ* hybridization, these four appeared to have a much lower virus load than control unvaccinated macaques infected in parallel. Of the four vaccinated–challenged macaques that became infected, only one (221-86) has died, and the pathology findings suggest that this death may not have been related to the SIV infection. Three of the four control macaques inoculated in parallel with the same doses of live SIV have died; pathology findings in these animals were consistent with AIDS (20). Since the two vaccinated macaques that appear uninfected (201-86 and 204-77) received challenges of 1000 and 200 animal infectious doses, their lack of infection cannot be simply explained by the lack of infectious virus in the inoculum. In total, eight naive macaques (four in titration and four controls in vaccine studies) were inoculated with between 1 and 1000 animal infectious doses of this same stock of SIV and all eight became infected. More recently, nine additional macaques were inoculated with 200 animal infectious doses of this stock as part of a separate study and all nine became infected.

Is there any reason to gain encouragement from these partial protections in such an idealized, optimized laboratory setting? We think so. To our knowledge this represents the first apparent vaccine protection against AIDS virus infection; it provides hope that an HIV vaccine will be possible. Furthermore, the disrupted whole virus used for vaccination in these studies is not a particularly good immunogen because of the underrepresentation of the gp120 envelope protein. It should be possible to make better immunogens that would provide better protection. It must be emphasized that a disrupted, inactivated virus preparation such as that used in this report would probably not be practical for use in humans.

The delayed anamnestic response following challenge of macaque 221-86 was certainly surprising and it should serve as a warning to the interpretation of the results described in this study. Evidence for SIV infection could conceivably surface at some future time in the macaques that were apparently protected against infection (201-86 and 204-77).

It is not clear why some of the vaccinated macaques clearly became infected upon challenge while others were apparently protected. If it is neutralizing antibodies that are eliminating incoming cell-free virus, the levels of neutralizing antibodies in the vaccinated animals on the day of challenge may have been just at the borderline of their ability to completely neutralize the dose of incoming virus. If this is true, large increases in the level of neutralizing antibodies prior to challenge or large decreases in the dose of live virus challenge might be expected to enhance the success rate.

The road to a safe, effective AIDS vaccine is not likely to be easy. Careful, systematic comparison of a variety of vaccine approaches as well as the variables associated with each approach will be needed if we are to develop the best possible vaccines; this will certainly require large numbers of experimental animals. Macaques, SIV, and other lentiviral models will have to assume prominent roles in this process.

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