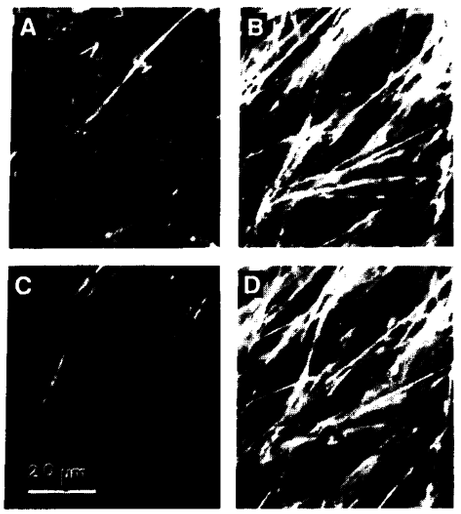

Fig. 4. Oscillating microtubules seen by dark-field video microscopy (*11*). (**A**) Beginning of the first assembly phase (135 s after the temperature shift). Note the numerous short rods; because of their high contrast they probably correspond to short microtubule bundles. (**B**) Maximum of first assembly (t = 258 s). Note the dense meshwork of microtubules (mostly bundles) and the high background (containing additional single microtubules which have a weak contrast and are not resolved). (**C**) Assembly minimum following the first maximum (t = 403 s). Note the decrease in the microtubule density and the lower background. A fraction of microtubule bundles remains through the disassembly phase. (**D**) Second assembly maximum (t = 482 s). Note that some of the prominent bundles retain their relative positions during the cycles.

cally built up and destroyed. Some of the stabler elements (for example, microtubule bundles) may persist through the oscillations and change only in apparent width, contrast, and flexibility. These elements remain nearly stationary. The intermediate-range polygons could correspond to hydrodynamic instabilities caused, for example, by the Marangoni effect. In the present case the viscosity is strongly modulated by microtubule assembly, so that the pattern of convection cells may in turn depend on the pattern of microtubules. The polygons also seem to be stationary in space, that is, when they are visible (just before or after a wave) they appear at the same positions. On the macroscopic levels the spatial patterns are dominated by the traveling waves of microtubule assembly. They broadly resemble the trigger waves of the BZ reaction. These have been explained by three conditions [reviewed in (*12*)]: (i) the solution must be in an excitable state; (ii) the reaction is started at a nucleation site and then proceeds autocatalytically by diffusion coupling; and (iii) waves occur because the initial reaction is followed by a transient refractory state. Analogous arguments can be applied to the present case: (i) The solution is initially in an excitable state because tubulin is ready to polymerize at 37°C; (ii) in the case of a circular layer of solution the nucleation barrier appears to be lowest at the periphery; and (iii) the refractory state (following microtubule disassembly) is dominated by tubulin oligomers that transiently trap the protein in an assembly-incompetent form (*5*).

What conclusions can one draw for pattern formation in living cells? Microtubules are known to be important for their shape and motility, but the mechanism and the interactions with other cellular elements are not well understood. Thus the mechanisms that generate the spatial patterns of living cells are difficult to explain in molecular terms. The point to be learned from the present results is that microtubules alone are capable of organizing themselves in time and space. The patterns we observe are simple, and moreover they are generated from only two types of molecules, tubulin and GTP. This simplicity opens the way of studying the mechanisms underlying microtubule self-organization that could lead to a better understanding of cellular pattern formation.

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A Formalin-Inactivated Whole SIV Vaccine Confers Protection in Macaques

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A vaccine against human immunodeficiency virus (HIV) would be highly effective in stopping the acquired immunodeficiency syndrome (AIDS) epidemic. A comprehensive evaluation of potential vaccine methodologies can be made by means of the simian model for AIDS, which takes advantage of the similarities in viral composition and disease potential between simian immunodeficiency virus (SIV) infection of rhesus macaques and HIV infection in humans. Immunization with a formalin-inactivated whole SIV vaccine potentiated with either alum and the Syntex adjuvant threonyl muramyl dipeptide (MDP) or MDP alone resulted in the protection of eight of nine rhesus monkeys challenged with ten animal-infectious doses of pathogenic virus. These results demonstrate that a whole virus vaccine is highly effective in inducing immune responses that can protect against lentivirus infection and AIDS-like disease.

DEVELOPMENT OF A VACCINE FOR HIV, the causative agent of AIDS, is the most effective means of combating this lethal disease. Since many viral vaccines do not actually prevent initial infection, but act by limiting virus dissemination and establishment after infection (*1–4*), evaluation of the efficacy of a vaccine for HIV solely on the basis of preventing infection may be limiting and misleading. The development of an HIV vaccine for the prevention of AIDS is greatly facilitated by the availability of a nonhuman primate model, the rhesus macaque (*Macaca mulatta*), in

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which SIV induces not only infection but also a disease remarkably similar to AIDS (*5–7*). The antigenic, genetic, morphologic, and functional similarities shared by HIV and SIV (*8–14*), together with the close immunological relationship of primate genera, argue that a vaccine proven to be effective in protecting rhesus monkeys from infection and disease after experimental challenge with SIV will likely be effective in protecting humans at risk for HIV infection and AIDS.

Using the SIV-macaque model, we tested the capacity of a formalin-inactivated whole virus vaccine to prevent infection or block the development of disease, or both. We examined killed whole virus vaccines before initiating studies with virion components not only because they have been successful in preventing many viral diseases (*1, 15*) but also because the whole of a substance must work for a part derived thereof to be effective. Virions harvested from low passage SIV/Delta$_{B670}$-infected H9 cells were used as a source for vaccine material (*16*). A compositional analysis of this preparation by reversed-phase high-performance liquid chromatography (HPLC) revealed that the complete virus particle was represented, with 2% to 3% of the total protein consisting of the external viral glycoprotein (gp110) and both full-length and truncated forms of the transmembrane glycoprotein (gp41 and gp35, respectively), along with the predicted stoichiometric amounts of the remaining viral core proteins (p61/65, p26, p17, p14, and p9). Introduction of a stop codon in the transmembrane glycoprotein gene (resulting in a truncated gene product) occurs as a result of adaptation of SIV to growth in continuous human T cell lines (*17*); gp41 was visible when virus was harvested from low-passage cells. The protein composition of the whole virus protein is shown by Coomassie blue staining and immunoblot analysis (Fig. 1).

Purified virions were inactivated by formalin treatment (*18, 19*). A whole virus vaccine inactivated in this manner was found highly effective in preventing infection of macaques with a type D retrovirus capable of inducing a similar AIDS-like syndrome (*19*). Formalin treatment rendered SIV noninfectious since measurements of reverse trancriptase activity in culture supernatants did not reveal infectious virus in cocultures of primary human phytohemagglutinin (PHA)-stimulated lymphoblasts. Assessment of residual infectivity was also performed by culturing the peripheral blood lymphocytes of the monkeys after immunization. Since 100 animal-infectious doses are equivalent to 1 tissue culture–infectious dose of the SIV isolate used in this study, assaying monkeys after immunization is the most sensitive measure-

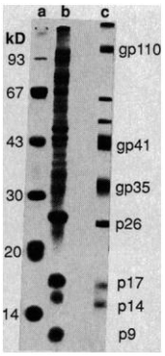

Fig. 1. SDS-PAGE analysis of SIV/Delta$_{B670}$ gradient-purified whole virus. The total protein profile, visualized by staining with Coomassie blue, is shown in lane b. Immunoblot analysis of whole virus, evaluated on a separate gel, is shown by immunoperoxidase staining, with a reference serum from an SIV-infected macaque in lane c. Protein standards are shown in lane a with molecular sizes as indicated. The characteristic migration positions of each of the SIV polypeptides are designated to the right of lane c.

Table 1. Chronology of whole virus immunizations. Whole virus preparations were inactivated after purification by incubation at 4°C for 24 hours with 0.8% formalin and combined with adjuvant before immunization. The amount of MDP administered to each monkey varied from 90 to 144 μg per kilogram of body weight per dose. Virus recovery was attempted at 2, 3, and 4 weeks after challenge, and monthly intervals thereafter by coculture with human PHA blasts. Monkeys were given physical examinations at biweekly intervals, at which time blood was obtained for serology and complete blood counts. Examinations included evaluation of changes in lymph nodes and spleen to determine lymphadenopathy (Lad) and splenomegaly (Spl), respectively. Lymphocyte subsets were monitored at monthly intervals by immunofluorescent staining and flow cytometry. Moribund animals were euthanized and complete necropsies were performed to determine cause of death. No manipulations other than routine examinations were performed from day 100 to day 505; during this time two vaccinees died from causes unrelated to SIV (G068 died with gastric dilatation and G066 with hypoglycemia). Pathological examination and culture of lymphoid tissues obtained at necropsy did not reveal any evidence of SIV in these monkeys. Vaccinees were given a 1000 μg of booster immunization potentiated with the respective adjuvants on day 505 and challenged 2 weeks later as indicated. No further manipulations other than routine examinations and viral cultures were performed from day 523 until day 785, at which time an inguinal lymph node was surgically removed for inoculation of naïve recipients as described. Abbreviations: ↑ ↓, increase or decrease, respectively, in lymphocyte populations; PI, postinfection; Ab, antibody; d, day; T4/4B4, OKT4$^+$4B4$^+$-staining lymphocytes.

Animal	Challenge (ID_{50}) day 100	Virus recovery	Immunological changes	Clinical signs	Challenge ID_{50} Day 519	Challenge ID_{50} Day 523	Virus recovery	Immunological changes day 785	Clinical signs day 785	In vivo transfer day 785
				Nonimmunized controls						
G010	1	Yes	Ab$^+$; ↑ B1	Died d 396 PI						
G158	1	Yes	Ab$^+$	None	10		No	Ab$^+$	None	NT
G529	1	No	No	None	10		Yes	↓ T4/4B4 by d 56	Died d 194 PI	NT
G625	1	No	IgM Ab (trans)	None		10	No	↑ B1	None	NT
G524					10		Yes	↓ T4/4B4 by d 28	Died d 186 PI	NT
G142						10	Yes	↓ T4/4B4 by d 56	Died d 217 PI	NT
G063						10	Yes	Ab$^+$	Lad, Spl	NT
G744						10	Yes	Ab$^+$	Lad, Spl	NT
				Whole virus + MDP + alum						
G497	1	Yes	↓ T4/4B4 by d 150	Died d 285 PI						
F799	1	No	No	None	10		No	None	None	No
G071	1	No	No	None	10		No	None	None	No
G163	1	No	No	None	10		No	↓ T4/4B4 by d 158	None	Yes
F803					10		No	None	None	No
G531					10		No	None	None	No
G581						10	No	None	None	No
G068		Unrelated death								
				Whole virus + MDP						
F928						10	No	No	None	No
G582						10	No	No	None	No
G160						10	No	No	None	No
G066		Unrelated death								

ment of residual infectivity of treated vaccine material currently available.

Several experiments with different doses of a cryopreserved preparation of SIV/Delta$_{B670}$ were performed over a 2.5-year period to determine the minimum infectious dose (ID_{50}) and minimum lethal dose (LD_{50}) required to infect and induce disease, respectively, in 50% of the animals tested. From 4 to 17 monkeys per dose were inoculated intravenously with 1 ml of serial tenfold dilutions of cryopreserved virus spanning a range from 1×10^{0} to 1×10^{-4}; 1 to 6 monkeys per group were inoculated with dilutions ranging from 1×10^{-5} to 1×10^{-9}. Virus isolations were performed on each inoculated animal by coculture of monkey peripheral blood lymphocytes (PBLs) with human PHA-stimulated lymphoblasts (*20*). Cultures were assayed for reverse transcriptase activity at 14 and 21 days; all reverse transcriptase–positive cultures were confirmed by an enzyme-linked antigen capture assay (*16*). We observed 100% infection and 70% to 100% disease in monkeys inoculated with $\leq 1 \times 10^{-3}$ dilution of virus; 40% infection and disease was found in five monkeys inoculated with a 1×10^{-4} dilution; neither infection nor disease was observed in monkeys inoculated with dilutions $\geq 1 \times 10^{-5}$. These experiments show the stability of the cryopreserved inoculum over a period of years, as well as determine that both the ID_{50} and the LD_{50} are in approximately 1 ml of a 1×10^{-4} dilution of cryopreserved material. Inoculation of a dilution of 1×10^{-3} (10 to 99 ID_{50}) gave reproducible results in 17 monkeys, resulting in 100% infection and a 75% mortality within 7 months. On the basis of these data, this dilution was selected as a reliable inoculum for subsequent challenge experiments of immunized monkeys.

A chronology of the whole virus vaccine trial is shown in Table 1. Two groups of eight and four monkeys, respectively, ranging from 1 to 2 years of age, were given intramuscular injections of formalin-inactivated whole virus potentiated with threonyl muramyl dipeptide (MDP) (Syntex) (*21*) and aluminum hydroxide (alum), or MDP alone. It was anticipated that the use of the combined adjuvants might enhance the induction of both humoral and cellular arms of the immune response. Both groups were given 650, 650, and 560 μg of inactivated total viral protein combined with adjuvants on days 1, 28, and 71, respectively.

Challenge with 1 ID_{50} within a month after the initial series of immunizations (day 100) resulted in infection and disease in one of four challenged vaccinees; disease was predicted in this animal by 150 days after inoculation by a selective decline in the helper-inducer lymphocyte population ($CD4^+CD29^+$ cells double stained with the monoclonal antibodies OKT4 and 4B4) (*22*). One of four control animals challenged similarly became infected and died with AIDS. Two additional controls seroconverted but remained healthy; one of these responded to virus exposure by the transient production of immunoglobulin M (IgM), but not IgG-specific antibody to SIV. No evidence for viral infection was observed in the fourth control monkey.

Thirteen months later, the vaccinees that survived the earlier challenge, along with the remaining monkeys in the study, were given booster immunizations potentiated with the respective adjuvants and challenged 2 weeks later (days 519 and 523) with 10 ID_{50}. Nonimmunized control animals were challenged similarly. Freshly prepared inocula were used within 2 hours after dilution of cryopreserved material, and control monkeys were inoculated after the immunized monkeys to assure that each inoculum contained infectious material throughout the time required for the inoculations.

SIV was readily recovered from five of seven controls beginning at 2 weeks after challenge. Of these, three showed signs of disease progression by a selective decline in $OKT4^+4B4^+$-staining lymphocytes by 56 days after inoculation (Fig. 2A). These animals died of AIDS by 217 days after infection. The two remaining virus-positive control monkeys have persistent lymphadenopathy and splenomegaly, clinical indicators of chronic disease (*16*). The two control monkeys from which virus could not be recovered after challenge (G158 and G625) were those that had seroconverted as a result of the earlier challenge with 1 ID_{50}. At the time of the second challenge, both were clinically normal and appeared to be either latently infected or virus-negative, since previous attempts at virus isolation had failed in both. The failure to establish a productive infection after challenge in both animals suggests that protective immunity may have been induced by the earlier exposure to the low dose of infectious virus.

Of the nine immunized monkeys challenged with 10 ID_{50}, three had been challenged previously with 1 ID_{50} and thus may have been primed with infectious virus before the second challenge. However, the remaining six monkeys were naive with respect to infectious virus. Attempts at virus isolation after challenge were repeatedly unsuccessful in all immunized monkeys, even when the monkey PBLs were depleted of $CD8^+$ lymphocytes by panning (*23*), followed by lysis in the presence of antibody and complement (*24*). $CD8^+$ cell depletion has been shown to remove cells that could

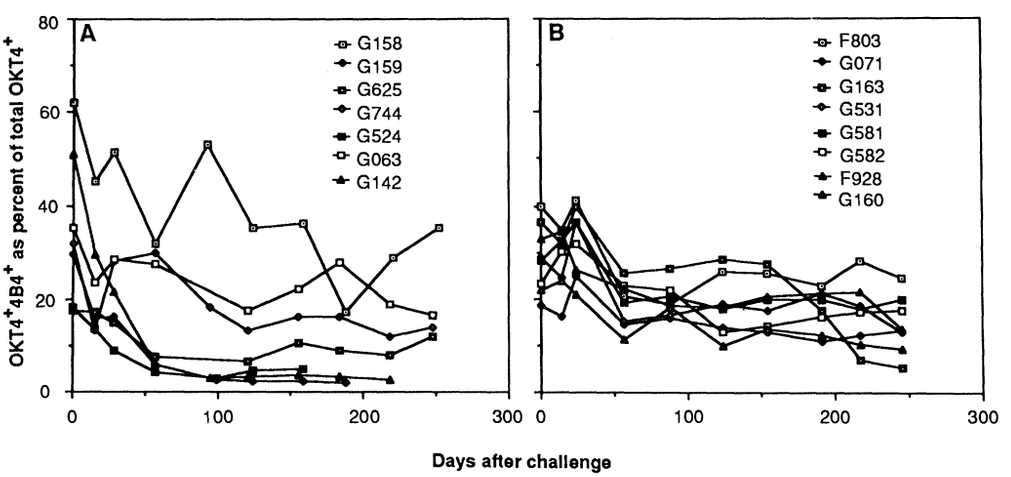

Fig. 2. Evaluation of the percentage of $OKT4^+$ lymphocytes doubly stained with 4B4 ($OKT4^+4B4^+$) in control (**A**) and immunized (**B**) monkeys after infectious challenge. Whole blood samples in EDTA anticoagulant were doubly stained with fluorescein-conjugated OKT4 (Ortho Diagnostics) and phycoerythrin-conjugated 4B4 (Coulter Immunology). Erythrocytes were lysed with NH_4Cl, after which samples were washed in RPMI medium, fixed with 1% paraformaldehyde, and analyzed by flow cytometry (Epics 541; Coulter) within 48 hours. The percentages of lymphocytes stained with OKT4 alone and doubly stained with OKT4 and 4B4 were determined by analyzing 5000 lymphocytes. The percentage of doubly stained $OKT4^+$ cells was calculated as the percentage of $OKT4^+4B4^+$ cells/percentage of total $OKT4^+$ cells × 100. Sustained decreases below 8% are considered predictive of disease. The data from individual monkeys are plotted with the symbols shown on the figure.

potentially suppress virus replication in vitro (*25*). All nine monkeys immunized with whole virus remain clinically normal 1 year after challenge. Eight of these have shown no consistent changes in lymphocyte subsets. A selective decline in $OKT4^+4B4^+$-staining lymphocytes, which is suggestive of viral infection, was observed in monkey G163 by 158 days after challenge (Fig. 2B).

The failure to isolate virus from the peripheral circulation of immunized monkeys does not necessarily mean that virus is not sequestered in lymphoid organs. To address this possibility, as well as to overcome potential suppression of virus replication in vitro by cytotoxic T lymphocytes contained in mononuclear cell preparations, lymph nodes were surgically removed from all immunized animals from which virus could not be cultured. Cell suspensions containing 1×10^7 to 1×10^8 cells prepared from each lymph node were inoculated intravenously into naive rhesus monkeys. Transfer of lymphocytes to naive animals not only has been successful in identifying inapparent infections in horses infected with another lentivirus [equine infectious anemia virus (*26*)], but also has been highly efficient in transmitting SIV from monkey to monkey (*5*). Eight of nine of the recipients have had no signs of viral infection. One monkey, inoculated with cells from monkey G163, became viremic within 7 days after infection. These data suggest that the formalin-killed whole virus vaccine prevented the establishment of SIV infection in eight of nine animals challenged with infectious virus.

Immunoblot analysis of the serial antibody response to immunization with the whole virus vaccine is shown in Fig. 3. Monkey G158, a nonimmunized, infected control, illustrates the typical response to experimental infection observed in asymptomatically infected rhesus monkeys (lane a) (*16*), with a strong antibody response throughout the infection to the core proteins p17 and p26, the glycoproteins gp35 and gp110, and p61/65, the putative reverse transcriptase or gag polyprotein. Sera obtained at the time of the initial challenge (day 100) from three monkeys immunized with whole virus potentiated with either the combined adjuvants (Fig. 3, lanes b and f) or MDP alone (lane j) showed a similar response. No apparent difference in the antibody responses was observed when monkeys immunized with the combined adjuvants or MDP alone were compared.

The antibody response to immunization declined over the next 13 months, with antibody to core proteins persisting longer than envelope-specific antibody (lanes c, g, and k). The booster immunization, delivered 2 weeks before challenge, induced anamnestic responses that were qualitatively indistinguishable at the time of challenge from that initiated by earlier immunizations (lanes d, h, and l). By 8 months after challenge, these responses, particularly with respect to gp35 and gp110, had declined in monkeys protected by immunization (lanes e and m). These results are in contrast to those for monkey G163, the single animal in this group that showed a predictive decline in helper-inducer T lymphocytes (lane i). Not only were antibodies to viral proteins maintained in this animal, but antibodies to gp35 and gp110 appeared stronger, presumably as a result of immune stimulation by viral replication.

Table 2. Glycoprotein-specific antibody responses after whole virus immunization and challenge with infectious virus. Glycoprotein-specific antibody titers were obtained over time after immunization by analysis of serial twofold dilutions of sera. Determinations were assayed by enzyme-linked immunosorbent assay with purified recombinant SIVmac gp140 expressed in baculovirus and assayed as described (*34*).

Animal	Titer (reciprocal dilution)			
	Day 100	Day 505	Day 519 to 523	Day 785
F803	3,200	200	12,800	400
F799	1,600	100	6,400	200
G531	1,600	200	3,200	100
G071	1,600	50	6,400	800
G581	3,200	100	12,800	3,200
G163	6,400	100	12,800	102,400
F928	1,600	100	3,200	200
G582	400	0	6,400	400
G160	800	50	5,300	100

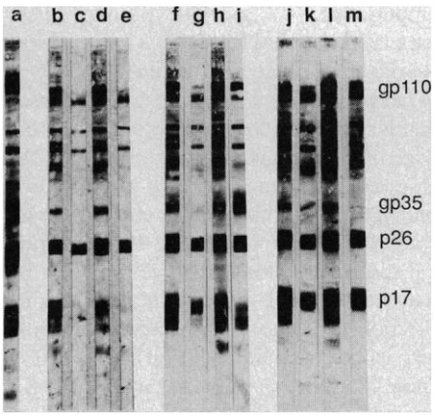

Fig. 3. Qualitative analysis of humoral immune responses to immunization with whole virus. Immunoblot analysis of sera obtained after immunization was performed by incubation of a 1:50 dilution of each serum sample with gradient-purified virus adsorbed onto nitrocellulose strips and stained with immunoperoxidase as described (*16*). Monkey G158, an infected monkey, is shown for comparative purposes in lane a. Monkeys G071 (lanes b to e) and G163 (lanes f to i) were immunized with whole virus + MDP + alum. Monkey G160 (lanes j to m) was immunized with whole virus + MDP. Determinations were performed on day 100 (lanes b, f, and j), day 505 (lanes c, g, and k), day 519 (lanes d and h), day 523 (lane l), and day 785 (lanes e, i, and m).

The level of glycoprotein-specific antibody induced by the initial series of immunizations (shown on day 100 at the time of the first challenge) was somewhat lower than that ordinarily observed in experimental infection (Table 2). In the majority of immunized monkeys this titer was less than 1:4000; whereas in monkey G158, the asymptomatically infected nonimmunized control, the titer exceeded 1:12,000 (*27*). During the rest period, titers decreased to the level observed before the booster immunization (day 505) in all immunized monkeys. The booster immunization induced glycoprotein-specific titers that, at the time of challenge (days 519 and 523), exceeded those produced by the initial series of immunization. No correlation between antibody titer and protection was observed, however, since monkey G163 was not protected despite his higher than average response.

A comparison of the peak titers at challenge with those obtained 9 months later (day 785) in immunized monkeys clearly distinguished between the single animal infected as a result of challenge (G163) and the eight that were protected. The titer in the infected animal at 9 months after challenge was four times that observed 2 weeks after the booster immunization, whereas the titers in the remaining monkeys had fallen during this time as would be predicted for uninfected animals.

The neutralizing antibody titer was also assessed in sera obtained at the time of challenge (*28*). In contrast to the significant gp140 titers achieved overall, neutralizing antibody titers in sera from immunized monkeys varied from less than 1:20 to 1:80. For comparison, a reference immune serum from an SIV-infected macaque displayed a neutralizing antibody titer of 1:640.

Immunization with formalin-inactivated whole SIV potentiated with either MDP or MDP combined with alum has protected

eight of nine juvenile rhesus monkeys against viral infection and nine of nine against disease for at least 1 year after challenge. These monkeys show no immunological signs of viral infection and remain clinically normal. In contrast, five of five nonimmunized, seronegative control animals challenged concomitantly with these monkeys and 12 of 12 additional monkeys receiving the same dose of cyropreserved virus became infected as a result of inoculation, with 12 of 17 (70%) showing predictive immunological changes and death due to immunodeficiency disease within 7 months after infection. Transfer of lymph node–derived lymphocytes from each immunized monkey to naive rhesus monkeys 7 months after challenge revealed the presence of SIV in the lymph node tissue of only one of nine. Since the SIV-infectious dose in the rhesus monkey is 100 times less than that required to establish an infection in vitro, these data suggest that the formalin-killed whole virus vaccine prevented the establishment of SIV infection in eight of nine monkeys challenged with infectious virus.

Desrosiers *et al.* (*29*) have reported apparent protection of two of six monkeys by immunization with a detergent-disrupted whole virus vaccine. The protection of eight of nine monkeys described in our study, however, is stronger evidence that immunization can be of practical use in the development of a vaccine for use in humans. In further support of these data, Montelaro *et al.* (*30*) have reported protection with a formalin-killed whole virus vaccine against infection by 1000 animal-infectious doses of equine infectious anemia virus in 9 of 12 horses.

The apparent success achieved by immunization with formalin-inactivated whole virus can likely be attributed to several reasons. (i) A high dose of highly purified material was used for all immunizations. (ii) The vaccine contained all major virion proteins, including gp110 and both full-length and truncated transmembrane glycoproteins. (iii) A rest period sufficient to establish appropriate memory cells was allowed before exposure to live virus. The value of immunological memory and time for its development for the induction of protective immunity has been well documented with the formalin-killed poliovirus vaccine developed by Salk (*31*) and may have been important in these experiments. Challenge with 1 ID_{50} within 1 month after the third immunization failed to protect one monkey from either infection or disease, whereas booster immunizations 13 months later protected eight of nine against challenge with tenfold more virus.

(iv) The virus used to prepare the vaccine material was derived from the cryopreserved stock used for challenge. That both vaccine and challenge preparations are genetically identical, however, is unlikely since adaptation of the pathogenic cryopreserved virus occurred as a consequence of growth in the H9 cell line used for propagation of the vaccine material. This adaptation minimally selects for variants containing a truncation of the transmembrane glycoprotein (*17, 31*). In addition, the original starting material was neither biologically nor molecularly cloned before these studies. These experiments suggest that epitopes critical in protective immunity nevertheless were conserved in the manipulations used in vaccine production. It will be necessary to evaluate the efficacy of this vaccine against exposure to genetically diverse SIV isolates.

(v) In contrast to the 200 to 1000 animal-infectious doses used by Desrosiers *et al.* (*29*), a smaller dose consisting of 10 to 99 ID_{50} was used to challenge immunized monkeys. As a result of inoculation of over 20 monkeys with this dose, we have shown it to be as infectious and pathogenic as the undiluted stock material that contains 1000 times more infectious particles. Unlike the SIV stock material, however, this dose is closer to the nominal levels of virus exposure in humans at risk for HIV infection.

The apparent low level of neutralizing antibody titers induced by immunization suggests that neutralization mediated by antibody may not be the sole mechanism for protection with a killed whole virus vaccine. Adequate protection may require multiple determinants, and these may be found on more than one viral protein. Maximal induction of protective immune responses may also require conformational determinants that are preserved by formalin. Either of these observations may explain the inability of purified HIV gp120 vaccines to prevent infection in chimpanzees (*32, 33*).

Thorough analysis of the responses induced in animals protected by immunization should identify components of the immune response that are critical to protection and direct further research in the development of viral subunit as well as whole virus vaccines.

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20. The Delta Primate Center takes responsibility for humane care and use of laboratory animals used in projects awarded by the Public Health Service. We are committed to comply with the Principles for Use of Animals, the *Guide for the Care and Use of Laboratory Animals*, the Provisions of the Animal Welfare Act, and other applicable laws and regulations. The Center's statement of assurance is on file with the PHS, Office for Protection from Research Risks (Assurance number A3701-01). This facility is accredited by the American Association for Accreditation of Laboratory Animal Care. Animals are anesthetized with ketamine prior to all procedures that require the removal of animals from their cages. No restraining devices are necessary during these procedures. When necessary, moribund animals are euthanized by intravenous inoculation of a lethal dose of sodium pentabarbitol.
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28. Virus neutralization assays were performed in duplicate as follows: 100 μl of twofold dilutions of heat-inactivated serum were incubated with 1×10^{-2} tissue culture ID_{50} of SIV/$Delta_{B670}$ in 100 μl for 1 hour at 37°C. Thereafter, the virus-serum mixture was added to 1×10^6 primary human PHA blasts and the incubation continued for an additional hour at 37°C. The cells were plated at a density of 5×10^5 cells per milliliter in 24-well microtiter trays. The neutralizing titer was determined as the minimum dilution of serum to induce 100% inhibition of reverse transcriptase activity in culture supernatants at 10 days after infection.
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34. Microtiter trays (96 wells) were coated overnight at 4°C with 200 ng of recombinant gp140 per well in 0.01*M* sodium carbonate, *p*H 9.5. Excess protein was removed by washing with saline and 0.05% Tween 20, 100 μl of each serum dilution in phosphate-buffered saline and 0.05% Tween 20 was added to each well, and the plates were incubated for 1 hour at room temperature on a rocking platform. Plates were washed, 50 μl of a 1:1000 dilution of urease-conjugated antibody to human IgG or IgM (Sigma) was added, and the incubation was continued for 30 min at 37°C. After washing, 100 μl of substrate (8 mg of bromecresol purple in 1.48 ml 0.01*M* NaOH, 0.1 g urea, and 200 μl of 0.1*M* EDTA, *p*H 5) was added to each well, and the optical density at 590 was measured with a Dynatech plate reader. Titers were determined 29 days after the third immunization (day 100), immediately before the final booster immunization (day 505), on the day of challenge with ten times the ID_{50} infectious doses (day 519 and 523), and 9 months after challenge (day 785).

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