Protective Effects of a Live Attenuated SIV Vaccine with a Deletion in the nef Gene

Muthiah D. Daniel, Frank Kirchhoff, Susan C. Czajak, Prabhat K. Sehgal, Ronald C. Desrosiers*

Vaccine protection against the human immunodeficiency virus (HIV) and the related simian immunodeficiency virus (SIV) in animal models is proving to be a difficult task. The difficulty is due in large part to the persistent, unrelenting nature of HIV and SIV infection once infection is initiated. SIV with a constructed deletion in the auxiliary gene nef replicates poorly in rhesus monkeys and appears to be nonpathogenic in this normally susceptible host. Rhesus monkeys vaccinated with live SIV deleted in nef were completely protected against challenge by intravenous inoculation of live, pathogenic SIV. Deletion of nef or of multiple genetic elements from HIV may provide the means for creating a safe, effective, live attenuated vaccine to protect against acquired immunodeficiency syndrome (AIDS).

There are good reasons for believing that development of an effective vaccine for AIDS will be a difficult task. Infection of humans with human immunodeficiency virus type-I (HIV-1) and of rhesus monkeys with SIVmac is fatal most or all of the time despite an apparently strong host immune response to the infecting virus. Infected individuals maintain vigorous humoral and cellular immune responses for months or years only to succumb eventually to the virus. The ineffective nature of the natural immune response suggests that a vaccine will have to provide highly stringent protective immunity, perhaps even sterilizing immunity, in order to achieve protection. These difficulties are compounded by the large number of HIV-1 strains that are non- or minimally cross-neutralizing.

The predicted difficulty in achieving protection against HIV and SIV has been borne out to varying degrees by vaccine trials in animal models. Inactivated whole virus has protected rhesus monkeys against challenge by live, pathogenic SIV (1–4) but primarily under highly specific conditions that use human cells for the production of both vaccine antigen and challenge virus. Even formalin-fixed, uninfected human cells can provide protection under these conditions (5). Apparently, an immune response to some cellular antigen or antigens is critical for this protection. The inactivated whole virus vaccine approach has been largely unsuccessful against SIV grown in rhesus monkey lymphocytes (6, 7). Priming with vaccinia recombinants followed by boosting has protected rhesus monkeys against challenge by homologous cloned SIV (8), but little or no protection has been observed against homologous uncloined SIV (9–11). Tests of several products have shown only limited success in chimpanzee trials (12). What is most disappointing about these studies is that the numerous failures have occurred despite extensive efforts to maximize, in an unrealistic fashion, the likelihood of vaccine protection. The vast majority of studies have used a minimal dose of challenge virus, matched to the strain used for vaccination, at or near the peak of vaccine-induced immune response.

We investigated an approach that uses live attenuated SIV as a vaccine. Six rhesus monkeys that were infected with cloned SIVmac239 that contained a constructed deletion in the auxiliary gene nef have maintained extremely low virus burdens and normal CD4+ lymphocyte concentrations and have remained healthy for more than 3 years after experimental inoculation with the mutated virus (13, 14). Eleven of twelve rhesus monkeys infected with wild-type SIV in parallel have died over this same period. The rhesus monkeys infected with SIVmac239/nef-deletion have shown no clinical signs whatsoever over the entire period of observation.

Four of the rhesus monkeys infected with nef-deleted SIV were challenged with

<table>
<thead>
<tr>
<th>Rhesus monkey</th>
<th>SIV recovery</th>
<th>Antibody titer</th>
<th>Challenge virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>353-88</td>
<td>–</td>
<td>1:1280</td>
<td>SIVmac239/</td>
</tr>
<tr>
<td>397-88</td>
<td>–</td>
<td>1:1280</td>
<td>nef-open</td>
</tr>
<tr>
<td>71-88</td>
<td>–</td>
<td>1:320</td>
<td>SIVmac251</td>
</tr>
<tr>
<td>255-88</td>
<td>–</td>
<td>1:2560</td>
<td>SIVmac251</td>
</tr>
</tbody>
</table>

*To whom correspondence should be addressed.

Table 1. Challenge of rhesus monkeys immunized with SIV deleted in nef. All four rhesus monkeys received a single inoculation of SIV-mac239/nef-deletion 2.25 years before challenge. Attempts to recover SIV from 10⁶ PBMCs on the day of challenge yielded negative results (dashes). The titers of neutralizing antibodies were measured on the day of challenge (7).

**1938**

SCIENCE • VOL. 258 • 18 DECEMBER 1992

**REFERENCES AND NOTES**

20. Sponsored in part by the National Cancer Institute (NCI), Department of Health and Human Services (DHHS), under contract NOI1-CO-74102 with Program Resources, Inc./DynCorp. The contents of this publication do not necessarily reflect the views or policies of the DHHS, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government. We thank S. M. Hewes, D. G. Johnson, R. W. Hill, and W. B. Knott (NCI-FCRDC) for technical assistance; C. M. Dinterman (NCI-FCRDC) for editorial comments; P. C. Grove (NCI-FCRDC) for clerical support; P. Cresswell (Yale University) for providing antisera to HLA DR, B. F. Haynes (Duke University) for monoclonal antibodies to HLA DR, HLA DP, and HLA DQ; and R. C. Gallo, NCI, for HIV-1-infected cells.
6 August 1992; accepted 6 November 1992
wild-type, pathogenic SIVmac 2.25 years after the initial inoculation with the mutated virus (Table 1). No booster immunizations of any type were used. Two of the vaccinated rhesus monkeys were challenged with cloned pathogenic SIVmac239/nef-open (intact nef), and two were challenged with uncloned pathogenic SIVmac251, a strain distinct from but closely related to SIVmac239 (15). SIVmac239 shares approximately 93% amino acid identity in the gp120 Env protein with clones derived from SIVmac251-infected cells (16). In both cases, virus stocks were prepared in primary rhesus monkey peripheral blood mononuclear cell (PBMC) cultures (17), and aliquots of the frozen virus stocks were carefully titered in rhesus monkeys before the challenge experiment (18). In both cases, ten rhesus monkey infectious doses were used for the challenge. Neutralizing antibody titers on the day of challenge ranged from 1:320 to 1:2560 in the four rhesus monkeys previously vaccinated with live nef-deletion virus (Table 1). Virus loads, measured by limiting dilution co-culture and semiquantitative polymerase chain reaction (PCR) (13, 14), were extremely low before challenge. We were unable to recover SIV from 10^6 PBMCs of the four rhesus monkeys on the day of challenge (Table 1). Two naïve rhesus monkeys served as controls for each of the challenge viruses.

Several parameters were monitored for evidence of protection against the challenge viruses. All four control monkeys showed a spike in plasma antigenemia 2 weeks after challenge, as we have always seen previously with infection by these viruses (Table 2). In some cases, the plasma antigenemia persisted and in others it dipped below detectable levels only to reappear at a later time. No plasma antigenemia was detected at any time in any of the four previously vaccinated monkeys (Table 2).

We also evaluated virus loads by measuring the numbers of PBMCs required to recover SIV by limiting dilution co-culture. All four unvaccinated control monkeys exhibited high virus burdens, as we have always seen previously with infection by these wild-type viruses (Table 3). Generally, 1,000 to 20,000 PBMCs were needed for SIV recovery from the control animals. One test monkey, 397-88, showed a spike in virus recovery at 4 weeks after the challenge in that SIV was recovered with 74,000 or more PBMCs. SIV recovery in subsequent weeks from monkey 397-88, however, was negative even with 10^6 cells. Virus loads with this measurement were also stably low in the other three previously vaccinated animals because virus recovery required ≥10^6 PBMCs (Table 3).

We also performed genetic analysis of viral DNA present in cells infected with recovered virus and in DNA directly from PBMCs. The nef sequences were amplified by PCR from DNA obtained from cells infected with SIV recovered from animal 255-88 at 12, 24, and 31 weeks after challenge (19). The size of the amplified product at all three times was shorter than the product expected from amplification of wild-type, nef-open sequences used for challenge and also shorter than the product expected from amplification of nef-deletion.

![Fig. 1. Analysis of nef gene sequences present in virus reisolated from rhesus monkey 255-88. DNA was prepared from cells infected with recovered virus and used for PCR amplification.](image)

Table 2. Protective effects of live attenuated nef-deletion vaccine on plasma antigenemia. The limit of detection of the assay used was approximately 0.05 ng/ml. W, weeks after challenge; D, dead.

<table>
<thead>
<tr>
<th>Rhesus monkey</th>
<th>Virus/challenge virus</th>
<th>Plasma antigenemia (nanograms of p27 per milliliter of plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W0</td>
</tr>
<tr>
<td>148-88</td>
<td>None/251</td>
<td>0.0</td>
</tr>
<tr>
<td>369-90</td>
<td>None/251</td>
<td>0.0</td>
</tr>
<tr>
<td>208-90</td>
<td>None/251</td>
<td>0.0</td>
</tr>
<tr>
<td>397-88</td>
<td>None/251</td>
<td>0.0</td>
</tr>
</tbody>
</table>
sequences present in the vaccine strain (Fig. 1A). Sequence analysis of this product revealed deletion of base pairs 9251 to 9432, which are the sequences in nef deleted in the vaccine strain, as well as deletions of base pairs 9476 to 9586 and 9650 to 9800. Thus, SIV recovered from animal 255-88 at weeks 12, 24, and 31 after challenge contained the original deletion present in the vaccine strain plus additional deletions in the region of the nef gene that overlaps the right long terminal repeat. The purine tract, the NF-kB binding site, and the Sp1 binding sites, critical for virus replication, were all retained in this virus. The important point is that we were not able to detect any of the wild-type nef-open viral sequences in the recovered SIV either by ethidium bromide staining of the amplified products (Fig. 1A) or by Southern (DNA) blot hybridization of the products (Fig. 1B).

The detection of DNA sequences directly from PBMCs required sensitive amplification procedures because of the extremely low virus burdens present in the vaccinated and challenged monkeys. We were able to amplify DNA sequences directly from the PBMCs of all four test animals by using a PCR amplification protocol that included both nested reactions (20, 21) and turbo-boosting (22) (Fig. 2). Negative control DNA prepared at the same time tested negative for the presence of viral DNA sequences. Multiple PCR reactions were performed with PBMC DNA samples taken from each test animal 36 and 40 weeks after challenge; five or more PCR reactions were performed with at least one DNA sample from each animal. Viral DNA was detected in nine out of nine amplifications from animal 71-88 and in five out of seven amplifications from animal 397-88, and the amplified product in all cases except one corresponded to the nef-deletion used originally for vaccination. Viral DNA in the one exception contained the original deletion in nef plus an additional 138-bp deletion. Viral DNA was also detected in 15 out of 15 amplifications from the PBMCs of animal 255-88. The predominant product from the PBMCs of animal 255-88 at 36, 37, and 40 weeks after infection was the triplic weighted variant that was also observed in the recovered virus from this same animal 12, 24, and 31 weeks after infection. Multiple amplifications with different sets of primers revealed by nucleotide sequence analysis smaller amounts of the original single nef-deletion and of variants with additional deletion of 111, 141, or 151 bp. Viral DNA was usually not detected from the PBMCs of animal 353-88 (Fig. 2), but in the two reactions in which it was detected both the original nef-deletion and additionally deleted variants were detected. Thus, sequences that correspond to wild-type nef-open challenge virus were not detected in any of the samples analyzed from the four test animals.

Control animals 148-88, 246-90, and 388-90 showed declines in CD4+ lymphocyte concentrations after the challenge, and by 36 weeks all control animals had either died (148-88 and 208-89) or had to be killed when moribund (388-90 and 246-90). All four previously vaccinated animals are still alive, healthy, and have shown no declines in CD4+ lymphocyte concentrations.

At 37 weeks after the initial challenge, two of the test animals (71-88 and 255-88) were challenged with 1000 rhesus monkey infectious doses of the uncloned SIV-mac239 stock. At weeks 3, 5, and 12 after this high-dose challenge, SIV was not recovered from their PBMCs even with the use of 106 cells, and PCR analysis revealed no evidence of wild-type nef-open challenge virus (Fig. 2). Thus, early data suggest that these vaccinated monkeys may even be resisting high-dose challenge.

Rhesus monkeys vaccinated with SIV-mac239/nef-deletion appear by all criteria used to have resisted challenge by wild-type pathogenic SIV. Previously vaccinated animals showed no evidence of plasma antigenemia at any time after challenge and maintained extremely low virus burdens as was observed before the challenge. Furthermore, genetic analysis of recovered virus and viral DNA present in their PBMCs revealed no evidence of wild-type, nef-open challenge virus. All four unvaccinated controls died, whereas all four previously vaccinated animals are alive and healthy and show no signs of the presence of wild-type virus. These are the most impressive protective effects we have seen in any of our vaccine experiments, including those experiments with inactivated whole virus (1), vaccinia recombinants expressing SIV env (9), multivalent vaccinia recombinant expressing gag, pol, and env that can be assembled into SIV pseudovirions particles (9), and multivalent vaccinia recombinant priming followed by SIV particle boosting (9).

We do not know the mechanisms that are responsible for the protective immunity observed in these experiments. In our previous vaccine experiments with vaccinia recombinant priming and SIV particle boosting, we were able to achieve neutralizing antibody titers that were 4 to 12 times higher on the day of challenge than those present in the monkeys vaccinated with SIV/nef-deletion (9). Yet, we have been unable to achieve a single vaccine protection with these priming-boosting protocols.
(9), including challenges performed with the same doses of the same challenge stocks that were protected against by the SIV/ nef-deletion vaccine. Either the nature of the neutralizing antibodies being elicited is different with the different approaches or the key protective component is something other than neutralizing antibodies. A possible role of cytotoxic T lymphocytes is certainly worthy of intensive investigation. Most viral vaccines currently in use in humans are of the live attenuated variety. It is simply difficult to match live virus infection for the strength, breadth, nature, and duration of the immune response that is generated. Our results suggest that live attenuated HIV-1 may also be the most potent, effective vaccine for the prevention of AIDS. Concern for safety is likely to be the key issue for the eventual development of this approach. In addition to nef, other genetic elements can be deleted from HIV-1 to help ensure long-term safety. Gibbs et al. (23) have identified an SIV mutant deleted in three genetic elements that replicates well in cell culture, infects rhesus monkeys, and induces neutralizing antibody responses that are only slightly less than those induced by the SIVmac239/ nef-deletion mutant. The use of deletion mutations is an important part of this strategy. The use of deletion mutants eliminates the possibility of reversion and helps to ensure the safety of inoculation with the attenuated virus. If other vaccine approaches indeed show little or no efficacy under field conditions, limited safety testing of live, multiply deleted HIV-1 in high-risk human volunteers seems warranted.

17. Two rhesus monkeys were preselected as negative for antibodies to SIV, type D retrovirus, simian T-lymphotropic virus type I (STLV-I), and foamy retrovirus. We prepared PBMCs from each by banding over Ficol-Hypaque and stimulated them with phytohemagglutinin (PHA; 1 μg/ml) for 2 to 4 days in complete RPMI 1640 medium with 20% fetal calf serum. Cells were then cultured in complete media that contained interleukin-2 but lacked PHA. Aliquots of each monkey's PBMCs were infected separately with cloned SIVmac239/ nef-open (13, 24) (produced by transfection of cloned DNA into wild-type mouse PBMC cultures) and with uncloned, original, early passage SIVmac251 (15). Culture supernatants were pooled at day 8, filtered through a 0.45-μm filter, and stored in aliquots in the vapor phase of liquid nitrogen (~160°C). SIVmac239/ nef-open stock contained p27 antigen (46 ng/ml) and 2.5 × 10^4 median tissue culture infectious doses (TCID50) per milliliter as determined on CEM×174 cells. SIVmac251 stock contained p27 antigen (96 ng/ml) and 5 × 10^6 TCID50 per milliliter in CEM×174 cells. Virus stocks were negative for type D virus on Raji cells and negative for foamy virus on Vero cells.
19. SIV recovered from animal 397-88 4 weeks after challenge was unfortunately not saved for subsequent genetic analysis.
25. We are particularly grateful to L. Arthur, J. Sullivan, G. Eddy, and the limited number of other scientists who gave encouragement for pursuit of this approach. We also thank D. Silva and D. Xia for technical assistance, B. Blake for critical reading, and J. Newton and T. McDonnell for manuscript preparation. Supported by U.S. Public Health Service grants AI25328, AI26463, AI26507, and RR00168 and a fellowship from the Deutsches Krebsforschungszentrum (F.K.).


**Targeted Degradation of c-Fos, But Not v-Fos, by a Phosphorylation-Dependent Signal on c-Jun**

Athanasios G. Papavassiliou, Mathias Treier, Catherine Chavrier, Dirk Bohrmann*

The proto-oncogene products c-Fos and c-Jun heterodimerize through their leucine zipper domains to form the AP-1 transcription factor. The transcriptional activity of the heterodimer is regulated by signal-dependent phosphorylation and dephosphorylation events. The stability of c-Fos was found to be also controlled by intracellular signal transduction. In transient expression and in vitro degradation experiments, the stability of c-Fos was decreased when the protein was dimerized with phosphorylated c-Jun. c-Jun protein isolated from phorbol ester–induced cells did not target c-Fos for degradation, which suggests that c-Fos is transiently stabilized after stimulation of cell growth. v-Fos protein, the retroviral counterpart of c-Fos, was not susceptible to degradation targeting by c-Jun.

The AP-1 family consists of dimeric, sequence-specific DNA binding transcription factors that are part of the pathway by which intracellular signals are converted into changes of gene activity. Stimulators of AP-1 include tumor-promoting agents [phorbol 12-myristate 13-acetate (TPA)], growth factors, and oncogenic products, all of which have been implicated in the stimulation of cell growth (1). Two components of AP-1, the products of the c-jun and the c-fos proto-oncogenes (c-Jun and c-Fos, respectively), are required for the stimulation of growth of quiescent cells and for continuous cell proliferation (2). These proteins also function in the transformation of cells by nonviral oncogenes, such as activated alleles of ras (3). The function of c-Jun and c-Fos as dominant regulators of cell growth is under stringent control. Inducible phosphorylations and dephosphorylations of c-Jun may modulate its DNA binding and transcription activation potential (4–6). We have found that the stability of c-Fos is also regulated by phosphorylation of c-Jun.

We expressed human c-Jun in HeLa and 293 cells to measure the stability of differentially phosphorylated forms of the c-Fos–c-Jun heterodimer in an in vitro protein degradation assay. Tissue culture cells were transiently transfected with cytomegalovirus (CMV)-based plasmid vectors that encode a c-Jun derivative with an artificial hexahistidine sequence at its NH2-terminus that allows rapid purification (7, 8) while preserving the posttranslational phosphorylation of the protein (6). This expression system yields a c-Jun protein (cmv-Fl35Jun) that is phosphorylated in the TPA-responsive manner described for the endogenous