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Infectivity of Wild-Type and Deleted Proviral SIV DNA

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Abstract: Live attenuated lentiviruses are potentially effective candidate HIV vaccines; however, delivery of these viruses in the field would be problematic. Delivery of attenuated lentiviruses as proviral DNA would be a simple means of immunization, but the efficiency of this method of delivery is not known. In this study, macaques were readily infected following inoculation of plasmid DNA encoding proviral simian immunodeficiency virus (SIVmac239), whether given i.m. (300μg) or epidermally (15μg), with all four animals succumbing to AIDS at a mean of 26 weeks following inoculation. Using a human skin explant model, we found that the 50% infectious dose (ID50) of proviral SIV or HIV-1 plasmid may be as low as 1μg when delivered to skin by gold particle bombardment using a gene gun. An infectious proviral clone of SIV mac239 with a 105bp deletion in the 3' *nef*/LTR overlap region was engineered (SIVsbbcΔ3), analogous to the initial common *nef*/LTR deletion in HIV-1 strains isolated from an Australian cohort of long-term slow-progressors. Two further macaques were also readily infected with SIVsbbcΔ3 after i.m. injection of 300μg of highly purified plasmid DNA. Unexpectedly, in one macaque inoculated with SIVsbbcΔ3 DNA, SIV strains isolated three to six weeks after infection had completely repaired the *nef*/LTR deletion with wild-type sequence, and eventually progressed to AIDS. The mechanism used to rebuild this deletion with wild-type sequence, presumably derived from an intact 5' LTR, is unclear, but possibilities include RNA read-through errors from the plasmid DNA and recombination with residual plasmid DNA at the inoculation site.

Introduction

The human toll of HIV is staggering with more than 21.8 million already dead from AIDS. HIV now kills more people worldwide than any other infectious disease. More than 36 million people are living with HIV and an estimated 5.3 million people were newly infected in 2000. Most of this toll is in underdeveloped countries whose economies cannot answer this threat. Prevention programmes have slowed the spread of HIV, but have not stopped it. The only answer for this world crisis is a safe and effective HIV vaccine.

A number of candidate HIV vaccines have been proposed, including subunit, live vector and live attenuated vaccines. Live attenuated vaccines attempt to induce protective immune responses without causing disease. Studies of live attenuated HIV vaccines have focussed on deletions in the *nef* and LTR genes such as the deletions found in a Sydney Blood Bank cohort (sbbc) of patients with very slowly progressive HIV infection derived by transfusion from a common blood donor [1,2]. Live attenuated SIV vaccines in monkeys have induced strong humoral and cellular immune responses, and protected monkeys from virulent SIV challenge [3]. Evidence suggests that prolonged (> 20 weeks) replication of an attenuated SIV vaccine is required for efficient generation of protective immunity [4,5]. However, live attenuated SIV vaccines have caused AIDS in neonatal monkeys [6] and HIV-1 strains with *nef* deletions have been reported to cause immunodeficiency in a few humans [7,8]. Live attenuated SIV strains can rebuild small deletions in the *nef* gene, regenerating the pathogenic wild type strain [9]. HIV-1 and SIV strains with *nef* deletions may delete other genetic material and potentially become more "fit" with time [3,10]. Although further deletions can be engineered into live attenuated SIV vaccines, there is a risk that the vaccine may become too attenuated to allow efficient replication and protective immunity [11].

If live attenuated vaccines were ever to prove safe enough for human trials, there are many logistical problems in producing and delivering a live attenuated HIV-1 vaccine. To produce sufficient stocks of an attenuated HIV strain by cell culture would be difficult and would also require human cell lines not yet approved for use in vaccine production. Quality control of an attenuated HIV-1 strain, which might rapidly mutate in tissue culture, would be very difficult. Live HIV-1 would require transport and storage at -70°C. All of these problems would be overcome by delivering the virus as infectious proviral DNA.

The aims of this study were to determine (i) if SIV DNA could efficiently initiate SIV infections in macaques when delivered either intramuscularly at a high dose of DNA or epidermally via gene gun at a low dose of DNA, (ii) the kinetics of SIV infection initiated as SIV DNA in comparison to those initiated by virus, (iii) whether initiation of attenuated SIV infections can occur with delivery of *nef*/LTR-deleted SIV DNA, (iv) analyze the resulting SIV infections for evidence of repair of the SIV deletion and (v) assess the infectivity of SIV DNA in human skin explant culture. A full report of the macaque study has recently been submitted for publication (Kent et al, submitted) and this report will summarize the findings, assess the purity of the inoculated SIVsbbcΔ3 DNA, further analyze the kinetics of SIV infections initiated from proviral SIV DNA inoculation in macaques, and study SIV and HIV-1 infections in cultured human skin following SIV or HIV-1 DNA inoculation.

Materials and Methods

SIV plasmids

Plasmids containing the 5' and 3' halves of SIVmac239 provirus were obtained from the NIH AIDS Reference Reagent Program (ARRP, www.aidsreagent.org), contributed by Dr. R. Desrosiers. Full length SIVmac239 provirus plasmid was kindly provided by Dr. K. Peden, (CBER, FDA, Bethesda, MD) and was constructed into a low copy number vector, pKP55, derived from pBR322. The *nef*/LTR deletions from the deleted SIV plasmids (ARRP numbers 2477, 2478) were prepared by first introducing the distal half of SIVmac239 3' from the *SphI* site at position 6446 (where 1 = the start of U3 from Genbank accession M33262) to a flanking *Eco*RI site and ligated into the pKP55 vector, kindly provided by Dr. K. Peden,

(CBER, FDA, Bethesda, MD), that was linearized with *SphI* and *Eco*RI [12,13]. A 105bp *nef*/LTR deletion from position 9657, that removed U3 sequences always changed or deleted in HIV-1 strains from the sbbc [2,10], was made into pKP-SIV-3'Nef-open using appropriate oligonucleotides (Kent et al, manuscript submitted). To make full-length proviral clones, the 5' half of SIV was cut from p239SpSp5' (ARRP #829) with *SphI* and cloned into *SphI*-linearized SIV 3' plasmids (Fig. 1).

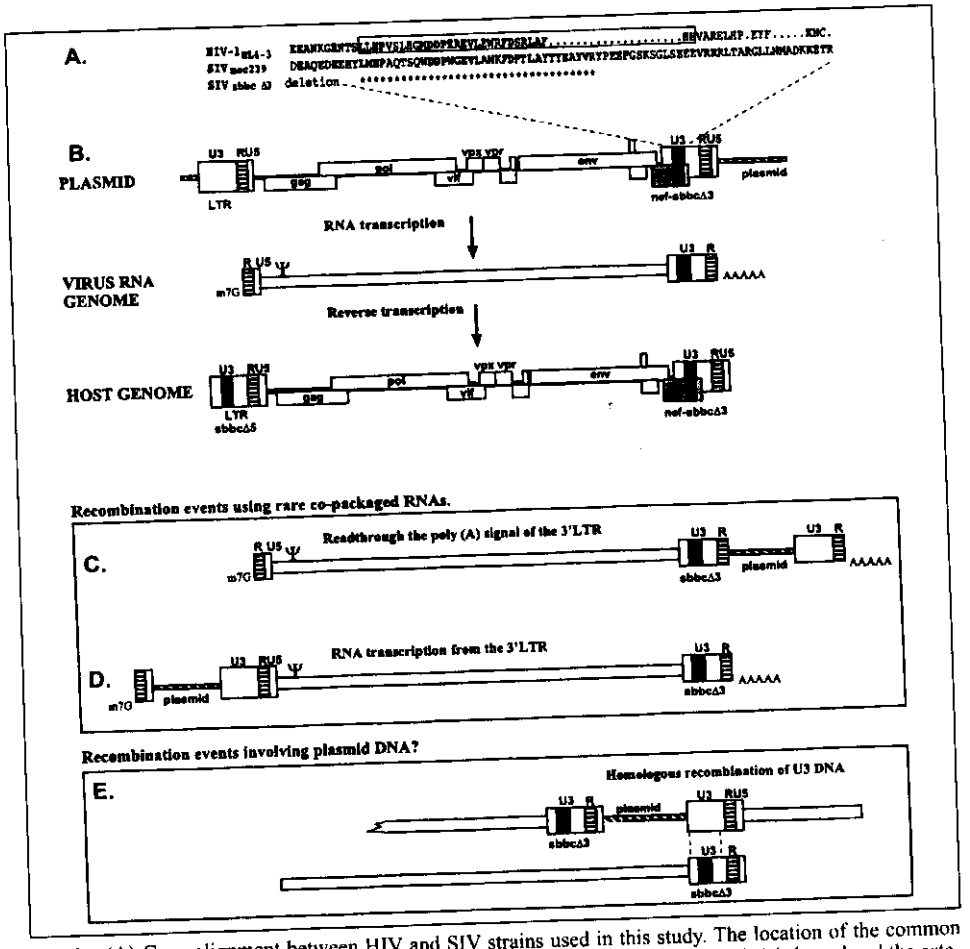

Fig. 1: (A) Gene alignment between HIV and SIV strains used in this study. The location of the common deletion of the HIV-1 strains infecting the Sydney Blood Bank Cohort (sbbc) is boxed and the asterisks denote the 105bp (35 amino acid) deletion introduced into the 3' *nef*/LTR overlap region of SIVmac239 to make the construct SIVsbbcΔ3 for inoculation into macaques. A deletion of identical sequence in the 5' LTR was made for SIVsbbcΔ3Δ5. The deletion in SIV *nef*/LTR is analogous to most of the shared *nef*/LTR sequence deletions present in the Sydney blood bank cohort of humans infected with *nef*/LTR deleted HIV-1 (Kent et al, manuscript submitted). (B) Shows the mechanism of transduction of the 3' U3 (*nef*/LTR) deletion into the 5' LTR after reverse transcription in the first round of replication of SIVsbbcΔ3. The reverse transcription of rare RNA species (C) reading through the 3' LTR and terminating at the poly(A) signal of the 5' LTR in circular plasmid, or (D) initiating in the 3' LTR, would include wild type U3 sequence that could be co-packaged into particles to promote repair of the deletion in U3 during reverse transcription. An alternate explanation is (E) that homologous DNA-based recombination between SIVsbbcΔ3 DNA (or RNA) and the intact 5' DNA from the residual plasmid inoculum.

Infection of macaques with SIV

Pigtailed macaques (*M. nemestrina*, aged 10-16 months) were free from SRV and STLV-1 infection. The studies were approved by the institutional Animal Ethics Committee. All macaques were vaccinated with tetanus toxoid (CSL, Parkville, Australia) i.m. at the time of SIV DNA inoculation. For i.m. injection, 300μg of SIV DNA were delivered in 1ml of saline to the left quadriceps (Table 1). For gene gun immunisations (Bio-Rad, Hercules, CA), plasmid DNA was attached to 1μm gold particles [14]. Macaques immunised by gene gun received 15 non-overlapping administrations of 1μg DNA to shaved abdominal skin at 200-350psi of He pressure. Virus isolation was performed by stimulating dilutions of macaque PBMC with 10μg/ml of PHA and 50U/ml rIL-2 for three days and co-culturing the cells with 10^6 CEM×174 cells for a further 21days and monitored for RT activity.

Table 1: Summary of SIV DNA inoculated animals.

Animal	Plasmid	Dose	Route of inoculation	Peak SIV RNA in plasma (copies/ml)	$CD4^+$ T cell decline	Final status
M11	SIVmac 239	300 μg	i.m.	7.6×10^7	Yes	Died, week 11
M12	SIVmac 239	300 μg	i.m.	1.4×10^8	Yes	Died, week 53
M13	SIVmac 239	15 μg	Epidermal	1.2×10^7	Yes	Died, week 20
M15	SIVmac 239	15 μg	Epidermal	9.4×10^7	Yes	Died, week 19
M14	SIVsbbcΔ3	300 μg	i.m.	2.3×10^6	No	Alive, week 70
M16	SIVsbbcΔ3	300 μg	i.m.	4.7×10^5	Yes	Died, week 61

Examination of SIV deletions in vivo

DNA extracted from PBMC of macaques inoculated with proviral SIV was examined for SIV sequences as previously described (Kent et al, manuscript submitted). Briefly, PBMC were lysed into 100μl of 0.45% NP40, 0.45% Tween 20, 50mM KCl, 2.5mM $MgCl_2$, 0.1mg/ml gelatin, 60μg/ml proteinase K, and 10mM Tris-HCl (pH 8.3) and incubated at 56°C for 1h, then 95°C for 10 min. Nested PCR analysis of SIV DNA was performed using oligonucleotide primers Odp.239, CAGAGACTCTTGCGGGCGC (position 9129-9148), and Odp.193, AGTGCTGGAGAGAACCTCCC (position 10016-10036), for the first round of 30 cycles and Odp.191, ATACTCGCAATCCCCAGG (position 9191-9208), and Odp.192, TGTGGAAAGTCCCTGCTG (position 9872-9890), for the nested round of 30 cycles. PCR was performed with 1U of Amplitaq (Perkin Elmer Cetus, Norwalk, CA), 0.8mM dNTPs and 1μM of primers in 10mM Tris-HCl, 50 mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, together with 2μl of the PBMC lysate in the first round, or 2μl of first round product diluted $1:10^3$ for the second round PCR. For thermocyling we first incubated for 5 min at 94°C then 30 cycles of 94°C (1.5 min), 65°C (1 min), 72°C (2.5 min), before finally incubating at 72°C for 7 min. The sensitivity of the PCR was ≤1 copy of SIV DNA in 1μl. Representative clones were made into the pCRII-topo vector (Invitrogen, San Diego, CA). Inserts were sequenced using the dideoxy method on an automated sequencer (Applied Biosystems, Foster City, CA).

Analysis of the purity of the SIVsbbcΔ3 DNA inoculated into macaques

To determine if wild-type *nef*/LTR sequences were contaminating SIVsbbcΔ3 DNA, we first used primers that amplify both wild type and sbbcΔ3 SIV DNA using oligonucleotide primers Odp.205, GGGAGACTCTTAGGAGAGG (position 9163-9182), and Odp.193, for the first round of 30 cycles (Fig. 4A, PCR – 1). For the nested round of 30 cycles, we used Odp.191, ATACTCGCAATCCCCAGG (position 9191-9208), and Odp.206, GTCATCCCACTGGGAAGTTTGAGCTG (position 9667 – 9693) that primes internal to the deletion (Fig. 4A, PCR –2). As control a titration series added 10-fold dilutions from 1×10^{10} copies of SIVmac239 plasmid into 1×10^{10} copies of SIVsbbcΔ3 plasmid, and a test sample of the SIVsbbcΔ3 plasmid used to inoculate the macaques.

Inoculation of human skin with proviral SIV DNA

Human skin was obtained with patient consent and human ethics approval from the Victoria Plastic Surgery Unit as normal skin otherwise discarded at the time of breast reduction surgery. Skin was stored at 4°C and processed within 2 hours of collection as previously described [15]. After incubation for 30 minutes on

ice in RPMI medium containing 250ug/ml gentamycin (Durlock, Melbourne, Australia) the full thickness skin was spread on a sterile surface and transfected with 1μg of proviral SIV or HIV-1 plasmids by a gene gun (Helios, Bio-Rad) at a pressure of 350psi. Split thickness skin was prepared from the transfected skin using a Froud skin graft knife and placed in culture in 6-well plates overnight. Split skin sheets were treated with dispase (5mgml Worthington) for 6-8 hours at 4°C and the epidermal sheets from each transfection were separated from dermis, divided into two and placed in duplicate culture in 24-well plates. After a further day, 10^6 human PBMCs previously activated with staphylococcal entertoxin B (40ng/ml, Sigma) were added to each well to expand any production of HIV-1. Epidermal skin sheets were removed on day 3 and supernatants harvested from day 4 through 16. Virus production was determined by a reverse transcriptase assay described previously [15] and modified to use a phosphoimager (FLA 2000, Fuji Photo Film Co., Tokyo, Japan) to quantify incorporated radioisotope.

RESULTS

SIV infection of macaques inoculated with SIV DNA

Six pigtail macaques were inoculated with wild type or *nef*/LTR deleted SIV DNA constructs by either i.m. or epidermal routes of administration (Fig. 1, Table 1, Kent et al manuscript submitted). All animals, including those receiving only 15μg of SIV DNA by gene gun, became infected with SIV as evidenced by (i) seroconversion to HIV-2 antigens by western blot (not shown), (ii) detection of SIV RNA in the plasma of all animals (Kent et al, manuscript submitted), and (iii) recovery of SIV from PBMC co-cultures (Fig. 2 and Kent et al, manuscript submitted). Quantitative co-cultures two weeks following administration demonstrated that SIV was recovered from 10^5 PBMC from all six animals (Fig. 2A), but when 10^4 PBMC were co-cultured, SIV was only recovered from animals receiving wild-type SIV DNA and not those receiving the attenuated SIV plasmid (Fig. 2B). Similarly, wild-type SIV DNA-inoculated macaques had higher peak plasma SIV RNA levels at 2-3 weeks following inoculation, ranging from 1.2×10^7 to 1.4×10^8 copies/ml in wild type SIVmac239-inoculated animals and 1.4×10^5 to 2.3×10^6 copies/ml in SIVsbbcΔ3-inoculated animals (Kent et al, manuscript submitted). SIVmac239-inoculated animals maintained SIV RNA levels of $>10^6$ copies/ml and macaques given the SIVsbbc plasmids had SIV RNA levels that fell below detection by week seven following inoculation (Kent et al, manuscript submitted). However, SIV RNA levels in one macaque (M16) inoculated with SIV sbbcΔ3 subsequently rose to $>4\times10^5$ copies/ml by week 46. Although recovery of SIV by co-culture from PBMC of M16 was sporadic between 10 and 40 weeks following inoculation, co-cultures became consistently positive after week 46 (Kent et al, manuscript submitted). Following acute infection, the other macaque inoculated with SIVsbbcΔ3 (M14) had SIV infrequently recovered by co-culture and undetectable or low, stable levels of SIV RNA (Kent et al, manuscript submitted). There were no apparent differences in the kinetics of SIV infection in animals receiving wild-type SIV DNA by either the i.m. (300g) or epidermal (15μg) routes.

Wild-type SIV DNA induced a marked decline in $CD4^+$ lymphocytes followed by clinical findings consistent with AIDS in all four animals (Kent et al, manuscript submitted). The four animals inoculated with wild type SIVmac239 DNA were euthanised at weeks 11, 19, 20, and 53, with SIV-associated symptoms (Kent et al, manuscript submitted). No attenuation of virulence was observed in the gene gun-inoculated animals despite the low dose of DNA administered. Of the two animals inoculated with the SIVsbbcΔ3 DNA, one animal (M16) had a decline in

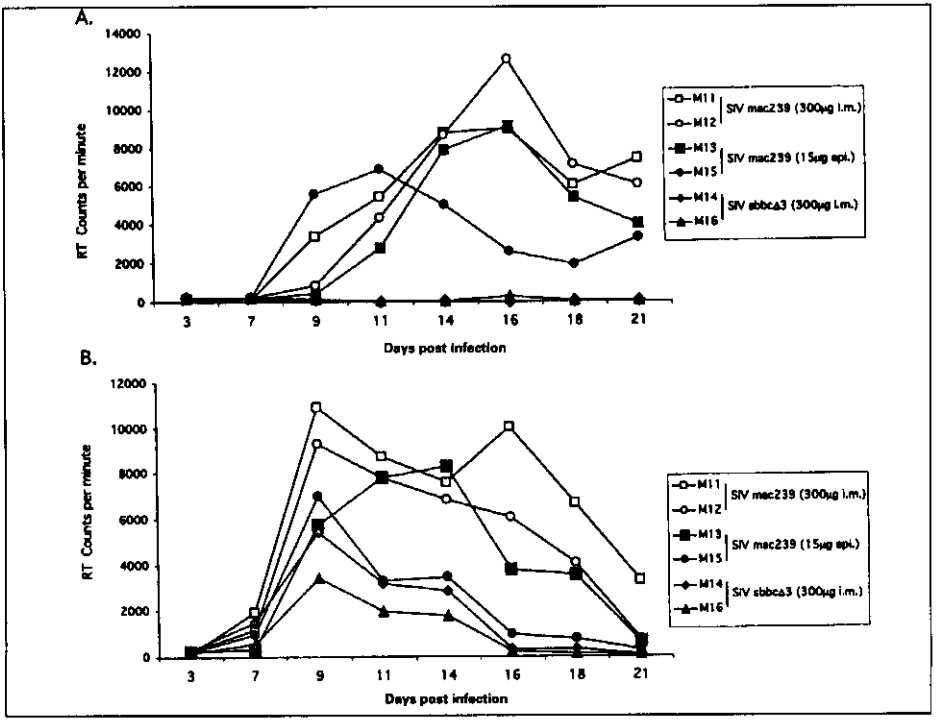

Fig. 2: Quantification of cell-associated SIV following SIV DNA inoculation of macaques. Dilutions of PHA/IL-2 stimulated PBMC from inoculated macaques were co-cultured with CEMx174 cells and assayed for RT activity. Two dilutions of input PBMC are shown two weeks following SIV DNA inoculation. Animals are described in Table 1. (A) at 10^4 PBMC, SIV is recovered only from wild-type SIV DNA inoculated animals, not *nef*/LTR SIV DNA inoculated animals, but (B) at 10^5 input PBMC SIV is recovered from all animals.

$CD4^+$ T cells and was euthanised with weight loss at week 61; the other macaque inoculated with the SIVsbbcΔ3 DNA remained healthy to over 65 weeks (Kent et al, manuscript submitted).

SIV DNA recovery and evidence of recombination

To assess the stability of the inserted deletions, DNA from serial PBMC samples of all six inoculated animals was extracted and subjected to nested PCR and cloning and sequencing across the *nef*/LTR overlap region (Kent et al, manuscript submitted). In all four animals inoculated with wild type SIV size DNA, PCR amplified wild-type sized *nef*/LTR from PBMC at every time point examined from week 2-3 following DNA inoculation until death of the animals detected (Kent et al, manuscript submitted). By nested PCR, DNA of a size consistent with the deleted *nef*/LTR region was amplified from both M14 and M16 early (three weeks) following inoculation (Fig. 3, Kent et al, manuscript submitted). However, after three weeks, amplification of SIV DNA in PBMC from M14 and M16, inoculated with the same SIVsbbcΔ3 construct, yielded different results. Three weeks after

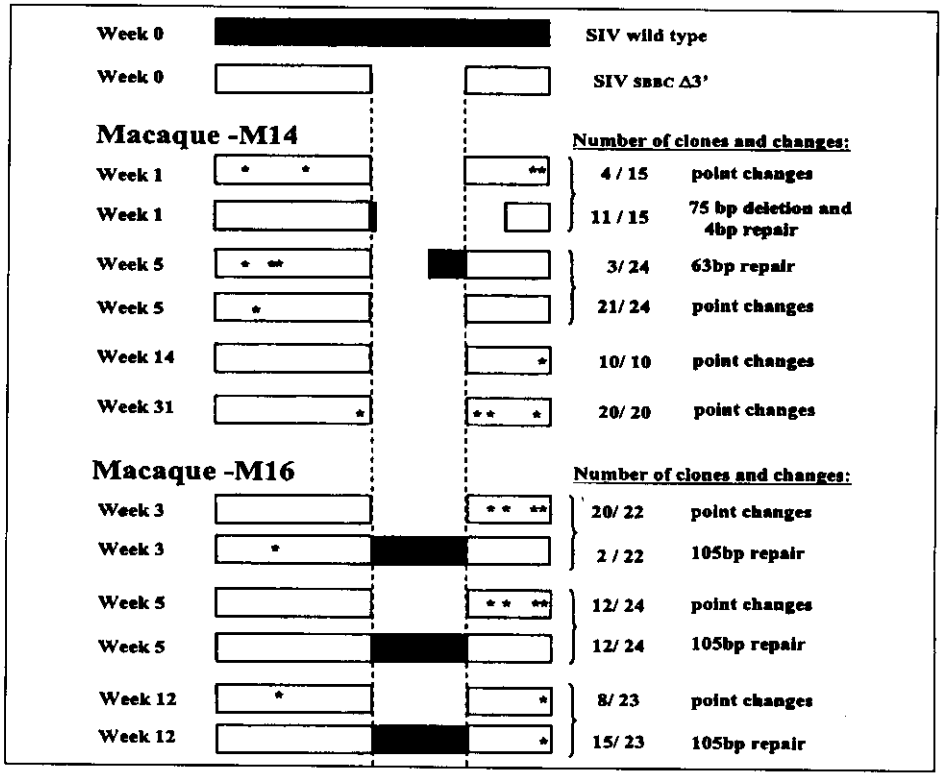

Fig. 3: Diagram of the *nef*/LTR deletion size over time in two macaques, M14 and M16, inoculated with SIVsbbcΔ3. Serial PBMC samples were lysed and extracted DNA analyzed by nested PCR and cloning and sequencing across the 105bp *nef*/LTR deletion (Kent et al, manuscript submitted). The number of clones demonstrating particular mutations is shown on the right.

M14 had been inoculated with SIVsbbcΔ3 DNA, PCR amplimers were of a size consistent with SIV *nef*/LTR deleted sequences, with rare (three of 24) clones isolated five weeks following inoculation in which the 105bp *nef*/LTR deletion had been partially rebuilt with a 63bp insertion exactly homologous with wild type sequence (Kent et al, manuscript submitted). With animal M16, however, wild-type size SIV *nef*/LTR was amplified by nested PCR from week five following SIV DNA inoculation. The wild-type size band became predominant in PCR products from M16 PBMC and was temporally associated with a rise is SIV viraemia and loss of CD4+ T cells in this animal. Sequencing of the *nef*/LTR region demonstrated that a complete reversion to wild-type sequence had occurred (Fig. 3, Kent et al, manuscript submitted).

Absence of wild-type SIV contaminating inoculated SIVsbbcΔ3

These data show that partial and full revertant mutants arose over time in animals inoculated with the *nef*/LTR deleted SIVsbbcΔ3. A possible explanation was that wild-type SIV was contaminating the SIVsbbcΔ3 inoculum. We therefore went back to the inoculated SIVsbbcΔ3 stock and performed a nested PCR using

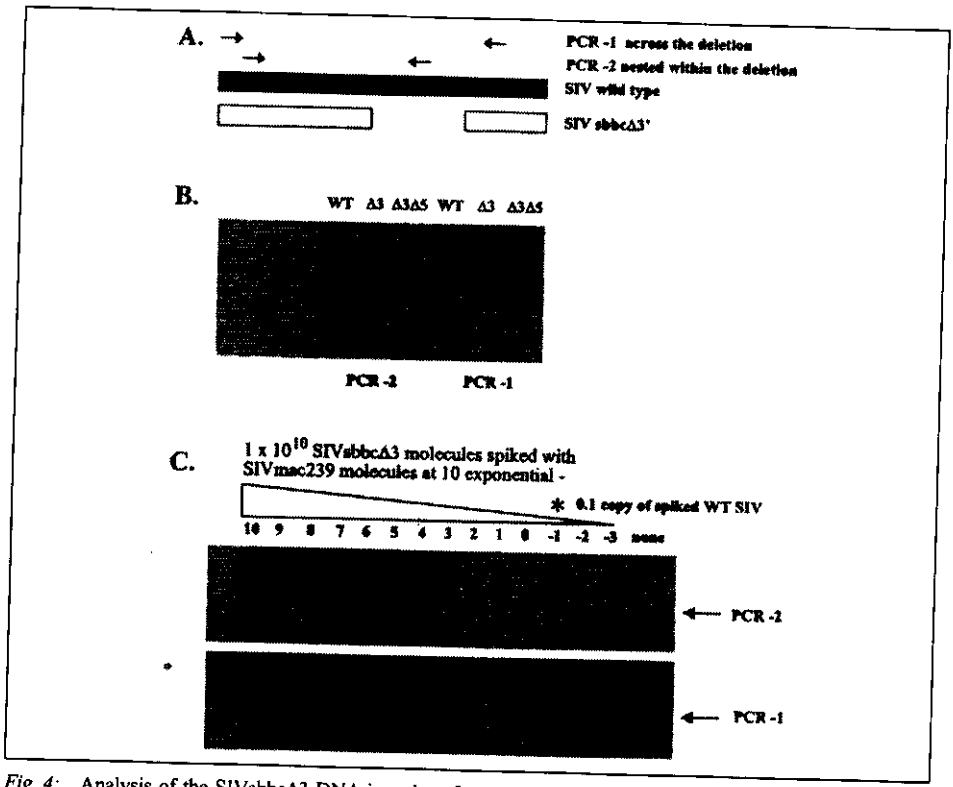

Fig. 4: Analysis of the SIVsbbcΔ3 DNA inoculum for contaminating wild-type sized SIV nef/LTR bands by PCR. (A) Shows the two PCR reactions that detect either both wild type and nef/LTR deleted forms of SIV (PCR – 1), or specifically detected the wild type form of SIV using a nested set of primers (PCR – 2) with template from PCR - 1. (B) Validation of the specificity of these PCR reactions in distinguishing SIV wild type from SIVsbbcΔ3 (noted Δ3), or an additional SIV plasmid containing an identical 105bp LTR deletion at both 3' and 5' ends (SIVsbbcΔ3Δ5, noted Δ3Δ5) using the PCR – 1 and the sensitive nested PCR-2b. (C) A series of 10 fold dilutions of wild-type SIVmac239 (starting at 1×10^{10} copies) was spiked into a constant amount of SIVsbbcΔ3 (1×10^{10} copies) to determine the sensitivity of the assay. Wild-type SIV is detected when a predicted 0.1 copies has been spiked in (denoted with an asterisk).

primers internal to the deletion to detect contaminating SIVmac239 sequence (Fig. 4A, PCR-2). To assess the sensitivity of such a PCR, serially diluted SIVmac239 DNA was spiked into SIVsbbcΔ3 (Fig. 4C). Our results suggested the sensitivity of the assay was ≤1 copy of SIVmac239/10^{10} copies of SIVsbbcΔ3. With this assay, no contamination with wild-type SIVmac239 was detected in the inoculated SIVsbbcΔ3 stock (Fig. 4B and 4C - none), suggesting that nef/LTR recombinations occurred in vivo in the SIVsbbcΔ3 inoculated macaques.

SIV infections in human skin cells following SIV DNA inoculation

Since the macaque studies demonstrated that as little as 15μg of SIV DNA could be used to initiate infection, we then assessed whether even less DNA (1μg) could initiate infection in a human skin explant model. This model uses human epidermis discarded at operation; previous studies have demonstrated that skin cell

emigrants (predominantly Langerhans cells) transmit HIV-1 infection following application of HIV-1 virus to the skin surface [15]. In this study we used the gene gun to transfect human epidermis with 1μg of wild-type SIV, *nef*-deleted proviral SIV, or a control DNA not containing SIV genes and assessed whether SIV could be recovered by co-culture of the emigrating skin cells with activated human PBMC. SIV infections could be initiated in this human skin explant model with 1μg of wild-type SIVmac239 DNA (Fig. 5 bottom panel), but not with a nef-deleted SIV (Fig. 5 third panel). In five of the six replicates RT activity was found in the co-cultures of emigrants from skin transfected with SIVmac239 and activated T cells. A similar frequency of transmission was found with the skin transfected with HIV-1 NL(AD8) chimeric virus (Fig. 5, second panel).

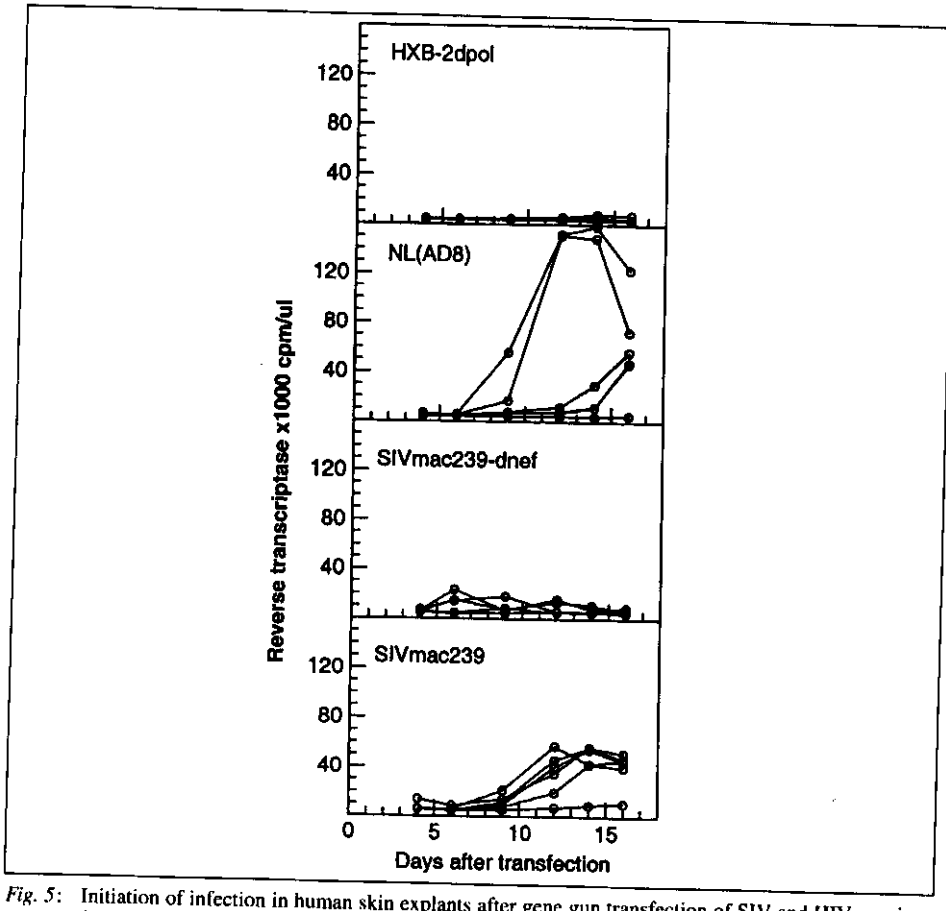

Fig. 5: Initiation of infection in human skin explants after gene gun transfection of SIV and HIV provirus. Skin explants were transfected with SIVmac239, nef deleted SIVmac239, HIV-1 strain NL(AD8) and control DNA HXB2dpol, which does not express infectious HIV-1, as indicated in each frame. The emigrating cells from epidermis were cultured with activated T cells and reverse transcriptase activity in the coculture supernatants determined from days 4 to 16. A similar number of the replicate cultures were productively infected after transfection with full length HIV and SIV proviral plasmids.

Discussion

This report summarizes and extends a study of the delivery or wild-type and attenuated SIV infections initiated via proviral DNA inoculation (Kent et al, manuscript submitted). Pathogenic SIV infections in macaques were initiated following inoculation of wild-type SIVmac239 DNA by either i.m. delivery of 300μg of DNA or epidermal administration of 15μg of DNA. Delivery of these constructs as DNA resulted in an infection which was as virulent as infection with virus, with animals demonstrating high levels of SIV replication, a loss of CD4+ T cells, and death at a mean of 26 weeks following SIV DNA inoculation. Previous studies have also demonstrated the infectivity and pathogenicity of wild-type SIV DNA [16-18], although these studies have used higher doses of SIV DNA delivered i.m. Epidermal delivery of SIV DNA may efficiently induce SIV infections with low amounts of SIV DNA. Supporting this concept was the recovery of wild-type SIV from cells migrating from human skin following inoculation of only 1μg of SIV DNA (Fig. 5). The delivery of proviral HIV-1 or SIV DNA to the epidermis, rather than i.m., is likely to lead to uptake and infection of skin dendritic cells (Langerhans cells), transfer of virus to local lymph nodes, and high levels of replication in dendritic cell-T cell clusters in lymph nodes, suggesting that this route may be more efficient that i.m. delivery [19].

A novel proviral SIV construct with a deletion only in the 3' *nef*/LTR overlap region was constructed and inoculated into two macaques. One animal (M16), sustained a complete reversion of a minority of clones to wild type SIVmac239 *nef*/LTR sequence within three weeks following inoculation. The minority wild type SIV*nef* population slowly but effectively competed against the attenuated SIV population until dominating the recovered *nef*/LTR sequences by 31 weeks following inoculation (Fig. 4). $CD4^+$ T cell counts declined thereafter and the animal was euthanised at week 61. The other animal (M14) remains healthy but sustained a partial, but transient repair of the *nef*/LTR deletion at an early time.

The precise, complete and rapid reversion event to the identical wild type sequence in monkey M16 suggests that either wild-type sequence was contaminating the inoculum or that recombination with homologous sequence at the 5' LTR in the inoculated DNA occurred (Kent et al, manuscript submitted). A sensitive PCR assay excluded the presence of wild-type SIV in the inoculum to the limits of the assay. We believe this is the first demonstration of homologous recombination in vivo following administration of a single SIV strain. The mechanism driving these recombination events in unclear but possibilities include: (i) recombination with a co-packaged rare RNA transcript that reads through the deleted 3' LTR and terminates at the poly(A) signal site in the R region of the wild type 5' LTR in the plasmid, thereby including a wild type U3 (Fig. 1C), (ii) recombination with co-packaged transcripts initiating from the 3' LTR and reading through the 5' U3 to the poly(A) signal sequence in the 3' LTR (Fig. 1D), or (iii) recombination between the wild type 5' LTR of plasmid DNA and viral DNA or RNA forms (Fig. 1E). These recombination events may be avoided if the 5' LTR contains the same deletion inserted into the 3' LTR.

In summary, live attenuated SIV vaccines can be delivered as proviral DNA, potentially providing a feasible mechanism to deliver such vaccines in the future. Further, epidermal administration of SIV DNA appears to be efficient and requires small doses of DNA, both in macaques and in human skin. Using the skin explant

model, we show that a 50% infectious dose (ID50) of wild type proviral SIV or HIV-1 plasmid may be as low as 1μg when delivered to skin by gold particle bombardment using a gene gun. Attenuating mutations in the *nef*/LTR of an HIV-1 or SIV plasmid require a higher DNA dose for infection. However, in vivo recombination events after live attenuated SIV DNA inoculation can reconstitute wild type pathogenic SIV, presumably by recombination events between the deleted 3' nef/LTR and an intact 5' LTR.

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Discussion

Clive PATIENCE: Did you see a marked decrease in the CD4 count following intradermal administration of your variants after one week? If so, do you have any explanation why the CD4 count went down about four-fold?

Damian PURCELL: We were measuring the percent of CD4s, which is a percent of the pre-inoculation percentage. Rather than counting total CD4 cells, we ran the samples through the FACS and examined the ratio of CD4s at a set time point, comparing that with the pre-inoculation time point. It is looking at the ratio rather than the actual numbers.

Clive PATIENCE: Do the real numbers follow the same pattern as well, or is it disturbance of the ratio of your CD4?

Damian PURCELL: When we started, we did not have the technology to count the numbers in the primates, but we shall be able to do so in the next set of animals.

Ben BERKHOUT: The unusual recombination event that you see may still have occurred through RNA in two ways. Perhaps the transcription is fired from your plasmids starting at the 3' LTR. Transcripts also start at the 5' LTR, but you get read-through transcription.

Damian PURCELL: It is possible that transcription is initiated from the 3' LTR and read through an RNA transcript to the 5' LTR, which would then be terminated and polyadenylated.

Ben BERKHOUT: But if polyadenylation is not efficient?

Damian PURCELL: The other more likely mechanism is a read-through of the polyadenylation site in the 5' LTR down to the second one, making quite a long RNA, which might be co-packaged. We have not completely excluded it, but we need a second experiment. The selective pressure to pick up that piece of DNA would be greater than for any cellular DNA.