Prevention of SIV Rectal Transmission and Priming of T Cell Responses in Macaques after Local Pre-exposure Application of Tenofovir Gel

Martin Cranage[1*], Sally Sharpe[2], Carolina Herrera[1], Alethea Cope[1], Mike Dennis[2], Neil Berry[3], Claire Ham[3], Jonathan Heeney[4,5], Naser Rezk[6], Angela Kashuba[6], Peter Anton[7], Ian McGowan[7¤], Robin Shattock[1]

1 Centre for Infection, Division of Cellular & Molecular Medicine, St George's University of London, London, United Kingdom, **2** Centre for Emergency Preparedness and Response, Health Protection Agency, Porton Down, Salisbury, United Kingdom, **3** Division of Retrovirology, National Institute for Biological Standards & Control, South Mimms, United Kingdom, **4** Department of Virology, Biomedical Primate Research Centre, Rijswijk, The Netherlands, **5** Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom, **6** University of North Carolina Centers for Aids Research (CFAR) Clinical Pharmacology and Analytical Chemistry Core, Chapel Hill, North Carolina, United States of America, **7** Center for Prevention Research, David Geffen School of Medicine at the University of California Los Angeles (UCLA), Los Angeles, California, United States of America

Funding: This work was supported by a National Institute of Allergy and Infectious Diseases National Institutes of Health (NIH) U19 award AI060614–01, the University of North Carolina (UNC) CFAR (AI 50410; AK and NR), and by the Sir Joseph Hotung Trust (MC and AC). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors declare no competing financial interests.

Academic Editor: Florian Hladik, University of Washington, United States of America

Citation: Cranage M, Sharpe S, Herrera C, Cope A, Dennis M, et al. (2008) Prevention of SIV rectal transmission and priming of T cell responses in macaques after local pre-exposure application of tenofovir gel. PLoS Med 5(8): e157. doi:10.1371/journal.pmed.0050157

Received: November 13, 2007
Accepted: June 9, 2008
Published: August 5, 2008

Abbreviations: AI, anal intercourse; ARV, antiretroviral; IFN-γ, gamma interferon; MNC, mononuclear cells; SFU, spot forming unit; SIV, simian immunodeficiency virus; PBMC, peripheral blood mononuclear cells; RT, reverse transcriptase; VI, virus isolation; vRNA, viral RNA

* To whom correspondence should be addressed. E-mail: mcranage@sgul.ac.uk

¤ Current address: Magee Womens Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America

ABSTRACT

Background

The rectum is particularly vulnerable to HIV transmission having only a single protective layer of columnar epithelium overlying tissue rich in activated lymphoid cells; thus, unprotected anal intercourse in both women and men carries a higher risk of infection than other sexual routes. In the absence of effective prophylactic vaccines, increasing attention is being given to the use of microbicides and preventative antiretroviral (ARV) drugs. To prevent mucosal transmission of HIV, a microbicide/ARV should ideally act locally at and near the virus portal of entry. As part of an integrated rectal microbicide development programme, we have evaluated rectal application of the nucleotide reverse transcriptase (RT) inhibitor tenofovir (PMPA, 9-[(R)-2-(phosphonomethoxy) propyl] adenine monohydrate), a drug licensed for therapeutic use, for protective efficacy against rectal challenge with simian immunodeficiency virus (SIV) in a well-established and standardised macaque model.

Methods and Findings

A total of 20 purpose-bred Indian rhesus macaques were used to evaluate the protective efficacy of topical tenofovir. Nine animals received 1% tenofovir gel per rectum up to 2 h prior to virus challenge, four macaques received placebo gel, and four macaques remained untreated. In addition, three macaques were given tenofovir gel 2 h after virus challenge. Following intrarectal instillation of 20 median rectal infectious doses (MID_{50}) of a noncloned, virulent stock of $SIV_{mac251/32H}$, all animals were analysed for virus infection, by virus isolation from peripheral blood mononuclear cells (PBMC), quantitative proviral DNA load in PBMC, plasma viral RNA (vRNA) load by sensitive quantitative competitive (qc) RT-PCR, and presence of SIV-specific serum antibodies by ELISA. We report here a significant protective effect ($p = 0.003$; Fisher exact probability test) wherein eight of nine macaques given tenofovir per rectum up to 2 h prior to virus challenge were protected from infection ($n=6$) or had modified virus outcomes ($n = 2$), while all untreated macaques and three of four macaques given placebo gel were infected, as were two of three animals receiving tenofovir gel after challenge. Moreover, analysis of lymphoid tissues post mortem failed to reveal sequestration of SIV in the protected animals. We found a strong positive association between the concentration of tenofovir in the plasma 15 min after rectal application of gel and the degree of protection in the six animals challenged with virus at this time point. Moreover, colorectal explants from non-SIV challenged tenofovir-treated macaques were resistant to infection ex vivo, whereas no inhibition was seen in explants from the small intestine. Tissue-specific inhibition of infection was associated with the intracellular detection of tenofovir. Intriguingly, in the absence of seroconversion, Gag-specific gamma interferon (IFN-γ)-secreting T cells were detected in the blood of four of seven protected animals tested, with frequencies ranging from 144 spot forming cells (SFC)/10^6 PBMC to 261 spot forming cells (SFC)/10^6 PBMC.

Conclusions

These results indicate that colorectal pretreatment with ARV drugs, such as tenofovir, has potential as a clinically relevant strategy for the prevention of HIV transmission. We conclude that plasma tenofovir concentration measured 15 min after rectal administration may serve as a surrogate indicator of protective efficacy. This may prove to be useful in the design of clinical studies. Furthermore, in vitro intestinal explants served as a model for drug distribution in vivo and susceptibility to virus infection. The finding of T cell priming following exposure to virus in the absence of overt infection is provocative. Further studies would reveal if a combined modality microbicide and vaccination strategy is feasible by determining the full extent of local immune responses induced and their protective potential.

The Editors' Summary of this article follows the references.

Introduction

The development of an effective vaccine against HIV is still thought to be a long-term endeavour; meanwhile the HIV pandemic continues relentlessly, fuelled primarily by sexual transmission. The relative ease by which HIV is transmitted rectally [1–4] makes this a particularly important, although relatively neglected, route to target with prevention strategies. Unlike the vagina, the rectal canal has only a single layer of columnar epithelium overlying tissue rich in activated lymphoid cells [5,6] and therefore for reasons of both anatomy and immunological status presents a particular challenge for preventative modalities. It is difficult to estimate the prevalence of anal intercourse (AI) in the heterosexual population, but recent studies have indicated that it may be far more common than had been thought. In a population-based study of 2,547 Northern Californian women aged 18–29 y, AI was reported in 21.7% of sexually active individuals [7]. Higher rates of AI have been described in women at particular risk of HIV infection through drug use, forced intercourse, and prostitution [8–11]. Data from Africa are very limited, but levels in excess of 40% incidence have been described in commercial sex workers in South Africa [12,13]. These figures alone demonstrate the need for the development of rectal microbicides, a focus catalyzed by the more visible need of the population of men who have sex with men, which still accounts for the majority of new infections in North America, South America, and Europe. Moreover, many of these men who have sex with men are having unprotected AI irrespective of their HIV status [14]. In the absence of vaccines, increasing effort is being applied to the parallel approach of preventative microbicides/pre-exposure use of antiretrovirals (ARVs). To date preclinical studies of tenofovir in the macaque rectal challenge model using either simian immunodeficiency virus (SIV) or recombinant chimeric simian HIV (SHIV) have focussed on the use of the orally bio-available prodrug tenofovir disoproxil fumarate (TDF) [15,16]. Although partial protection was observed against multiple low dose mucosal virus challenge, it appeared that the dose of available drug may have been suboptimal. We have taken a different approach, reasoning that the optimal route to protect cells of the rectum and possibly beyond may be through local application of tenofovir given, in effect, as a rectal microbicide. As well as testing this hypothesis, we sought to determine if protective efficacy correlated with drug uptake and whether exposure to virus in the absence of overt infection stimulated virus-specific T cell responses.

Methods

Macaques, Tenofovir Gel, and Virus

A well-established macaque rectal challenge model using uncloned SIVmac$_{251/32H}$; a virus that results in high cell-associated and plasma viral RNA (vRNA) loads shortly after a single application to naïve macaques [17–20] was used for this study. Purpose-bred male rhesus macaques (*Macaca mulatta*) of Indian origin aged 4–6 y were obtained from a UK breeding colony. All animals were housed according to the Code of Practice of the UK Home Office (1989) and were sedated with ketamine hydrochloride prior to inoculation with virus and/or venepuncture. Animals were killed humanely by an overdose of anaesthetic. All procedures involving animals were approved by the Ethical Review Committee of the Centre for Emergency Preparedness and Response, Salisbury, UK. None of the animals had been used previously for experimental procedures and all animals were shown to be SIV negative (by virus isolation [VI], PCR, and serology) before entering the experiment. Tenofovir gel (1%) or control placebo gel, a proprietary formulation containing purified water with edetate disodium, citric acid, glycerin, methylparaben, propylparaben, and hydroxyethylcellulose (pH 4.5) supplied by Gilead Sciences was transferred to 5-ml syringes and administered in 3-ml volumes via a 10 FG soft catheter introduced 8 cm into the rectum. The process was atraumatic with no obvious leakage. Assignment of macaques to different treatment groups is shown in Table 1.

Table 1. Assignment of Macaques to Experimental Groups

Study	Group	Animal Number	Procedure
Evaluation of protective efficacy of rectal tenofovir	A	D3, D30, D37, D39, D43, D79	Tenofovir 15 min before virus
	B	C57, D9, D26, D68	Placebo 15 min before virus
	C	D77, D83, E73, E81	No treatment before virus
	D	D14, D69, D56	Tenofovir 2 h before virus
	E	D15, D18, D29	Tenofovir 2 h after challenge
Explant studies	F	M3, M6	No treatment before necropsy
	G	M1, M5, M32, M38	Tenofovir 3 h before necropsy

doi:10.1371/journal.pmed.0050157.t001

All macaques were challenged with 20 median rectal infectious doses MID_{50} of SIVmac $_{251/32H}$ 11/88 stock equivalent to 18,974 50% tissue culture doses ($TCID_{50}$) as determined by previous titration [18]. Virus inoculum was diluted aseptically shortly before challenge and administered in 3-ml volumes with a 6 FG soft catheter 8 cm into the rectum. At 1, 2, 6, 12, 16, and 20 wk postchallenge macaques were bled to provide 15 ml of heparinised blood for VI and proviral DNA assay, 2 ml of EDTA blood for plasma vRNA load determination, and 2 ml of clotted blood for serum antibody analysis. Animals were observed for adverse effects immediately after injection of test substance, followed by daily observation for the duration of study. At each time of sedation the following clinical data were taken for each animal: body weight, temperature, axillary and inguinal lymph node scores, and haemoglobin concentration. Necropsies were performed on all the animals from which SIV had not been recovered and on two SIV-infected animals. At necropsy, the animals were assessed for gross pathology and the following specimens collected for analysis: heparinised blood, spleen, inguinal, mesenteric, and iliac lymph nodes, 20-cm sections of ileum/jejunum, and colorectum tissue. The gut tissues were washed to remove faecal material and each sample placed into 20 ml of RPMI supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and gentamicin (80 μg/ml) before rapid transport to the laboratory (3–4 h) on water ice for explant culture. All other tissues, as well as small samples of the gut tissues, were frozen at −70 °C for later analysis by PCR.

Two other SIV-naïve rhesus macaques were used to obtain gut tissues for explant studies of tenofovir antiviral activity in vitro (see below). A further four SIV-naïve macaques were

dosed with tenofovir gel as described above 3 h prior to necropsy, and gut tissues used for ex vivo analysis of intracellular drug concentration and viral inhibitory activity (Table 1).

Virus Isolation

At each time point of sampling, 2×10^6 peripheral blood mononuclear cells (PBMC) were cocultured with C8166 cells essentially as described previously [21]. Cultures were examined at the time of feeding every 3–4 d for cytopathic effect (cpe). Cultures where cpe was not seen were kept for at least 28 d. Virus growth was confirmed by immunofluorescent staining of acetone-methanol fixed cells for the detection of SIV antigens using a polyclonal antiserum from an SIV infected macaque. A similar procedure was used for back titration of the challenge stock virus, where 10-fold dilutions were added to four replicate flasks of C8166 cells for each dilution tested.

Plasma vRNA

Plasma vRNA concentrations were determined by quantitative competitive (qc) real time reverse transcriptase (RT)-PCR with a cut-off sensitivity of 40 RNA equivalents/ml [22].

Cell Associated Proviral DNA

Levels of proviral SIV *gag* DNA were determined using a highly sensitive PCR assay. Genomic DNA was extracted from whole blood or lymphoid samples by proteinase K digestion and phenol/chloroform using standard protocols, and the amount included in each PCR determined retrospectively by microfluorometry using a total DNA quantification kit on the basis of calf-thymus DNA standards (Sigma). One microlitre aliquots of DNA extract were assayed, in triplicate, using a Taqman Universal PCR Master mix (Applied Biosystems) with primers and probe located in conserved regions of *gag*. Ten-fold serial dilutions of a p2-LTR plasmid [23], which encompasses the target *gag* sequences and diluted in 60 μg/ml herring sperm DNA, were included in each run as controls. Primer sequences were 5′-AGTGCCAACAGGCTCAGAAAA-3′ (forward, 1253–1273) and 5′- TGCGTGAATGCACCA-GATG-3′ (reverse, 1304–1322) numbering based on SIV_{mac239}, at an optimal concentration of 900 nM for each primer. Taqman hydrolysis probe sequence 5′-(6′-FAM-TTAAAAAGCCTTTATAATACTGTCTGCG-BHQ1)-3′ (forward, 1275–1301) was used at a concentration of 225 nM. Amplifications were performed on an Mx3000P genetic analyser (Stratagene) with a thermoprofile of initial HotStart of 95 °C/10 min, followed by 95 °C/30 s, and 60 °C, 1 min/45 cycles. Quantitative SIV DNA levels were calculated using the Mx3000P software, expressed as copies of SIV DNA per 10^5 PBMC/mononuclear cells (MNC). The absolute detection limit of the assay, as determined by Poisson statistics, was a single SIV DNA copy. Similar data were generated on DNA extracted from low-copy number SIV-infected cell preparations where the actual copy number per PCR reaction was determined to be 1.48 copies of SIV gag DNA. Each assay was controlled internally with a spiked DNA preparation and validated by detection of <three copies of SIV DNA.

Tenofovir Quantification

Plasma samples were assayed for tenofovir concentration by the Clinical Pharmacology and Analytical Chemistry Core of the University of North Carolina Center for AIDS Research. Drug concentrations in plasma were determined by a validated high pressure, liquid chromatography (HPLC) method with ultraviolet detection [24]. This method utilized a dynamic range of 10–10,000 ng/ml, with intra- and interday variability of <10% across this range. Total tenofovir concentrations were assayed in tissues using a fully validated HPLC method with mass spectrometry detection (S. Choi, et al., unpublished data). Briefly, internal standard (tolbutamide) was added to tissue prior to complete homogenization using a Fast prep-24 Homogenizer and Lysing Matrix A (MP Biomedicals). Samples were subjected to solid phase extraction on a Varian Bond Elut-C_{18} column. Chromatographic separation of TNF and the internal standard (tolbutamide) was achieved with a Varian Polaris $3C_{18}$-A, 150 mm × 2 mm, reversed phase analytical column, using a gradient method of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Detection of TNF and tolbutamide was achieved by electrospray ionization MS in the positive mode using 288.05 and 271.00 m/z, respectively. Linear tenofovir calibration curves were obtained between 1.0 and 1,000.0 ng/ml, with a correlation coefficient (r^2) greater than 0.999. Intra- and interday accuracy for tenofovir ranged from 89.7% and 109.4% and from 97.3% and 104.9%, and precision ranged from 1.3% and 10.9% and 2.6% and 9.0%, respectively. Assays were performed with and without incubating tissue with phosphatase, to enable comparison between total parent tenofovir concentrations, and indirect measures of the combination of mono- and diphosphate (active compound) concentrations. Both approaches were validated according to Food and Drug Administration (FDA) standards (http://www.fda.gov/cder/guidance/) with respect to calibration curve performance, recovery, intra- and interday accuracy, and precision of the method, stability of the analytes at various test conditions (determined by TNF and TNF-DP QC performance), evaluation of specificity and matrix effect, and comparisons of differences between fresh and frozen tissue.

Gamma Interferon Elispot and Antibody Assay

Freshly isolated tissue-derived MNC [25] and cryo-preserved PBMC were used in the analysis of T cell function. The frequency of SIV specific T cells secreting gamma interferon (IFN-γ) was determined by ELISpot essentially as described previously [26] but using pools of $SIVmac_{251/32H}$ 15 mer peptides, made on the basis of epitope prediction: Gag pool (EVA7066.1–16); Nef pool 1 (EVA7067.1–16); Nef pool 2 (EVA7067.17–29); Tat pool (EVA7069.1–10); Rev pool (EVA7068.1–8). Peptides were made as part of Programme EVA and supplied from the Centralised Facility for AIDS Reagents, National Institute of Biological Standards & Control, UK (www.nibsc.ac.uk/catolog/aids-reagent). All assays were carried out in triplicate, using 2×10^5 cells/well and a final peptide concentration of 10 μg/ml for each component of the pool. The frequency of spot forming units (SFUs) was determined with a reader (AID). Background-subtracted frequencies of SIV-specific IFN-γ–secreting T cells above 50 SFU)/10^6 cells and at least twice the standard deviation of the control were considered as positive.

In Vitro Assay of Infectivity Inhibition in Indicator Cells and Explant Cultures

Full-length, replication and infection competent proviral HIV-1 clones pYU2 [27,28] and pNL4–3 [29] were provided by

the NIH AIDS Research and Reference Reagent Program (http://www.aidsreagent.org/).

TZM-bl cells, a CXCR4-positive HeLa cell clone engineered to express CD4 and CCR5 and to contain integrated reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under the control of an HIV long terminal repeat sequence, were obtained from the NIH AIDS Research and Reference Reagent Program and were grown at 37 °C in Dulbecco's Minimal Essential Medium containing 10% foetal calf serum, 2 mM L-glutamine, and antibiotics (100 U of penicillin/ml, 100 μg of streptomycin/ml). For quantification of virus infectivity cells were seeded at 3×10^3 cells/well 24 h before addition of virus. After incubation with virus for 2 d, the cells were washed with PBS and treated with 100 μl of luciferase cell culture lysis reagent. As previously described [30], 50 μl of each lysate was transferred to a white, opaque assay plate for luciferase quantification in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments) using 50 μl of luciferase assay reagent (Promega). The extent of luciferase expression was recorded in relative light units (r.l.u.). Virus infectivity inhibition assays were performed using a standardized amount of virus culture supernatants normalized for infectivity. Cells were incubated with serial dilutions of drugs for 1 h at 37 °C, and then virus was added to cells and left for 48 h. Fifty percent inhibitory concentrations (IC_{50}) were calculated from the sigmoidal dose response curves.

Colorectum and ileum–jejenum explants were prepared and cultivated at an air-media interface as described previously for human tissue [31]. Briefly, after two washes and removal of the muscle from the resected tissue, 2–3 mm^3 explants were cut. Explants from nontreated animals were incubated with or without PMPA at 100 μg/ml for 1 h and then incubated with $SIVmac_{251/32H}$ for 2 h without removal of the drug. Explants from tenofovir-treated animals were incubated directly with virus. After four washes in PBS, the explants were cultivated on gelfoam (Pharmacia & Upjohn) for 15 d with Dulbecco's Minimal Essential Medium (Sigma) containing 10% foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 80 μg/ml gentamicin. Assays were performed with four replicate wells per sample each well containing three explants. Supernatants were harvested at 3, 7, 11, and 15 d and Gag p27 concentrations were measured by ELISA (p27 antigen ELISA, Zeptometrix Corporation) following the manufacturer's instructions.

Statistical Analysis

The significance of the protection data was determined using the two-tailed Fisher exact probability test by manual calculation. Drug activity titration curves were compared by cross-sectional time series analysis using Stata v10 (Stata Corp). p-values of <0.05 were considered to be significant.

Results

Comparison of Tenofovir-Mediated Inhibition of HIV-1 and SIV Replication In Vitro

Having shown that tenofovir had no evidence of toxicity on TZM-bl cells, as assessed by MTT viability assay at the highest dose tested (unpublished data), we initially compared the activity of tenofovir, formulated as a solution from PMPA powder or formulated as a gel, as supplied by Gilead Sciences, against a panel of HIV-1 isolates with alternative secondary receptor usage (CXCR4-using RF, IIIB, and NL4.3; CCR5-using BaL, YU.2, and R8BaL) and against $SIVmac_{251/32H}$; the latter being used for the macaque challenge experiments. There was no evidence of any difference in the mean average level of inhibition of $SIV_{mac251/32H}$ replication with either formulation at any dose ($p = 0.89$). This result was determined allowing for the inherent correlation across doses within the same intervention group using cross-sectional time series analysis. Furthermore, tenofovir IC_{50} values for $SIV_{mac251/32H}$ were of the same order of magnitude as those measured against both R5 and X4 restricted isolates of HIV (Figure 1).

Dosing and Outcome of Virus Challenge

We used a 1% tenofovir gel formulation made and supplied by Gilead Sciences as part of a programme on the development of a vaginal microbicide. Although this formulation had not been optimised for rectal use, we considered that it would provide a suitable starting point for preclinical evaluation. No adverse effects were seen following rectal gel administration. Virological analysis over a 20-wk period after rectal challenge with 20 MID_{50} showed that four of six animals in group A (tenofovir 15 min before challenge) and two of three animals in group D (tenofovir 2 h before challenge) were protected from systemic infection on the basis of failure to (a) recover virus from PBMC, (b) to detect proviral DNA in PBMC, (c) to detect vRNA in plasma, and (d) to detect SIV-specific antibodies in serum. All the naïve/not-dosed animals and three of four animals receiving placebo gel were infected and had high levels of circulating vRNA, and proviral DNA and virus was recoverable from the PBMC at all times of testing (Figure 2). This protective effect just reached statistical significance at $p = 0.05$ (Table 2). Furthermore, in comparison with the controls, two of three animals given tenofovir rectally prechallenge that became infected had modified virological outcomes. Virus was recovered from the PBMCs of animal D43 only at weeks two and six corresponding to the only times that vRNA was detected in plasma. Interestingly, the proviral DNA load remained below 50 copies/10^5 PBMC and by week 20, the last point of sampling, had declined to just above the limit of detection. In animal D56 the earliest time of virus detection by any of the methods used was 12 wk after challenge; a delay not seen in other animals in this study or in control animals rectally infected with the same virus stock in previous studies. Taken together, these data showed that tenofovir gel administered at least up to 2 h prior to virus challenge had a significant protective effect ($p = 0.003$) (Table 3). Inclusion of another 17 naïve controls that had been challenged intrarectally with the same virus challenge stock [17–20] revealed a highly significant level of protection from detectable virus infection ($p < 0.001$) (Table 4). One of three animals given similar intrarectal dosing 2 h after virus challenge (group E) was protected from infection. Back titration of the virus challenge stock in C8166 cells confirmed infectivity titre had been maintained upon storage.

Of the eight animals that remained VI/PCR negative, seven were clinically normal throughout the study, and one, D68, which had received placebo gel, whilst clinically normal at necropsy had enlarged axillary and inguinal nodes at week 12. In contrast, all of the animals in which virus was recovered at high frequency had some clinical signs and/or necropsy

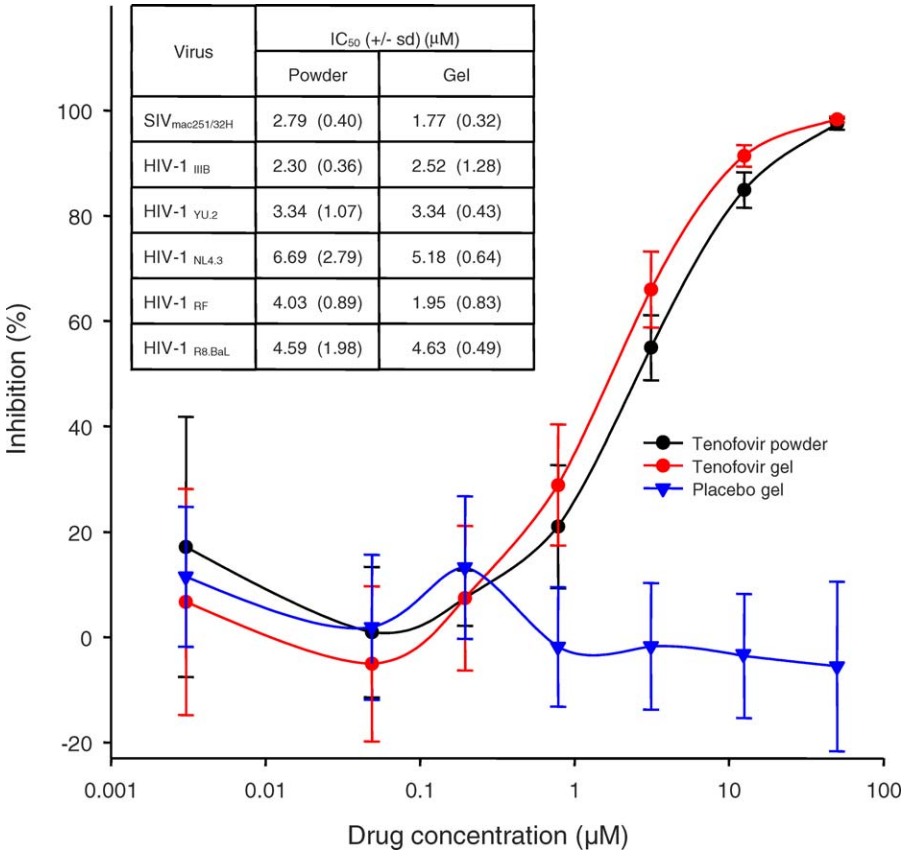

Figure 1. Solution and Gel Formulated Tenofovir Inhibited $SIV_{mac251/32H}$ Infectivity In Vitro at Similar Doses and in the Same Range as for Representative HIV-1 Isolates

Infection of TZM-bl indicator cells in the presence and absence of tenofovir was compared by luminescence analysis of cell lysates and the results expressed as percent inhibition. The graph shows the full titration of drug formulations on infection with $SIV_{mac251/32H}$; the virus stock used in subsequent challenge experiments in vivo. Each point represents the mean of three independent experiments performed in triplicate +/− standard deviation. The results for a panel of HIV-1 strains in comparison to $SIV_{mac251/32H}$ are shown as IC_{50} values in the inset.

doi:10.1371/journal.pmed.0050157.g001

findings consistent with SIV infection, such as enlarged iliac, axillary and inguinal lymph nodes, and splenomegaly. One animal, D83, had evidence of progression to AIDS including loss of weight, tenting of skin, lung abnormalities including, grey discolouration, numerous petechial haemorrhages on the surface, and adhesions to the thoracic wall. Animal D43, which had been given tenofovir 15 min prior to virus challenge and showed only a weak and transient viraemia, remained clinically normal throughout the study.

To determine if virus was sequestered in tissue associated with the virus challenge, quantitative proviral PCR was used to examine MNC isolated from rectum and ileum, as well as iliac, inguinal, and mesenteric lymph nodes. In the apparently protected animals, there was no evidence of infection whereas in animal E81, a naïve challenged control, proviral loads of 26, 5, 130, 2,100, and 105 proviral DNA copies/10^5 MNC equivalents were detected in each tissue respectively.

T Cell Responses in Protected Animals

To determine if mucosal exposure to virus in the absence of overt infection had stimulated T cell immunity, SIV-specific IFN-γ ELISpot analysis was performed on PBMC from protected animals and one infected animal (E81) taken 20 wk after challenge. Four of seven protected animals had IFN-γ-secreting Gag-specific T cells at frequencies ranging from 144 to 261 SFU/10^6 cells, whereas the infected animal E81 had both Gag and Tat-specific circulating T cells (Figure 3A). In contrast, no significant reactivity was seen in 12 naïve macaques tested (<40 SFU/10^6 cells). The finding of T cell responses in the virus exposed macaques was found to be statistically significant; $p = 0.009$ (Fisher exact test). Interestingly, the presence of Gag-specific IFN-γ secreting T cells in animal D68 (pre-exposed to placebo gel) confirmed that challenge virus gained access to antigen presenting cells. Interpretation of T cell reactivity in MNC from colorectal tissue was precluded by a very high spontaneous background; however, MNC from the small intestine of three of four protected animals contained SIV-specific IFN-γ-secreting T cells with broader antigen specificity (including specificity for nonstructural virus-encoded antigens) and in two of these cases no responses in PBMC (Figure 3B). Thus the local mucosal immune system may have been primed by SIV antigens produced de novo during drug-modulated abortive or limited infection. Despite evidence of T cell priming, none of the protected animals had detectable SIV-specific serum antibodies (Figure 3C).

Plasma Tenofovir Concentration and Protection Status

Analysis of plasma tenofovir concentration at the time of virus challenge, 15 min after gel administration, revealed a

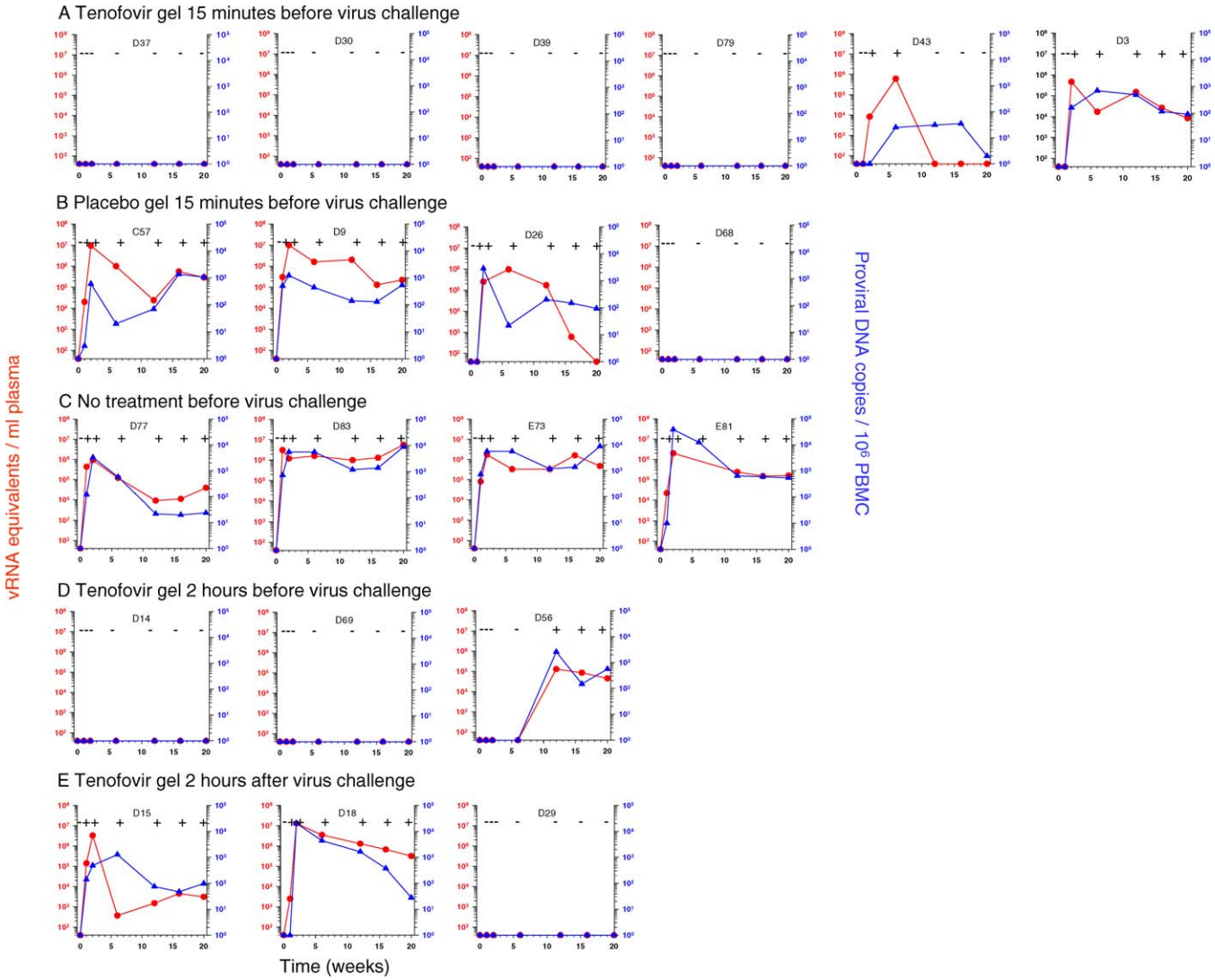

Figure 2. Rectal Administration of Tenofovir Gel Protected a High Proportion of Macaques against Subsequent Acquisition of SIV by Rectal Transmission
The results of VI from PBMC are shown as + or − for each animal. The temporal profiles of plasma vRNA concentration (red dot) and frequency of PMBC-associated proviral DNA (blue triangle) are shown for each animal in the study.
doi:10.1371/journal.pmed.0050157.g002

strong association with protective efficacy. The lowest concentration of plasma tenofovir associated with protection as defined by failure to isolate and/or detect virus in PBMC and plasma and lack of seroconversion, was 119.9 ng/ml (Table 5). Taking into account estimated plasma volume, protection was associated with as little as 0.11% of the total tenofovir applied. Moreover, an effect upon plasma viraemia was observed with as little as 0.06% of applied tenofovir detected in plasma at 15 min. In animals given tenofovir 2 h prior virus challenge plasma tenofovir, at the time of challenge ranged between below the 10 ng/ml limit of detection to 23.3 ng/ml. These results suggested therefore that drug concentration peaked rapidly after rectal dosing.

Ex Vivo Modelling of Tenofovir Activity

To further address the possible mechanism of the protection observed we utilised our recently described in vitro colorectal explant model [31] and adapted it to macaque tissue. Using tissues from another group of SIV-naïve macaques (group F), first, we demonstrated that $SIVmac_{251/32H}$ replicated in this system and that replication was sensitive to addition of tenofovir in vitro (Figure 4A). We also found similar replication in ileum–jejunum explants (unpublished data). Interestingly, replication in macaque explants peaked earlier (day seven) and at a lower level than that seen for HIV in human explants as determined by Gag p27/24 production (C. Herrera, unpublished data and [31]), regardless of virus dose. Next, we investigated the replication kinetics in intestinal tissue explants taken from another four SIV naïve macaques that were given tenofovir gel in vivo 3 h prior to necropsy (group G). In colorectal explants from three of four animals, complete or nearly complete inhibition of virus replication was seen and in the other animal a high level of variability between replicate samples resulted in lower mean

Table 2. Contingency Table for Dosing and Outcome of Virus Challenge: Analysis for "Complete" Protection (i.e., No Virus Detection) Associated with Tenofovir Gel Administered Intrarectally Prior to Virus Challenge; $p = 0.05$

Dosing	Infected	Not Infected
Tenofovir	3	6
No tenofovir	7	1

doi:10.1371/journal.pmed.0050157.t002

inhibition (Figure 4B). In contrast, inhibition of virus replication was not seen in explants from the small intestine (unpublished data) suggesting that tenofovir was, at least in part, acting on cells at the virus portal of entry. Analysis of intestinal tissue samples collected at necropsy showed that all tenofovir-dosed animals had measurable concentrations of drug in lysates of colorectal tissue at concentrations between 20.8 and 54.2 μg/g protein but no drug was detected in lysates of homogenates from the small intestine (Table 6). Tissues from untreated animals (group F) acted as negative controls. To indirectly estimate the amount of intracellular phosphorylated tenofovir in tissues, samples were analysed with (to measure the combination of tenofovir + tenofovir monophosphate + tenofovir diphosphate) and without (to measure tenofovir only) phosphatase hydrolysis. Subtracting the concentration of tenofovir obtained from tissue samples without phosphatase, from the concentration of tenofovir obtained from tissue samples with phosphatase, demonstrated that between 46%–75% of total tenofovir in tissues was present as the intracellular mono- and diphosphate forms. On the basis of intracellular data describing tenofovir monophosphate:diphosphate ratios [32–34], it was estimated that approximately 30%–60% of total tenofovir in tissues was present as the intracellular diphosphate form.

Discussion

Summary of Results

The preclinical study reported here using the SIV-macaque challenge model, showing a statistically significant protective effect, strongly supports the notion that local application of an ARV agent can efficiently protect against subsequent intrarectal challenge with virus. In addition, the study revealed a potential metric of protective efficacy wherein, above a critical threshold, the concentration of tenofovir detectable in the plasma 15 min after rectal application was positively associated with protection. Topical use of tenofovir prior to virus exposure also facilitated the priming of SIV-specific T cell responses in a proportion of macaques, opening up the possibility that this type of prophylaxis may be able to prime and/or boost immune responses elicited with experimental vaccines.

Table 3. Contingency Table for Dosing and Outcome of Virus Challenge: Analysis for Modified Outcome (i.e., "Complete" Protection or Reduced Frequency of Virus Detection) Associated with Tenofovir Gel Administered Intrarectally Prior to Virus Challenge; $p = 0.003$

Dosing	Infected (High Frequency Detection > 80%)	Not Infected or Modified Infection
Tenofovir	1	8
No tenofovir	7	1

doi:10.1371/journal.pmed.0050157.t003

Table 4. Contingency Table for Dosing and Outcome of Virus Challenge: Analysis for "Complete" Protection, Including Historical Controls Challenged with the Same Virus Stock Intrarectally, Associated with Tenofovir Gel Administered Intrarectally Prior to Virus Challenge; $p < 0.001$

Dosing	Infected	Not Infected
Tenofovir	3	6
No tenofovir	24	1

doi:10.1371/journal.pmed.0050157.t004

Protection from Infection

It has become increasingly evident that current experimental approaches to the development of an HIV vaccine, largely based upon the generation of T cell immunity, are, at best, likely only to reduce the level of viraemia following exposure to virus. Although this may have partial benefit, in the longer term, as shown in the SIV model, virus frequently escapes from immune control following the selection of variants mutated in T cell epitopes [35,36]. The development of mucosal targeted vaccines, that may be able to completely prevent infection, is a major roadblock to progress and has refocused the field on prevention of infection at the viral portal of entry. The concept of using ARVs topically to prevent infection with HIV is gaining momentum and several clinical trials are currently in progress [37]. The findings reported here showing protection from overt infection in six of nine macaques against rectal transmission of SIV after topical application of tenofovir gel, suggest that this approach has high potential for clinical translation. Interestingly, even in two of three unprotected animals a delay in appearance of viraemia (D56) or a low, transient viraemia (D43) was seen. Although it is not possible to rule out that these effects were due to natural animal-to-animal variability, the VI profiles were highly atypical compared to those observed in our previous studies with this stock of virus [17,18]. We suggest that there may have been low level seeding of virus into a sparse population of cells containing suboptimal concentrations of drug and that the majority of target cells were blocked by tenofovir providing only limited potential for local replication of virus.

The long intracellular half-life of tenofovir diphosphate, the pharmacologically active metabolite of tenofovir [32,33], potentially makes tenofovir an ideal compound for topical use. Indirect analysis to measure total intracellular phosphorylated tenofovir (mono- and diphosphate combined) concentration in rectal tissue, taken from animals dosed in vivo, indicated that up to approximately 60% of the drug had been converted to the active form. It is known that the metabolism of tenofovir varies in different cell types, at least

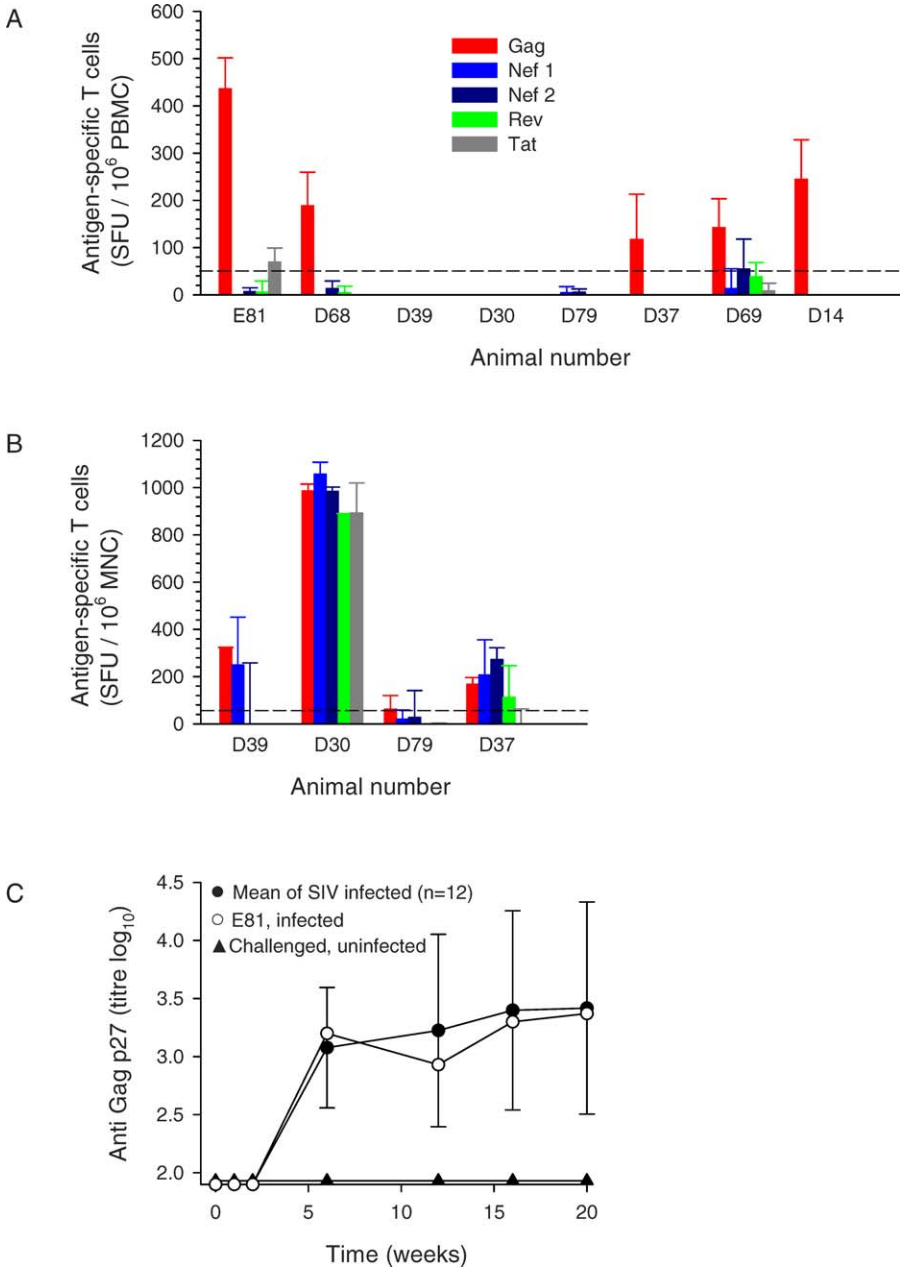

Figure 3. SIV-Specific IFN-γ Secreting T Cells Were Detected in SIV-Challenged Macaques in the Absence of Serum Antibody Responses and Evidence of Overt Infection

(A) IFN-γ secreting T cell frequencies in PBMC from protected animals (D68–D14) compared to those in an SIV-infected animal (E81) measured 20 wk after virus exposure measured by ex vivo ELISpot. The mean frequencies of three replicate determinations plus one standard deviation are shown for each peptide pool used.

(B) SIV-specific IFN-γ secreting T cell frequencies in MNC isolated from ileum–jejunum tissue of four protected animals measured post mortem at 21 wk after virus challenge by ex vivo ELISpot.

(C) The group mean +/– standard deviation profile of anti-SIV Gag p27 binding antibody titres (measured by ELISA) from animals infected with SIV (●) and the individual profile for an SIV-infected macaque E81 (○), in which T cell ELISpot was analysed was compared with animals from which no virus was detected following challenge (▲).

doi:10.1371/journal.pmed.0050157.g003

in vitro [33,34]. Of particular relevance to the present study, it has been reported that in dendritic and Langerhans cells the mono- to diphosphate ratio is approximately 1:3 dependent upon time of sampling [34]. It was encouraging that two of three animals given tenofovir 2 h prior to virus challenge were protected against overt infection. Further experiments are now required to fully determine the longevity of protection and its relationship of tenofovir metabolism in rectal tissue.

Another critical consideration is whether protective efficacy can be increased to 100%. Of particular interest in the present study was the observation of a positive association between the concentration of tenofovir measured in the circulation 15 min after topical application and the outcome

Table 5. Plasma Tenofovir Levels and Association with Protection from Infection

Animal Number	Virus Status	Plasma Tenofovir Concentration (ng/ml)	Weight (kg)	Estimated Total Plasma Tenofovir (ng)	Proportion of Tenofovir Applied Detected in Plasma at 15 min (%)
D30	Protected	180.9	8.18	48,843	0.16
D37	Protected	130.4	10.46	44,988	0.15
D39	Protected	130	7.63	32,760	0.11
D43	Modified profile	73.3	8.02	19,424	0.06
D79	Protected	119.9	14.45	57,192	0.19
D3	Not protected	60.2	7.33	14,568	0.05

Plasma tenofovir concentration was determined 15 min after rectal administration at the time of virus challenge. Weights on the day of challenge were calculated by interpolation of measurements taken 1 wk before and 1 wk after challenge. Total plasma tenofovir was estimated assuming a plasma volume of 33 ml/kg.
doi:10.1371/journal.pmed.0050157.t005

of virus challenge. Our working hypothesis is that this metric provides a surrogate measure of local uptake of tenofovir; thus only in animals where the local tissue concentration is beyond a critical threshold is protection achieved. Using the newly developed assay for intracellular tenofovir it should now be possible to test this hypothesis directly. If indeed the rate limiting step to 100% protective efficacy is local uptake of tenofovir, several possibilities require further investigation, including: is efficacy increased (a) at higher concentrations of administered tenofovir gel; (b) with larger volumes of administered gel; (c) with optimised gel formulations; (d) with combinations of ARTs having differing pharmacokinetics; (e) with prior washing to remove residual faecal matter? Thus, the quantification of plasma tenofovir following rectal administration could be used to inform the optimisation of rectal dosing in terms of concentration, volume, formulation, and timing to accelerate the progression of this approach to clinical trials.

Utility of the Ex Vivo Explant System

Our study has also further validated the use of the ex-vivo rectal explant system by, for the first time, extending its use to macaques. Not only is this system useful for the evaluation of antiviral compounds and combinations in vitro; as we have shown here, it may also be used to measure antiviral activity following application of drug in vivo. Detailed pharmacokinetic studies are required to determine when the peak concentration of tenofovir appears in the plasma, but our results from this preliminary study suggest that the peak is early. Interestingly ileum/jejunum tissue taken from dosed macaques remained susceptible to infection. Indeed, the lack of drug in the small intestine was confirmed by analysis of intracellular tenofovir concentration, suggesting that secondary adsorption at this site is insignificant. The relatively low rectal dose of tenofovir applied, equating to an average of 10 μg/kg, of which a maximum of 0.19% was detected in plasma 15 min later, was far below the dose used in oral pre-exposure prophylaxis [16]. Rectal administration of tenofovir gel may be less likely to select for RT escape mutants even when inadvertently used by HIV-infected individuals; however, this possibility requires further investigations to determine the cumulative concentration of drug entering the circulation. Even though, following optimisation, rectal use of tenofovir alone may prove to be sufficient to confer protection, use in combination with other topical ARVs/microbicides may be advantageous. Experiments are in progress in vitro to model synergistic or additive effects of drug combinations in the explant system. Furthermore, we have recently shown that drug combinations can protect colorectal explants against infection with rarely transmitted RT escape mutants (C. Herrera, unpublished data).

Possible Mechanisms of Distal Protection

At the doses used in the present study it is predicted that the protective effects will be confined to the rectal portal of entry; however, the high degree of protection against rectal challenge seen in the present study, including the absence of SIV detection by highly sensitive qPCR in regional lymph nodes was perhaps surprising given that virus may translocate rapidly in dendritic cells [38]. An interesting possibility that requires further investigation is that tenofovir may be reaching local nodes through lymphatic drainage. The interior iliac lymph nodes are known to be a site of early virus replication and have common drainage of the female genital tract and rectum. Indeed, targeting an SIV vaccine preparation to the vicinity of these nodes has conferred significant protection from rectal challenge [39].

Although our study was not powered to allow statistical analysis of rectal postexposure efficacy, in the group of animals receiving gel 2 h after virus exposure only one of three animals was protected. Given that the virus replication cycle is approximately 24 h, this preliminary observation is in accord with the hypothesis that virus is translocated rapidly from the portal of entry and is sequestered either in a cell in which tenofovir is metabolised only slowly and/or that the virus may be held in trans in a nonreplicating state, as has been proposed for dendritic cell-mediated infection [40,41]. Thus, topical application of ARVs such as tenofovir following local viral challenge could be used to further investigate early virus trafficking in vivo, an area of study in which there is still a critical paucity of information.

Priming of Specific T Cells

The priming of SIV-specific T cells detectable in the circulation in the absence of seroconversion is reminiscent of similar responses seen in so-called highly exposed, persistently seronegative individuals (reviewed in [42]). The restriction of significant reactivity to Gag, a structural component of the virion, in the protected animals, suggested the virus inoculum itself, rather than viral proteins synthes-

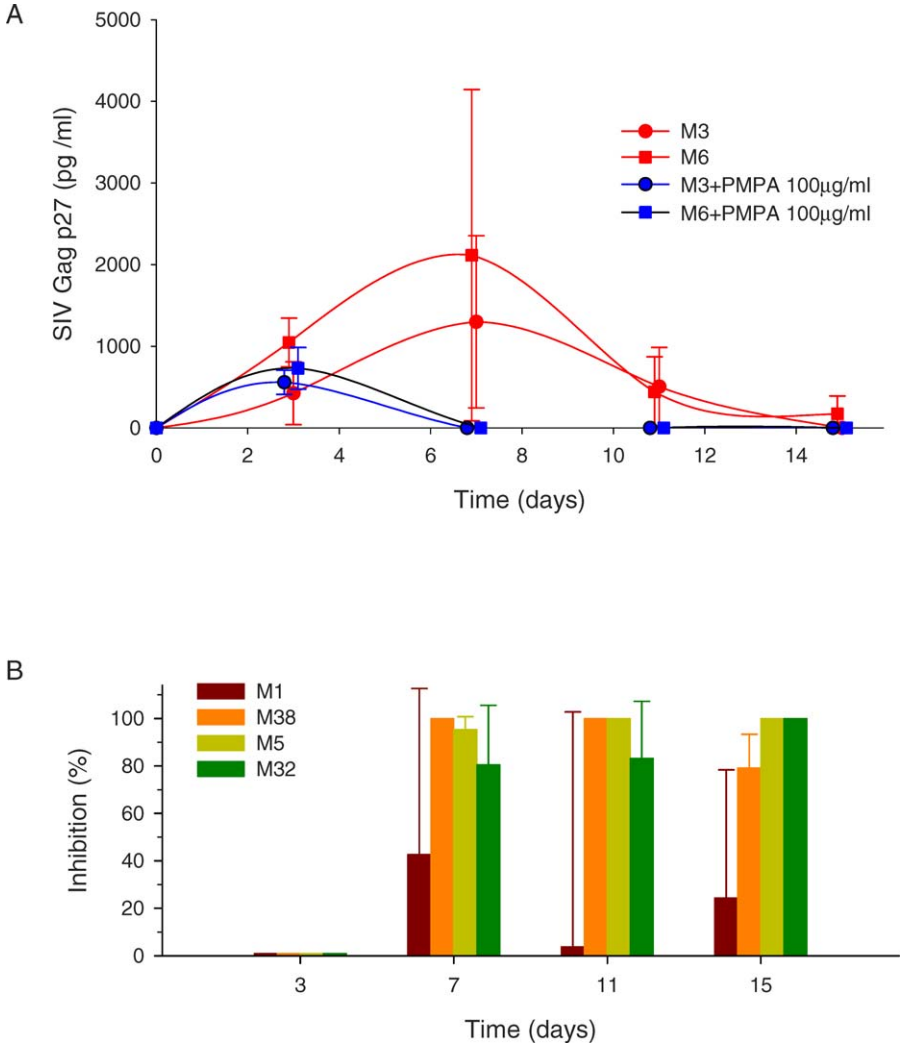

Figure 4. Colorectal Explants from Macaques Supported Replication of SIV That Was Inhibited by Pretreatment with Tenofovir In Vitro and In Vivo

(A) Replication dynamics of $SIVmac_{251/32H}$ in explants from two untreated animals (group F: M3, M6) in the presence or absence of exogenously added tenofovir at 100 µg/ml. A total of 10^4 $TCID_{50}$ of virus was added to each well containing three explants in a total volume of 200 µl of medium. Virus replication was assayed by SIV Gag p27 production and mean values +/– standard deviations are shown for four replicates of each tissue.

(B) Colorectal explants from four animals (group G: M1, M38, M5, M32) dosed in vivo with tenofovir per rectum 3 h before tissue removal were exposed to virus in vitro (as described above) and culture supernatants assayed for Gag p27. Mean percent inhibition of SIV Gag p27 production plus standard deviations are shown.

doi:10.1371/journal.pmed.0050157.g004

ised de novo, may have been processed by antigen presenting cells either for presentation to $CD4^+$ T cells via MHC class II or following processing through cross-presentation pathways, for presentation to $CD8^+$ T cells via MHC class I. It is possible that the tenofovir gel, which had not been formulated for rectal application, resulted in transient tissue damage, as reported for some sexual lubricants [43], resulting in seepage of virus inoculum into the submucosa where local DC could pick up antigen. Nonetheless, the majority of animals were protected from infection in the current study. Alternatively a low level of viral replication may have occurred locally with virus spread curtailed by tenofovir-blockade of secondary target cells. Indeed, transient early postexposure treatment of SIV-infected macaques with tenofovir has been associated with the stimulation of T cell responses, sometimes in the absence of SIV-specific serum binding antibodies [44]. Interestingly, in this instance, $CD8^+$ lymphocytes were also associated with resistance to rechallenge. If limited replication of virus did occur locally in the present experiment, this may explain the apparent broadening of T cell responsiveness in cells recovered from the intestine to include recognition of virally encoded nonstructural antigens. The latter observation however needs cautious interpretation because of the relatively high level of spontaneous background reactivity. Indeed in the large intestine, spontaneous IFN-γ production was so high as to exclude analysis for antigen-specific cells, and although considerably lower in cells recovered from the small intestine, more work using cells recovered from SIV-naïve macaques is required to define a robust cut-off level for specific responses. We have reported previously that single low dose exposure to SIV by the rectal route can induce T and B cell priming in the absence of overt infection and a serum antibody response [25]. It is however, far from clear whether such responses have any antiviral significance or

Table 6. Intracellular Concentrations of Base and Phosphorylated Tenofovir in Colorectal and Ileum–Jejunum of Macaques Given Tenofovir Gel Rectally

Tissue	Animal Number	Drug Concentration with and (without) Phosphatase (μg/g Protein)	Proportion as Intracellular, Phosphorylated Drug (%)
Colorectum	M1	54.2 (24.0)	55.7
	M3	<1	na
	M5	20.8 (7.3)	64.9
	M6	<1	na
	M32	36.8 (9.3)	74.7
	M38	35.9 (19.3)	46.2
Ileum–Jejunum	M1	<1	na
	M3	<1	na
	M5	<1	na
	M6	<1	na
	M32	<1	na
	M38	<1	na

Macaques M1, M5, M32, and M38 were dosed with tenofovir gel intrarectally. Macaques M3 and M6 remained undosed; tissues from these animals were used as negative controls to calibrate the tenofovir assay. na, not applicable.
doi:10.1371/journal.pmed.0050157.t006

whether they represent a "footprint" of abortive virus infection. Further detailed investigation is needed to determine if B cells are primed by virus exposure of tenofovir-predosed animals and whether B and T cell responses can be boosted and broadened in specificity upon subsequent exposure to antigen. The apparent longevity of the T cell response seen in the present study was interesting and further work is required to analyse the dynamics of T cell priming in this context. It is possible that the persistence of responsiveness was due to low level boosting by sequestered virus or antigen. Although proviral DNA was undetectable in tissues of protected animals it is not possible to absolutely rule out the possibility of a very low level occult infection as has been described in macaques following intrarectal exposure to low doses of virus (N. Polyanskaya et al., unpublished data) [44,45]. On-going and planned HIV-preventative clinical trials should provide further insight into the generation and nature of T cell responses in the absence of overt infection. This is of importance, as theoretically, although not supported by data on primed, repeatedly exposed macaques (N. Polyanskaya et al., unpublished data), activated $CD4^+$ T cells at the portal of entry could facilitate or enhance infection by providing a pool of target cells for virus replication. It is imperative that further rechallenge experiments are undertaken in appropriate macaque models before informed decisions can be made regarding advancement of this approach to clinical trial.

Limitations of the Study

The study reported here has several limitations. Firstly, the ARV-gel was delivered controllably under idealised conditions. Ensuring that this occurs in practice will require careful development. Secondly, finding an acceptable form of administration may require different strategies for different target populations. For some users application of a rectal gel may be acceptable, for others, formulation of the ARV into a suppository may be more acceptable. Compatibility with other products that may be used before AI is a further consideration. Thirdly, cell-free challenge virus was applied in a controlled, atraumatic manner in the absence of semen. Fourthly, the predictive value of the SIV-macaque model requires further investigation. This issue was highlighted recently by the much publicized failure of two large clinical trials in HIV prevention (the Merck STEP vaccine trial, and a trial of the vaginal microbicide cellulose sulphate), using modalities that had apparently shown promise in the macaque model. However, in the case of the recombinant replication deficient adenovirus vaccine (STEP trial), reduction in virus load was originally demonstrated following immunization with a construct expressing SIV Gag and challenge with simian HIV (SHIV)-89.6P [46]. This challenge virus has a highly atypical biological phenotype [47], and in a subsequent study with SIVmac239 a marginal effect was only seen in animals expressing the Mamu-A*01 MHC class I allele [48]. Although an enhanced effect was shown in a further study using adenovirus constructs expressing SIV Gag, Rev, Tat, and Nef, this approach was tested only in the more sensitive Mamu-A*01 macaques [49]. Likewise, in the evaluation of cellulose sulphate as a vaginal microbicide, the macaque protection data were not definitive with the majority of treated animals showing evidence of virus infection by PCR [50].

Conclusions

Given the caveats discussed above, our data nonetheless suggest that rectal mucosal dosing with tenofovir shows promise for protection against rectal transmission of an immunodeficiency virus. Moreover, given the functional IFN-γ T cell responses induced, there may be potential for synergy between topical ARV/microbicide use and vaccination as a two-pronged strategy for preventing infection with HIV.

Acknowledgments

We thank Jim Rooney, Gilead Sciences for providing tenofovir gel; Carol Fraser (St. George's University of London [SGUL]) for serological analysis; Sharon Leach and Stuart Dowall (Centre for Emergency Preparedness and Response [CEPR]) for virus isolation and specimen dispatch; and Zara Fagrouch (Biomedical Primate Research Centre [BPRC]) for quantitative competitive (qc)-real time RT- PCR. We are grateful to Neil Almond (National Institute for Biological Standards & Control [NIBSC]) for making laboratory facilities and training available; to Harvey Holmes and the staff of the Centralised Facility for AIDS Reagents, NIBSC, for SIV peptide pools

and recombinantly expressed p27 antigen; and to Jim Turpin (NIH) for useful discussion. We thank Alicja Rudnicka (SGUL) for statistical advice and analysis.

Author contributions. MC, SS, and RS designed the experiments; SS and MD were responsible for the in vivo work; C. Herrera performed the explant experiments; AC performed the ELISpot experiments; NB, C. Ham, and JH performed the quantitative PCR analyses; NR and AK were responsible for the assay of tenofovir; PA coordinated the overall Microbicide Development Programme; IMcG had oversight of the preclinical testing project; and MC wrote the manuscript.

References

1. Vittinghoff E, Douglas J, Judson F, McKirnan D, MacQueen K, et al. (1999) Per-contact risk of human immunodeficiency virus transmission between male sexual partners. Am J Epidemiol 150: 306–311.
2. Kalichman SC, Pompa D, Luke W, Austin J (2002) HIV transmission risk behaviours among HIV-positive persons in serodiscordant relationships. Int J STD AIDS 13: 677–682.
3. Padian NS, Shiboski SC, Glass SO, Vittinghoff E (1997) Heterosexual transmission of human immunodeficiency virus (HIV) in northern California: results from a ten-year study. Am J Epidemiol 146: 350–357.
4. Leynaert B, Downs AM, De V I (1998) Heterosexual transmission of human immunodeficiency virus: variability of infectivity throughout the course of infection. European Study Group on Heterosexual Transmission of HIV. Am J Epidemiol 148: 88–96.
5. Poles MA, Elliot J, Taing P, Anton PA, Chen IS (2001) A preponderance of CCR5(+) CXCR4(+) mononuclear cells enhances gastrointestinal mucosal susceptibility to human immunodeficiency virus type 1 infection. J Virol 75: 8390–8399.
6. Shattock RJ, Moore JP (2003) Inhibiting sexual transmission of HIV-1 infection. Nat Rev Micobiol 1: 25–34.
7. Misegades L, Page-Shafer K, Halperin D, McFarland W (2001) Anal intercourse among young low-income women in California: an overlooked risk factor for HIV? AIDS 15: 534–535.
8. Alves K, Shafer KP, Caseiro M, Rutherford G, Falcao ME, et al. (2003) Risk factors for incident HIV infection among anonymous HIV testing site clients in Santos, Brazil: 1996–1999. J Acquir Immune Defic Syndr 32: 551–559.
9. Alarcon JO, Johnson KM, Courtois B, Rodriguez C, Sanchez J, et al. (2003) Determinants and prevalence of HIV infection in pregnant Peruvian women. AIDS 17: 613–618.
10. Gross M, Holte SE, Marmor M, Mwatha A, Koblin BA, et al. (2000) Anal sex among HIV-seronegative women at high risk of HIV exposure. The HIVNET Vaccine Preparedness Study 2 Protocol Team. J Acquir Immune Defic Syndr 24: 393–398.
11. Weber AE, Boivin JF, Blais L, Haley N, Roy E (2002) HIV risk profile and prostitution among female street youths. J Urban Health 79: 525–535.
12. Karim SS, Ramjee G (1998) Anal sex and HIV transmission in women. Am J Public Health 88: 1265–1266.
13. Van Damme L, Ramjee G, Alary M, Vuylsteke B, Chandeying V, et al. (2002) Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet 360: 971–977.
14. Koblin BA, Chesney MA, Husnik MJ, Bozeman S, Celum CL, et al. (2003) High-risk behaviours among men who have sex with men in 6 US cities: baseline data from the EXPLORE study. Am J Public Health 93: 926–932.
15. Sabbarao S, Otten RA, Ramos A, Kim C, Jackson E, et al. (2006) Chemoprophylaxis with tenofovir disoproxil fumarate provided partial protection against infection with simian human immunodeficiency virus in macaques given multiple virus challenges. J Inf Dis 194: 904–911.
16. Van Rompay KK, Kearney BP, Sexton JJ, Colón R, Lawson JR, et al. (2006) Evaluation of oral tenofovir disoproxil fumarate and topical tenofovir GS-7340 to protect infant macaques against repeated oral challenges with virulent simian immunodeficiency virus. J Acquir Immune Defic Syndr 43: 6–14.
17. Cranage MP, Baskerville A, Ashworth LAE, Dennis M, Cook N, et al. (1992) Intrarectal challenge of macaques vaccinated with formalin-inactivated simian immunodeficiency virus. Lancet 339: 273–274.
18. Cranage MP, Baskerville A, Ashworth LAE, Dennis M, Cook N, et al. (1992) Mucosal infection and vaccine studies with simian immunodeficiency virus. Vaccine Res 1: 311–318.
19. Benson J, Chougnet C, Robert-Guroff M, Montefiori D, Markham P, et al. (1998) Recombinant vaccine-induced protection against the highly pathogenic simian immunodeficiency virus $SIV_{(mac251)}$: dependence on route of challenge exposure. J Virol 72: 4170–4182.
20. Patterson LJ, Robey F, Muck A, Van Remoortere K, Aldrich K, et al. (2001) A conformational C4 peptide polymer vaccine coupled with live recombinant vector priming is immunogenic but does not protect against rectal SIV challenge. AIDS Res Hum Retroviruses 10: 837–849.
21. Cranage MP, Sharpe SA, Whatmore AM, Polyanskaya N, Norley N, et al. (1998) *In vivo* resistance to simian immunodeficiency virus superinfection depends on attenuated virus dose. J Gen Virol 79: 1935–1944.
22. ten Haaft P, Verstrepen B, Uberla K, Rosenwirth B, Heeney J (1998) A pathogenic threshold of virus load defined in simian immunodeficiency virus- or simian-human immunodeficiency virus-infected macaques. J Virol 72: 10281–10285.
23. Clarke S, Almond N, Berry N (2003) Simian immunodeficiency virus Nef gene regulates the production of 2-LTR circles in vivo. Virology 306: 100–108.
24. Rezk NL, Crutchley RD, Kashuba ADM (2005) Simultaneous quantification of emtricitabine and tenofovir in human plasma using high-performance liquid chromatography after solid phase extraction. J Chromatog B 822: 201–208.
25. Polyanskaya N, Bergmeier LA, Sharpe SA, Cook N, Leech S, et al. (2001) Mucosal exposure to subinfectious doses of SIV primes gut-associated antibody-secreting cells and T cells: lack of enhancement by nonneutralizing antibody. Virology 279: 527–538.
26. Sharpe SA, Cope A, Dowall S, Berry N, Ham C, et al. (2004) Macaques infected long-term with attenuated simian immunodeficiency virus (SIVmac) remain resistant to wild-type challenge, despite declining cytotoxic T lymphocyte responses to an immunodominant epitope. J Gen Virol 85: 2591–2602.
27. Li Y, Hui H, Burgess CJ, Price RW, Sharp PM, et al. (1992) Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in vivo: evidence for limited defectiveness and complementation. J Virol 66: 6587–6600.
28. Li Y, Kappes JC, Conway JA, Price RW, Shaw GM, et al. (1991) Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication competent and –defective viral genomes. J Virol 65: 3973–3985.
29. Adachi A, Gedelman HE, Koenig S, Folks T, Willey R, et al. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 59: 284–291.
30. Herrera C, Klasse PJ, Michael E, Kake S, Barnes K, et al. (2005) The impact of envelope glycoprotein cleavage on the antigenicity, infectivity, and neutralizing sensitivity of Env-pseudotyped human immunodeficiency virus type 1 particles. Virology 338: 154–172.
31. Fletcher PS, Elliot J, Grivel JC, Margolis L, Anton P, et al. (2006) Ex vivo culture of human colorectal tissue for the evaluation of candidate microbicides. AIDS 20: 1237–1245.
32. Robbins BL, Srinivas RV, Kim C, Bishofberger N, Fridland A (1998) Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphanate 9-R-(2-Phosphonomethoxypropyl)adenine (PMPA), Bis (isopropyloxymethylcarbonyl) PMPA. Antimicrob Agents Chemother 42: 612–617.
33. Delaney WE 4th, Ray AS, Yang H, Qi X, Xiong S, et al. (2006) Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. Antimicrob Agents Chemother 50: 2471–2477.
34. Balzarini J, van Herwege Y, Vanham G (2002) Metabolic activation of nucleoside and nucleotide reverse transcriptase inhibitors in dendritic and Langerhans cells. AIDS 16: 2159–2163.
35. Barouch DH, Kunstman J, Kuroda MJ, Schmitz JE, Santra S, et al. (2002) Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 415: 335–339.
36. Friedrich TC, Dodds EJ, Yant LJ, Vojnov L, Rudersdorf R, et al. (2004) Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat Med 10: 275–281.
37. US National Institutes of Health (2008) Clinicaltrials.gov. Available: http://clinicaltrials.gov. Accessed 1 July 2008.
38. Steinman RM, Granelli-Piperno A, Pope M, Trumpfheller C, Ignatius R, et al. (2003) The interaction of immunodeficiency viruses with dendritic cells. Curr Top Microbiol Immunol 276: 1–30.
39. Lehner T, Wang Y, Cranage M, Bergmeier LA, Mitchell E, et al. (1996) Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques. Nat Med 2: 767–775.
40. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, et al. (2000) DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100: 587–597.
41. Pöhlmann S, Baribaud F, Lee B, Leslie GJ, Sanchez MD, et al. (2001) DC-SIGN interactions with human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus. J Virol 75: 4664–4672.
42. Kulkami PS, Butera ST, Duerr AC (2003) Resistance to HIV-1 infection: lessons learned from studies of highly exposed persistently seronegative (HEPS) individuals. AIDS Rev 5: 87–103.
43. Fuchs EJ, Lee LA, Torbenson MS, Parsons TL, Bakshi RP, et al. (2007) Hyperosmolar sexual lubricant causes epithelial damage in the distal colon: potential implication for HIV transmission. J Infect Dis 195: 703–710.
44. Lifson JD, Rossio JL, Piatak Jr M, Parks T, Li L, et al. (2001) Role of $CD8^+$ lymphocytes in control of simian immunodeficiency virus infection and resistance to rechallenge after transient early antiretroviral treatment. J Virol 75: 10187–10199.
45. Pauza CD, Emau P, Salato MS, Triverdi P, MacKenzie, et al. (1993) Pathogenesis of SIVmac251 after atraumatic inoculation of the rectal mucosa in rhesus monkeys. J Med Primatol 22: 154–161.
46. Shiver JW, Fu T-M, Chen L, Casimiro DR, Davies M-E, et al. (2002) Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 415: 331–335.

47. Feinberg MB, Moore JP (2002) AIDS vaccine models: challenging challenge viruses. Nature Med 8: 207–210.
48. Casimiro DR, Wang F, Schleif WA, Liang X, Zhang Z-Q, et al. (2005) Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with DNA and recombinant adenoviral vaccine vectors expressing Gag. J Virol 79: 15547–15555.
49. Wilson NA, Reed J, Napoe GS, Piaskowski S, Szymanski A, et al. (2006) Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. J Virol 80: 5875–5885.
50. Saifuddin M (2008) Intravaginal administration of 6% cellulose sulfate (CS) gel prevented systemic infection in rhesus macaques in a multiple dose R5/X4 SHIV vaginal challenge model. Late-breaker presentation. In: Proceedings of Microbicides 2008 Conference; 24–27 February, 2008, Delhi, India.

Editors' Summary

Background. About 33 million people are now infected with the human immunodeficiency virus (HIV), which causes AIDS by killing immune system cells. As yet, there is no cure for AIDS, although HIV infections can be held in check with antiretroviral drugs. Also, despite years of research, there is no vaccine available that effectively protects people against HIV infection. So, to halt the AIDS epidemic, other ways of preventing the spread of HIV are being sought. For example, pre-exposure treatment (prophylaxis) with antiretroviral drugs is being investigated as a way to prevent HIV transmission. In addition, because HIV is often spread through heterosexual penile-to-vaginal sex with an infected partner, several vaginal microbicides (compounds that protect against HIV when applied inside the vagina) are being developed, some of which contain antiretroviral drugs.

Why Was This Study Done? Because HIV can cross the membranes that line the mouth and the rectum (the lower end of the large intestine that connects to the anus) in addition to the membrane that lines the vagina, HIV transmission can also occur during oral and anal sex. The lining of the rectum in particular is extremely thin and overlies tissues rich in activated T cells (the immune system cells that HIV targets), so unprotected anal intercourse carries a high risk of HIV infection. Anal intercourse is common among men who have sex with men but is also more common in heterosexual populations than is generally thought. Tenofovir (an antiretroviral drug that counteracts HIV after it has entered human cells) given by mouth partly protects macaques against rectal infection with simian immunodeficiency virus (SIV; a virus that induces AIDS in monkeys and apes) so the researchers wanted to know whether this drug might be effective against rectal SIV infection if applied at the site where the virus enters the body.

What Did the Researchers Do and Find? To answer this question, the researchers rectally infected several macaques with SIV up to 2 h after rectal application of a gel containing tenofovir, after rectal application of a gel not containing the drug, or after no treatment. In addition, a few animals were treated with the tenofovir gel after the viral challenge. Most of the animals given the tenofovir gel before the viral challenge were partly or totally protected from SIV infection, whereas all the untreated animals and most of those treated with the placebo gel or with the drug-containing gel after the viral challenge became infected with SIV. High blood levels of tenofovir 15 min after its rectal application correlated with protection from viral infection. The researchers also collected rectal and small intestine samples from tenofovir-treated macaques that had not been exposed to SIV and asked which samples were resistant to SIV infection in laboratory dishes. They found that only the rectal samples were resistant to infection and only rectal cells contained tenofovir. Finally, activated T cells that recognized an SIV protein were present in the blood of some of the animals that were protected from SIV infection by the tenofovir gel.

What Do These Findings Mean? These findings, although based on experiments in only a few animals, suggest that rectal treatment with antiretroviral drugs before rectal exposure to HIV might prevent rectal HIV transmission in people. However, results from animal experiments do not always reflect what happens in people. Indeed, clinical trials of a potential vaginal microbicide that worked well in macaques were halted recently because women using the microbicide had higher rates of HIV infection than those using a control preparation. The finding that immune-system activation can occur in the absence of overt infection in animals treated with the tenofovir gel additionally suggests that a combination of a local antiretroviral/microbicide and vaccination might be a particularly effective way to prevent HIV transmission. However, because HIV targets activated T cells, viral rechallenge experiments must be done to check that the activated T cells induced by the virus in the presence of tenofovir do not increase the likelihood of infection upon re-exposure to HIV before this potential microbicide is tried in people.

Additional Information Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0050157.

- Read the accompanying *PLoS Medicine* Perspective by Florian Hladik
- An overview of HIV infection and AIDS is available from the US National Institute of Allergy and Infectious Diseases
- HIVInSite has comprehensive information on all aspects of HIV/AIDS, including an article on safer sex, which includes information on the risks associated with specific types of sex and on microbicides and other methods to prevent the sexual transmission of HIV
- Information on all aspects of HIV/AIDS is available from Avert, an international AIDS charity, including information on HIV prevention and on microbicides
- The World Health Organization has a fact sheet on microbicides
- The UK charity NAM also provides detailed information on microbicides
- PrEP Watch is a comprehensive information source on pre-exposure prophylaxis for HIV prevention
- Global Campaign for Microbicides is an international coalition of organisations dedicated to accelerating access to new HIV prevention options