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Preliminary studies on the use of pertussis toxin for the modulation of intra-vaginal SIV transmission in rhesus macaques

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Abstract

Background—Pertussis toxin (PTX) blocks GPCR signaling resulting in the inhibition of chemotaxis /cell adhesion. It was reasoned that inhibition of cell trafficking may be an approach to prevent HIV/SIV transmission.

Methods—In this study PTX in HEC gel was applied to the vaginal wall of monkeys that were then challenged intravaginally with SIVmac251.

Results—Results of these studies showed that 2/4 animals were resistant to infection. Furthermore, infection was correlated with a marked increase in the plasma and cervico-vaginal lavage levels of select chemokines and cytokines.

Conclusions—Results from this preliminary feasibility study dictate that further studies that include a larger number of animals are required to optimize this protocol and establish the efficacy of this approach. In addition, such future studies will provide important information on the role of specific chemokines that play a role in lymphocyte trafficking within the genital tract and serve as additional therapeutic targets.

Keywords

Pertussis toxin; Vaginal transmission; SIVmac251; Rhesus macaques. G-protein coupled receptors

Introduction

Currently 36.7 million people are living with HIV worldwide and although antiretroviral chemotherapy is highly effective in controlling viral replication and reducing transmission in developed countries, HIV continues to remain a major public health issue in resource-limited settings [1]. A majority of HIV infections worldwide occur through mucosal exposure and recent UNAIDS estimates that globally, 30–40% of HIV infection occur

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through the heterosexual route via the female genital tract (FGT) [2]. Currently, in sub-Saharan Africa, more than half the population are infected with HIV and of those 76% are young females aged 15–24 [3]. Therefore, measures to prevent heterosexual mucosal transmission of HIV are urgently needed to reduce the burden of infection [4]. It is reasoned that naturally occurring physical barriers that include the presence of mucus associated chemicals and the composition of the epithelial wall of the FGT followed by local innate immune mechanisms must play a vital role during acute HIV infection [5]. Previous studies have documented the ability of PTX to inhibit the intracellular signaling pathway mediated by the interaction of GPCR with its ligand leading to inhibition of chemotaxis [6].

Pertussis toxin (PTX) is the major virulence factor of *Bordetella pertussis*, the causative agent of whooping cough. PTX is a pentameric protein, which is functionally composed of A and B subunits. The A subunit (active) protomer exhibits adenine diphosphate ribosyltransferase activity responsible for ribosylation and inactivation of G_i-like proteins [7]. The B-oligomer consists of four subunits (S2 to S5) that bind to extracellular cell surface molecules that allow the toxin to enter the cells and includes molecules such as Toll-like receptor 4. The ligation of TLR-4 activates an intracellular signal transduction cascade. Such activity maybe the basis by which PTX-B inhibits HIV replication in vitro in primary cultures of monocyte derived macrophages (MDM) and inhibits cytokine-induced virus expression in chronically infected U1 cells [8]. Furthermore, the in vivo intravenous administration of PTX has been shown to induce significant lymphocytosis in addition to a decrease in the number of SIV infected cells [9] without any noted toxicity. These previous findings coupled by the ability of PTX to inhibit lymphoid cell recruitment prompted us to carry out the studies reported herein.

Materials and Methods

Animals

Four adult female rhesus macaques (*Macaca mulatta*) of Indian origin were used for the studies reported herein. The animals were born and housed at the Yerkes National Primate Research Center (YNPRC) of Emory University (Atlanta, GA) and were maintained according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Department of Health and Human Service guideline and Use of Laboratory Animals. These animals were fed monkey diet (Purina) supplemented daily with fresh fruit or vegetables and water ad libitum. The studies reported herein were performed under IACUC protocol #2001725 “Gut homing cells in SIV infection” which was reviewed and approved by the Emory University IACUC and the biosafety review Committee. The YNPRC has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International since 1985.

Pertussis toxin preparation and administration

Pertussis toxin in HEC gel form was prepared according to the protocol described by Moench et al [10]. Briefly, the lyophilized form of pertussis toxin (Cat # 180, List Biological Laboratories, Inc., Campbell, CA,) was re-suspended in 1.5 ml of sterile water. On day zero,

10 µg/kg of PTX was incorporated in 1 ml of HEC gel and administered intra-vaginally using a nasogastric PVC feeding tube. This was followed immediately by the insertion of a sterile small glass tube (round end first) that was used to spread the gel along the vaginal vault. One hour following the first exposure of PT toxin a second dose of 10µg/kg of the toxin in 1 ml of HEC gel was similarly administered.

Virus Infection

Two hours after the last application of the PTX in HEC gel, each rhesus macaque was challenged by intra-vaginal exposure to 1 ml neat virus stock of SIV mac251 (100,000 TCID₅₀ containing 720 ng of p27). The virus stock of SIVmac251 was obtained courtesy of Dr. Nancy Miller (NIAID, NIH). Data from the initial intra-vaginal titration studies showed that 1 ml of a 1/20 dilution of the stock virus (5000 TCID₅₀ containing 36 ng of p27) was sufficient to infect 50% of monkeys via the IVAG route. The 1/20 dilution of the stock SIVmac251 dose was used in our previous studies to successfully infect female rhesus macaques via the IVAG route [11].

Sample Collection

Blood was collected from anesthetized monkeys into EDTA collection tubes and plasma separated after centrifugation. Cervico-vaginal lavage (CVL) was collected by irrigation of the vaginal vault with 2 ml of sterile DPBS, followed by aspiration from the posterior fornix.

Plasma Viral loads

Plasma viral loads were monitored in samples every week for 6 weeks, and every two weeks up to 10–15 weeks, using previously published protocols. Macaques were only considered infected when their plasma viral loads showed >1000 vRNA copies/ml at 2 consecutive weekly intervals. The threshold for the assay was 50 vRNA copies/ml of plasma [11]

Levels of cytokines and chemokines

Plasma and CVL samples from each of the monkeys were analyzed for levels of a series of cytokines and chemokines using commercially available kits (I-FABP, IL-6, SCD14, MIP-1 α, IL-10, IP-10, IFN-α, TNF-α-R & D Systems; IFN-γ-Mabtech; CRP – Abnova). Raw values for each of the cytokines/chemokines were normalized to the mean of the baselines, log₂-transformed, and then subjected to hierarchical clustering and heat maps were generated using *heatmap.2* of R package *gplots*. Values denoted as -3 to +3 represent a decrease to an increase in the levels relative to base line values for each of the chemokine/ cytokine analyzed.

Results

Experimental design

The objective of this study was to generate preliminary data aimed at assessing the feasibility and efficacy of the vaginal application of PTX as a topical microbicide against SIV. As described in Figure 1, following 2 baseline collections, PTX (10 µg/kg) was applied to the vaginal wall incorporated in HEC gel at 0 and at 1 hour followed two hours later by a

single high dose intra-vaginal challenge with SIVmac251 to a group of 4 adult rhesus macaques. None of the animals were treated with Depo-provera. Infection was defined as plasma positive viremia at 2 consecutive weeks. Macaques were bled weekly for 6 weeks, biweekly until 12 weeks thereafter to measure chemokines/cytokines and plasma viral loads.

Plasma viral loads

Two of the four female rhesus macaques exposed to PTX followed by challenge with SIVmac251 via the IVAG route showed readily detectable virus at week one that was confirmed by results of plasma viremia on samples collected on the second week post challenge (see Fig. 2). The two infected RM showed plasma viral loads with peak viral loads of 7.5×10^6 copies/ml. The pattern of viremia thereafter followed a pattern seen in general with SIV infection of Indian origin rhesus macaques. Plasma samples from the other two macaques showed undetectable viremia (below detection limit of the current viral load assays of <50 copies/ml) (Figure 2) for up to 14 weeks following challenge.

Levels of cytokines/chemokines

ELISA based assays were performed in efforts to measure the effect of PTX on levels of chemokines and cytokines in the plasma and CVL samples collected from the monkeys prior to (baseline) and post challenge. Levels of the cytokines and chemokines in the plasma and CVL of the uninfected animals did not show much difference before after the gel application (data not shown) suggesting that the application of the PTX-HEC gel in uninfected animals by itself had no detectable “inflammatory” effect. The plasma levels of 9 of the 10 cytokines/chemokines from the two infected macaques showed increased levels as early as 1-week post infection that was sustained up to 6 weeks p.i. (Figure 3A). However, the plasma levels of MIP-1 α levels remained the same throughout the study. The levels of I-FABP and sCD14 levels showed a slight increase as compared to baseline samples.

Next, we analyzed the levels of the same panel of chemokines/cytokines in aliquots of the CVL samples obtained from both the infected and uninfected RMs. Plasma samples from the uninfected animals did not show any increase in CVL cytokines/chemokines similar to the profile noted for the plasma from the same animals. However, CVL samples from the infected animals showed that most of the cytokines/chemokines were up regulated as compared to baseline levels but only at week 1 and 2 (Figure 3B) except for IFN γ and IFN- α . Unlike, plasma samples MIP-1 α levels were clearly up regulated at week 1 and 2 but at later time points the levels were similar to baseline values (Figure 3B).

Discussion

It is to be kept in mind that the present study was conducted to provide some preliminary data as to the feasibility of the use of PTX as an agent that could be utilized to test as a potential prevention strategy of HIV/SIV transmission. It is clear from the results of the study that a much larger cohort of animals is needed to derive meaningful data. More importantly, it is to be noted that the present study utilized a relatively high dose of highly pathogenic SIVmac251 (100,000 TCID₅₀/dose) primarily because it is a well-known fact that intra-vaginal challenge studies require high doses and are notorious for resulting in

resistance of animals to infection. Thus, we reason that a much lower dose in a repeated challenge model may be more optimal to provide meaningful data. The present study, however, does provide data on the feasibility of the approach and indicates that at the doses utilized it is probably safe since there were no recognized signs of toxicity associated with the procedure. Previously, it has been suggested that PTX has an effect on the innate immune system by inhibiting the early recruitment of neutrophils and macrophages, and shown to interfere with early chemokine production [12]. In addition, It has been shown that PTX-B possess anti-HIV activity in vitro and may have therapeutic value in the prevention and/or therapy of HIV infection [8]. Therefore, we have tested the application of PTX in an IVAG challenge model of SIVmac251 infection and attempted to understand the role of PTX in transmission and early events of chemokines/cytokines during transmission. In the current study the changes in the levels of cytokine/chemokine response were noted only in SIV infected animals and associated with levels of viremia. Clearly the application of PTX alone did not alter any changes in chemokines/cytokines in both plasma and CVL providing suggestive evidence that there is no detectable inflammation by such application. It has been documented that susceptibility to HIV-1 infection and disease progression is associated with the expression of certain chemokines [13]. In several studies CVL samples were used to analyze susceptibility to infection and high levels of interleukin (IL)-8, RANTES, secretory leukocyte protease inhibitor, and interferon- α (IFN- α) were shown to be associated with protection from HIV-1 infection [14]. Following establishment of infection, monocyte chemotactic protein-1 (MCP-1) was shown to positively correlate with viral load. Higher systemic levels of the chemokines such as the monokine induced by interferon- γ (MIG/CXCL9) and interferon- γ -induced protein (IP)-10 have been observed in HIV-positive commercial sex workers (CSWs) compared with HIV-1-uninfected CSWs [15]. In our study, pro-inflammatory cytokines like IL-6 and TNF α , IFN γ and inflammatory marker CRP were also up regulated both in plasma and CVL samples suggesting that these are also induced by SIV infection and may contribute to the dissemination of SIV infection.

It will be very interesting to test whether additional dose or repeated administration of PTX will lead to adverse effects on genital mucosa or helps to protect against SIV infection. This information will be very valuable to model the cohort of commercial sex workers (CSW) who are highly exposed to potential HIV infection [16] and whom we submit will theoretically utilize such gel applications on a regular basis to control HIV infection. Currently it is unclear whether repeated administration of PTX is immunogenic leading to the production of anti-PTX antibodies and the potential of such antibodies to neutralize the anti-HIV effects of the PTX when applied locally and therefore will be intriguing to test these concepts in future studies. In this context, it is important to note that pertussis vaccine is routinely administered during childhood and is followed by booster immunization during adolescence and thus individuals could have such anti-PTX antibodies in their circulation that could neutralize the PTX when administered as a gel formulation. However, it is not clear whether such antibodies are present in CVL or locally within the vaginal mucosa [17, 18]. This should therefore be taken into consideration before deciding on any large-scale studies.

In conclusions, the results of this preliminary study provides a foundation for more extended studies aimed at the evaluation of PTX as a potential agent for inhibiting HIV/SIV

transmission. In addition, this study warrants extensive large-scale animal studies involving low dose IVAG challenge and repeated administration of PTX.

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Pertussis Schema

Figure 1.

Schema of the study design. Four RMs were treated with PTX at day 0 with two doses of PT toxin in the HEC gel 10ug/kg, one hour apart followed by challenge with 1ml SIVmac251 virus via IVAG route 2 hrs later. Blood and CVL fluids were collected at various time points as indicated in the figure.

Figure 2.

Plasma SIV RNA levels are expressed as copies/ml (cut-off value of 50 copies/ml, dashed line) for each individual animal over the time as described in figure. The animal RW014 remained uninfected and was exposed again with two doses of PTX and challenged with SIVmac251 virus.

Figure 3. Hierarchical clustering of differentially expressed chemokines and cytokines were presented in the form of heat maps in plasma (3A) and CVL (3B) among the infected macaques. In heat maps, red and blue represent increased and decreased levels relative to the base line level in each of the chemokine/cytokine analyzed.