Pertussis toxin B-oligomer inhibits HIV infection and replication in hu-PBL-SCID mice

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Keywords: anti-viral, R5 HIV-1

Abstract

* Bordetella pertussis* toxin B-oligomer (PTX-B) has been shown to inhibit HIV infection and replication in vitro. The potential anti-viral effect of PTX-B was tested here in an in vivo surrogate model of HIV infection, i.e. SCID mice reconstituted with human peripheral blood leukocytes (PBL) (hu-PBL-SCID) and infected with a CCR5-dependent (R5) HIV-1 strain. SCID mice inoculated intra-peritoneal (i.p.) with PTX-B and then infected with the R5 strain SF-162 were sacrificed 7 days later and analyzed for human PBL (hu-PBL) lymphoid tissue reconstitution, infection of hu-PBL, plasma viremia and viral rescue from ex vivo-cultivated i.p. hu-PBL. Unlike mice treated with 500 ng per animal of PTX-B showing no evidence of viral inhibition, daily administration of PTX-B (50 ng per mouse) strongly inhibited virus infection and replication, as determined by undetectable viremia, absence of infected hu-PBL and lack of rescue of infectious HIV in most animals. Furthermore, PTX-B injection 2 h before and twice after infection prevented HIV-1 infection and replication in all (10/10) tested animals. Thus, PTX-B potently inhibited virus infection and replication in hu-PBL-SCID mice, supporting the hypothesis that it may represent a new pharmacological agent against HIV-1 infection.

Introduction

The B-oligomer of pertussis toxin (PTX-B) is a non-toxic molecule responsible for binding of pertussis toxin (PTX) to target cells (1). Functionally, PTX-B possesses several biological properties originally ascribed to the holotoxin (2–4), such as the ability of stimulating DNA synthesis in B cells (5), proliferation of T lymphocytes (6–8) and both Tn1- and Tn2-dependent immune responses (9–13). In vivo, PTX-B has been shown to protect mice from experimental autoimmune encephalomyelitis (14) and to represent an effective adjuvant of an anti-influenza vaccine (11, 15). Concerning retroviruses, PTX was reported to exert a profound anti-viral activity both in in vitro-infected peripheral blood leukocytes (PBL) (16, 17) and in macaques chronically infected with simian immunodeficiency virus (SIV) (18). Of note, similar anti-viral effects were observed in vitro with PTX-B; in particular, both PTX and PTX-B inhibited both entry and post-entry steps of the HIV-1 life cycle inactivated primary T cells (16, 19), monocyte-derived macrophages (MDM) (20), ex vivo cultures of human lymphoid tissues (M. Alfano et al. manuscript in press) and cell lines either acutely or chronically infected with HIV (20–22). These findings suggest that PTX-B possesses the potential features of a novel preventive and/or therapeutic molecule against HIV infection; however, no information is available thus far on the potential in vivo anti-HIV activity of PTX-B.

Since the discovery of HIV as the etiological agent of AIDS (23, 24), many efforts have been devoted towards developing relevant animal models with the double purpose of studying viral-induced pathogenesis and of testing potential anti-viral agents and vaccines. In this regard, mice affected by SCID are characterized by a genetic defect in their DNA repair system not allowing the rearrangement of both Ig and TCR genes (25). Consequently, SCID mice do not efficiently reject...
allografts and xenografts (25), and can be transplanted with human organs, such as fetal liver and thymus, lymph nodes (LN) and PBL, thus generating xenochimeric SCID mice. SCID mice can be reconstituted by intra-peritoneal (i.p.) injection of human PBL (hu-PBL) (26, 27), which rapidly acquire an activated phenotype and spread to liver, LN, spleen and bone marrow within 24 h after injection (28–31). SCID mice reconstituted with hu-PBL (hu-PBL-SCID mice) are highly susceptible to HIV-1 infection and, together with the model of hu-SCID mice reconstituted with human fetal liver hematopoietic cells, fetal thymus or LN (32–34), represent the only animal model allowing the study of the in vivo interaction between HIV-1 and human cells (35–38). HIV actively replicates in these animals causing a prompt dysfunction of human immune cells (39) thus allowing the monitoring of selected virological and immunological parameters.

Therefore, we investigated whether PTX-B was effective in controlling infection and replication of R5 HIV-1 in hu-PBL-SCID mice.

Methods

SCID mice

CB17 scid/scid female mice (Harlan Srl, Udine, Italy) of 4 weeks of age were housed in microisolator cages and kept under sterile conditions. All food, water and bedding were autoclaved prior to use.

Hu-PBL

Hu-PBL were obtained from peripheral venous blood of healthy donors who tested negative for HIV-1 and hepatitis B virus infections. Thirty million hu-PBL, purified by Ficoll-Paque (Pharmacia, Piscataway, NJ, USA) density gradient centrifugation, were re-suspended in 0.5 ml of RPMI 1640 medium (BioWhittaker, Verviers, Belgium) and injected i.p. in recipient mice.

PTX-B administration and HIV-1 infection

PTX-B (Calbiochem, San Diego, CA, USA) was dissolved in PBS (BioWhittaker) and stored at 4°C until use. Two weeks after animal reconstitution, 1 ml of PTX-B in PBS was injected i.p. 2 h before infection and then either daily for six consecutive days (protocol 1, P1) or twice, 3 and 6 days after infection (protocol 2, P2), unless otherwise specified. The R5 strain HIV-1SF162 (10^5 50% tissue culture infective dose), previously expanded in PHA-activated PBL (T cell blasts), was injected i.p. The average weight of the mice at the time of PTX-B treatment and infection was 16–17 g. Animal toxicity was evaluated by means of hair ruffling, weight loss and mortality at the time of mice sacrifice.

Human cell recovery from the peritoneal cavity and lymphoid organs of hu-PBL-SCID mice

Hu-PBL-SCID mice were sacrificed either 7 or 14 days after infection and cells were collected from the peritoneal cavity, spleen and LN. A two-step peritoneal lavage was performed first with 1 ml of RPMI 1640 medium followed by a second 4-ml wash. Spleen and LN were disrupted with the blunt end of a 5-ml plastic syringe plunger, the connective tissue and debris were allowed to settle and the single-cell suspensions were diluted in RPMI 1640 medium, centrifuged twice at 1200 r.p.m. min^-1 for 10 min and re-suspended in 1 ml of RPMI 1640 medium (30).

Mouse organ reconstitution and determination of virus infection

The presence of human cells in the mouse lymphoid organs was monitored by PCR amplification of the HLA-DQα human gene fragments, as previously published (30). The presence of HIV DNA was also detected by specific amplification of HIV-1 gag sequences in 1 μg of total DNA extracted from spleens and LN of the hu-PBL-SCID mice (30). The sensitivity and linearity of the assay were tested by amplifying serial dilutions of DNA prepared from the 8E5 cell line (which harbors one proviral copy per cell) serially diluted into SCID mouse cell DNA (30). Latent infection by replication-competent virus was demonstrated by co-cultivation of cell suspensions from peritoneal lavage with human allogeneic T cell blasts re-suspended in RPMI 1640 medium plus 10% FCS (HyClone, Logan, UT, USA) in the presence of recombinant IL-2 (50 U ml^-1, Chiron Corp., Emeryville, CA, USA) for 10 days. Virus replication was determined by detection of HIV p24Gag antigen by a commercial ELISA kit (Dupont, Bruxelles, Belgium) in culture supernatants (sensitivity < =20 pg ml^-1). Plasma viremia of hu-PBL-SCID mice was assayed by the Ultrasensitive Amplicor Monitor HIV-1 RNA kit (Roche Diagnostics Inc., Branchburg, NJ, USA) with a sensitivity =<50 copies of HIV RNA ml^-1.

Flow cytometry analysis

Cells recovered from the peritoneal cavity of the hu-PBL-SCID mice were re-suspended in PBS and incubated with the appropriate fluorochrome-conjugated anti-human CD3, CD4 and CD8 (Becton Dickinson, San Jose, CA, USA) mAb for 30 min. Cells were then washed with a mixture of PBS, 2% FCS and 0.1% sodium azide and fixed with 2.5% PFA. Two-color flow cytometry was performed on cells gated based on the light-scatter properties of human T cells with a FACSort cytometer (Becton Dickinson), and up to 5000 cells per sample were analyzed with the Cell Quest software (Becton Dickinson) according to forward and side scatter in order to gate only the live lymphocyte population and to exclude erythrocytes, dead cells and tissue debris.

Statistics

All results are expressed as mean ± SD, whereas the statistical analysis was performed by one-way ANOVA.

Results

High-dose PTX-B (500 ng per mouse) does not influence either the reconstitution of mouse lymphoid tissues with human cells or HIV infection and replication

We have previously observed that in vitro PTX-B at the concentration of 1 nM (corresponding to ~75 ng ml^-1)
efficiently inhibits HIV-1 infection, replication and/or expression in infected primary T cell blasts, MDM and cytokine-stimulated chronically infected U1 cells re-suspended at concentrations of 0.5–1 $\times$ 10^6 cells ml$^{-1}$ (16, 19, 20). In order to identify an appropriate non-toxic dose to be tested in the hu-PBL-SCID mouse model, we have initially administered 500 ng of PTX-B per mouse i.p. 2 weeks after their reconstitution with 30 $\times$ 10^6 hu-PBL. Two hours later, the HIV inoculum was injected i.p. and PTX-B was then administered daily, according to P1; the animals were sacrificed on the seventh day. Seven days after infection, spleen and LN of PTX-B-treated animals ($n$ = 7), either uninfected or HIV infected, were found to be normally reconstituted with human cells, as determined by DNA-PCR amplification for the human antigen HLA-DQ$\alpha$ (Fig. 1A).

HIV infection was not inhibited by this regimen (P1) of PTX-B administration, as demonstrated by PCR analysis of HIV DNA performed on the reconstituted mouse lymphoid tissue (Fig. 1A), determination of HIV-1 p24$\text{Gag}$ antigen in culture supernatants of hu-PBL harvested from the peritoneal cavity and co-cultivated with allogeneic human T cell blasts (Fig. 1B) and determination of plasma viremia (Fig. 1C). These last parameters were actually moderately increased in mice treated daily with high-dose PTX-B (P1), although not significantly. Also the intermittent protocol of PTX-B administration (P2) did not modify the reconstitution of lymphoid organs (data not shown, $n$ = 7) and failed to inhibit HIV infection and replication, although the boosting effect on both HIV p24$\text{Gag}$ antigen expression and plasma viremia was reduced in comparison with P1 (Fig. 1B and C, respectively).

Thus, this high dose of PTX-B (500 ng per mouse) failed to inhibit HIV infection and replication.

**Inhibition of HIV-1 infection and replication by low-dose PTX-B (50 ng per mouse) regimens**

Having failed to observe an anti-viral effect of a relatively high dose of PTX-B (500 ng per mouse), we investigated whether this could occur with a lower dose of PTX-B (50 ng per mouse), according to P1 and P2.

Normal reconstitution with human cells, as determined by DNA amplification for the human HLA-DQ$\alpha$, was demonstrated in animals receiving low-dose PTX-B according to both P1 ($n$ = 10) and P2 ($n$ = 10) 7 days after infection (Fig. 2A). PTX-B prevented HIV infection in two out of four animals following P1 in terms of detection of HIV DNA in lymphoid tissues (Fig. 2A and Table 1). Plasma viremia and ex vivo production of p24$\text{Gag}$ antigen were suppressed in all animals negative for HIV DNA, whereas undetectable plasma viremia was also observed in one additional animal found positive for both HIV DNA and ex vivo virus replication (Fig. 2B and C, respectively, and Table 1).

Intermittent injection of PTX-B (P2) was more effective than daily administration (P1) in that it completely prevented HIV infection of human cells in mouse lymphoid organs (Fig. 2A) as well as virus recovery from hu-PBL harvested from the peritoneum (Fig. 2C) and plasma viremia (Fig. 2B) in all tested animals (Table 1).

Fig. 1. Daily administration of a high dose of PTX-B (500 ng per mouse) does not influence the reconstitution of lymphoid tissues or prevent HIV infection. PTX-B was injected i.p. 2 h before and every 24 h after infection (P1). (A). Mouse lymphoid tissue reconstitution by huPBL and HIV infection was monitored by DNA-PCR; PCR positive control is represented by 10^4 8E5 cells, representing the highest linear point of the standard curve, run in the same assay; the results shown were obtained from 1 out of 2 independent experiments with similar effects. (B). HIV replication was detected by co-cultivation of cells recovered from the animal peritoneal cavity 7 days after infection and allogeneic T cell blasts. (C). Plasma viremia was measured 7 days after infection in mice that received PTX-B by i.p. injections 2 h before and every 24 h after infection (P1) or 2 h before and twice after infection (P2). Panels (B) and (C) show average ± SD calculated on seven animals per each condition (data are from two independent experiments executed with human cells obtained from two different donors).
This lower dose of PTX-B did not influence either the percentage or the ratio of human CD4+ and CD8+ T cells (1.26 ± 0.48 versus 1.30 ± 0.13 in untreated versus PTX-B-treated animals, respectively, n = 6, Fig. 3) harvested from the peritoneal cavity of uninfected animals. HIV infection reduced the percentage of CD4+ but not that of CD8+ T cells rescued from the peritoneal cavity and significantly inverted the ratio of human CD4/CD8 T cells (1.26 ± 0.48 versus 0.46 ± 0.23 in uninfected versus HIV-infected animals, respectively, P = 0.03; n = 6, Fig. 3). PTX-B treatment alone did not modify either the percentage of CD4+ and CD8+ T cells or the CD4/CD8 ratio in both uninfected and infected animals (Fig. 3).

A single injection of PTX-B does not prevent HIV infection but inhibits viral replication

We finally evaluated the effect of a single PTX-B injection administered either before or after HIV infection of hu-PBL-SCID mice. Mice were injected a single time with a low dose (50 ng per mouse) of PTX-B 2 h before or 72 or 144 h post-infection; the animals were sacrificed 2 weeks after viral inoculation. The presence of human cells and of HIV DNA in spleen and LN was demonstrated in all animals by PCR amplification of HLA-DQα and HIV gag DNA, respectively. Co-cultivation of cells harvested from the peritoneal cavity with allogeneic T cell blasts resulted in a productive virus infection both in control and PTX-B-treated animals, with comparable peak levels of HIV p24 gag antigen release (2133 ± 88 versus 2141 ± 45 pg per ml in control and PTX-B-treated animals, respectively). However, only 7 out of 19 PTX-B-treated animals (37%) showed detectable plasma viremia versus 19 out of 23 (83%) controls (Table 1). Furthermore, plasma viremia was statistically reduced in the animals that received PTX-B compared with the untreated controls (610 ± 2191 versus 44 016 ± 23 592 copies per ml, respectively, P = 0.04, one-way ANOVA). This effect was independent of whether
PTX-B was inoculated before or after infection (Table 1). Thus, a single PTX-B injection was not sufficient to prevent HIV infection, but it partially interfered with viral replication in vivo.

All PTX-B doses and regimens of administration were not toxic to the animals, as evaluated by means of hair ruffling, weight loss and mortality (data not shown).

Discussion

In the present study, a low dose of PTX-B (50 ng per mouse), unlike a higher dose (500 ng per mouse), could prevent HIV infection and replication in HIV-1-infected hu-PBL-SCID mice as a function of the schedule of administration. Daily administration of PTX-B prevented HIV-1 infection in 7 out of 10 animals in the absence of toxicity, whereas a regimen of three i.p. administrations of PTX-B was fully effective in 10 out of 10 animals. Partial prevention and inhibition of plasma viremia, although not of infection, was observed in 19 animals that received a single injection of PTX-B either 2 h before or at different times after infection. Therefore, this study provides the first evidence that PTX-B could act as a potent anti-HIV agent in vivo.

Concerning the efficacy of PTX or PTX-B as anti-viral agents in vivo, it was previously shown that intravenous inoculation of a single bolus (25 μg kg⁻¹) of the holotoxin PTX to chronically SIV-infected rhesus macaques resulted in both T cell activation and decreased viral replication in lymphoid tissue together with a diminished frequency of infected cells in peripheral blood (18). On the basis of this study as well as the abundant in vitro evidence (16, 20), it is conceivable that most of the observed effects of the holotoxin were indeed exerted by its non-toxic B-oligomeric subunit.

PTX-B has been characterized in vitro as an inhibitor of different steps of the HIV life cycle, including viral fusion and entry of R5 HIV-1 via heterologous desensitization and uncoupling of CCR5 from CD4 (16, 19). PTX-B was also shown to interfere with post-entry events common to both R5- and CXCR4-dependent viruses in T cells and primary MDM (19), and inhibited cytokine- and Tat-induced viral expression in chronically infected U1 cells and HL3T1 cells (19, 20, 40). In addition, it has been independently reported that PTX-B may act as a negative modulator of nuclear factor-κB p65 activation in tumor necrosis factor-α-stimulated U937 cells (22).

HIV infection of hu-PBL-SCID mice, as well as of SCID mice reconstituted with human fetal liver or thymus, has been broadly used to investigate the pathogenic effects of infection and to explore the potential efficacy of anti-viral compounds (41–47). Intermittent administration of PTX-B given three times i.p. (2 h before and 72 and 144 h after infection) was fully effective in preventing infection of 30 × 10⁶ huPBL. Since its maximal inhibitory concentration in vitro was previously defined to be ~75 ng per 10⁻⁶ cells (16, 20), the optimal dose of PTX-B required to inhibit HIV infection in vivo may indeed be significantly lower than that required in vitro. In this regard, it should be underscored that anti-HIV agents proven to be efficacious in infected individuals have also been tested in reconstituted SCID mice by using similar protocols (41, 45–47). Based on these results, a potential dose of PTX-B capable of inhibiting 90% of HIV infection or replication could be estimated to be ~2.5 μg kg⁻¹. In terms of molarity, PTX-B [molecular weight (MW) 75 000] is predicted to be effective at 0.033 nmol kg⁻¹, a concentration significantly lower than that required by current anti-retroviral agents. For example, 3'-azido-3'-deoxythymidine with an MW of 267.24 is administered at daily doses ranging from 160 to 480 mg kg⁻¹ (42, 45). Fifty milligrams of nevirapine (MW 266.3) corresponds to 0.18 mmol kg⁻¹ day⁻¹ (47). Furthermore, by targeting cellular events, viral mutations are not expected to represent a major obstacle to the potential use of PTX-B or equivalent molecules, such as PT-9K/129G (20, 48), in humans.

An interesting observation is the major efficacy of PTX-B when administered intermittently (P2) instead of daily (P1). Although there is no obvious explanation for these differences, it should be underscored that PTX-B is endowed with a strong cell-signaling capacity (16). Continuous PTX-B administration may lead to receptor desensitization, whereas this event may not occur if sufficiently long intervals are scheduled between each injection. In this regard, another signaling molecule, IL-2, is currently being evaluated for clinical efficacy based on its capacity of promoting a significant expansion of CD4⁺ T cells when administered intermittently (49), whereas these effects are either blunted or not present at all when the cytokine is delivered continuously (50). As for PTX-B in the present study, the specific correlates of this functional dichotomy of IL-2-induced effects are essentially unknown.

**Fig. 3.** Low-dose PTX-B does not influence T cell sub-populations recovery. Percentage of human T cell sub-populations harvested from the peritoneal cavity in control and HIV-infected animals treated or not with PTX-B at the time of sacrifice. Shown are results (mean ± SD) of the percentage of cells harvested from six animals per each experimental group pooled from two independent experiments performed with human cells from two different donors.
PTX-B inhibits HIV infection in vivo

We have found that a higher dose of PTX-B (500 ng per mouse) lacks the anti-HIV activity observed with the lower dose (50 ng per mouse), independent of the schedule of administration. This finding could potentially be attributed to the presence and eventual boosting of PTX-B-induced antibody endowed with neutralizing capacity against the molecule acting as an immunogen. An open question concerning the potential use of PTX-B in humans is indeed its immunogenicity and the fact that a substantial portion of the population may already possess anti-PTX antibodies as a consequence of either infection or vaccination. In this regard, a detoxified PTX (PTX-9K/129G), retaining all the PTX-B properties including the anti-HIV effect in vitro (20, 51), has been successfully used in a vaccine formulation to prevent Bordetella pertussis infection (48). Of note is the fact that PTX-9K/129G alone was insufficient to induce substantial neutralizing activity and that adjuvants were required in order to achieve a significant titer of neutralizing antibody (52). Therefore, since HIV-infected individuals are characterized by a poor response to recall antigen (53, 54), PTX-B administration in the absence of adjuvants is expected to be poorly immunogenic in these individuals. Finally, it has been recently reported that the mitogenic activity of PTX-B is retained by a smaller portion of the molecule (7), likely endowed with reduced immunogenicity than PTX-B. Therefore, smaller PTX-B-related agents could also be tested for efficacy against HIV infection, both in vitro and in hu-PBL-SCID mice.

In conclusion, we have observed that repeated administration of a relatively low dose of PTX-B (50 ng per mouse) prevented HIV infection and replication in hu-PBL-SCID mice. PTX-B should therefore be considered a potential new anti-HIV agent that should be tested for preventing HIV infection or curtailing its replication in virus-exposed or early infected individuals.

Acknowledgements

This study was supported in part by grant number 40D.5 (to M.A.) of the IV National Program of Research on AIDS of the Istituto Superiore di Sanità, Rome, Italy.

Abbreviations

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<tr>
<td>hu-PBL</td>
<td>human peripheral blood leukocytes</td>
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<td>i.p.</td>
<td>intra-peritoneal</td>
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<td>LN</td>
<td>lymph nodes</td>
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<td>MDM</td>
<td>monocyte-derived macrophages</td>
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<td>SIV</td>
<td>simian immunodeficiency virus</td>
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References


