Pertussis toxin alters the innate and the adaptive immune responses in a pertussis-dependent model of autoimmunity

Rajeev K. Agarwal, Shu Hui Sun, Shao Bo Su, Chi-Chao Chan, Rachel R. Caspi *

Laboratory of Immunology, National Eye Institute, National Institutes of Health, 10 Center Drive, 10/10N222, Bethesda, MD 20892-1857, USA

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Abstract

Pertussis toxin (PTX) is used to promote development of autoimmune diseases. The mechanism(s) are still incompletely understood. We dissected the innate and adaptive immune responses in a PTX-dependent model of autoimmune retinal disease, experimental autoimmune uveoretinitis (EAU), a Th1-driven disease of the neural retina elicited in F344 rats with a peptide derived from the retinal antigen interphotoreceptor retinoid binding protein (IRBP). Our results showed that optimal doses of PTX led to strongly increased innate cytokine responses, followed by enhanced adaptive Th1 immunity and disease. At supraoptimal doses of PTX, EAU was suppressed, the animals exhibited persistent lymphocytosis and had an inhibited chemotactic response to chemokines. We suggest that the suppressive effect of PTX at supraoptimal doses is due to inhibition of lymphocyte emigration from the blood into the target tissue, secondary to inhibition of Gi-protein-coupled chemokine receptor signaling, that persists into the effector phase of disease.

Keywords: Autoimmune uveitis; EAU; Chemokines; Pertussis toxin

1. Introduction

Experimental autoimmune uveoretinitis (EAU) is an autoimmune disease whose target tissue is neural retina, characterized by massive ocular inflammation, vasculitis, destruction of photoreceptor cells and ultimately causing visual handicap. EAU can be induced in mice or rats by active immunization with evolutionarily conserved retinal proteins such as interphotoreceptor retinoid binding protein (IRBP) or by adoptive transfer of CD4+ MHC class II restricted Th1 cells specific to those antigens (Caspi et al., 1986; Gery et al., 1986a; Rizzo et al., 1996; Sanui et al., 1989). CD4+ T cells are further divided into two distinct categories, Th1 or Th2, depending on the cytokines they produce (Mosmann et al., 1986; Mosmann and Coffman, 1989; Romagnani, 1994). Th1 type cells secrete IFN-γ, IL-2 and lymphotoxin, and are responsible for cell-mediated immune responses, whereas Th2 type clones produce IL-4, IL-5, IL-10 and IL-13, and are involved in humoral immunity and allergic responses. Our previous studies have shown that in EAU the pathogenic effector cells are of Th1 phenotype, while Th2-type response may be counterregulatory and protective (Caspi, 1994; Caspi et al., 1996).

Bordetella pertussis in the form of whole heat-killed bacteria or of purified pertussis toxin (PTX) has for many years been used to promote development of organ-specific autoimmune diseases in animal models, such as EAU, experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune orchitis (EAO) (Caspi et al., 1988; Gery et al., 1986b; Hart et al., 1987; Kerlero de Rosbo et al., 1995; McAllister et al., 1986; Mendel et al., 1995; Munoz et al., 1984; Munoz and Mackay, 1984; Raine, 1984; Tuohy, 1994). Administration of PTX permits expression of disease in resistant strains, and enhances it in susceptible strains (Arimoto et al., 2000; Caspi et al., 1996; Silver et al., 1999). In the EAU resistant F344 rat treatment with PTX is necessary to induce EAU (Caspi et al., 1996).

Despite its use for over two decades to enhance induction of autoimmune disease, the mechanism of action of PTX is still not fully understood. It has been accepted for a long time that PTX alters the adaptive immune system, but little is known about the innate immune response.
time that much of the enhancing effects are due to changes in vascular permeability, thus facilitating the breakdown of blood–tissue barriers and permitting infiltration of inflammatory cells into the target organ (Linthicum and Frelinger, 1982; Linthicum et al., 1982). In addition, our more recent studies showed that when administered at the time of uveitogenic immunization, PTX promotes development of the Th1 response (Caspi et al., 1996; Silver et al., 1999). Others subsequently demonstrated that effects of PTX on antigen-presenting cells may underlie this phenomenon (He et al., 2000 and Hofstetter et al., 2002). However, researchers who use PTX for enhancement of cell-mediated autoimmune diseases are well aware that higher doses of the toxin can result in reduced disease. The mechanism of the strict dose dependency of the effect of PTX is unknown.

The goal of the present study was to dissect the innate and adaptive responses associated with different doses of PTX in the F344 rat, in which administration of PTX is a prerequisite for EAU induction. Our results showed that optimal doses of PTX led to strongly increased innate IFN-γ, IL-10 and a slightly increased IL-12p40 response, followed by enhanced adaptive Th1 immunity and disease. At supraoptimal doses of PTX, EAU was suppressed without inhibition of Th1 responses. Rats given supraoptimal doses of PTX exhibited leukocytosis long after blood leukocyte numbers in animals given lower doses of PTX had returned to normal, and had inhibited chemotactic responses to chemokines. Our data suggest that the inhibitory effect of PTX at higher doses is due to inhibition of lymphocyte emigration from the blood into the target tissue, which may be secondary to inhibition of Gi-protein-coupled chemokine receptor signaling.

2. Materials and methods

2.1. Animals

Female F344 rats 6–10 weeks old were procured from Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). All the rats were housed under specific pathogen-free conditions and were used in accordance to the Institutional guidelines as well as of the Association for Research in Vision and Ophthalmology.

2.2. Antigen and reagents

Peptide R16 of bovine IRBP (sequence ADGSSWEGVGVPDV, residues 1177–1191) that constitutes a major pathogenic epitope for the F344 rat (RT1-1^{v1}) (Sanui et al., 1989) was synthesized using conventional solid phase chemistry on an Applied Biosystems peptide synthesizer (Foster City, CA) as described earlier (Silver et al., 1995). Murine recombinant SDF-1α and RANTES were generously provided by J.L. Gao and P.M. Murphy, NIAID, NIH, (Bethesda, MD). Pertussis toxin (PTX) was purchased from Sigma (St. Louis, MO). Complete Freund’s adjuvant (CFA) and Mycobacterium tuberculosis strain H37RA were purchased from Difco (Detroit, MI).

2.3. Induction and scoring of EAU

F344 rats were immunized in one hind footpad with 0.1 ml of emulsion of 30 μg of R16 peptide in CFA (1:1, v/v), containing 2.5 mg/ml of M. tuberculosis H37RA and were given graded doses (0–10 μg) of B. pertussis toxin (PTX) in a total volume of 0.1 ml i.p. at the same time. Freshly enucleated eyes were collected for histopathology 14–16 days after immunization (5–7 days after onset of disease), and were fixed in methacrylate. The incidence and severity of EAU was scored on an arbitrary scale of 0–4 on hematoxylin and eosin-stained sections, according to a semiquantitative system described earlier (Caspi, 1997; Chan et al., 1990).

2.4. Delayed type hypersensitivity (DTH)

DTH responses were evaluated by ear assay. Two days prior to termination of the experiment, the thickness of each ear was recorded with a spring-loaded micrometer. Then, the ear pinna was injected with 10 μg of R16 peptide in a total volume of 10 μl using a 30-gauge needle. After 48 h, the thickness of each ear was measured and the Ag-specific DTH response was calculated as the difference between the ear thickness before and after challenge.

2.5. Lymphocyte proliferation assay

Cells from lymph nodes draining the site of immunization (popliteal) were collected 14–16 days after immunization. Triplicate wells of 0.2 ml culture were incubated in round-bottomed 96-well tissue culture plates (3 × 10^5 cells/well). The cultures were incubated with or without R16 peptide (2 μg/ml) in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented as described earlier (Caspi et al., 1986) and containing 1.5% syngeneic rat serum. After 48 h, the cultures were pulsed with 1 μCi [3H]-thymidine for an additional 16 h, and were harvested and counted using standard liquid scintillation.

2.6. Cytokine production

Lymph node cells collected and processed as for the proliferation assay were cultured at 1 × 10^6 cells/well in flat-bottomed 96-well plates with R16 peptide (5 μg/ml). Supernatants were collected after 48 h and were tested for presence of cytokines by ELISA using commercial kits. IFN-γ was estimated by the ELISA kit from Invitrogen, whereas IL-4, IL-6, IL-10, TNF-α and TGF-β were assayed, using ELISA kits from Biosource International (Camarillo, CA).
2.7. Leukocytosis determination

Rats immunized as described and given different doses of PTX were bled at different time intervals from the retro-orbital plexus into Microtainer tubes with EDTA (Becton Dickinson, Franklin Lakes, NJ), and complete blood counts (CBC) were performed using a cell counter (Abbott Cell Dyn 3500, Abbott Park, IL).

2.8. Multiprobe ribonuclease protection assay (RPA)

Spleens harvested from immunized rats treated with different doses of PTX 24 h after immunization were snap frozen on dry ice and stored at −70 °C until used. Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH) following manufacturer’s instructions. Total RNA (5 μg) from individual samples was used for RPA using the Riboquant (multiprobe RPA) kit from Pharmingen (San Diego, CA). The assay was performed according to the instruction manual of the kit without modifications. The gel was exposed to a phosphor screen (Storm 860, Molecular Dynamics, Sunnyvale, CA) and the intensities of the bands were compared.

2.9. Chemotaxis assay

Spleen cells were collected on day 10 and day 15 after immunization, corresponding to onset and peak of disease, respectively, under optimal conditions. Migration of cells to chemokines was evaluated using a 48-well microchemotaxis chamber as described earlier (Falk et al., 1980; Su et al., 1999). Briefly, 25-μl volumes of chemoattractant solution diluted in chemotaxis medium (RPMI-1640, 1% BSA, 25 mM Hepes) were pipetted in the wells of lower compartment of the chamber (Neuro Probe, Gaithersburg, MD). Cell suspension (50 μl of 2 × 10⁶ cells/ml) was layered in the wells of the upper compartment of the chamber. The two compartments were separated by a polycarbonate filter (5 μm pore size, Neuro Probe) coated with 20 μg/ml Fibronectin (Sigma) at 4 °C overnight. The chamber was incubated for 4 h at 37 °C in humidified air with 5% CO₂. After the incubation, the filter was removed, fixed and stained with Diff-Quik (Harlew, Gibbstown, NJ). The number of migrated cells in three high-powered fields (×400) was counted by light microscopy in coded samples. Average values of triplicate samples were calculated and the values of samples with chemokine were normalized to controls without chemokine at the same timepoint.

2.10. Reproducibility, statistical analysis and data presentation

Experiments were repeated at least twice and usually more times. Response patterns were highly reproducible. Where appropriate, significance of EAU scores was evaluated by frequency analysis, using Snedecor and Cochran’s (1967) test for linear trend in proportion. Statistical analysis of immunological responses was by two-tailed independent test. Differences of p ≤ 0.05 were considered significant and are marked on the graphs.

3. Results

3.1. Enhancing effect of PTX on induction of EAU follows an optimum curve

We have previously shown that PTX enhances the Th1 response and permits development of EAU in resistant F344 rats when given concurrently with immunization (Caspi et al., 1996). In order to test this effect at different doses of PTX resistant F344 rats were immunized with 30 μg of R16 peptide and were given graded concentrations of PTX from 0 to 10 μg/rat. Histopathologic examination of eyes harvested at peak of disease (days 14–16, corresponding to 5 days after onset), revealed that maximal disease occurred in the presence of 1 μg PTX. Surprisingly, higher PTX doses had an inhibitory effect, down to complete inhibition at 10 μg PTX per rat (Fig. 1). Histopathological examination of eyes of rats given 10 μg PTX revealed a normal architecture of the eye similar to those in rats that received no PTX.

3.2. Effect of graded doses of PTX on cellular responses

Rats immunized with R16 peptide in the presence of graded doses of PTX were DTH-tested by ear challenge 12 days after immunization as described in Materials and methods, and the responses were measured on day 14. DTH responses to peptide R16 followed the same optimum curve as the EAU scores (Fig. 1).
curve as seen for the disease, i.e. maximum at 1 μg PTX, and minimum at 0 and 10 μg PTX (Fig. 2).

Proliferation to peptide R16 (2.0 μg/ml) of splenocytes collected 14 days after immunization showed considerable enhancement in rats given PTX, but there was little difference between the different doses of PTX (Fig. 3). This shows that efficient priming had taken place at all PTX doses. The Ag-specific proliferation was significantly higher than the 0 μg PTX, but had no prominent difference between 0.1, 1.0, 3.0 or 10 μg PTX.

3.3. Effect of PTX on the innate response as reflected by early cytokine gene expression

Since PTX is administered at the time of priming, its effect on the innate immune response will in large measure determine the direction of the adaptive response. To study the effects of PTX on innate immunity, spleens were collected 24 h after immunization in the presence of graded PTX doses and cytokine mRNA levels were measured using the ribonuclease protection assay (RPA). Transcripts of IFN-γ and IL-12p40 (Fig. 4a), and IL-1β and IL-1Ra (Fig. 4b) showed characteristic changes with increasing concentrations of PTX. IFN-γ and (surprisingly) IL-10 showed a positive dose response, IL-12p40 showed only a mild (but statistically significant) rise. In contrast, IL-1β and IL-1 receptor antagonist (IL-1Ra) both showed a negative dose response. Surprisingly, we observed the elevation in mRNA levels of IL-10 with increasing concentration of PTX (Fig. 4a and b).

3.4. Effect of PTX on adaptive response as reflected by Ag-driven cytokine production

At the termination of the experiment 14–16 days after immunization, splenocytes were collected, pooled within each group and stimulated with R16 peptide. Cytokines were assayed after 48 h by ELISA as described in Materials and methods. Results showed that adaptive IFN-γ levels were low in the group that did not receive PTX and were high in rats that received 1.0 μg PTX. Similarly, immunized Lewis controls that developed EAU without PTX treatment produced an average of 8730 pg/ml of IFN-γ, roughly comparable to PTX-treated F344. The intensity of the
IFN-γ response paralleled disease scores and is in line with our previous results (Caspi et al., 1996). Interestingly, IFN-γ production remained high even at 10 µg PTX even though these rats did not develop disease.

In contrast, adaptive IL-10 production was highest in F344 rats that did not receive PTX and progressively declined with rising doses of PTX (Fig. 5a and b). Interestingly, IL-10 production in Lewis controls that developed EAU without PTX treatment was 1642 pg/ml, higher even than in unimmunized F344. This suggests that the resistance of F344 to EAU is due more to lack of a Th1 response (as evidenced by low IFN-γ) than to presence of an IL-10 response. Antigen-specific production of TNF-α, IL-4, IL-6 and TGF-β1 did not appear to be affected by PTX treatment (not shown). These data indicated that the Th1 response at the highest dose of PTX that inhibited disease was not reduced.

3.5. Effect of graded doses of PTX on leukocytosis

In view of the normally tight association between Ag-driven IFN-γ production and EAU scores in this model, we were surprised that EAU inhibition at higher doses was not accompanied by reduction in the Th1 response. Because our previous data showed that EAU can be inhibited if PTX is administered during the effector phase of disease due to inhibition of leukocyte infiltration into the target organ (Su et al., 2001), we hypothesized that at higher doses of PTX given at immunization, cell migration may still be inhibited even at later timepoints. Towards that end, we evaluated the recirculation ability of leukocytes in rats treated with PTX. CBC was performed on anticoagulated blood drawn from immunized rats that received optimal or supraoptimal doses of PTX at different times after immunization. While in rats that received no PTX, total WBC counts were decreased on day 1 after immunization compared to baseline, in the groups that received PTX, WBC counts were significantly elevated, more so at the higher PTX dose. By day 10 (disease onset) the levels of WBCs returned to normal in rats treated with optimal dose of PTX, but rats treated with the supraoptimal 10-µg dose still exhibited lymphocytosis even at 15 days after immunization (Fig. 6).

3.6. Chemotactic response of leukocytes from rats treated with high-dose PTX is reduced

Chemokines play an important role in cell migration and extravagation into the tissues and their signaling, mediated by G-protein-coupled receptors, is sensitive to PTX (Rossi and Zlotnik, 2000; Sallusto et al., 2000). We therefore hypothesized that leukocytes of rats given the high dose of PTX might have a persisting defect in their response to chemokines that would be detectable at the time when disease was present in controls. To test this notion, we examined migration of splenocytes from rats treated with the optimal (1 µg) or supraoptimal (10 µg) dose of PTX to
two chemokines representing the CC and CXC chemokine groups (RANTES and SDF1-α, respectively). These two chemokines were chosen because we know that the recombinant mouse chemokine is able to attract rat leukocytes. Since all chemokine receptors are Gi-protein dependent and affected by PTX, we considered response to RANTES and SDF1-α to be representative of their respective chemokine groups. A standard chemotaxis assay was performed in Boyden chambers as described in Materials and methods and the cells migrating in the presence of chemokine or medium were enumerated. The results showed that leukocytes from rats treated with high-dose PTX had severely impaired migration to SDF1-α in comparison to untreated rats, as well as in comparison to rats treated with the optimal dose, on both days 10 and 15 after immunization. Migration to RANTES in high dose-treated rats appeared only minimally affected compared to the optimal or no-PTX treatment (Fig. 7).

### 4. Discussion

In the present study, we undertook to examine the innate and adaptive immune responses in a PTX-dependent rat model of EAU at different doses of PTX, from sub- to supraoptimal, and correlate them to the elicited EAU scores. The data showed a clear optimum curve in terms of disease scores that developed at the different PTX doses, with 0.1 μg being suboptimal, 1 μg being optimal and 3 μg or more being supraoptimal. At 10 μg of PTX, EAU pathology was essentially eliminated. DTH responses, which in this model are usually correlated with pathogenicity, showed the same kinetics. Inhibition of EAU at the high doses was not due to cessation of priming, because antigen-specific lymph node cell proliferation even at the highest PTX dose of 10 μg was only marginally reduced, indicating that priming still took place.

We then proceeded to examine the type of cytokine responses generated as a function of PTX dose. Evaluation of innate cytokine responses 24 h after immunization by the RPA assay in explanted but otherwise unmanipulated spleenocytes revealed a sharp rise in IFN-γ with increasing PTX doses. We also detected a mild but statistically significant rise in IL-12p40 mRNA with increasing PTX doses. This confirms and extends the data of He et al. (2000), who showed enhanced IL-12 production in vitro by splenocytes of PTX-treated mice. IL-12 is known to trigger IFN-γ release from NK cells and it is likely that the higher IL-12 response may have contributed to the enhanced IFN-γ responses. Although the rise in IL-12 mRNA that was detected at 24 h was modest, it is possible that even this modest increase was biologically relevant. Alternatively, maximal IL-12 mRNA induction may have occurred before 24 h, to be followed at 24 h by robust induction of IFN-γ mRNA. It may be of note in this context that the innate IL-10 response at 24 h was modest, it is possible that even this modest increase was biologically relevant. Alternatively, maximal IL-12 mRNA induction may have occurred before 24 h, to be followed at 24 h by robust induction of IFN-γ mRNA. It may be of note in this context that the innate IL-10 response at 24 h was progressively upregulated with increasing PTX doses. IL-10, whose production is induced by IL-12, is a negative regulator of IL-12 transcription (Aste-Amezaga et al., 1998; Ma et al., 1997). Thus, its induction may have contributed to reduced expression of the IL-12 gene already by 24 h after PTX administration. Two other innate response mediators, IL-1β and IL-1RA, showed progressive inhibition with increasing doses of PTX.

The adaptive response of the F344 rat at increasing doses of PTX became increasingly skewed towards Th1, as evidenced by increased IFN-γ vs. progressively decreased IL-10 responses. Because the nature of the adaptive response in a large measure determined by the preceding innate response, the increased adaptive IFN-γ is in line with the upward trend in innate IFN-γ with increasing PTX doses. However, the decreasing adaptive IL-10 is in an apparent contradiction to the increase observed in innate IL-

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**Fig. 7.** Chemotactic response of leukocytes as a function of PTX dose. Spleens obtained from the rats whose leukocyte counts are shown in Fig. 6 were subjected to a standard chemotaxis assay in Boyden chambers. Shown are values of samples with chemokine normalized to controls without chemokine at each time point. Results are an average of four rats evaluated individually in two separate experiments. Differences that attained statistical significance (p ≤ 0.05) relative to no-PTX control are marked with asterisks. Actual counts of migrated cells ranged between 7 and 50 cells per high-power field for control and test samples, respectively.
10. The reason for this is unknown and may stem from effects that were not evaluated in the present study. It is worth noting that the PTX-enhanced IFN-γ response of the F344 is roughly equivalent to the IFN-γ response of the Lewis rat that develops EAU without the need for PTX treatment. On the other hand, the IL-10 response in the absence of PTX is present in both strains, and is even higher in the Lewis than in the F344. This suggests that it is the enhancement of the Th1 response, as manifested by adaptive IFN-γ, rather than inhibition of the IL-10 response that is associated with EAU-promoting effect of PTX. These data confirm and extend our previous observations in this model (Caspi et al., 1996).

Because of the strongly increased innate IFN-γ response, we initially hypothesized that inhibition of disease at high-dose PTX could be due to feedback inhibition by excess inflammatory mediators. Systemically produced endogenous IFN-γ serves to limit the severity of EAU in various strains of mice (Caspi et al., 1994). In a previous study, we demonstrated directly that elevated levels of innate IFN-γ secondary to administration of exogenous IL-12 at the time of immunization lead to protection from disease development (Tarrant et al., 1999). However, under these circumstances, the adaptive IFN-γ response is severely curtailed, which was not the case in the present study. Additionally, this level of IFN-γ was comparable to what was produced by the Lewis rats in the absence of PTX treatment, further arguing against such a mechanism of feedback inhibition here.

We therefore examined an alternative hypothesis. Recently, we showed that PTX administered at the time of cell migration to the target organ rather than at the time of immunization has an inhibitory effect on EAU (Su et al., 2001). This was found to be due to inhibition of lymphocyte extravasation and infiltration into the target tissue secondary to blockade of chemokine receptor signaling. Thus, PTX has both enhancing and inhibitory effects on disease expression, and one or the other predominates depending on the stage of disease pathogenesis. Because the inhibitory effect on migration persists for several days after a single PTX administration (Su et al., 2001), we hypothesized that PTX administered at the time of immunization at sufficiently high doses might still be inhibiting cell migration during the effector phase of disease. The observation that DTH in the high-dose animals was also severely inhibited, although lymphocyte proliferation was only marginally reduced (indicating that priming has occurred) and adaptive IFN-γ production was unreduced (indicating that a Th1 response was generated), is in line with this interpretation. This was further borne out by evidence of persistent leukocytosis into the effector phase of disease, indicating that lymphocyte recirculation in these animals was inhibited. Finally, splenocytes of the same rats were deficient in their ability to migrate to SDF1-α, and to a lesser extent to RANTES, further supporting the conclusion that persistent inhibition of chemokine responses was at least in part responsible for protection from EAU at high PTX doses.

In summary, the results of the present study confirm and extend our previous data regarding the role of PTX in an autoimmunity model that depends on PTX for its induction. While optimal doses promote disease and the Th1 response, supraoptimal doses inhibit disease and DTH without diminishing the Th1 response. The mechanism of inhibition by supraoptimal doses of PTX appears to be due to inhibition of lymphocyte recirculation secondary to persistent inhibition of chemokine responses into the effenter phase of disease.

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