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Pertussis Toxin Inhibits Induction of Tissue-Specific Autoimmune Disease by Disrupting G Protein-Coupled Signals

Shao Bo Su, Phyllis B. Silver, Meifen Zhang, Chi-Chao Chan, and Rachel R. Caspi

Pertussis toxin (PTX) has been used for many years as an adjuvant that promotes development of tissue-specific experimental autoimmune diseases such as experimental autoimmune encephalomyelitis, experimental autoimmune uveitis (EAU), and others. Enhancement of vascular permeability and of Th1 responses have been implicated in this effect. Here we report a surprising observation that, in a primed system, PTX can completely block the development of EAU. Disease was induced in B10.RIII mice by adoptive transfer of uveitogenic T cells, or by immunization with a uveitogenic peptide. A single injection of PTX concurrently with infusion of the uveitogenic T cells, or two injections 7 and 10 days after active immunization, completely blocked development of EAU. EAU also was prevented by a 1-h incubation in vitro of the uveitogenic T cells with PTX before infusing them into recipients. Uveitogenic T cells treated with PTX in vitro and lymphoid cells from mice treated with PTX in vivo failed to migrate to chemokines in a standard chemotaxis assay. Neither the isolated B-oligomer subunit of PTX that lacks ADP ribosyltransferase activity nor the related cholera toxin that ADP-ribosylates G\textsubscript{i} (but not G\textsubscript{s}) proteins blocked EAU induction or migration to chemokines. We conclude that PTX present at the time of cell migration to the target organ prevents EAU, and propose that it does so at least in part by disrupting signaling through G\textsubscript{i} protein-coupled receptors. Thus, the net effect of PTX on autoimmune disease would represent an integration of enhancing and inhibitory effects. The Journal of Immunology, 2001, 167: 250–256.

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1 Abbreviations used in this paper: EAE: experimental autoimmune encephalomyelitis; EAU: experimental autoimmune uveitis; PTX: pertussis toxin; IRBP, interphotoreceptor retinoid-binding protein; CT, cholera toxin; MCP, monocyte chemoattractant protein; MIP, macrophage-inflammatory protein.

Peptide SGIPYISYLHPGNTILHVD representing residues 161–180 of human interphotoreceptor retinoid-binding protein (IRBP)
was synthesized by Fmoc chemistry on a peptide synthesizer (model 461; PE Applied Biosystems, Foster City, CA). PTX, CT, CFA, and Mycobacterium tuberculosis strain H37RA were purchased from Sigma (St. Louis, MO). PTX, CT, and H37RA were purchased from Research Biochemicals (Natick, MA). Murine recombinant SDF-1α, macrophage-inflammatory protein (MIP)-1α, MIP-1β, and RANTES were generously provided by J. L. Gao and P. M. Murphy, National Institute of Allergy and Infectious Diseases, National Institutes of Health, (Bethesda, MD).

**Uveoretinitis T cell line**

A long-term T cell line specific to the IRBP-derived p161–180 was established from B10.RIII mice and was propagated as described previously (15). Briefly, a 25-cm² Th1 cell line was established from lymph node cells of B10.RIII mice primed with p161–180 by initially stimulating the cells with the immunizing peptide in the presence of IL-12 and anti-IL-4 Abs. The cells subsequently were propagated by alternating cycles of stimulation with p161–180 and expansion in IL-2 containing medium every 2–3 wk. This cell line produces a typical Th1 cytokine profile and induces EAU with a clinical onset of 4–6 days after transfer.

**Primary culture of IRBP-specific lymph node cells**

Donor B10.A mice were immunized with 50 μg of IRBP. Lymph node cells and spleen cells collected on day 14 after immunization were pooled.

The cell suspension was adjusted to 10⁵ cells/ml in 1% normal mouse serum-RPMI 1640 medium, and the cultures were stimulated in 75-cm² moattractant solutions diluted in chemotaxis medium (RPMI 1640, 1% mouse serum). The cultures were stimulated in 75-cm² moattractant solutions diluted in chemotaxis medium (RPMI 1640, 1% mouse serum) and divided among three sites: base of tail and both thighs. In some groups, 0.5 ml aliquots of chemotactic solutions were injected into the peritoneal cavity of syngeneic recipients.

**Cell migration assay**

Auxiliary and inguinal lymph node cells of mice were harvested 24 h after i.v. administration of PTX, CT, or B oligomer. Uveoretinitis T line cells were collected 1 h after in vitro treatment with PTX. Migration of cells to chemokines was assessed by using a 48-well microchemotaxis chamber technique as described previously (18, 19). Briefly, a 25-μl aliquot of chemotactant solutions diluted in chemotaxis medium (RPMI 1640, 1% BSA, 25 mM HEPES) was placed in the wells of the lower compartment. A 50-μl cell suspension (2 × 10⁶ cells/ml in chemotaxis medium) was placed in the wells of the upper compartment of the chamber (Neuroprobe, Cabin John, MD). The two compartments were separated by a polycarbonate filter (5-μm pore size; Neuroprobe) coated with 20 μg/ml Fibronectin (Sigma) at 4°C overnight. The chamber was incubated at 37°C for 4 h in humidified air with 5% CO₂. At the end of the incubation, the filter was removed, fixed, and stained with Diff-Quik (Harleu, Gibbstown, NJ). The number of migrated cells in three high-powered fields (×400) was counted by light microscopy after coding the samples. Results are expressed as the mean ±SD value of the migration in triplicate samples.

**Results**

PTX administered at the time of effector cell migration aborts induction of EAU

B10.RIII mice immunized with IRBP and with its major epitope p161–180 are able to develop EAU without PTX treatment. However, treatment with 0.2–1 μg of PTX concurrently with immunization enhances disease scores (21). For EAU to develop, Ag-specific effector cells primed in the periphery must migrate to the target organ, extravasate into the tissue, and recruit inflammatory leukocytes, a process that should be facilitated by enhancement of vascular permeability and breakdown of the blood-organ barrier.

To evaluate the effect of PTX administered at the time of effector cell migration on induction of EAU, B10.RIII mice immunized with a uveoretinitis dose of p161–180 were given PTX on day 7 after active immunization. This is the time considered to be critical for effector cell migration, as clinical onset of EAU typically occurs on day 8–9 after immunization. Eyes were collected on day 14. Unexpectedly, a single infusion of 0.5 μg of PTX on day 7 inhibited EAU scores in most mice, and if followed by a second dose on day 10, largely prevented EAU in all mice (Fig. 1A). It is important to point out that this is a dose that reproducibly enhances EAU in a variety of mouse strains when administered on day 0, concurrently with immunization.

In a second approach, based on adoptive transfer of Ag-specific effector T cells, a single infusion of 1 μg of PTX was given to B10.RIII mice concurrently with adoptive transfer of 2 × 10⁶ cells from a uveoretinitis T cell line. EAU scores were assessed 10 days later by histopathology. Again, rather than enhancing disease scores, PTX completely inhibited expression of EAU (Fig. 1B). An identical effect was observed with IRBP-primed lymph node cells from B10.A mice after primary culture (data not shown).

Long-term follow-up of mice treated or not with PTX was performed by weekly fundoscopy for up to 35 days after adoptive transfer of the T cell line. The control group developed full-blown disease (scores of 4) within the first week (with onset occurring ~4 days after transfer), and remained at or close to that grade throughout the observation period. The PTX-treated group remained negative. After as long as 21 days, only about half of the mice developed minimal retinal signs initially in one eye (grade 0.5), and subsequently their diseases stabilized with little further progress (Fig. 2).

In a subsequent series of experiments, the effects of different doses and timing of PTX administration on EAU induced by adoptive transfer of uveoretinitis T cells were studied. Fig. 3 demonstrates that inhibition of adoptive EAU by PTX is dose-dependent.
and gradually diminishes over a 1-log dose difference from 1 to 0.1 \( \mu \text{g} \). Fig. 4 illustrates that the protective effect of a single 0.5-\( \mu \text{g} \) dose of PTX is detectable for almost 2 wk after its administration. The maximal inhibitory effect was observed when PTX was given on day 0 relative to adoptive transfer, and near maximal effect was seen when PTX was given 3 days before adoptive transfer. Protection was still apparent with PTX given as long as 10 days before infusion of the uveitogenic cells. Thus, the effect of PTX is long-lived.

### Inhibition is dependent on the enzymatic A-protomer subunit of PTX and can be exerted directly on the T cell

The biological activities of PTX are to ADP-ribosylate \( \text{Gi} \) type G proteins, to down-regulate cAMP production, inhibit \( \text{Ca}^{2+} \) flux, and interfere with cell migration (9). PTX is composed of an enzymatically active subunit (A protomer) responsible for the biological activities, and a membrane binding subunit (B oligomer) that delivers the A subunit into the cell (9, 10). The structurally and functionally related CT ADP-ribosylates \( \text{Gs} \) type G proteins; it increases cAMP production, and does not inhibit cell migration and \( \text{Ca}^{2+} \) flux (9, 11). To examine whether the protective effects of PTX involved ADP ribosylation of \( \text{Gi} \) proteins, we compared the ability of intact PTX, of the enzymatically inactive B oligomer, and of CT to inhibit adoptive transfer of EAU. Fig. 5A shows that in vivo administration of the B oligomer or of CT to mice subsequently injected with uveitogenic cells did not prevent EAU, suggesting that the protective effect requires the ADP-ribosylating activity of the A subunit.

We next examined whether PTX could act directly on the uveitogenic T cells, by preincubating line cells about to be adoptively transferred into mice with PTX in vitro for 1 h. Fig. 5B

### FIGURE 1. Inhibition of EAU induction by PTX. A, B10.RIII mice were immunized with 20 \( \mu \text{g/mouse} \) of IRBP p161–180 to induce EAU. PTX was injected by i.v. at the indicated times relative to Ag immunization. EAU score was assessed 14 days later by histopathology. The EAU scores of PTX-treated mice at all points differ significantly from the respective controls (\( p < 0.05 \)). B, B10.RIII mice received \( 2 \times 10^6 \) cells from a uveitogenic T cell line by i.p. injection concurrently with 1 \( \mu \text{g} \) of PTX by i.v. EAU score was assessed 10 days later by histopathology.

### FIGURE 2. Long-term effects of PTX on adoptively transferred EAU. B10.RIII mice received \( 2 \times 10^6 \) cells from a uveitogenic T cell line i.p. and 0.5 \( \mu \text{g} \) of PTX i.v. at the same time. Mice were followed weekly by fundoscopic examination. The EAU scores of PTX-treated mice vs their respective controls are significantly different at each of the time points (\( p < 0.05 \)).

### FIGURE 3. Dose response of inhibitory effect of PTX on EAU. B10.RIII mice received \( 2 \times 10^6 \) cells from a uveitogenic T cell line i.p. and the indicated amount of PTX i.v. at the same time. EAU score was assessed 10 days later by histopathology. The EAU scores of PTX-treated mice are different from controls for the 1-, 0.5-, and 0.1-\( \mu \text{g} \) doses (\( p < 0.05 \)).

### FIGURE 4. Time dependence of protective effect of PTX. Groups of B10.RIII mice were given 0.5 \( \mu \text{g} \) of PTX i.v. at the indicated time relative to adoptive transfer of \( 2 \times 10^6 \) cells from a uveitogenic T cell line i.p. EAU score was assessed 10 days later by histopathology. The EAU scores of PTX-treated mice are significantly different from untreated controls for all points between 0 and \(-10 \) days (\( p < 0.05 \)).
shows that treatment of uveitogenic T cells with PTX before transfer was sufficient to inhibit adoptive EAU. Microscopic examination of the cells after the incubation for vital dye exclusion indicated that they were fully viable, making it unlikely that the protective effect of PTX involved toxicity. This is in line with the observations of others concerning lack of toxicity of PTX on lymphoid cells (22, 23). Again, the enzymatically inactive B oligomer and the Gs protein-targeting CT were unable to inhibit the ability of the T cell line to induce disease.

**Uveitogenic T cells incubated with PTX as well as lymphocytes of PTX-treated mice are unable to migrate to chemokines**

Chemokines signal through G_i protein-coupled receptors, are sensitive to PTX, and play important roles in cell migration and extravasation into the tissues (24, 25). Therefore, it was logical to suspect that PTX inhibits EAU due at least in part to effects on chemokine signaling. To test this hypothesis, the migration of PTX-treated T cells and of lymph node cells from PTX-treated mice to chemokines was examined by a standard chemotaxis assay in a Boyden chamber (18). Uveitogenic T cells incubated with PTX did not migrate to chemokines, including MIP-1α and RANTES (Fig. 6A). Incubation with B oligomer or CT did not affect the chemotactic responses of the uveitogenic line cells. Similarly, lymph node cells from B10.RIII mice injected 24 h earlier with PTX were compromised in their ability to respond to chemokines, but B oligomer and CT had no inhibitory effect (Fig. 6B). These data support the notion that the protective effect of PTX is attributable to its enzymatic activity that disrupts G_i protein-coupled chemokine receptor signaling.

**Interference with migration of either the Ag-specific T cells or of the recruited inflammatory leukocytes is sufficient to abort EAU induction**

Previous studies on the EAU model demonstrated that induction of EAU after adoptive transfer of (fluorescently labeled) uveitogenic T cells involves two discrete waves of cell entry into the eye (26). Small numbers of labeled Ag-specific T cells infiltrate the retina within 24 h after cell transfer and disappear by 72 h. This is followed at 96 h by a massive and prolonged influx of unlabeled host-derived leukocytes (thought to have been recruited by the earlier wave of Ag-specific cells) with a small proportion of labeled cells mixed in, and induction of the tissue damage typical of EAU. We were interested in dissecting the effect of PTX on the
first and second stage of cell entry in terms of protection from disease. Toward this end, mice were treated with PTX concurrently with adoptive transfer of the uveitogenic T cell line (day 0), on day 3, on day 3 and day 7. EAU was assessed on day 10. Fig. 7 shows that, as before, a single injection of PTX on day 0 (corresponding to the first wave of infiltration) was protective, as were two injections on day 3 and 7 (corresponding to the second wave of infiltration). A single injection of PTX on day 3 did not prevent emergence of disease on day 10, consistent with the more prolonged kinetics of the second phase of infiltration. Thus, inhibition of either the first wave (Ag-specific T cells) or the second wave (mostly recruited host leukocytes) of cellular infiltration aborts development of EAU.

Discussion

Many rodent strains require PTX concurrently with immunization to develop EAU, and in the ones that do not require it, PTX enhances disease scores. The enhancing effects of PTX on autoimmunity also have been well documented in EAE and autoimmune orchitis, but the mechanism has been the subject of some controversy. Some early studies indicated effects on vascular permeability, and connected susceptibility and the effect of PTX in the EAE model to histamine sensitization genes and the disruption of the blood-brain barrier (12–14). Other early studies presented evidence favoring effects on the sensitization phase of immunity (27).

Our studies addressing the role of PTX in EAU were based on two hypotheses. We reasoned that because most of the PTX injected at the time of immunization must be gone by the time that migration of effector cells takes place into the target organ 1–2 wk later, it is logical to look for effects of PTX on early events in the immune response that coincide temporally with presence of PTX in the system. Our previous work on the effect of PTX on Th1/Th2 phenotype commitment of effector T cells in animals immunized for EAU induction indeed showed that both in mice and in rats, PTX treatment strongly enhanced Th1 responses to the immunizing Ag in parallel to enhancing disease scores (21, 28). More recent data from other laboratories confirmed this and suggested that activation of Ag-presenting cells and induction of IL-12 production are involved in this process (Ref. 29 and T. Forsthuber, unpublished observations). These results are in line with the early observations of Lando et al. (27) describing effects on the Ag sensitization phase.

The second hypothesis, which is specifically examined in the present study, was that if enhancement of vascular permeability is indeed a major mechanism, the effect should be strongest if PTX is administered at the time of effector cell migration. To our surprise, the data showed that PTX administered to animals coincident with effector cell trafficking and extravasation into the target organ, aborts development of disease. The same is true if the eliciting Ag-specific T cells are pretreated with PTX before their infusion into animals. The phenomenon is dependent on the ADP-ribosyltransferase activity of PTX, indicating that G, proteins are a necessary target, and is accompanied by compromised responses to chemokines of the affected lymphoid cells. Because migration, adhesion, and extravasation of leukocytes is highly dependent on chemokine signals (24, 25, 30), the data are consistent with the interpretation that prevention of EAU involves at least in part the disruption by PTX of effector cell migration and infiltration into the target organ, secondary to blockade of chemokine signaling through G protein-coupled receptors. Our data also are consistent with a recent study by Blankenhorn et al. (31) who demonstrated (in mice concordant for MHC and for the locus controlling histamine sensitization) a single locus on chromosome 9 that controls EAE severity and monocyte infiltration into the spinal cord. This locus (eae9) includes a number of genes, among them the chemokine receptor CXCR5. The same group also presented evidence that the locus eae7 on mouse chromosome 11 contains genes encoding the chemokines TCA3, monocyte chemoattractant protein (MCP)-1, and MCP-5 (32).

The inhibitory effect of PTX administered to mice at different times after transfer of uveitogenic T cells sheds new light on the cellular events involved in EAU pathogenesis. Our previous study (26) indicated that cellular infiltration into the eye during adoptively transferred EAU occurs in two discrete phases: 1) within 24 h of transfer, small numbers of activated uveitogenic T cells enter the intact eye, apparently at random, and disappear by 72 h; and 2) at 96 h, there begins a massive influx of host-derived bloodborne leukocytes (and additional Ag-specific T cells) that is quickly followed by appearance of pathology. The result that PTX injected either at 0 or 3 days inhibits disease indicates that EAU can be prevented by interfering with either the first or the second wave of cellular infiltration. The ability to prevent disease by inhibiting the second wave of infiltration alone supports the hypothesis that the Ag-specific T cells cannot by themselves cause pathology, and that tissue damage is dependent on the subsequently recruited host effector cells. It is also in line with our previous observation that recruited Ag-nonspecific T cells are required for EAU pathogenesis, as athymic animals develop strongly attenuated disease after adoptive transfer of uveitogenic T cells (33). In view of the critical importance of recruited host leukocytes for EAU induction, it could be argued that treatment on day 0 prevented disease simply by inhibiting the secondary recruitment. This possibility is argued against by experiments not shown here, demonstrating that PTX prevented entry of fluorescently tagged activated T cells into the retina at 24 h (S.B. Su et al., unpublished results). Interestingly, although a single injection of PTX on day 0 (first wave of infiltration) was sufficient to abort disease, administration of PTX on day 3 (second wave of infiltration) appeared to require a second dose several days later. We propose that the activated Ag-specific T cells must be allowed to enter the eye within a very brief period of time after adoptive transfer or (in the absence of continued stimulation) they will revert to a resting state in which they are unable to induce disease (Ref. 34 and R. Caspi, unpublished results). Once the uveitogenic T cells have entered the eye...
and initiated inflammatory changes, including chemokine production by ocular cells, a second dose of PTX is needed to prevent disease development.

Our data do not address the question whether interference by PTX with the first wave vs the second wave of cellular infiltration into the eye involves the same mechanism(s). The chemokines MCP-1, inflammatory protein 10, RANTES, and MIP-1β are expressed in ocular tissues during EAU and are thought to be important in recruitment of inflammatory cells into the eye (35). Although it is generally accepted that cellular infiltration into inflamed tissue is dependent on chemokines to trigger adhesion of effector leukocytes to activated vascular endothelium, arrest, and diapedesis (24, 25), this applies only to the second wave of cellular infiltration. The first wave of activated T cells traffics to a healthy eye that has no detectable production of chemokines. Although the ability of PTX (as treatment in vivo or as preincubation in vitro) to interfere with this stage suggests involvement of G protein-dependent processes(es), the actual molecular targets remain to be identified and will be the subject of a separate investigation. Our data in no way exclude the possibility that secondary effects of PTX on G protein-dependent processes, such as G protein-dependent adhesion molecule activation, may also contribute to its negative effects on EAU. Data reported by Schenkel and Pauza (36) indicate that PTX treatment reduces expression of leukocyte function Ag-1 on the cell surface. Of note in this context are our previous observations that leukocyte function Ag-1 is involved in EAU pathogenesis in mice and that its blockade ameliorates disease expression (37).

Our findings appear to be at variance with the well-documented enhancing effects of PTX on autoimmunity. In EAE, PTX is needed not only for disease induced by active immunization, but also in many cases for disease induced by adoptive transfer of primed T cells. How then, is EAU different in this aspect from EAE? We believe that in principle it is not different. The inhibitory effects of PTX on lymphocyte recirculation and homing have been well documented and are not restricted to particular disease models or to selected autoimmune situations (38–41). The early report by Spangrude et al. (23), describing inhibition of the adoptive transfer of contact sensitivity by PTX and attributing it to inhibitory effects on cell migration, is a case in point. To observe inhibitory effects of PTX, a model is needed where disease can be achieved without it. EAU in the B10.RII mouse is such a model. In the case of some EAE combinations, where adoptive transfer by itself is not sufficient to elicit disease expression, only the enhancing effects of PTX can be documented. In line with this interpretation is the report of Munoz and Mackay (42) describing an adoptively transferred EAE model that is dependent on PTX but appears after an extended onset and an atypical clinical course, induced in PL/J mice by myelin oligodendrocyte glycoprotein (MOG)-derived peptide: preliminary analysis of MOG T cell epitopes. Eur. J. Immunol. 25:565.

Other investigators reported inhibitory effects of PTX on EAE, but the mechanism of that inhibition remained unidentified. Robinson et al. and Ben-Nun et al. (22, 43) reported that pretreatment of animals 2 wk before immunization with PTX protected from EAE. Ben-Nun et al. (44) attributed the protection to the nonenzymatic B oligomer, whereas Robinson et al. (22) localized the phenomenon to the A subunit, but felt that it involved a TCR-mediated pathway. However, in these studies, protection by PTX pretreatment could have been attributable at least in part to formation of Abs to PTX, which would then inhibit its biological activity when it subsequently was used as part of the immunization protocol (45).

Our data certainly do not exclude delayed effects of PTX on vascular permeability. As we show here, the effect of PTX, at least for inhibition of EAU, is long lived. It stands to reason that the same may be true for other cellular effects of PTX, possibly including histamine sensitization of endothelial cells that affects cellular permeability. Thus, our data suggest that the effect of PTX on autoimmune disease combines inhibitory as well as enhancing influences, with one or the other predominating at different stages. The net observed outcome would thus represent an integration of these opposing effects.

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References


