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Dissociating the Enhancing and Inhibitory Effects of Pertussis Toxin on Autoimmune Disease

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Pertussis toxin (PT), a bacterial toxin elaborated by Bordetella pertussis, which is the causative agent of the disease whooping cough in human, is a noncovalently linked heterohexameric protein. It is structurally and functionally divided into A and B subunits (1–3). The A subunit (A protomer, PT-A) is composed of a single peptide (S1) with ADP-ribosyltransferase activity that modifies GTP-binding regulatory proteins (G proteins), thus interfering with G protein-dependent signal transduction in mammalian cells. The B subunit (B oligomer, PT-B), composed of a pentameric protein complex (S2, S3, two S4 and S5), confers the binding capacity of PT to cell surface glycoprotein receptors and delivers the A protomer into the cell (1, 2, 4, 5).

PT has been used for many years as an adjuvant to enhance induction of organ-specific autoimmune diseases elicited by immunization with tissue Ags. Such models include experimental autoimmune encephalomyelitis, experimental autoimmune orchitis, experimental autoimmune uveitis (EAU), and others (6–11). Previous work by us and by others showed that enhancing effect of PT on autoimmunity is due at least in part to promotion of the Th1 response owing to effects on APCs (12–14). In contrast, we recently showed in the EAU model that PT also has potent inhibitory effects on disease that are especially prominent when it is administered at a later stage in disease development. The disease-inhibitory activity required the PT-A subunit, and was attributed to inhibition of effector cell migration to target organ secondary to disruption of G protein-coupled signals by PT-A (15, 16).

The present study was designed to dissociate the inhibitory from the enhancing effects of PT on EAU by evaluating the effects of purified PT-B on the induction of disease. The effect of purified PT-A could not be evaluated, as PT-A can only enter into the cell as a complex with PT-B. Mice given PT-B or whole PT concurrently with immunization developed the disease with similar maximal scores. PT-B treatment also increased delayed-type hypersensitivity (DTH), enhanced lymphocyte proliferation, and elevated IFN-γ production to the uveitogenic Ag. PT-B induced mRNA and protein expression of IL-12 p70 by splenocytes. These data indicate that PT-B mimics the activity of whole PT in triggering Th1-polarizing innate immunity responses.

Materials and Methods

Animals
Six- to 8-wk-old C57BL/6, C3H/HeN Cr, and C3H/HeJ mice supplied by The Jackson Laboratory (Bar Harbor, ME) were kept under specific pathogen-free conditions and given standard laboratory chow and water ad libitum. Animal care and use were in compliance with institutional guidelines.

Ag and reagents
Whole bovine interphotoreceptor retinoid-binding protein (IRBP) was purified from retinas by Con A-Sepharose affinity chromatography and HPLC (17). Whole PT or PT-B isolated from whole PT was purchased from Sigma-Aldrich (St. Louis, MO).

SDS-PAGE
SDS-PAGE was performed in a standard Laemmli system using 10% gel. The bands were visualized by Coomassie blue staining and scanned. The ratio of contamination of PT-B with A protomer was analyzed with NaOH treatment. 

Chemotaxis assay
Cell migration was assessed by using a 48-well microchemotaxis chamber, as described previously (18, 19). 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.

LPS measurement
The amount of LPS contamination in PT and PT-B preparations was measured using the Chromogenic Limulus Amebocyte Lysate Test Kit (Cambrex, Walkersville, MD), according to manufacturer’s instructions. The kit yields LPS levels in units. Levels of LPS in pg/ml were calculated from a calibration curve constructed from serial dilutions of a known concentration of LPS.
Treatment with inhibitors

PT (50 μg/ml), PT-B (50 μg/ml), or LPS (10 μg/ml) was pretreated with protease K (25 μg/ml) for 45 min at 37°C in Dulbecco’s PBS (containing Ca^{2+} and Mg^{2+}). The final concentrations of PT, PT-B, LPS, and the LPS inhibitor polymyxin B added to the cultures were 100 ng/ml, 100 ng/ml, 10 ng/ml, and 25 μg/ml, respectively.

EAU induction and scoring

EAU was induced by immunization with 150 μg bovine IRBP in CFA, as described before (20). At the same time, graded doses of PT-B or PT were injected i.p. Eyes were harvested 21 days after immunization. H&E-stained tissue sections of the whole eye were prepared and were graded by a masked observer on a scale of 0–4 in half-point increments, using the criteria described previously (12).

Delayed-type hypersensitivity

A total of 10 μg IRBP in 10 μl PBS or 10 μl PBS was injected into the pina of right or left ear. Ear thickness was measured 48 h later using a spring-loaded micrometer.

Lymphocyte proliferation assay

Draining (inguinal and iliac) lymph nodes from immunized mice were collected 21 days after immunization, and were pooled within the group. Lymphocyte proliferation was assessed by tritiated thymidine uptake, as described previously (20). The data are shown as cpm.

Cytokine assays

Lymph node cells from IRBP-immunized mice or splenocytes from naive mice were cultured in 24-well flat-bottom plates (5 × 10^5 cells/ml culture medium per well) with the specified stimulants. Supernatants were collected 48 h later and were kept frozen in small aliquots at −70°C. Cytokine production was measured by ELISA using Ab pairs from Endogen (Boston, MA) for IFN-γ, as described previously (21). IL-12 p70, the bioactive heterodimer, was determined using a kit (Quantikine M) from R&D Systems (Minneapolis, MN).

**IL-12 p40 mRNA determination**

Total cellular RNA was extracted from splenocytes for IL-12 p40 mRNA amplification, as follows: splenocytes (5 × 10^5/ml) from naive C57BL/6 mice were suspended in DMEM supplemented with 1% normal mouse serum and incubated with the specified concentrations of PT, PT-B, or LPS at 37°C overnight. In other experiments, naive mice were injected with the specified amounts of LPS, PT, or PT-B. The spleens were removed 2 h later. Total cellular RNA was extracted from collected spleens. Reverse transcription was performed as per instructions in the kit (Clonetics, Palo Alto, CA). PCR amplifications were performed for 25 cycles on the resultant cDNA for the expression of IL-12 p40 mRNA. The sequences of the specific primers used in the detection of IL-12 p40 are as follows: sense, 5'-ATCGTTTGTGTTGTTCTCC-3'; antisense, 5'-AGTCCCTTCTGTTCAATTTC-3'. PCR amplification products were electrophoresed and viewed on a 1% agarose gel and stained with ethidium bromide. Bands were scanned and were quantified using National Institutes of Health Image.

Reproducibility and data presentation

Experiments were repeated at least twice and usually three or more times. Results were highly reproducible. Figures show representative experiments.

Results

The preparation of PT-B has no detectable contamination by intact PT

Bioactive PT ADP-ribosylates G_i-type G proteins and interferes with cell migration by blocking chemokine receptor signaling. This activity is mediated by the enzymatic A subunit. In the present study, we wished to evaluate the effects of PT-B, free of the A subunit, on induction of EAU. Because commercially available PT-B is purified after dissociation of the A and the B subunits of PT, it was necessary to first ascertain that the PT-B preparation was free of contamination with whole PT. Fig. 1, A and B, demonstrates that contamination with PT-A was virtually undetectable by SDS-PAGE. Because biological assays are in general more sensitive, we examined the PT-B preparation for inhibition of cell migration in a standard Boyden chamber chemotaxis assay. Unlike intact PT, the tested preparation of PT-B did not inhibit cell migration to the chemokine, monocyte chemoattractant protein-1. Human PBMC suspension treated with 100 ng PT or with graded doses of PT-B at 37°C for 1 h was submitted to chemotactic assay using a Boyden chamber. The number of migrated cells in three high-powered fields was counted by light microscopy after coding the samples. Results shown are representative of three independent experiments.

**PT-B subunit mimics the effects of whole PT on induction of EAU and associated immunological responses**

PT administered at the time of immunization with the autoantigen promotes induction of EAU and strongly enhances its associated immunological responses, including lymphocyte proliferation and DTH (22, 23). To evaluate the effects of PT-B, C57BL/6 mice immunized with IRBP in CFA were given a concurrent i.p. injection of an optimal dose of whole PT (determined to be 0.5 μg; Fig. 2A), graded doses of PT-B, or vehicle. Evaluation of eyes collected for histopathology on day 21 showed that vehicle-treated mice failed to develop disease (Fig. 2B). In contrast, PT-B facilitated development of EAU in a dose-dependent manner, ultimately reaching the same maximal disease scores as mice treated with an optimal dose of PT. The same mice challenged 2 days before the termination of the experiment for DTH responses to IRBP exhibited strongly augmented DTH scores (Fig. 2C). Similarly to the DTH response, lymphocyte proliferation was markedly enhanced by inclusion of PT-B in the immunization regimen (Fig. 2D). Thus, PT-B was able to mimic the enhancing effect of the whole PT on EAU, DTH, and Ag-specific lymphocyte proliferation.
PT-B promotes development of a Th1 response

Our previous data indicated that EAU is dependent on the presence of a type 1 response to the uveitogenic Ag. We therefore compared the IFN-γ production in response to IRBP of mice immunized and treated, or not, with PT or PT-B. Draining lymph node cells were collected 21 days after immunization. Ag-stimulated supernatants were generated from the different groups and were assayed for content of IFN-γ by ELISA. The results showed that the lymphocytes from mice treated with PT-B produced at least as much IFN-γ as mice treated with an optimal dose of PT-B, and both groups made considerably more IFN-γ than vehicle-treated mice (Fig. 3). Taken together, these data suggest that similarly to intact PT, PT-B enhances disease expression by promoting cellular responses and increasing Th1 polarization.

PT-B induces IL-12 expression

IL-12 is a pivotal cytokine of the innate immune response that promotes cellular immunity (24). In the EAU model, it is required to generate uveitogenic effector cells and to maintain their function (25). It was therefore important to examine the effect of PT and PT-B on induction of innate IL-12. Induction of IL-12 was assessed in vitro and in vivo by expression of IL-12 p40 message, and by secretion of the IL-12 p70 heterodimer. LPS served as positive control.

Splenocytes of naive mice stimulated in culture with PT or with PT-B overnight expressed IL-12 p40 mRNA, and supernatants collected from these cells at 48 h contained IL-12 p70 protein detectable by ELISA (Fig. 4, A and B). More importantly, IL-12 was also induced in vivo. Splenocytes from mice 2 h after in vivo challenge with PT or PT-B expressed IL-12 p40 message (Fig. 4C). Splenocytes from parallel mice, placed in culture for 48 h without further stimulation, secreted IL-12 p70 protein into the supernatants (Fig. 4D). These results indicate that PT-B can closely mimic the effect of whole PT in triggering an innate IL-12 response, which could in part explain the observed skewing of the adaptive anti-IRBP response toward the Th1 phenotype and the enhancement of cellular responses and disease scores.

The biological effects of PT-B cannot be attributed to LPS contamination

LPS is a bacterial component with adjuvant effects, and is a common contaminant in biological preparations. To rigorously exclude the possibility that any of the results shown above might be caused, or contributed to, by LPS contamination of the PT-B preparation, a number of experiments were performed. First, we quantitated the amount of endotoxin in the PT-B preparation and compared it with the minimal amount of LPS needed to induce production of IL-12 from mouse splenocytes in culture. The amount of LPS in the stock solution (100 μg/ml) of PT-B preparation was less than 0.3 endotoxin units (less than 0.1 ng), as measured by the Chromogenic Limulus Amebocyte Lysate Test. More than 5 ng/ml of LPS was needed to trigger IL-12 message and IL-12 protein production.
splenocytes: splenocytes (5 × 10^6/ml) were cultured with PT (100 ng/ml), PT-B (100 ng/ml), or LPS (10 ng/ml) at 37°C overnight. RT-PCR for IL-12 the gel. Bands were scanned and quantitated using National Institutes of Health Image. The fold increase over stimulated control was calculated after normalization to β-actin. B. Protein expression by parallel cultures of splenocytes incubated for 48 h. IL-12 p70 in the supernatants was analyzed by ELISA. C. mRNA expression in spleens of mice treated in vivo with LPS (100 μg), PT (2 μg), or PT-B (2 μg). The spleens were removed after 2 h, and RT-PCR was performed as in A. Bands were scanned and quantitated using National Institutes of Health Image. The fold increase over untreated control was calculated after normalization to β-actin. D. IL-12 protein in 48-h supernatants of splenocytes from mice treated in vivo as in C and incubated without further stimulation. Shown is IL-12 p70 protein, as assayed by ELISA in 48-h supernatants. Detection limit in this assay was 7.8 pg/ml. The data are representative of three repeat experiments.

from splenocytes, which is 250-fold more than the amount of LPS present in the 2 μg/ml solution of PT-B used for the assay (Fig. 5, A and B). Second, induction of IL-12 by the PT-B preparation was abrogated completely by proteinase K, attesting to the protein nature of the IL-12-inducing factor, whereas LPS-induced IL-12 production was resistant to proteinase K treatment (Fig. 5C). Conversely, the LPS inhibitor polymyxin B did not lower IL-12 production induced by the PT-B preparation, whereas it completely blocked IL-12 induction by LPS (Fig. 5C). Third, PT-B triggered IL-12 production by splenocytes of C57/HeJ mice, which do not respond to LPS due to a Toll-like receptor 4 defect (Fig. 5C). Fourth, LPS was administered to mice concurrently with IRBP immunization in graded doses up to several orders of magnitude more than would be present as contaminant in PT-B. LPS was unable at any dose to reproduce the enhancing effect of PT-B on EAU and its associated immunological responses, including DTH, Ag-specific lymphocyte proliferation, and Ag-specific IFN-γ production (Fig. 5, D, E, F, and G). These experiments indicated that none of the observed effects of PT-B could be attributed to traces of LPS present in the preparation.

Discussion

PT has been used routinely in conjunction with immunization with tissue-specific Ags as an adjuvant to enhance induction of autoimmune diseases (6–11). However, it is not clear which subunit of PT elicits these disease-enhancing effects. The report of He et al. (14) showing enhancement of IL-12 production in mouse splenocytes secondary to blockade of G protein signaling would appear to implicate the enzymatic PT-A subunit in this effect. In contrast, we (15) previously demonstrated that PT-A is responsible for inhibitory effects of PT on EAU, secondary to blockade of G protein-coupled receptors and disruption of chemokine signaling, and a subsequent study by others pointed out similar inhibitory effects of PT also on a Th2-driven model of allergic lung inflammation (26). In the present study, we dissociate the inhibitory from the enhancing effect of PT, by demonstrating that purified PT-B, free of biologically significant contamination with whole PT or with LPS, is able to closely mimic the effects of whole PT on EAU induction, on enhancement of cellular responses, and on promotion of Th1 polarization as judged by autoantigen-induced production of IFN-γ. Furthermore, PT-B mimicked the effect of whole PT in being able to induce IL-12 message and IL-12 protein synthesis in splenocytes both in vitro and in vivo. This demonstrates that there is another route for inducing IL-12 by PT than through inhibition of G protein signaling.

Ours is not the first study that examines various activities of the PT-B subunit independent of the enzymatic A subunit. PT-B binds to cell surface glycoproteins, such as fetuin, haptoglobin, and the Lewis’ blood group determinant on the cell surface; triggers phospholipase C and tyrosine kinase-dependent signal transduction events; and inhibits HIV replication and virus expression in human macrophages (3–5, 27, 28). Tamura et al. (29) reported that the B subunit is directly mitogenic to lymphocytes in vitro. We too observed that PT as well as PT-B enhanced proliferation when added directly to lymphocyte cultures (data not shown). Notably, Ryan et al. (30) found that a mutated PT with an enzymatically inactive A subunit exhibited adjuvant activities, including Ag-specific lymphocyte proliferation, cytokine and Ab production in vivo, and enhanced expression of B7 molecules on APC in vitro. Our study confirms and extends these data to an autoimmune disease model in which tissue pathology is dependent on Th1 polarization (31). In contrast, our data are at variance with Ben-Nun et al. (32), who reported that pretreatment of animals with PT-B 2–3 wk before an encephalitogenic challenge protected them from experimental autoimmune encephalomyelitis. We believe that in this case protection may have been due to induction of Abs in response to the pretreatment, which neutralized or eliminated the PT that was administered 2–3 wk later as necessary component of the encephalitogenic immunization protocol.

To understand exactly how PT-B exerts its immunostimulatory effects, the specific receptor(s) for PT-B will need to be identified. Although PT-B delivers PT-A into the cell, enabling it to block ADP glycosylation, one cannot assume that PT-B interacts directly with G protein-coupled receptors. Additional experiments, which are beyond the scope of the present study, are needed to identify the cellular receptor(s) for PT-B involved in this process and the signaling pathway(s) they activate.

The dose response of the enhancing effect of PT-B demonstrated that on a molar basis it was less than that of whole PT: to obtain a maximal enhancing effect, 2 μg of PT-B was needed vs 0.5 μg of whole PT. Because contamination of the PT-B preparation was excluded as a possible reason for this, two nonmutually exclusive explanations could be invoked. If all the enhancing activity of PT resides in the B subunit, the weaker adjuvanticity of PT-B compared with PT might be caused by a subtle configurational change of the B subunit as a result of dissociation from the A subunit, conceivably resulting in reduction of biological activity. Our data do not negate the possibility that a part of the enhancing activity might reside in the A subunit, as suggested by the data of He et al. (14), showing that inhibition of G protein signaling up-regulates IL-12 and promotes the Th1 response. It should be kept in mind, however, that the same G protein inhibition by the A subunit also...
strongly suppresses disease expression due to inhibition of responses to chemokines and prevention of effector cell migration to the target organ (15, 16). All these considerations notwithstanding, an important conceptual advance that stems from the present study is that neither the ADP-ribosyltransferase activity of PT-A, nor the IL-12-enhancing effects of Gi protein blockade that it causes, are necessary for Th1 polarization of immunological responses and for disease induction in the EAU model.

In summary, the present data, in conjunction with our previous reports (15, 16), demonstrate that the enhancing and inhibitory effects of PT on experimental autoimmunity can be dissociated from each other. Although the A subunit is largely responsible for the inhibitory effects of PT on EAU secondary to inhibition of cell migration by Gi-coupled receptor inhibition, the enhancing effect can be duplicated by the B subunit that mimics the effects of whole...
bioactive PT on induction of innate IL-12, enhancement of adaptive IFN-γ, potentiation of cellular immunity, and facilitation of autoimmune tissue pathology.

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