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Pertussis Toxin Is Superior to TLR Ligands in Enhancing Pathogenic Autoimmunity, Targeted at a Neo-Self Antigen, by Triggering Robust Expansion of Th1 Cells and Their Cytokine Production

Chiaki Fujimoto,* Cheng-Rong Yu,2* Guangpu Shi,2* Barbara P. Vistica,* Eric F. Wawrousek, ‡ Dennis M. Klinman,‡ Chi-Chao Chan,* Charles E. Egwuagu,* and Igal Gery3*

Microbial products are assumed to play a major role in triggering pathogenic autoimmunity. Recently accumulated data have shown that these products stimulate the immune system by interacting with TLRs, expressed on APCs. To examine the capacity of various TLR ligands to trigger pathogenic autoimmunity, we used a system in which naive CD4 cells, specific against hen egg lysozyme (HEL), are injected into recipient mice expressing HEL in their eyes. Only when stimulated, the naive cells acquire pathogenic capacity and induce ocular inflammation. Seven TLR ligands were tested in this system: lipoteichoic acid/peptidoglycan, zymosan, poly(I:C), LPS, pertussis toxin (PTX), flagellin, and CpG oligodeoxynucleotide. Treatment of recipient mice with HEL alone stimulated proliferation of the transferred cells, but no disease, whereas ocular inflammation did develop in recipient mice coinjected with HEL and any one of the seven TLR ligands. Inflammation induced by PTX surpassed by its severity those induced by all other tested TLR ligands and was accompanied by a dramatic increase in number of the transferred cells that acquired features of effector Th1 lymphocytes. Ocular inflammation and number of transferred cells in recipients injected with PTX and HEL were substantially reduced by treatment with Abs against IFN-γ or IL-12, thus indicating the role of these cytokines in the PTX effect. Overall, our observations demonstrate that various TLR ligands are capable of triggering pathogenic autoimmunity and that PTX surpasses other microbial products in this activity, by stimulating excessive proliferation and polarization toward Th1 of naïve T cells. The Journal of Immunology, 2006, 177: 6896–6903.

The negative selection process in the thymus is incomplete, allowing T cells with specificity toward self Ags to escape deletion (1–4). Such cells are present in healthy individuals in a “resting” state, but when activated, these resting cells acquire effector features and the capacity to invade the target tissue and initiate pathogenic autoimmune processes (3, 5, 6). The mechanisms whereby self-specific T cells are activated are not completely clear, but there is ample indirect evidence to suggest that microbial infection plays a major role in this process (3, 7–9). This notion has been supported by the well-known requirement for microbial components in adjuvants used to promote pathogenic autoimmunity; PTX was found to enhance immune responses and have been used in various forms of experimental autoimmune uveitis (EAU, experimental autoimmune uveitis). This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Abbreviations used in this paper: PGN, peptidoglycan; LTA, lipoteichoic acid; ODN, oligodeoxynucleotide; PTX, pertussis toxin; poly(I:C), polyriboinosinic polyribo nic acid; Tg, transgenic; ALPC, allophycocyanin; HEL, hen egg lysozyme; EAU, experimental autoimmune uveitis.
with H&E. The severity of inflammation was scored as described elsewhere (28). Briefly, the level of inflammation was evaluated separately in the anterior segment, vitreous, and retina, on a scale of 0–3. The final score consisted of the sum of the three subscores on a final scale of 0–9.

**CFSE dilution assay**

Naïve CD4 cells (5 × 10⁶ cells/ml) were labeled with CFSE (Molecular Probes) as described by Bird et al. (32). CFSE-labeled cells were injected i.v. into HEL-Tg mice (5 × 10⁶ cells in 200 μl). Recipient mice were euthanized on the indicated days postadoptive transfer, and splenocytes were collected and stained with allophycocyanin (ALPC)-conjugated anti-CD4 mAb (BD Pharmingen). Cell suspensions were analyzed on a FACScan Cytometer (BD Biosciences) and were gated for CFSE° dye dilution peaks, with 10⁶ events being acquired in a live cell gate.

**Flow cytometry analysis**

mAbs against murine CD4-ALPC (L3T4), CD49d-PE (R1-2), CD2L-PE (ME-14), and isotype rat IgG controls-FITC, -PE, or -ALPC were purchased from BD Pharmingen. A clonotypic mAb specific for the Tg TCR of the 3A9 mice, designated 1G12, a gift from E. Unanue (Washington University, St. Louis, MO), was used conjugated with FITC. Anti-CD16/CD32 Abs (2.4G2; BD Pharmingen) was used to block FcRs in all stainings.

Spleen cells of recipient mice were collected at different time points following adoptive transfer of donor cells and single-cell suspensions were prepared by conventional methods. Collected cells were treated with ammonium chloride potassium (“ACK”) buffer (Cambrex) and washed in staining buffer. For cell surface staining, incubation steps were performed for 30 min at 4°C. Flow cytometry analysis was performed on a FACS-Calibur (BD Biosciences) using FlowJo (Tree Star).

**Measurement of cytokine production**

Spleen cells of recipient mice were cultured in 24-well plates at 5 × 10⁶ cells/well in 1 ml of RPMI 1640 medium, supplemented with HL-1 serum replacement (Cambrex), antibiotics, and 2-ME (50 μM), with or without stimulants, as indicated. Supernatants were collected after incubation for 48 h and their cytokine levels were determined by the Pierce, using Multiple SearchLight technology.

**Real-time RT-PCR analysis**

Total RNA was extracted with TRIzol from whole eyes of recipient mice on day 7 postadoptive transfer of CD4 cells. RNA (10 μg), SuperScript II Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dT)₁₂–₁₆ were used for first-strand cDNA synthesis as previously described (33). Real-time 5′-nuclease fluorogenic RT-PCR analysis was performed on an iCycler iQ Real-Time PCR Sequence Detection System (Bio-Rad). PCR was conducted with the following primers: IFN-γ, 5′-CAGCAACAGCAAGGGCAA-3′ and 5′-CTGAGACTTTGTTGGTTGTGAC-3′; IL-4, 5′-ACAGGAGGAAGGCAGCCAT-3′; 5′-CTTGGTGTCTTGTTGC-3′, β-actin, 5′-CGGTTCGAGATGGCTCC-3′, 5′-CACGAACAGCAAGGGCAA-3′. Fluorescence-labeled probes used are: IFN-γ, 5′-FAM-CCCCCAAGAGGAAGCTGG TGA-3′; 5′-FAM-AAGGATGCATTCAATGAGATTTGCCAAGTITGGA-AHQ-13′; β-actin, 5′-TET-ACGGGATGTAGTTGCAACGCTCTC-3′; 5′-TET-ACGGGATGTAGTTGCAACGCTCTC-3′. Triplicate samples of 10-fold serial dilutions of cDNA were assayed and used to construct standard curves. PCR parameters were as recommended for the TaqMan Universal PCR master mix kit (Applied Biosystems). β-actin was used as an external copy number standard to enable the measurement of relative amounts of IFN-γ and IL-4 mRNA. It should be emphasized that the standard curves generated from cDNA dilution series showed excellent linearity indicating precise quantitative relationship between cDNA copy number and fluorescence intensity within the dynamic range of the assay.

**Treatment with anti-IL-12 Ab and anti-IFN-γ Ab**

Rat mAbs against murine IL-12, clone C17.8, a gift from G. Trinchieri (Scherling-Plough Research Institute, Dardilly, France) and rat mAb against murine IFN-γ (clone R4–62A), purchased from American Type Culture Collection, were administered i.p., at 0.5 mg/mouse on days 1, 3, and 5 postadoptive transfer of naïve CD4 cells. Control mice were injected with normal rat IgG2a according to the same schedule. Recipient mice were euthanized on day 7 and pathological changes in their eyes, as well as numbers of donor cells in their spleens were determined as described above.
Statistical analysis

Data are shown as the mean values ± SEM. For histological scores, each mouse (average of both eyes) is shown and treated as one event for the purpose of statistical analysis, using the Mann-Whitney rank sum test. Statistical significance of differences for all other assays was analyzed by independent Student’s t test. Differences of p < 0.05 were considered significant.

Results

PTX is superior to TLR ligands in triggering pathogenicity by naive CD4 cells

To examine the capacity of PTX and other TLR ligands to trigger pathogenicity in naive T cells, we used an experimental system in which naive CD4 cells specific against HEL are transferred into Tg-recipient mice in which HEL is expressed in the lens. No inflammatory ocular changes are detected in recipients of naive CD4 cells, but severe inflammation develops in eyes of recipients injected with CD4 cells activated with Th1 cytokines in culture before transfer (6, 28). This system thus made it possible to assess the capacity of agents to trigger pathogenicity in CD4 cells in vivo, by treating recipients of naive CD4 cells with the tested agents and assessing the development of ocular inflammatory changes in these recipient mice. The tested agents included PTX and six TLR ligands, i.e., the combination of PGN and LTA, poly(I:C), LPS, flagellin, zymosan, and CpG ODN. Data accumulated in repeated experiments are summarized in Fig. 1A. All tested agents were injected with or without HEL.

No ocular changes were seen in recipients treated with any of the agents without HEL (data not shown). Marginal or no inflammatory changes were detected in recipients injected with HEL alone, whereas ocular inflammation of different severity levels was observed in recipient mice injected with the combination of HEL and any of the tested agents (Fig. 1A). Treatment with PTX was superior to all six TLR ligands, with the majority of PTX-treated recipient mice developing remarkably more severe inflammatory changes than recipients treated with any of the tested TLR ligands.

Typical changes in eyes of mice treated with LPS, CpG, or PTX are depicted in Fig. 1B. The moderate ocular changes in the eyes of recipient mice treated with LPS or CpG included mainly infiltration of inflammatory cells, mostly lymphocytes. The infiltration characterized in the entry sites of the cells, i.e., large vessels at the optic nerve head, limbus, and retinal blood vessels. Inflammatory cells were also seen in most cases in the vitreous as well. Dramatically more severe inflammation was seen, in contrast, in eyes of recipient mice treated with PTX. Typically, the major changes in these eyes included heavy cellular infiltration in most ocular tissues, severe retinal detachment, with serous and cellular exudates in the subretinal space, various degrees of destruction to the retina, edema of the cornea, and protein material in the vitreous.

PTX stimulates vigorous donor cell proliferation, with unique kinetics

The severity of ocular inflammation in recipient mice in the experimental system used here is determined to a large extent by the number of transferred effector cells (28). To examine the possibility that the superior activity of PTX in the transferred inflammation system is due to vigorous proliferation of donor cells in the recipient mouse, we monitored the division level of donor cells in recipient mice by the CFSE assay. Data collected in mice treated with CpG ODN were also included, for comparison. As seen in Fig. 2, essentially no division was observed on day 3 postcell transfer in donor cells in the spleen of mice treated with either PTX or CpG ODN alone, but active proliferation did develop in mice injected with these molecules in combination with HEL. Interestingly, the division rate in mice treated with PTX or CpG ODN and HEL did not differ much from that seen in mice injected with HEL alone (Fig. 2). The division rate in all mice injected with HEL was rapid and the CFSE was diluted beyond clear detection as soon as day 5 (data not shown). These results thus indicate that the division rate of donor cells at the early phase following cell transfer does not explain the unusual severity of ocular inflammation in mice treated with PTX.

Another approach we used to monitor the effect of PTX and other microbial products on the number of donor cells in the recipient mice, at different time points, was to actually count these
cells in the recipient spleen. This approach was made possible by the availability of a clonotypic Ab, designated ‘1G12,’ that is specific for the 3A9 mouse TCR (34, 35). Fig. 3A shows the flow cytometric data, on day 7 postcell injection, when the mouse eyes were collected for examination. The percent of 1G12+ cells in spleens of PTX-treated mice (6.4%) was ~13-fold higher in this experiment than that in CpG ODN-treated mice (0.5%) (Fig. 3A). The calculated actual numbers of 1G12+ cells in spleens of recipient mice treated with all tested agents, plus HEL, are shown in Fig. 3B. Treatment with all six TLR ligands initiated increases in donor cell numbers, as compared with PBS-injected controls, but the effect of treatment with PTX profoundly exceeded that of other ligands. Also of interest are the findings that 1) the effect of all stimuli was completely dependent on the accompanying injection with HEL (data not shown) and that 2) HEL alone induced only a marginal increase in donor cell number on day 7, despite its activating cell division immediately following injection, as shown in Fig. 2.

The uniqueness of PTX effect on the number of donor cells was further demonstrated when comparing its kinetics to that of the other stimuli. Fig. 4A records the number of donor cells in spleens of recipient mice at different time points following treatment with HEL alone, or in combination with PTX or CpG ODN. A marginal increase in donor cells was seen on days 3 and 5 in mice treated with HEL alone. A moderate increase in 1G12+ cells was recorded in mice treated with CpG ODN plus HEL, reaching a peak on day 5 and declining thereafter. In contrast, donor cell numbers in PTX-treated mice reached a high peak on day 7 and decreased only slightly on day 9. Examination for inflammation of eyes of the mice tested for donor cell number (Fig. 4B) revealed that the peak of severity was reached on day 7 in both groups, treated with PTX or CpG ODN, with a moderate decrease by day 9.

Donor cells in PTX-treated mice efficiently acquire adhesion molecule profile characteristic for tissue invasion

The inflammation-inducing capacity of T cells is determined to a large extent by the profile of molecules on their surface. Of particular importance are the adhesion molecules CD49d (“VLA4”) and CD62L (“L-selectin”); studies of our group and others have shown that tissue invading T lymphocytes are characterized by high expression of CD49d and low expression of CD62L (6, 36, 37). To examine the effect of treatment with PTX and the TLR ligands on the profile of surface molecules on donor cells, we determined the expression of CD49d and CD62L on 1G12+ cells in spleen of recipient mice. Fig. 5 summarizes the flow cytometric data collected in repeated experiments. Spleen cells of recipients treated with most ligands exhibited increased expression of CD49d and reduction in CD62L, but these changes in mice treated with PTX surpassed those induced by all other agents.

**PTX treatment promotes Th1 cytokine production by donor cells**

Naive CD4 cells undergo polarization toward Th1 or Th2 immune types following antigenic stimulation. To examine the type and level of polarization of donor cells in recipient mice of the different groups, we cultured spleen cells of these recipient mice and measured the level of type-specific cytokines following stimulation with HEL (Fig. 6). Levels of IL-12 and IFN-γ, Th1-specific cytokines, were dramatically higher in cultures from PTX-treated mice than in cultures of all tested TLR ligands. In contrast, production of IL-10 in cultures from the PTX-treated mice was one of
The PTX effect is neutralized by Abs against IFN-γ and IL-12. In view of the vigorous production of IL-12 and IFN-γ in mice treated with PTX, we examined the role of these Th1 cytokines in the pathogenic process by treating PTX-injected recipient mice with Abs against these cytokines. As shown in Fig. 8, treatment with either one of these Abs reduced both the level of ocular inflammation (Fig. 8A) and the number of donor cells in the recipients’ spleen (Fig. 8B and C). These observations thus suggest that both IL-12 and IFN-γ play active roles in the PTX-induced pathogenic process.

**Discussion**

The present study compared, for the first time, several major known TLR ligands for their capacity to trigger CD4-mediated pathogenic autoimmune processes. The ligands’ activities were assessed by an experimental system we developed, in which naive CD4 cells, specific against HEL, are adoptively transferred into recipient mice that express HEL in their eyes (28, 29). The transferred naive cells do not induce disease in untreated recipients, but do so in mice treated with agents capable of stimulating the naive CD4 cells to become effector cells (6, 28). Our system thus provides an experimental model that imitates the presumable process by which pathogenic autoimmunity is initiated by infection. Our system has several advantages over other systems that have been shown: 1) the population of adoptively transferred naive CD4 cells in our system is homogeneous, 2) the number of cells used can be modified at will, 3) the transferred cells can be identified by a clonotypic Ab and any changes in their number or surface markers can be readily monitored, and 4) the immune response of the transferred T cells can be differentiated from that of the host cells, since only the former cells recognize HEL.

All tested TLR ligands were found capable of triggering pathogenic autoimmune processes in our system, thus indicating that autoimmunity may be stimulated by a wide spectrum of microbial
products. Whereas moderate levels of ocular inflammation were found in mice treated with six of the seven tested TLR ligands, exceptionally severe changes were seen in eyes of recipients treated with PTX (Fig. 1A), reaching severity levels comparable only to those observed in mice injected with 10⁶ or more in vitro-stimulated Th1 cells (28). Data we collected in the present study suggest that the strikingly severe ocular changes in PTX-treated recipient mice could be attributed to the dramatic increase in number of transferred cells, which acquired features of "activated" Th1 cells and produced very high levels of Th1 type cytokines.

The observation concerning the increase in number of transferred cells in the PTX-treated mice sheds new light on the activity of this microbial product in triggering pathogenic autoimmunity. Our observation was made possible by the availability of the clostridial gene, rather than by the availability of the microbial product itself (28). The data we collected in the present study suggest that the strikingly severe ocular changes in PTX-treated recipient mice could be attributed to the dramatic increase in number of transferred cells, which acquired features of "activated" Th1 cells and produced very high levels of Th1 type cytokines.

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These two cytokines play major roles in shifting the immune response toward the Th1 pole, and their participation in the pathogenic process is indicated by the reduced ocular inflammation seen in recipient mice treated with Abs against IFN-γ or IL-12 (Fig. 8). Furthermore, the actual involvement of IFN-γ in the inflammatory response promoted by PTX was indicated by the finding of high expression of the IFN-γ transcript in eyes of PTX-treated recipient mice (Fig. 7). Our finding concerning the selective stimulation of Th1 cytokine production in PTX-treated mice is in line with data reported by Hou et al. (26). It should be mentioned, however, that IL-12 shares a major component, p40, with another cytokine, IL-23 (40) and therefore, treatment with the IL-12 Ab could neutralize IL-23 as well. Because IL-23 drives the subset of Th17 lymphocytes (40, 41), this Ab treatment could also have affected the immunopathogenic Th17 population. This possibility is currently under investigation.

Analysis of the Th1/Th2 profiles of the infiltrating cells in inflamed eyes of recipient mice treated with the different TLR ligands (Fig. 7) revealed remarkable selectivity in the effect of the different ligands. Whereas PTX and CpG ODN promoted “Th1 response” (high IFN-γ, low IL-4), treatment with PGN, poly(I:C), and LPS skewed the response toward the Th2 type (low IFN-γ, high IL-4). Our data are thus in line with recent observations by the groups of Pulendran (42, 43) and Raz (44), showing that different TLR ligands differentially regulate the Th1/Th2 balance.

Recent publications indicated that the enhancing effect of PTX on the immune response is mediated mainly via the activation of TLR4 (7, 27). Data collected in the present study suggest, however, that the effect of PTX in our system could not be entirely attributed to this mode of action. LPS, the “hallmark” ligand for TLR4, was included in our study and its stimulatory activity differed remarkably from that of PTX by all tested parameters, i.e., severity of ocular inflammation, increase in number of donor cells, and, in particular, the profile of cytokines produced by the donor cells in culture. It is conceivable, therefore, that the unique activity of PTX in the system we used in this study is mediated mainly via a mechanism other than the “conventional” activation of TLR4. It is also of note that TLR4 was found not to be essential for another immunopathogenic process in the eye in which PTX plays an essential role, i.e., induction of experimental autoimmune uveitis (EAU). Su et al. (45) reported that mice deficient in TLR4 developed EAU similarly to their wild-type control, whereas no disease could be induced in mice deficient in IL-1R.

It is noteworthy that the PTX effect in our experimental system depended on cotreatment of the recipient mice with HEL, because no inflammation was detected in recipients of naive CD4 cells treated with PTX alone (data not shown). This observation suggests that PTX does not directly affect the naive CD4 cells, but rather, this molecule possibly enhances the pathogenic process by promoting the antigenic stimulation of these cells by a unique mechanism. It is also of interest that marginal or no ocular inflammation was seen in recipient mice injected with HEL alone, with no additional stimulus, despite the proliferation of donor cells observed in these mice (Fig. 2). This finding indicates that the process of Ag-induced cell division by itself does not elicit pathogenic capacity in naive T cells specific against self Ags. It is also of note that Thompson et al. (46) have recently found in a similar cell transfer system that T cells stimulated by the Ag alone divided well, but exhibited poor survival, as compared with cells stimulated with the combination of the Ag and a TLR ligand.

Our finding that PTX triggers a powerful pathogenic autoimmune process by stimulating naive CD4 cells to vigorously proliferate and acquire effector Th1 cell features sheds new light on the mode of action of this microbial product as an adjuvant for induction of pathogenic autoimmunity. Treatment with PTX is essential for induction of experimental autoimmune diseases such as experimental autoimmune encephalomyelitis (13, 21) or EAU (22, 23) and its mode of action has been attributed to several mechanisms, in particular increasing vascular permeability (5, 14), activation of the innate immunity via TLR4 (7, 27), reduction in number of Treg cells (47, 48), and enhancement of Th1 responses by stimulation of dendritic cells (26). Our observations suggest that enhanced proliferation of Th1 cells and, perhaps, of Th17 cells, specific against the target self Ag combines with the other effects of PTX to produce the unique adjuvant activity of this molecule.

In summary, using an experimental system in vivo that detects the conversion of naive CD4 cells into pathogenic effector Th cells, we found that all tested TLR ligands exhibited this capacity, thus supporting the notion that infection plays a major role in triggering autoimmunity. PTX, also a TLR ligand, surpassed by far all other ligands in triggering severe inflammation, following vigorous cell proliferation and Th1 cytokine production. Data collected...
here thus extend our knowledge about the capacity of PTX to promote pathogenic autoimmunity.

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