The Journal of Immunology

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J Immunol 2002; 169:117-125; ;
doi: 10.4049/jimmunol.169.1.117
http://www.jimmunol.org/content/169/1/117

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Pertussis Toxin Modulates the Immune Response to Neuroantigens Injected in Incomplete Freund's Adjuvant: Induction of Th1 Cells and Experimental Autoimmune Encephalomyelitis in the Presence of High Frequencies of Th2 Cells[1]

Harald H. Hofstetter, Carey L. Shive, and Thomas G. Forsthuber[2]

Pertussis toxin (PT) has been widely used to facilitate the induction of experimental autoimmune encephalomyelitis (EAE) in rodents. It has been suggested that this microbial product promotes EAE by opening up the blood-brain barrier and thereby facilitates the migration of pathogenic T cells to the CNS. However, PT has other biological effects that could contribute to its activity in EAE, such as enhancing the cytokine production by T cells and induction of lymphocytosis. In this work, we investigated the effects of PT on the pathogenicity, cytokine differentiation, and clonal sizes of neuroantigen-reactive T cells in EAE in mice. Our results show that PT prevented the protection from EAE conferred by injection of PLPp139–151 in IFA and induced high frequencies of peptide-specific Th1 cells and disease. Interestingly, the mice developed EAE despite the simultaneous vigorous clonal expansion of PLPp139–151-specific Th2 cells. The data indicate that the Th2 cells in this model neither were protective against EAE nor promoted the disease. Furthermore, the results suggested that the effects of the toxin on neuroantigen-reactive T cells were promoted by the PT-induced activation of APCs in lymphoid tissues and the CNS. Together, the results suggest that microbial products, such as PT, could contribute to the initiation of autoimmune disease by modulating the interaction between the innate and adaptive immune system in the response to self Ags. ***The Journal of Immunology*****, 2002, 169: 117–125.**

Experimental autoimmune encephalomyelitis (EAE)[3] is an animal model recapitulating the human disease multiple sclerosis (1). It is characterized clinically by neurological deficits and histologically by inflammatory infiltrates in the CNS (2–4). In most animal models of EAE, the disease is induced actively through immunization with myelin proteins such as myelin basic protein (MBP) or proteolipid protein (PLP) in CFA, or passively by transfer of activated neuroantigen-specific T cells. In contrast, injection of the same myelin Ags in IFA is highly efficient in protecting from EAE (5, 6). Interestingly, the incidence and severity of the disease induced by immunization with neuroantigens in CFA is enhanced by the coinjection of pertussis toxin (PT) (7–9).

PT belongs to the A-B structure class of bacterial toxins (10). Its B-subunit binds to a receptor on the cell surface, and the enzymatically active A-subunit disrupts intracellular signaling by irreversible ADP ribosylation of the G_i-subclass of G proteins (10, 11). PT has pleiotropic effects on the immune system, such as T cell mitogenesis, augmentation of cytokine and Ab production, and the promotion of delayed type hypersensitivity responses (12–15).

The mechanism by which PT facilitates the induction of EAE has mostly been attributed to its breaking down the blood-brain barrier (BBB) (16, 17). This interpretation has recently come under scrutiny, and the promotion of autoimmune Th1 immunity has been alternatively postulated as the primary mode of its action (8, 9, 18, 19). While PT seemed to induce polarized Th1 immunity in the response to autoantigens, it has recently been reported to further clonal expansion of both Th1 and Th2 cells in the immune response to foreign Ags (20, 21).

To take a new look at this issue, we have taken advantage of the sensitivity of the cytokine ELISPOT assay and tested the effects of PT on clonal sizes and cytokine differentiation of neuroantigen-reactive T cells in EAE directly ex vivo at the single-cell level (22).

The data show that PT prevents the protection conferred by injection of neuroantigens in IFA and promotes the clonal expansion of both neuroantigen-specific Th1 and Th2 cells and EAE. Furthermore, the results suggested that this effect of the toxin was mediated via the activation of APCs in lymphoid tissues and the CNS.

Materials and Methods

Animals, Ags, and treatments

Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at Case Western Reserve University (Cleveland, OH) under special pathogen-free conditions. All animal procedures were conducted according to guidelines of the Institutional Care and Use Committee of Case Western Reserve University. Female SJL/J mice were injected at 6–10 wk of age with the Ag in IFA or CFA. PT (200 ng; List Biological

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Received for publication October 18, 2001. Accepted for publication April 30, 2002.

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[1] This work was supported by grants from the National Institutes of Health (NIH AI-41609) and the National Multiple Sclerosis Society (Harry Weaver Neuroscience Scholarship JF-2092-A-1), and a fellowship of the Studienstiftung des Deutschen Volkes (to H.H.H.).

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; PT, pertussis toxin; BBB, blood-brain barrier; MBP, myelin basic protein; PLP, proteolipid protein.

0022-1767/02/$02.00

Laboratories, Campbell, CA) was injected i.p. in 500 μl saline at 0 and 24 h, or at the time points indicated. PLP peptide AA139–151 (PLPp139–151, HSLGKWLGHPDKF) was synthesized by Princeton Biomolecules (Langhorne, PA). MBP was prepared as described (23). IFA was purchased from Life Technologies (Grand Island, NY), and CFA was made by mixing *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) at 5 mg/ml into IFA. Ags were mixed with the adjuvant to yield a 2 mg/ml emulsion, of which 50 μl was injected either i.p. or s.c. as specified.

Evaluation of clinical disease

Mice were monitored daily for 30+ days after injection of neuroantigen, and the severity of disease was recorded according to the following scale (24): grade 0, no abnormality; grade 1, limp tail; grade 2, moderate hind limb weakness; grade 3, complete hind limb paralysis; grade 4, quadriplegia or premoribund state; grade 5, death. If necessary, food was provided on the cage floor.

Cell preparations from the organs tested and T cell separation

Single-cell suspensions of cells from the spinal cord were prepared as previously described (25). Afterward, the cells were plated at various concentrations ranging from 2×10^4 to 3×10^5cells/well in HL-1 serum-free medium (BioWhittaker, Walkersville, MD) together with Ag and 5×10^5 irradiated naive spleen cells as APCs. Single-cell suspensions from the spleen were prepared as previously described (20). The cells were counted and plated with Ag at 1×10^6 cells/well. For the cell separations, $CD4^+$ T cells were obtained by passing the spleen cells through a murine $CD4^+$ or $CD8^+$ T cell enrichment column (R&D Systems, Minneapolis, MN) following the manufacturer's suggested protocol, or by FACS sorting. This protocol usually results in 90–99% enrichment for $CD4^+$ T cells. Afterward, the cells were plated at 5×10^5 cells/well in HL-1 serum-free medium together with Ag and 3×10^5 irradiated naive spleen cells as APCs.

Adoptive transfer of PLPp139–151-specific T cells

For adoptive transfer experiments, splenic mononuclear cells from mice immunized (as indicated in Fig. 4) were prepared as described above. These cells were subsequently preactivated by incubation with PLPp139–151 at the previously established optimal stimulatory concentration (30 μg/ml) in complete DMEM for 3 days before i.p. injection into the recipient animals. PT (200 ng) was injected (as detailed in Fig. 5) into the donor or recipient mice. The recipients were monitored daily after the injection, and clinical disease was assessed according to the standard scale outlined above. For the experiments shown in Fig. 4, measurements for all cytokines by ELISPOT as described below were conducted before the incubation to assess the number of cytokine-producing T cells at the single-cell level. Subsequently, the total number of T cells injected was adjusted according to the number of IFN-γ-producing cells calculated from the preincubation ELISPOT assays as a function of the total number of cells recovered.

Cytokine measurements by ELISPOT and computer-assisted ELISPOT image analysis

ELISPOT plates (ImmunoSpot; Cellular Technology, Cleveland, OH) were coated overnight with IFN-γ-specific (R46A2; 4 μg/ml) or IL-5-specific (TRFK5; 5 μg/ml) capture Ab, or with a combination of both for double-color ELISPOT assays as described (20). The plates were blocked with 1% BSA in PBS for 1 h at room temperature, then washed four times with PBS. Spleen cells were plated at 10^6 cells/well alone or with Ag (7 μM) in HL-1 medium and cultured for 24 h for IFN-γ, 48 h for IL-5, or 36 h for double-color assays. Subsequently, the cells were removed by washing and the biotinylated detection Ab XMG1.2-biotin (2 μg/ml) for IFN-γ and TRFK4-biotin (2 μg/ml) for IL-5 were added and incubated overnight. The plate-bound second Ab was then visualized by adding streptavidin-alkaline phosphatase (DAKO, Carpinteria, CA) and nitroblue tetrazolium (Bio-Rad, Hercules, CA)/5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich, St. Louis, MO). Image analysis of ELISPOT assays was performed on a Series 1 ImmunoSpot Image Analyzer (Cellular Technology). In brief, digitized images of individual wells of the ELISPOT plates were analyzed for cytokine spots, based on the comparison of experimental wells (containing T cells and APCs with Ag) and control wells (T cells and APCs but no Ag; usually fewer than five spots per well). After separation of spots that touched or partially overlapped, nonspecific noise was gated out by applying spot size and circularity analysis as additional criteria. Spots that fell within the accepted criteria were highlighted and counted. Additionally, spot-size histograms were generated, reflecting the distribution of spots according to the cytokine output per cell. The stimulation index was calculated by dividing the number of cytokine spots detected in experimental wells (T cells, APCs, and relevant Ag) by the number of cytokine spots in control wells (T cells, APCs, no Ag). The spot number in unimmunized or control mice (immunized with irrelevant Ag) was in the same range as in the medium controls (fewer than five spots). Where indicated, statistical analysis was performed with the paired *t* test or the Mann-Whitney *U* rank sum test using SigmaStat software (SPSS, Chicago, IL).

Flow cytometry analysis

Single-cell suspensions were incubated at 1×10^6 cells per sample with 0.5–1 μg of anti-I-A^s, anti-B7-1, anti-B7-2, CD40, or MAC-1 Abs, (BD PharMingen, San Diego, CA) for 1 h on ice. Cells were washed with PBS. Erythrocytes were lysed with Immuno-lyse (Coulter, Miami, FL) and the cells were fixed in Coulter Clone fixative reagent and analyzed on a FACSScan (BD Biosciences, Mountain View, CA).

Histopathology

At the time of the experiment the brain and spinal cord of the mice were removed and either preserved in Z-Fix or snap-frozen in 2-methyl-butane. Thin slices of the CNS tissue were prepared and stained with H&E. The tissue was then examined by light microscopy in a blinded fashion by a neuropathologist and evaluated for the extent of inflammation and graded as follows: 0, no inflammation; 1, a few mononuclear cells; 2, organization of inflammatory infiltrates around positive vessels; 3, extensive perivascular cuffing with extension into the subarachnoid space; 4, extensive perivascular cuffing with increasing subarachnoid inflammation (24).

Results

Coinjection of PT prevents the protection from EAE conferred by injection with neuroantigens in IFA

Injection of neuroantigens in IFA is an efficacious way to induce tolerance and to protect rodents from EAE (5, 6). Although the mechanisms underlying this IFA-mediated protection have remained unresolved, immunoregulation, induction of immune deviation (neuroantigen-specific Th2 immunity), and a decrease in the clonal sizes of encephalitogenic neuroantigen-specific Th1 cells have been implicated (5, 6). Thus, we used the injection of neuroantigens in IFA as an experimental system to investigate the mechanism by which PT contributes to the induction of EAE.

Shown in Fig. 1*A* are groups of SJL mice injected with the immunodominant H-2^s-restricted PLPp139–151 peptide in IFA, with or without PT, and observed for up to 6 mo for clinical symptoms of EAE. SJL mice injected with PLPp139–151:CFA and PT (a standard protocol for EAE induction in this mouse strain) served as the positive control for disease in these experiments (Fig. 1*A*, ●).

The results show that PLPp139–151:IFA:PT-injected mice (Fig. 1*A*, ○) developed relapsing remitting EAE, with similar onset and severity to the disease observed in PLPp139–151:CFA:PT-injected mice (Fig. 1*A*, ●; $p > 0.05$). Confirming previous reports, injection of SJL mice with PLPp139–151:IFA without PT did not induce EAE (Fig. 1*A*, ▼) (5, 6). Histologic examination of brain sections revealed extensive perivascular and periventricular mononuclear inflammatory infiltrates in both the PLPp139–151:IFA:PT- and the PLPp139–151:CFA:PT-injected mice, but not in mice that received PLPp139–151:IFA in the absence of PT (data not shown).

Previously it was reported that SJL mice possess high frequencies of PLPp139–151-specific precursor T cells (26), which could have contributed to the effect of PT on EAE induction in this model. Therefore, we tested SJL mice for the induction of EAE with MBP, for which an increase in the frequencies of Ag-specific precursor T cells was not reported. As shown in Fig. 1*B*, EAE could also be induced in SJL mice immunized with MBP:IFA:PT (Fig. 1*B*, ○). However, the onset of disease was delayed in MBP:IFA:PT-injected mice as compared with MBP:CFA:PT-immunized animals ($p < 0.005$), whereas EAE severity was similar in both groups. In contrast, mice injected with MBP:IFA without PT never developed any clinical signs of EAE (data not shown).

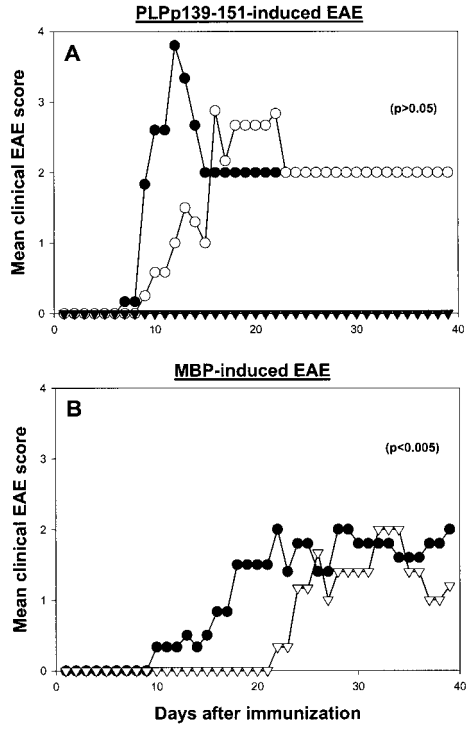

FIGURE 1. Coinjection of PT with neuroantigens in IFA prevents the protection conferred by IFA and induces EAE. *A*, Six- to 8-wk-old SJL/J mice were immunized with PLPp139–151 in IFA (▼), CFA and PT (●), or IFA and PT (○) and observed for clinical signs of EAE. Experiments were performed as outlined in *Materials and Methods*. Shown are the mean EAE scores for one representative experiment ($n = 10$). Similar results were obtained in four independent experiments. *B*, Experiments were performed as in *A*, but mice were immunized with MBP in IFA:PT (▽) or CFA:PT (●) ($n = 6$).

Taken together, the results showed for the first time that PT prevented IFA-mediated protection from EAE in mice, expanding observations in experimental autoimmune uveitis and EAE in rats (9, 19, 27).

PT induces clonal expansion of PLPp139–151-specific Th1 and Th2 cells

The injection of prototypic foreign and self Ags such as HEL, MBP, or PLP in IFA elicited highly polarized Th2 responses (IL-4 and IL-5, but not IFN-γ production; IgG1 and IgE, but not IgG2a Abs) (6, 28). However, when PT was coinjected, Ag-specific Th1 and Th2 immune responses to foreign Ags were induced (20, 21). In contrast, coinjection of PT with self Ags has been reported to sway the immune response toward a Th1 pattern of cytokine production (8, 9, 19). Thus, we tested whether the induction of EAE in our model was associated with a Th1 polarization of the autoimmune T cell response.

Shown in Fig. 2 are the results of SJL mice injected with PLPp139–151 in CFA or IFA, with or without coinjection of PT. The mice were tested 2–7 wk later for PLPp139–151-induced cytokine production. As shown in Fig. 2*A*, mice injected with PLPp139–151 in IFA mounted a strong Th2 recall response (Fig. 2*A*, *left panel*, IL-5$^+$IFN-γ$^-$), whereas mice immunized with the peptide in CFA (prototypic Th1 adjuvant) mounted a Th1 response (Fig. 2*A*, *middle panel*, IFN-γ$^+$IL-5$^-$). Interestingly, the mice immunized with PLPp139–151:IFA and coinjected with PT demonstrated a vigorous concurrent peptide-specific Th1 and Th2 response (Fig. 2*A*, *right panel*, both IFN-γ and IL-5). As shown in Fig. 2*B*, cell separation in conjunction with serial dilution experiments demonstrated that the cytokine response was mediated by individual CD4$^+$ T cells, consistent with previous reports (29–31). Similar results were obtained when the mice were tested at various time points after immunization, or when different mouse strain and Ag combinations were tested (e.g., B10.PL or C57.BL/6 mice with MBP or myelin oligodendrocyte glycoprotein, respectively; data not shown), indicating that the results were not particular for the SJL/PLP model.

Thus, in contrast to the reports mentioned (8, 19), our results demonstrated a vigorous concurrent clonal expansion of neuroantigen-specific CD4$^+$ Th1 and Th2 cells after PT injection.

PT-induced Th2 cells migrate to the CNS but fail to prevent EAE

In the presented experiments, EAE was induced in PLPp139–151:IFA:PT-injected mice in the face of high frequencies of neuroantigen-specific Th2 cells (Fig. 2). Therefore, the PLPp139–151-reactive Th2 cells failed to provide protection in this model, which could have been the result of PT-induced changes, such as impaired production of regulatory cytokines or a failure to migrate to the brain. To test whether the PLPp139–151-specific Th2 cells actually migrated to the CNS and exerted effector functions, we isolated spinal cord cells from these mice and performed ELISPOT assays for Th1 and Th2 cytokines as described previously (25). As shown in Fig. 3, both PLPp139–151-specific Th1 and Th2 cells were detected in the spinal cord isolates of PLPp139–151:IFA:PT-primed mice, as indicated by vigorous IFN-γ and IL-5 production (Fig. 3, *right bars*; also IL-2 and IL-4, not shown), but not in PLPp139–151:IFA- or PLPp139–151:CFA:PT-injected animals. However, the ratio of Th1:Th2 cells in the CNS was increased as compared with the spleen (IFN-γ:IL-5 ratio of 0.6 in the spleen and 3.4 in the CNS). To rule out that IFN-γ and IL-5 were actually produced by the same T cells (Th0 cells), we performed two-color cytokine ELISPOT assays on the spinal cord isolates (data not shown). The results demonstrated that IFN-γ and IL-5 were produced by individual Th1 or Th2 cells, but not by Th0 cells, producing both cytokines simultaneously (data not shown).

Several lines of evidence supported the conclusion that these cytokines were produced by tissue-resident, CNS-infiltrating T cells, and not by contaminating cells from blood contained within the vasculature of the CNS. First, no T cell responses were detected in the CNS of PLPp139–151:IFA-immunized mice (Fig. 3, *left bars*) or in the CNS of PLPp:IFA:PT- or PLPp:CFA:PT-injected mice before the onset of EAE (data not shown). Second, the frequencies of PLPp139–151-reactive cytokine-producing cells in the blood were similar to the frequencies noted in the spleen (data not shown). However, the clonal sizes detected in the spinal cord isolates were 5- to 15-fold higher than in the blood or the spleen (Fig. 2*A* vs Fig. 3). Finally, the ratio of CD4$^+$ T cells to erythrocytes in the spinal cord isolates was 1:2, whereas in the peripheral blood it was usually $<$1:3500, indicating that only approximately one of 1000 cytokine-producing cells in the CNS was derived from the peripheral blood (25).

Hence, the Th2 cells migrated to the CNS, and they were not impaired in their ability to release Th2 cytokines. The data suggest that an increased ratio of neuroantigen-specific Th1:Th2 cells may have accounted for the inability of the Th2 cells to prevent disease in this model. Alternatively, PT could have contributed to the induction of EAE via a novel, hitherto unidentified mechanism that allowed pathogenic Th1 cells to evade the regulatory effects of Th2 cells.

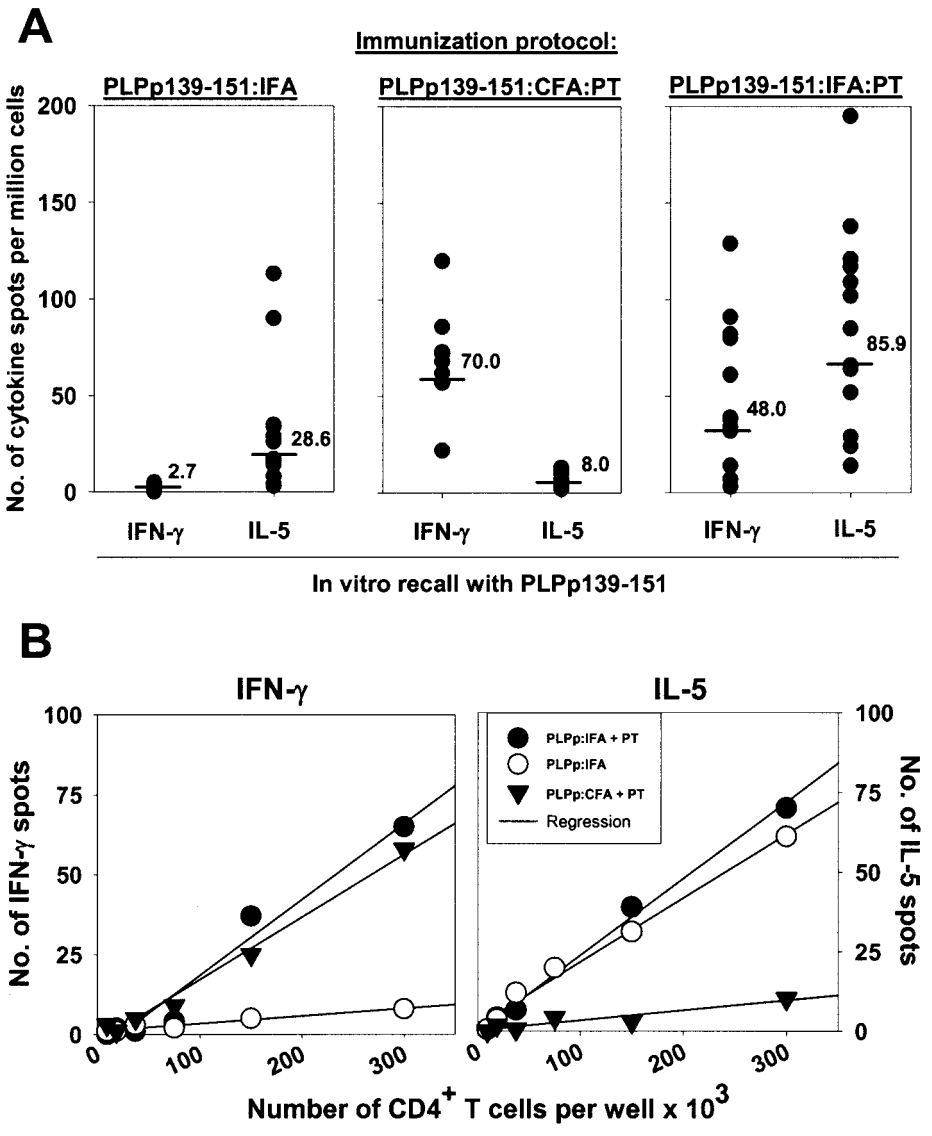

FIGURE 2. PT induces clonal expansion of PLPp139–151-specific Th1 and Th2 cells. Six- to 8-wk-old SJL/J mice were immunized with PLPp139–151 in IFA (*left panel*), CFA and PT (*middle panel*), or IFA and PT (*right panel*). Two to 7 wk later, frequencies of PLPp139–151-specific IFN-γ- and IL-5-producing T cells were measured by cytokine ELISPOT assay in single-cell suspensions of spleen cells. *A*, Cytokine-producing cells per million spleen cells. Horizontal bars indicate means of groups of 7–13 mice tested in three independent experiments. The values shown are the means of triplicate wells, with the background subtracted (usually fewer than five spots). Stimulation index (SI) of >3 is considered positive (see *Materials and Methods*). *B*, PLPp139–151-specific IFN-γ (*left panel*) or IL-5 (*right panel*) production by $CD4^+$ T cells. T cells were separated as outlined in *Materials and Methods*. No Ag-specific cytokine production was seen in unimmunized mice or mice injected with PT alone (data not shown).

The encephalitogenic potential of PLPp139–151-reactive Th1 cells induced by injection with neuroantigen in IFA and PT is similar to that of T cells induced by CFA

To test whether PT had altered the effector function of neuroantigen-specific Th1 cells, we primed SJL mice with PLPp139–151:IFA:PT, or with PLPp139–151:CFA:PT, and tested for EAE induction by adoptive transfer of spleen cells to naive SJL recipients. To directly compare the encephalitogenic potential of the T cells generated by either immunization protocol, we measured the number of PLPp139–151-specific cytokine-producing T cells recovered from the donor mice. For this, the recovered cells were tested in cytokine ELISPOT assays for PLPp139–151-specific T cell responses in parallel to the in vitro restimulation culture of the cells with the PLP peptide. Subsequently, we calculated the total number of activated PLPp139–151-specific T cells in culture, as outlined in *Materials and Methods*, and transferred identical numbers of PLPp139–151-reactive IFN-γ-producing T cells from either group to the respective naive SJL recipients. Shown in Fig. 4 are the results for SJL mice that received increasing numbers of cells from either PLPp139–151:IFA:PT- or PLPp139–151:CFA:PT-primed donors. Interestingly, transfer of as few as 500 activated PLPp139–151-specific IFN-γ-secreting T cells from either group was sufficient to induce EAE (Fig. 4, ◇). Furthermore, disease severity increased similarly as a function of the number of transferred cells, and disease onset was identical. In contrast, adoptive transfer of PLPp139–151:IFA-primed T cells (no PT coinjection of the donor mice at the time of primary immunization) did not induce EAE (data not shown).

Taken together, the results demonstrate that T cells from PLPp139–151:IFA:PT-primed mice were as efficient in inducing EAE as were T cells from PLPp139–151:CFA:PT-primed animals. Furthermore, the data show that once PLPp139–151-specific Th1 cells were generated, PT was no longer required for the induction of EAE in the adoptive hosts. Thus, the results suggest that PT acted in the induction of EAE in this model independently of the BBB, and that the effector function of the PT-induced pathogenic Th1 cells was not changed.

IFA-induced PLPp139–151-specific Th2 cells are not rendered pathogenic by PT

Autoantigen-reactive Th2 cells have mostly been associated with protection from autoimmune disease (32–34). However, it has recently been shown that Th2 cells can mediate autoimmune pathology in some models (35, 36). Because we failed to demonstrate protection by the PLPp139–151-specific Th2 cells in our system, we asked whether or not they contributed to the disease. Because

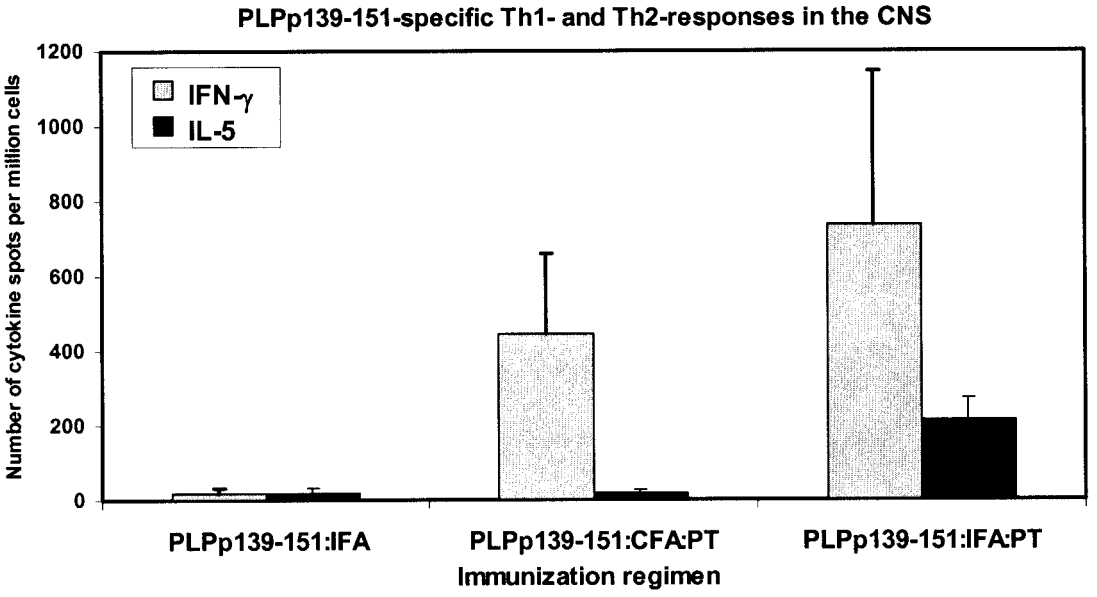

FIGURE 3. Concurrent presence of Ag-specific Th1 and Th2 cells in the CNS of PLPp139–151:IFA:PT-injected SJL mice. Shown are the mean cytokine spot numbers of spinal cord cells isolated from groups of SJL mice immunized with PLPp139–151 in IFA, CFA and PT, or IFA and PT tested in IFN-γ or IL-5 ELISPOT assays. Mice were tested 2–7 wk after immunization, and experiments were performed as outlined in *Materials and Methods*. Results shown represent groups of three to seven mice tested in three independent experiments and are expressed as the number of spots per million.

the separation of PT-induced Th2 cells from the concurrently induced Th1 cells was not feasible, we alternatively generated PLPp139–151-specific Th2 cells by priming with the peptide in IFA and then tested the effects of PT on these cells in vivo or in vitro. To test PT effects on Th2 cells in vivo, we injected SJL mice with the PLP peptide in IFA or CFA and waited until PLPp139–151-specific Th2 or Th1 immunity was firmly established (after 2 wk). On days 18 and 19 after immunization, the mice were injected with PT. As shown in Fig. 5*A*, injection of PT into PLPp139–151:IFA-primed mice at this late time point did not induce EAE (Fig. 5*A*, ○) or Th1 immune responses (data not shown). In contrast, mice injected with PLPp139–151 in CFA developed EAE, which was not significantly altered by the coinjection of PT (Fig. 5*A*, △ vs ●). Similar results were obtained when PT was injected into the recipients at the time of adoptive transfer of PLPp139–151:IFA-primed Th2 cells into naive SJL mice (Fig. 5*B*, ○). Similarly, in

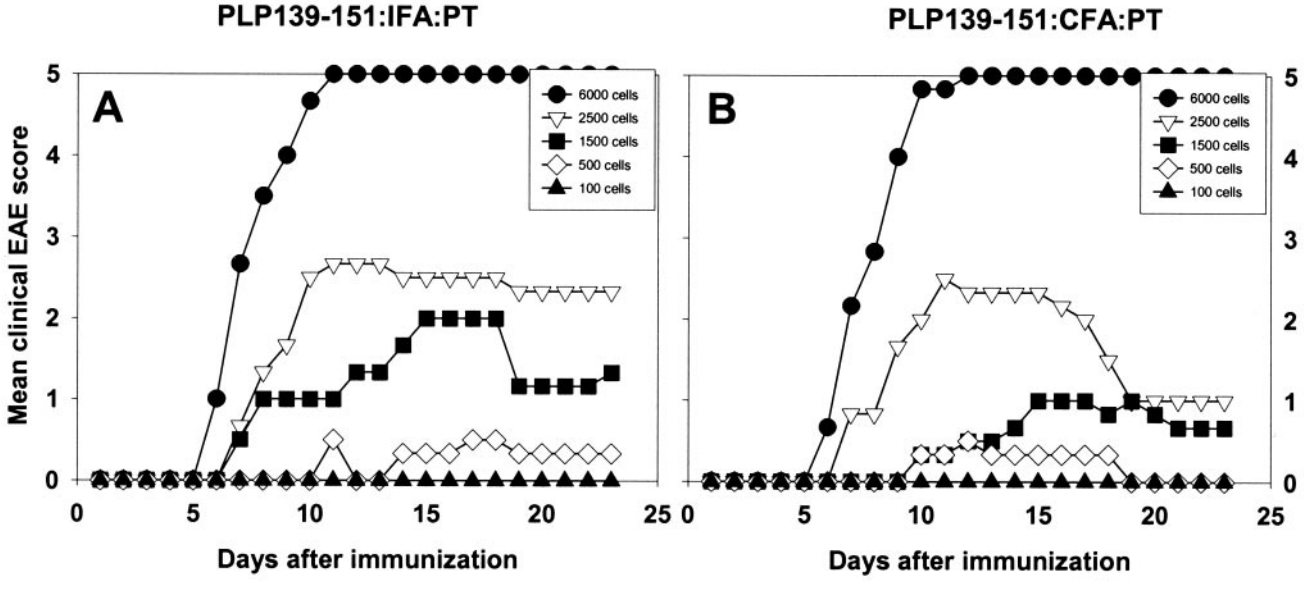

FIGURE 4. Similar encephalitogenicity of IFA:PT or CFA:PT-induced PLPp139–151-specific T cells. Spleen cells from PLPp139–151-immunized SJL mice were restimulated in vitro and adoptively transferred to naive SJL recipients as outlined in *Materials and Methods*. In parallel to the preactivation culture, the number of activated PLPp139–151-specific T cells in culture was measured by cytokine ELISPOT assays as outlined in *Materials and Methods*. The total number of activated PLP peptide-specific T cells was calculated (number of spots times total number of cells recovered), and the transferred cells were adjusted accordingly, starting at 6000 IFN-γ-producing cells injected per recipient mouse (●). *A*, Mean EAE scores of recipients of cells from PLPp139–151:IFA:PT-injected donors. *B*, Mean EAE scores of recipients receiving cells from PLPp139–151:CFA:PT-injected donors. Mann-Whitney rank sum test results comparing adoptive transfer of PLPp139–151:CFA:PT- with PLPp139–151:IFA:PT-induced T cells were as follows: $p = 0.878$ (6000 cells); $p = 0.047$ (2500 cells); $p = 0.003$ (1500 cells); $p = 0.52$ (500 cells); $p = 0.991$ (100 cells).

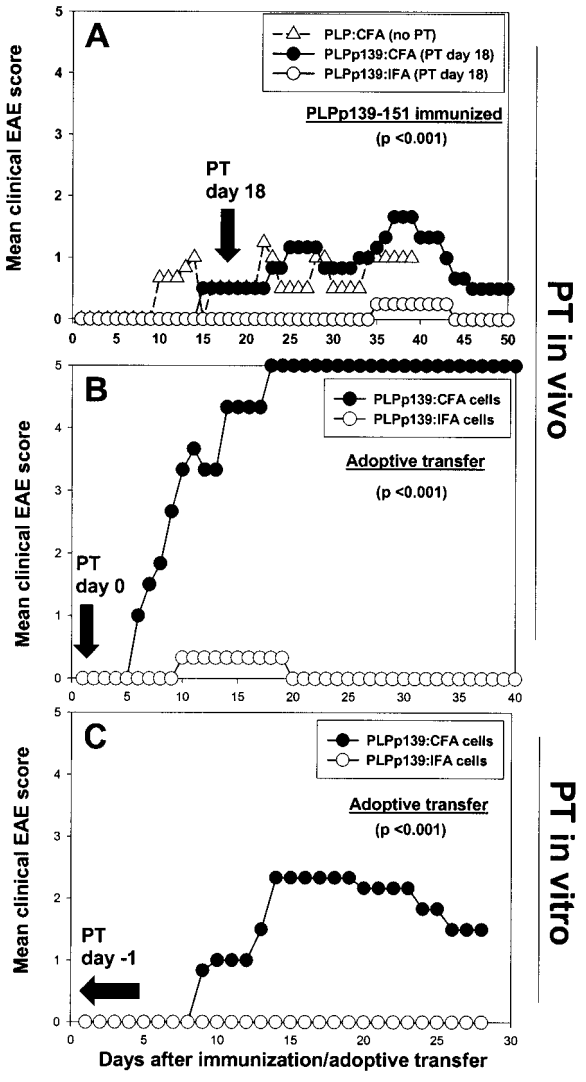

FIGURE 5. PLPp139–151-specific Th2 cells are not rendered encephalitogenic by PT. Experiments were performed as outlined in *Materials and Methods*. Shown are the mean EAE scores for groups of three to six mice, repeated once. *A*, Six- to 8-wk-old SJL mice were immunized with PLPp139–151 in IFA (○) or CFA (●). Both groups of mice were injected with PT at days 18 and 19 thereafter, and the animals were observed for EAE. *B*, PT was injected in the recipients at the time of adoptive transfer of spleen cells from PLPp139–151:IFA-primed (○) or CFA-primed (●) SJL mice, and 24 h thereafter. *C*, Adoptive transfer of spleen cells from PLPp139–151:IFA-primed (○) or CFA-primed (●) SJL mice. PT was added to the culture medium during the last 24 h of the in vitro restimulation period. Cells were extensively washed and transferred to the recipient SJL mice.

vitro incubation of PLPp139–151:IFA-induced Th2 cells with PT and adoptive transfer to naive recipients did not induce EAE (Fig. 5*C*), even at concentrations of the toxin that were mitogenic in vitro (data not shown) (12).

Taken together, the data show that IFA-induced PLPp139–151-specific Th2 cells were not encephalitogenic in this model, and exposure of the Th2 cells to PT in vivo and in vitro did not render them pathogenic. Further supporting this conclusion was the fact that the severity of EAE in the presented experiments was absolutely dependent on the frequency of transferred PLPp139–151-specific Th1 cells (Fig. 4), irrespective of the presence of Th2 cells in PLPp139–151:IFA:PT-primed donors.

PT activates APCs in the spleen and CNS

The presented experiments showed that PT induced Th1 differentiation and clonal expansion of both self (neuroantigen)-specific Th1 and Th2 cells, similar to its effects in the T cell response to prototypic foreign Ags (20, 21). It was suggested that the underlying mechanism involved G protein-mediated activation of APCs (20). Hence, we tested whether PT acted similarly on APCs in the autoimmune response to neuroantigens, which could be a mechanism contributing to its efficacy in the induction of EAE.

Shown in Fig. 6 is the expression of surface molecules on spleen and spinal cord cells of SJL mice after a 24-h incubation with medium (Fig. 6, *upper rows*) or PT (Fig. 6, *lower rows*, 400 ng/ml). The results show that PT enhanced the expression of MHC class II, B7-1, and B7-2 molecules on spleen cells 4- to 6-fold over medium control (Fig. 6*A*). Moreover, it enhanced the expression of MHC class II, CD40, and MAC-1 molecules on spinal cord cells (Fig. 6*B*). Similar results were obtained with microglia and astrocyte cell lines (data not shown), and we have also noted the enhanced production of IL-12 and IL-6 by these cells.

Taken together, the data suggest that PT activates APCs in lymphoid tissues and the CNS and facilitates the priming of autoreactive T cells and their reactivation in the target organ. Thus, the results suggest that this could be a mechanism by which the toxin facilitates the induction of EAE.

Discussion

In this study we have shown that PT prevented the protection of mice from EAE conferred by injection of neuroantigens in IFA. Moreover, it induced vigorous clonal expansion of neuroantigen-specific Th1 and Th2 cells and EAE. The mechanism by which PT acted on T cell differentiation and clonal expansion in this model

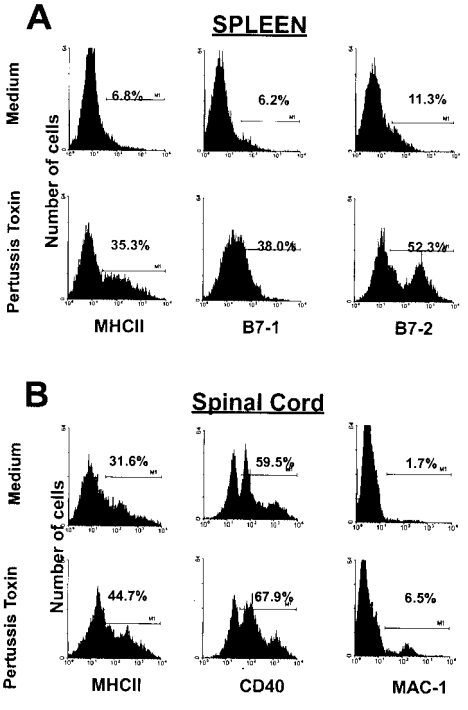

FIGURE 6. PT enhances the expression of MHC class II and costimulatory molecules on APCs. Single-cell suspensions of SJL/J spleen or spinal cord cells were cultured for 24 h with either medium (*upper rows*) or 400 ng PT (*lower rows*). Surface molecule expression was analyzed by flow cytometry as outlined in *Materials and Methods*. Shown are representative animals from groups of three mice, repeated with similar results in three experiments.

appeared to be via the activation of APCs in lymphoid tissues and the CNS, probably by providing stronger costimulatory signals and/or growth factors for the autoreactive T cells.

To induce Th1 immunity and EAE, the delivery of PT was required early after injection of the myelin Ags in IFA; administration of the toxin several weeks after immunization had no effect. Furthermore, PT was injected i.p., and thus was remote from the s.c. site of Ag deposition. Hence, this microbial product acted systemically in the induction of EAE. This effect of the toxin has been interpreted in the past as a result of its action on the BBB. The model held that PT opened up the BBB by histamine-induced vascular leakage (17) and thereby facilitated the transmigration of T cells and the induction of disease (7, 37). However, this interpretation has been challenged (18, 38), and our data support an additional mechanism of its action as well. The data show that the induction of EAE was critically associated with the Th1 differentiation and clonal expansion of neuroantigen-specific T cells. Once a neuroantigen-specific Th1 response was established, PT was no longer required for the propagation of disease, as demonstrated by the adoptive transfer of EAE with spleen cells from PLPp139–151:IFA:PT-injected mice. These results are consistent with studies in the rat, where PT induced Th1 immunity and EAE after immunization with MBP in IFA, and with studies in experimental allergic orchitis and experimental autoimmune uveitis that demonstrated an enhancing effect of PT on autoimmune disease and Th1 responses (8, 19, 27, 39). Furthermore, mapping of EAE-modifying loci in mice has identified *eae9* as a PT-controlled locus that overrides genetic checkpoints in the pathogenesis of EAE and is independent of the proposed PT-mediated changes in vascular permeability (40). Interestingly, *eae9* is located in a region encoding CXCR5 and IL-18 (IFN-γ-inducing factor).

Our data show that as few as 6000 IFN-γ-producing PLPp139–151-specific effector T cells induce lethal EAE, and many fewer activated cells were sufficient to induce clinically significant disease. Thus, the number of autoreactive T cells required to induce autoimmune disease may be much lower than generally assumed.

However, in contrast to the above-mentioned studies, we have additionally observed a vigorous clonal expansion of myelin-specific Th2 cells, which led us to investigate the role of these cells.

Th2 cells have been associated with protection from autoimmune disease (32, 33, 41, 42). However, this notion has come under scrutiny. In particular, marmoset monkeys immunized with myelin oligodendrocyte glycoprotein developed EAE that appeared to be Th2 mediated (35, 43). Furthermore, unopposed Th2 immunity, such as adoptive transfer of Th2 cells into immunocompromised hosts (36) or induction of Th2 responses in IFN-γ knockout mice (44), furthered autoimmune pathology. Therefore, the PT-induced Th2 cells in our experiments could have ameliorated the disease, could have had no effect, or could even have contributed to the autoimmune pathology.

Several lines of evidence argue against a protective effect of the PT-induced Th2 cells. First, disease severity and kinetics were similar in Th1 polarized PLPp139–151:CFA:PT-injected and PLPp139–151:IFA:PT-primed mice. The latter showed concomitant high frequencies of Th1 and Th2 cells. Interestingly, both neuroantigen-specific Th1 and Th2 cells were detected in the CNS of PLPp139–151:IFA:PT-injected mice (Fig. 3), indicating that Th2 cells indeed migrated to the CNS and released Th2 cytokines but did not prevent or ameliorate the disease. Adoptive transfer of spleen cells from both groups induced disease with similar severity and kinetics in the recipients, despite the apparent cotransfer of Th2 cells with the cells from PLPp139–151:IFA:PT-injected mice. These latter observations are consistent with earlier reports that have failed to detect a protective effect of Th2 cells when cotransferred with Th1 cells, such as in insulin dependent diabetes mellitus models and EAE (45, 46).

Along with a lack of protection by the PT-induced Th2 cells, we found no evidence for their contribution to the CNS pathology. Directly testing PT-induced Th2 cells was not feasible; however, using IFA-induced Th2 cells as a model, we noticed no effect of the toxin on the promotion of EAE by Th2 cells (Fig. 5).

Hence, how could the lack of protection by Th2 cells in this model be explained? First, PT could have prevented the migration of Th2 cells to the CNS, e.g., by blocking chemokine receptors (47). However, the presence of high frequencies of neuroantigen-reactive Th2 cells in the CNS in PLPp139–151:IFA:PT-primed mice argued against this hypothesis, and MBP-reactive Th2 cells can migrate to the CNS (48). Second, Th2 cells migrated to the CNS but arrived too late to prevent Th1-mediated immunopathology via negative feedback regulation of APCs by secretion of IL-4 and IL-10. Finally, the induced Th2 cells may not be efficiently activated under the Ag-presenting conditions in the CNS, and/or these cells could have released smaller amounts of regulatory cytokines. Although we did not detect differences in IL-5 or IL-4 production in IFA:PT- or IFA-induced T cells, the data may not fully reflect the conditions in situ in the CNS. Furthermore, there could be differences in the release of the regulatory cytokines IL-10 and TGF-β, which we have not tested (49–51).

Alternatively, PT could have rendered the neuroantigen-specific Th1 cells resistant to regulatory mechanisms, such as the action of Th2 cytokines or Fas-Fas ligand-mediated apoptosis in the CNS. Consistent with this hypothesis, PT has been reported to interfere with induction of apoptosis in T cells (52, 53).

Thus, the effects of PT on the induction of EAE could be the result of direct action of the toxin on T cells or, indirectly, its effects on the CNS, or both.

PT-sensitive G proteins are expressed by a variety of cells in the CNS, including macrophages, microglia, astrocytes, and oligodendrocytes. Furthermore, previous reports indicated that the toxin exerted some of its immunological effects via activation of APCs in lymphoid tissues (20). Hence, it is possible that PT-activated APCs in the CNS contribute to the T cell-mediated pathology. Consistent with this hypothesis, we observed an increased expression of MHC class II and costimulatory molecules on PT-stimulated cell isolates from the spinal cords of SJL mice. Similar, albeit not as pronounced, effects were noted on PT-treated microglia and astrocyte cell lines (C. L. Shive and T. G. Forsthuber, unpublished observations). The concentrations of PT used in the in vitro studies were most likely higher than what APCs would be exposed to in mice in vivo. However, the toxin could be more efficacious in vivo because of additional effects of the histamine pathway, which could indirectly contribute to APC activation (54).

Thus, the data suggest the Th1 differentiation and clonal expansion of encephalitogenic T cells via PT effects on APCs as the mechanism for the efficacy of the toxin in EAE. The toxin could furthermore contribute to this process by promoting Ag recognition by pathogenic T cells on PT-activated microglia in the CNS. Finally, there could be additional effects of the toxin on the BBB, which could contribute to the disease process.

Importantly, the results show that EAE can be induced in this model in the complete absence of CFA. CFA injections generally result in severe skin pathology, including granuloma formation and necrosis, which are absent following IFA:PT injection (data not shown). Thus, induction of EAE with neuroantigens in IFA and PT could provide a useful alternative to the standard CFA-based EAE protocols.

Taken together, the data suggest an alternative mechanism by which microbial products, such as PT, could contribute to the initiation of human autoimmune disease in the absence of molecular mimicry. In this model, the priming or reactivation of autoreactive T cells may be facilitated by structurally unrelated microorganisms that have in common the activation of APCs. Therefore, therapies aimed at deactivation of APCs in target organs of the autoimmune attack could provide an alternative approach for the treatment of autoimmune disease.

Acknowledgments

We thank Drs. Paul V. Lehmann, Magdalena Tary-Lehmann, and Peter Heeger for their valuable comments on this work, and Rocio Guardia for excellent technical assistance.

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