The Requirement for Pertussis to Induce EAU Is Strain-Dependent: B10.RIII, but Not B10.A Mice, Develop EAU and Th1 Responses to IRBP without Pertussis Treatment

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PURPOSE. Experimental autoimmune uveoretinitis (EAU) in mice is an important model for elucidating basic mechanisms in autoimmune eye disease. The need for pertussis toxin (PTX) as an additional adjuvant to elicit EAU has limited the usefulness of this model in some types of studies by introducing a pleiotropic factor with confounding effects on the immune response.

METHODS. In the present study the authors examined the ability of B10.RIII mice, the most susceptible strain known so far, to develop EAU in response to the retinal antigen, interphotoreceptor retinoid-binding protein (IRBP), and to a major uveitogenic epitope of IRBP, peptide (p)161-180, in the absence of PTX treatment.

RESULTS. The data indicate that high disease scores in response to IRBP and p161-180 were found in B10.RIII mice, without the need for PTX as part of the immunization protocol. Unlike the B10.A strain in which appreciable disease did not develop without PTX, B10.RIII mice mounted a high IFN-γ response to IRBP in the absence of PTX treatment. Interestingly, and unlike the effect with IRBP, in vitro recall response to p161-180 was low in IFN-γ, despite good EAU scores.

CONCLUSIONS. The data indicate that an important mechanism through which PTX facilitates induction of cell-mediated autoimmunity is by promoting a Th1 polarization of the immune response. The propensity of B10.RIII mice to mount a more polarized Th1 response to IRBP than other strains may contribute to their ability to develop EAU without pertussis adjuvant. Nevertheless, the induction of EAU by p161-180 in the context of a relatively limited IFN-γ production indicates that non-Th1- and Th-related mechanisms are likely to act in concert to determine the outcome of disease. (Invest Ophthalmol Vis Sci. 1999;40:2898–2905)
treatment in all cases. Antigen-specific production of IFN-γ indicated that the cellular response of B10.RIII mice to IRBP in the absence of PTX was more polarized toward type 1 than that of B10.A, and that PTX treatment elevated IFN-γ production in B10.A mice in response to IRBP to the level found in B10.RIII mice. We propose that this explains in part the tendency toward development of EAU in the B10.RIII mouse after IRBP immunization without PTX treatment. We further propose that one of the mechanisms that contribute to the well-documented property of PTX to promote cell-mediated autoimmunity is its ability to polarize the immune response toward the Th1 pathway.

MATERIALS AND METHODS

Animals

Six- to 8-week-old B10.A and B10.RIII mice were supplied by Frederick Cancer Research Facility (Frederick, MD) and by Jackson Laboratories (Bar Harbor, ME), respectively. Animal care and use were in compliance with institutional guidelines and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antigens

Whole bovine IRBP was purified from retinas by concanavalin A-Sepharose-affinity chromatography and high-performance liquid chromatography. Human p161-180 (sequence SGIPYII-SYLHPGNTILHVD) was synthesized on a peptide synthesizer (model 461; Applied Biosystems; Foster City, CA) using Fmoc chemistry.

EAU Induction and Scoring

EAU was induced by active immunization with graded doses of IRBP or p161-180 in phosphate-buffered saline (PBS) emulsified 1:1 vol/vol in complete Freund’s adjuvant (CFA) that had been supplemented with Mycobacterium tuberculosis strain H37RA (Sigma, St. Louis, MO) to 2.5 mg/ml. A total of 200 μl emulsion was injected subcutaneously, divided among three sites: base of tail and both thighs. In some groups, 0.5 μg Bordetella pertussis toxin (PTX) (Sigma) was injected intraperitoneally at the same time. EAU by adoptive transfer was induced by intraperitoneal injection of pooled spleen and lymph node cells obtained from primed donors and stimulated in culture with 20 μM p161-180 in the presence or absence of 50 ng/ml interleukin (IL)-12, essentially as previously described. In some adoptive transfer recipients, 1 μg PTX was administered intravenously just before the adoptive transfer. Clinical EAU was evaluated by fundoscopy under a binocular microscope after dilation of the pupil and graded on a scale of 0 to 4 in half-point increments, using the criteria described previously, which are based on the type, number, and size of lesions present. Incidence was shown as the number of positive animals of all animals in the group. Severity of disease was the average score of eyes from those animals in which disease developed (if disease was unilateral, both eyes were averaged).

Delayed-Type Hypersensitivity

To assess delayed-type hypersensitivity (DTH), 10 μg IRBP or peptide in 10 μl PBS was injected into the ear pinna. Ear-thickness increment was measured 48 hours later using a spring-loaded micrometer. The response was calculated as the difference between ear thickness before and after challenge.

Lymphocyte Proliferation Assay

Draining (inguinal and iliac) lymph nodes were collected after 21 days, and were pooled within the group. Triplicate 0.2-ml cultures containing 5 × 10⁵ cells were seeded in round-bottomed 96-well microtiter plates. The RPMI medium (Biowhittaker, Walkersville, MD) was supplemented with mouse serum, mercaptoethanol, antibiotics, glutamine, and nonessential amino acids, as described, and contained 30 μg/ml IRBP or 20 μM p161-180 as stimulants. The cultures were incubated for a total of 60 hours. Tritiated thymidine (1 μCi/well) was added during the last 18 hours. The data are shown as Δcpm (Δcpm = mean cpm in cultures with antigen, minus the mean cpm in control cultures without antigen).

Cytokine Assays

Lymph node and spleen cells were cultured in 96-well flat-bottomed plates (1 × 10⁶ cells/0.2 ml culture medium per well) either alone or with stimulants at the concentrations mentioned earlier. Supernatants were collected after 48 to 72 hours and were kept frozen in small aliquots at −70°C. Cytokine production was measured by enzyme-linked immunosorbent assay (ELISA) using antibody pairs from Pharmingen (La Jolla, CA) for IL-4, or from Endogen (Boston, MA) for IL-5 and IFN-γ, or from R&D (Minneapolis, MN) for TNF-α, as described previously.

Adoptive Transfer of EAU

Donor B10.RIII mice were immunized with 50 μg p161-180. Lymph node and spleen cells collected on day 14 after immunization were pooled. The cell suspension was adjusted to 10⁷ cells/ml in RPMI medium supplemented as for the proliferation assay, and the cultures were stimulated in 75-cm² flasks for 72 hours with 20 μM p161-180 in the presence or absence of 50 ng/ml IL-12. To remove excess adherent cells (macrophages), the stimulating cultures were transferred into new flasks after 24 hours and again after 48 hours. After 5 days, the lymphocytes were separated from erythrocytes and debris by discontinuous density gradient centrifugation on Ficoll (Lympholyte M; Accurate, Westbury, NY) and counted. Each recipient mouse was injected intraperitoneally with the specified number of cells. Some recipient mice were injected with 1 μg PTX intravenously just before the adoptive transfer. Eyes were collected from the recipients after 10 days and were evaluated for EAU by histopathology.

Reproducibility and Data Presentation

Experiments were repeated at least twice. Results were highly reproducible. Figures show pooled data from repeat experiments, or representative experiments, as indicated.
RESULTS

Induction of EAU with IRBP in B10.RIII, but Not in B10.A Mice, without Pertussis Treatment

To induce EAU, B10.A and B10.RIII mice were immunized with graded doses of IRBP in CFA, with or without a concomitant intraperitoneal injection of 0.5 μg PTX (Fig. 1). Although both strains had high disease scores after the immunization regimen that included PTX, only B10.RIII mice had high disease scores without pertussis treatment, which at 25 μg IRBP per mouse were almost equal to those in mice immunized with PTX. At the lower and higher limits of the dose-response curve, pertussis appeared to lower the threshold dose required for EAU induction and to forestall reduction of disease scores at higher IRBP doses. B10.A mice showed trace disease at best, which consisted of mild vitritis without retinal damage, even at the high antigen dose of 200 μg IRBP per mouse. That appreciable disease did not develop in B10.A mice without PTX treatment largely confirms data in our previously published and unpublished data.2

To pinpoint the time of disease onset, groups of four mice were immunized with 50 μg IRBP, with or without 0.5 μg PTX, and were observed daily by fundoscopy. Kinetics of disease development are shown in Table 1. B10.RIII mice showed clinical signs as early as 7 days after immunization if treated with PTX, or 8 days if untreated with PTX. Onset of disease in PTX-treated B10.A mice lagged by 2 days behind the corresponding B10.RIII group. Untreated B10.A mice began to show clinical signs only after day 12, and full-blown histologic disease did not develop even on day 21 (as shown in Fig. 1). It should be pointed out that fundoscopy scores did not translate directly to histology scores, but in general there was good correlation between both types of evaluations when performed in parallel.

Induction of EAU in B10.RIII Mice with P161-180 without Pertussis Treatment

We have previously defined the human sequence of IRBP p161-180 as a major pathogenic epitope for B10.RIII mice. Animals immunized with peptide using a regimen that included PTX had disease scores that approached scores obtained after immunization with IRBP and PTX. Because experiments described in the previous section showed that EAU in B10.RIII could be elicited with IRBP without PTX, we wanted to test whether the same was true in response to the peptide.

B10.RIII mice were immunized with graded doses of p161-180 in CFA, with or without concomitant administration of 0.5 μg PTX. Mice immunized without PTX had good EAU scores that at peptide doses between 10 and 25 μg peptide per mouse were only slightly lower than those in mice immunized with PTX (Fig. 2). However, use of PTX appeared to lower the threshold of antigen dose required for EAU induction and to eliminate the plateau of maximal achievable disease score observed at the highest doses of immunizing peptide. Thus, similar to EAU induced by whole IRBP, EAU induced by p161-180 in B10.RIII mice appears relatively independent of PTX as an additional adjuvant, except at extremely low or high antigen doses.

Kinetics of disease development in response to 25 μg p161-180 in CFA, with or without 0.5 μg PTX, are shown in Table 1.

Dependence on PTX Treatment of DTH Scores and Lymphocyte Proliferation in Response to IRBP, Irrespective of Disease Development

The same mice that were immunized for development of EAU were challenged 2 days before the end of the experiment for DTH responses to the immunizing antigen (IRBP or p161-180). The ear-swelling responses 48 hours after challenge showed that in all cases DTH scores were highly dependent on inclusion of PTX in the immunization regimen (Fig. 3). This was irrespective of the antigen, mouse strain, and disease scores resulting from the immunization.

Lymphocyte proliferation to the immunizing antigen was tested on draining lymph node cells that were pooled within each group. Similarly to the DTH responses, lymphocyte proliferation was strongly enhanced by inclusion of PTX in the immunization regimen, whether or not it was needed for elicitation of EAU (Fig. 4).

Thus the pattern of DTH and lymphocyte proliferation showed an apparent dissociation from EAU development in PTX-untreated B10.RIII mice; even mice that had good disease scores without PTX had relatively low DTH and proliferative
responses. A strong enhancing effect of PTX on DTH responses has previously been noted by others in various experimental situations\(^9\),\(^10\) and could be influenced by combined effects of PTX on IFN-\(\gamma\) production, lymphocyte migration, and vascular permeability, that may tend to keep IFN-\(\gamma\)–producing, antigen-specific lymphocytes in the circulation for a longer than normal period, while facilitating their egress into inflammatory sites.\(^9\),\(^10\),\(^12\),\(^13\) Similarly, effects on recirculation may inhibit emigration of primed lymphocytes from the draining lymph node, accounting for higher proliferative responses in vitro.

**Development of a More Polarized Type 1 Response to IRBP in B10.RIII Mice Than in B10.A Mice in the Absence of PTX Treatment**

Our previous data indicate that EAU is strongly dependent on the presence of a type 1 response to the uveitogenic antigen.\(^19\),\(^20\) We therefore examined the cytokine profile of the response to IRBP and to p161-180 in mice immunized in the presence and absence of PTX. Draining lymph node cells were collected 21 days after immunization, antigen-stimulated supernatants were generated from the different groups and were assayed for content of IFN-\(\gamma\), IL-4, IL-5, and TNF-\(\alpha\) by ELISA. The results showed that neither strain produced detectable IL-4 titers in response to IRBP and both had low antigen-specific IL-5 responses, indicating absence of a significant type 2 response. This response pattern was previously observed by us to be associated with genetic resistance to EAU\(^19\),\(^20\) and contrasts with the usual response to uveitogenic antigen in the susceptible strains. Nevertheless, culturing primed lymph node cells of (PTX-untreated) p161-180–immunized mice in the presence of IL-12 strongly enhanced their ability to produce IFN-\(\gamma\) as well as their ability to transfer disease adoptively to naive recipients (Table 4 and Fig. 5).

**The Paradox of p161-180: Uveitogenicity without a High IFN-\(\gamma\) Response**

Interestingly, in the case of the peptide the relationship between IFN-\(\gamma\) response in culture and disease development did not hold up. Although this peptide was uveitogenic even without PTX treatment, the in vitro response to the peptide was low in IFN-\(\gamma\), indicating a low type 1 response (Table 3). It was also low in IL-4 and IL-5, indicating absence of an appreciable type 2 response (Table 3). Such a null cytokine pattern was previously observed by us to be associated with genetic resistance to EAU\(^19\),\(^20\) and contrasts with the usual response to uveitogenic antigen in the susceptible strains. Nevertheless, culturing primed lymph node cells of (PTX-untreated) p161-180–immunized mice in the presence of IL-12 strongly enhanced their ability to produce IFN-\(\gamma\) as well as their ability to transfer disease adoptively to naive recipients (Table 4 and Fig. 5).

![FIGURE 2. EAU scores of B10.RIII mice immunized with p161-180 with or without concomitant pertussis treatment. The score at each dose is the average of all the mice in the group ± SE. EAU incidence (total/positive) is shown next to each point. The data are a composite of two experiments.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933585/ on 04/29/2017)
enhancement of uveitogenicity on adoptive transfer was particularly apparent at limiting cell numbers (Fig. 5). Thus, 5 million cells from cultures treated in vitro with IL-12 and showing a predominant Th1 cytokine profile induced close to maximal disease, whereas the same number of cells without IL-12 treatment and showing the IFN-γ low cytokine profile were unable to transfer disease. Thus, although an effector response low in IFN-γ production induced EAU disease in the case of this peptide, polarization of the response toward Th1 enhanced disease expression.

Effects of PTX on Adoptively Transferred EAU

Because PTX is a bacterial product that stimulates innate immunity, we treated some recipient mice with PTX just before infusion of peptide-primed cells from PTX-untreated donors, on the assumption that PTX would stimulate production of endogenous IL-12 in vivo and thus promote uveitogenicity of the p161-180–primed cells, similar to the effect of IL-12 treatment in vitro. Interestingly, pretreatment of recipient mice with PTX 8 hours before infusing 20 or 40 million primed cells, which caused severe disease in recipients that did not receive...
PTX, completely prevented development of EAU. The PTX-treated adoptive transfer recipients were still free of disease 10 days after the adoptive transfer, corresponding to 5 to 6 days after EAU onset in PTX-untreated recipients, at which point the experiment was terminated (Fig. 5). A delay in onset of adoptively transferred EAU in rats after pretreatment of recipients with pertussis adjuvant (bacteria) has previously been reported by others.21 Thus, PTX has dramatically different effects when administered at the induction, rather than at the expression stage of disease.

**DISCUSSION**

The present article reports that B10.RIII mice can develop EAU without PTX as an additional adjuvant. Our previous studies indicated that the development of EAU in rats and mice is closely correlated with their ability to mount a Th1-dominated response to IRBP.19,20 We therefore believe that the development of EAU in the B10.RIII strain without PTX as additional adjuvant is at least in part because of its propensity to mount a Th1-dominated response to IRBP in the absence of such treatment. PTX-induced enhancement of the Th1 response in association with abrogation of resistance to disease has been seen in the rat EAU model.19 The present study also points to a role for influences other than the Th1–Th2 balance in susceptibility, such as an ability to produce high levels of TNF-α.22 The observation that EAU can be induced in B10.RIII mice without PTX treatment increases the usefulness of the mouse model for basic studies of uveitis, by eliminating a pleiotropic adjuvant substance with many known and unknown effects on the immune system.

Our results shed light on the long-debated effects of pertussis adjuvant on cell-mediated autoimmunity. Although it has long been known that pertussis administered concurrently with immunization promotes induction of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and EAU, the mechanism by which this comes about is controversial. Some investigators have attributed enhancement of EAE by pertussis to histamine sensitization of vascular endothelial cells and increase of vascular permeability, whereas others have proposed effects on the sensitization phase of EAE.4–6,12 Because pertussis is administered at the time of immunization, it should be gone long before effector cell infiltration into the target organ occurs 7 to 9 days later. We therefore believe that the effects are more likely to be exerted on early events that coincide temporally with presence of PTX in the system, such as priming of effector T cells and their commitment to the Th1 pathway.

Support for this interpretation is also provided by the observation that PTX completely abrogated disease when administered with adoptive transfer of exogenously generated uveitogenic effector cells. If effects on vascular permeability were a primary mechanism, enhancement of disease would be expected. We propose that inhibition of adoptively transferred EAU is related to the documented inhibitory effects of PTX on recirculation and homing of lymphocytes.7–13 Because PTX is a known uncoupler of G-proteins, we hypothesize that these effects may be secondary to blocking of chemokine signaling through G-protein–coupled receptors, which is necessary for migration and extravasation of effector lymphocytes and recruited leukocytes into the target organ.23–24 This hypothesis is currently under investigation in a separate study.

Interestingly, B10.RIII mice appeared to produce less IFN-γ in response to p161-180 of IRBP than to the whole IRBP molecule. Furthermore, a Th1-dominant response to this epitope did not appear to be a prerequisite for its ability to induce disease, except under conditions of suboptimal uveitogenic challenge (insufficient or excessive immunization dose).

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**Table 2. Antigen-Specific Cytokine Production by Lymph Node Cells of B10.RIII and B10.A Mice Immunized with IRBP with or without PTX**

<table>
<thead>
<tr>
<th>Strain</th>
<th>PTX Treatment</th>
<th>IFN-γ (ng/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-4 (ng/ml)</th>
<th>IL-5 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.RIII</td>
<td>–</td>
<td>56.4</td>
<td>585</td>
<td>&lt;0.078</td>
<td>&lt;0.156</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>174.0</td>
<td>624</td>
<td>&lt;0.078</td>
<td>0.232</td>
</tr>
<tr>
<td>B10.A</td>
<td>–</td>
<td>4.8</td>
<td>213</td>
<td>&lt;0.078</td>
<td>&lt;0.156</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>52.9</td>
<td>682</td>
<td>&lt;0.078</td>
<td>0.322</td>
</tr>
</tbody>
</table>

Supernatant for cytokine assays was collected after 48 hours.

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**Table 3. Antigen-Specific Cytokine Production by Lymph Node Cells of B10.RIII Mice Immunized with p161-180 with or without PTX**

<table>
<thead>
<tr>
<th>PTX Treatment</th>
<th>IFN-γ (ng/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-4 (ng/ml)</th>
<th>IL-5 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>1.1</td>
<td>391</td>
<td>&lt;0.078</td>
<td>&lt;0.156</td>
</tr>
<tr>
<td>+</td>
<td>8.5</td>
<td>566</td>
<td>&lt;0.078</td>
<td>&lt;0.156</td>
</tr>
</tbody>
</table>

Supernatant for cytokine assays was collected after 48 hours.

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**Table 4. Antigen-Specific Production of IFN-γ in the Presence or Absence of IL-12 in Culture by Pooled Spleen and Lymph Node Cells of B10.R.III Mice Immunized with p161-180**

<table>
<thead>
<tr>
<th>Time in culture</th>
<th>IL-12</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hours</td>
<td>–</td>
<td>0.419</td>
<td>0.752</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9.340</td>
<td>9.738</td>
</tr>
<tr>
<td>72 hours</td>
<td>–</td>
<td>1.056</td>
<td>1.401</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9.456</td>
<td>10.327</td>
</tr>
</tbody>
</table>

*The cells were infused into recipient mice after 72 hours in culture with 20 μM p161-180 ≥ 50 ng/ml IL-12. EAU scores of the recipients are shown in Figure 5.
or a limiting number of adoptively transferred effector cells). One reason for this apparent paradox may be the identity of the antigenic epitopes involved. It is known that the type of response evoked is in part dependent on the epitope itself, and is affected by the affinity of the interaction with T cell receptor and major histocompatibility complex (MHC). High-affinity (or high-avidity) interactions in several antigen systems have been seen to promote development of type 1 responses, whereas low-affinity interactions may encourage type 2 responses. We hypothesize that the response to p161-180 involves mostly the self-specific repertoire from which high-affinity cells have been deleted, and therefore results in an IFN-γ-poor response. In contrast, the bovine IRBP molecule contains also multiple nonconserved, bovine-specific epitopes that can interact with the T-cell receptor with high affinity and cause abundant production of IFN-γ. Although these arguments may explain the difference in response phenotype to the peptide and the whole IRBP molecule, they do not explain the dissociation between the type of response to the peptide in vitro (low Th1) and the response to the same peptide in vivo (uveitis). The ability of p161-180 to induce disease in the context of a response relatively low in IFN-γ supports the interpretation that factors besides Th1-Th2 balance influence pathogenicity.

In summary, EAU induction in the B10.RIII mouse strain by active immunization with IRBP or with its immunodominant epitope 161-180 was found not to require the use of pertussis adjuvant. Unlike B10.A, B10.RIII mice showed strong Th1 response and high TNF-α levels to IRBP without PTX treatment, although even in this strain the disease was enhanced by PTX under suboptimal or supraoptimal conditions of induction. Although in the past it has been proposed that PTX promotes induction of cell-mediated autoimmunity because of its effects on vascular permeability, the present data, as well as data published by us previously, indicate that PTX-driven Th1 polarization may play an important role. Nevertheless, the ability of p161-180 to elicit EAU, apparently without inducing a strong Th1 response, remains a paradox and points to the involvement of additional factors besides Th1-Th2 balance in pathogenicity. The present investigation broadens the usefulness of the mouse EAU model and permits its use in studies in which pertussis treatment is undesirable or would confound the conclusions.

References
12. Sudweeks JD, Todd JA, Blankenhorn EP, et al. Locus controlling Bordetella pertussis-induced histamine sensitization (Bphs), an autoimmune disease-susceptibility gene, maps distal to T-cell re-


