Genetic Analysis of the Influence of Pertussis Toxin on Experimental Allergic Encephalomyelitis Susceptibility: An Environmental Agent Can Override Genetic Checkpoints

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Genetic Analysis of the Influence of Pertussis Toxin on Experimental Allergic Encephalomyelitis Susceptibility: An Environmental Agent Can Override Genetic Checkpoints

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Pertussis toxin (PTX) is a potent ancillary adjuvant used to elicit several different autoimmune diseases, including experimental allergic encephalomyelitis (EAE). To delineate the genetics of PTX effect in EAE, we mapped EAE-modifying (eae-m) loci in cohorts of backcross mice immunized with and without PTX. In this study, we analyzed the genetic basis of EAE susceptibility and severity and the intermediate phenotypes of mononuclear cell infiltration, suppuration, and demyelination. In animals immunized with PTX, one major locus, eae9, controls disease susceptibility and severity. Eae9 also regulates the extent of mononuclear cell infiltration of the spinal cord in male mice. Without PTX, five eae-m loci were noted, including three new loci in intervals on chromosomes 8 (eae14), 10 (eae17), and 18 (eae18). Taken together, these results suggest that eae9 controls the effects of PTX in EAE susceptibility, and is capable of overriding the other genetic checkpoints in the pathogenesis of this disease.

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As an initial step in understanding the genetic control of PTX effects in regulating EAE susceptibility, we mapped the locus (Bphs) controlling PTX-induced hypersensitivity to histamine (12). This phenotype has been reported to be associated with susceptibility to EAE (13) and to experimental allergic orchitis (14). Bphs is on chromosome 6 (12) and has been physically located within a yeast artificial chromosome contig encoding Eno2, Tnfrsf7 (Cd27), Libr, Tnfrsf1a (Tnfr1), and Vwf (15). However, Bphs does not determine EAE susceptibility in all PTX-dependent strains of mice, and some strains of mice differ in EAE susceptibility but do not differ in histamine sensitivity or Bphs alleles. Therefore, additional loci controlling PTX effects must exist. The purpose of the present study was to examine the genetic control of PTX effects in EAE by using strains of mice that are concordant for both Bphs and H2. Examination of EAE in (B10.S/DvTe × SJL/J) × B10.S/DvTe backcross (BC1) mice conditioned with PTX allows the determination of whether PTX elicits more frequent and/or more severe clinical signs of EAE and the identification of additional loci mediating its effects. We report here that the use of PTX significantly increases the proportion of backcross mice with clinical signs of EAE. One major locus on chromosome 9 controlling PTX effects in EAE susceptibility was found, in contrast to the multiple eae-m loci that are involved in EAE in the absence of PTX. Our results support the hypothesis that PTX is capable of circumventing or overriding many of the genetically determined checkpoints associated with EAE in this strain combination.

Materials and Methods

Animals and immunizations

(B10.S/DvTe × SJL/J) × B10.S/DvTe backcross mice (BC1) were generated continuously over 12 mo from the same pool of B10.S/DvTe and (B10.S/DvTe × SJL/J) F1 hybrid breeding stock. Inoculation of groups of mice, ranging in age from 6 to 12 wk, was staggered over the same period. Mice were infected s.c. at two sites on the posterior flank (0.15 ml/injection site) with 1.0 mg of SJL/J spinocord homogenate (SCH), in 0.15 ml PBS, emulsified with an equal volume of CFA (16). A booster injection of SJL/J...
Linkage of marker loci to disease susceptibility was tested by munized with SCH and CFA alone (PTX
number of days that it exhibited clinical symptoms. Liver tissue was col-
ected for histological evaluation, were chosen based on previous studies
reagent for demyelination. Representative areas of the brain and SC, se-
ited with hematoxylin and eosin for routine evaluation and luxol fast blue-periodic acid
m, and mounted on glass slides. Sections were stained with hematoxylin
phocytic response. CNS tissue response ranged from mice with no
mice: 40% clinically unaffected PTX
by sex. Each permutation was done by randomly shuffling and reassigning trait
values among the BC1 animals, while holding the genotypic information
ixed. Linkage analysis was then done for each permuted data set, and the
distribution of the maximum test statistic from each of the 1000 permutations
hances in experimental disease severity for both males and females as
pared to the PTX− group (PTX− vs PTX+: males, p = 0.0001; females, p = 0.016; males, p = 0.016; females, p = 0.03).
PTX−

<table>
<thead>
<tr>
<th>Group</th>
<th>Unaffected</th>
<th>Affected</th>
<th>% Affected</th>
<th>Day Onset</th>
<th>Average Peak Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX+</td>
<td>Total</td>
<td>28</td>
<td>62</td>
<td>69</td>
<td>17.3 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>20</td>
<td>37</td>
<td>65</td>
<td>16.9 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8</td>
<td>25</td>
<td>76</td>
<td>18.0 ± 2.4</td>
</tr>
<tr>
<td>PTX−</td>
<td>Total</td>
<td>69</td>
<td>53</td>
<td>43</td>
<td>19.3 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>38</td>
<td>29</td>
<td>43</td>
<td>18.9 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>31</td>
<td>24</td>
<td>44</td>
<td>19.9 ± 3.4</td>
</tr>
</tbody>
</table>

* PTX+ vs PTX−: total, p < 0.0001; males, p = 0.016; females, p = 0.03.

** PTX− vs PTX+: total, p < 0.0001; males, p = 0.002; females, p = 0.03.

*** PTX+ vs PTX−: total, p = 0.002; males, p = 0.3; females, p < 0.0001.

Results

Incidence of EAE in the two treatment groups

Signs of EAE were more frequent, and the latency to onset was
significantly shorter, in both males and females in the PTX+ group
(69% affected) compared with the PTX− group (43% affected)
(Table I). Although the incidence of disease was higher in PTX+ mice, there was no difference in susceptibility between males and
females within either group. However, disease as measured by
average peak score (or by a severity index, data not shown) was
less severe in the PTX+ group, especially in females (Table I). A
substantial cohort of mice in the PTX− group developed neuro-
pathology in the absence of detectable clinical signs. We have
recently termed this type of disease benign EAE (25). Benign EAE
was prominent in PTX− mice: 40% clinically unaffected PTX−
mice showed monocytes and lymphocytes in their SCs without any
outward signs of EAE, whereas only 16% of unaffected PTX+ mice
had this phenotype. This was not influenced by sex.

Neuropathology in the two treatment groups

SC and brain samples were obtained from and scored for the oc-
currence and degree of mononuclear infiltration, suppuration, and
demyelination. The character and distribution of CNS histopatho-
logical lesions observed in this study were consistent with those
reported in previous studies (18, 19, 25). Inflammatory responses
in susceptible animals ranged from those with a predominantly
neutrophilic response admixed with a smaller mononuclear/lympho-
cytic component to those with a predominantly mononuclear/lym-
phocytic response. CNS tissue response ranged from mice with no
injury to those with loss of myelin, reactive gliosis, swollen
axon sheaths, and swollen axons. In all mice with histopathology,
the inflammatory response had a perivascular distribution that was
predominantly observed in the meninges and in the white matter.
In the SC, predilection for the nerve root entry zone was observed, as reported previously (19).

When mice from the two treatment groups were examined for the presence of histopathology in the CNS (brain or SC), the PTX+ group again had a higher incidence of affected mice than the PTX− group for either CNS site (data not shown). Differences between males and females were not observed for histopathological traits in the brain or SC of PTX+ mice. In contrast, female mice in the PTX− group had significantly greater brain involvement than males (Table II). Because animals were not all euthanized at the same time points subsequent to the manifestation of their disease, overall between-group comparisons could not be made for histopathology. For PTX+ mice, linkage to the binary trait of susceptibility was found in an interval that is marked by D9 Mit105 (χ2 values were 11.7 and 9.3, indicating probability of linkage at α = 0.05 and α = 0.1, respectively). This effect came largely from male mice (χ2 = 17.0). A QTL designated eae9 has previously been identified in the same interval in an F2 intercross with the same parental strains (16). In the PTX− cohort, linkage to susceptibility was found to markers on chromosome 8, peaking at D8 Mit190 (χ2 = 18.6, α = 0.1).

Loci that showed evidence of linkage to severity or histopathological signs of EAE are given in Table III. In the PTX+ cohort, we again found linkage to a single major QTL (eae9) between D9 Mit22 and D9 Mit24. The SJL-derived allele at eae9 is associated with increased severity. Stratification by sex revealed that one phenotype of eae9 is to increase monocyte/lymphocyte infiltration in the SC in males. No linkage to any other locus, including previously identified eae-m loci, was seen in PTX− mice. In contrast, genetic control of EAE

Table II. CNS histopathology in male and female PTX+ or PTX− BC1 mice

<table>
<thead>
<tr>
<th>Trait</th>
<th>PTX+</th>
<th>PTX−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Demyelination</td>
<td>1.04</td>
<td>1.03</td>
</tr>
<tr>
<td>Suppression</td>
<td>0.68</td>
<td>0.70</td>
</tr>
<tr>
<td>Monocytic/lymphocytic infiltration</td>
<td>1.44</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demyelination</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>Suppression</td>
<td>0.47</td>
<td>0.33</td>
</tr>
<tr>
<td>Monocytic/lymphocytic infiltration</td>
<td>1.16</td>
<td>1.51</td>
</tr>
</tbody>
</table>

a Mean value for the specified trait.
b p value from Student’s t test comparing male and female mice.

Table III. Quantitative trait loci linked to EAE susceptibility in PTX-treated vs untreated backcross mice

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Marker</th>
<th>cM</th>
<th>eae</th>
<th>Trait</th>
<th>LRT</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX+ Total</td>
<td>D9Mit105</td>
<td>35</td>
<td>eae9</td>
<td>High score</td>
<td>13.3a</td>
<td>SJL</td>
</tr>
<tr>
<td>Females</td>
<td>D9Mit105</td>
<td>35</td>
<td>eae9</td>
<td>Severity index</td>
<td>15.1a</td>
<td>SJL</td>
</tr>
<tr>
<td>Males</td>
<td>D9Mit105</td>
<td>35</td>
<td>eae9</td>
<td>Mono/lymph SC</td>
<td>12.8a</td>
<td>SJL</td>
</tr>
<tr>
<td>PTX− Total</td>
<td>D8Mit190</td>
<td>21</td>
<td>eae14</td>
<td>High score</td>
<td>22.9b</td>
<td>B10.S</td>
</tr>
<tr>
<td>Females</td>
<td>D8Mit190</td>
<td>21</td>
<td>eae14</td>
<td>Severity index</td>
<td>25.9b</td>
<td>B10.S</td>
</tr>
<tr>
<td>Males</td>
<td>D8Mit190</td>
<td>21</td>
<td>eae14</td>
<td>Demyelination in SC</td>
<td>11.6b</td>
<td>B10.S</td>
</tr>
<tr>
<td></td>
<td>D11Mit98</td>
<td>58</td>
<td>eae7</td>
<td>High score</td>
<td>12.8a</td>
<td>SJL</td>
</tr>
<tr>
<td></td>
<td>D11Mit98</td>
<td>58</td>
<td>eae7</td>
<td>Severity index</td>
<td>13.7a</td>
<td>SJL</td>
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<tr>
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<td>D8Mit190</td>
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<td>eae14</td>
<td>High score</td>
<td>19.8a</td>
<td>B10.S</td>
</tr>
<tr>
<td></td>
<td>D8Mit190</td>
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<td>eae14</td>
<td>Severity index</td>
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<td>B10.S</td>
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<td>eae14</td>
<td>Demyelination in SC</td>
<td>13.3b</td>
<td>B10.S</td>
</tr>
<tr>
<td></td>
<td>D10Mit31</td>
<td>36</td>
<td>eae17</td>
<td>High score</td>
<td>16.2a</td>
<td>SJL</td>
</tr>
<tr>
<td></td>
<td>D10Mit31</td>
<td>36</td>
<td>eae17</td>
<td>Severity index</td>
<td>15.8a</td>
<td>SJL</td>
</tr>
<tr>
<td></td>
<td>D16Mit12</td>
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<td>eae11</td>
<td>Mono/lymph SC</td>
<td>13.1a</td>
<td>SJL</td>
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<tr>
<td></td>
<td>D18Mit3</td>
<td>54</td>
<td>eae18</td>
<td>Mono/lymph SC</td>
<td>17.8a</td>
<td>SJL</td>
</tr>
<tr>
<td></td>
<td>D18Mit3</td>
<td>54</td>
<td>eae18</td>
<td>Demyelination in SC</td>
<td>20.4a</td>
<td>SJL</td>
</tr>
</tbody>
</table>

a LRT is significant for experimentwise threshold of α < 0.05. Cut-offs for the α = 0.05 level of significance range from LRT 12.9 to 15.6. The level of significance indicated by an LRT > 15.6 was not established in this experiment.
b LRT statistic given for each trait is significant for experimentwise threshold of α = 0.1. Cut-offs for this level of significance range from LRT 11.5 to 12.5.
c Indicates which parental allele of the EAE-modifying locus is associated with greater severity of clinical signs.
five linkages were seen in the PTX− cohort, including two QTL previously shown to play a role in this strain combination (16, 25). The most significant QTL in the PTX− cohort is on chromosome 8 between D8 Mit3 and D8 Mit31. Susceptibility, severity, and SC demyelination are associated with the B10.S-derived allele at this locus, which we have designated eae14. In addition to eae14, a locus on chromosome 11 that colocalizes with eae7 (16) contributed to severity in this cohort. As in the F2 intercross between these same strains, sex-specific linkages were seen in the PTX− mice. Females from the PTX− group showed linkage to chromosome 10 for severity of clinical signs in an interval between D10 Mit126 and D10 Mit10. We have designated this locus as eae17. A QTL controlling severity of mononuclear infiltrates in the SC was mapped to a broad region of chromosome 16. Increased severity of inflammation was associated with inheritance of the SJL allele at this locus, which was previously designated as eae11 (25). In PTX− males, a strong QTL was mapped to chromosome 18. The SJL-derived allele at this locus (eae18) resulted in greater infiltration and demyelination in the SC. No QTL for brain histopathology met experiment-wise cutoffs for suggestive (α = 0.10) or significant (α = 0.05) linkage in either cohort.

**Multiple linear regression analysis**

Multiple linear regression was used to test the hypothesis that significant QTL interact. Significant marker loci for disease severity (D8 Mit190, D11 Mit98, and D10 Mit42), SC demyelination (D8 Mit190 and D18 Mit3), and SC monocyte/lymphocytic infiltration (D8 Mit190, D16 Mit50, and D18 Mit3) were analyzed as independent variables in multiple linear regression analyses with the appropriate phenotype as the dependent variable. To investigate possible interactions between significant marker loci, two-locus interaction terms were added to the regression models as independent variables. None of the interaction terms was significant (p > 0.05). Without interaction variables, statistical significance was achieved for disease severity (F = 13.59, p < 0.0001), SC demyelination (F = 10.54, p < 0.0001), and SC monocyte/lymphocytic infiltration (F = 6.31, p = 0.0005).

**Discussion**

In our study, EAE was more frequent in PTX-treated backcross mice. However, disease symptoms were milder. These findings are consistent with previous studies demonstrating that PTX can “convert” some EAE-resistant rodent strains to susceptible ones, indicating that autoimmune resistance is environmentally sensitive (26). The ability to convert otherwise resistant mice implies that some checkpoints in autoimmunity may be controlled by environmentally sensitive loci (27). For example, if a particular locus maintains the integrity of the BBB, administration of PTX might overcome that mechanism of EAE resistance. Thus, the activity of any EAE resistance alleles at that locus could be bypassed by PTX in a genetic cross where those alleles were segregating, and thus linkage to that locus would be minimized. Characterization of the loci controlling the activity of PTX in EAE will be helpful in understanding the action of environmentally sensitive loci in susceptibility to autoimmune disease.

PTX has many effects on the immune system, aside from its vasoactive amine-sensitizing activity (6, 7). Because our animals were not segregating for the Bphs phenotype, which is believed to be associated with BBB permeability changes, we expect that other genetically regulated PTX-mediated phenotypes will be present in our cross. The mechanism by which PTX affects EAE in this backcross could therefore be related to one of its other known activities (5). For example, PTX causes a generalized lymphocytosis that is accompanied by a depletion of lymphocytes in the spleen, lymph node, and thymus due to the failure of cells to migrate back to the peripheral lymphoid tissue (28, 29). PTX is also reported to be preferentially mitogenic for T cells (30), and it has been reported to enhance delayed-type hypersensitivity responses in an Ag-specific manner (31, 32). The enhanced delayed-type hypersensitivity due to PTX correlates with elevated Ag-specific production of IFN-γ by these cells (33). Additionally, PTX can induce both Th1 and Th2 immune responses (34, 35) and can increase expression of the costimulatory molecules B7-1 and B7-2 on macrophages and B cells, and CD28 on T cells (35). In this regard, PTX is capable of preventing the induction of peripheral T cell anergy to murine encephalitogenic peptides, again in an Ag-specific manner (36); central mechanisms of tolerance may also be affected by PTX (37). Any of these or other known roles of PTX might be critical to the development of clinical signs or neuropathology.

In the genetic analysis of the PTX− group, only one locus (eae9) shows evidence of linkage to disease susceptibility in comparison to five loci in the PTX+ group. Our results therefore suggest that PTX overcomes these critical genetic checkpoints in the etiology of autoimmune disease. Eae9 was associated with the extent of mononuclear infiltration in the SC and severity of EAE clinical signs. This QTL is located in an interval encoding Birl/CXCR5, Il10ra α-chain, and Il18 (IFN-γ-inducing factor). Any of these candidate genes could be responsible for the eae9-mediated phenotypes mentioned above. Given that PTX can induce increased IFN-γ production by T cells, it is interesting to consider the possibility that eae9 is Il18. Another possibility is that lymphocytosis could be affected by the differential action of the chemokine receptor Birl/CXCR5 alleles. This is particularly intriguing given that the chemokines Sca1 (T cell activation 3), Sca2 (monocyte chemoattractant protein (MCP)-1), and Sca12 (MCP-5) are candidates for eae7 on chromosome 11 (38), which was also identified in the PTX− backcross (Table III). In three separate crosses, therefore, we observed linkage to either chemokines or their receptors (eae9 and eae7 in the BC PTX+ and PTX−, and eae7 in the F2 in Ref. 38), and thus allelic differences in the chemokine-signaling pathways may control susceptibility to EAE by regulating the migration of inflammatory cells and their access into the CNS.

The five QTL in the PTX− cohort include a novel eae-m locus we have named eae14. eae14 demonstrates significant linkage to disease incidence and severity and to the extent of SC demyelination. This QTL maps to an interval containing several genes of possible relevance to EAE, two of which are glutathione reductase (Grl1) and caspase-3 (Casp3). Glutathione reductase (EC 1.6.4.2) is an enzyme in the cytosol that maintains high levels of reduced glutathione in the cell, which provides significant antioxidant activity. It is expressed in macrophages and in brain. A deficiency in glutathione reductase in either of these tissues could render them susceptible to the toxic effects of free oxygen radicals in the inflammatory site (39). C57BL strains have the Grl+ allele and are known to have lower enzyme activity, whereas SJL/J mice have the Grlb allele and higher enzymatic activity (40). This difference in enzyme activity could explain why B/B homozygotes for eae14 develop more severe disease in the PTX− group. The second candidate gene in this interval is caspase-3, a downstream effector molecule of cellular apoptosis. The active form of caspase-3 is highly expressed in the spleen and less so in the brain (41). It has been hypothesized that apoptosis of T cells might play a role in the resolution of EAE (42–44).

The PTX− cohort displayed sexual dimorphism. One linkage to EAE severity was found on chromosome 10 only in PTX− females. This interval contains several interesting candidate genes, including matrix metalloproteinase 11 (Mmp11 at 41 cM), tissue
inhibitor of metalloprotease 3 (Timp3 at 49 cM), insulin-like growth factor 1, (Igf1 at 48 cM), migration inhibitory factor (Mif at 41 cM), and the aire locus (Ref. 4 and R. Roper et al., manuscript in preparation). AIRE is a known autoimmune susceptibility gene in the human autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (45). In addition, PTX− females showed linkage to a QTL on chromosome 16, which colocalizes with eae11 seen in an F2 cross between these same parental strains (25). The apparent female specificity of eae11 in the backcross for one of the SC traits is not the same as in the F2 cross, where only males displayed linkage to markers in the eae11-defining region (D16 Mit110-D16 Mit140 at ~20–40 cM). This could be due to one of several reasons: the genetic environment for the F2 and backcross are different, and we would not expect all of the genetic effects to be replicated between the experiments. If so, a significant interaction in the F2 between eae11 and some other locus/loci (a combination not present in the BC1) could account for the difference. Alternatively, the larger population studied for the F2 provides more statistical power to detect any QTL, particularly one with small effects, and perhaps the effects of eae11 in the backcross males are not observed due to less power. These are not necessarily the same QTL, because the eae11 interval is quite broad.

In males from the PTX− cross, the strongest QTL, located on distal chromosome 18, is associated with significant inflammation and demyelination in the SC. This QTL, eae18, resides in an interval that contains the melanocortin receptor (Mc2r at 37 cM) and the myelin basic protein gene (Mbpl at 55 cM). In an earlier report, Baker et al. (46) found an eae-m locus in this region in an (ABH × NODF1 × NOD) backcross. It is noteworthy that B10.S and SJL/J and some other locus/loci (a combination not present in the BC1) could account for the difference. In summary, we have identified two new EAE-modifying loci, one of which is a new locus controlling the effects of PTX in EAE pathogenesis. The precise mechanism of action of this gene remains unknown, but presumably reflects an intermediate phenotype other than PTX-induced vascular permeability changes. The molecular characterization of this QTL will potentially aid in the understanding of environment-gene interactions in autoimmune disease.

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References


