



An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide

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EAE can refer either to experimental autoimmune encephalomyelitis or experimental allergic encephalomyelitis. Although EAE is classically a prototypic T helper 1 (T_H1) cell-mediated autoimmune disease, it can also be induced by T_H2 cells. Characteristically, the most severe manifestation of allergy, anaphylaxis, is associated with exposure to a foreign antigen that is often derived from medication, insect venom or food. We report here that, after self-tolerance to myelin is destroyed, anaphylaxis may be triggered by a self-antigen, in this case a myelin peptide. "Horror autotoxicus", which was initially described by Ehrlich, may not only include autoimmunity to self, it may also encompass immediate hypersensitivity to self, which leads to shock and rapid death.

Experimental autoimmune encephalomyelitis (EAE) is the prototypical animal model for T cell autoimmunity and shares many clinical and pathological features with the human disease multiple sclerosis (MS). CD4⁺ T cells that are reactive to myelin antigens are the primary mediators of the disease^{1–3}. Naturally circulating in the periphery, these cells must undergo activation and clonal expansion to cause EAE¹. Production of T helper 1 (T_H1)-associated pro-inflammatory cytokines such as interferon γ (IFN- γ), interleukin 2 (IL-2) and tumor necrosis factor α (TNF- α) play a critical role in the development of EAE and in subsequent brain inflammation². Antibodies are the critical mediators of allergic reactions, which, in their most extreme manifestation, result in anaphylactic shock^{4,5}. Immediate hypersensitivity and allergy are associated with the action of a set of cytokines that include IL-4, IL-5 and IL-13 (T_H2 cytokines), which induce both a shift in antibody isotype and eosinophilia^{3,5}. Although in humans immunoglobulin E (IgE) is the antibody isotype that is involved in immediate-type hypersensitivity^{4–6}, both IgE and IgG1 can elicit allergic reactions in mice^{7,8}. They do this by binding to Fc ϵ RI and Fc γ RIII, respectively, on mast cells and basophils⁸.

T_H1 responses are associated with autoimmune disease such as EAE and MS. Conversely, T_H2 responses protect animals from EAE and may ameliorate MS⁹. Nevertheless, adoptive transfer of myelin-reactive T_H2 cells to immunodeficient mice induces EAE with an eosinophilic infiltrate in the brain¹⁰. This suggests that a T_H2-type immune response could play a role in the development of this disease. Mast cells represent major effector cells of T_H2-associated immune responses^{3–5}, yet several lines of evidence indicate that mast cells also may contribute to the development of EAE and participate in the pathology of MS^{11–21}. Localized release of vasoactive amines, cytokines, chemokines and other mediators from mast cells that are found at the sites of inflammatory demyelination in EAE^{10,14} and MS^{15–17} may promote the entry of autoreactive T cells into the central nervous system (CNS)²². They may also contribute to other

aspects of the pathology of EAE or MS, including demyelination^{19,20}. In agreement with a potentially important role for mast cells in the expression of EAE, mice that are genetically deficient in mast cells exhibit significantly reduced incidence and severity of EAE²¹.

In the course of developing peptide-based antigen-specific therapy in EAE, we encountered animals that died very rapidly after the administration of the self-antigen myelin proteolipid protein (PLP) amino acids 139 through 151, or PLPp(139–151). We report here our investigations into the mechanisms that underlie this unexpected phenomenon.

Results

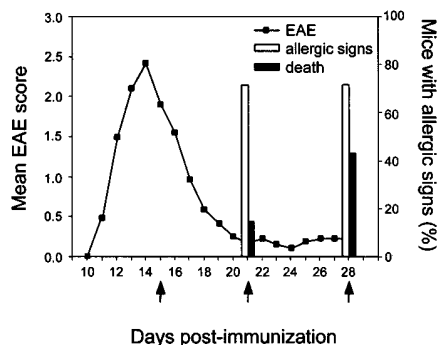
Induction of anaphylaxis with a self-peptide

In three consecutive experiments, EAE was induced with PLPp(139–151) in SJL/J mice. EAE occurred in 79% of mice (23 of 29) with disease onset at day 11.7 $\pm$ 0.14 (mean $\pm$ s.e.m.) and a disease score at day 14 of 2.4 $\pm$ 0.28. Mice were challenged with PLPp(139–151) 15, 21 or 28 days after induction of EAE. In 71% of mice challenged after 4 weeks (10 of 14) and 71% of mice challenged after 3 weeks (5 of 7), but in none of the mice challenged after 2 weeks (0 of 8), reactions that had the characteristics of immediate hypersensitivity occurred within a few minutes of injection (**Fig. 1**). Affected mice developed piloerection; prostration; erythema of the tail, ears and footpads; were unable to move after stimulation; and presented with dyspnea and shallow breathing. Death occurred within 30 min in 43% of the mice challenged after 28 days (6 of 14) and in 14% of the mice challenged after 21 days (1 of 7).

The nature and time-course of these symptoms were highly suggestive of anaphylactic shock^{4,5,8,23}. In addition, as is characteristic of mice that exhibit IgE- or IgG1-dependent anaphylaxis^{8,24}, the mice also developed a significant drop in body temperature (**Fig. 2a**) and evidence of significantly increased airway resistance (**Fig. 2b**). In addition, histopathological examination of tissues obtained from autopsies of these mice showed extensive vascular congestion in lungs, liver and heart (**Fig. 3**).

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Figure 1. Challenge with soluble PLPp(139–151) after the acute phase of EAE causes signs of anaphylactic shock. In three consecutive experiments, EAE was induced in 29 SJL/J female mice with subcutaneously injected PLPp(139–151) in CFA. Mice were then challenged with the same peptide (100 µg in PBS) intraperitoneally (arrows) after 15 (8 mice), 21 (7 mice) or 28 (14 mice) days. The majority of mice developed allergic symptoms and died when challenged at days 21 and 28, but not day 15.



upon challenge at day 21 and 91% at day 28. (Reactions developed in 10 of 11 mice with elevated IgG1 versus 0 of 3 mice without elevated IgG1; $P=0.011$ by Fisher's exact test.) These findings suggest a correlation between high titers of anti-PLPp(139–151) IgG1 and the expression of immediate hypersensitivity reactions to peptide challenge, particularly on day 28.

We did not detect IgE antibodies specific for PLPp(139–151) with our enzyme-linked immunosorbent assay (ELISA) method. However, total IgE, which was not detectable in the sera of strain-, age- and gender-matched normal mice, was elevated in 33% of mice at day 15 (3 of 8), 29% of mice at day 21 (2 of 7) and 21% of mice at day 28 (3 of 14) (Fig. 4c). Total IgE concentration

did not increase during the course of EAE. Taken together, these findings suggest that the allergic reactions that can be expressed in these mice after the acute phase of EAE are mediated by IgG1 antibodies.

Characterization of the self-allergen and its adjuvants

An allergic response to self-PLPp(139–151) could also be detected when the immunization did not induce clinical EAE. Mice were immunized, via the intraperitoneal (i.p.) route, with PLPp(139–151) in incomplete Freund's adjuvant (IFA) or in complete Freund's adjuvant (CFA). Challenge after 4 weeks caused anaphylactic shock and death in 100% of the mice immunized with the peptide in IFA (5 of 5) and 75% of the mice immunized with the peptide in CFA (6 of 8) (Table 1).

In many of our experiments immunization and challenge was carried out with a PLPp(139–151) amino acid sequence that included a substitution, at position 140, of the native amino acid, cysteine, with serine (Cys¹⁴⁰→Ser¹⁴⁰). This substitution is commonly used as a "pathogenic sequence" to induce EAE²⁵. However, to evaluate whether the allergic responses can be induced to the "true-self-antigen", we also examined mice in which EAE was induced with the peptide that contained the native cysteine at position 140, referred to hereafter as native PLPp(139–151). Challenge with this soluble self-peptide 3 or 4 weeks after the induction of EAE, elicited allergic responses in 45% of the mice (13 of 29) and death in 24% (7 of 29). This indicated that allergic

To confirm that anaphylactic shock was triggered specifically by PLPp(139–151), we challenged some mice with a control peptide of 13 amino acids 28 days after EAE induction. The absence of allergic reactions, even after repeated challenges with the control peptide, suggested that the allergic response in mice with EAE was specific for PLPp(139–151). (Anaphylaxis developed in 10 of 14 mice after challenge with this peptide versus 0 of 12 after challenge with the control peptide; $P<0.0002$ with Fisher's exact test; Table 1 and Fig. 2a,b.) In addition, mass spectroscopic analysis of the peptides indicated a single peak at the appropriate molecular size, which rules out aggregation of these molecules (data not shown).

Analysis of the humoral response

The humoral response was studied at different time-points during the course of EAE by analyzing serum that had been collected a few hours before peptide challenge (Fig. 4). Titers of IgG antibody to PLPp(139–151) increased during the course of EAE. Among these antibodies, and in all the mice, titers of IgG1 exceeded those of IgG2a (Fig. 4a,b). Although only one of eight mice (12.5 %) had an elevated titer of IgG1 antibodies to PLPp(139–151) at day 15, 71% (5 of 7) or 79% (11 of 14) of the mice developed high titers of this antibody isotype by day 21 or 28, respectively. Of the mice with elevated titers of IgG1 antibodies to PLPp(139–151), 60% (3 of 5) exhibited allergic reactions

Figure 2. The reactions that occur after challenge with PLPp(139–151) are associated with a drop in body temperature and an increase in enhanced respiratory pause. EAE was induced in SJL/J female mice with PLPp(139–151) or native PLPp(139–151) 3–4 weeks before challenge with the respective peptides or, as a negative control, with a control peptide. As an additional negative control, naïve SJL/J female mice that had not been immunized with the peptide were challenged with PLPp(139–151). As a positive control for IgE-dependent anaphylaxis, SJL/J female mice that had received anti-DNP IgE 24 h before were challenged with DNP-HSA. Data are mean±s.e.m. values and the number of mice in each group is shown. (a) Body temperature was recorded at multiple intervals after the challenge. (**** $P<0.001$ by ANOVA versus either negative-control group; *** $P<0.005$ versus either negative-control group; **** $P<0.001$ versus positive-control group; * $P<0.05$ versus positive-control group.) (b) The average enhanced respiratory pause (Penh, a measure of airway resistance) at 3 min intervals after challenge is expressed for each time-point as a percentage of baseline Penh values (baseline is defined as 100%). (*** $P<0.005$ by ANOVA versus either negative-control group; * $P<0.05$ versus either negative-control groups; n.s., nonsignificant differences, where $P>0.05$ among the three indicated groups.)

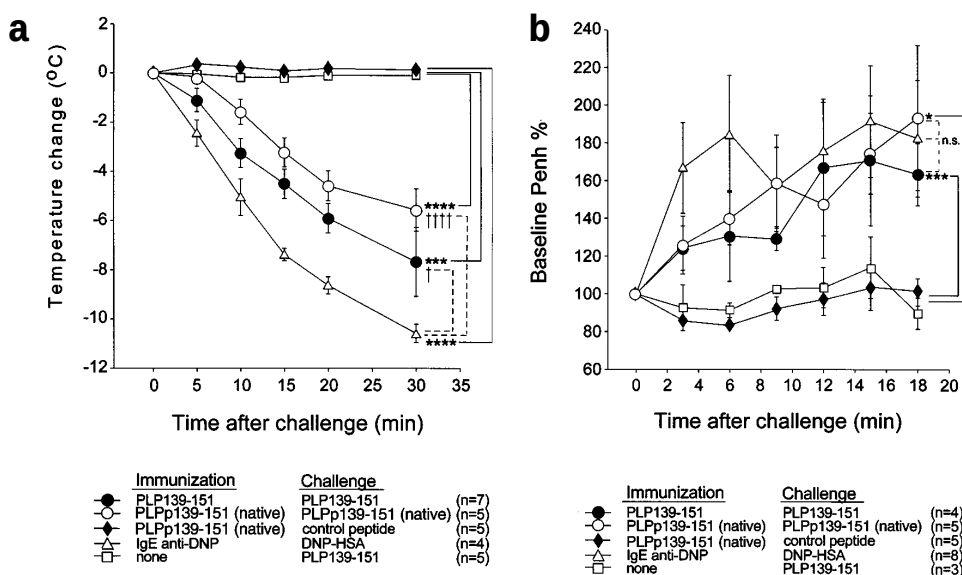


**Table 1. Incidence of allergic reactions in mice challenged with various peptides**

Mouse strain	Peptide of primary immunization (100 µg)	Immunization protocol		Number of mice with EAE	Peptide used for challenge ^a	Number of mice with allergic reactions at the challenge ^b
		adjuvant	route			
SJL/J	PLPp(139–151)	CFA	s.c.	13 of 14	PLPp(139–151)	10 of 14 ^e
SJL/J	PLPp(139–151)	CFA	s.c.	11 of 12	Control peptide	0 of 12
SJL/J	PLPp(139–151)	CFA	i.p.	1 of 8	PLPp(139–151)	6 of 8
SJL/J	PLPp(139–151)	IFA	i.p.	0 of 5	PLPp(139–151)	5 of 5
SJL/J	Native PLPp(139–151)	CFA	s.c.	27 of 30	Native PLPp(139–151)	13 of 29
SJL/J	PLPp(139–151)	CFA	s.c.	0 of 10	PLPp(139–151)	0 of 10
SJL/J	Leu ¹⁴⁵ →Lys ¹⁴⁵	CFA	s.c.	0 of 10	Leu ¹⁴⁵ →Lys ¹⁴⁵	0 of 10
					MOG(35–55)	
					MOG(35–55) ^d	
SJL/J	PLPp(178–191)	CFA	s.c.	13 of 15	PLPp(178–191)	0 of 14
C57BL/6	MOG(35–55)	CFA	s.c.	0 of 20	MOG(35–55) ^d	1 of 20 ^f , 2 of 20 ^g , 16 of 20 ^h
C57BL/6	PLPp(139–151)	CFA	s.c.	0 of 10	PLPp(139–151)	0 of 10
PL/J	MBPAc(1–11)	CFA	s.c.	8 of 10	MBPAc(1–11) ^d	0 of 7
		Ptx	i.v. ^c			
PL/J	MBPAc(1–11)	CFA	s.c.	0 of 10	MBPAc(1–11) ^d	0 of 10

^aUnless otherwise specified, challenge occurred 4 weeks after immunization with i.p. injection of 100 µg of the different peptides in PBS. ^bAbsence of allergic signs was confirmed by three weekly challenges. ^c*Bordetella pertussis* toxin (Ptx) was given as an adjuvant in a 200 ng dose at days 0 and 2 after immunization. ^dChallenge by i.p. injection with 500 µg of the peptide in PBS. ^e $P < 0.0002$ by Fisher's Exact test challenge with PLPp(139–151) versus challenge with control peptide. ^fChallenge at 4 weeks. ^gChallenge at 5 weeks. ^hChallenge at 6 weeks. (s.c., subcutaneous; i.p. intraperitoneal; i.v., intravenous.)

responses to “true-self” could still induce anaphylaxis, albeit at a somewhat lower frequency compared to the modified PLPp(139–151) sequence (**Table 1**). In addition, the key pathophysiological features of these reactions are strikingly similar to those observed in IgG1-FcγRIII-associated passive systemic anaphylaxis^{8,24}. A marked drop in body temperature, a significant increase in airway resistance and little or no morphological evidence of mast cell degranulation were observed in mice that developed anaphylaxis in response to native PLPp(139–151) (**Figs. 2a,b** and **5**).

To induce an allergic type- T_H2 response, as well as a T_H1 response capable of eliciting EAE, a peptide antigen that is complexed with a major histocompatibility (MHC) molecule must be presented to T cells^{23,26}. In SJL/J mice (H-2^s), PLPp(139–151) binds with high affinity to the I-A^s MHC class II molecule²⁷. PLPp(139–151)-reactive T cells are normally found in naïve SJL/J mice, probably because of a lack of thymic deletion of these autoreactive T cells²⁸. The residues at positions 145 (leucine) and 148 (proline) of PLPp(139–151) are important for MHC class II (I-A^s)-binding. The Leu¹⁴⁵→Lys¹⁴⁵ analog of PLPp(139–151) is unable to bind to I-A^s and to stimulate T cells hybridomas specific to PLPp(139–151)²⁵. As might be expected, mice immunized with the Leu¹⁴⁵→Lys¹⁴⁵ analog of PLPp(139–151) did not express immediate

hypersensitivity reactions when challenged with the same peptide 28 days after immunization (**Table 1**).

To determine whether another self-antigen could induce immediate hypersensitivity in another strain of mice, we explored the induction of allergic responses in H-2^b mice that had been immunized with myelin oligodendroglial glycoprotein (MOG). C57BL/6 (H-2^b) mice, but not SJL/J (H-2^s) mice, are susceptible to EAE induced by MOG, which binds to I-A^b (ref. 29). In two consecutive experiments, SJL/J and C57BL/6 mice were immunized with the peptide MOG(35–55) and 4 weeks later, at a time when self-tolerance to MOG has been broken and anti-MOG responses are present (data not shown), challenged with the same peptide. MOG did not induce clinical EAE unless *Bordetella Pertussis* was used as an adjuvant. No SJL/J mice (0 of 20) developed any allergic reactions after challenge with MOG (**Table 1**). However, in the C57BL/6 mice, 5% (1 of 20) developed anaphylaxis after the first challenge. This increased to 10% (2 of 20) after the second injection and to 80% (16 of 20) after the third challenge with a 0.5 mg dose of the peptide. Of the mice with anaphylaxis, 25% died (5 of 20).

Compared to SJL/J mice, C57BL/6 are less sensitive to the effects of histamine and perhaps to other mast cell-derived mediators^{13,18,30}. Strain

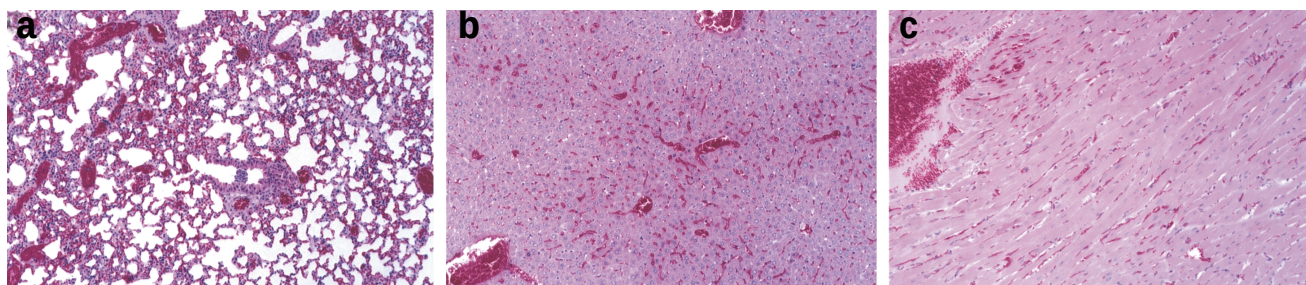
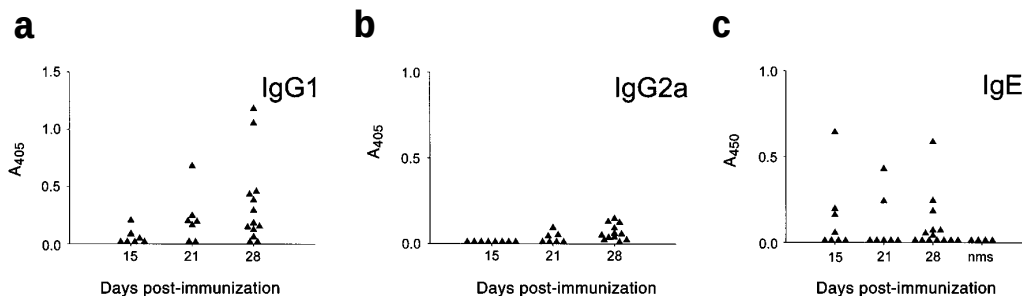


Figure 3. Histopathology of tissue sections obtained from a 12-week-old SJL/J mouse that was killed when it became moribund 20 min after apparent anaphylaxis. EAE had been induced, with PLPp(139–151), 4 weeks previously. The mouse was then challenged with 100 µg of i.p. PLPp(139–151). There was extensive vascular congestion in all tissues examined. Tissues were stained with hematoxylin and eosin and are shown at magnification $\times 300$. (a) Marked pulmonary congestion and focal emphysematous changes in the lung. (b) Sinusoidal as well as vascular congestion in a liver section. (c) Vascular congestion in cardiac muscle.

Figure 4. Titers of anti-PLPp(139–151) of the IgG1 isotype progressively increased during the course of EAE compared to IgG2a and to total IgE. Sera were collected from mice at different time-points after EAE induction, 2–3 h before challenge with PLPp(139–151). Antibodies to PLPp(139–151) of isotypes (a) IgG1 and (b) IgG2a as well as (c) total IgE were measured by ELISA. Each data point represents the antibody titer of an individual mouse, at the time-point indicated, at a dilution of 1:100 for IgG1 and IgG2a and of 1:20 for IgE. (nms, normal mouse serum.)



variation in these mice could explain why anaphylactic shock appeared in C57BL/6 mice only after repeated challenges with a higher dose of MOG(35–55). Nevertheless, these results clearly show that another self-peptide, MOG(35–55), was able to induce immediate hypersensitivity in a mouse strain (C57BL/6) that can present this antigen to the immune system. Immunization of C57BL/6 mice with PLPp(139–151) in CFA did not induce immediate hypersensitivity, as it did in SJL/J mice, as shown by the absence of allergic reactions (0 of 10 mice) after challenge with repeated doses of PLPp(139–151). These experiments also rule out the possibility that anaphylaxis developed because of some contaminant in the antigen preparation. This is because PLPp(139–151), though able to induce anaphylaxis in SJL/J mice, was not able to do so in C57BL/6 mice.

Role of thymic tolerance in allergy to self

The peptides tested, PLPp(139–151) and MOG(35–55), are self-antigens that are found in the myelin sheath. PLPp(139–151) is not expressed in the thymus and thus does not produce thymic, or central, tolerance. There is no evidence that MOG is expressed in the thymus of mice (C. Bernard, personal communication). We therefore considered

whether there was a correlation between the ability a self-antigen to induce thymic tolerance and the ability to induce anaphylaxis. To evaluate this possibility, we tested two self-antigens that are expressed in thymus and induce some thymic tolerance. PLPp(178–191) is expressed in the thymus of SJL/J mice, where it induces some degree of thymic tolerance²⁸. We found that although PLPp(178–191) binds to I-A^s and induces EAE, it did not induce anaphylaxis (Table 1 and Fig. 6).

Similarly myelin basic protein (MBP) acetylated 1–11 peptide, MBPac(1–11) is expressed in the thymus of PL/J mice^{31,32}. With peptide-MHC multimers it has been shown that MBPac(1–11)-specific T cells are below the limit of detection in PL/J mice, which suggests that they have been deleted by thymic selection³³. However, after immunization with MBPac(1–11), some residual T cells undergo rapid expansion and can be easily detected in the periphery. T cell interaction with

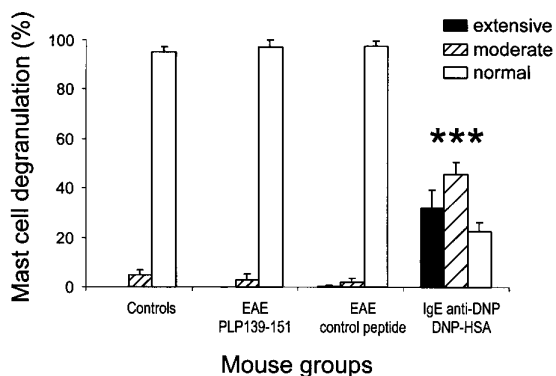


Figure 5. Mice with EAE that develop anaphylactic shock after challenge with native PLPp(139–151) exhibit a much lower mast cell degranulation compared to mice that developed IgE-dependent anaphylaxis. SJL/J female mice in which EAE had been induced 3 weeks previously with native PLPp(139–151) were challenged via the i.p. route with this same peptide (EAE PLP139–151) or with a control peptide (EAE control peptide). For IgE-dependent anaphylaxis, SJL/J female mice that had received anti-DNP IgE 24 h previously were challenged with DNP-HSA (IgE anti-DNP DNP-HSA). As control, tissues from strain-, age- and gender-matched naïve mice were collected (control). Epon-embedded Giemsa-stained sections that were 1 μ m thick were examined to assess the extent of mast cell degranulation in the tissues (ears). Data are the mean $\pm$ s.e.m. values from five mice per group. (*** P < 0.001 by the χ^2 test data from IgE-anti-DNP-sensitized, DNP-challenged mice versus data from any of the other groups of mice.)

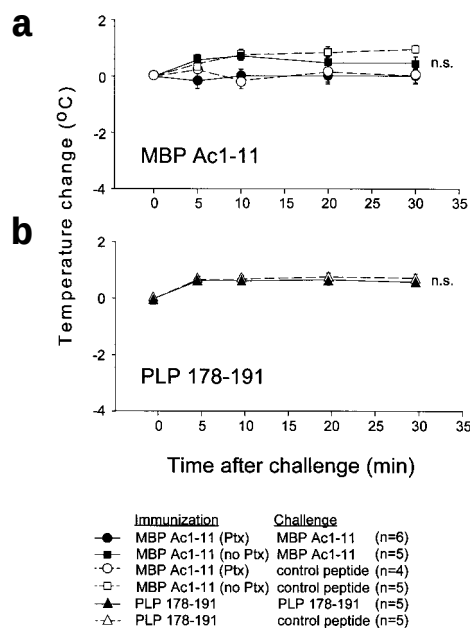


Figure 6. Absence of allergic reactions after challenge with MBPac(1–11) or with PLPp(178–191) is confirmed by the absence of changes in body temperature. (a) PL/J female mice immunized with MBPac(1–11) with (Ptx) or without (no Ptx) pertussis toxin (which was used as an adjuvant) were challenged with MBPac(1–11) or with a control peptide. (b) SJL/J female mice immunized with PLPp(178–191) were challenged with PLPp(178–191) or with a control peptide. (a,b) Data were obtained from measurement of body temperature during the third weekly challenge and the number of mice in each group are shown. (n.s., nonsignificant differences, where P > 0.05, as shown by ANOVA tests comparing challenge with myelin-specific peptides versus challenge with control peptide.)

**Table 2. Treatment of EAE with cyproheptadine**

Treatment	Incidence (%)	Disease score (at day 13) ^a	Disease onset (day) ^a	Peak disease severity ^a
Cyproheptadine ^b	80 (8/10) ^c	0.8±0.4 ^d	13±0.8 ^e	1.8±0.4 ^f
PBS	100 (10/10)	2.4±0.4	11.3±0.5	3.1±0.3

^aData shown as mean±s.e.m values. ^bCyproheptadine was administered i.p., 15 mg/kg/day in 0.5 ml of PBS.

^cNumbers in parentheses are the number of sick animals of the total tested. ^dP=0.01, ^eP=0.05, ^fP=0.03. All *P* values are in comparison to the PBS-treated group as assessed by Student's *t* test.

MBPAC(1–11) is a low-avidity interaction that permits escape from central tolerance^{34–36}. Immunization with MBPAC(1–11) effectively triggered EAE but did not induce anaphylaxis (Table 1 and Fig. 6). Thus, with two examples of self-antigens, PLPp(178–191) and MBPAC(1–11), which are expressed in thymus and do induce some degree of thymic tolerance, there appears to be resistance to the induction of anaphylaxis, even after self-tolerance is broken and EAE is induced.

Potential role of histamine and serotonin

Suppression of EAE with inhibitors of mast cell degranulation or with antagonists of histamine and serotonin (which can be derived from mast cells or other sources) has been reported in a passive EAE model in rats¹² and in an active EAE model in mice, where *Bordetella pertussis* was used as an adjuvant¹¹. We decided to investigate the potential involvement of histamine or serotonin in the development of PLPp(139–151)-induced EAE in our model (in which *Bordetella pertussis* was not used). Ten SJL/J mice were treated daily with cyproheptadine, an anti-histamine–anti-serotonin drug. Amelioration of acute clinical disease was observed in the cyproheptadine-treated group, compared with the control PBS-treated group (Table 2). The onset of disease was delayed compared to the control PBS group (*P*<0.05) and mean peak disease-severity (*P*<0.03) and mean disease score (*P*<0.01) were reduced.

Discussion

The findings reported here show that, after recovery from EAE, mice can exhibit severe allergic reactions, including fatal anaphylaxis, to a self-myelin peptide. The mechanisms that account for the development of such responsiveness are not fully understood. It has been reported that, during the course of EAE, there is a significant decline in T_H1-type cytokine secretion, although T_H2-type cytokine secretion rises³⁷. IL-4, in particular, peaks during the remission of EAE³⁸. We hypothesize that the elevated secretion of IL-4 during the remission phase of EAE promotes the production of IgG1 antibodies to PLPp(139–151) that capable of mediating anaphylaxis³⁹.

Allergy is classically defined as an immunological reaction to a foreign antigen⁵. Immediate hypersensitivity reactions have been reported in response to the administration of peptide competitors, which contain some D-amino acids, for I-A in the treatment of insulin-dependent diabetes in the nonobese diabetic (NOD) mouse²⁶. The peptides used, however, were not self-peptides. There are reports of anaphylaxis in response to “self-antigens” such as insulin and factor VIII. However, in each of these reports, it appears that a nonself molecule may actually have triggered anaphylaxis. Anaphylaxis in response to human insulin preparations that had been modified by protamine have been reported^{40,41}. However, radioallergosorbent tests revealed that the IgE reaction was directed to protamine and not to insulin^{40,41}. Anaphylaxis has also been reported in response to factor VIII, administered as either the purified or the recombinant protein. But both

preparations required purification by affinity chromatography with a mouse-derived monoclonal antibody to factor VIII or to von Willebrand factor^{42,43}. The reactions to factor VIII may have been due to a human anti-mouse response or to preexistent “natural” antibodies to mouse⁴⁴. In addition, in subjects that are deficient in factor VIII, the factor VIII itself may have been “foreign”.

Our findings show that allergic responses, which are typically T_H2-mediated, can be

induced in mice with EAE, despite the well known T_H1 bias of this disease. Indeed, even CFA, which is well known to polarize the immune response towards T_H1, could be used to induce immediate hypersensitivity. As an aside, a paucity of allergic disease in MS patients, compared to a control cohort, has been reported⁴⁵. We found that allergic responses could not be elicited 2 weeks after EAE induction, during the peak of disease. Instead, they appeared only after 3 weeks, during the recovery from the acute attack or at about the time of the first relapse, when T_H2 responses are known to occur³⁸. This suggests that immediate hypersensitivity reactions that are strong enough to produce anaphylaxis generally occur late in the course of this EAE model. On the other hand, the findings that treatment with cyproheptadine could significantly reduce mean disease score at day 13, reduce mean disease severity and delay mean day of disease onset raise the possibility that allergic mechanisms may promote the development and severity of EAE. Nevertheless, cyproheptadine treatment may have effects on EAE that are unrelated to allergic mechanisms, for example, the inhibition of actions of histamine or serotonin that is derived from sources other than mast cells.

In addition to their implications for understanding the pathogenesis of EAE in mice, our findings offer a cautionary tale with regard to efforts to develop immune therapies for MS that aim to shift a pathogenic T_H1 response towards T_H2 immunity. Indeed, in a recent phase II clinical trial in which MS patients received an altered peptide ligand (APL) from an epitope of myelin basic protein, repeated injections of the APL induced immediate hypersensitivity reactions in 9% of the subjects⁴⁶. Although none of these patients exhibited anaphylaxis, the findings support our data in that T_H2 immunity to self or “altered self” molecules may produce a spectrum of undesirable reactions, including allergy⁴⁶ and, in marmoset EAE, a worsening of disease⁴⁷. In MS patients, use of APLs may reduce disease activity on magnetic resonance (MR) scans. Allergic reactions were not associated with a worsening of MS⁴⁶.

Myelin antigens, including PLP, are expressed in the thymus of normal mice during development and tolerance to them may develop at this site²⁸. It is worth noting that only the DM-20 isoform of PLP is expressed in the thymus of SJL/J mice and the PLPp(139–151) fragment is expressed in the white matter of the brain. During EAE an immune response to this epitope, which lies outside of the region encoded by DM-20^{28,48,49}, is elicited. Hence tolerance to this epitope may not have

Table 3. Myelin antigens that cause anaphylaxis

Antigens	Causes EAE	Expressed in thymus	Causes anaphylaxis
PLPp(139–151)	Yes	No	Yes
MOG(35–55)	Yes	No	Yes
PLPp(178–191)	Yes	Yes	No
MBPAC(1–11)	Yes	Yes	No



developed in the thymus, which may explain why profound allergic reactions can develop to this self-constituent⁴⁹. In support of this hypothesis, two antigens—MBPac (1–11) and PLPp(178–191), which are expressed in the thymus and induce central tolerance—did not induce anaphylaxis, even after self-tolerance was broken and EAE was induced (Table 3).

Ehrlich's "horror autotoxicus" may thus involve allergy to self, as well as autoimmunity to self-antigens that escape thymic tolerance. Recognition of self-molecules may lead therefore to organ-specific autoimmunity, systemic autoimmunity, protective autoimmunity⁵⁰ and allergy.

Methods

Immunization protocol. EAE was induced in 8- to 12-week-old SJL/J female mice (The Jackson Laboratory, Bar Harbor, ME) with PLPp(139–151) as described⁵¹ but without the use of *Bordetella pertussis* toxin. Mice were scored as follows: 0, healthy; 1, tail weakness or paralysis; 2, paraparesis (incomplete paralysis of one or two hind limbs or plegia of one hind limb); 3, paraplegia extending to the thorax; 4, forelimb weakness, paralysis with hind limb paraparesis or paraplegia; 5, moribund or dead animal. Blood was collected from the tails of mice 2, 3 and 4 weeks after immunization and analyzed for antibody responses. Mice were challenged 2, 3 or 4 weeks after i.p. immunization with 100 µg of PLPp(139–151) in PBS and observed for 1 h to evaluate symptoms of hypersensitivity. The same protocol was used to immunize and challenge SJL/J, C57BL/6 and PL/J mice (The Jackson Laboratory) with the different myelin, analog or control peptides. In PL/J mice i.v. *Bordetella pertussis* toxin (200 ng) was given as an adjuvant at the moment of the immunization, as well as 2 days later, to induce EAE⁵². To evaluate the efficacy of EAE treatment with anti-histamine and/or anti-serotonin drugs, cyproheptadine (Sigma, St Louis, MO) at a dose of 15 mg/kg was injected daily via the i.p. route in 0.5 ml of PBS. Injections started on day 2 after EAE was induced in SJL/J mice with PLPp(139–151). All animal protocols were approved by the Division of Laboratory Medicine at Stanford and conformed to National Institutes of Health guidelines.

Measurement of peptide-specific serum Ig responses. Peptide-specific IgG1, IgG2a and IgE antibodies were measured in mice sera samples by ELISA. Briefly, for IgG1 and IgG2a ELISA, 96-wells microtiter plates (Immunolon, Dynex, Chantilly, VA) were coated overnight at 4 °C with PLPp(139–151) (0.1 ml) that was diluted in NaHCO₃ buffer (0.1 M at pH 8.0) at a concentration of 0.010 mg/ml. The plates were blocked with 10% fetal calf serum in PBS buffer for 2 h. Samples were diluted in blocking buffer to 1:100 for IgG1 and IgG2a ELISA and to 1:20 for IgE ELISA, then incubated for 2 h at room temperature. Antibody binding was tested by the addition of alkaline phosphatase-conjugated monoclonal goat anti-mouse IgG1, IgG2a or IgE (Southern Biotechnology Associates, Birmingham, AL), each at a 1:1000 dilution in blocking buffer. Enzyme substrate was added and plates were read at 405 nm on a microplate reader. Antibody titer was considered elevated if optical density ≥ 0.1.

Measurement of total IgE production. Total IgE was measured by sandwich ELISA (PharMingen, San Diego, CA) according to the manufacturer's instructions⁵³. Serum samples were analyzed in duplicate at a 1:20 dilution.

Antigens. Peptides were synthesized on a model 9050 peptide synthesizer (Milligen, Burlington, MA) by standard 9-fluorenylmethoxycarbonyl chemistry. Peptides were purified by HPLC and tested for endotoxin with a limulus amoebocyte lysate qualitative test (BioWhittaker, Walkersville, MD). Endotoxin concentrations in all preparations were always <0.03 endotoxin units (EU)/ml. Size was confirmed by mass spectroscopy, which also indicated that no aggregates had formed. Peptides used for experiments were: PLPp(139–151), HSLGKWLGHDPKF; native PLPp(139–151), HCLGKWLGHDPKF; MOG(35–55), MEVGWYRSPFSRVVHLYRNGK; PLPp(178–191), NTWTTCCQSIAPFSK; MBPac(1–11), Ac-ASQKRPSQRHG; Leu¹⁴⁵→Lys¹⁴⁵ analog of PLPp(139–151), HSLGKWKGHPDKF; and control peptide, CAVPLKTMQSGSSFM.

Pathological studies. For routine histopathological studies, the lungs, liver and heart of mice that died after antigen challenge were stored in 10% formalin for 1 week. Sections were then obtained and stained with hematoxylin and eosin. For the quantification of mast cell degranulation, the presence of mast cells and their state of degranulation were assessed in 1-µm, Epon-embedded, Giemsa-stained sections⁵⁴. Tissues were removed and fixed, as described⁵⁴, immediately after death. Death was induced by antigen challenge with the native PLPp(139–151) or IgE anti-DNP + DNP-HSA or, in mice that received control peptide or survived challenge with the native PLPp(139–151) or dinitrophenyl-conjugated human serum albumin (DNP-HSA), by CO₂ inhalation. As a normal control, tissues from strain-, age- and gender-matched normal mice killed by CO₂ inhalation were also collected. Sections of the ear skin dermis were examined by an observer who was unaware of the identity of individual sections and evaluated for mast cell number and the extent of mast cell degranulation as described⁵⁴. Mast cells were classified as extensively degranulated, moderately degranulated or normal as described⁵⁴.

Elicitation of passive systemic anaphylaxis. Mice received an i.p. injection of anti-DNP IgE (20 µg) dissolved in HMEM (200 µl, Gibco-BRL, Gaithersburg, MD) that contained PIPES buffer (0.47 g/l, Sigma) and were challenged 24 h later by an i.v. injection of DNP-HSA (200 µg, Sigma) in saline (200 µl)⁵.

Determination of airway responsiveness. Airway responses were assessed using a single-chamber whole-body plethysmograph (Buxco, Troy, NY) as described⁵⁵. Enhanced respiratory pause (Penh) was used as the measure of airway resistance. Mice were placed individually in the plethysmograph, baseline readings were then taken and averaged for 10 min. After challenge with various peptides or i.p. DNP-HSA, readings were taken and averaged every 3 min for 30 min. For each time point the Penh values measured during each 3-min sequence are expressed as a percentage of baseline Penh values.

Temperature measurement. Baseline temperature was established for each animal using a rectal probe (Physitemp, Clifton, NJ). Rectal temperature was recorded in each mouse 5 min after each challenge with i.p. peptide or i.v. DNP-HSA.

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