and/or to apoptosis, as observed in osteoclasts [2]. As described above, the relative potencies with which NBPs inhibit FPP synthase in vitro are zoledronate >> risedronate > alendronate > pamidronate. Thus, zoledronate has the strongest activity for the inhibition of FPP synthase, and this may explain why this agent has the strongest necrotic action.

However, the potency with which an NBP induces inflammation and/or necrosis may also be determined by the amount of it that is incorporated into cells within a given tissue. The relative potencies with which NBPs other than zoledronate induced necrosis in the ear skin were pamidronate ≥ alendronate > risedronate, suggesting that pamidronate may enter the cells in the ear tissue more easily than alendronate and risedronate. Taking into consideration the length of time for which the various NBPs have been available for clinical use (see "approval years" in Fig. 1) and the number of ONJ cases reported for each of them in those periods, it seems likely that the relative potencies with which NBPs induce ONJ are zoledronate >> pamidronate > alendronate > risedronate [16]. This is nearly in agreement with the order of relative potencies obtained for the necrotic actions observed in the present study. Inhibition of FPP synthase by alendronate has been suggested to lead to inflammatory reactions (including the induction of histidine decarboxylase) of various degrees in mice [39], and the relative potencies for the induction of histidine decarboxylase in mouse tissues (liver, lung, and spleen) are reportedly alendronate > pamidronate [24]. These results suggest that (i) the amounts of an NBP incorporated into cells may be variable among different types of cells or tissues, and (ii) the step entailing incorporation of NBPs into cells is very important for the induction of inflammation and/or necrosis.

Mechanism underlying inhibition by clodronate.

We previously reported that clodronate can prevent or reduce the inflammatory effects of various NBPs in various tissues in mice (both clodronate and NBPs were intraperitoneally injected), and this protective effect was greatest when clodronate was injected simultaneously with the NBP [29]. As described above, under neutral conditions, NBPs may enter various cells (possibly through a specific mechanism), but clodronate (a non-NBP) cannot enter them (this is an important property of clodronate). Nevertheless, we found that clodronate strongly inhibited the necrotic actions (but not ABREs) of NBPs in mouse ears. Thus, one explanation is that clodronate may inhibit the entry of NBPs into cells. This could mean that the administration of clodronate with an NBP would make the NBP act selectively on bone (i.e., osteoclasts). Identification of the specific mechanism or molecules responsible for the entry of NBPs into cells is the next concern of our laboratory.

Possible clinical application of the findings.

The ABRE of clodronate is much weaker than those of the NBPs (Fig. 1). Hence, NBPs (such as zoledronate, risedronate, alendronate, and pamidronate) are in clinical use worldwide, whereas the use of clodronate has been approved in only a few places (Europe and Canada). In the present study, we found that clodronate strongly inhibited the inflammatory and necrotic actions of NBPs, and that (very conveniently) the ABREs of NBPs were almost unaffected by clodronate. Indeed, their combined administration sometimes resulted in an enhanced ABRE (Fig. 5). This finding suggests that combined administration of an NBP with clodronate may be effective at reducing or preventing the inflammatory and/or necrotic actions of NBPs while retaining their powerful ABREs. Recent studies have shown that both NBPs and clodronate have anti-tumor activities against bone-metastatic tumors [4, 5, 40]. As mentioned above, the molecular mechanisms

by which NBPs and clodronate exert their cytotoxic effects are different. Thus, if both an NBP and clodronate are simultaneously incorporated into tumor cells on bone surfaces, it might be expected that their anti-tumor effects would at least summate. This very interesting hypothesis will need to be tested in future studies. Whether or not that hypothesis proves to be correct, the present results support the use of clodronate as a combination drug for use with NBPs. Another possibility that might be considered is the use of clodronate as a substitution drug for NBPs in patients who have already used NBPs and are at risk of ONJ.

Conclusion.

Although the experiment used in the present study to examine necrosis involves a model of necrosis (not of ONJ), the results, taken together with others already in the literature, suggest that (i) clodronate inhibits the inflammatory and necrotic actions of NBPs by reducing the incorporation of NBPs into cells related to these reactions, (ii) clodronate could potentially be used as a combination drug with an NBP to limit or prevent the necrotic actions of NBPs while retaining their ABREs, and (iii) clodronate might also be useful as a substitution drug for NBPs in patients at risk of ONJ.

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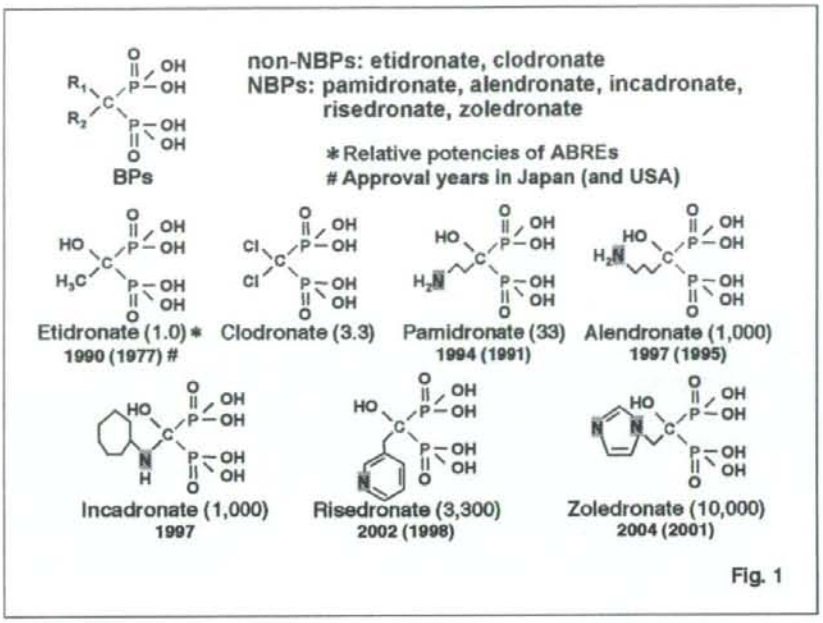

Fig. 1

Fig. 1. Structures of non-nitrogen-containing bisphosphonates (non-NBPs) (etidronate and clodronate) and nitrogen-containing bisphosphonates (NBPs) (pamidronate, alendronate, incadronate, risedronate, and zoledronate). Also shown are their relative potencies in terms of anti-born-resorptive effects (ABREs) [1] and their approval years in Japan and USA.

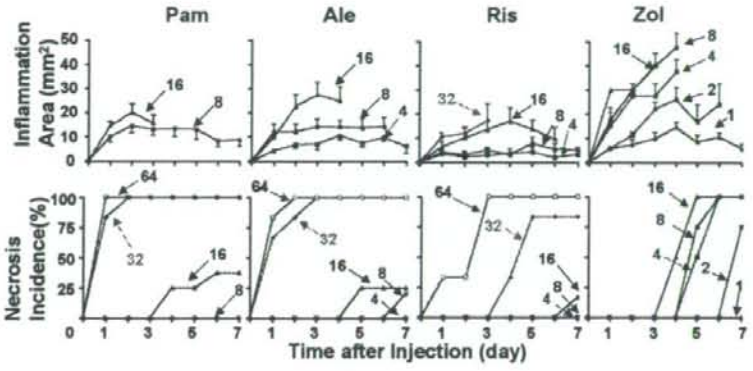

Fig. 2

Fig. 2. Ear inflammation and necrosis induced by nitrogen-containing bisphosphonates (NBPs). Various concentrations of NBPs [pamidronate (Pam), alendronate (Ale), risedronate (Ris), or zoledronate (Zol)] were subcutaneously injected into ear-pinnas (20 μl/ear) (i.e., injection of 1 mM solution corresponding to 20 nmol/ear). The values are mean ± SD from 6 ears. The numbers attached to the respective data indicate mM of the relevant NBP. Saline injection produced no detectable inflammation, or necrosis, at the indicated times (data not shown).

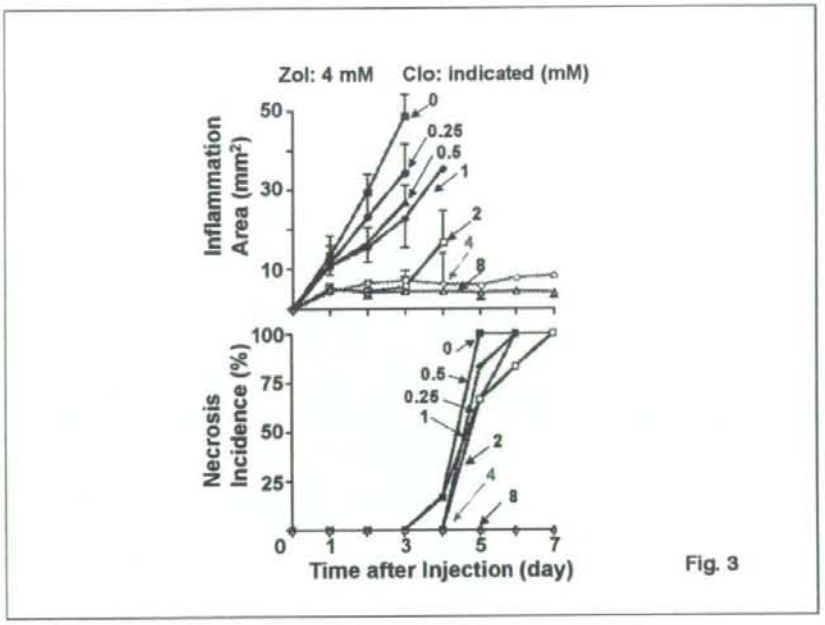

Fig. 3. Effects of clodronate (Clo) on the ear inflammation and necrosis induced by zoledronate. Solutions containing zoledronate (4 mM) plus various concentrations of clodronate (indicated in the figure) were subcutaneously injected into ear pinnas (20 μl/ear) (i.e., 80 nmol/ear). The values are mean ± SD from 6 ears. The numbers attached to the respective data indicate mM of clodronate.

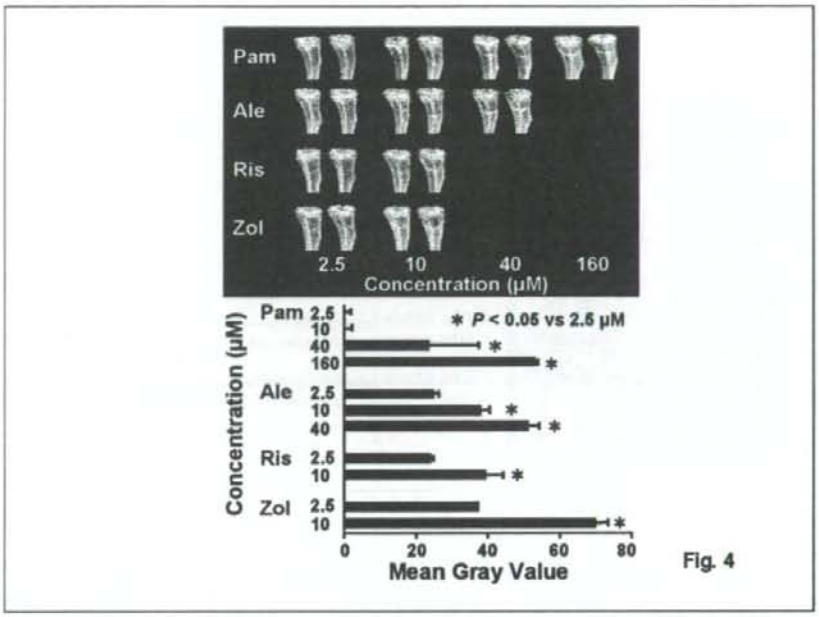

Fig. 4. Bisphosphonate (BP) band formation by nitrogen-containing bisphosphonates (NBPs). A solution containing the indicated concentration of pamidronate (Pam), alendronate (Ale), risedronate (Ris), or zoledronate (Zol) was intraperitoneally injected (0.2 ml/mouse) (i.e., 0.5-32 nmol/mouse) into young male mice. Two weeks later, tibias were removed and BP bands formed in right tibias were compared. BP bands are shown for 2 of the 3 mice in each group. Mean gray values are means ± SD from 3 mice.

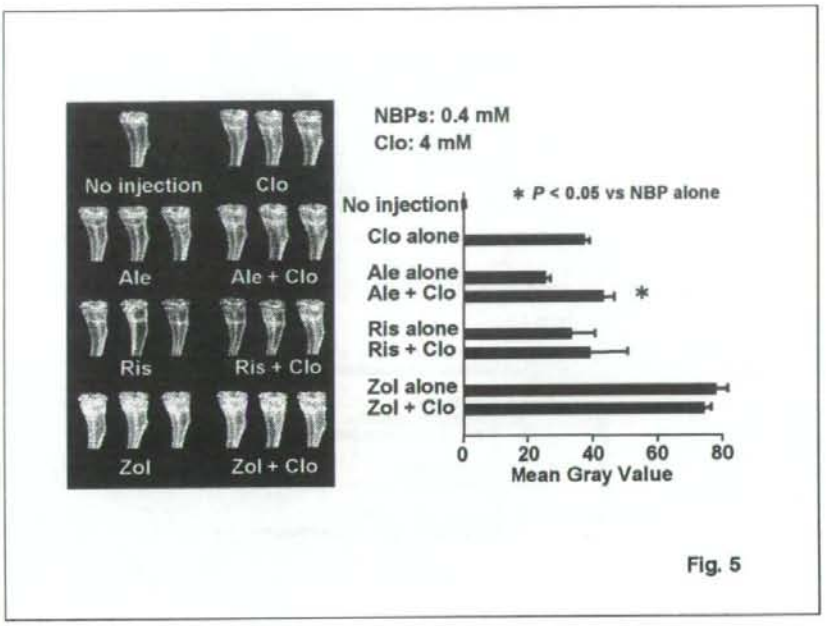

Fig. 5. Effects of 4 mM clodronate (Clo) on the bisphosphonate (BP) band formation induced by 0.4 mM of various nitrogen-containing bisphosphonates (NBPs) [alendronate (Ale), risedronate (Ris), or zoledronate (Zol)]. A mixture containing Clo (4 mM) and an NBP (0.4 mM) was intraperitoneally injected (0.2 ml/mouse) (i.e., Clo 800 nmol/mouse and an NBP 80 nmol/mouse) into young male mice. Two weeks later, tibias were removed and subjected to BP band analysis. BP bands formed in the right tibias are shown for all 3 mice in each group. Mean gray values are means ± SD from 3 mice.

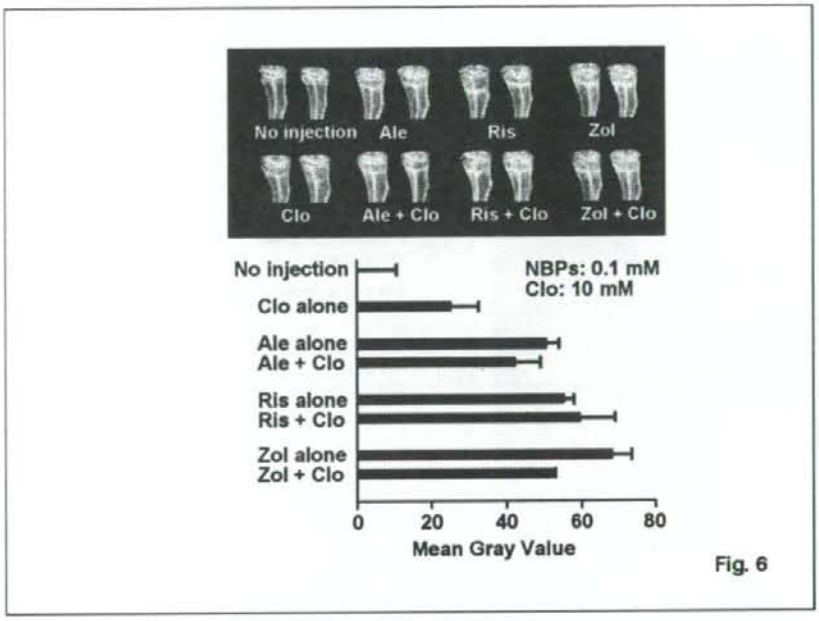

Fig. 6. Effects of 10 mM clodronate (Clo) on the bisphosphonate (BP) band formation induced by 0.1 mM of various nitrogen-containing bisphosphonates (NBPs) [alendronate (Ale), risedronate (Ris), or zoledronate (Zol)]. A mixture containing Clo (10 mM) and an NBP (0.1 mM) was intraperitoneally injected (0.2 ml/mouse) into young male mice (i.e. Clo 2 μmol/mouse and an NBP 0.02 μmol/mouse). Two weeks later, tibias were removed and subjected to BP band analysis. BP bands formed in the right tibias are shown for 2 of the 3 mice in each group. Mean gray values are means ± SD from 3 mice. There was no significant difference between any NBP alone and the same NBP in combination with clodronate.

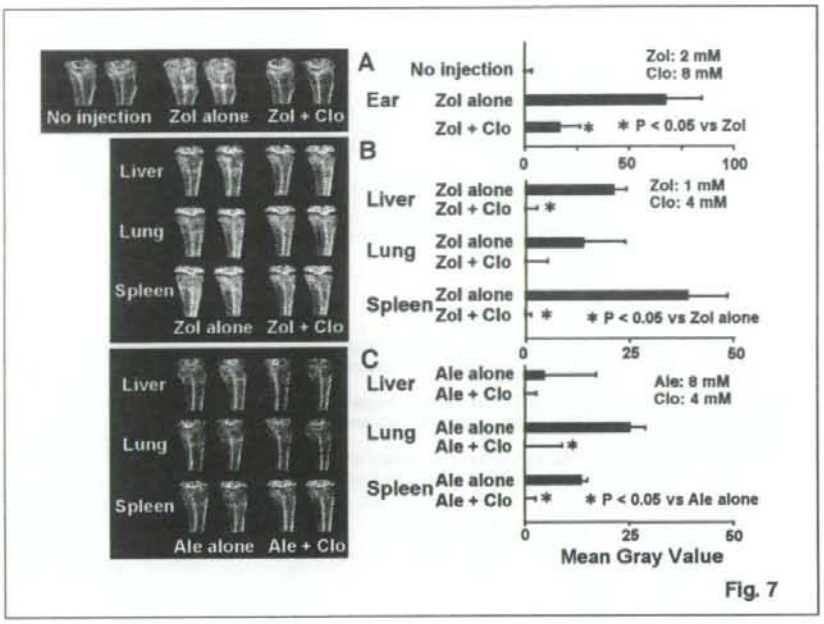

Fig. 7. Effects of clodronate (Clo) on the retention of nitrogen-containing bisphosphonates (NBPs) [zoledronate (Zol) and alendronate (Ale)] in various tissues. A solution containing Clo alone, an NBP alone, or Clo + an NBP was injected into mice (3 mice in each group) as follows: (**A**) Zol (2 mM) or Zol (2 mM) + Clo (8 mM) was subcutaneously injected into the ear-pinna (20 μl/ear), (**B**) Zol (1 mM) or Zol (1 mM) + Clo (4 mM) was injected intraperitoneally (0.2 ml/mouse), or (**C**) Ale (8 mM) or Ale (8 mM) + Clo (4 mM) was injected intraperitoneally (0.2 ml/mouse). Three hours later, ears, livers, lungs, and spleens were removed. The ears, lungs, spleens (those from 3 mice were combined), or livers (portions from each of 3 mice were combined, making a total of 0.5 g) were subjected to preparation of tissue extracts (see Methods). Each tissue extract was injected into young mice (0.5 ml/mouse). Two weeks later, tibias were removed and subjected to bisphosphonate (BP) band analysis. BP bands formed in the right tibias are shown for 2 of the 3 mice in each group. Mean gray values are means ± SD from 3 mice. In these experiments (A, B, and C), tissue extracts from mice injected with Clo alone (4 mM) did not produce detectable BP bands (data not shown).

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Anaphylaxis to a self-peptide in the absence of mast cells or histamine

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Induction of T helper 1 (Th1) to Th2 deviation through administration of self- or altered self-peptides holds promise for treatment of autoimmunity. However, administration of self-peptides in models of autoimmunity can result in anaphylactic reactions. Although both IgE and IgG1 antibodies might be involved in the development of anaphylaxis to myelin peptides in experimental autoimmune encephalomyelitis in mice, the effector cells and molecules involved are not fully understood. Here we show that systemic anaphylaxis to the self-antigen myelin oligodendrocyte glycoprotein (MOG) 35–55 can occur in mice lacking mast cells ($Kit^{W}/Kit^{W\text{-}v}$ mice) or histamine (histidine decarboxylase-deficient mice), but is prevented in mice lacking IL-4. Treatment of mice with CV6209, a platelet-activating factor antagonist, slightly reduced the incidence of anaphylaxis to self-MOG35–55 in this model, but more effectively protected mice against anaphylaxis to this peptide when self-MOG35–55 was administered in a different immunization protocol that omitted the use of *Bordetella pertussis* toxin as an adjuvant at the time of immunization. Thus, anaphylactic reactions to self-MOG can occur in the absence of mast cells or histamine, key elements of the classical IgE-, mast cell-, and histamine-dependent pathway of anaphylaxis.

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anaphylaxis; experimental autoimmune encephalomyelitis; histamine; IL-4; mast cells; self-peptide

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are immune-mediated diseases of the central nervous system in which T helper 1 (Th1) $CD4^+$ cells reacting to myelin are considered to have a leading role.[1] Therapeutic approaches aimed to shift the immune response against myelin antigens from Th1 to Th2 by i.v. injection(s) of soluble myelin peptides or proteins have been shown to induce antigen-specific immune tolerance and amelioration of EAE (reviewed in Fontoura *et al*[2]). However, such therapeutic approaches have substantial potential risk.[3] Mice with EAE can develop fatal anaphylaxis on re-exposure to certain self-peptides of the myelin sheath.[4–8] Repeated administration of self- or altered self-peptides resulted in immediate hypersensitivity reactions in a subset of patients with MS treated with NB5788, an altered peptide ligand (APL) of myelin basic protein,[9] and in 9% of MS patients treated with glatiramer acetate, a random four-amino-acid non-self-polymer widely used for the treatment of this disease.[10]

Anaphylaxis in humans is thought to be mediated largely (if not solely) by a pathway consisting of IgE antibodies, the high-affinity receptor for IgE (FcεRI), mast cells and basophils, and histamine is thought to represent a major mediator of such anaphylactic reactions (reviewed in Kawakami *et al*[11]). However, in mice, anaphylaxis can be elicited either by IgE- or by IgG1-dependent mechanisms.[12] The second, IgG1-dependent, pathway is IgE-independent, but requires the low-affinity receptor for IgG (FcγRIII) and, depending on the setting, can involve basophils and perhaps macrophages, and platelet-activating factor (PAF).[12–15] In a chronic EAE model, induced in C57BL/6 mice by immunization with the self-myelin peptide oligodendrocyte glycoprotein (MOG)

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35–55, we and others found that anaphylaxis to self was abrogated in mice lacking the γ-chain common to Fc receptors (FcR),[5,8] but not in mice lacking the α chain of FcγRIII (FcγRIII α chain$^{-/-}$), suggesting that the IgE-FcεRI-mast cell/basophil–histamine pathway might contribute to the development of anaphylaxis in this model.[5,8] However, except for the findings obtained in various FcR-knockout mice, the effector cells and molecules involved in anaphylaxis to this self-myelin peptide are not fully understood. A better understanding of the mechanisms contributing to such reactions may help in the development of strategies aimed at preventing and/or treating undesired allergic reactions associated with peptide therapies for autoimmune disorders. We therefore analyzed the importance of mast cells, histamine and IL-4, key elements of IgE-dependent anaphylaxis, in the development of anaphylaxis to self-MOG35–55 in a chronic model of EAE. We also attempted to prevent anaphylaxis to this self-peptide by using CV6209, an antagonist of PAF, a key mediator in the development of IgG1-dependent anaphylaxis. Here we show that anaphylaxis to self-MOG35–55 requires neither mast cells nor histamine, but it does require IL-4. Our results also suggest that PAF can contribute to the development of anaphylaxis to this self-peptide, but PAF probably is not the only mediator involved.

MATERIALS AND METHODS

Mice

Eight- to twelve-week-old mast cell-deficient WBB6F$_1$-*Kit*$^{W/Wv}$ (*W/W*^{v}) and congenic wild-type (WBB6F$_1$-*Kit*$^{+/+}$) female mice (Jackson Laboratory, ME, USA), C57BL/6J-IL-4$^{-/-}$ and wild-type C57BL/6J mice (Jackson Laboratory), histidine decarboxylase-deficient mice (HDC$^{-/-}$) backcrossed six generations onto C57BL/6 (provided by Dr H Ohtsu, Sendai, Japan)[16] and wild-type C57BL/6N (Charles-Rivers, Calco, Italy) were used in this study. Experiments were conducted in the animal facilities of the Neurological Institute Foundation Carlo Besta or of Stanford University. All procedures involving animals were approved by the ethical committee of the Neurological Institute Foundation Carlo Besta and the Division of Comparative Medicine at Stanford University, and carried out according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC) and the National Institutes of Health guidelines.

Peptides

MOG35–55 (MEVGWYRSPFSRVVHLYRNGK), control peptide-1 (random-CAVPLKTQMSGSSFM), and control peptide-2 (acetylcholine receptor, AchR, 97–116; DGDFAIVKFTKVLLDYTGHI) were synthesized and purified to >95% by analytical reverse-phase HPLC.

Immunization Protocol

EAE was induced as described earlier[5] by subcutaneous immunization with MOG35–55 (100 μg per mouse) in Complete Freund's Adjuvant (CFA; Difco Laboratories, Detroit, MI, USA), containing 4 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). All mice also received two intravenous injections of *Bordetella pertussis* (*B. pertussis*) toxin (200 ng/mouse) (List Biological Laboratories, Campbell, CA, USA) on day 0 and day 2 post immunization. The clinical features of EAE, which developed in all of the mice used in this study, have already been described.[6,17–19] In another model of MOG35–55-induced EAE, mice were immunized without using *B. pertussis* toxin as an adjuvant (see Results section for details).

Induction of Active Systemic Anaphylaxis

We evaluated whether mice exhibited active systemic anaphylaxis by challenging them 6 weeks after induction of EAE with an i.p. injection of MOG35–55 (100 μg) dissolved in PBS (100 μl). Control mice were challenged with an i.p. injection of a control peptide at the same dose. Mice were observed for 1 h after peptide challenge for clinical signs of anaphylaxis, and for each mouse body temperature was recorded with a rectal probe (Physitemp Instruments, Clifton, NJ, USA) at baseline and at 5, 10, 20, 30, and 60 min after challenge.[5] Data are shown as mean ± s.e.m. for all mice in each group. In the groups in which some mice died during the observation period of 1 h, mean values were determined on the basis of data for the surviving mice. Mice were considered to have anaphylaxis when suggestive clinical signs (ie, reddening of the skin, piloerection, prostration, reduced or lack of response to stimuli, and death) were accompanied by a decrease in body temperature of at least 1°C. In some experiments, mice were treated with an i.p. injection of the PAF antagonist CV6209 (Biomol International Inc., Plymouth Meeting, PA, USA), given at the dose of 100 μg 5 min before peptide challenge, and/or with the histamine receptor 1 (H1R) antagonist triprolidine (Sigma, St Louis, MO, USA), given i.p. at the dose of 200 μg 30 min before peptide challenge. Control mice were treated i.p. with PBS. Reagents were diluted in PBS to a final volume of 200 μl.

Measurement of Serum Ig Responses

Blood was collected from the tail 6 weeks after the induction of EAE and sera were stored at −20°C until analyzed. Peptide-specific IgG1, IgG2a, and IgE antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described.[5,6,16] Briefly, 96-well microtiter plates (Immunol, Thermo Labsystems) were coated overnight at 4°C with MOG35–55 diluted in coating buffer (0.010 mg/ml). Plates were blocked with PBS 10% FCS for 2 h. Samples were diluted in blocking buffer at 1:100 for IgG1 and IgG2a, and at 1:25 for IgE, and antibody binding was tested by the addition of peroxidase-conjugated monoclonal goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL, USA). Enzyme substrate was added and plates were read at 450 nm on a micro plate reader. Total IgE was measured by sandwich ELISA (BD PharMingen) following the manufacturer's instructions.[16]

Statistical Analysis

Differences among groups in the time course of body temperature were examined by ANOVA. Differences among groups in the number of mice exhibiting systemic allergic reactions were analyzed by the Fisher's exact test. Two-tailed student's *t*-test was used to compare results between two groups. In all tests, $P<0.05$ was considered statistically significant.

RESULTS

Expression of Anaphylaxis to MOG35–55 in Mast Cells- or Histidine Decarboxylase-Deficient Mice

$WBB6F_1$-$Kit^{W/Wv}$ (W/W^v) mice, which virtually lack mast cells in all tissues, have been shown to be resistant to the development of IgE-dependent passive systemic anaphylaxis,[12,15,20] but to be susceptible to passive IgG1-mediated or active anaphylaxis.[12,15] Challenge with MOG35–55 6 weeks after peptide immunization induced anaphylaxis in both W/W^v and congenic $Kit^{+/+}$ wild-type mice (Figure 1a and Table 1), indicating that mast cells are not required for the expression of anaphylaxis in this model. Consistent with earlier observations,[12,19] serum titers of total IgE and, to a lesser extent, of peptide-specific IgG1 antibodies were higher in W/W^v *vs* $Kit^{+/+}$ mice (Figure 1b). We failed to detect antigen-specific IgE with our ELISA method in sera of these mice, as well as in those of all of the other strains used in this study.

$HDC^{-/-}$ mice, which are profoundly histamine-deficient in all tissues,[16,21] cannot exhibit hypothermia in association with IgE-dependent passive anaphylaxis.[22] A paucity of mast cells and abnormalities in mast cell cytoplasmic granules have been described among the phenotypic abnormalities in $HDC^{-/-}$ mice,[21] whereas no major differences in basophil number have been observed. We found that $Fc\varepsilon RI^+$, $B220^-$ cells (regarded as basophils) represented $2.9\pm0.5\%$ of the total blood cells in $HDC^{-/-}$ mice and $2.8\pm0.7\%$ in $HDC^{+/+}$ mice (data not shown); similarly, Dr Elke Schneider—Necker Hospital, Paris—has found that $CD49^+$, $Fc\varepsilon RI^+$ cells (regarded as basophils) represented from 0.6 to 1.2% of bone marrow cells in $HDC^{-/-}$ or $HDC^{+/+}$ mice (Elke Schneider, personal communication). Challenge with MOG35–55 induced anaphylaxis in both $HDC^{-/-}$ and wild-type mice, interestingly with $HDC^{-/-}$ mice developing a greater drop in body temperature than wild-type mice (Figure 2a and Table 1). As we reported earlier,[16] total serum IgE titers were significantly increased in $HDC^{-/-}$ *vs* wild-type mice, whereas no significant differences were observed in titers of peptide-specific IgG1 (Figure 2b).

The results obtained in mast cell-deficient W/W^v mice and histamine-deficient $HDC^{-/-}$ mice indicate that neither mast cells nor histamine, key players in IgE-dependent anaphylaxis,[14] are necessary for the development of anaphylaxis to MOG35–55 in mice.

Expression of Anaphylaxis to MOG35–55 in IL-4-Deficient Mice

IL-4 is required for isotype switching to IgE response and can also promote isotype switching to IgG1.[23] Active IgE-dependent anaphylaxis does not develop in IL-4- or IL-4 receptor-deficient mice.[13,14] Moreover, the development of IgG1 antibodies with anaphylactic activity requires IL-4,[24] suggesting that this cytokine is also involved in the development of IgG1-dependent anaphylaxis. As shown in Figure 3a and Table 1, IL-4-deficient mice exhibited little or no anaphylaxis to MOG35–55. As expected in these IL-4-deficient mice, which have a general impairment of Th2

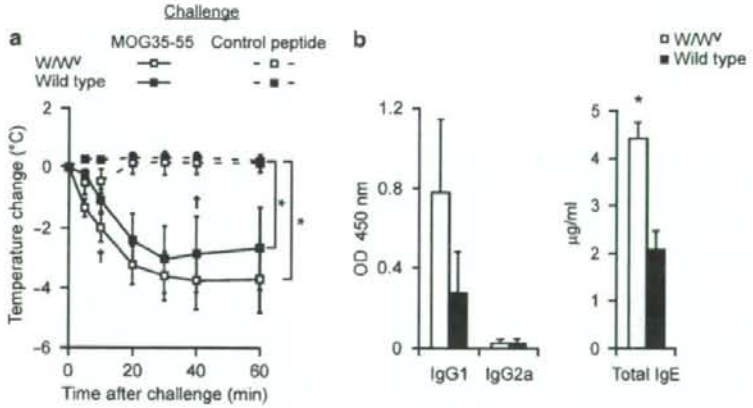

Figure 1 Expression of anaphylaxis to self-MOG35–55 in mast cell-deficient mice. (**a**) Changes in body temperature in mast cell-deficient WBB6F1-$Kit^{W/Wv}$ (W/W^v) and congenic WBB6F1-$Kit^{+/+}$ ($Kit^{+/+}$) mice on i.p. challenge with MOG35–55 or control peptide-1. †One mouse dead from anaphylactic shock at that time point. The exact numbers of challenged mice in the various groups are given in Table 1. *$P<0.001$. (**b**) MOG35–55-specific IgG1 and IgG2a, and total IgE antibody titers in sera of W/W^v and congenic $Kit^{+/+}$ mice ($n=6$–8 mice per group). *$P<0.005$.

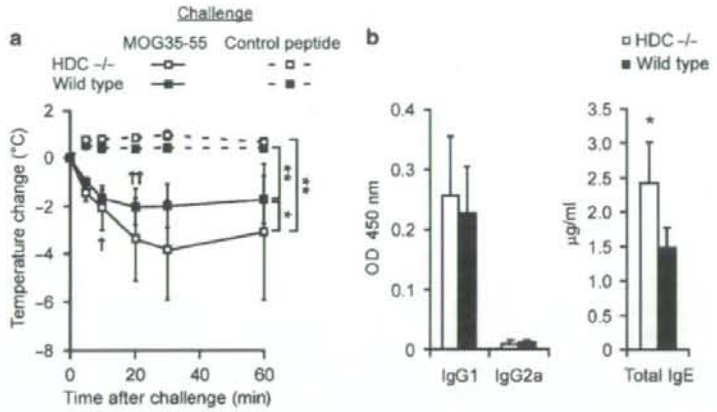

Figure 2 Expression of anaphylaxis to self-MOG35–55 in histamine-deficient mice. (**a**) Changes in body temperature in C57BL/6-HDC$^{-/-}$ (HDC$^{-/-}$) and control C57BL/6 mice on i.p. challenge with MOG35–55 or control peptide-2. †Three and ††four mice dead from anaphylactic shock at that time point. The exact numbers of challenged mice in the various groups are given in Table 1. *$P<0.05$ and **$P<0.001$. (**b**) MOG35–55-specific IgG1 and IgG2a, and total IgE antibody titers in sera of HDC$^{-/-}$ and control C57BL/6 mice ($n=8$–11 mice per group). *$P<0.05$.

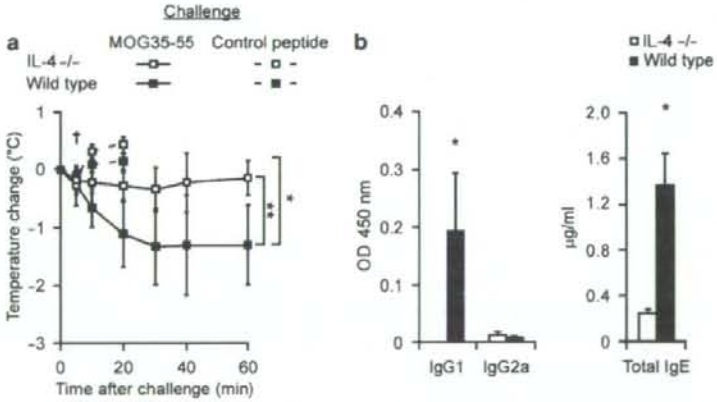

Figure 3 Expression of anaphylaxis to self-MOG35–55 in IL-4-deficient mice. (**a**) Changes in body temperature in C57BL/6-IL-4$^{-/-}$ (IL-4$^{-/-}$) and control C57BL/6 mice on i.p. challenge with MOG35–55 or control peptide-1. †One mouse dead at that time point. *$P<0.05$ and **$P<0.005$. The exact numbers of mice in the various groups are given in Table 1. (**b**) MOG35–55-specific IgG1 and IgG2a, and total IgE antibody titers in sera of IL-4$^{-/-}$ and control C57BL/6 mice ($n=5$–7 mice per group). *$P<0.005$.

responses and a significant reduction of IgE and IgG1 antibody production in response to nematode infection,[25–27] total IgE antibody titers were significantly lower in the sera of IL-4$^{-/-}$ *vs* wild-type mice, and peptide-specific IgG1 antibodies were below the detection limit (Figure 3b). Accordingly, we hypothesize that the resistance against anaphylaxis to MOG35–55 in these IL-4-knockout mice reflects, at least in part, the absence of a peptide-specific IgG1 (or IgG1 and IgE) response. Indeed, anaphylaxis to MOG35–55 requires antibody binding to FcR, as anaphylaxis to the peptide is abrogated,[5] or significantly reduced,[8] in mice that lack the FcR common γ-chain, and therefore lack FcεRI (that binds IgE) and FcγRIII (that binds IgG1 immune complexes). However, other mechanisms might also have contributed to resistance against anaphylaxis observed in IL-4$^{-/-}$ mice, such as reduced sensitivity to mediators of anaphylaxis (eg, histamine, PAF, etc) in the absence of this cytokine (reviewed in Finkelman *et al*[13]) or the absence of antigen binding to basophils, which has recently been described in these knockout mice.[28]

Effects of Blockade of PAF on the Incidence and Severity of Anaphylaxis to MOG35–55

PAF has been shown to play a major role in IgG-dependent anaphylaxis in the mouse,[14] and blockade of this mediator can prevent IgG1-mediated passive anaphylaxis in mice.[15] As

Table 1 Anaphylaxis in mast cell-, histamine- or IL-4-deficient mice and their controls on challenge with MOG35–55

Mouse strain	I.p. challenge (100 μg per mouse)	No. of mice with anaphylaxis (%)	No. of mice dead from anaphylaxis	Lowest drop in body temperature[a]	
				All mice	Mice with anaphylaxis
W/W^v	MOG 35–55	7/9 (78)	1/9	−3.9 ± 0.9[b]	−5.0 ± 0.8
	control peptide 1	1/4 (25)	0/4	−0.7 ± 0.3	−1.3
Kit$^{+/+}$	MOG 35–55	5/10 (50)	1/10	−3.7 ± 1.2[c]	−5.9 ± 0.9
	control peptide 1	0/5	0/5	−0.1 ± 0.1	—
HDC$^{-/-}$	MOG 35–55	7/8 (88)	3/8	−3.8 ± 1.6[c]	−4.3 ± 1.8
	control peptide 2	0/8	0/8	0	—
C57BL/6	MOG 35–55	11/14 (79)	4/14	−2.5 ± 0.6[b]	−3.1 ± 0.7
	control peptide 2	0/14	0/14	−0.1 ± 0.0	—
IL-4$^{-/-}$	MOG 35–55	1/10[d] (10)	0/10	−0.7 ± 0.3[e]	−3.4
	control peptide 1	1/7 (14)	1/7	−0.4 ± 0.3	−2.2
C57BL/6	MOG 35–55	7/15 (47)	0/15	−2.1 ± 0.6[b]	−3.8 ± 0.7
	control peptide 1	0/7	0/7	−0.2 ± 0.1	—

[a]Data represent mean ± s.e.m.
[b]$P < 0.01$ by Student's *t*-test *vs* mice challenged with control peptide.
[c]$P < 0.05$ by Student's *t*-test *vs* mice challenged with control peptide.
[d]$P < 0.05$ by Fisher's exact test *vs* corresponding control C57BL/6 mice challenged with MOG35–55.
[e]$P > 0.05$ by Student's *t*-test *vs* mice challenged with control peptide and $P < 0.05$ by Student's *t*-test *vs* wild-type C57BL/6 mice challenged with MOG35–55.

we observed that anaphylaxis to MOG35–55 was not prevented in mice lacking histamine (Figure 2a) or mast cells (Figure 1a), we analyzed the role of PAF in the development of anaphylaxis to this self-peptide. As shown in Table 2, treatment with the PAF antagonist CV6209 slightly reduced the incidence of anaphylaxis to self-MOG35–55 (although this effect was not statistically significant in the number of mice studied), but such treatment did not affect the severity of anaphylaxis, as defined by the mean lowest body temperature developed by mice treated with CV6209 compared with mice treated with vehicle. Treatment with H1R receptor antagonist triprolidine was not effective in preventing anaphylaxis to self-MOG35–55, confirming the results obtained in HDC$^{-/-}$ mice, whereas treatment with CV6209 and triprolidine in combination was slightly more effective than treatment with CV6209 alone in reducing the incidence of anaphylaxis to this peptide (Table 2). Although the effects detected did not achieve statistical significance, these results suggest that PAF plays a role in anaphylaxis to MOG35–55, but is not the only mediator involved.

We also evaluated the role of PAF in another model of anaphylaxis to MOG35–55, elicited in C57BL/6 mice that were immunized with this peptide in CFA, but without *B. pertussis* toxin (PTX) used as an adjuvant. As reported earlier,[6] under this protocol of immunization, mice developed anaphylaxis with higher doses of antigen as compared with mice that had received PTX at the time of peptide immunization (500 *vs* 100 μg). Also, compared with mice that received PTX at the time of immunization, these mice had significantly lower titers of total IgE in their serum (total IgE levels were 204.2 ± 13 ng/ml in mice that did not receive PTX *vs* 1.0 ± 0.25 μg/ml in mice that received PTX; $P = 0.007$) and higher antigen-specific IgG1 titers (OD was 1.137 ± 0.191 in mice that did not receive PTX *vs* 0.724 ± 0.074 in mice that received PTX; $P = 0.037$). In mice subjected to this immunization protocol (Table 3), treatment with the PAF antagonist was more effective in reducing the severity of anaphylaxis to MOG35–55 than in our standard protocol. Anaphylaxis occurred in 3 of 10 mice treated with CV6209 *vs* 6 of 10 mice treated with vehicle ($P = 0.353$), and the mean maximum drop in body temperature was 0.8 ± 0.4° in mice treated with CV6209 *vs* 2.6 ± 0.7° in mice treated with vehicle ($P = 0.037$). Moreover, in this immunization protocol, treatment with both CV6209 and triprolidine was more

Table 2 Anaphylaxis in wild-type C57Bl/6 mice treated with a PAF antagonist and/or an H1R antagonist before peptide challenge (in mice immunized to MOG35–55 with *Bordetella pertussis* toxin)

Treatment	No. of mice with anaphylaxis (%)	No. of mice dead from anaphylaxis	Lowest drop in body temperature[a]	
			All mice	Mice with anaphylaxis
CV6209	3/8 (37)	1/8	−2.0 ± 1.0	−4.9 ± 1.5
Triprolidine	4/8 (50)	0/8	−2.2 ± 1.0	−4.3 ± 1.3
CV6209+Triprolidine	2/8 (25)	1/8	−1.3 ± 0.7	−4.2 ± 2.0
Vehicle	6/10 (60)	2/10	−2.0 ± 0.8	−3.2 ± 1.0

[a]Data represent mean ± s.e.m.

Table 3 Anaphylactic shock in wild-type C57Bl/6 mice treated with a PAF antagonist and/or an H1R antagonist before peptide challenge (in mice immunized to MOG35–55 without *Bordetella pertussis* toxin)

Treatment	No. of mice with anaphylaxis (%)	No. of mice dead from anaphylaxis	Lowest drop in body temperature[a]	
			All mice	Mice with anaphylaxis
CV6209	3/10 (30)	0/10	−0.8 ± 0.4[b]	−2.2 ± 0.9
Triprolidine	4/10 (40)	0/10	−1.5 ± 0.6	−3.4 ± 1.0
CV6209+Triprolidine	1/10 (10)[c]	0/10	−0.3 ± 0.2[b]	−1.8 ± 0.0
Vehicle	6/10 (60)	0/10	−2.6 ± 0.7	−4.2 ± 0.6

[a]Data represent mean ± s.e.m.
[b]$P<0.05$ by Student's *t*-test *vs* vehicle-treated mice.
[c]$P<0.05$ by Fisher's exact test *vs* vehicle-treated mice.

effective in preventing anaphylaxis than was treatment with CV6209 or with triprolidine alone (Table 3). Anaphylaxis occurred in 1 of 10 mice treated with CV6209 and triprolidine *vs* 6 of 10 mice treated with vehicle ($P=0.028$); the mean maximum body temperature drop was $0.3 \pm 0.2^{\circ}$ in mice treated with CV6209 and triprolidine *vs* 2.6 ± 0.7 in mice treated with vehicle ($P=0.010$). Taken together, our data suggest that PAF may have a more significant role in this model of anaphylaxis to self-MOG35–55 (Table 3) compared with the model in which PTX is used (Table 2). However, in both models, our data suggest that mediators other than PAF are also involved. Indeed, our results with triprolidine are consistent with the possibility that even though it is not essential for the responses, histamine may contribute to some of the changes seen in association with anaphylaxis to MOG35–55, especially in the model in which immunization was elicited with PTX.

DISCUSSION

Our results indicate that anaphylaxis to self-MOG35–55 can be induced in mice in the absence of mast cells or histamine, but does require IL-4. The findings in this study are consistent with the possibility that the pathway of anaphylaxis to self-MOG35–55 involves IgG1, FcγRIII, macrophages and/or basophils, and PAF.[5] However, the PAF antagonist CV6209 was not fully effective in abrogating the changes in body temperature associated with anaphylaxis to MOG35–55 in either of the two models tested, indicating that other mediators are also probably involved. Prior work by us[5] and others[8] found that anaphylaxis to self in this model was not eliminated in mice lacking the α chain of FcγRIII (FcγRIII α chain$^{-/-}$). Taken together with the results presented here, this finding suggests that, if IgG1 antibodies indeed contribute importantly to anaphylaxis in this model, such IgG1 antibodies may be functioning through mechanisms other than binding as immune complexes to FcγRIII.

However, our results do not rule out possible contributions in this model of anaphylaxis of an alternative pathway that involves IgE but that requires neither mast cells nor histamine. Like mast cells, basophils express FcεRI and FcγRIII, and therefore might be activated in this model by either IgE- or IgG1-dependent mechanisms. Basophils are present in the mast cell-deficient W/W^{v} mice that we used in our experiments, although in some settings in somewhat lower numbers than in the corresponding wild-type mice, as well as in mast cell-deficient W^{Sh}/W^{Sh} mice.[29–32] Indeed,

depletion of basophils in W^{Sh}/W^{Sh} mice *in vivo* with a monoclonal antibody suppressed IgG1-mediated passive systemic anaphylaxis, revealing an important role for basophils in that model of IgG1-mediated anaphylaxis.[15] Thus, although we cannot exclude an involvement of other cells, such as macrophages, in the models of MOG35–55-induced anaphylaxis that we tested, basophils activated by IgE (and/or IgG1) might represent important effector cells in anaphylaxis to self-MOG, especially in mast cell-deficient mice. In future studies, selective depletion of basophils and/or macrophages *in vivo* might help to define the role of these cells in anaphylaxis to self-MOG35–55.

Blockade of PAF with the antagonist CV6209, which effectively blocked IgG1-dependent passive systemic anaphylaxis,[15] only partially reduced the incidence of active systemic anaphylaxis to MOG35–55 in the main model of immunization to MOG35–55 that was used in our study, and this effect did not achieve statistical significance. However, CV6209 seemed to be more effective in substantially reducing the severity of anaphylaxis to this self-peptide in a model that omitted PTX as an adjuvant at the time of immunization. These results suggest that PAF plays a role in anaphylaxis to this self-peptide in the two immunization models examined, but is unlikely to be the only mediator involved. Our results also indicated that a combination of both CV6209 and the H1R antagonist triprolidine was more effective in inhibiting anaphylaxis than was treatment with CV6209 or with triprolidine alone, especially in the immunization model that omitted PTX as an adjuvant (Table 3). These findings are consistent with those reported in a model of self-anaphylaxis induced in a non-obese diabetic (NOD) mouse model of human diabetes mellitus, in which only the treatment with both PAF antagonist and H1R antagonist in combination, but not with either of them used alone, was effective in preventing anaphylaxis to self-insulin peptides.[33] One way to interpret all of our data is that even though anaphylaxis to MOG35–55 in mice does not require either mast cells or histamine, and PAF is one of the mediators that contribute to the pathology in this setting, histamine (or another mediator whose effects may be reduced by treatment with triprolidine) also can contribute to the pathology.

The data presented here were derived entirely from studies in mouse models, and their implications for the clinical management of MS and other autoimmune disorders remain to be ascertained. Even though IgE is thought to be the main (or only) Ig isotype that contributes significantly to anaphylactic and allergic responses in humans (reviewed in Finkelman *et al*[13]), anaphylaxis has been reported in people in whom there was no evidence of antigen-specific IgE antibodies or mast cell degranulation (reviewed in Finkelman *et al*[13]). Some of such cases might reflect the existence of IgG-dependent, but IgE-independent, pathways of anaphylaxis. It is worth mentioning here that MS patients who developed allergic reactions to NB5788, which usually occurred in those receiving the highest doses of this APL, presented with elevated titers of peptide-specific IgG1 antibodies, but not of peptide-specific IgE antibody levels.[9] Also, despite the frequent appearance of allergic reactions in glatiramer acetate-treated patients, glatiramer acetate-specific IgE was detected only in one patient.[34] It is possible that the allergic reactions to peptide therapies occurring in such individuals might have components of IgG-, but not IgE-, dependent pathways of anaphylaxis. However, the relationship of these clinical findings to the data reported herein from our studies in mice remains to be ascertained. Indeed, in humans, the antibody isotype thought to be most similar to IgG1 in mice is not IgG1, but IgG4.[35,36]

In conclusion, we have shown here that anaphylaxis to self-MOG35–55 in mice can occur in the absence of mast cells and histamine, key elements of the classical IgE-, mast cell-, and histamine-dependent pathway of anaphylaxis. Our pharmacological data suggest that PAF can contribute to the pathology associated with anaphylaxis to MOG35–55, especially in a model in which mice are immunized to the peptide without the use of PTX as an adjuvant, but that other mediators are likely also to be involved. Although one must always be cautious in extrapolating the results of mouse studies to humans, our data raise the possibility that drugs that block effector mechanisms of the classical IgE-dependent pathway of anaphylaxis, such as anti-histamines or inhibitors of mast cell degranulation, might be ineffective in preventing certain forms of peptide-induced anaphylaxis in human subjects.

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DISCLOSURE

The authors declare no conflicts of interest.

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ORIGINAL INVESTIGATION

Effects of the H_3 receptor inverse agonist thioperamide on cocaine-induced locomotion in mice: role of the histaminergic system and potential pharmacokinetic interactions

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Abstract
Rationale Previous studies have shown that intraperitoneal injections of thioperamide, an imidazole-based H_3 receptor inverse agonist that enhances histamine release in the brain, potentiate cocaine-induced hyperlocomotion. The present study examined the involvement of the histaminergic system in these effects of thioperamide in mice.
Materials and methods We investigated whether immepip, a selective H_3 agonist, could reverse the potentiating effects of thioperamide. Moreover, the non-imidazole H_3 inverse agonist A-331440 was tested on the locomotor effects of cocaine. Using high-performance liquid chromatography with ultraviolet detection, cocaine plasma concentrations were measured to study potential drug–drug interactions between thioperamide and cocaine. Finally, thioperamide was tested on the locomotor effects of cocaine in histamine-deficient knockout mice in order to determine the contribution of histamine to the modulating effects of thioperamide.
Results Thioperamide potentiated cocaine-induced hyperlocomotion in normal mice, and to a higher extent, in histamine-deficient knockout mice. A-331440 only slightly affected the locomotor effects of cocaine. Immepip did not alter cocaine-induced hyperactivity but significantly reduced the potentiating actions of thioperamide on cocaine's effects. Finally, plasma cocaine concentrations were more elevated in mice treated with thioperamide than in mice that received cocaine alone.
Conclusions The present results indicate that histamine released by thioperamide through the blockade of H_3 autoreceptors is not involved in the ability of this compound

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