Hydroxyzine inhibits experimental allergic encephalomyelitis (EAE) and associated brain mast cell activation

Violetta Dimitriadou a,1, Xinzhu Pang b, Theoharis C. Theoharides a,*

a Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA

b Department of Pathology, New England Medical Center, 2850 Washington Street, Boston, MA 02111, USA

Received 1 February 2000; accepted 15 March 2000

Abstract

Experimental allergic encephalomyelitis (EAE) has been used as an animal model for the human demyelinating disease multiple sclerosis (MS). In acute MS or EAE, early disruption in the integrity of the blood–brain-barrier (BBB) precedes brain infiltration by inflammatory cells or any clinical evidence of disease. BBB permeability could be affected by vasoactive mediators and cytokines released from perivascular brain mast cells. We investigated the number and degree of activation of brain mast cells in EAE and the effect of the heterocyclic histamine-1 receptor antagonist hydroxyzine, a piperazine compound known to also block mast cells. Acute EAE was induced in Lewis rats by immunization with whole guinea pig spinal cord homogenate and complete Freund’s adjuvant (CFA). A second group of animals were treated orally with hydroxyzine for one day before immunization and then continuously for 14 days. Control rats were treated with CFA or hydroxyzine alone. The clinical progression of EAE was assessed on days 10, 12 and 14 after immunization. The number of metachromatic mast cells and the degree of degranulation was assessed in the thalamus with light microscopy. At day 14, there was a three-fold increase in the number of brain mast cells with EAE, as compared to controls. These cells were positive for the immunoglobulin E binding protein (FcεRI), while those from control rats were not. Over 40% of all thalamic mast cells studied in EAE showed partial staining or extruded secretory granule indicative of secretion. Hydroxyzine treatment inhibited the progression and severity of EAE by 50% and the extent of mast cell degranulation by 70% (p < 0.05). These findings indicate that brain mast cells are associated with EAE development and that inhibition of their activation correlates positively with the clinical outcome.

Keywords: Brain; Demyelination; Hydroxyzine; Inflammation; Mast cells; Secretion; Encephalomyelitis; Multiple sclerosis
1. Introduction

Experimental allergic encephalomyelitis (EAE) has been used extensively as an animal model for the human demyelinating disease multiple sclerosis (MS) [1]. Rats with acute EAE develop brain edema and perivascular infiltrates ('cuffing') of mononuclear cells accompanied by various degrees of paralysis, urinary incontinence and even death [2]. In autoimmune brain demyelination [3], one also finds mast cells as in brain lesions of EAE [4,5] and of (MS) [6–10]. Mast cells can be activated by myelin basic protein (MBP) [8,11] leading to syngeneic brain demyelination [12] and myelin degradation in vitro [13]. Moreover, MBP and estradiol show a synergistic action in triggering mast cell secretion and brain demyelination [14], both of which are more prominent in EAE susceptible rats [14].

Mast cells derive from a distinct precursor in the bone marrow [15] and migrate from the circulation into tissue sites where they mature in the local microenvironment [16]. Mast cells are located perivascularly, often in close association to neurons, and are critical for allergic [16] and possibly neuroinflammatory reactions [17]. During neonatal development in the rat, mast cells appear to enter the brain from the leptomeninges along penetrating vessels [18]. Intracranial mast cells of the adult rat are most abundant in dura mater throughout the adult life [19] and the thalamus, with considerable numbers also in the hypothalamus and median eminence [4,20–25]. Two brain mast cell populations can be distinguished [26]: connective tissue mast cells (CTMC) characterized by the presence of rat mast cell protease (RMCP)-I and mucosal-like mast cells (MMC) identified by their content of RMCP-II; a third cell type contains heterogenous secretory granules and lipid bodies, features that have led to the term neurolipomastoid [19,23]. Adult rat hypothalamic mast cells were recently characterized definitively and were shown to contain histamine, heparin, RMCP-I, as well as mRNA for immunoglobulin E (IgE) binding protein (FceRI) [27]. Mast cells in the meninges are found in apposition to neurons [19,28]) and can be activated by neurotransmitters [19], as well as by antidromic sympathetic [29] or trigeminal nerve stimulation [30] leading to vasodilation [30]. Such evidence has led to the suggestion that mast cells may regulate blood–brain barrier (BBB) permeability and leukocyte infiltration into the brain [31]. In fact, mast cell derived molecules were shown to induce increased permeability of the BBB [32,33]. Such action may be relevant for the pathophysiology of MS in which disruption of the BBB precedes any pathological or clinical findings [34,35].

In the present study, intracranial mast cell number and state of activation were investigated in EAE, with and without treatment with the heterocyclic piperazine histamine-1 receptor antagonist hydroxyzine which had previously been shown to inhibit neurogenic mast cell activation [36]. The results support the involvement of mast cells in EAE and suggest that drugs that inhibit mast cell function may be useful therapeutic agents.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBB</td>
<td>blood–brain barrier</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CTMC</td>
<td>connective tissue mast cell</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental allergic encephalomyelitis</td>
</tr>
<tr>
<td>MMC</td>
<td>mucosal mast cells</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>RMCP</td>
<td>rat mast cell protease</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
</tbody>
</table>
2. Experimental

2.1. Induction of EAE

Female Lewis rats (12 in each group) weighing about 165 g each (Taconic, Germantown, NY) were injected in both hind footpads with 0.2 ml of one of the following: (a) 0.9% NaCl, (b) CFA (DIFCO Laboratories, West Molesay, Surrey, UK) with 30 mg/ml Mycobacterium tuberculosis (H37Ra from DIFCO), (c) CFA with H37Ra and 50 mg GRSCH. Others were treated as in (b) or (c), but their drinking water contained $10^{-4}$ M hydroxyzine HCl (UCB, Belgium) from one day prior to immunization until the 14th day when the animals were sacrificed. Animals were allowed food and water ad libitum (addition of hydroxyzine to the water did not affect the taste). Food was placed on the wire top and inside the cage while water bottles were outfitted with long snouts to permit even rats with EAE to eat and drink. Rats were maintained in a 14:10 light–dark cycle in a modern animal facility, supervised by veterinarians from Tufts School of Veterinary Medicine.

2.2. Grading of EAE

The clinical appearance of all animals was evaluated daily throughout the experimental period, but results are shown for days 12–14 post-immunization, when most of the animals became sick. The weight of the animals and amount of water consumed was also recorded daily for the EAE and the hydroxyzine-treated EAE animals, but there was no statistical difference. The clinical disability index for EAE was calculated using scores from 1 to 5 as follows: 1 = decrease of tail tone, 2 = abnormal gait, 3 = paraparesis/hemiparesis, 4 = quadriplegia and 5 = quadriplegia +1 point added for urinary incontinence.

2.3. Fixation for light microscopy

On day 14, all animals were first anesthetized with a single intraperitoneal injection (0.1 ml) of xylazine and ketamine (100 mg/ml each) and were perfused intracardially with 50 ml 0.9% NaCl for 30 min, followed by 100 ml of a fixative solution containing formaldehyde:acetic acid:methanol (FAM, 1:1:8) for another 30 min at room temperature. Rats were then decapitated and tissue samples for light microscopy were taken from the thalamus which had previously been shown to be particularly rich in mast cells.

2.4. Staining of mast cells

Fixed thalamus was frozen, sections were cut 5 μm thick using a vibratome and stained with 0.25% toluidine blue, pH 2.5, for 45 min at room temperature [37]. Three non-sequential sections were chosen from one random block from each thalamus for examination. All sections were evaluated at ×200, while some sections were photographed at ×400 using a Nikon inverted microscope (Don Santo, MA). Mast cell degranulation was assessed by <50% staining and/or presence of extruded secretory granules. Results are expressed as number of mast cells per mm².

2.5. FccRI immunocytochemistry

*Immunoglobulin E binding protein.* We used a mouse monoclonal antibody (mAB BC4, a gift from Dr. R.P. Siraganian) which binds to rat FccRI as long as the FccRI is not occupied by IgE. After 30 min treatment with 5% normal horse serum, the samples were incubated with the anti-rat FccRI antibody at 1:1000 dilution for 1 h at room temperature followed by three washes in PBS. The slides were then exposed to horse anti-mouse immunoglobulin G-biotin at 1:200 dilution for 30 min. After three washes, the sections were incubated in streptavidin–rhodamine at 1:200 dilution at 1:200 dilution for 30 min and then washed again. Finally, the slides were mounted in aqueous mounting medium (Biomedica Immunocochemicals, Foster City, CA). This antibody failed to stain purified rat peritoneal mast cells after the mast cells had been preincubated with 0.1 μg/ml rat IgE (Zymed, San Francisco, CA) for 60 min at 37°C to occupy all available FccRI on the surface and washed with PBS.
2.6. Calculation of amount of hydroxyzine consumed

A crude approximation of the amount of hydroxyzine ingested by an 165 g female rat was calculated as follows: the amount of water consumed was recorded as 10±2 ml per female rat per 24 h; this amount was slightly less than the average volume known to be consumed by normal male rats which is calculated as 110 ml/kg/24 h. Given this volume of water consumed, the amount of hydroxyzine ingested from a 10^{-4} M solution was calculated by the formula:

\[
37.5 \text{ mg in 10 ml} \times \frac{1000 \text{ ml}}{110 \text{ ml/kg/24 h}} = 1000 \times 0.375 \text{ mg}.
\]

If this amount were to be extrapolated to an average 70 kg human, the corresponding amount of hydroxyzine consumed would be \(0.375 \text{ mg} \times \frac{70,000 \text{ g}}{165 \text{ g (rat)}} = 159 \text{ mg} \).

2.7. Statistical analysis

Statistical analysis was carried out using the non parametric Mann–Whitney U test; significance is denoted by \(p < 0.05\).

3. Results

Clinical signs of EAE were present in 7/12 (58%) of immunized \((n = 12)\) animals on day 12 and in 100% by day 14. The mean clinical score was 3.8±1.5 on day 12 and rose to 4.7±0.5 on day 14 when the animals were sacrificed (Table 1). In contrast, in the immunized group treated with hydroxyzine \((n = 12)\), only 4/12 (33%) animals were clinically affected on day 12 with a mean score of 0.75±0.1 \((p < 0.05\) compared to EAE) and 8/22 (36%) by day 14 with a mean score of 2.0±1.2 \((p < 0.05\) compared to EAE). The control groups were unaffected (results not shown). Thus, treatment with hydroxyzine substantially modified the extent and progression of clinical signs of EAE. The amount of hydroxyzine ingested by each rat per day was approximated to be 0.375 mg, assuming complete absorption.

Thalamic mast cells were immunocytochemically strongly positive for FcεRI in animals with EAE, but only faintly so in control animals treated with complete Freund’s adjuvant (CFA) alone (Fig. 1) indicating that EAE had led to increased synthesis of FcεRI. Light microscopic observations indicated that metachromatic mast cells in the thalamus (Fig. 2) numbered \(5±1\) mast cells/mm^2 in control rats, but increased (Fig. 2) to \(17±3\) mast cells/mm^2 \((p < 0.05)\) in animals with EAE (Table 2). The number of mast cells in the CFA alone group was \(7±2\) mast cells/mm^2 and in the hydroxyzine alone group was \(4±1\) mast cells/mm^2; these values were similar to that present in control rats \((5±1\) mast cells/mm^2). Hydroxyzine did not affect \((p > 0.05)\) the number of mast cells \((16±2)\) present in EAE animals (Table 2). Treatment with hydroxyzine reduced \((p < 0.05)\) activation of the thalamic mast cells to 11.7% (Table 2).

Mast cells in the thalamus were mostly perivascular (Fig. 3) and the secretory granules of mast

### Table 1

<table>
<thead>
<tr>
<th>Day of sacrificea</th>
<th>Clinical scores of treated groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EAE</td>
</tr>
<tr>
<td>12</td>
<td>3.8±1.5 (7/12)</td>
</tr>
<tr>
<td>13</td>
<td>4.6±0.8 (9/12)</td>
</tr>
<tr>
<td>14</td>
<td>4.7±0.5 (12/12)</td>
</tr>
</tbody>
</table>

a Days after immunization.

b Number of animals (from 12 total) showing signs of EAE. The control, sham-immunized and hydroxyzine alone groups had no clinical evidence of EAE and are not included in this table.

c \(p < 0.05\) compared to EAE.

### Table 2

<table>
<thead>
<tr>
<th>Conditionsa</th>
<th>Number mm^2</th>
<th>Activation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ((n = 12))</td>
<td>5±1</td>
<td>7.8</td>
</tr>
<tr>
<td>EAE ((n = 12))</td>
<td>17±3c</td>
<td>43.9c</td>
</tr>
<tr>
<td>HXZ + EAE ((n = 12))</td>
<td>16±2d</td>
<td>11.7d</td>
</tr>
</tbody>
</table>

a Examined at day 14 after immunization.

b Total pixels per six areas examined for each animal.

c \(p < 0.05\) compared to EAE.

d \(p < 0.05\) compared to control.
cells studied in control animals appeared intact in 92.2% (Fig. 4A). There was no difference in the CFA (93.1% intact mast cells) and hydroxyzine (91.9% intact mast cells) groups (p > 0.05). In contrast, 43.9% of mast cells in the thalamus of animals with EAE contained secretory granules with altered or absent content consistent with secretion (Fig. 4B and C). On day 12, mast cells had clear signs of degranulation with loss of toluidine blue staining and extruded granule content (Fig. 4B). At day 14, many mast cells had lost most of the staining with toluidine blue and gave the appearance of ‘ghost’ cells (Fig. 4C).

4. Discussion

The present results indicate that brain mast cell numbers increased and that a substantial number of these, degranulated in EAE. Treatment with hydroxyzine substantially reduced the progression and severity of EAE, as well as the extent of intracranial mast cell activation. These actions could not be due simply to hydroxyzine’s H1-receptor antagonistic effect because vascular permeability in rodents is mediated both by serotonin and histamine [38]; moreover, the typical histamine 1-receptor antagonist diphenhydramine did not have any effect (results not shown). Hydroxyzine is a heterocyclic, piperazine H1-receptor antagonist which penetrates the BBB [39] and has been shown to inhibit secretion from CTMC [40,41], as well as rat basophil leukemia (RBL) cells [42]. Hydroxyzine was also shown to inhibit neurogenic mast cell activation [36]. The amount of hydroxyzine consumed by each rat per 24 h was approximated at 0.375 mg, assuming complete absorption. This amount could be approximated to about 150 mg/day for a 70 kg human.

Fig. 1. Immunofluorescence light photomicrographs of thalamic mast cells at day 14 post-immunization following immunocytochemistry for FcγRI. (A) Control animals treated with CFA alone; (B) animals with EAE. Magnification = ×1000.
Fig. 2. Light photomicrographs of thalamic perivascular mast cells at day 14 post-immunization stained with acidified toluidine blue. (A) Control animals treated with CFA alone; (B) EAE animals; note the presence of a much greater number of mast cells as compared to controls (A). Magnification = ×100. Open curved arrow = venule, solid arrow head = mast cell.
Fig. 3. Light micrographs of thalamic perivascular mast cells at day 14 post-immunization stained with toluidine blue. (A) Control animals treated with CFA alone; (B) EAE animals; note the increased number of mast cells as compared to control (A). Magnification $\times 400$. Open curved arrow = venule, solid arrowhead = mast cell.
an amount within the allowable dose of hydroxyzine for allergic conditions.

Mast cells have previously been identified both in EAE [4,5,43] and in MS [6–10]. However, there is some controversy concerning the number of mast cells in EAE. For instance, one study reported a modest reduction (8.4 ± 1.4 in controls compared to 6.7 ± 0.9 mast cells/mm², p < 0.05) in dura mast cells at day 10 of EAE development. This reduction was considered to be due to mast cell degranulation during earlier stages of EAE even though all remaining mast cells were intact by light microscopy [44]. A later study reported that the percentage of apparently degranulated intracranial mast cells observed with light microscopy was increased in acute EAE and was maximal when inflammation reached the thalamus, even though no change in mast cell numbers was noted [5]. A more recent study reported a significant reduction of mast cell numbers in the thalamus of EAE rats with 55–70% of the remaining mast cells appearing degranulated by light microscopy [45]. The reason for this apparent ‘decrease’ may be the increased presence of ‘ghost’ cells we reported which are difficult to identify, but had previously been noted in scleroderma and were termed ‘phantom’ mast cells [46]. By electron microscopy, ‘immature’ mast cells were noted in EAE brains, a sign of secretory activity [45]; mast cell degranulation was also observed in experimental allergic neuritis [47]. The apparent brain mast cell proliferation we observed could be either due to: (a) increased number of precursors coming from the bone marrow [48]; (b) increased availability of stem cell factor (SCF) as has been shown in cutaneous mastocytosis [49], or (c) production of nerve growth factor (NGF) in response to neuronal demyelination and damage. However, demyelination is not extensive in the rat EAE model [3,50] in contrast to some chronic EAE models that resemble, to a greater extent, human MS [51]. Nevertheless, NGF has been shown to both stimulate mast cell proliferation [52,53] and degranulation [53,54], as well as induce growth of hemopoietic cells [55]. Hydroxyzine may be effective in reducing EAE because it not only inhibits mast cell secretion [40], but also neuronal depolarization [56], thus possibly limiting the availability of neuron-derived mast cell growth factors.

Increased permeability of the BBB is now recognized as a key early event which precedes demyelination or clinical signs in MS [34,35,57,58]. Acute stress by immobilization was recently shown to lead to mast cell-dependent increase in BBB permeability [59] which may be relevant in view of the fact that acute stress has been correlated to new episodes and brain lesions in MS patients [60]. The molecular basis of BBB

Fig. 4. Light micrographs of perivascular thalamic mast cells: (A) control animal at day 14 post-immunization showing three intact mast cells staining intensely with toluidine blue and (B) and (C) EAE animal; (B) note the degranulated mast cells at day 12 post-immunization with obviously reduced staining and extruded granule content; (C) at day 14 post-immunization, mast cells have lost their secretory granule content giving the impression of ‘ghost’ cells. Magnification = ×1000. Arrowheads = blood vessel; curved arrow = mast cell.
alterations is unknown [57]. However, mast cell-derived molecules [61] such as histamine [62], arachidonic acid [63], bradykinin [64] and serotonin [65] have been shown to decrease BBB integrity. Mast cells are also a rich source of most known cytokines including tumor necrosis factor TNF-[alpha] [66]; moreover, mast cell-derived TNF-[alpha] induces endothelial leukocyte adhesion molecule 1 [67]. Brain mast cells were recently shown to release TNF-[alpha] upon immunologic stimulation [68] and EAE could not be developed in TNF-[alpha] knockout mice [69]. Soluble TNF receptor [70] and adhesion molecules were detected in cerebrospinal fluid (CSF) of MS patients [71,72] and may correspond to BBB impairment [73]. Adhesion molecules are expressed on brain endothelial cells and are known to play a key role in trafficking of immune cells across the BBB [74]. In EAE, the expression of vascular adhesion molecules was increased [75,76] and EAE was inhibited by an antibody to ICAM-1 [77]. Developmental studies in the rat indicate that mast cells migrate from the leptomeninges along penetrating blood vessel into the thalamus [18]. Most of these mast cells are CTMC-like [78] as are those found in the hypothalamus [27]. Mast cells may contribute directly or indirectly by either being recruited to the brain parenchyma or by secreting cytokines and other mediators leading to diapedesis of inflammatory cells in the subarachnoid space [79]. In fact, RANTES was recently shown to induce mast cell recruitment [80] and perivascular T cells express RANTES in MS lesions [81].

Of importance is the present observation that brain mast cells from EAE were positive for Fc[alpha]RI, while control animals were not. Lack of Fc[alpha]RI was noted previously in control animals [27]. Moreover, brain mast cells from normal rodents were also shown to lack c-kit, the receptor for SCF [82]. These reports suggest that brain mast cells do not proliferate and do not get activated immunologically under normal circumstances, but can do so during an inflammatory process.

The present results complement earlier pharmacological findings implicating biogenic amines in EAE. For instance, intracisternal administration of the mast cell secretagogue compound 48/80 before immunization reduced the extent of EAE presumably due to depletion of biogenic amines [44]. The mixed serotonin and H1-receptor antagonist cyproheptadine inhibited Bordetella pertussis-induced vascular permeability and EAE development in mice [32] and reduced EAE [83]. However, the effect of cyproheptadine may not be due only to its antagonism of biogenic amines receptors, but also due to its ability to inhibit mast cell secretion [40]. Inhibition of EAE was also reported by the experimental mast cell ‘stabilizer’ picroximil [83].

It would be interesting to know whether hydroxyzine could have a beneficial effect in humans with MS since the level of the specific human mast cell enzyme tryptase was increased in the CSF of MS patients, indicating that intracranial mast cell activation also occurs in this human demyelinating disease [84]. The amount of hydroxyzine extrapolated from the present study to a human was about 150 mg/day, assuming complete absorption. Preliminary evaluation of a randomized, double-blind, placebo-controlled clinical trial using 50 mg/day hydroxyzine in relapsing-remitting MS indicated that it slowed disease progression substantially [85].

Acknowledgements

This work was supported in part by grant RG-1961-A-1 from the National Multiple Sclerosis Society and a grant from Muro Pharmaceutical (Tewksbury, MA) to TCT. Thanks are due to Dr. R.P. Siraganian (Johns Hopkins University) for the antibody against Fc[alpha]RI, Dr. Luca Massacesi (U. Florence, Italy) for help with the EAE protocol and Dr. S.L. Hauser (UCSF) for useful discussions. We also thank Ms. Linda Tamulaites and Ms. Sharon Titus for their word processing skills.

References


Sharma HS, Olsson Y, Dey PK. Changes in blood–brain barrier and cerebral blood flow following elevation of


