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A Key Regulatory Role for Histamine in Experimental Autoimmune Encephalomyelitis: Disease Exacerbation in Histidine Decarboxylase-Deficient Mice

Silvia Musio,* Barbara Gallo,* Stefano Scabeni,* Marilena Lapilla,* Pietro L. Poliani,† Giuseppe Matarese,‡ Hiroshi Ohtsu,§ Stephen J. Galli,**# Renato Mantegazza,* Lawrence Steinman,** and Rosetta Pedotti*¹

Histamine can modulate the cytokine network and influence Th1 and Th2 balance and Ab-isotype switching. Thus, pharmacological blockade or genetic deletion of specific histamine receptors has been shown to reduce the severity of experimental autoimmune encephalomyelitis (EAE), a prototypic Th1-mediated disease with similarities to human multiple sclerosis. To study the comprehensive contribution of endogenous histamine to the expression of EAE, we attempted to induce EAE in histidine decarboxylase-deficient mice, which are genetically unable to make histamine. In this study, we show that EAE is significantly more severe in HDC−/−, histamine-deficient mice, with diffuse inflammatory infiltrates, including a prevalent granulocytic component, in the brain and cerebellum. Unlike splenocytes from wild-type mice, splenocytes from HDC−/− mice do not produce histamine in response to the myelin Ag, whereas production of IFN-γ, TNF, and leptin is increased in HDC−/− splenocytes in comparison to those from wild-type mice. Endogenous histamine thus appears to regulate importantly the autoimmune response against myelin and the expression of EAE, in this model, and to limit immune damage to the CNS. Understanding which receptor(s) for histamine is/are involved in regulating autoimmunity against the CNS might help in the development of new strategies of treatment for EAE and multiple sclerosis. * The Journal of Immunology, 2006, 176: 17–26.

Experimental autoimmune encephalomyelitis (EAE),¹ the most commonly used animal model for human multiple sclerosis (MS), is generally thought to be a Th1-mediated disease (1–3). CD4⁺ T cells reactive to myelin, which produce proinflammatory cytokines, such as IFN-γ, osteopontin and TNF, are known to play a key role in disease pathogenesis and progression (3). Despite several lines of evidence that support a role for Th2 cells, which are classically associated with allergic responses, in suppressing EAE and MS (4), recent observations suggest that EAE can elicit, in the same subjects, both Th1- and Th2-associated immune responses (5–7). For example, mice with EAE develop anaphylaxis upon re-exposure to certain “self-peptides” of myelin that are also targets of autoimmune attack in EAE (5). Furthermore, several components of classical allergic responses can also significantly influence the pathogenesis of EAE (6, 8–12), suggesting that both Th1- and Th2-associated mechanisms and mediators might contribute to the pathogenesis of EAE and MS.

Histamine is one of the main mediators of human and murine anaphylaxis, but histamine can also significantly influence the immune response by modulating cytokine secretion, T cell proliferation, and APC functions. Histamine (β-imidazolylethylamine), a biogenic amine with both neurotransmitter and vasoreactive functions, plays an important role in several physiological and pathological processes. Synthesized from histidine by a unique enzymatic reaction mediated by L-histidine decarboxylase (HDC), histamine mediates multiple biological activities through four types of receptors (histamine receptors (HRs)): H1R, H2R, H3R, and the recently cloned H4R (13; for reviews, see Refs. 14–16). Histamine has been shown to influence T cell polarization by having effects which favor the development of a Th2 response (17–28), whereas, in polarized T cells, depending on the receptor engaged, histamine can promote Th1 responses through H1R and down-regulate both Th1 and Th2 responses through H2R (29).

These and other lines of evidence support the hypothesis that histamine can influence the development of EAE (for review, see Ref. 10). Blockade of histamine with specific H1R antagonists has been shown to reduce the pathology associated with EAE (6, 30), and, recently, inhibition of early-phase EAE has been reported in mice deficient for either H1R or H2R (9, 12). We have previously shown that H1R and H2R are expressed on mononuclear cells within the inflammatory foci in the brains of mice with EAE, whereas encephalitogenic Th1 cell lines activated against myelin proteolipid protein 139–151 expressed more H1R and less H2R compared to Th2 T cell lines (6). Interestingly, by gene microarray analysis, we found relative overexpression of the H1R gene in the chronic plaques of MS patients (31). Furthermore, Bordetella
pertussis histamine sensitization (Bphs), a gene controlling the susceptibility to EAE and other autoimmune diseases, has been recently reported to be H1R (9). Taken together, this evidence suggests that histamine can have an important role in the development of EAE and that blockade of specific HRs, such as H1R or H2R, can ameliorate EAE. However, the influence of histamine on the expression of EAE during the chronic phase of the disease has not yet been established.

In this study, we used HDC-deficient (HDC−/−) mice, which are unable to synthesize histamine, to investigate the role of endogenous histamine in the development and progression of EAE. We report in this study that myelin oligodendrocyte glycoprotein (MOG) 35–55-induced chronic EAE is more severe in a setting of profound histamine deficiency. Furthermore, MOG35–55-reactive T cells from HDC−/− mice produced more IFN-γ, TNF, MCP-1, and leptin compared with those of wild-type mice. We also show that the CNS inflammatory infiltrates that develop in the brain parenchyma in a setting of histamine deficiency are more diffuse, with a large component of polymorphonuclear leukocytes and eosinophils.

Materials and Methods

**Animals**

The production of mice with targeted mutations that result in failure of production of HDC, and many of the phenotypic characteristics of these mice, have been described in detail previously (32). In our study, we used 8- to 12-wk-old female HDC knockout (HDC−/−) mice backcrossed for six generations with C57BL/6 mice and used age/gender-matched C57BL/6N mice (Charles Rivers Breeding Laboratories) as controls. Mice used for this study were generated by breeding of homozygous HDC−/− mice in the facilities of the National Neurological Institute Besta (Milan, Italy) or of Tohoku University (Sendai, Japan). Shipped mice were kept for 2–3 wk in our facility before being entered into the experiments. To confirm the genotypes of the mice used with respect to the HDC gene, genomic DNA was extracted from peripheral blood or tail samples and analyzed by PCR as described elsewhere (32, 33). Both HDC−/− and wild-type mice were kept on a normal diet (16% protein; 2.5% fat; Mucedola, Milan, Italy) containing less than 0.3 mg (= 2.7 μM) of histamine/kg of food. All procedures involving animals were approved by the ethical committee of the Institute and performed according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

**Peptides**

MOG35–55 (MEVGWYRSPFSRVVHLRYNGK) and control peptide acetylcholine receptor 97–116 (negative control), or medium alone. Cells were cultured in 96-well microtiter plates at a density of 4 × 10^5 cells/well in 200 μl of RPMI 1640 (EuroClone) supplemented with l-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5 × 10^-4 M), HEPES buffer (0.01 M), and 1% congeneric normal mouse serum. After 72 h of incubation at 37°C with 5% CO2, cultures were pulsed for 18 h with 0.5 μCi of [3H]thymidine per well before harvesting. Results are expressed as mean cpm ± SEM from triplicate cultures.

**Cytokine and chemokine analysis**

Splenocytes were harvested from HDC−/− and wild-type mice 10–14 days after the induction of EAE and cultured in a 24-well plate (3.5 × 10^5 cells/well) with MOG35–55, Con A, or medium alone in the same conditions as described above. For cytokine quantification, supernatants from in vitro-cultured splenocytes were analyzed by ELISA with capture and detection Abs for IL-2, IL-4, IL-6, IL-10, TNF, and IFN-γ according to the manufacturer’s protocols (anti-mouse OptEIA ELISA Set; BD Pharmingen) (36). For measurements of cytokine levels, supernatants were collected at different time points: 24 h for IL-2, 48 h for IFN-γ and TNF, and 96 for IL-4, IL-6, and IL-10. SEM of duplicates were always within 10% of the mean. A semiquantitative analysis for the simultaneous detection of 32 mediators, including cytokines, chemokines, and growth factors, was also performed on splenocyte supernatants with the Mouse Cytokine Array II (RayBiotech) following the manufacturer’s instructions (37). Briefly, after overnight blocking at 4°C with blocking buffer, membranes were incubated for 2 h at room temperature with cell-free supernatants (1 ml from splenocytes of HDC−/− and wild-type mice collected after 48 h of stimulation with MOG35–55 [20 μg/ml], Con A [4 μg/ml], or medium alone. After a 2-h incubation with 1 ml of biotin-conjugated Abs, each membrane was incubated for 30 min with HRP-conjugated streptavidin. After washings, the membranes were developed with an ECL-type system (provided by the kit), exposed to x-ray film (Kodak), and processed by autoradiography (Kodak). Autoradiographs of the arrays were scanned to determine the density of the protein array spots and analyzed with a TINA 2.0 program (Raytest, Strasbenhardt, Germany). Relative protein concentrations of different samples were analyzed by comparing densities resulting from subtraction of the blank and normalization for the positive controls (upper left corner of the array).

**Leptin measurement in sera and supernatants**

Serum samples were obtained from tail veins of HDC−/− and wild-type mice and stored at −80°C for serum leptin measurement. All serum samples (dilution 1/20) were tested in a mouse leptin-specific ELISA (R&D Systems) according to the manufacturer’s instructions (capture Ab clone AF496; detection Ab clone BA-F198, both from R&D Systems); the detection limit of the assay was typically <22 pg/ml, and the intra- and interassay variability was 4.3 and 7.6%, respectively. In vitro leptin seclrecion by T cells was measured using the same mouse leptin-specific ELISA (R&D Systems) on cell culture supernatants (dilution 1/2) derived from 96-h MOG35–55-stimulated splenocytes.

**Assay for histamine**

Histamine levels were measured in tissues, plasma, and cell-free supernatants from splenocytes of HDC−/− and wild-type mice by using an enzyme immunoassay kit (ImmunoTech; Beckman Coulter) according to the manufacturer’s instructions (38). Briefly, 50 μl of the samples added to 25 μl of acylation solution and 25 μl of acylation buffer was incubated in precoated wells (provided by the kit) for 18 h at 2–8°C. After washing, the

**Induction of EAE**

EAE was induced with MOG35–55 as previously described (34). Briefly, MOG35–55 peptide was dissolved in PBS to a concentration of 1.2 mg/ml and emulsified with an equal volume of IFA supplemented with 8 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco). Mice were injected s.c. with 0.3 ml of the peptide emulsion (for a total of 200 μg of MOG35–55/mouse) and, on the same day and 48 h later, were injected i.v. with 0.1 ml containing 500 ng of B. pertussis toxin (Ptx; Sigma-Aldrich) dissolved in PBS. Mice were assessed daily for clinical signs of EAE according to the following 5-point scale: 0, healthy; 1, tail weakness or paralysis; 2, paraparesis (incomplete paralysis of one or both hind limbs/plegia of one hind limb); 3, paraplegia extending to the thoracic level; 4, forelimb weakness or paralysis with hind limbs paraparesis or paraplegia; and 5, moribund or dead animal.

**Pathological studies**

For histological evaluation of EAE in HDC−/− and wild-type mice, six to seven animals per group were sacrificed 2 or 6 wk after the induction of EAE, and the brain and spinal cord were removed and fixed in 10% Formalin. Tissue samples were embedded in paraffin and 4-μm sections were cut on a microtome and stained for histological examination. Routine H&E staining was used to study basic histopathological changes and sections were analyzed by a pathologist unaware of the identity of individual sections (P.L.P.). The frequency of perivascular inflammatory infiltrates was expressed as the number of inflammatory infiltrates per square millimeter (35). Giemsa staining was used to identify the component cells within inflammatory infiltrates. Demyelinated areas were detected by Luxol Fast Blue staining and immunohistochemical staining of MBP was performed using a rabbit anti-MBP Ab. Chirp polyclonal Ab (at 1/50 dilution; Chemicon) revealed by a biotin-labeled secondary goat anti-rabbit Ab (at 1/200 dilution; Amersham) using 3,3′-diaminobenzidine tetrahydrochloride (0.05%) as a chromogen.
reaction was terminated by the addition of 200 μl of substrate and incubated at room temperature. After neutralization with the stop solution, the plate was read at 405 nm. The concentration was determined by interpolation with the standard curve and correction for the dilution factor. The detection limit of the assay was 0.5 nM.

For histamine quantification in mouse tissues, specimens of brain, spleen, lymph nodes, and skin were collected and analyzed according to previously reported methods (32, 38, 39). Briefly, tissues were snap frozen in liquid nitrogen, weighed, and homogenized in 0.1 M perchloric acid. Homogenates were centrifuged at 10,000 × g for 5 min at 4°C, and supernatants were collected and neutralized with an equal volume of 0.14 M sodium borate buffer. The resulting supernatants were tested with a histamine enzyme immunoassay kit as described above and the concentration of histamine was determined by interpolation with the standard curve and correction for the dilution factor and the starting weight of the samples.

Measurement of serum Ab responses
Blood was collected from the tail of HDC−/− and wild-type mice before and 6 wk after the induction of EAE. Sera were stored at −20°C until analyzed for Ab responses. MOG35–55-specific IgG, IgG1, IgG2a, IgG2b, and IgG3 Abs were measured by ELISA as described elsewhere (40). Briefly, 96-well microtiter plates (Immunol; Thermo Labsystems) were coated overnight at 4°C with 0.1 ml of MOG35–55 diluted in 0.1 M NaHCO3 buffer (pH 8) at a concentration of 0.010 mg/ml. The plates were blocked with PBS/1% BSA (blocking buffer) for 2 h. Samples were diluted in blocking buffer at 1/10.000. Ab binding was tested by the addition of peroxidase-conjugated monoclonal goat anti-mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates), each at a 1/5000 dilution in blocking buffer. Enzyme substrate was added and plates were read at 450 nm on a microplate reader. Total IgM and IgG Abs were measured in naive sera by ELISA as described previously (40). Briefly, 96-well flat-bottom microtiter plates (Maxisorp; Nunc) were coated over-night at 4°C with 0.1 ml of goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories) diluted in 0.1 M NaHCO3 buffer (pH 8) at a concentration of 0.010 mg/ml. The plates were blocked with PBS/1% BSA for 2 h, and samples were diluted in blocking buffer at 1/10,000. Ab binding was tested by the addition of peroxidase-conjugated monoclonal goat anti-mouse IgM or IgG (Southern Biotechnology Associates), each at a 1/5000 dilution in blocking buffer. Enzyme substrate was added and plates were read at 450 nm on a microplate reader. Total IgE was measured by sandwich ELISA (BD Pharmingen) following the manufacturer’s instructions (41).

H2R expression
Total RNA was isolated from brain, spleen, and stomach of HDC−/− and wild-type mice using Trizol reagent (Invitrogen) under RNAse-free conditions according to the instructions of the manufacturer. cDNA was synthesized from total mRNA using Superscript II reverse transcriptase (Invitrogen) and random primers as described by the manufacturer. mRNA expression of H2R was analyzed by nested RT-PCR by using the following primers: H2R-forward primer, 5’-tca ttt cca tca ccc tct cc-3’; H2R reverse primer, 5’-ttg tga gag ttg tgg ctt gc-3; nested H2R forward primer, 5’-gga aca gca gaa atg gga cc-3; and nested H2R reverse primer, 5’-tgt ctc atg ggt gct gc-3 (42). H2R PCR and nested H2R PCR products were 508 and 214 bp, respectively. β-actin was used as the housekeeping gene. Primers (forward, 5’-cat cgt gg ggg ccc etc tag gca c; reverse, 5’-cgc ggc age cag gtc
cag aac e-3’) amplified a 436-bp PCR product. After an initial denaturation step at 94°C for 2 min, a 35-cycle profile of 30 s at 94°C, 30 s at 55°C for H2R primers (or at 58°C for nested H2R primers), and 2 min at 72°C was performed. After the final cycle, the temperature was maintained at 72°C for 10 min to allow complete synthesis of the amplified products. PCR products were identified by 1.5% agarose gel electrophoresis. Nested PCR products were sequenced and showed 97% homology with the reported nucleotide sequences (42, 43).

Results
Exacerbation of EAE in HDC−/− mice
To study the clinical expression of EAE in histamine deficiency, we induced chronic progressive EAE in HCD−/− and wild-type mice, each of them bearing the H-2b haplotype. All HDC−/− and wild-type mice developed EAE upon immunization with MOG35–55 (Table I). EAE developed earlier in HDC−/− mice (onset was at 12 ± 0.5 days in HDC−/− vs 14.8 ± 0.6 days in wild-type mice; p = 0.0012 by Mann-Whitney U rank sum Test) and the development and progression of the disease was significantly more severe in HDC−/− mice compared with wild-type mice (Fig. 1a and Table I). For example, disease score at day 17 was 3.6 in HDC−/− vs 1.9 in wild-type mice (p < 0.0001 by Student’s t test), and mean peak disease severity was 3.9 ± 0.1 in HDC−/− vs 2.6 ± 0.2 in wild-type mice (p < 0.0001 by Student’s t test). We also observed an exacerbation of EAE in HDC−/− vs wild-type mice when a lower dose of MOG35–55 peptide (100 μg) and of B. pertussis toxin (200 ng i.v., twice) were used to immunize the mice, a protocol that induced significantly milder EAE in each of the groups (Fig. 1b and Table I).

Severe brain inflammation and abundant granulocytic infiltration in HDC−/− mice
We then studied CNS inflammation in HDC−/− vs wild-type mice. Histopathological analysis of brain and spinal cord obtained from mice 6 wk after the induction of EAE revealed more inflammatory foci in the spinal cord of the knockout mice compared with wild-type mice (4.59 ± 1.59 vs 1.99 ± 0.57/mm3, respectively; p = 0.0154 by Mann-Whitney U rank sum test). In HDC−/− mice, we observed more severe and diffuse inflammation, characterized by inflammatory cells that more deeply infiltrated the CNS parenchyma, with more poorly defined perivascular cuffing and more diffuse demyelination (Fig. 2, a and b). Notably, in contrast to the classical mononuclear cell infiltrates observed in the lesions of the wild-type mice, in the knockout mice the infiltrates of EAE were characterized by a prominent and diffuse granulocytic component (Fig. 2, c and d), including many eosinophils (Fig. 2, inset in c). An atypically large number of infiltrates was also observed in brain and cerebellum of HDC−/− vs wild-type mice (Fig. 2, e and f).

Table I. EAE in HDC−/− and wild-type mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>EAE Induction</th>
<th>Incidence (%)a</th>
<th>EAE Onset (day)b</th>
<th>Disease Score at Peakc,d</th>
<th>Peak Disease Severityc,d</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDC−/−</td>
<td>MOG35–55, 200 μg s.c.</td>
<td>14/14 (100)</td>
<td>12.0 ± 0.5e</td>
<td>3.6 ± 0.3f</td>
<td>3.9 ± 0.1f</td>
</tr>
<tr>
<td>Wild type</td>
<td>MOG35–55, 200 μg s.c.</td>
<td>18/18 (100)</td>
<td>14.8 ± 0.6</td>
<td>1.9 ± 0.2</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>HDC−/−</td>
<td>MOG35–55, 100 μg s.c.</td>
<td>7/10 (70)</td>
<td>23.7 ± 2.7</td>
<td>1.0 ± 0.2e</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Wild type</td>
<td>MOG35–55, 100 μg s.c.</td>
<td>3/10 (30)</td>
<td>20.0 ± 3.9</td>
<td>0.2 ± 0.2</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

a Data are shown as mean ± SEM values.
b Peak of EAE was at day 17 for mice immunized with MOG35–55 at 200 μg s.c and PTX 1 μg i.v., and at day 35 for those immunized with MOG35–55 at 100 μg s.c. and PTX 400 μg i.v.

p < 0.0001.
p < 0.0012.
p < 0.05 (for HDC−/− vs. wild-type mice by Mann-Whitney U rank sum test).
Reduced in HDC
d MOG35–55 peptide, and no significant differences in T cell proliferation against MOG35–55 in vivo in a setting of profound histamine deficiency, and therefore in the presence of a reduced possibility for histamine to regulate the immune responses through any of its receptors, we first examined the ability of T cells from HDC
d wild-type mice to respond to the Ag MOG 35–55. To reduce possibility for EAE, spleen cells from HDC
d mice contain- 
ed increase in HDC
d IL-4 were found in either group (data not shown). Many of the granulocytes present in the inflammatory lesions of HDC
d mice are eosinophils, which exhibit strong eosinophilic cytoplasm in H&E-stained sections (inset of c; arrows indicate eosinophils; original magnification, ×60) in contrast to lesions in wild-type mice, which show classic lymphocytic perivascular cuffing with infiltration of monocytes and macrophages (d, H&E staining; original magnification, ×60). Most of the granulocytes, such as TNF (Fig. 3c) and IL-6 (Fig. 3d), were similar in the two groups. No detectable levels of IL-2 (Fig. 3c) and IL-2 (Fig. 3f) were similar in the two groups. No detectable levels of IL-4 were found in either group (data not shown).

To characterize further the immune response against MOG35–55 generated in HDC
d mice with a profound histamine deficiency, using a different platform we studied the simultaneous relative expression of 32 proteins, including cytokines, chemokines, and growth factors (37). Such cytokine array analyses, conducted on supernatants from splenocytes of HDC
d mice immunized for EAE, detected 4 proteins (IFN-γ, IL-3, MCP-1, and tissue inhibitor of metalloproteinases) using a different platform we studied the simultaneous relative expression of 32 proteins, including cytokines, chemokines, and growth factors (37). Such cytokine array analyses, conducted on supernatants from splenocytes of HDC
d mice immunized for EAE, detected 4 proteins (IFN-γ, IL-3, MCP-1, and tissue inhibitor of metalloproteinases) upon Ag-specific stimulation and 10 proteins (6CKine/CCL21, cutaneous T cell-attracting chemokine, IL-2, IL-3, IL-6, IL-17, IFN-γ, MIP-2α, RANTES, tissue inhibitor of metalloproteinases 1) upon Con A stimulation. Beside confirming an increase in IFN-γ upon either Ag-specific or

Similar histopathological findings were observed in CNS tissue samples from animals sacrificed during the acute phase of EAE 2 wk after induction of the disease (data not shown).

Increased production of proinflammatory cytokines in HDC
d mice

To study the immune response against myelin in histamine deficiency, we first examined the ability of T cells from HDC
d wild-type mice to respond to the Ag MOG35–55. To reduce possible effects of histamine deriving from fetal bovine or normal mouse sera in these experiments, only congeneric nonimmunized mouse serum (with plasma obtained from HDC
d mice containing about one-tenth of the histamine contained in that from wild-type mice; Table II) was added to the culture medium used for all in vitro stimulations. As shown in Fig. 3a, LNCs from both HDC
d and wild-type mice responded vigorously to the MOG35–55 peptide, and no significant differences in T cell proliferation were observed between the two groups.

Several lines of evidence support a role for histamine in T cell polarization. To evaluate the immune profile of T cells activated against MOG35–55 in vivo in a setting of profound histamine deficiency, and therefore in the presence of a reduced possibility for histamine to regulate the immune responses through any of its receptors, we measured cytokine production upon in vitro stimulation of spleen cells of HDC
d and wild-type mice immunized for EAE. Spleen cells from HDC
d mice produced significantly more IFN-γ when stimulated with MOG35–55 as compared with those of wild-type mice (Fig. 3b). Other proinflammatory cytokines, such as TNF (Fig. 3c) and IL-6 (Fig. 3d), were also increased in HDC
d mice, while levels of IL-10 (Fig. 3e) and IL-2 (Fig. 3f) were similar in the two groups. No detectable levels of IL-4 were found in either group (data not shown).

To characterize further the immune response against MOG35–55 generated in HDC
d mice with a profound histamine deficiency, using a different platform we studied the simultaneous relative expression of 32 proteins, including cytokines, chemokines, and growth factors (37). Such cytokine array analyses, conducted on supernatants from splenocytes of HDC
d mice immunized for EAE, detected 4 proteins (IFN-γ, IL-3, MCP-1, and tissue inhibitor of metalloproteinases) upon Ag-specific stimulation and 10 proteins (6CKine/CCL21, cutaneous T cell-attracting chemokine, IL-2, IL-3, IL-6, IL-17, IFN-γ, MIP-2α, RANTES, tissue inhibitor of metalloproteinases 1) upon Con A stimulation. Beside confirming an increase in IFN-γ upon either Ag-specific or

**FIGURE 1.** Exacerbation of EAE in HDC
d mice. a, EAE was induced in HDC
d (n = 14) and wild-type mice (n = 18) with MOG35–55 (20 µg s.c. and PTX 500 ng i.v. on days 0 and +2), and mice were scored daily as described (see Materials and Methods). b, EAE was induced in HDC
d (n = 10) and wild-type mice (n = 10) with lower doses of MOG35–55 (100 µg) and PTX (200 ng i.v. twice on days 0 and +2) (see Results). Data represent mean clinical score ± SEM. *p < 0.05 by Mann-Whitney U rank sum test for comparison at that time point between HDC
d and wild-type mice.

**FIGURE 2.** Distribution and phenotype of the lesions in the CNS of HDC
d mice. a and b, Cross-sections of the whole spinal cord (MBP staining; original magnification, ×4) highlight the widespread distribution of the inflammatory lesions as well as demyelinated areas (asterisks) in the HDC
d mice (a) compared with the multifocal perivascular and meningeal localization of the lesions (asterisks) in wild-type mice (b). c and d, In the inflammatory foci of the HDC
d mice, there is an abundance of granulocytes (c, H&E staining; original magnification, ×60) in contrast to lesions in wild-type mice, which show classic lymphocytic perivascular cuffing with infiltration of monocytes and macrophages (d, H&E staining; original magnification, ×60). e and f, HDC
d mice show diffuse demyelination in the brain (e, representative section from cerebellum, MBP staining; original magnification, ×10) and large inflammatory foci (f, representative section from cerebellum, H&E staining; original magnification, ×60), with a cellular composition similar to that observed in spinal cord lesions.
Con A stimulation in HDC−/− cells, our cytokine array analysis revealed an increase of MCP-1 and IL-3 in Ag-stimulated HDC−/− spleen cells compared with those from wild-type mice (Fig. 4, a, upper panels, and b, left panel). Furthermore, in Con A-stimulated spleen cells of HDC−/− mice, IL-17 and IL-6 were also increased compared with levels from cells of wild-type mice (Fig. 4, a, lower panels, and b, right panel). A lower sensitivity (i.e., higher detection limits) of the mouse cytokine array might explain why IL-2, IL-10, and TNF, detected in supernatants with the capture and detection ELISA method, could not be detected on the array.

Activated splenocytes from wild-type mice produce histamine

It has been shown that immune cells are able to produce histamine, and both CD4+ and CD8+ T cells can produce histamine upon Con A stimulation (44). We measured histamine in supernatants of stimulated splenocytes from HDC−/− and wild-type mice. As shown in Fig. 5, spleen cells from wild-type mice, but not HDC−/− mice, produced histamine when stimulated with either MOG35−55 or Con A. Such histamine might have been derived from either lymphocytes or basophils, an alternative source of histamine that would be expected to be present in populations of mouse splenocytes (45).

Leptin is increased in stimulated splenocytes from HDC−/− mice

A bidirectional regulatory loop between histamine and leptin has been described elsewhere (46, 47), and leptin has been shown to be an important proinflammatory cytokine in EAE (48–50). Confirming previous observations (47), HDC−/− naive mice showed higher amounts of leptin in their sera compared with control mice (Fig. 6a). Because the effects of histamine on leptin secretion from immune cells have never been studied, we measured leptin production from spleen cells of HDC−/− and wild-type mice with EAE. Splenocytes from immunized HDC−/− mice produced significantly more leptin than wild-type mice (Fig. 6b). Interestingly, leptin production also was higher in unstimulated spleen cells of HDC−/− vs wild-type mice, suggesting that T cells (and/or other potential sources of leptin in these splenocyte populations) from HDC−/− immunized mice produce more leptin not only after Ag-specific stimulation but also at baseline before stimulation.

Ab responses in HDC−/− mice

Histamine has been shown to influence B cell responses, either directly or through modulation of secretion of cytokines, including IL-13 or IL-4, from T cells (9, 12, 29, 51). Analysis of Ab responses in sera from HDC−/− and wild-type mice with EAE (Figs. 7, upper panel) revealed no difference in the IgG Ab titers against MOG35−55 in HDC−/− vs wild-type mice (mean OD, 0.665 ± 0.200 in HDC−/− vs 0.612 ± 0.110 in wild-type mice; p > 0.05 by Student’s t test). Analysis of the IgG isotypes showed that only IgG1 (mean OD, 0.502 ± 0.252 in HDC−/− vs 0.265 ± 0.134 in wild-type mice) and IgG2a (mean OD, 0.257 ± 0.157 in HDC−/− vs 0.080 ± 0.019 in wild-type mice) were increased, albeit not significantly so, in HDC−/− vs wild-type mice. No differences were observed between the two groups for IgG2b (0.621 ± 0.237 mg/ml), or medium alone.

**Table II. Histamine content in tissues of HDC−/− and wild-type mice**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Wild-Type Mice</th>
<th>HDC−/− Mice</th>
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<tbody>
<tr>
<td>Brain</td>
<td>32.9 ± 1.3</td>
<td>2.4 ± 1.9a</td>
</tr>
<tr>
<td>Plasma</td>
<td>15.2 ± 2.5</td>
<td>1.8 ± 0.49a</td>
</tr>
<tr>
<td>Spleen</td>
<td>336.7 ± 97.4</td>
<td>28.6 ± 8.6a</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>11.4 ± 3.6</td>
<td>0.4 ± 0.2c</td>
</tr>
<tr>
<td>Skin</td>
<td>7.2 ± 1.0</td>
<td>0.2 ± 0.0f</td>
</tr>
</tbody>
</table>

*a* Histamine levels of four mice per group (mean ± SEM) expressed in nanograms per gram of tissue for brain, plasma, and spleen and in micrograms per gram of tissue for lymph nodes and skin.

*p* < 0.0005.

*p* < 0.05 by Student’s t test for comparison between HDC−/− and wild-type mice.

**FIGURE 3**. T cell activation and cytokine production in HDC−/− mice. LNCs and splenocytes were obtained from HDC−/− and wild-type mice 10–14 days after EAE was induced with MOG35−55 and were stimulated in vitro with serial dilutions of the specific Ag, Con A (4 μg/ml), or medium alone. a, T cell proliferative response to MOG35−55 in HDC−/− and wild-type mice (LNCs from three mice in each group). Mean cpm ± SEM were calculated from triplicate wells. Representative of similar results obtained in three consecutive experiments. IFN-γ (b), TNF (c), IL-6 (d), IL-10 (e), and IL-2 (f) concentrations in supernatants of individual cultures of splenocytes from HDC−/− (n = 5) and wild-type (n = 5) mice were determined by capture and detection ELISA method in duplicate (see Materials and Methods). Results are shown as means ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.005 by Mann-Whitney U rank sum test for comparison between HDC−/− and wild-type mice. Representative of similar results obtained in three consecutive experiments.

**FIGURE 4**. Histamine production in supernatants of wild-type and HDC−/− mice stimulated with Ag. Histamine was measured in supernatants of either lymphocyte or basophilic cultures of splenocytes from wild-type mice (a) or HDC−/− mice (b). Histamine production was calculated and represented as the amount of histamine produced per gram of tissue for lymph nodes and skin and in micrograms per gram of tissue for brain, plasma, and spleen. Significant differences were obtained by Student’s t test (c). Results are shown as means ± SEM. a, p < 0.05; b, p < 0.0005 by Mann-Whitney U rank sum test for comparison between HDC−/− and wild-type mice.
in HDC−/− vs 0.542 ± 0.158 in wild-type mice) or IgG3 isotypes (0.043 ± 0.024 in HDC−/− vs 0.032 ± 0.005 in wild-type mice). Total IgE titers were significantly increased in HDC−/− vs wild-type mice (2.1 ± 0.4 μg/ml in HDC−/− mice vs 1.4 ± 0.4 μg/ml in wild-type mice; \( p = 0.043 \) by Mann-Whitney \( U \) rank sum test).

As previously reported (40), the absence of endogenous histamine did not affect levels of IgG and IgM Abs in naive mice, because similar levels of Abs were detected in the sera of HDC−/− and wild-type mice and the IgG: IgM ratio was not significantly different in the two groups (Fig. 7, lower panel). Furthermore, no differences were observed between the two groups for total IgE levels. Natural IgG Ab titers to MOG35–55 were very low in sera of nonimmunized mice and, similar to what has been reported (40), did not differ significantly between HDC−/− and wild-type mice.

**H2R expression in HDC−/− mice**

According to RT-PCR analysis and binding assays with a specific radioligand, a down-regulation of H2R expression in tissues of HDC−/− mice kept under a histamine-free diet (i.e., <0.6 nM histamine/g tissue) (32, 39) has been previously reported (52). H2R has been shown to play an important role in the regulation of the immune response (29) as well as in the development of EAE (12). To examine the possibility that a functional deficiency of this receptor could have contributed to our results, we analyzed by RT-PCR the expression of H2R in several tissues of HDC−/− and wild-type mice. Despite being maintained with a normal diet (i.e., not histamine free or low), HDC−/− mice were still profoundly histamine deficient, with levels in various tissues that were 10-
MOG35–55-specific IgG were tested by ELISA in sera from 9-wk-old mice. To study the role of histamine in EAE, we compared HDC KitW/KitW-v mice to wild-type mice. Histamine deficiency, however, has different effects on the characteristics of EAE. In the virtual absence of mast cells, in the tissues of HDC KitW/KitW-v mice, EAE is mediated by Th17 cells, as demonstrated by the increased production of IL-17 and IFN-γ, which is not seen in wild-type mice. Furthermore, HDC KitW/KitW-v mice lack histamine synthesis and, therefore, virtually cannot undergo histamine-dependent activation of any of the four known histamine receptors (32). These mice have been used to clarify the role of histamine in anaphylactic responses and allergic inflammation (53–55), as well as in several neurophysiological functions (33, 56). Because it is difficult to achieve complete and long-lasting elimination of the effects of histamine in vivo using pharmacological approaches (32), HDC KitW/KitW-v mice provide a good model in which to study autoimmune responses against CNS myelin in the virtual absence of any kind of regulation mediated by histamine. Of note, histamine can be ingested by HDC KitW/KitW-v mice through the diet; nevertheless, these mice have been kept under a normal diet in several previous studies (38, 46, 54, 55, 57–59). We analyzed the histamine content of several tissues of HDC KitW/KitW-v mice and clearly showed that under a normal diet these mice are still profoundly histamine deficient compared to the wild-type mice. Furthermore, using RT-PCR we detected no differences in H2R expression at the mRNA level between HDC KitW/KitW-v and wild-type mice under the diet used in our studies. Although our RT-PCR results cannot rule out the possibility that there were quantitative differences in the levels of H2R protein expression in the HDC KitW/KitW-v and wild-type mice, our results suggest that, under the diet provided, both HDC KitW/KitW-v and wild-type mice expressed H2R, at least at a mRNA level.

A paucity of mast cells and abnormalities in mast cell cytoplasmic granules have been described among the phenotypic abnormalities in HDC KitW/KitW-v mice (32). However, the absence of mast cells, as opposed to the absence of HDC KitW/KitW-v mice, has different effects on the characteristics of EAE. In the virtual absence of mast cells in KitW/KitW-v mice, EAE develops later and has a significantly more benign course than it does in the congenic wild-type (KitW/KitW-v) mice (8, 11, 60). In contrast, we observed a worsening of clinical EAE in HDC KitW/KitW-v mice. Thus, it is unlikely that in HDC KitW/KitW-v mice the effects of a lack of HDC and of a profound histamine deficiency on the clinical expression of the disease, or on the immune response against myelin, is related to the reduction in numbers of mast cells or other abnormalities affecting mast cells, in the tissues of HDC KitW/KitW-v mice. Furthermore, unlike wild-type C57BL/6 mice, splenocytes from HDC KitW/KitW-v mice did not produce and secrete histamine upon Ag or Con A stimulation, and the HDC KitW/KitW-v cells produced more IFN-γ and TNF, effects that might be related to the lack of H2R-dependent effects of histamine (14, 23, 29).

Two loci linked to EAE at the telomeric end of chromosome 2 and one at the distal p fragment of this chromosome have been reported (61–63). Our microsatellite marker analysis showed that the mouse strain 129-derived portion of chromosome 2 resided within 28.0–84.0 cM, while the eae loci described on this chromosome were located in portions of the chromosome that already were replaced by C57BL/6 chromosomal DNA by the sixth generation of backcrossing to C57BL/6. However, we of course cannot rule out formally some possible effects in our experiments of other “residual,” as yet uncharacterized, non-C57BL/6 genes.

It has been shown that histamine can play an important role in the regulation of immune responses (reviewed in Refs. 14 and 15). Histamine promotes a Th2 environment by reducing IL-12 production and increasing the secretion of IL-10 in human monocytes (17, 18), as well as in immature dendritic cells (19–21). In human monocytes, histamine can inhibit production of TNF (22), a key

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**FIGURE 7.** Serum Abs in HDC KitW/KitW-v and wild-type mice. Upper panel. Anti-MOG35–55-specific IgG as well as IgG isotypes and total IgE Abs were tested by ELISA in sera from HDC KitW/KitW-v and wild-type mice obtained 4 wk after the induction of EAE. Each mouse was tested individually in duplicate at a dilution of 1/100. Bars represent average titers for each group and wild-type mice. Lower panel, Total IgG, IgM, IgE, and anti-MOG35–55-specific IgG were tested by ELISA in sera from 9-wk-old HDC KitW/KitW-v and wild-type mice before the induction of EAE. Bars represent mean and SEM of values of 10 mice from each group, with individual samples tested in duplicate. A value of $p < 0.05$ by Student’s $t$ test for comparison between HDC KitW/KitW-v and wild-type mice was considered significant.

**FIGURE 8.** Expression of H2R mRNA in tissues of HDC KitW/KitW-v and wild-type mice. cDNA was synthesized from total RNA prepared from brain, stomach, and spleen and used as template in a PCR. The PCR products were run on a 1.5% agarose gel and detected under UV light. As controls, mouse β-actin primers were used in a parallel PCR.
cytokine in the pathology of EAE and MS (64), and can diminish IL-18–induced IFN-γ, TNF, and IL-12 production by human PBMC (23). Histamine-dependent enhancement of Th2 cytokines can also be demonstrated in mouse immune cells (24–27), and histamine can increase the production of IL-10 by mouse splenocytes (28). Several reports, investigating both human and mouse cells in vitro, suggest that histamine induces these Th2–polarizing effects through H2R. The increased production of IFN-γ and TNF that we observed in cells from HDC+/+ mice might be related to the profoundly reduced histamine regulation, perhaps through H2R, during Ag presentation, resulting in an increase of Th1 responses compared with those in wild-type mice. MCP-1 was also increased in splenocytes of HDC−/− mice (Fig. 4). Interestingly, MCP-1 was also found to be increased in the plasma of HDC−/− mice in an experimental model of peritonitis (57) and in the splenocytes of HR2-deficient mice (12). Because MCP-1 is one of the chemoattractants for phagocytes, an increase in levels of this molecule might help to explain the accumulation of more intense leukocytic infiltrates in the CNS of mice that are profoundly deficient in histamine.

Our results are consistent with the observations of Sonobe et al. (44), who recently reported that CD8+ T cells from either H1R+/− or H2R−/− mice produce more IFN-γ than wild-type CD8+ T cells when stimulated with Con A. We found that splenocytes from HDC−/− mice also produced larger amounts of IL-17 than did the corresponding wild-type cells. An important role for IL-17-producing Th cells, whose development is favorized by IL-23, in the induction of EAE has just been described (65). Interestingly, gene microarray analysis, IL-17 was increased in the chronic plaques of MS patients (31). Leptin, which we found was increased in splenocytes of HDC−/− mice compared with those of wild-type mice upon Ag stimulation, might also play an important role in the exacerbation of EAE observed in these mice (reviewed in Ref. 66). The increased levels of leptin in HDC−/− mice might be related to the reduced effects of histamine on H3R, since it was reported that H3R knockout mice show an obese phenotype related to increased levels of leptin (67).

Pharmacological studies have shown that it is possible to inhibit the development or severity of EAE by the specific blockade of H1R with antagonists for this receptor, such as pyrilamine or hydroxyzine (6, 30), or by triggering H2R with a specific agonist for this receptor (68). In addition, the work of Teuscher and colleagues (9), confirming the results of pharmacological studies, reported amelioration of early acute EAE in H1R-deficient mice. These H1R-deficient mice exhibited an immune deviation of the response against myelin toward a less encephalitogenic Th2 phenotype, with suppression of IFN-γ and up-regulation of IL-4 secretion by T cells that had been activated by myelin (9). Surprisingly, contrary to the pharmacological study of Emerson et al. (68), EAE was also ameliorated in H2R-deficient mice (12), probably because of altered APC activity in the absence of H2R-dependent effects of histamine.

It should be noted that in our study, in contrast to previous reports (9, 12, 30), mice were observed for clinical signs of EAE for up to 8 wk, i.e., during both the early acute and chronic phase of EAE. Furthermore, in our work, EAE was studied in mice exhibiting a profound deficiency of histamine and therefore a deficiency of the histamine–mediated triggering of either H1R or H2R. In our experiments, a complete lack of HDC and histamine synthesis resulted in increased expression of features of Th1 responses and an exacerbation of EAE. In HDC−/− mice, there would be also a reduction of the effects of histamine mediated via H3R or H4R, or perhaps through other, unknown receptors for histamine; it is possible that a deficiency of such effects also may have contributed to the alterations in EAE observed in the HDC−/− mice. For example, Teuscher and colleagues observed a worsening of MOG35–55–induced EAE in H3R-deficient mice and postulated an important role for H3R in the regulation of T cell entry into brain parenchyma through the blood-brain barrier (C. Teuscher, personal communication). We observed an atypical abundance of inflammatory infiltrates in the cerebellum and brain in HDC−/− mice, with a large granulocytic component, including, as has been reported in H2R-deficient mice (12), eosinophils. It is noteworthy that eosinophil infiltration was increased in this EAE model in mice with a significant histamine deficiency, since it has been proposed, based on studies with anti-histamines (69) or histamine-binding proteins (70), that histamine can, at least in some circumstances, contribute to the eosinophilia that develops during mouse models of allergic asthma. Other mechanisms, such as a reduced triggering of histamine through H2R, which has been shown to play an important role in limiting leukocytic infiltration in an air pouch-type allergic inflammation model (53), as well as possible unknown compensatory mechanisms leading to activation of G protein–coupled receptors by cross-stimulation, also could have contributed to the features of the inflammatory infiltrates that we observed in the CNS of HDC−/− mice.

We have demonstrated here that the HDC−/− mice used in our studies had extremely low levels of histamine on the diets we provided to these mice and that there was no difference between HDC−/− and wild-type mice in the levels of mRNA for H2R, as detected by RT-PCR. However, levels of histamine in HDC−/− mice are not zero, nor can we formally rule out that some phenotypic difference between the HDC−/− mice and the wild-type mice, which is a consequence of the lack of HDC other than the histamine deficiency per se, such as a difference in the levels of H2R protein or some other result(s) of the animals’ profound deficiency in histamine, may have contributed to our results. However, our findings strongly support the conclusion that HDC and histamine play an important role in limiting the extent of immune damage to the CNS, an effect that may be mediated, at least in part, by the ability of histamine to regulate Th1 cytokine secretion from T cells. Understanding which receptors are involved in the protective effects mediated by histamine in this model of EAE might help in designing new treatments for EAE and, possibly, for MS.

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Disclosures
The authors have no financial conflict of interest.

References


