

Human Skin Mast Cells Express H2 and H4, but not H3 Receptors

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Mast cells generate and release histamine during anaphylactic reactions, and there is pharmacological evidence that histamine regulates this process via specific receptors. Therefore, we examined human leukemic (HMC-1) and normal skin mast cells for the expression of all four currently known histamine receptors. Both cell types expressed H2 and H4 receptors at mRNA and protein levels, whereas H3 receptor specific mRNA and receptor protein was undetectable. Similarly, immunohistochemistry of cutaneous tissue showed an absence of H3 receptor in these cells. Despite transcription of mRNA, H1 receptor protein was only moderately expressed in HMC-1 cells and was virtually absent in skin mast cells. Furthermore, only H1, H2, and H4 receptors were detectable by Western blot analysis of HMC-1 cells. Radiolabeled histamine binding was strongly inhibited only by H2 (ranitidine)- and H3/H4 (FUB 108)-specific antagonists. Histamine-induced increase of cAMP was inhibited by the H2 receptor antagonist famotidine, whereas induction of IP3 was not observed, making signaling via the H1 receptor unlikely. These data show that human mast cells constitutively express primarily H2 and H4 receptors and that H2 receptors are functionally linked to cellular processes. They provide new insights into the mechanisms that govern auto- and paracrine histamine-induced mast cell functions.

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Histamine is a multifunctional, ubiquitously distributed biogenic amine that is stored abundantly in and is rapidly released from tissue mast cells and blood basophils, inducing vasodilatation, vascular permeability, and smooth muscle contraction in target organs of anaphylactic reactions (Jurkiewicz, 1998). Although H1 receptors have been extensively studied for their effects on allergic reactions (Hill, 1990), H2 receptors are best known for their function in gastric acid secretion and in immunomodulation. Besides the well-known H1 and H2 histamine receptors, two additional types of histamine receptors have been described by molecular cloning of cDNAs encoding G protein coupled receptors (Gantz *et al*, 1991; Lovenberg *et al*, 1999; Nakamura *et al*, 2000; Oda *et al*, 2000). In the 1980s, the H3 receptor was identified as a pre-synaptic heteroreceptor that is primarily expressed in neurons of the central and peripheral nervous system where it mediates inhibition of neurotransmitter release, including histamine release, with a concomitant decrease of cAMP (Hill, 1990).

The most recently described H4 receptor is more widely distributed, especially in organs associated with the immune system (Liu *et al*, 2000; Morse *et al*, 2001). Its abundant expression in cells associated with allergic

reactions, such as eosinophils and T lymphocytes, is of particular interest (Morse *et al*, 2001). Data regarding the type of histamine receptor expressed by mast cells in different organs and species are variable and in part even contradictory (Masini *et al*, 1982; Wescott *et al*, 1982; Alm *et al*, 1984). This also holds for recent pharmacological studies using human leukemic mast cells (HMC-1 cells) or RBL cells where H2 and/or H1 receptors were found to modulate cytokine secretion (Lippert *et al*, 1995; Hide *et al*, 1997; Lippert *et al*, 2000). H3 receptors were initially found on rat peritoneal and cutaneous (Ohkubo *et al*, 1994; Bissonnette, 1996) as well as on human adenoidal mast cells. (Bent *et al*, 1991) However, according to more recent data, they seem to be restricted to the central nervous system (Rozniecki *et al*, 1999). Expression of the H4 receptor has so far been described at the mRNA level with commercially available probes from cultured bone marrow-derived mast cells (Morse *et al*, 2001), and very recently also indirectly in receptor-deficient mice (Hofstra *et al*, 2003). In a preliminary report, we have also reported its presence at protein level in human skin mast cells.¹

Abbreviations: FACS, flow cytometric analysis; FITC, fluorescein isothiocyanate; H receptors, histamine receptors; HMC-1 cells, human leukemic mast cells; PBS, phosphate-buffered saline; PE, phycoerythrin

¹Expression of histamine receptor types 1, 2, and 4 but not type 3, on human mast cells. Lippert U, Artuc M, Grützkau A, Haase I, Blaschke V, Zachmann K, Neumann Ch. and Henz B.M. Arch Dermatol Res 2002; 293:172.

In addition to its effects in allergic responses, there is increasing evidence for numerous other functions of histamine, such as inhibition of mediator secretion and involvement in cell growth and adhesion (Jurkiewicz, 1998; Cuss, 1999). Furthermore, recent studies have described the expression of functional histamine receptors in human T cells and dendritic cells, emphasizing the potential immunomodulatory effects of this molecule (Jutel *et al*, 2001; Gutzmer *et al*, 2002; Idzko *et al*, 2002).

In order to clarify the presence of histamine receptors in human mast cells, we examined the expression of all four currently known receptor subtypes in the human mast cell line HMC-1 and in isolated skin mast cells. Furthermore, we included an investigation of mast cells localized within the tissue by immunohistochemical staining. Using specific H1, H2, and H3/H4 antagonists, functional activities of the histamine receptors were explored. Besides the clear demonstration of a functional H2 receptor, the demonstration of a constitutively strong H4 receptor protein expression in human mast cells is intriguing in light of its supposed role in allergic and immunological processes.

Results

Demonstration of histamine receptor expression by PCR Initially, we examined whether mast cells express mRNA for all four currently known histamine receptors. Using RT-PCR analysis, constitutive H1, H2, and H4 receptor expression was detectable in immature HMC-1 cells as well as in skin mast cells (Fig 1A, B). In contrast, the H3 receptor was undetectable by RT-PCR in both cell types, although it could be demonstrated in human brain tissue (Fig 1B). As reported before (Liu *et al*, 2000; Morse *et al*, 2001), the H4 receptor was not detected in brain tissue, underlining the specificity of the PCR products (Fig 1B).

Furthermore, we compared the expression level of the different histamine receptors in HMC-1 and isolated human skin mast cells at RNA level as a possible first indication of their distribution in these cell types. β -Actin served as an internal standard. As demonstrated in Fig 2C, expression of H2 and H4 receptor RNA exceed that for the H1 receptor in both cell types. Interestingly, the major difference in RNA expression level between both cell types was observed for the H4 receptor type, which was clearly expressed more strongly in skin mast cells.

Protein expression of histamine receptors in the HMC-1 cell line as well as in isolated human skin mast cells by flow cytometric analysis (FACS)-analysis In a next step, we tried to examine the expression and distribution of histamine receptor protein in HMC-1 cells and isolated human skin mast cells. On flow cytometry, almost 90% of HMC-1 cells exhibited binding of the H2 receptor antibody, which could be blocked to about 80% on pre-treatment with the corresponding control peptide (Fig 2A). H1 and H4 receptors were detected in 43% and 49% of HMC-1 cells, respectively, and this binding was also suppressed by the corresponding control peptides, as shown in Fig 2A. In agreement with our PCR data, no binding was noted with the H3 receptor antibody. As positive control for H1

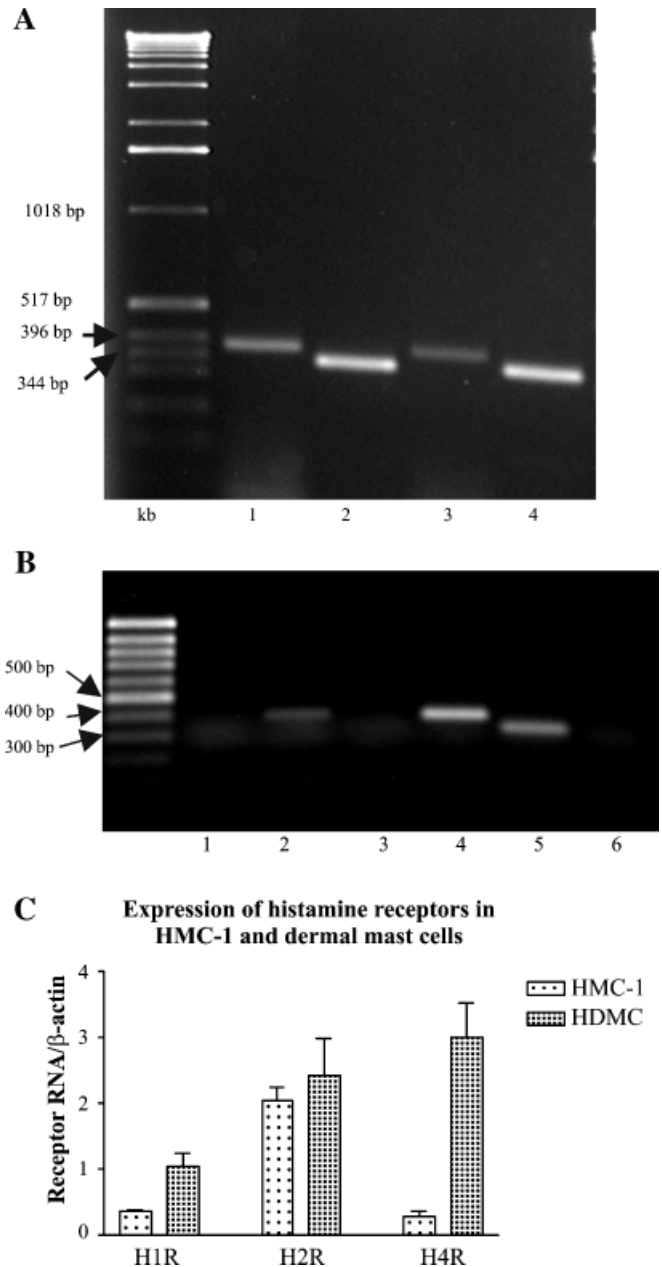
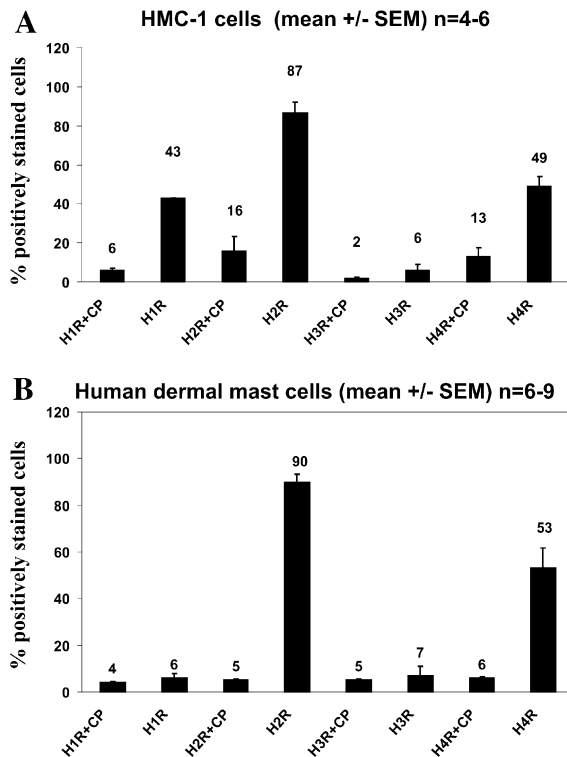


Figure 1
Studies of H1 and H2 receptor mRNA expression in human mast cells by RT-PCR. (A) Shows H1 (lanes 1 and 3) and H2 (lanes 2 and 4) receptor expression by RT-PCR in HMC-1 cells (lanes 1 and 2) and cutaneous mast cells (lanes 3 and 4). (B) Investigation of H3 and H4 receptor mRNA in HMC-1 cells and human skin mast cells. Neither HMC-1 cells (lane 1) nor human skin mast cells (lane 3) express the H3 receptor mRNA. The H3 receptor, however, was clearly detected in brain tissue, which served as positive control, (lane 5). In contrast, H4 receptor expression was found in HMC-1 cells (lane 2) as well as in skin mast cells (lane 4) whereas it remained undetectable in the brain tissue (lane 6). (C) Comparison between the mRNA expression of histamine receptors in HMC-1 cells and human dermal mast cells (HDMC). The values were normalized by using β -actin and analyzed with the help of an image analyzer software. The bars represent the means of three independent experiments, including the standard error.

expression, the histiocytic lymphoma cell line U937 (Davio *et al*, 1995) was studied and showed 22% binding, with a 94% inhibition by the control peptide. Human erythrocytes served as negative controls and, as expected, showed no

**Figure 2**

(A) Protein expression and distribution of histamine receptors on HMC-1 cells by flow cytometry. Statistical analysis from four to six independent experiments with HMC-1 cells are shown, using specific H1–H4 histamine receptor antibodies (HR). In addition, the specificity of the binding was proved by its inhibition after pre-incubation with the corresponding control peptides (CP). **(B)** Presence of histamine receptor protein on human skin mast cells by flow cytometry. Isolated human skin mast cells were stained with histamine receptor antibodies (HR) and, in corresponding experiments, also with the antibody and the control peptide (CP). The columns show the mean data from six to nine independent experiments.

binding for any of the four types of histamine receptors (data not shown).

In human skin mast cells, no H3 receptor and no significant expression of H1 receptor was seen on flow cytometry although, in the case the H1 receptor, its expression at mRNA level was observed (Fig 2B). In contrast, and as in HMC-1 cells, a high percentage of H2 receptor-positive skin mast cells was however found (Fig 2B). Interestingly, a strong constitutive expression of the H4 receptor protein (about 50%) was detected in skin mast cells as well (Fig 2B).

Demonstration of mast cell histamine receptors *in situ* In order to validate the findings on isolated skin mast cells with cells localized within their tissue environment, we performed double immunofluorescence staining with the well-established mast cell marker tryptase and the specific histamine receptor antibodies.

As can be seen in Fig 3A–C, the H1 receptor protein was hardly detectable on mast cells whereas almost all mast cells expressed the H2 receptor protein (Fig 3D–F). In contrast, no H3 receptor-positive mast cells were detected, although some non-specific binding was noted in the skin tissue (Fig 3G–I), underlining our results obtained with HMC-1 and isolated skin mast cells. As expected from the

data obtained so far by PCR and FACS analysis, the majority of skin mast cells also expressed the H4 receptor protein (Fig 3J–L).

Taken together, these findings support the concept that also within human tissue, the H2 and H4 receptors represent the main pool of histamine receptors in mast cells.

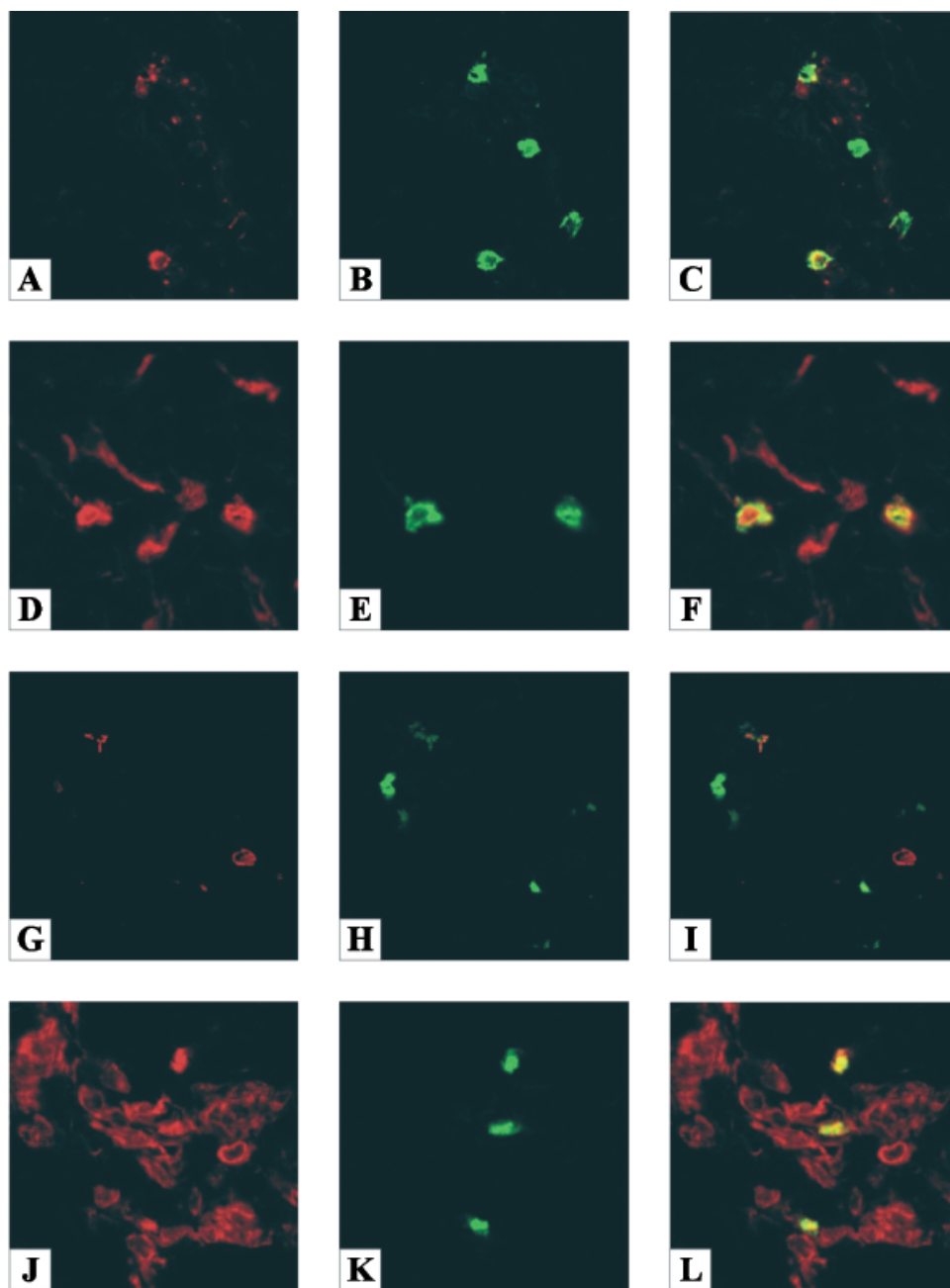
Determination of histamine receptors protein size by western blot In order to determine the protein size of histamine receptors in human mast cells and at the same time to provide evidence for the quality of the antibodies used in this study, HMC-1 cells were chosen as a model since these cells express marked amounts of the H1 receptor protein and since it is very difficult to isolate sufficient skin mast cells for these experiments. As shown in Fig 4, expression of two bands for the H1 receptor could be demonstrated (Fig 4A, lane 1). The band at 60 kDa is of the expected size, and the smaller one (40 kDa) corresponds most likely to a proteolytic product, as previously described in other cells by Ruat *et al* (1988). Both bands disappeared after pre-incubation of the antibody with its control peptide (Fig 4A, lane 2). For the H2 receptor, six bands of 87, 59, 49, 48.5, 37.5, and 30.5 kDa were detected (Fig 4A, lane 3), with the five latter disappearing after pre-incubation with the control peptide. Since the largest band (87 kDa) could not be displaced by the control antibody, this band must be viewed as being non-specific (Fig 4A, lane 4).

As predicted, no binding was found for the H3 receptor (Fig 4B, lane 1, antibody alone and lane 2, antibody and control peptide). In contrast, two bands were detected for the H4 receptor (90 and 78 kDa) (Fig 4B, lane 3), and both could be displaced by the control peptide (Fig 4B, lane 4). These data confirm our findings of histamine receptor protein expression obtained by FACS analysis.

Histamine binding and its displacement As a next step, we examined whether mast cell histamine receptors can indeed bind their histamine ligand, using two different sets of binding experiments. Initially, we used fluorescence labeled histamine and FACS. Baseline binding of fluorescence labeled histamine to its receptors on HMC-1 cells showed a concentration-dependent histamine binding of maximally 30% (Fig 5).

In the second, more classical, set of experiments, [³H]thymidine labeled histamine was used, together with specific histamine receptor antagonists in order to determine histamine binding to specific types of receptors. Unlabelled histamine inhibited the binding of ³H-histamine dihydrochloride to HMC-1 cells by about 70% (Fig 6). Non-specific binding in the presence of 10 μ M histamine was less than 25%. The IC₅₀ and K_i values were 0.838 and 0.7 nM, respectively. Binding was inhibited by about 50% using either the H2 receptor antagonist ranitidine or the H3 and H4 receptor antagonist FUB 108. In comparison, the inhibition of histamine binding to the H1 antagonist was significantly lower.

Functional aspects of histamine receptors A final set of experiments was addressed at the question of functional activity of the H1 and H2 receptors. Since histamine binding to its H2 receptor was marked, and since an increase of

**Figure 3****Demonstration of histamine receptor protein in human tissue mast cells by confocal laser microscopy.**

Human skin sections were stained for the different histamine receptor subtypes (red) (shown in A, D, G, J) and for the mast cell marker tryptase (green) (shown in B, E, H, K). Cells stained yellow on double staining in the overlay of both markers (C, F, I, L) are mast cells that are positive for the respectively stained H receptor. Note that only few cells stained for the H1 receptor (C), whereas nearly all mast cells expressed the H2 receptor (F). The H3 receptor was undetectable in human tissue mast cells (I), but the H4 receptor (L) was again expressed in almost all mast cells.

intracellular cAMP has been described in association with H2 receptor ligand interaction (Hill *et al*, 1990), this effect was studied as evidence for functionality of this histamine receptor subtype on HMC-1 cells. As expected, histamine caused a concentration-dependent increase in cAMP which was, however, moderate compared with cholera toxin (positive control) (Fig 7). An almost complete abrogation of this response was noted on pre-incubation of the cells with the H2 antagonist famotidine. In contrast, the potent H1 antagonist desloratadine caused an enhancement of cAMP-levels that was most pronounced at the highest doses tested (Fig 7).

Although minor binding of histamine to the H1 receptor was observed, activation of HMC-1 cells via this receptor was also studied with regard to the generation of the IP3 response, which represents a typical signaling event for this

receptor in numerous tissues and cell types (Hill *et al*, 1990). Histamine at varying concentrations, however, failed to induce this effect (not shown).

Discussion

The present data provide evidence for the expression of H2 and H4 receptors on HMC-1, on isolated human skin mast cells and in addition on skin mast cells located within peripheral human tissue. These receptors seem to account for most of the histamine binding on human mast cells since distinct binding was observed on flow cytometry and with displacement studies using radioactive histamine and specific receptor antagonists. Against the background of the well documented differences between murine and

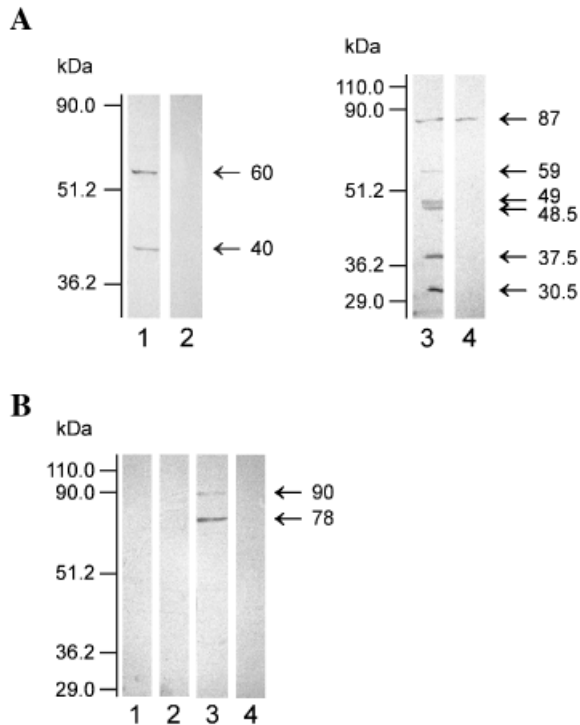


Figure 4
Investigation of histamine receptor protein size in HMC-1 cells by western blot analysis. (A) Shows the expression of the H1 and H2 receptor. For the H1 receptor to bands were detected (lane 1) that disappeared by pre-incubation of the antibody with the corresponding peptide (lane 2). Lanes 3 and 4 represent the expression of the H2 receptor (lane 3: antibody alone; lane 4: antibody and control peptide). Since the 87 kDa band was not inhibited by the corresponding control peptide, it has to be viewed as unspecific. (B) Detection of the H4 receptor (lane 3) that disappears after pre-incubation with the control peptide (lane 4). As expected, no expression of the H3 receptor type was found neither for the antibody alone (lane 1) nor for antibody plus control peptide (lane 2).

human mast cells, the present data seem of major importance in view of the applicability of findings in mice on mast cell function in human disease.

When interpreting the present results, it is important to keep in mind that H3 and H4 receptors exhibit 40% homology and are functionally related (Liu *et al*, 2000; Morse *et al*, 2001). H3 inhibitors also bind to the H4 receptor, as noted in preliminary studies for the H3 antagonist FUB 181 that was used here (Stark *et al*, 1998). In view of the lack of the H3 receptor on skin mast cells, the histamine displacement noted with FUB 181 strongly suggests the presence of H4 receptors on mast cells. This assumption was confirmed by the demonstration of H4 receptor protein expression on HMC-1 and skin mast cells. Our findings, using western blot analysis and flow cytometry, and above all the reactivity of the H4 receptor-specific antibody with mast cells in cutaneous tissue, confirm and extend the recent data of Morse *et al* who demonstrated H4 mRNA using commercially available cDNA of human mast cells cultured from bone marrow precursors. The cross-reactivity of H3 antagonists with H4 receptors also offers an explanation for previous data on H3 receptor expression on peripheral mast cells (Bissonnette *et al*, 1996) and is in line with recent animal studies that failed

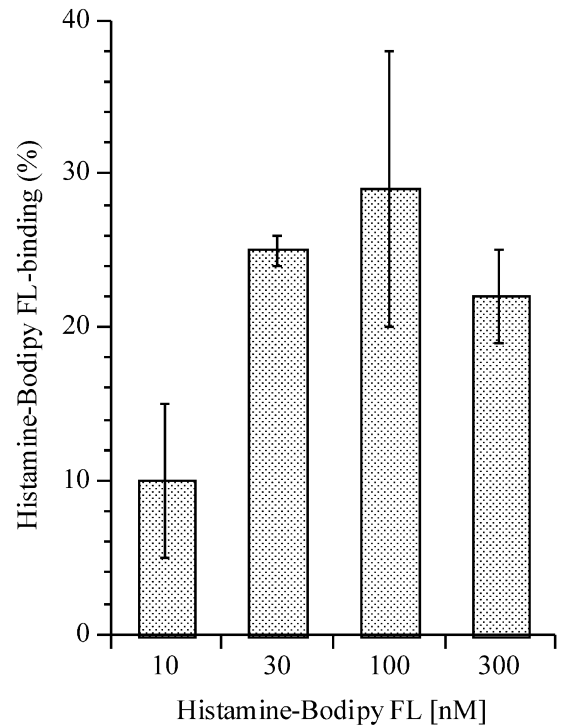


Figure 5
Binding of fluorescence-labeled histamine (Bodipy FL histamine) to HMC-1 cells. Cells were incubated for 60 min at 4°C with various concentrations of fluorescence labeled histamine in the presence or absence of 1 μ M unlabeled histamine. At least 10^4 cells were analyzed by an Epics XL flow cytometer.

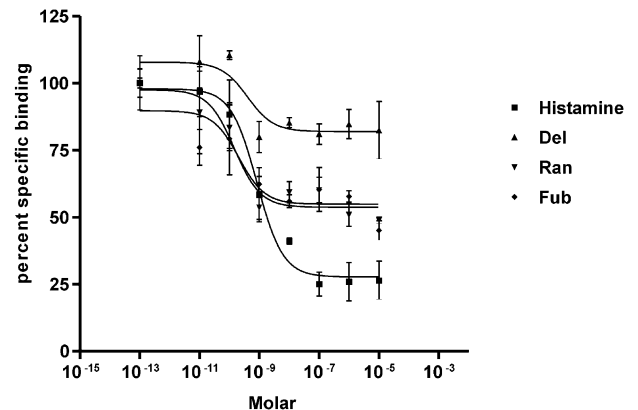


Figure 6
Competition for specific ^{3} H-histamine binding in HMC-1 cells. In this case, different histamine receptor antagonists also were included in the study. Data show means $\pm$ SD of three independent experiments. The curves were calculated from the computed parameters using Prism2.

to show expression of H3 receptors on peripherally located mast cells (Nemmar *et al*, 1999; Rozniecki *et al*, 1999).

In functional studies, we were able to demonstrate a histamine-induced increase of cAMP, which was inhibited by pre-incubation with an H2 antagonist. These findings suggest that histamine regulates its own secretion also in HMC-1 cells, as shown before with H2 antagonists in association with an intracellular increase of cAMP for human lung mast cells (Lichtenstein *et al*, 1973). A similar

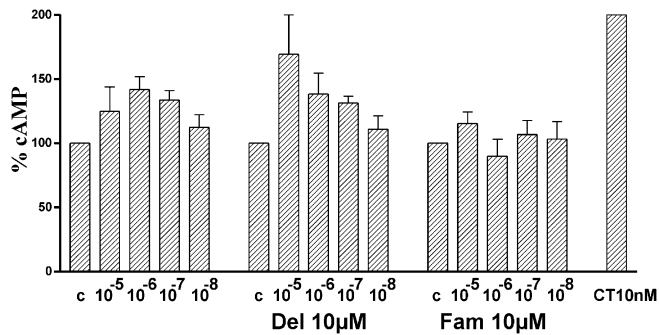


Figure 7
cAMP measurements in HMC-1 cells after treatment with histamine and pre-incubation with an H1 or an H2 antagonist. Cells were pre-incubated with buffer, desloratadine or famotidine (both at 10 µM) for 30 min and then stimulated with histamine at 10^{-5} – 10^{-8} M. The value obtained with cells kept in buffer alone was set at 100%, and cholera toxin was used as positive control. Data represent means $\pm$ SD of three independent experiments.

situation may also apply to secretion of mediators different from histamine, as recently demonstrated for TNF- α , IL-3, and IL-8 (Lippert *et al*, 2000).

The data showing that pre-incubation with an H1 antagonist caused an increase of histamine-induced cAMP seem surprising at first sight, but they probably indicate a competitive antagonism between H1 and H2 receptors on the same cells, as proposed before for other cell types (Palacios *et al*, 1978). Overall, H1 receptor expression on human skin mast cells seems less distinct. Although the expression of this receptor appears convincing on the basis of our PCR, western blot and FACS data in HMC-1 cells, the inhibition of histamine binding on HMC-1 cells by the H1 antagonist is only moderate and may explain why a rise in IP3 was not noted. In cutaneous mast cells, however, there was only a very weak expression of the H1 receptor, indicating that this receptor may be of less importance in mature or normal mast cells, as holds at least for mast cells in the skin.

Taken together, functional H2 receptors are expressed by mast cells where they may exert immunomodulatory effects, in analogy to findings with lymphocytes and monocytes (Elenkov *et al*, 1998; Jutel *et al*, 2001).

In addition, isolated human mast cells as well as tissue mast cells also showed a strong constitutive expression of the H4 receptor protein. This finding is of great interest in view of the suggested participation of the H4 receptor in allergic reactions. Although the regulatory function of H4 receptors is not well understood until now, first data indicate that these receptors may be involved in inhibitory mechanisms, as described before for the closely related H3 receptor (Liu *et al*, 2000; Morse *et al*, 2001). On the other hand, recent reports describe H4 receptor involvement in migration of murine mast cells, CD4⁺ T cells, and of eosinophil granulocytes (Gantner *et al*, 2002; O'Reilly *et al*, 2002; Hofstra *et al*, 2003).

Taken together, H4 receptors might act, together with H2 receptors, in the downregulation of allergic reactions, but they may also facilitate these reactions by the attraction of potent allergic effector cells. In view of the currently available limited and in part even contradictory data regarding

the biological function of H4 receptors, the beneficial or enhancing roles of these molecules in allergic processes need to be further elucidated. On the basis of the information available so far, one may however expect that new agents targeted at H4 receptors will soon be developed providing novel therapeutic regimens for the modulation of allergic diseases.

Materials and Methods

Cells Human leukemic mast cells (HMC-1, from H. J. Butterfield, Minneapolis, Minnesota) were cultured as described (Butterfield *et al*, 1988). Skin mast cells were isolated from human foreskin and purified by positive sorting using the monoclonal anti-c-Kit antibody YB5B8 (kindly provided by L. Ashman, Adelaide, Australia) and a secondary magnetic microbead-coupled antibody (Miltenei Biotec, Bergisch Gladbach, Germany), as previously detailed (Grützka *et al*, 2000). Cells used for RT-PCR were >95% c-Kit positive on flow cytometry with a phycoerythrin (PE)-conjugated anti-c-Kit antibody (Coulter Immunotech, Hamburg, Germany). Histiocytic lymphoma cells (U937, purchased from the ATCC, Rockville, Maryland, number CRL 1593) was cultured in RPMI 1640 medium supplemented with 10% FCS. Human erythrocytes were isolated from whole blood of healthy blood donors using gradient centrifugation (Prieto *et al*, 2001) (purity of 99.5%, viability >99%).

Skin specimens were obtained during surgical breast reductions, with informed consent. Human brain tissue (hypothalamus) was from the tissue bank of the Neuropathology Department of the Charité, Berlin, Germany.

Reagents Histamine dihydrochloride (Sigma, Deisenhofen, Germany) was dissolved at 1 mM in an aqueous stock solution. The following histamine antagonists were studied with the stock solutions indicated: H1 antagonists: cetirizine, 10^{-2} M (UCB, Brussels, Belgium) and diphenhydramine hydrochloride, 1 M (ICN, Eschwege, Germany); desloratadine, 10^{-2} M in DMSO (Essex Pharma, Munich, Germany); H2 antagonists: ranitidine, 1 M (ICN), famotidine, 10^{-2} M in DMSO (Fluka, Deisenhofen, Germany); the H3/H4 antagonist FUB 181 (Stark *et al*, 1998) at 10^{-2} M in DMSO (from W. Schunack, Institute of Pharmacology, Free University, Berlin, Germany).

For flow cytometry and western blot, commercially available H2, H3, and H4 receptor antibodies were used (all from Alpha Diagnostic International, San Antonio, Texas). Since no specific binding with a commercially available H1 receptor antibody was detected, we produced and affinity purified an H1 receptor antibody in rabbits, using a keyhole limpet hemocyanin coupled peptide synthesized on the basis of the sequence of the human H1 receptor (C-N-E-N-F-K-K-T-F-K-R-I-L-H) (Schneider *et al*, 1983), corresponding to the H1 receptor located at the 471–485 C-terminal intracellular domain (Hill, 1990).

RT-PCR analysis RNA was extracted according to the instructions of the manufacturer (RNeasy, Qiagen, Hilden, Germany) and reverse transcribed, as reported before (Lippert *et al*, 1998). Oligonucleotide primer sequences for H receptors were designed (MWG Biotech, Ebersberg, Germany) according to the following sequences published in Genbank, with their accession numbers given in square brackets: H1 receptor [Z34897], H2 receptor [M64799], H3 [AB045369], and H4 [AB04370]. PCR products were subjected to gel electrophoresis and detected by gel analysis software (Herolab, Wiesloch, Germany). Negative controls with water instead of cDNA were always included. PCR conditions were as follows: HIS1-F: ACC-ATC-CAA-AAC-CCC-CAA-GG; HIS1-R: TCT-TGA-ATG-CGA-GCG-GAG-CC, 373 bp, 2 min 95°C, 36 cycles of 45 s 95°C, 45 s 59°C, 1 min 72°C. HIS2-F: GAC-TCT-ACC-GCA-TGC-AAG, HIS2-R: GCC-AGT-GGG-TCC-ATG-AC, 336 bp, 2 min

95°C, 38 cycles of 45 s 95°C, 45 s 59°C, 1 min 72°C. HIS3-F: CGG-CTG-TCA-ACC-CTG-TCC-TCT-ACC, HIS3-R: CCC-AGC-CTC-CAG-TCC-AGC-CAG-TGA, 334 bp, 2 min 95°C, 35 cycles of 45 s 95°C, 45 s 65°C, 1 min 72°C. HIS4-F: GGT-ACA-TCC-TTG-CCA-TCA-CAT-CAT, HIS4-R: ACT-TGG-CTA-ATC-TCC-TGG-CTC-TAA, 396 bp, 2 min 95°C, 35 cycles of 45 s 95°C, 45 s 59°C, 1 min 72°C. All reactions were terminated by a final elongation step of 10 min at 72°C. The PCR products were identified by cycle sequencing, using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems GmbH, Weiterstadt, Germany).

Quantitative PCR Expression levels of different histamine receptor cDNAs in HMC-1 and skin mast cells were compared by semi-quantitative PCR. For this purpose, cDNA levels were first normalized using β -actin as an internal standard. PCR conditions for β -actin were as follows: β -actin-F: TC TTG TTT TCT GCG and β -actin-R: CCT TCC TGG GCA TGG AGT CCT 400 bp 5 min 94°C, 23 cycle of 1 min 94°C, 1 min 58°C, 1 min 72°C. PCR was terminated by a final elongation step of 10 min at 72°C. Expression levels of histamine receptors were analyzed by image analyzer software (UVP Inc, Cambridge, UK).

Flow cytometry Protein expression of histamine receptors was studied using specific polyclonal rabbit antibodies against the H1, H2, H3, and H4 receptors and a PE-conjugated F(ab')₂ fragment of goat anti-rabbit IgG (Biosource, Solingen, Germany) as secondary antibody. After pre-incubation with human AB serum (Behringwerke AG, Marburg, Germany) (30 min, 4°C) to block non-specific binding, cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany), permeabilized with 0.025% saponin (Sigma) and analyzed by flow cytometry (PAS-III, Partec, Münster, Germany), as described (Grützkau *et al*, 1997). In some experiments, cells were pre-incubated with a 10-fold excess of the respective control peptides (Alpha Diagnostic International) for 1 h min at 4°C.

Double immunofluorescence staining of skin sections for confocal laser microscopy Skin biopsies were fixed in acetone for 24 h, followed by an incubation in methyl benzoate (30 min) and in xylene (45 min) under negative pressure, and 3 μ m sections were cut from paraffin embedded tissue. Prior to staining, endogenous peroxidase was blocked with hydrogen peroxide (0.3%). Non-specific binding of the antibodies was inhibited by incubation with TNB buffer (Nen Life Science Products, Boston, Massachusetts) according to the manufacturer's instructions. Subsequently, the sections were washed and incubated with the primary antibodies against H1, H2, H3, and H4 receptors (all diluted 1:50) overnight at 4°C. In further steps, slides were treated with peroxidase labelled goat anti rabbit/goat anti mouse immunoglobulins (DAKO, Hamburg, Germany), washed and incubated with biotinylated thyramide (1:50 in amplification diluent, Nen Life Science Products). Reactive sites were detected with indocarbocyanine (Cy3)-conjugated streptavidine (1:1000 in phosphate-buffered saline (PBS), Dianova, Hamburg, Germany, 60 min). Slides were washed thoroughly in PBS, and the second step primary antibody mAb human mast cell tryptase (diluted 1:300 in PBS, clone AA1, DAKO) was added for 60 min. Subsequently, after washing, Cy5-conjugated goat anti-mouse immunoglobulins (1:500 in PBS, Dianova) were applied for 60 min. Omission of first or second step antibody and both antibodies served as negative controls to prove successful blocking of additional binding sites of the first step primary antibody and unspecific cellular antigens.

Analysis of dual-color fluorescence signals was digitally performed with confocal laser microscopy on a Leica TCS NT laser scanning microscope (Heidelberg, Germany) with 400-fold magnification.

Immunoblot HMC-1 cells were resuspended in homogenization buffer (280 mM sucrose, 50 mM TRIS, 1 mM EDTA, pH 7.4 containing protease inhibitors) and homogenized using a ball cracker. A post-nuclear supernatant was generated by centrifugation for 15 min at 400 $\times$ g. Membranes were sedimented using

aTLA100.3 rotor (30 min, 400.000 $\times$ g) (Beckmann, Fullerton, California). SDS-PAGE was done on a 10% acrylamide gel, with 50 μ g of protein sample per slot and a low range molecular weight standard (Bio-Rad Laboratories GmbH, München, Germany). The protein was transferred to nitrocellulose membranes by electro-blotting and stained with the anti-H1 (diluted 1:250), -H2, -H3, and -H4 receptor antibodies (diluted 1:50). As secondary antibody, a goat anti-rabbit-HRP (horse radish peroxidase) labeled antibody (diluted 1:1500, Dianova), was used and stained with chloronaphthol (Merck), as described before (Brand *et al*, 1991).

Receptor binding studies Initially, binding studies were performed by flow cytometry, as described (Lippert *et al*, 1998), using a commercially available fluorescence labeled histamine (Bodipy FL histamine; Molecular Probes, Leiden, Netherlands). HMC-1 cells were incubated (60 min, 4°C) with increasing reagent concentrations in the presence of 1 μ M unlabeled histamine and analyzed with an Epics XL flow cytometer (Coulter, excitation wavelength 488 nm, emission wavelength 525 nm). The percentage of Bodipy FL histamine binding was calculated as follows: % histamine Bodipy FL binding = (MFI1 – MFI2)/MFI2 $\times$ 100 (MFI1 = mean fluorescence intensity in the absence, MFI2 = the same in the presence of 1 μ M unlabeled histamine).

HMC-1 cells (2 $\times$ 10⁶ per well) were also incubated at 4°C with 1 nM ³H-histamine (Amersham Pharma Biotech Europe, Freiburg, Germany) and increasing concentrations of competing ligand (unlabeled histamine, desloratadine, ranitidine, FUB 181) in binding buffer (50 mM Tris/HCl, pH 7.4; 100 mM NaCl, 5 mM MgCl₂, 0.25% bovine serum albumin). Unbound radioactivity was removed by washing cells on glass fiber filters with ice-cold PBS. Radioactivity of the filters was counted in a liquid scintillation counter (Beckmann). Data were analyzed with the non-linear curve-fitting program Prism2.

cAMP and IP3 assay For assessment of intracellular cAMP, HMC-1 cells were diluted in 500 μ L serum-free basal Iscove's containing 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) (Calbiochem-Novabiochem, Bad Soden, Germany). For stimulation in the presence of H1 or H2 antagonists, cells were pre-incubated with desloratadine or famotidine (10 μ M) for 30 min at 37°C, followed by incubation with histamine (0.01–10 μ M) for another 30 min at 37°C. Subsequently, cells were suspended in lysis-buffer, and aliquots were assayed for cAMP using a specific EIA-kit (Amersham Pharmacia Biotech). Cells stimulated with cholera toxin (1 nM) (Calbiochem-Novabiochem) served as positive control.

Histamine-induced effects on generation of inositol 1,4,5-trisphosphate (IP3) were measured in HMC-1 cells, as reported before (Haase *et al*, 1996). Briefly, cells labeled with 5 μ Ci per mL or 20 μ Ci per mL ³H-inositol for 24 h were stimulated for 20 s to 30 min and then lysed in 10% perchloric acid. The supernatant was neutralized using KOH and analyzed by HPLC.

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