Cell. Mol. Life Sci. 65 (2008) 1585 – 1595
1420-682X/08/101585-11
DOI 10.1007/s00018-008-8106-2
© Birkhäuser Verlag, Basel, 2008

Cellular and Molecular Life Sciences

Research Article

Activation-induced cellular accumulation of histamine in immature but not mature murine mast cells

L. J. Fitz[a,b,*], A. Brennan[a], C. R. Wood[b,+], S. J. Goldman[a] and M. T. Kasaian[a,b]

[a] Wyeth Research, 200 CambridgePark Drive, Cambridge, MA 02140 (USA), Fax: +1 617 665 5499, e-mail: LFitz@wyeth.com
[b] Pharmacology Department, Boston University School of Medicine, Boston, MA 02138 (USA)

Received 29 February 2008; received after revision 25 March 2008; accepted 26 March 2008
Online First 14 April 2008

Abstract. Mast cell activation involves the rapid release of inflammatory mediators, including histamine, from intracellular granules. The cells are capable of regranulation and multiple rounds of activation. The goal of this study was to determine if there are changes in the content of pre-formed mast cell mediators after a round of activation. After 24 h, the histamine content of bone marrow-derived mast cells (BMMC), but not that of peritoneal mast cells, exceeded the amount in resting cells. Accumulation of histamine in BMMC peaked at 72 h of activation, and returned toward preactivation levels by 96 h. The increase in histamine content was accompanied by an increase in the gene expression of histidine decarboxylase. No increases in β hexosaminidase or murine mast cell protease-6 were observed. These findings indicate that BMMC respond to activation by increasing total cell-associated histamine content. This increase may be important to the response of these cells upon subsequent exposure to antigens.

Keywords. Histidine decarboxylase, FcεRI, granule, mast cell, histamine.

Introduction

Mast cells respond rapidly to activation by releasing histamine and other pre-formed mediators from granules, by synthesis of leukotrienes and prostaglandins, and by induction of cytokine and chemokine gene transcription [1, 2]. Mast cells are activated by a wide range of inflammatory stimuli such as bacteria, parasites, and multivalent allergens, which bind IgE molecules that are precomplexed to high-affinity IgE receptors on the mast cell surface. Activation and degranulation are followed by regranulation, so that the cells become responsive to rechallenge [3]. Mast cells are long-lived and therefore it is possible for mast cells to undergo multiple activation events, such as occurs during chronic inflammatory conditions. The process of mast cell regranulation is not completely understood. Changes in the total cellular content of mast cell mediators after activation may affect their response to sequential activation in a rapid time frame, such as may occur in an ongoing immune response. To study this process, total cell-associated histamine was measured at various times after activation.

Histamine is a 111 Da, multifunctional, basic amine that is stored in mast cell granules. By binding and signaling through its four different receptors (H_1, H_2, H_3, and H_4), it induces smooth muscle cell contraction, increases vascular permeability, stimulates angiogenesis, and modulates T cell cytokine production [4–7]. Histamine is synthesized from histidine by a single intracellular enzyme, histidine decarboxylase (HDC)

* Corresponding author.
+ Present address: Dyax, Cambridge, MA (USA)

[8–10]. The importance of histamine to mast cell function is evident from HDC gene deletion experiments. Mice deficient in HDC are unable to produce histamine and have impaired mast cell development and function [11, 12].

Histamine regulation in mast cells following their activation is not well understood. In this study, we examined changes in histamine content associated with activation of bone marrow-derived mast cells (BMMC) and peritoneal mast cells (PMC) following cross-linking of the high-affinity IgE receptor or following IgE-independent activation. We found that BMMC responded to activation by a transient increase in total histamine but no increase in another granule component, β-hexosaminidase. This transient increase in histamine was preceded by an increase in HDC gene expression.

Materials and methods

BMMC. Primary mast cells were cultured from femoral and tibial bone marrow of female 6–8-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid (HEPES), 2 mM glutamine (Mediatech, Herndon, Virginia), 50 μM 2-mercaptoethanol (Sigma) (complete DMEM), 20 ng/ml murine interleukin-3 (IL-3), and 50 ng/ml murine stem cell factor (SCF) (R&D systems, Minneapolis MN). Non-adherent cells were passaged every 5–6 days for 4 weeks (10% CO_2 at 37°C). After 4 weeks in culture, the cell population expressed characteristic morphology of mast cells, and cultures contained greater than 95% KIT positive and IgE-binding cells as determined by flow cytometry (data not shown).

Mouse PMC. For experiments with mast cells obtained from the peritoneum, C57BL/6J mice were killed by inhalation of CO_2. Peritoneal lavage was collected by injecting 5 ml 25 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) pH 7.2 buffer containing 0.05 mM ethylenediaminetetraacetic acid (EDTA) into the peritoneum, and recovering the resident cells. The mixed peritoneal cells, which contain mast cells, were cultured overnight in complate DMEM supplemented with IL-3 (20 ng/ml) and murine IgE (0.1 μg/ml) (clone SP-E/7, Sigma). For some experiments, the mast cells were enriched from other peritoneal cells by layering cells over a cushion of 50% Percoll:PIPES EDTA (Sigma-Aldrich, St. Louis MO). After centrifugation (1290 rpm, 20 min), the mast cells separated to the bottom of the Percoll, and remaining cells were at the interface between the Percoll and the PIPES-EDTA. Mast cell-enriched and -depleted populations were separately cultured overnight in DMEM as described above.

Mast cell activation. For BMMC experiments, 50 000 cells/well were activated with either 1 μg/ml ionomycin (Sigma) or various concentrations of dinitrophenol-bovine serum albumin (DNP-BSA) added to 10% DMEM in a total volume of 0.2 ml. Cultures were incubated overnight or for various times, as indicated in Results, in 10% CO_2, 37°C. To assess the amount of histamine released after a second challenge, BMMC were activated for 24 h with murine IgE directed against DNP (0.1 μg/ml) and DNP-BSA (10 ng/ml), washed free of the activating agents, rested overnight without activating agents, then rechallenged with the IgE and DNP-BSA for 24 h. More than 90% of the BMMC were viable throughout the course of activation as determined by their ability to exclude trypan blue. For total peritoneal cells and enriched mast cells from the peritoneum (PMC), 10 000 cells/well were activated with either various concentrations of antibody directed against IgE (BD Pharmingen, San Jose CA) or the polycationic mast cell activator, compound 48/80 (Sigma). For both BMMC and PMC, cells were centrifuged (400 *g*, 10 min) after various times in culture, and the products of mast cell released into supernatants were collected. For determination of total cell-associated histamine, cells were washed free of supernatant, and total cellular content of histamine was determined following lysis of the cells in 200 μl 0.2% Triton X-100. More than 90% of the total peritoneal cells and enriched mast cells from the peritoneum were viable throughout the 48 h of culture as determined by their ability to exclude trypan blue.

β-hexosaminidase substrate assay. An enzymatic assay was used to quantitate β-hexosaminidase in mast cell supernatants and total cell lysates. The β-hexosaminidase substrate, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (50 μl/well, 1.3 mg/ml in 0.08 M sodium citrate pH 4.5; Sigma), was added to 10 μl supernatant or lysate from mast cells and incubated at 37°C overnight. Sodium hydroxide (50 μl/well, 1 N) was added to stop the reaction, and the absorbance at 405 nM was determined using a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA). Spontaneous release was determined by incubating cells with PACM buffer alone. Percent of maximum release was calculated compared to the total amount of β-hexosaminidase from control cells lysate after treatment with 0.2% Triton X-100 using the following formula:

Table 1. Primers used for semi quantitative RNA analysis of BMMC

Gene	Forward primer	Reverse primer	probe
GAPDH	CGTGTTCCTACCCCCAATGT	GTCATCATACTTGGCAGGTTTCTC	CGTGGATCTGACGTGCCGCC
β-hexos-aminidase	TCACTGACCTAGAAAATGCC TACAA	TCCACGGCTGACCATTCTG	CGACTGGCCGTGCACCGC
mMCP-6	TGGGACCGCACATCAAAAG	AGGAGCTGGTCCCCATAGTATAGA	ACAGCTCTTCCGGGTGCAGCT
HDC	AACCCCATCTACCTCCGACAT	AACCCCATCTACCTCCTCCGACAT	CCAACTCTGGTGCAGCCACGGAC

$$\% \text{ maximal } \beta \text{ hex release} = [(\text{sample} - \text{background})/(\text{total cell lysate} - \text{background})] \times 100.$$

Histamine detection assay. The histamine concentration of various samples was determined using a competition-type enzyme-linked immunosorbent assay (ELISA) kit purchased from ImmunoTech (Beckman Coulter, Fullerton, CA), according to the manufacturer's instructions. Mast cell supernatant or lysate samples from various stimulation and incubation conditions were assayed in replicates of three to five. Statistical analysis was performed by Student's *t*-test. Values of $p < 0.05$ were considered significant. The limit of assay detection was 3 nM.

Proliferation. BMMC (50 000 cells/well) that were treated under the same FcεRI cross-linking or ionomycin activation conditions described previously were assayed for proliferation by two methods. In the first method, cells were incubated with 0.5 μCi/well [^{3}H]thymidine (Amersham, Piscataway, NJ) for 6 h. Cells were then washed and cell lysate was collected on a filterplate. A trilux β plate reader (PerkinElmer, Boston, MA) was used to quantitate incorporated [^{3}H]thymidine. In the second method of proliferation measurement, BMMC from cultures treated by FcεRI cross-linking or ionomycin were counted daily using a hemocytometer.

Densitometric analysis. The density of mast cell granule content was evaluated using LaserPix image analysis software (Bio-Rad, Hercules, CA). Mast cells were activated by IgE receptor cross-linking, harvested at various time points post activation, and spun onto microscope slides using a Cytospin (Thermo Fisher Scientific, Waltham, MA), and stained with Wright-Geimsa. Thirty individual cells per time point were defined as areas of interest, and relative integrated optical density (IOD) was quantitated for each cell.

Quantitative real-time PCR. Gene expression levels in various BMMC cultures were measured using quantitative (q) real-time RT-PCR. Cells (10 ml, 5×10^5 cells/ml) from resting or FcεRI cross-linked BMMC cultures were harvested by centrifugation (400 *g*, 10 min). RNA was prepared using an RNeasy kit, following the manufacturer's instructions (Qiagen, Valencia, CA). The GenBank accession numbers for each gene analyzed by qRT-PCR were: murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AK132984), β-hexosaminidase (NM010422), murine mast cell protease-6 (mMCP-6; NM010781), and HDC (NM008230). Oligonucleotide primers and probes were designed using Primer Express software (Applied Biosystems Division of Perkin-Elmer, Foster City, CA) and synthesized by Eurogentec (San Diego, CA). The primer and probe sequences are shown in Table 1. Probes were labeled on the 5′ end with the reporter dye, 6-carboxyfluorescein (FAM) and on the 3′ end with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA), and HPLC purified (Eurogentec). A Prism 7000 Sequence Detection System (Applied Biosystems) thermocycler instrument was used for reverse transcription followed by 40 PCR cycles. Reactions used buffer provided by the manufacturer (Eurogentec MasterMix buffer) and contained *Thermus aquaticus* (Taq) DNA polymerase with 5′ nuclease activity, 0.25 U/μL RNase inhibitor, and 300 nM each of forward primer, reverse primer and probe. Reporter fluorescence threshold cycle (Ct) values were plotted *versus* a sixfold serial dilution of each RNA sample. The standard curve for each gene of interest was used to determine the relative amount of target sequence in the RNA from resting and activated BMMC and each was normalized to the relative amount of GAPDH. Relative gene expression was reported as a fold difference between resting and degranulated BMMC. The fluorescence signal for each unknown sample was detected within the sixfold serial standard range of each gene.

Results

Activation of BMMC increases cell-associated histamine. To examine effects of mast cell activation on cell-associated histamine content, BMMC were activated with ionomycin (1 μg/ml). Mast cell degranulation occurs within minutes, but for these experiments,

to allow for the full extent of degranulation, and to allow cells to recover from degranulation, the released histamine was measured 24 h later. As expected, ionomycin treatment resulted in the release of preformed histamine, whereas only a low amount of histamine was released from resting cells (Fig. 1A). The total cellular histamine content was measured after lysing cells with 0.2 % Triton X-100. Surprisingly, the amount of cell-associated histamine in the ionomycin-treated BMMC was increased 12-fold compared to that in resting BMMC (Fig. 1C). To determine if other mast cell activation methods also induced an increase in total cell-associated histamine, BMMC were subjected to FcεRI cross-linking. After 24 h, histamine content in supernatants and cells was quantitated and compared to that in resting BMMC. Released histamine increased relative to control cells, as expected (Fig. 1B) and cell-associated histamine increased 3-fold (Fig. 1D). Thus, total BMMC histamine content is increased following activation by FcεRI-dependent and -independent methods. This response to activation was studied in more detail.

Histamine released from BMMC was dependent on the dose of DNP-BSA added to cross-link FcεRI and showed a characteristic bell-shaped curve (Fig. 2A). The increase in the total cell-associated histamine that occurs 24 h following activation of BMMC is also dependent on the concentration of DNP-BSA added to cross-link FcεRI (Fig. 2B). BMMC treated with the lowest dose of DNP-BSA tested, 0.3 ng/ml, showed a small but significant increase in total cell-associated histamine concentration (1.4-fold increase). The maximal histamine accumulation occurred at 3 ng/ml, the same dose that was optimal for histamine release. The increase in total cell-associated histamine was dose dependent, with maximal cell-associated histamine increased 4.5-fold relative to resting cells.

To determine the kinetics of cell-associated histamine accumulation, and to investigate activation effects on amounts of another granule component, cell-associated histamine content was measured at several time points following BMMC activation and compared to the content of β-hexosaminidase. The histamine released from FcεRI-activated mast cells was 83 ± 14 ng/10^6 BMMC 15 min after activation and reached a maximum of 183 ± 21 ng/10^6 BMMC at 48 h (Fig. 3A). As expected after degranulation, cell-associated histamine at 15 min (91 ± 15 ng/10^6) and 1 h (91 ± 11 ng/10^6) was decreased compared to resting BMMC (272 ± 38 ng/10^6) (Fig. 3B, inset). At 4 h after activation, the total cell-associated histamine amount recovered was no different from the amount in resting cells. After this point, the cell-associated histamine continued to accumulate in activated cells. At 24 h after activation, the amount of histamine in activated cells (726 ± 19 ng/10^6 BMMC) exceeded that in resting cells (258 ± 9 ng/10^6 BMMC) cultured for the same amount of time (Fig. 3B). At 72 h after activation, cell-associated histamine reached a peak value that was 12-fold increased compared to resting BMMC. By 96 h, the cell-associated histamine had diminished, but was still significantly higher than found in the control resting cells (Fig. 3B). The increased histamine remained cell-associated, and was not released into the supernatant (Fig. 3A). In contrast to the increased cell-associated histamine content, there was no significant change in β-hexosaminidase activity relative to resting cells at any time point (Fig. 3C). The lack of a parallel increase in the quantity of the granule enzyme, β-hexosaminidase, suggests that the increased histamine content is not a consequence of increased granule number or overall accumulation of granule components.

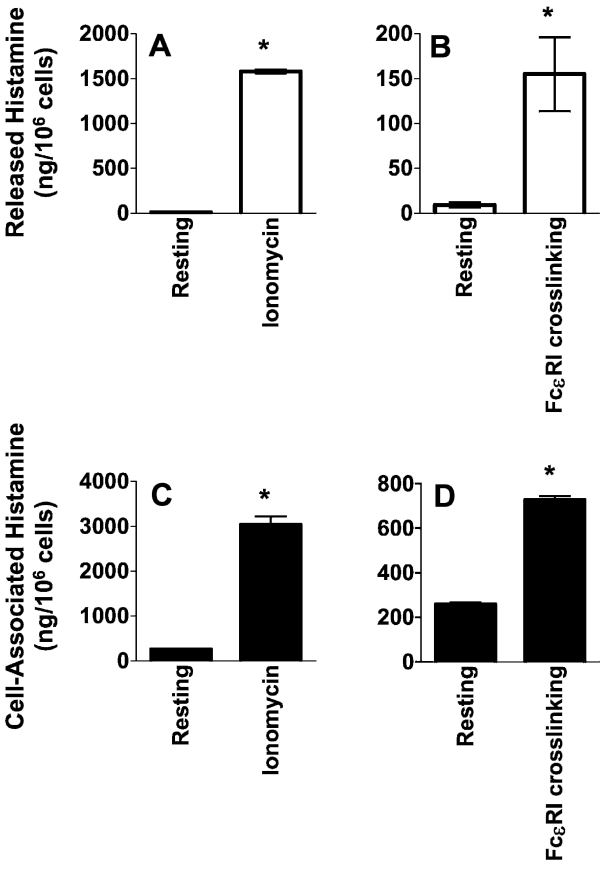

Figure 1. Activation increases BMMC total cell-associated histamine content. Histamine released into supernatants was quantitated 24 h after culture in IL-3/SCF plus, either (*A*) without (resting), or with 1 μg/ml ionomycin, or (*B*) with FcεRI cross-linking (100 ng/ml anti-DNP IgE and 3 ng/ml DNP-BSA). Cells were washed free of supernatant, and total cellular content of histamine was determined following lysis of the cells with 0.2 % Triton X-100 after 24 h culture with IL-3/SCF, either (*C*) without (resting), or with ionomycin, or with (*D*) FcεRI cross-linking. Data are shown as mean ± SD of five replicates per point. * $p < 0.05$.

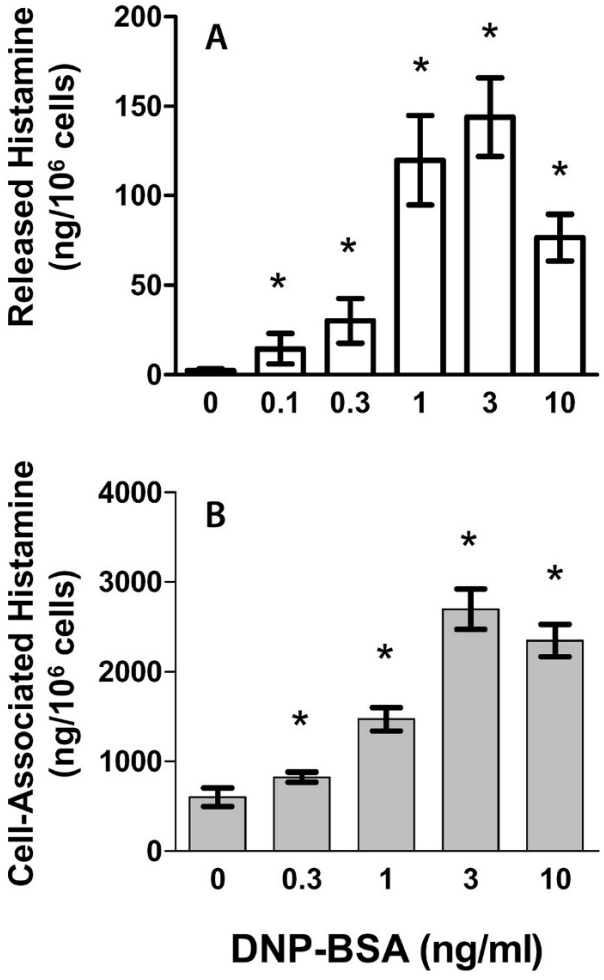

Figure 2. Accumulation of histamine in BMMC is dependent on DNP-BSA concentration. BMMC were cultured in IL-3/SCF alone (0 DNP-BSA) or presensitized with IgE and cultured 24 h with the indicated concentrations of DNP-BSA. (*A*) Histamine released into supernatants was quantitated over the dose titration of DNP-BSA. (*B*) Cells were washed free of supernatant, and total cellular content of histamine was determined following lysis of the cells with 0.2% Triton X-100. Data shown are mean ± SD of five replicates per point. * $p < 0.05$.

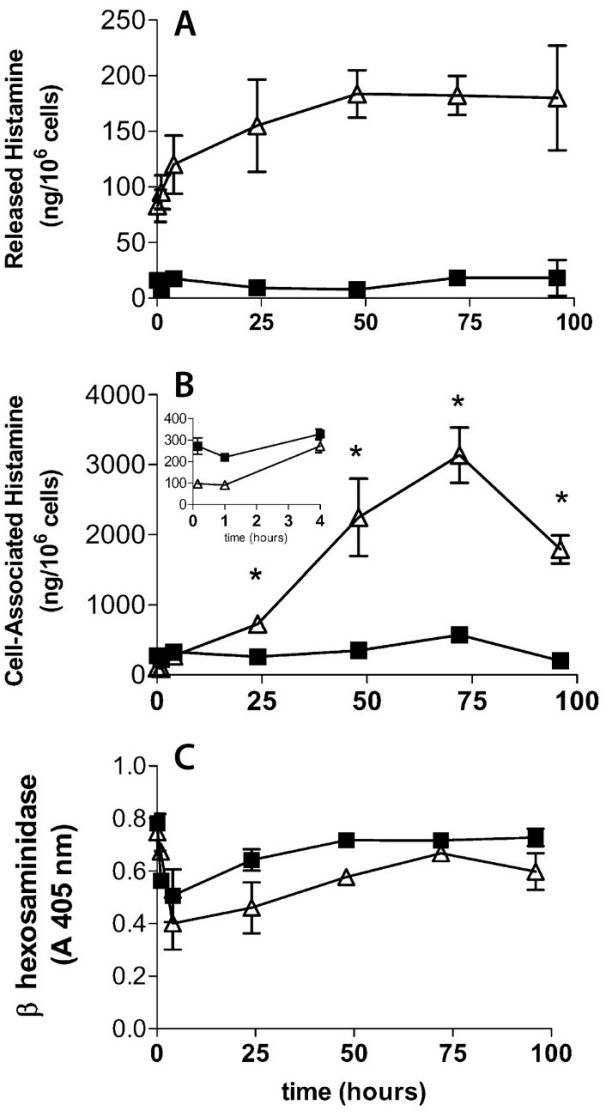

Figure 3. Time course of histamine accumulation after activation; total cell-associated amounts of histamine and β-hexosaminidase. BMMC were cultured with IL-3/SCF and activated by FcεRI cross-linking (100 ng/ml anti-DNP IgE and 3 ng/ml DNP-BSA) (△) or not activated (■) for the times indicated. (*A*) Histamine released into supernatants was quantitated at each time point. Cells were washed free of supernatant, and total cellular content of histamine (*B*) or β-hexosaminidase (*C*) was measured following lysis of the cells with 0.2% Triton X-100. (*B*, inset) Total cell-associated amounts of histamine after 15 min, and 1 and 4 h after activation (△) compared to the same culture times for unactivated BMMC (■). Data shown are mean ± SD of five replicates. * $p < 0.05$ compared to resting.

Increased cell-associated histamine is not due to mast cell proliferation or granule accumulation. Several experiments were done to further investigate the increase in BMMC histamine content that occurred 24–96 h after activation. We considered that an apparent accumulation of cell-associated histamine after BMMC activation could be secondary to proliferation. Within the 96-h time frame of these experiments, however, there was no evidence that activation of mast cells induced their proliferation. Consistent with previous reports [13, 14], there was only minimal incorporation of [^{3}H]thymidine in cultured cells and no differences between resting and activated cells (data not shown). In addition, there was no difference in cell number between cultures of resting BMMC and activated BMMC, up to 96 h after activation (data not shown). The lack of a proliferative response by BMMC following activation suggests that the increase in histamine content measured in these cultures is not due to an increase in the number of mast cells.

Histamine accumulation following activation might also be secondary to an accumulation of mast cell granules. To test this possibility, we visualized BMMC

granules by staining with Wright-Geimsa dye. Nearly all cells in the resting cultures contained granules, comparable in size and density per cell (Fig. 4A). Hours after FcεRI cross-linking, BMMC had granule content that appeared sparser and lighter in staining than those of resting cells, consistent with mast cell degranulation. Densitometric analysis showed a significant decrease in granule density at 1, 3, and 24 h, and no difference compared to resting cells at 48 h (Fig. 4B). Even though total cell-associated histamine was increased 8–12 fold by 48 h post activation, granule content was not increased relative to that found in resting cells.

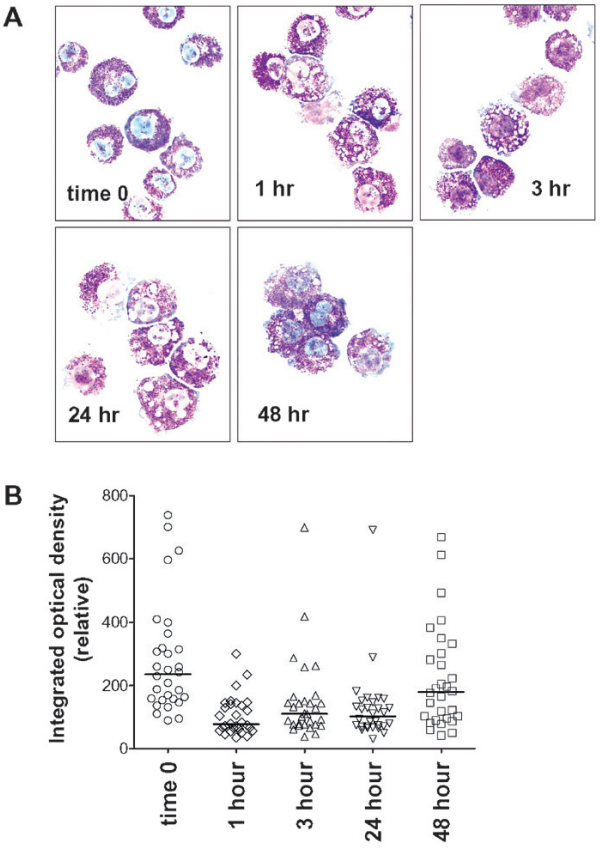

Figure 4. (*A*) Light microscopic photos of BMMC at time 0, and at 1, 3, 24, and 48 h post activation by IgE receptor cross-linking. Cells were centrifuged onto a slide and stained with Wright-Giemsa dye. (*B*) Densitometric analysis of mast cell granule content, measured as integrated optical density (IOD), for 30 individual cells per time point, using LaserPix software. Median values for the 1-, 3-, and 24-h time points were significantly different than time 0 ($p < 0.005$ by *t*-test).

Increased cell-associated histamine correlates with HDC induction. Histamine is synthesized by HDC, which is associated with mast cell granules [15]. The relative gene expression of HDC and two other granule components, β-hexosaminidase, and murine mast cell protease-6 (mMCP-6), was compared between resting and activated BMMC. Since gene induction is expected to precede protein expression, RNA was prepared from cells 1.5 h following FcεRI cross-linking and compared to RNA prepared from BMMC that were incubated with IgE but no DNP-BSA. The relative gene expression was measured by qRT-PCR and normalized to GAPDH. The relative gene expression for β-hexosaminidase was not different between resting and activated BMMC (Fig. 5). This finding is consistent with the lack of difference in the enzymatic activity of β-hexosaminidase between resting and FcεRI cross-linked BMMC (Fig. 3C). Similarly, there was no change in gene expression of another predominant granule enzyme, mMCP-6 (Fig. 5). In contrast, there was a significant increase in HDC gene expression in FcεRI-cross-linked BMMC compared to resting cells, indicating that the mechanism for an increase in cell-associated histamine after mast cell degranulation may involve a rapid increase in HDC transcription (Fig. 5).

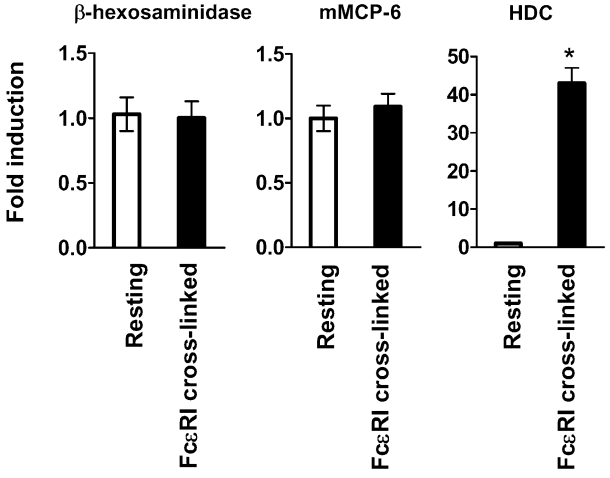

Figure 5. BMMC activation increases expression of HDC but not other granule components. Relative mRNA expression is shown for resting BMMC (open bar) and for BMMC activated for 1.5 h by FcεRI cross-linking (100 ng/ml anti-DNP IgE and 3 ng/ml DNP-BSA) (filled bars). RT-PCR measurements were normalized to GAPDH for: β-hexosaminidase, mMCP-6, and HDC, as indicated. Data are shown as mean ± SD of three replicates.

Histamine release after BMMC rechallenge. Throughout the time course of the experiments described previously, BMMC were continuously exposed to activating agents. To address whether BMMC are capable of releasing additional histamine under conditions of increased cell-associated histamine, BMMC were challenged with DNP-BSA for 24 h, washed free of the activating agents, rested overnight, and then rechallenged for 24 h. Resting BMMC (buffer, Table 2) had 190 ± 16 ng/10^6 cells of

Table 2. Released and cell-associated histamine in BMMC after one or two rounds of challenge.

Treatment for 24 h	Histamine (ng/10^6 cells)		Rechallenge for 24 h	Histamine (ng/10^6 cells)	
	Released	Cell associated		Released	Cell associated
Buffer	14 ± 4	190 ± 16	Buffer	12.8 ± 8.4	258 ± 9.1
			DNP-BSA	106 ± 61	534 ± 189
DNP-BSA	125 ± 24	494 ± 214	Buffer	3.5 ± 1.2	523 ± 106
			DNP-BSA	126 ± 30	1154 ± 181

Histamine was measured 24 h after BMMC were activated with either buffer or 10 ng/ml DNP-BSA on day 0. Cells were washed free of the DNP-BSA, rested overnight, and released and cell-associated histamine was measured 24 h after rechallenging the cells with a buffer control or DNP-BSA. Data shown are mean ± SD of four replicates.

cell-associated histamine. Cells activated for 24 h had 494 ± 214 ng/10^6 cells of cell-associated histamine (Table 2). BMMC that were challenged for 24 h, rested overnight after washing away excess DNP-BSA, and then rechallenged with DNP-BSA for 24 h had 1154 ± 181 ng/10^6 cells of cell-associated histamine. This was about the same amount as BMMC with continuous challenge for 48 h (Fig. 2B). Although the cell-associated histamine in activated BMMC was increased, there was no increase in the amount of histamine that was released from the rechallenged cells (126 ± 30 ng/10^6 cells) compared to cells activated for the first time (125 ± 24 ng/10^6 cells) (Table 2).

Total cell-associated histamine of PMC does not increase in response to activation. BMMC are an *in vitro* model of what may be considered an immature or mucosal mast cell phenotype, whereas mast cells located in the mouse peritoneum (PMC) have features of mature connective tissue mast cells. PMC store more histamine in their granules than BMMC [16]. Having observed increased histamine content in BMMC following activation, we asked whether the same phenomenon occurred in PMC. Unfractionated mouse peritoneal cells were activated by cross-linking the FcεRI with anti-IgE, or activated in an IgE-independent manner with compound 48/80. Released and total cell-associated histamine was quantitated 24 h later. As expected, histamine was released from the peritoneal cell population following activation with FcεRI cross-linking or compound 48/80 (Fig. 6A). In contrast to BMMC activation, neither FcεRI-cross-linking nor compound 48/80 treatment induced an increase in the total cell-associated histamine content of the peritoneal cell population (Fig. 6B). The lack of increase in cell-associated histamine following PMC activation is consistent with previous studies [17, 18].

To investigate this response to activation more fully, and to address whether the lack of increased total cell-associated histamine in peritoneal cells was due to the presence of other cell types, mast cells were enriched over a cushion of Percoll and examined for released

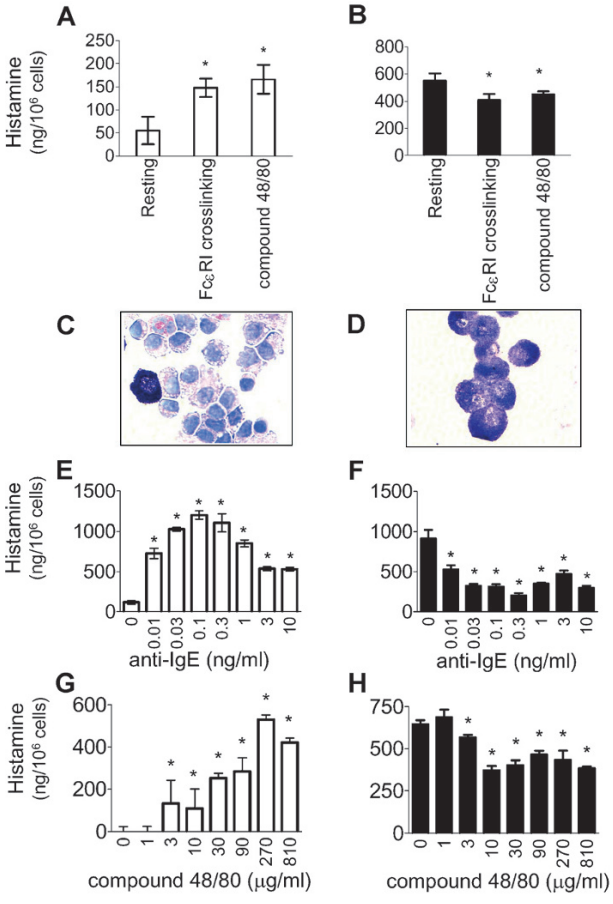

Figure 6. PMC do not accumulate total cell-associated histamine following activation. (*A*) A mixed cell population of PMC were cultured for 24 h in IL-3/SCF, (resting), or with FcεRI cross-linking (100 ng/ml anti-IgE and 1 μg/ml IgE) or with 90 μg/ml compound 48/80. Histamine released into supernatants. (*B*) Cells were washed free of supernatant, and total cellular content of histamine was measured following lysis of the cells with 0.2 % Triton X-100. Light microscopic photos of total cells from (*C*) mouse peritoneum and (*D*) enriched PMC. Cells were centrifuged onto a slide and stained with Wright-Giemsa dye. The PMC enriched to >98 % mast cells were cultured for 24 h in IL-3/SCF, plus (*E*, *F*) 100 ng/ml IgE and increasing concentrations of anti-IgE to cross-link FcεRI (0–10 μg/ml anti-IgE) or (*G*, *H*) increasing concentrations of compound 48/80 (0–810 μg/ml). (*E*, *G*) Histamine released into supernatants. (*F*, *H*) Cells were washed free of supernatant, and total cellular content of histamine was measured following lysis of the cells with 0.2 % Triton X-100. Data shown are means ± SD of triplicates. * $p < 0.05$.

and total cell-associated histamine following activation. Total cells in the peritoneum contain approximately 5% mast cells (Fig. 6C) and PMC recovered from a Percoll cushion were enriched to >98% (Fig. 6D). The mast cell-depleted fraction did not release histamine in response to activation and no histamine was detected in the total cell population (data not shown). The enriched PMC responded in the same way to activation as mast cells in unfractionated peritoneal cells. The enriched PMC released histamine after FcεRI cross-linking in a dose-dependent manner, and maximal release of histamine occurred at 0.1 μg/ml anti-IgE antibody (Fig. 6E). No concentration of anti-IgE induced an increase of cell-associated histamine from the enriched PMC (Fig. 6F). Instead, the doses of anti-IgE that stimulated histamine release, starting at 0.1 μg/ml, produced a corresponding significant decrease in total cell-associated histamine content. The amount of histamine released plus the total intracellular amount of histamine in the mixed peritoneal cell population following activation was approximately equivalent to the total histamine content in resting cultures, indicating that no additional histamine appeared to be induced. To further characterize histamine amounts in PMC, cells were activated in an FcεRI-independent manner using the polyamine compound 48/80. As expected, treatment with increasing concentrations of compound 48/80 from 0 to 810 μg/ml resulted in a dose-dependent release of histamine into the supernatant (Fig. 6G). Similar to FcεRI-dependent activation, no concentration of compound 48/80 induced an increase in the total cell-associated histamine amounts (Fig. 6H). Thus, unlike BMMC, PMC do not respond to activation by increasing the amount of cell-associated histamine.

Discussion

In this study, we show that between 24 and 96 h after activation, the histamine content of BMMC is replenished to an amount exceeding that in preactivated cells. Histamine is a basic amine that is stored in basophil and mast cell cytoplasmic granules and is released from these intracellular stores upon activation by a number of inflammatory stimuli, including agents that cross-link the high-affinity IgE receptor. Histamine contributes to symptoms of atopic diseases, anaphylaxis, and urticaria, has T cell immunomodulatory activity, and may support immune responses against bacteria [5, 6, 19]. The extent to which histamine is replenished in mast cells following a round of activation and degranulation provides insight into the functional capacity of a mast cell in an environment supporting sequential rounds of activation. The increase in total cell-associated histamine content suggests enhanced functional capabilities for mast cells in an ongoing immune response. Following the initial release of histamine that occurs within 15 min after activation, a further increase of histamine was released and reached a plateau 48 h after activation.

The direct correlation between the dose response of DNP-BSA required for both histamine release and an accumulation of cell-associated histamine suggests the increase in cell-associated histamine is due to activation. BMMC activation induces expression of HDC, the rate-limiting enzyme for histamine production. The magnitude of this increase in HDC transcription is comparable to that of histamine production. Tanaka et al. [20, 21] previously demonstrated a transient increase in HDC transcript and protein content following activation of BMMC with monomeric IgE, in the absence of IgE receptor cross-linking. Although the effect of this treatment on total cell-associated histamine content was not reported, the observation that cross-linking and thus degranulation were not required raises the possibility that 'stored' and 'inducible' histamine may have distinct biological roles [22]. The short-term increase in histamine may be due to a transient increase in HDC, leading to a transient increase in histamine. A transient increase in cell-associated histamine may allow mast cells to respond to multiple immunological challenges such as occur under conditions of inflammation. Because the nature of the increased cell-associated histamine is transient, the heightened response by mast cells resolves and these long-lived cells do not remain in a heightened response state.

The transient increase in cell-associated histamine following activation is consistent with an earlier observation showing no difference in cell-associated histamine 12 days after BMMC activation compared to unactivated cells [13]. The reduction in cell-associated histamine 72–96 hours post activation was not due to histamine release from the cell during this time frame. One possibility for the decrease is that the cell-associated histamine in BMMC is metabolized by the same cytoplasmic enzymes that metabolize histamine in tissues, histamine-*N*-methyl transferase (HMT) and diamine oxidase (DAO) [23, 24]. Mast cells and basophils are the only cell types that store histamine; however, other cell types can be induced to produce histamine. Neutrophils produce histamine in response to *Mycoplasma* [25]. In bone marrow progenitor cells [26] and macrophages [27], histamine is synthesized and stored in the cytoplasm and not in granules. Although cytoplasmic histamine has not been reported in mast cells, it is possible that

the increased histamine observed in the BMMC examined in the present study is not stored in granules, but may be cytosolic. This is consistent with our findings that higher amounts of histamine were not released upon rechallenge, that BMMC granule number did not increase, and that other granule constituents (β hexosaminidase and mMCP-6) did not accumulate under conditions associated with increased histamine. Accumulation of cytosolic histamine may give the cells additional effector capability against intracellular pathogens such as *Mycoplasma.* Mast cells directly recognize mycobacteria and are required for protection against these intracellular pathogens [28]. Cytosolic histamine in neutrophils has been shown to be important for innate immunity against *Mycoplasma,* and cytosolic histamine could play a similar role in mast cells [25]. Further experiments are needed to confirm if the high quantity of histamine observed in the present study includes histamine stored in the cytoplasm or in granules, or if it is associated with the cell surface.

Mast cell activation is associated with the release of an array of mediators, including histamine, proteolytic enzymes, leukotrienes, prostaglandins, cytokines, and chemokines, within a rapid time frame [1, 29]. The histamine accumulation response that we observed became apparent as early as 24 h post activation, and peaked at approximately 72 h post activation. This suggests the possibility that released mediators acting back on the mast cell could drive the histamine accumulation response. For example, GM-CSF, which may be produced by BMMC, has been reported to induce HDC gene transcription in U937 human monocytes [30]. An alternative possibility is that a signaling pathway triggered by the initial cell surface activation event leads to histamine accumulation. This interpretation would be consistent with the induction of HDC following stimulation of BMMC with monomeric IgE [20, 21], which would be expected to activate signaling pathways but would not trigger release of the full complement of mast cell mediators. Because of the complexity of mediator release associated with mast cell activation, as well as the complexity of signaling responses triggered downstream of IgE receptor cross-linking [29, 31], precise identification of the mechanism responsible for this phenomenon must await further study.

The increase in total cell-associated histamine observed in BMMC does not occur in PMC. All mast cells are derived from a common progenitor in the bone marrow [32]. They circulate to tissues where they undergo maturation and take on different characteristics, dependent on the surrounding tissue environment [33]. Mast cells derived from bone marrow *in vitro* have features of mucosal or immature connective tissue-type mast cells (CTMC) and PMC have features of mature CTMC [34–38]. BMMC and PMC differ in the composition of their granule constituents. PMC store more histamine in their granules than BMMC, and they contain higher amounts of heparan sulfate proteoglycan in their granules compared to BMMC [16], whereas BMMC granules are abundant in chondroitin sulfate proteoglycan [2]. Differences have been noted between BMMC and PMC in other phenotypic and activation responses. CTMC but not BMMC respond to the polybasic amine-activating agent, compound 48/80. The amount of histamine in resting PMC in the present study was about four times greater than the amount in resting BMMC, consistent with previous reports [16, 38]. The total amount of histamine released from the enriched PMC under maximal activation conditions was greater than the total amount released after maximal activation of BMMC, comparable to the greater histamine content of resting PMC. However, unlike the response in BMMC, activation of PMC did not induce an increase in the total amount of cell-associated histamine from either FcεRI-dependent or -independent activation. The lack of an increase in the total cell-associated histamine of mouse PMC 24 h after activation is consistent with previous reports that also show no increase in the total cell-associated histamine content of PMC co-cultured with 3T3 fibroblasts and activated between 1 and 14 days [17, 18]. Although PMC do not respond to activation by increasing their total cell-associated histamine content, the cells remain responsive to rechallenge [17, 18]. The differences in total histamine content following activation observed between PMC and BMMC may be related to differences in the functional capacities of these mast cell types.

In support of our observations with BMMC, Castellani et al. [39, 40] have recently reported that human umbilical cord blood-derived mast cells have increased levels of HDC transcript following IgE receptor cross-linking. One advantage of producing more histamine following activation may be to prepare for subsequent rounds of activation. One round of activation may serve as an inflammatory sensor so that the cells escalate their response upon reactivation. The increase in mast cell histamine content following a round of activation may yield new insights into mast cell functions during allergic reactions, wound healing, and host defense against pathogens, under conditions in which multiple rounds of mast cell activation are expected to occur.

Acknowledgements. We thank Karen Page for providing technical protocols, and helpful comments.

1 Williams, C. and Galli, S. (2000) The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. J. Allergy Clin. Immunol. 105, 847–859.
2 Galli, S. J. (1990) New insights into "The riddle of the mast cells": Microenvironmental regulation of mast cell development and phenotypic heterogeneity. Lab. Invest. 62, 5–33.
3 Gurish, M. F. and Boyce, J. A. (2002) Mast cell growth, differentiation, and death. Clin. Rev. Allergy Immunol. 22, 107–118.
4 Jutel, M., Watanabe, T., Klunker, S., Akdis, M., Thomet, O. A. R., Malolepszy, J., Zak-Nejmark, T., Koga, R., Kobayashi, T., Blaser, K. and Akdis, C. A. (2001) Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. Nature 413, 420–425.
5 Dy, M. and Schneider, E. (2004) Histamine-cytokine connection in immunity and hematopoiesis. Cytokine Growth Factor Rev. 15, 393–410.
6 Ohtsu, H. and Watanabe, T. (2003) New functions of histamine found in histidine decarboxylase gene knockout mice. Biochem. Biophys. Res. Commun. 305, 443–447.
7 Jutel, M., Blaser, K. and Akdis, C. A. (2006) The role of histamine in regulation of immune responses. Chem. Immunol. Allergy 91, 174–187.
8 Yamamoto, J., Yatsunami, K., Ohmori, E., Sugimoto, Y., Fukui, T., Katayama, T. and Ichikawa, A. (1990) cDNA-derived amino acid sequence of L-histidine decarboxylase from mouse mastocytoma P-815 cells. FEBS Lett. 276, 214–218.
9 Ohmori, E., Fukui, T., Imanishi, N., Yatsunami, K. and Ichikawa, A. (1990) Purification and characterization of L-histidine decarboxylase from mouse mastocytoma P-815 cells. J. Biochem. (Tokyo) 107, 834–839.
10 Yatsunami, K., Ohtsu, H., Tsuchikawa, M., Higuchi, T., Ishibashi, K., Shida, A., Shima, Y., Nakagawa, S., Yamauchi, K., Yamamoto, M., Hayashi, N., Watanabe, T. et al. (1994) Structure of the L-histidine decarboxylase gene. J. Biol. Chem. 269, 1554–1559.
11 Wiener, Z., Andrasfalvy, M., Pallinger, E., Kovacs, P., Szalai, C., Erdei, A., Toth, S., Nagy, A. and Falus, A. (2002) Bone marrow-derived mast cell differentiation is strongly reduced in histidine decarboxylase knockout, histamine-free mice. Int. Immunol. 14, 381–387.
12 Ohtsu, H., Tanaka, S., Terui, T., Hori, Y., Makabe-Kobayashi, Y., Pejler, G., Tchougounova, E., Hellman, L., Gertsenstein, M., Hirasawa, N., Sakurai, E., Buzas, E. et al. (2001) Mice lacking histidine decarboxylase exhibit abnormal mast cells. FEBS Lett. 502, 53–56.
13 Levi-Schaffer, F. and Shalit, M. (1993) Proliferation and functional responses of bone marrow-derived mast cells after activation. Cell. Immunol. 148, 435–443.
14 Slutsky, B., Jarvis, D., Bibb, P., Feldberg, R. and Cochrane, D. (1987) Viability and recovery from degranulation of isolated rat peritoneal mast cells. Exp. Cell Res. 168, 63–78.
15 Tanaka, S., Nemoto, K., Yamamura, E. and Ichikawa, A. (1998) Intracellular localization of the 74-and 53-kDa forms of L-histidine decarboxylase in a rat basophilic/mast cell line, RBL-2H3. J. Biol. Chem. 273, 8177–8182.
16 Stevens, R. L., Rothenberg, M. E., Levi-Schaffer, F. and Austen, K. F. (1987) Ontogeny of *in vitro*-differentiated mouse mast cells. FASEB J. 46, 1915–1919.
17 Levi-Schaffer, F. and Riesel, N. (1989) In vitro regeneration of activated rat peritoneal mast cells cocultured with 3T3 fibroblasts. Cell. Immunol. 119, 30–40.
18 Levi-Schaffer, F., Gare, M. and Shalit, M. (1990) Unresponsiveness of rat peritoneal mast cells to immunologic reactivation. J. Immunol. 145, 3418–3424.
19 Galli, S., Kalesnikoff, J., Grimbaldeston, M., Piliponsky, A., Williams, C. and Tsai, M. (2005) Mast cells as "tunable" effector and immunoregulatory cells: Recent advances. Annu. Rev. Immunol. 23, 749–786.
20 Tanaka, S., Mikura, S., Hashimoto, E., Sugimoto, Y. and Ichikawa, A. (2005) Ca^{2+} influx-mediated histamine synthesis and IL-6 release in mast cells activated by monomeric IgE. Eur. J. Immunol. 35, 460–468.
21 Tanaka, S., Takasu, Y., Mikura, S., Satoh, N. and Ichikawa, A. (2002) Antigen-independent induction of histamine synthesis by immunoglobulin E in mouse bone marrow-derived mast cells. J. Exp. Med. 196, 229–235.
22 Tanaka, S. and Ichikawa, A. (2006) Recent advances in molecular pharmacology of the histamine systems: Immune regulatory roles of histamine produced by leukocytes. J. Pharmacol. Sci. 101, 19–23.
23 Wang, L., Yan, L., McGuire, C., Kozak, C. A., Wang, M., Kim, U. J., Siciliano, M. and Weinshilboum, R. M. (2001) Mouse histamine *n*-methyltransferase: Cdna cloning, expression, gene cloning and chromosomal localization. Inflamm. Res. 50, 300–308.
24 Medina, M. A., Correa-Fiz, F., Rodriguez-Caso, C. and Sanchez-Jimenez, F. (2005) A comprehensive view of polyamine and histamine metabolism to the light of new technologies. J. Cell Mol. Med. 9, 854–864.
25 Xu, X., Zhang, D., Zhang, H., Wolters, P. J., Killeen, N. P., Sullivan, B. M., Locksley, R. M., Lowell, C. A. and Caughey, G. H. (2006) Neutrophil histamine contributes to inflammation in mycoplasma pneumonia. J. Exp. Med. 203, 2907–2917.
26 Salachas, F., Schneider, E., Lemoine, F. M., Lebel, B., Daeron, M., Navarro, S., Ziltener, H. and Dy, M. (1994) Aggregated IgE mimic interleukin-3-induced histamine synthesis by murine hematopoietic progenitors. Blood 84, 1098–1107.
27 Hirasawa, N., Murakami, A. and Ohuchi, K. (2001) Expression of 74-kDa histidine decarboxylase protein in a macrophage-like cell line raw 264.7 and inhibition by dexamethasone. Eur. J. Pharmacol. 418, 23–28.
28 Xu, X., Zhang, D., Lyubynska, N., Wolters, P. J., Killeen, N. P., Baluk, P., McDonald, D. M., Hawgood, S. and Caughey, G. H. (2006) Mast cells protect mice from mycoplasma pneumonia. Am. J. Respir. Crit. Care Med. 173, 219–225.
29 Gilfillan, A. M. and Tkaczyk, C. (2006) Integrated signalling pathways for mast-cell activation. Nat. Rev. Immunol. 6, 218–230.
30 Murata, Y., Tanimoto, A., Wang, K. Y., Tsutsui, M., Sasaguri, Y., De Corte, F. and Matsushita, H. (2005) Granulocyte macrophage-colony stimulating factor increases the expression of histamine and histamine receptors in monocytes/macrophages in relation to arteriosclerosis. Arterioscler. Thromb. Vasc. Biol. 25, 430–435.
31 Cao, L., Yu, K., Banh, C., Nguyen, V., Ritz, A., Raphael, B. J., Kawakami, Y., Kawakami, T. and Salomon, A. R. (2007) Quantitative time-resolved phosphoproteomic analysis of mast cell signaling. J. Immunol. 179, 5864–5876.
32 Chen, C., Grimbaldeston, M., Tsai, M., Weissman, I. and Galli, S. (2005) Identification of mast cell progenitors in adult mice. Proc. Natl. Acad. Sci. USA 102, 11408–11413.
33 Austen, K. and Boyce, J. (2001) Mast cell lineage development and phenotypic regulation. Leuk. Res. 25, 511–518.
34 Gurish, M. F., Ghildyal, N., McNeil, H. P., Austen, K. F., Gillis, S. and Stevens, R. L. (1992) Differential expression of secretory granule proteases in mouse mast cells exposed to interleukin 3 and c-kit ligand. J. Exp. Med. 175, 1003–1012.
35 Sredni, B., Friedman, M. M., Bland, C. E. and Metcalfe, D. D. (1983) Ultrastructural, biochemical, and functional characteristics of histamine-containing cells cloned from mouse bone marrow: Tentative identification as mucosal mast cells. J. Immunol. 131, 915–922.
36 Eklund, K. K., Ghildyal, N., Austen, K. F., Friend, D. S., Schiller, V. and Stevens, R. L. (1994) Mouse bone marrow-derived mast cells (mBMMC) obtained *in vitro* from mice that are mast cell-deficient *in vivo* express the same panel of granule proteases as mBMMC and serosal mast cells from their normal littermates. J. Exp. Med. 180, 67–73.
37 Serafin, W. E., Dayton, E. T., Gravallese, P. M., Austen, K. F. and Stevens, R. L. (1987) Carboxypeptidase a in mouse mast cells. Identification, characterization, and use as a differentiation marker. J. Immunol. 139, 3771–3776.

38 Malbec, O., Roget, K., Schiffer, C., Iannascoli, B., Dumas, A. R., Arock, M. and Daeron, M. (2007) Peritoneal cell-derived mast cells: An *in vitro* model of mature serosal-type mouse mast cells. J. Immunol. 178, 6465–6475.

39 Castellani, M. L., Perrella, A., Kempuraj, D. J., Boucher, W., Tagen, M., Salini, V., Vecchiet, J., Tete, S., Frydas, S., Theoharides, T. C. and Conti, P. (2007) Immunological activation of human umbilical cord blood mast cells induces tryptase secretion and interleukin-6, and histidine decarboxylase mRNA gene expression. Pharmacol. Res. 55, 57–63.

40 Castellani, M. L., Shaik, Y. B., Perrella, A., Frydas, S., Simeonidou, I., Salini, V., Tete, S., Conti, C. M., Vecchiet, J., Theoharides, T. C., Conti, P. and De Lutiis, M. A. (2007) Expression and secretion of CXCl8 (IL-8), release of tryptase and transcription of histidine decarboxylase mRNA by anti-IgE-activated human umbilical cord blood-derived cultured mast cells. Neuroimmunomodulation 14, 97–104.

To access this journal online:
http://www.birkhauser.ch/CMLS