**Article**

**Suppression of IFN-γ production in murine splenocytes by histamine receptor antagonists**

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**Abstract:** Accumulating evidence suggests that histamine synthesis induced in several types of tumor tissues should modulate tumor immunity. We found that a transient histamine synthesis was induced in CD11b^+Gr-1^+ splenocytes derived from BALB/c mice transplanted with a syngeneic colon carcinoma, CT-26, when they were co-cultured with CT-26 cells. Significant levels of IFN-γ were produced under this co-culture condition. We explored the modulatory roles of histamine on IFN-γ production and found that several histamine receptor antagonists, such as pyrilamine, diphenhydramine, JNJ7777120, and thioperamide, could significantly suppress IFN-γ production. However, suppression of IFN-γ production by these antagonists was also found when splenocytes were derived from the Hdc^-/- BALB/c mice. Suppressive effects of these antagonists were found on IFN-γ production induced by concanavalin A or the combination of an anti-CD3 antibody and an anti-CD28 antibody in a histamine-independent manner. Murine splenocytes were found to express H1 and H2 receptors, but not H3 and H4 receptors. IFN-γ production in the Hh1r^-/- splenocytes induced by the combination of an anti-CD3 antibody and an anti-CD28 antibody was significantly suppressed by these antagonists. These findings suggest that pyrilamine, diphenhydramine, JNJ7777120, and thioperamide could suppress IFN-γ production in activated splenocytes in histamine-independent manner.

**Keywords:** IFN-γ; histamine; splenocyte; histamine H1 receptor, histidine decarboxylase

1. **Introduction**

Histamine has diverse physiological roles, which are mediated by four subtypes of its specific receptors [1]. They are excellent and promising targets of therapeutic compounds for various diseases, such as type I allergy, peptic ulcer, and sleep disorders. Histamine is formed through decarboxylation of histidine by L-histidine decarboxylase (HDC), which is expressed in the limited kinds of cells, such as mast cells, enterochromaffin-like cells, and histaminergic neuron. Accumulating evidence suggests that HDC could be induced in some kinds of myeloid cells in addition to mast cells [2-5]. Activated neutrophils have also been identified as the source of histamine, which might be involved in lung and airway inflammation in response to mycoplasma pneumonia infection [6, 7].

It has been controversial how histamine should modulate tumor immunity. Jutel et al. demonstrated using the Hh2r^-/- mice that H2 receptor expressed in T cells should be involved in...
suppression of their cytokine production [8]. We previously raised the possibility that histamine should suppress tumor immunity through inhibition of the local expression of several cytokines that have potentials to antagonize tumor growth by acting on the H₂ receptors in a murine syngeneic tumor model [9,10]. Granulocytic myeloid cells expressing HDC were found to recruit FoxP3⁺ cells in murine colon cancer [11]. These findings suggest that histamine should contribute to suppression of immune responses. On the other hand, Yang et al. demonstrated that the frequency of chemical carcinogenesis was increased in the Hdc⁻/⁻ mice [12]. They hypothesized that histamine synthesis should induce maturation of CD11b⁺Ly6G⁺ cells in an autocrine manner, resulting in the impaired immunosuppressive functions. This concept has also been supported by the findings in a murine glioma model [13]. They suggested that suppression of the activity of the myeloid-derived suppressor cells (MDSC) by histamine might result in enhanced tumor immunity. However, it remains unknown how HDC should be induced in such myeloid cells and how HDC⁺ MDSC could maintain in the tumor tissues.

It is of great significance to clarify how histamine should modulate tumor immunity because various histamine receptor ligands have been developed and some of them are approved for clinical use. We found that histamine synthesis was induced in murine splenocytes derived from the tumor-bearing mice when they were co-cultured with the tumor cells. We here determined the identity of histamine-forming cells in the splenocytes and investigated the roles of histamine using various histamine receptor antagonists.

2. Results

2.1. Induced histamine synthesis in the splenocytes derived the tumor-bearing mice

We previously demonstrated that histamine synthesis was induced in the tumor tissue in a murine syngenic tumor model, in which CT-26 cells were transplanted in the dorsal skin of male BALB/c mice [9]. We then asked whether splenocytes derived from the tumor-bearing mice could produce histamine in the presence of CT-26 cells in vitro to identify the population responsible for histamine synthesis in tumor immunity. Significant levels of HDC activity were observed in the splenocytes derived from the tumor-bearing mice when they were co-cultured with mitomycin c-treated CT-26 cells (Fig. 1a). Histamine synthesis was detected neither in the splenocytes alone nor in the splenocytes derived from the control, tumor-free mice. We then investigated the effects of the conditioned medium obtained from the co-culture on histamine synthesis in the other freshly prepared splenocytes derived from the tumor bearing mice. The conditioned medium obtained from the co-culture of the splenocytes derived from the tumor-bearing mice and CT-26 cells was found to induce HDC in the splenocytes derived from the tumor-bearing mice, not in the control splenocytes (Fig. 1b). Flow cytometric analysis indicated that HDC was induced in CD11b⁺Gr-1⁻ populations (Fig. 1c). Very low levels of basal HDC expression were also found in the CD11b⁺Gr-1low population.

2.2 IFN-γ production mainly in spleen CD8⁺ T cells

IFN-γ production during the co-culture period was also measured. IFN-γ production was detected only when the splenocytes derived from the tumor-bearing mice were co-cultured with CT-26 cells as well as HDC (Fig. 2a). Significant levels of IFN-γ were released 12 hr after the onset of the co-culture and were abolished by depleting CD8⁺ T cells (Fig. 2b and 2c).

2.3 Suppression of IFN-γ production during the co-culture period by several histamine receptor antagonists

Histamine receptors have been found to be involved in modulation of cytokine production from various immune cells including T cells [14]. We then investigated the effects of histamine produced by the splenocytes on IFN-γ production during the co-culture period. Relatively high concentrations
of H₁ antagonists, pyrilamine and diphenhydramine, and H₂ antagonists, JNJ7777120 and thioperamide, significantly suppressed IFN-γ production during the co-culture period (Fig. 3a and 3b). An H₃ antagonist, cimetidine, had no effects on the IFN-γ production. We then asked whether these compounds suppressed IFN-γ production by antagonizing endogenous histamine by using the splenocytes derived from the tumor-bearing HDC⁻/⁻ mice. Unexpectedly, these compounds suppressed IFN-γ production by the HDC⁻/⁻ splenocytes in a similar fashion to that by the control splenocytes (Fig. 3c and 3d). The amounts of IFN-γ production during the co-culture periods were comparable between the wild type and HDC⁻/⁻ splenocytes (Wild type; 980 ± 300 pg/ml, HDC⁻/⁻; 1,400 ± 680 pg/ml, n=4).

2.4 Effects of histamine receptor ligands on IFN-γ production in the activated splenocytes

We then asked whether suppressive effects of several histamine receptor antagonists should be specific for IFN-γ production induced in the immune responses against tumor expansion. Concanavalin A (Con A), which is a lectin with potentials to activate T cells, induced IFN-γ production in the wild type and HDC⁻/⁻ splenocytes (Wild type; 830 ± 160 pg/ml, HDC⁻/⁻; 900 ± 240 pg/ml, n=4). Pyrilamine, diphenhydramine, JNJ7777120, and thioperamide dose-dependently suppressed Con A-induced IFN-γ production in the splenocytes derived from the wild type and HDC⁻/⁻ mice, whereas cimetidine had no effects on it (Fig. 4). Histamine exhibited partial suppressive effects on Con A-induced IFN-γ production, which reached to a plateau at 1 µM. We investigated using the antibodies raised against CD3 and CD28 whether IFN-γ production induced by T cell receptor activation should be also suppressed by these histamine receptor antagonists. Because previous studies demonstrated that H₁ receptor should be involved in augmented Th1 responses [8, 15], we investigated the effects of these compounds using the splenocytes derived from Hh₁r⁻/⁻ mice [16] in addition to Hdc⁻/⁻ mice. Production of IFN-γ upon T cell receptor activation was significantly suppressed by pyrilamine, diphenhydramine, JNJ7777120, and thioperamide in the splenocytes derived from BALB/c, C57Bl6, HDC⁻/⁻ (backcrossed to BALB/c), and Hh₁r⁻/⁻ strains (Fig. 5). The amounts of IFN-γ were comparable between the wild type and HDC⁻/⁻ mice (BALB/c; 5,900 ± 3,800 pg/ml, HDC⁻/⁻; 2,000 ± 400 pg/ml, n=4) whereas those were higher in Hh₁r⁻/⁻ mice than in the wild type mouse (C57Bl6; 225 ± 53 pg/ml, Hh₁r⁻/⁻; 1,400 ± 45 pg/ml, n=3).

2.5 Effects of histamine receptor ligands on IL-2 production in the activated splenocytes

We observed decreases in the number of aggregated splenocyte colonies in the presence of these histamine receptor antagonists, raising the possibility that they might suppress IL-2-mediated proliferation of T cells. We then investigated the effects of these compounds on IL-2 production in the activated splenocytes. Pyrilamine, diphenhydramine, and JNJ7777120 significantly suppressed IL-2 production in the splenocytes stimulated with Con A whereas cimetidine, thioperamide, and histamine did not affect IL-2 production (Fig. 6a). IL-2 production induced upon T cell receptor activation was found to be insensitive to these compounds except diphenhydramine (Fig. 6c). The absence of HDC did not affect the potencies of these compounds (Fig. 6b and 6d).
Figure 1. (a) Splenocytes were collected from the control (open symbols) and tumor-bearing mice (closed symbols) and then cultured in the absence (circles) or presence of CT-26 cells (squares) for the periods indicated. HDC activity was measured in the cultured splenocytes. (b) Culture of splenocytes were performed under various conditions as described above for 6 hr and the conditioned media were then collected. Splenocytes from the control (open symbols) and tumor-bearing mice (closed symbols) were incubated in each conditioned medium for 6 hr and HDC activity was measured. HDC activity in the splenocytes co-cultured with CT-26 cells was measured as the reference. (c) Expression levels of HDC in the splenocytes derived from the tumor-bearing mice co-culture without or with CT-26 cells were measured using flow cytometry with the antibodies raised against CD11b, Gr-1, and HDC. Expression of HDC was analyzed in the subpopulations of the splenocytes. Multiple comparisons were performed using one-way ANOVA with the Tukey post test. Values with *p<0.05 and **p<0.01 are regarded as significant.
Figure 2. (a) Splenocytes were collected from the control and tumor-bearing mice and then cultured in the absence or presence of CT-26 cells for 24 hr. The amounts of IFN-γ in the medium were measured. (b) Splenocytes were collected from the tumor-bearing mice and co-cultured with CT-26 cells for the periods indicated. The amounts of IFN-γ in the medium were measured. (c) Total splenocytes derived from tumor-bearing mice or those depleted with CD8+ T cells were co-cultured with CT-26 cells for 24 hr. The amounts of IFN-γ in the medium were measured.

Figure 3. Splenocytes derived from the tumor-bearing wild type mice (a and b) and HDC−/− mice (c and d) were co-cultured with CT-26 cells for 24 hr in the presence of the indicated concentrations of pyrilamine (a, c, open circles), diphenhydramine (a, c, closed circles), cimetidine (b, d, open squares), JNJ7777120 (b, d, open circles), and thioperamide (b, d, closed circles). The amounts of IFN-γ in the medium are presented as the percentages of the control. Values are presented as the mean ± SEM (n=3).
Figure 4. Splenocytes derived from the wild type mice (a, b, and c) and HDC⁻/⁻ mice (d, e, and f) were stimulated with concanavalin A (5 µg/ml) for 24 hr in the presence of the indicated concentrations of pyrilamine (a, d, open circles), diphenhydramine (a, d, closed circles), cimetidine (c, f, open squares), JNJ7777120 (b, e, open circles), thioperamide (b, e, closed circles), and histamine (c, f, closed squares). The amounts of IFN-γ in the medium are presented as the percentages of the control. Values are presented as the mean ± SEM (n=3).
Figure 5. Splenocytes derived from the BALB/c mice (a), HDC⁻ mice (b), C57Bl6 mice (c), and Hh1r⁻ mice (d) were stimulated in the culture plates coated with an anti-CD3 antibody and an anti-CD28 antibody for 24 hr in the presence of 100 µM of pyrilamine (Py), diphenhydramine (Di), cimetidine (Ci), JNJ7777120 (JNJ), thioperamide (Th), and histamine (H). The amounts of IFN-γ in the medium are presented as the percentages of the control. Multiple comparisons were performed using one-way ANOVA with the Tukey post test. Values with *p<0.05 and **p<0.01 are regarded as significant.
Figure 6. Splenocytes derived from the wild type mice (a and c) and HDC−/− mice (b and d) were stimulated with concanavalin A (5 µg/ml, a and b) or in the culture plates coated with an anti-CD3 antibody and an anti-CD28 antibody (c and d) for 24 hr in the presence of 100 µM of pyrilamine (Py), diphenhydramine (Di), cimetidine (Ci), JNJ7777120 (JNJ), thioperamide (Th), and histamine (H). The amounts of IL-2 in the medium are presented as the percentages of the control. Multiple comparisons were performed using one-way ANOVA with the Tukey post test. Values with *p<0.05 and **p<0.01 are regarded as significant.
3. Discussion

Accumulating evidence suggests that MDSC should be the major source of histamine in the tumor tissues, although it remains to be clarified how HDC is induced. We reproduced here the process of induction of histamine synthesis by murine myeloid cells in the tumor tissues by establishing a system, in which splenocytes derived from the tumor-bearing mice were co-cultured with mitomycin c-treated tumor cells. HDC-expressing cells were found to be CD11b+Gr-1+ cells, raising the possibility that histamine synthesis was induced in MDSC in our model. Induction of histamine synthesis was limited in the situation, in which tumor immunity was established. It is likely that CT-26 cells triggered acquired immunity against themselves during the co-culture period, such as stimulated production of a wide variety of cytokines including IFN-γ. Because the conditioned medium could induce histamine synthesis in the splenocytes derived from the tumor-bearing mice, it is likely that soluble mediators, such as cytokines, induced upon tumor immunity should act on the surface receptors on the splenocytes to induce HDC. Previous studies demonstrated that several cytokines should be involved in induction of histamine synthesis. Interleukin-3 could induce histamine synthesis in murine bone marrow [17]. Injection of IL-1 into mice was found to induce histamine synthesis in various tissues, such as bone marrow, spleen, lung, and liver, as well as lipopolysaccharide (LPS) injection [18]. Several studies suggested that GM-CSF could also induce HDC in bone marrow-derived cells [6,19]. Although we could not identify the cytokines that should be responsible for induced histamine synthesis, our co-culture model will be useful for identifying the putative HDC-inducing factors.

We then tried to determine the roles of histamine produced by MDSC in our system. Among several histamine receptor antagonists, H1 antagonists (pyrilamine and diphenhydramine) and H4 antagonists (JNJ777120 and thioperamide) were found to suppress IFN-γ production, whereas an H2 antagonist, cimetidine, had no effects on it. Unexpectedly, these inhibitory effects were also observed in the Hdc−/− splenocytes, indicating that the suppressive effects of these compounds should not be associated with their abilities to compete with histamine. Because no common target molecules of these compounds have been reported, it is rather difficult to identify their targets in addition to histamine receptors. These inhibitory effects were also observed when splenocytes derived from tumor-free, control mice were stimulated with concanavalin A or the combination of anti-CD3 and anti-CD28 antibodies. These results suggest that shared signaling pathways leading to IFN-γ production should be suppressed by these compounds, because CD4+ T cells should also be involved in IFN-γ production under these conditions. Interestingly, clozapine, which could function as an H4 agonist [20], was found to suppress IFN-γ production in activated human peripheral blood mononuclear cells through suppression of expression of T-bet, which is the master transcription factor of Th1 differentiation [21]. A recent study reported that some benzoxazole derivatives could suppress IFN-γ production in murine CD4+ T cells in a similar manner to clozapine [22]. Accumulating evidence suggests that T-bet should be involved in IFN-γ production both in CD4+ and CD8+ T cells, although T-bet-independent IFN-γ production was reported in CD8+ T cells [23-25]. The compounds with suppressive effects on IFN-γ production found here may also have potentials to modulate the expression levels and transcriptional activity of T-bet.

Among the compounds tested here, pyrilamine and diphenhydramine exhibited relatively strong effects. Because previous studies have indicated that several H1 antagonists including pyrilamine could function as inverse agonists [26-29], we verified this possibility using the Hh1r−/− mice. Previous studies demonstrated that H1 receptors should be involved in promotion of Th1 responses including IFN-γ production [8, 15]. We confirmed significant levels of H1 and H4 receptor mRNA in isolated splenocytes (data not shown). However, our results obtained using the Hh1r−/− splenocytes indicated that the H1 receptor should be unrelated to the actions of pyrilamine and diphenhydramine. Regarding the expression levels of H4 receptors, no or very low levels of mRNA expression were detected in the isolated splenocytes (data not shown), although the abundant levels of expression were confirmed in the bone marrow cells. Our finding might be inconsistent with the previous study demonstrating that murine H4 receptors are distributed in the hematopoietic system including bone marrow and spleen [30]. We speculate that JNJ7777120 might suppress IFN-γ
production in an H4 receptor-independent manner whereas it should be clarified how the expression levels of H4 receptors in splenocytes should be dynamically changed.

Suppressive effects of these histamine receptor antagonists on IFN-γ production were observed in relatively higher concentrations, indicating that a majority of the reported findings using these compounds should not be associated with suppressed IFN-γ production. This study will contribute in development of novel therapeutic compounds targeting IFN-γ which plays critical roles in pathology of various diseases, such as autoimmune diseases and inflammatory bowel diseases [31,32].

4. Materials and Methods

4.1. Materials

4.2. Animals Specific-pathogen-free 5-10-week-old male BALB/c mice and C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). Hdc⁻/⁻ mice [32] were backcrossed to BALB/c strain for more than 10 generations. Hh1r⁻/⁻ mice [16] were backcrossed to C57BL/6 strain for more than 10 generations. These gene-targeted male mice were used at 8-10 week of age. All mice were kept in a specific-pathogen-free animal facility at Okayama University. This study was approved by the Committee on Animal Experiments of Okayama University.

4.3. Murine syngenic tumor model Male BALB/c mice (5 weeks of age) were transplanted with a syngenic colon tumor cell line, CT-26 (1 x 10⁶ cells/mouse, at the dorsal skin) as previously described [9]. The spleen was collected 14 days after the transplantation, minced, and filtered with nylon mesh to obtain the splenocytes.

4.4. Co-culture of splenocytes and CT-26 cells Suspended splenocytes were treated with ACK buffer (150 mM NH₄Cl containing 10 mM KHCO₃, 1 mM EDTA) to eliminate red blood cells. CT-26 cells were treated with 1 µg/ml mitomycin C for 3 hr in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (complete RPMI medium) and then twice washed. Splenocytes suspended in the complete RPMI medium were seeded onto the monolayer of CT-26 cells.

4.5. Measurement of histamine Splenocytes were homogenized in the cell lysis buffer (10 mM potassium phosphate, pH 6.8 containing 10 mM KHCO₃, 1.5 mM MgCl₂, 0.2 mM dithiothreitol, 0.01 mM pyridoxal phosphate, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 0.1% Triton X-100) and centrifuged at 10,000 x g for 15 min at 4°C. The resultant supernatant was subjected to the enzymatic assay of HDC. The reaction was performed in 0.1 M potassium phosphate, pH 6.8 containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal phosphate, 2% polyethylene glycol #300, 0.2 mM aminoguanidine, and 0.8 mM histidine, for 4 hr at 37°C and then terminated by addition of perchlorolic acid (fin. 3%). Histamine was fluoremetrically measured by HPLC with a cation exchange column, WCX-1 (Shimadzu, Japan), after derivatization with o-phtalaldehyde [33].

4.6. Flow cytometry Cultured splenocytes were collected and incubated with the 2.4G2 antibody (BD Biosciences, NJ) to block Fc receptors at 4°C for 15 min. The cells were then labeled with the antibodies raised against the surface antigens, CD11b or Gr-1. For intracellular staining, the cells were fixed with PBS containing 4% formaldehyde at 4°C for 30 min and then permeabilized with PBS containing 0.1% saponin and 1% FBS at room temperature for 10 min. The permeabilized cells were incubated with an anti-HDC antibody (ab37291, Abcam, UK) at 4°C for 30 min and visualized with a phycoerythrin-conjugated anti-rabbit IgG antibody. The cells were analyzed using FACS Calibur (BD Biosciences).

4.7. Measurement of cytokines The amounts of cytokines in the medium were measured using the ELISA kits (mouse IFN-γ ELISA Ready Set Go! And mouse IL-2 ELISA Ready Set Go!, Thermo Fisher Scientific, MA) according to the manufacture’s instructions.
4.8. Depletion of CD8+ T cells
Depletion of CD8+ T cells from total splenocytes was performed using CD8a+ T cell Isolation Kit II (Miltenyi Biotec, Germany) according to the manufacturer’s instruction.

4.9. Stimulation of T cell receptors
Splenic T cells were activated in the antibody-coated culture wells. Culture wells were coated with an anti-CD3 antibody (5 µg/ml, BD Biosciences) and an anti-CD28 antibody (1 µg/ml, BD Biosciences) at 4°C over night. In case of the splenocytes derived from the Hh1r-/- mice and the control C57BL/6 mice, 2 µg/ml of an anti-CD3 antibody and 0.4 µg/ml an anti-CD28 antibody were used for coating.

4.10. Measurement of histamine receptor mRNAs
Total RNAs were extracted from the splenocytes using NucleoSpin RNA Kit (TaKaRa Bio Inc., Japan) and reverse transcribed using PrimeScript™ RT Reagent Kit (TaKaRa Bio Inc.). First strand DNAs were subjected to quantitative PCR using KOD SYBR qPCR Mix (TOYOBO, Japan) or SYBR Green PCR Master Mix (Thermo Fisher Scientific) with the specific primer pairs as follows. Hh1r: 5’-TCA CTC CAG GCC TCA CAT GAC-3’, 5’-CAA AGT TCT CAT CCC AAG TTT CCA-3’, Hh2r: 5’-CAG TCC TAA GCG ACC CGG TA-3’, 5’-GGC ACT GCT GGA TGT A-3’, Hh3r: 5’-ATG ACC GAT TCC TGT CAG TCA CTC-3’, 5’-TTG ATG TAC AGA GGA-3’, Hh4r: 5’-TAC TGG CAT CTT GCC ACC AG-3’, 5’-ACG TGA GGG ATG TAC AGA GGA-3’, and Gapdh: 5’-TGT GTC CGT CGT GGA TCT GA-3’, 5’-TTG CTG TTG AAG TCG CAG GAG-3’.

4.11. Statistics
Statistical significance for comparison between two groups was determined by unpaired Student’s t-test. Statistical significance for comparisons among multiple groups was determined using one-way ANOVA. Additional comparisons were made with Dunnett multiple comparison test for comparison with the control groups or Tukey-Kramer multiple comparison test for all pairs of column comparison.


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Abbreviations

ANOVA Analysis of variance
Con A Concanavalin A
FBS Fetal bovine serum
GM-CSF Granulocyte-macrophage colony-stimulating factor
HDC L-Histidine decarboxylase
IFN Interferon
MDSC Myeloid-derived suppressor cells
PBS Phosphate buffered saline
PCR Polymerase chain reaction
T-bet T box-containing protein expressed in T cells
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