Cytokine responses of intraepithelial lymphocytes are regulated by histamine H2 receptor

Cytokine responses of intraepithelial lymphocytes are regulated by histamine H_2 receptor

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Running title: H_2R-mediated cytokine responses in IELs

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Abstract

Backgrounds. Histamine participates in the immune regulation of several gastrointestinal diseases. However, the effect of histamine on intestinal intraepithelial lymphocytes (IELs), the front line of intestinal mucosal immune system, is not well-understood. We examined whether histamine has a direct effect on cytokine production by IELs and the involvement of histamine receptor subtypes.

Methods. Murine IELs were activated by PMA plus ionomycin with/without histamine. Secreted cytokines were measured and compared with those of splenocytes. Intracellular cytokines were detected by flow cytometory. Expression of histamine receptor subtypes in IELs was examined by RT-PCR.

Results. Histamine H_1 receptor (H_1R), H_2R, and H_4R, but not H_3R mRNA were expressed on IELs. Histamine significantly decreased Th1-cytokine (IFN-γ, TNF-α, and IL-2) and also IL-4 production in IELs as well as splenocytes. The selective H_2R antagonist famotidine, but not the H_1R antagonist pyrilamine nor the H_3R/H_4R antagonist thioperamide, competes with the inhibitory effect of histamine on these cytokine production in IELs. These suppressive effects of histamine were mimicked by a selective H_2R/H_4R agonist dimaprit. Further, these suppressive effects of histamine for Th1-cytokine and IL-4 did not accompany with the enhancement of IL-10 production nor IL-10 mRNA level in IELs. Intracellular cytokine analysis revealed that the number of IFN-γ-producing αβ T cells was significantly reduced by histamine in IELs.

Conclusions. Histamine has a direct suppressive effect on IEL-derived cytokines via H_2R, which would have a crucial role in the suppression of local immunoregulation in the intestinal epithelium.

Key words: histamine, IEL, histamine H_2 receptor, IFN-γ, TCRαβ

Abbreviations: IEL, intraepithelial lymphocyte; H_2R, histamine H_2 receptor; IBD, inflammatory bowel disease; IBS,irritable bowel syndrome; GPCRs, G protein-coupled receptors; GALT, gastrointestinal associated lymphoid tissue; HDC, histidine L-decarboxylase; CBA, cytometric.bead array; DC, dendritic cell, reverse transcriptase-polymerase chain reaction, RT-PCR, lamina propria lymphocyte, LPL.

Introduction

Histamine, one of the most intensively studied biological amines, is involved in the immune regulation of the inflammatory response of several gastrointestinal disorders such as food allergy [1], inflammatory bowel disease (IBD) [2, 3] and irritable bowel syndrome (IBS)[4, 5]. Histamine exerts its effects through four receptors (H_1R, H_2R, H_3R and H_4R) which are members of the G protein-coupled receptor (GPCR) family [6, 7]. Recent studies revealed that histamine regulates innate and acquired immune responses through H_1R as well as H_2R [8] and shifts the systemic Th1/Th2 balance from Th1 to Th2 [9, 10].

Accumulating evidence suggests that histamine also affects gastrointestinal associated lymphoid tissue (GALT) and has an important role in the intestinal mucosal immune system. In the digestive tract, mast cells, basophils, and gastric enterochromaffin-like cells are the major source of "granule-stored" histamine, and recently the cells such as lymphocytes [11], macrophages [12] and colorectal cancer cells [13], which have high L-histidine decarboxylase (HDC) activity, are also thought to be a source of "inducible" histamine. In patients with IBD, urinary excretion of the histamine metabolite *N*-methylhistamine is enhanced and is related to clinical and endoscopic disease activity [14]. H_1R and H_2R mRNA levels in the small intestine are significantly elevated in patients with food allergies and IBS [5]. Based on these observation and others, there is a speculation that not only H_1R, but also H_2R may be related for the dysregulation of the mucosal immunity [8, 15, 16]. Furthermore, in colorectal cancer, histamine modulates the Th1/Th2 balance and attenuates anti-tumor cytokine expression [17]. Several clinical trials have been carried out with H_2R antagonists in colorectal cancer patients; both increased survival and immunological recognition of the tumor is reported [18].

Intraepithelial lymphocytes (IELs) bear the front line of intestinal mucosal immunity and are phenotypically and functionally distinct from T cells in peripheral blood or the spleen

[19-21]. Suggested functions for IELs are mediation of inflammatory reaction, surveillance of the intestinal epithelium and induction or maintenance of oral tolerance [22, 23]. It is plausible for IELs to play a crucial role in the development and progression of the intestinal diseases. Studies indicate that IELs secret several cytokines as immunoregulator cells in response to foreign antigens *in vivo* or stimulants *in vitro* [19, 24-29]. Secreted cytokines are varied by the condition of the stimulation. In murine IELs, IFN-γ and IL-5 are produced spontaneously and enhanced by stimulation [24]. Other studies shown that IFN-γ, TNF-α, TGF-β1, IL-2, IL-3 and IL-6 are produced in $CD8^+$ IELs by anti TCR mAb stimulation [25]. Yet, there are few reports studying how IELs are regulated, and no study has examined the regulatory effects of histamine on IEL function.

The aim of current study was to determine whether histamine contributes to regulate intestinal mucosal immune responses mediated by IELs. We examined whether histamine has direct effects on conventional Th1- or Th2-cytokine production by IELs *in vitro* and whether different effects are mediated by the different histamine receptor subtypes. Further, we determined which IELs subsets were involved in IFN-γ production by detecting intracellular cytokine production.

Materials and methods

Mice

Male C3H/HeN mice were obtained from Japan SLC (Hamamatsu, Japan) and were maintained under specific pathogen-free conditions. All mice in this study were used at 7-10 weeks of age and were allowed free access to food and water *ad libitum*. All experiments were performed according to the Guidelines and Regulations for Laboratory Animal Care of Hamamatsu University School of Medicine.

Materials

Phorbol myristate acetate (PMA), ionomycin, lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6), histamine dihydrochloride, pyrilamine maleate salt, famotidine, thioperamide maleate salt, and RPMI-1640 were purchased from Sigma (St. Louis, MO, USA). Dimaprit was purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) and Hank's Balanced Salt Solution (HBSS) were purchased from Gibco (Grand Island, NY, USA). Percoll was purchased from Amersham /GE Healthcare Biosciences (Little Chalfont, UK). PE-labeled anti-mouse TCRβ chain, FITC-labeled anti-mouse TCRγδ, FITC-labeled anti-mouse CD3, PE-labeled anti-mouse CD4, PerCP-labeled anti-mouse CD4, PE-labeled anti-mouse CD8a, PE-labeled CD11c, PE-labeled anti-mouse CD14, FITC-labeled anti-mouse IFN-γ, PE-labeled anti-mouse IL-4 were purchased from BD PharMingen (San Diego, CA, USA).

PMA and ionomycin were dissolved in dimethyl sulfoxide (DMSO), and diluted in RPMI-1640. Residual DMSO was <0.5% in culture medium. The other reagents such as histamine ligands and LPS were diluted in Milli Q water, and the stock solutions were stored at -20℃ until use.

Isolation of IELs and preparation of splenocytes

IELs were isolated from the small intestine according to a previously reported

method [30] with a minor modification [31]**.** Briefly, the small intestine was cut into pieces and stirred at 37°C for 45min in HBSS containing 5% FBS. Supernatants containing IELs were filtered through a glass wool column before centrifugation through a 44/70% percoll gradient. IELs at the interface were collected, washed and then resuspended in culture medium (RPMI-1640 supplemented with 10% FBS).

The spleen was removed from sacrificed mice and cut into several pieces. The cell suspension was then centrifuged and the cell pellet was resuspended in Dulbecco's phosphate buffered saline. The red blood cells were lysed in lysing buffer (NH_4Cl 150mM, $KHCO_3$ 1mM, EDTA 0.01mM). Splenocytes were washed two times and resuspended in the culture medium (RPMI-1640 supplemented with 10% FBS).

Analysis of H_1R, H_2R, H_3R, and H_4R mRNA expression on IELs

The mRNA expression of histamine receptor subtypes in IELs was detected by conventional reverse transcriptase-polymerase chain reaction (RT-PCR). Isolation of total RNA was performed using RNeasy mini kit (QIAGEN, Hilden, Germany) with DNase treatment (Ambion, Austin, TX, USA). After DNase treatment, reverse transcription was performed using random hexamer primers (TaKaRa BIO, Otsu, Japan) and M-MLV (TaKaRa BIO). The oligonucleotide primers used for reaction were as follows; for β-actin, 5′-tgttaccaactgggacgaca-3′ (forward) and 5′-ccatcacaatgcctgtggta-3′(reverse), fragment size 236 bp; for H_1R, 5′-gaccttggtggatcgacagt-3′ (forward) and 5′-tgtctggaatgtgagcgaag-3′ (reverse), fragment size 153 bp; for H_2R, 5′-ttccttacttcactgccttcg-3′ (forward) and 5′-ttgtgagagttgtggcttgc-3′(reverse), fragment size 199 bp; for H_3R, 5′-agcgcatgaagatggtatcc-3′ (forward) and 5′-agccagaaggacgtctcgta-3′ (reverse), fragment size 196 bp; and for H_4R, 5′-gaatcagctgcatctcgtca-3′ (forward) and 5′-gtgacctggctagcttcctg-3′ (reverse), fragment size 187 bp. The PCR condition was 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 37 cycles. PCR products were loaded on 3%

agarose gel to check the specificity of the reaction.

Analysis of IFN-γ and IL-10 mRNA expression on IELs

Total RNA extraction and reverse transcription was performed as described above. Real-time PCR was performed using LightCycler (Roche Applied Science, Indianapolis, IN, USA). The oligonucleotide primers used for reaction were as follows; for IFN-γ, 5′-ctcttcctcatggctgtttc-3′ (forward) and 5′-ttgctgatggcctgattgtc-3′ (reverse), fragment size 233 bp; for IL-10, 5′-gccaagccttatcggaaatg-3′ (forward) and 5′-atggccttgtagacaccttg-3′ (reverse), fragment size 235 bp. PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacture's instructions. The PCR condition was 95°C for 15 min for 1 cycle, 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 40 cycles. The data are normalized to β-actin from same sample.

Analysis of lymphocyte subtype by flow cytometry

The surface phenotype of IELs and splenocytes were analyzed using fluorescence-labeled anti-mouse mAbs. Cells were stained with mAbs for 15 min on ice and washed in PBS. Cells were examined using an EPICS XLII System (Beckman Coulter, Fullerton, CA, USA).

Analysis of secreted cytokines by cytometric bead array

Cells were adjusted to a concentration of 1×10^6 cells/mL in culture media, pretreated with or without histamine ligands and incubated for the indicated time at 37°C in a humidified atmosphere of 5% CO_2 in air with or without PMA (40 ng/mL) plus ionomycin (4 μg/mL), or LPS (1 μg/mL). The supernatants were collected at 48 h after incubation in IELs and at 24 h after incubation in splenocytes, respectively and IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10 were detected using the mouse cytometric bead array (CBA) kit (BD PharMingen). The CBA kit employs a series of beads with discrete fluorescence intensities that enable us to simultaneously detect multiple soluble cytokines. Each bead provides a capture surface for a

cytokine and is analogous to a coated well in an enzyme-linked immunosorbent assay.

Analysis of intracellular cytokines

Cells were adjusted to a concentration of 1×10^6 cells/mL in culture media, pretreated with or without histamine (10^{-4} M) and incubated with PMA (40 ng/mL) plus ionomycin (4 μg/mL) and brefeldin A (20 μg/mL) for 4 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Intracellular IL-4 and IFN-γ were detected in fixed and permeabilized cells using mAbs conjugated to PE or FITC. Data was acquired by flow cytometry using an EPICS XLII System.

Administration of histamine agonists and antagonists

To assess the effects of histamine via histamine receptor subtypes, we used the H_1R antagonist pyrilamine (10^{-5} M), the H_2R antagonist famotidine (10^{-5} M), the H_3R/ H_4R antagonist thioperamide (10^{-5} M) in the presence of histamine (10^{-4} M). The selective H_2R/ H_4R agonist dimaprit (10^{-4} M) was used for the further confirmation of H_2R selectivity with or without famotidine (10^{-5} M). These doses were chosen based on pharmacological characteristics according to previous studies in non-immune cells [32], and also immune cells: i.e., splenocytes [8, 10], and we have not observed any cell toxicity by these agents based on a trypan blue assay.

Statistical analysis

The Student's *t*-test was used for comparisons of secreted cytokine production between the control and stimulation groups with PMA plus ionomycin or LPS. To examine the effects of histamine, the data are shown as a percentage of each level in the presence of PMA plus ionomycin without any histamine receptor ligand and represent the means ± standard error of the mean. Comparison between IFN-γ producing $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ population was statistically analyzed with the Student's *t*-test. A level of $P < 0.05$ was considered to be statistically significant.

Results

Purity and phenotypic characterization of IELs

Cell viability, determined by trypan blue exclusion, of purified IELs and splenocytes were > 93% and >95%, respectively. Cell purity of the IELs compartment assessed by flow cytometry was 80.2 ± 4.7% (Figure 1), similar to the previous report [33]. To examine the phenotypic characteristics of purified IELs, we stained IELs with mAbs for the cell surface markers. Most IELs were $CD3^+$ T cells (88.2±3.2%, n=3) and predominantly expressed CD8 (85.6±0.9%) compared with CD4 (9.1±0.7%). IELs contained $CD4^+CD8^+$ double positive cells (4.0±1.0%) and $CD11c^+$ cells (7.9±0.1%). IELs had comparable numbers of $TCR\gamma\delta^+$ cells (38.6±2.4%) and $TCR\alpha\beta^+$ cells (48.7±1.6%). In splenocytes, there were low number of $CD3^+$ T cells (35.3±2.7%), and few number of $CD4^+CD8^+$ (0.3±0.02%) double positive T cells compared with IELs. Purity of whole splenocytes was 90.4 ± 1.0% (n=3). Splenocytes were predominantly expressed $TCR\alpha\beta^+$ cells (30.6±0.6%) compared with $TCR\gamma\delta^+$ cells (1.9±0.6%).

Profile of cytokine production derived from IELs compared with splenocytes

We compared cytokine production by IELs and splenocytes activated with or without either PMA plus ionomycin or LPS. As shown in Table 1, there was low cytokine production in the absence of stimulants (control group) both in IELs and splenocytes. Different profiles of cytokine production were observed between IELs and splenocytes in the presence of stimulants. Although IELs produced significantly higher levels of Th1-cytokines (IFN-γ, TNF-α, IL-2) and IL-4 when activated by PMA plus ionomycin, LPS did not yield any inducible cytokines. On the other hand, splenocytes did produce IFN-γ and TNF-α by the activation by LPS as well as PMA plus ionomycin while the production level was differs between the two conditions. Therefore, we subsequently used PMA plus ionomycin for the

following experiments.

Detection of H_1R, H_2R, H_3R, and H_4R mRNA expression on IELs

To elucidate the expression of known subtypes of histamine receptor in IELs, we examined RT-PCR to detect the H_1R, H_2R, H_3R, and H_4R mRNA. As shown in Figure 2, H_1R, H_2R, and H_4R mRNA were expressed on IELs, while H_2R mRNA expression seems to be more substantial compared to the expression of H_1R and H_4R mRNA. We did not observe any signal for H_3R mRNA.

Histamine decreases IFN-γ, TNF-α, IL-2 and IL-4 derived from activated IELs as well as splenocytes

To determine whether histamine has regulatory effects on cytokine production in IELs, we examined the cytokines in the presence or absence of histamine compared with in splenocytes. Both in IELs and splenocytes, without PMA plus ionomycin stimulation, histamine had no apparent stimulatory effects on production of these cytokines (data not shown). When the IELs were activated by PMA plus ionomycin, histamine significantly decreased IFN-γ, TNF-α, IL-2 and IL-4. The inhibitory effect of IFN-γ, TNF-α, IL-2 and IL-4 was 41, 48, 39 and 32% respectively at 10^{-4} M histamine. These effects were also observed in splenocytes; the inhibitory effect of IFN-γ, TNF-α, IL-2 and IL-4 was 30, 46, 49 and 16% respectively (Fig. 3). Dose-dependent effects by histamine, between 10^{-7} M and 10^{-4} M, were observed in Th1-cytokine (IFN-γ, TNF-α, and IL-2) production, but not in IL-4 in IELs which was secreted at a relatively low level.

Suppressive effects of histamine on Th1-cytokine and IL-4 secretion in IELs were mediated predominantly via H_2R

To determine the functional selectivity of histamine receptor subtypes on the histamine-mediated regulation of cytokine production, we examined the effects of selective histamine receptor antagonists on histamine treatment in IELs compared with splenocytes. We used following selective antagonists for our experiments; pyrilamine for H_1R, famotidine for H_2R, and thioperamide for H_3R/H_4R [32]. Selective histamine receptor antagonists did not influence cytokine production by themselves while the cells were activated by PMA plus ionomycin (data not shown). Then, cells were pretreated with pyrilamine (10^{-5} M), famotidine (10^{-5} M) or thioperamide (10^{-5} M) for 5 min and thereafter treated with histamine (10^{-4} M) according to previous reports [8, 9, 34]. As shown in Figure 4, in IELs, only famotidine, blocked the suppressive effect of histamine, and restored the cytokine response on Th1-cytokine (IFN-γ, TNF-α and IL-2) and IL-4 comparable to the levels without histamine treatment. In splenocytes, the restorable effect of famotidine was not observed on IL-4, and minute effects were observed on Th1-cytokine compared to those in IELs.

To further confirm whether H_2R signaling regulates cytokine production, we examined the effect of a selective agonist on IEL or splenocyte-derived IFN-γ, TNF-α, IL-2 and IL-4 production. As shown in Figure 5, dimaprit (10^{-4} M), a selective H_2R/H_4R agonist, mimicked the suppressive effect of histamine on these cytokine production derived from both IELs and splenocytes. Famotidine almost completely eliminated the effects of dimaprit on IEL-derived cytokine production. In splenocytes, the restorable effects by famotidine on the dimaprit-mediated effects on cytokine production were also significant; however, the restorations were rather small compared to those in IELs (Fig. 5).

Histamine decreases the number of INF-γ producing TCRαβ$^+$ cells estimated by intracellular cytokine production in IELs

We examined whether the effects of histamine on cytokine secretion were also seen

in intracellular cytokine production by IELs. Representative dot plots of intracellular IFN-γ/IL-4 staining performed after 4 hr are shown in Figure 6A. IFN-γ producing cells were detected in IELs activated by PMA plus ionomycin, whereas IL-4 producing cells were too few (0.1%) for further evaluation. The number of IFN-γ producing cells decreased in total IEL population when treated with histamine compared to the control (12% versus 18%). Further, we examined whether this effect of histamine on IFN-γ could be observed in $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$ IELs. Interestingly, IFN-γ producing cells were clearly in the $TCR\alpha\beta^+$ IEL population but not the $TCR\gamma\delta^+$ IEL population (Fig. 6B), and the number of IFN-γ producing cells significantly decreased in $TCR\alpha\beta^+$ IEL population when treated with histamine (Fig. 6B).

Suppressive effects of histamine on Th1-cytokine and IL-4 does not accompany with enhancement of IL-10 production in IELs

Since increase of IL-10 is well-known to induce peripheral T cell tolerance [15] and previous studies revealed histamine increased IL-10 secretion via H_2R in DC [35], Th2 cells and splenocytes [10], we examined whether suppressive effects of histamine on Th1-cytokine and IL-4 accompanied with enhancement of IL-10 production in IELs. IELs certainly produced significantly higher levels of IL-10 when activated by PMA plus ionomycin, compared to no stimulation control (261.7±48.3 versus. 53.3±24.0 pg/ml). Unexpectedly, histamine had no effect on secreted IL-10 level in activated IELs (Fig. 7). Further, in splenocytes there is no significant increase of secreted IL-10 when treated by PMA plus ionomycin and also no significant effect with histamine.

Histamine reduced IFN- γ mRNA expression, but not IL-10 mRNA expression in IELs

To evaluate the mechanism underlying histamine-mediated cytokine suppression,

the cytokine mRNAs levels were also tested to know whether the expression of were modulated by histamine under stimulation. We examined real-time PCR to reveal the IFN-γ and IL-10 mRNA expression in IELs. As shown in Figure 8, histamine significantly decreased the IFN- γ mRNA expression in activated IELs by PMA plus ionomycin at 6 h incubation although the difference with/without histamine at 24h incubation turn out to be small. On the contrary, IL-10 mRNA expression levels were not modulated by histamine treatment. These results suggested that the regulation of cytokine production by histamine is attained at mRNA level.

Discussion

In this study, we have examined for the first time, whether histamine has a direct effect on cytokine production in murine IELs *in vitro*. Our main findings were that histamine decreased IEL-derived Th1-cytokine production and also decreased IL-4 production at a low level. These suppressive effects of histamine did not accompany with enhancement of IL-10 production. A selective H_2R antagonist (famotidine), but not H_1R and H_3R/H_4R antagonists, competed with those suppressive effects of histamine. The effects of histamine were mimicked by a selective H_2R/H_4R agonist (dimaprit), and the effects of dimaprit were also competed by famotidine. Our findings involved that the suppressive effects of histamine on Th1-cytokine and IL-4 secretion in IELs were mediated predominantly via H_2R signaling. Further, we found the number of IFN-γ producing cells significantly decreased in $TCR\alpha\beta^+$ IEL population when treated with histamine and also the expression of IFN-γ significantly decreased in histamine treated IEL. The cytokine production by histamine was supposed to be attained at mRNA level.

IELs arise from both thymic-dependent and thymic-independent sources and they are phenotypically and functionally distinct from the T cells of peripheral blood or the spleen [20, 36]. As for the stimulation of IELs, we have tested PMA plus ionomycin and LPS. We also intended to use anti-CD3, experiments fail to show the enough stimulatory reaction, and variations among the experiments were too high to evaluate the effect of histamine and its ligands (data not shown). Although it is well known that LPS activates many cultured cells including peripheral blood monocytic cells, we found that LPS did not have an effect on the IEL-derived cytokines tested. While splenocytes are composed not only T cells and B cells, but also antigen presenting cells (APCs) such as macrophages and dendritic cells which express TLR4 [37], IELs are considered that they do not express TLR4.

Previous studies have shown that histamine regulates monocytes, dendritic cells, T

cells and B cells in lymphatic organs and subepithelial tissues, and different effects have been observed among these cell types [38]. In APCs, H_1R and H_3R signaling induce proinflammatory activity and increased APCs capacity, whereas H_2R signaling plays a suppressive role on monocytes [39] and monocyte-derived dendritic cells (DCs) [16, 35, 40]. In peripheral T cells, histamine enhances Th1-type responses by triggering H_1R, whereas both Th1- and Th2-type responses are negatively regulated by H_2R [8]. These distinct effects suggest roles for H_1R in autoimmunity and H_2R in peripheral tolerance. In digestive tract, the expression of H_1R, H_2R, and H_4R mRNA in the mucosa was reported [5]. These reports did not distinguish between IELs and other kind of cells such as enterocytes or LPLs. Our findings verify that dominantly H_2R, but also H_1R and H_4R, mRNA are expressed on murine IELs along with the facts demonstrated immunological response in case of H_2R.

In the present study, histamine suppressed Th1-cytokines and IL-4. H_2R-dependent effects of Th1-cytokine and IL-4 suppression in IELs are similar to those of peripheral T cells which previously reported [8, 41]. These effects may be the reflection of the functional characteristics of T cell tolerance in mucosal epithelium as IELs are crucial for the mucosal response to foreign antigens. Increase of IL-10 is well-known to induce peripheral T cell tolerance [15]. Since earlier reports have revealed histamine enhanced IL-10 secretion through H_2R in monocytes, DCs, PBMC and splenocytes [9, 10, 35, 40], we examined whether secreted IL-10 level in IELs was also affected by histamine. Contrary to our prediction, there are significant differences in IL-10 production in IELs compared to that of splenocytes. The suppressive effects of histamine did not accompany with enhancement of IL-10 production or IL-10 mRNA expression in IELs. Our findings suggested that the other mechanisms may exist to suppress the cytokine production for T cell tolerance in IELs. Since former report [42] suggested that the adherent cells were the key modulator of histamine-mediated effects on IL-10 production in splenocytes, small amount of IL-10 production in current experiments

was may be due to different condition of the cell stimulation; i.e., without adherent cells in our study.

In the present study, H_1R, H_3R or H_4R-mediated regulation of IELs cytokine production was not observed. H_2R antagonists are widely used to treat acid-related diseases such as peptic ulcer and reflux esophagitis. It is possible that H_2R antagonists may affect the clinical course of certain gastrointestinal diseases by way of regulating the local mucosal immunity rather through the systemic immune regulation. Enhancement of inflammatory cytokines and Th1/Th2 dysregulation are present in the intestinal T cells of patients with Crohn's disease [43]. Since the suppressive effect of histamine on the IELs is more remarkable in the Th1 cytokines rather than Th2, it is possible that histamine may have a protective effect on the inflammation of Crohn's disease which is known to be Th1-polarized [44, 45].

Upregulation of histamine and histidine decarboxylase are known to occurs in colorectal cancers [13]. Endogenous histamine in colon cancer tissues suppresses local tumor immunity and promotes tumor growth via H_2R signaling [46].The H_2R antagonist famotidine enhances lymphocytic infiltration in colorectal cancer [47] and cimetidine attenuates experimental tumor growth [48]. Further, some clinical studies have found survival benefits by administering H_2R antagonists for the treatment of colorectal cancer [49-51]. Given that IELs are crucial for immune surveillance of the intestinal epithelium [22, 23], direct regulation of IELs by histamine would be associated with the suppression of apoptosis of tumor cells.

In this study, we also examined whether the effects of histamine on cytokines were reflected in the intracellular cytokine production by IELs. Whereas there were too few IL-4 producing cells to evaluate the effect of histamine, IFN-γ producing cells were significantly reduced by treatment with histamine. Since IELs are heterogenous with regard to their phenotype and function [22], we further determined which cell subsets produced this cytokine. We found that almost all of the IFN-γ producing cells were $TCR\alpha\beta^+$ cells, bit not $TCR\gamma\delta^+$

cells. This suggests that there are functional differences, with regard to the cytokine production, in IEL subsets, whereas earlier studies have reported that TCR$\gamma\delta^+$ cells produced IFN-γ [19, 24, 25]. In the previous study, cells were stimulated by anti CD3 mAb, anti TCR$\alpha\beta$ mAb, or anti TCR$\gamma\delta$ mAb [25].

There are some limitations in this study. Our findings of regulatory effect by histamine on IELs were only observed in the particular condition stimulated PMA plus ionomycin *in vitro*, that is a generally recognized method to activate lymphocytes via protein kinase C pathway and calcium-dependent mechanisms. The purified IELs are relatively fragile to assess their functional characteristics, and are more difficult to examine their secreted cytokines than the other leucocyte's compartments such as PBMCs and splenocytes. Therefore, further examination in gastrointestinal disease models *in vivo* may be required to determine the extent of effects of our findings.

In summary, we have presented the basic mechanism of histamine-mediated immunoregulation of IELs *in vitro*. Histamine has a direct suppressive effect on IEL-derived Th1-cytokines and IL-4, which may have an important role in the local immunoregulation of the intestinal epithelium. The inhibitory effects of histamine on Th1-cytokines in IELs were mediated predominantly via H_2R signaling. Although further study is required to clarify the role of histamine-mediated immunoregulation in the intestinal mucosa *in vivo*, our findings would help to account for an important regulatory mechanism by endogenous histamine for the control of mucosal inflammatory functions in several gastrointestinal diseases.

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Figure Legends:

Figure 1

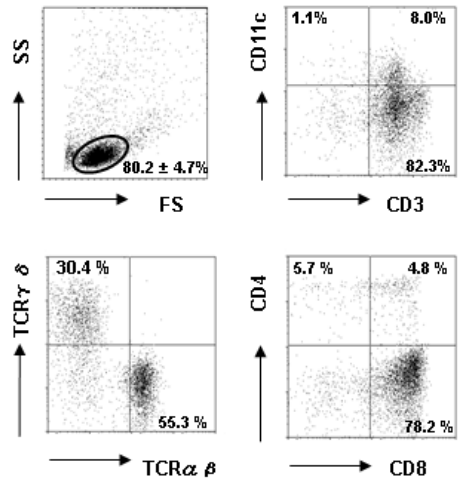

Figure 1. Purity and phenotypic characterization of IELs

Expression of CD3, CD4, CD8, CD11c, TCRαβ, TCRγδ on IELs stained with fluorescence-labeled anti-mouse mAbs were analyzed by flow cytometer. These experiments were performed three times with similar results. Side scatter (SS) depends on the inner complexity of the particle, and forward scatter (FS) correlates with the cell volume.

Figure 2

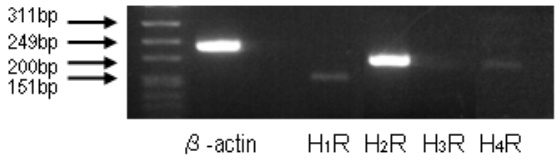

Figure 2. Expression of H_1R, H_2R, H_3R, and H_4R mRNA on IELs

The mRNA expression of histamine receptor subtypes in IELs was detected by conventional RT-PCR. PCR products were loaded on 3% agarose gel to check the specificity of the reaction. The bands were visualized by staining with ethidium bromide.

Figure 3

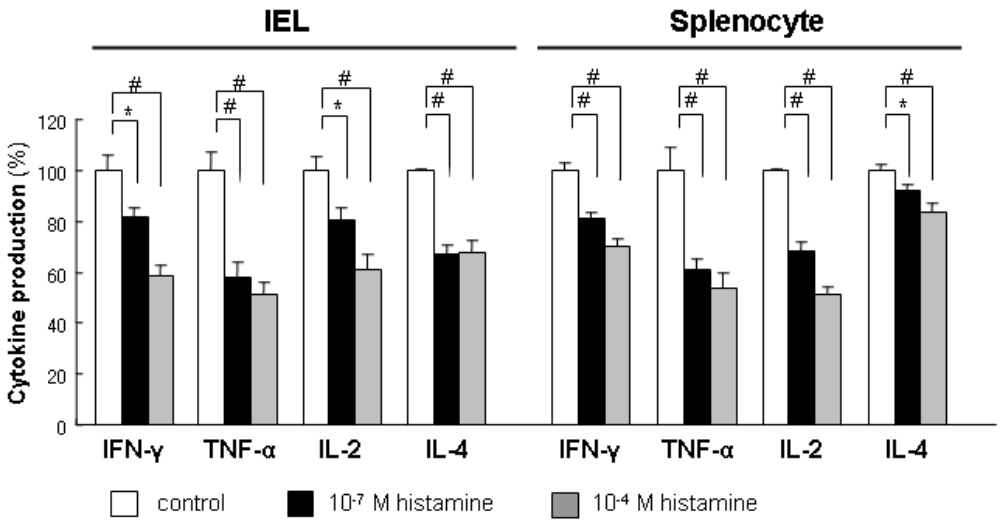

Figure 3. Histamine decreases Th1-cytokine and IL-4 derived from IELs and splenocytes

Cells were adjusted to a concentration of 1×10^6 cells/mL, treated with or without histamine (10^{-7} and 10^{-4} M) in the presence of PMA plus ionomycin. IELs were incubated for 48 h, and splenocytes were for 24 h. Cytokine levels were measured by the CBA system. The data are shown as a percentage compared to the level without histamine and represented as the means ± SEM in 9 independent experiments. The mean production of IFN-γ, TNF-α, IL-2 and IL-4 in the presence of PMA plus ionomycin without histamine in IELs were 3396.9 pg/mL, 396.9 pg/mL, 272.7 pg/mL and 45.2 pg/mL, respectively. In splenocytes, the mean production of IFN-γ, TNF-α, IL-2 and IL-4 in the presence of PMA plus ionomycin without histamine were 5116.1 pg/mL, 460.3 pg/mL, 392.3 pg/mL and 39.1pg/mL.*, $P < 0.05$ and #, $P < 0.01$ (compared to the control group)

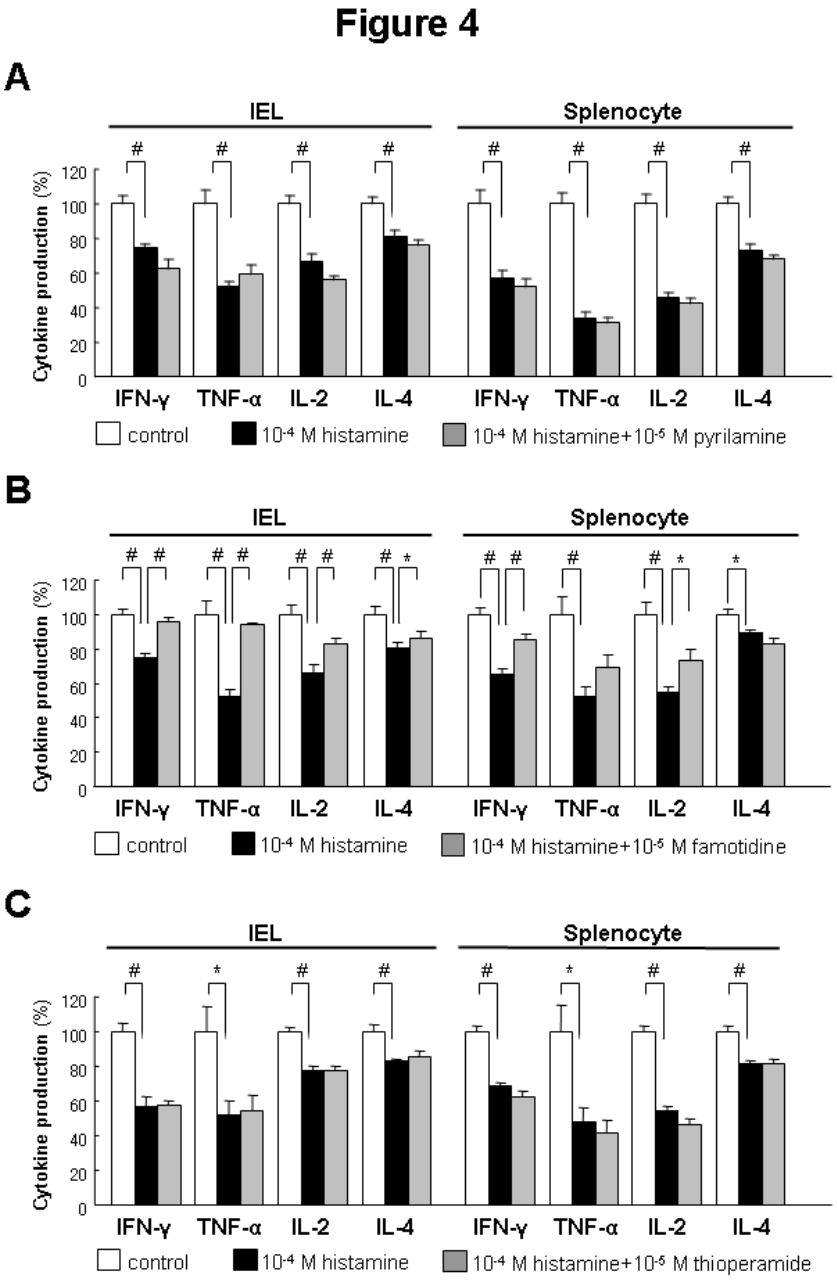

Figure 4. A selective H_2R antagonist, but not a selective H_1R or H_3R/H_4R antagonist, restored the suppressive effect of histamine on Th1-cytokine and IL-4 production on IELs

Effects of selective histamine receptor antagonists **(A)** pyrilamine; H_1R antagonist, **(B)** famotidine; H_2R antagonist, and **(C)** thioperamide; H_3R/H_4R antagonist on histamine-inducing inhibition of Th1-cytokine and IL-4 production in IELs and splenocytes were examined. Cells were adjusted to a concentration of 1×10^6 cells/mL, pretreated with pyrilamine (10^{-5} M), famotidine (10^{-5} M) or thioperamide (10^{-5} M) for 5 min. Cells were then treated with histamine (10^{-4} M) in the presence of PMA plus ionomycin. IELs were incubated for 48 h, and splenocytes were for 24 h. The levels of cytokine were measured by the CBA system. The data are shown as a percentage of the levels in the presence of PMA plus ionomycin without histamine, and represent the means ± SEM in 6~10 independent experiments. *, $P < 0.05$ and #, $P < 0.01$ (compared to the control group)

Figure 5

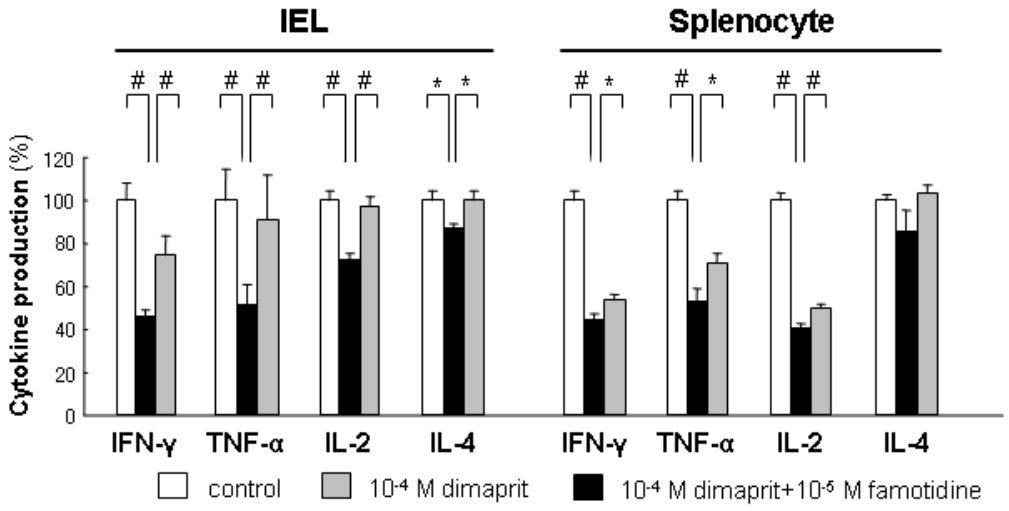

Figure 5. A selective H_2R/H_4R agonist mimicked the suppressive effects of histamine on Th1-cytokine and IL-4 production, that is blocked by a selective H_2R antagonist

Effects of a selective histamine H_2R/H_4R agonist (dimaprit) on Th1-cytokine and IL-4 production in IELs were examined. Cells were adjusted to a concentration of 1×10^6 cells/mL, pretreated with or without famotidine (10^{-5} M), a selective H_2R antagonst, for 5 min, then treated with dimaprit (10^{-4} M) in the presence of PMA plus ionomycin. IELs were incubated for 48 h, and splenocytes were for 24 h. The levels of cytokine were measured by the CBA system. The data are shown as a percentage compared to the levels without histamine in the presence of PMA plus ionomycin without histamine and represent the means ± SEM in 9 independent experiments. *, $P < 0.05$ and #, $P < 0.01$ (compared to the control group)

Figure 6

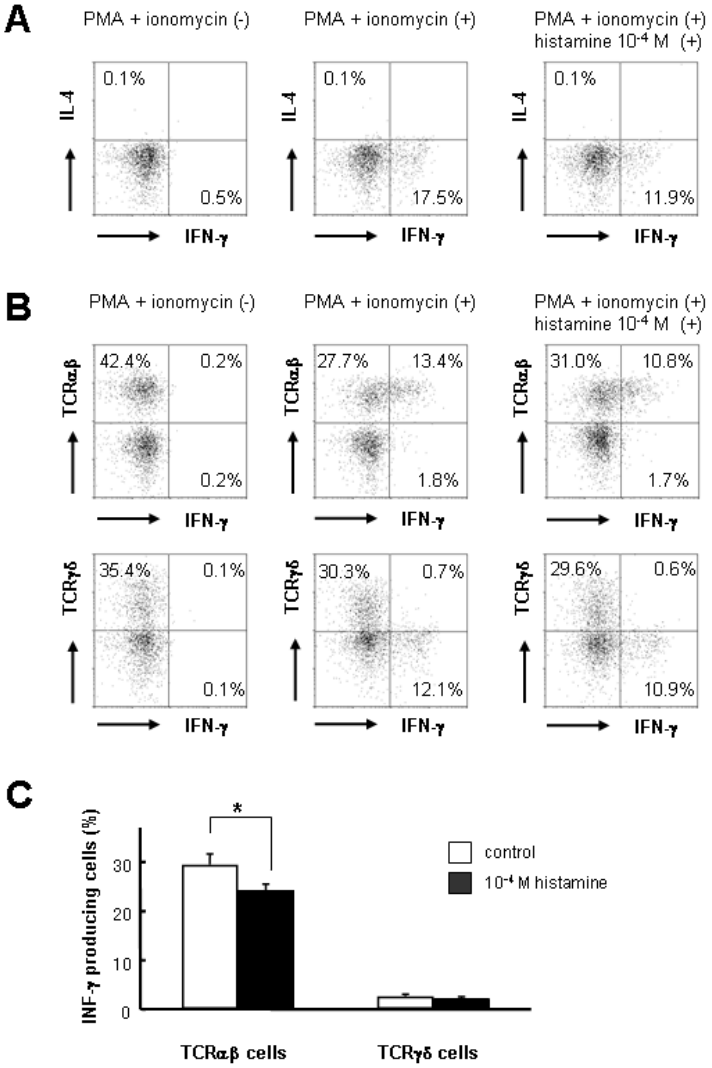

Figure 6. Effects of histamine on intracellular IFN-γ/IL-4 production in IELs

(A) Cells were treated with or without histamine (10^{-4} M), stimulated with or without PMA plus ionomycin and incubated for 4 hr. Expression of intracellular IFN-γ and IL-4 were detected with fluorescence-labeled anti-mouse mAbs and analyzed by flow cytometry. These experiments were performed three times with similar results. **(B)** Effects of histamine on intracellular IFN-γ production in $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ cells. IELs were pretreated with or without histamine (10^{-4} M), stimulated with or without PMA plus ionomycin, and incubated for 4 hr. Expression of $TCR\alpha\beta^+$, $TCR\gamma\delta^+$ and intracellular IFN-γ were labelled with fluorescence-labeled anti-mouse mAbs and analyzed by flow cytometry. These experiments were performed three times with similar results. **(C)** Quantitative analysis of INF-γ-producting cells with or without histamine in between $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ IELs. Intracellular IFN-γ positive cells were counted in $TCR\alpha\beta^+$ IELs or $TCR\gamma\delta^+$ IELs with or without histamine (10^{-4} M), stimulated with PMA plus ionomycin for 4 h. The data represent the means ± SEM in 3 independent experiments. *, $P < 0.05$ (compared to the control group)

Figure 7

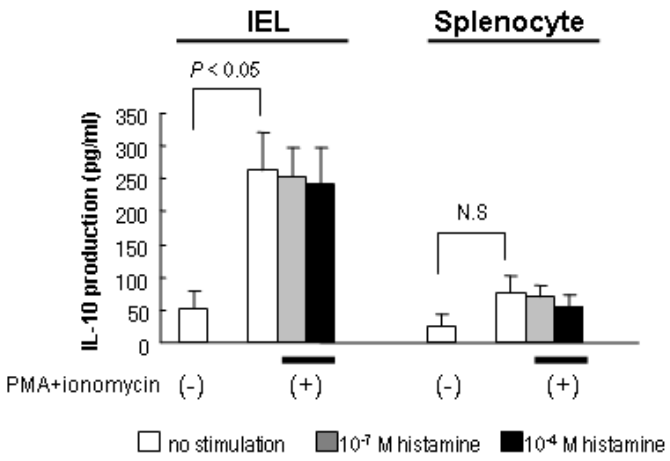

Figure 7. Production of IL-10 derived from IELs was not affected by histamine

Cells were adjusted to a concentration of 1×10^6 cells/mL, treated with or without histamine (10^{-7} and 10^{-4} M) in the presence of PMA plus ionomycin and incubated. The levels of IL-10 were measured by the CBA system. The data represent the means ± SEM in 5~10 independent experiments. Mean production of IL-10 in the presence of PMA plus ionomycin was 261.7 pg/mL on IELs and 76.7 pg/mL on splenocytes. No significant change was observed among the treatment groups.

Figure 8

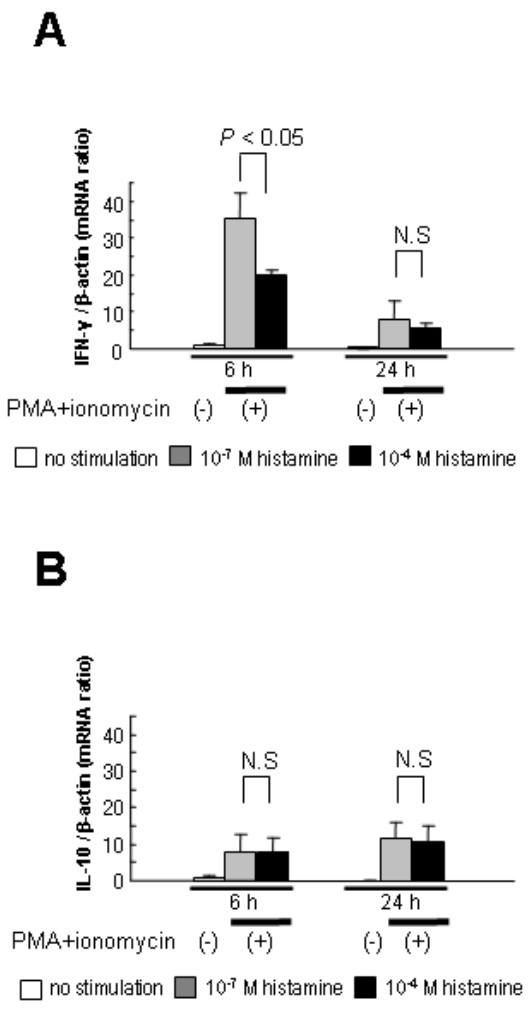

Figure 8. Histamine reduced IFN-γ mRNA expression, but not IL-10 mRNA expression in IELs

Effects of histamine on IFN-γ **(A)** and IL-10 mRNA **(B)** expression in IELs. Cells were adjusted to a concentration of 1×10^6 cells/mL, treated with or without histamine (10^{-4} M) in the presence of PMA plus ionomycin and incubated for 6 h or 24 h. Cytokine mRNA levels are normalized to β-actin from same sample. The data represent the means ± SEM in 3 independent experiments performed in triplicate. *, $P < 0.05$ (compared to the absence of histamine group)

Table 1 **Cytokine production in IELs compared with splenocytes**

	Control	**PMA** (40ng/mL) + **ionomycin** (4μg/mL)		**LPS** (1μg/mL)	
IELs	(pg/mL)	(pg/mL)		(pg/mL)	
IFN-γ	5.4 ± 0.4	3473.6 ± 578.9	($p < 0.01$)	4.7 ± 0.8	*(n.s.)*
TNF-α	40.6 ± 4.5	228.5 ± 71.4	($p < 0.05$)	40.0 ± 6.4	*(n.s.)*
IL-2	6.9 ± 1.8	133.8 ± 10.7	($p < 0.01$)	4.2 ± 0.8	*(n.s.)*
IL-4	10.5 ± 2.5	40.1 ± 6.3	($p < 0.01$)	6.2 ± 1.3	*(n.s.)*
IL-5	8.9 ± 4.2	13.8 ± 5.3	*(n.s.)*	7.1 ± 1.4	*(n.s.)*
Splenocytes	(pg/mL)	(pg/mL)		(pg/mL)	
IFN-γ	3.0 ± 0.5	4045.5 ± 725.9	($p < 0.01$)	127.9 ± 33.1	($p < 0.01$)
TNF-α	15.3 ± 2.5	207.3 ± 86.6	($p < 0.05$)	302.1 ± 42.4	($p < 0.01$)
IL-2	0.3 ± 0.3	113.6 ± 57.2	*(n.s.)*	1.4 ± 0.4	*(n.s.)*
IL-4	4.3 ± 0.3	48.5 ± 8.3	($p < 0.01$)	3.3 ± 0.7	*(n.s.)*
IL-5	5.0 ± 2.4	6.1 ± 2.7	*(n.s.)*	3.6 ± 1.9	*(n.s.)*

Data are represented as means ± SEM in 3 ~ 5 independent experiments. Cytokine production by the conditions were compared to that of control condition.