Table 1. Composition of Elental

Total	100 g (375 kcal)	Amino acids	17.6 g
Amino acids	17.6 g (66 kcal)	L-Isoleucine	803 mg
Carbohydrate (dextrin)	79.3 g (304 kcal)	L-Leucine	1124 mg
Lipid (soybean oil)	0.60 g (5 kcal)	L-Lysine-HCl	1110 mg
Vitamin A	810 IU	L-Methionine	810 mg
Vitamin D	64.0 IU	L-Phenylalanine	1089 mg
Vitamin B-1	0.24 mg	L-Threonine	654 mg
Vitamin B-2	0.25 mg	L-Tryptophan	189 mg
Vitamin B-6	0.33 mg	L-Valine	876 mg
Niacin	2.78 mg	L-Histidine-HCl-H_2O	626 mg
Pantotenic acid	1.38 mg	L-Arginine-HCl	1406 mg
Folic acid	55 μg	L-Alanine	1124 mg
Vitamin B-12	0.88 μg	Mg-K-L-Aspartate	1295 mg
Vitamin C	9.75 mg	Na-L-Aspartate-H_2O	1084 mg
Vitamin K	11.3 μg	L-Glutamine	2415 mg
Vitamin E	4.13 IU	Glycine	631 mg
Biotin	48.8 μg	L-Proline	788 mg
Choline	10.7 mg	L-Serine	1449 mg
Na	325 mg	L-Tyrosine	138 mg
K	272 mg		
Cl	646 mg		
Mg	50.0 mg		
Ca	197 mg		
P	152 mg		
Fe	2.25 mg		
I	19.0 μg		
Mn	375 μg		
Cu	250 μg		
Zn	2.25 mg		

BASIC—ALIMENTARY TRACT

Isolation of Lamina Propria Mononuclear Cells and Thioglycolate-Induced Peritoneal Exudate Cells

Lamina propria mononuclear cells (LPMCs) were isolated from colonic tissues as previously described.[22] One milliliter of 3% (wt/vol) thioglycolate was injected in the peritoneal cavity of mice, and the peritoneal exudate cells (TG-PECs) were harvested from the mice. TG-PECs were plated onto 96-well culture plates at 1×10^5 cells/well for 1 hour in DMEM, and then nonadherent cells were removed. The adherent cells were used as peritoneal macrophages.[25] These cells were cultured for an additional 2 hours in DMEM containing 10% (vol/vol) FBS, LPS (10 ng/mL), and various other reagents. Supernatants were collected and assessed for cytokine secretion by ELISA. Cell viability was determined using a water-soluble tetrazolium reagent, WST-8 (Nacalai Tesque, Kyoto, Japan).

ELISA

For the measurement of mouse TNF-α, IL-6 (BD Biosciences, San Jose, CA) and keratinocyte chemoattractant (KC) (R&D Systems, Minneapolis, MN), sandwich ELISAs were performed according to the manufacturer's instructions.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from the peritoneal macrophages (1×10^6 cells/well) using Isogen (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. For complementary DNA (cDNA) synthesis, 1 μg total RNA was reverse-transcribed using Superscript II and oligo(dT) 12–18 primers (Invitrogen, Carlsbad, CA). cDNA, equivalent to 10 ng RNA, was used as a template for real-time reverse-transcription polymerase chain reaction (RT-PCR) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following conditions: 5.85 μL SYBR Green PCR master mix (Applied Biosystems) and 500 nmol/L primers in a final volume of 16 μL. The PCR program consisted of a denaturing step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, and an annealing step at 60°C for 1 minute. Prior to the sample analysis, the efficiency of the PCR was assessed by the standard curve method. A standard curve was constructed from an amplified DNA of several concentrations of known standard. Because the efficiency of the amplification using each primer set was approximately equal, a comparative threshold cycle method was performed to process the data. The following PCR primers for β-actin, TNF-α, and IL-6 were used: β-actin sense, 5′-ggtaccaccatgtaccc-agg-3′, β-actin antisense, 5′-gtacttgcgctcaggaggag-3′; TNF-α sense, 5′-ccaccacgctcttctgtcta-3′, TNF-α antisense, 5′-agggtct-gggccatagaact-3′; IL-6 sense, 5′-cagaggataccactcccaaca-3′, IL-6 antisense, 5′-cagaattgccattgcacaac-3′.

Semiquantitative RT-PCR

After incubation with LPS in 24-well plates, total RNA was extracted from the peritoneal macrophages (8×10^5 cells/well) using Isogen. For cDNA synthesis, 0.36 μg total RNA was used. The PCR program was 1 cycle of denaturing at 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, annealing at 56°C for 1 minute, and extension at 72°C for 2 minutes. The following PCR primers for HDC (516 base pair [bp]), GAPDH (249 bp) were used: HDC sense, 5′-ggatccaagat-cagatttctacctgtggac-3′, HDC antisense, 5′-gtcgacgacatgtgctt-gaagattcttcac-3′; GAPDH sense, 5′-tgatgacatcaagaaggtggtgaag-3′, GAPDH antisense, 5′-tccttggaggccatgtaggccat-3′.

Immunoblotting Analysis

Cells were lysed with sample buffer containing 2% SDS (wt/vol). p65 Nuclear extracts were prepared using the Nuclear and Cytoplasmic Extraction Kit (Pierce Biotech, Rockford, IL). Samples were heated at 95°C for 5 minutes and centrifuged at 4°C, and the supernatant was analyzed by SDS-PAGE. Proteins were transferred to PVDF membranes, which were blocked with 10% nonfat dry milk. The membranes were blotted with the primary antibody, then with a horseradish peroxidase-conjugated secondary antibody, and detected by an ECL system according to the manufacturer's instructions.

Cytokine Array

The mouse inflammation antibody array I was purchased from Ray Biotech (Norcross, GA) and used according to the manufacturer's instructions. Briefly, cultured medium from TG-PECs was added to the membrane, which was incubated for 1 hour with biotin-conjugated cytokines (provided with the kit). After incubation, membranes were washed and incubated for 30 minutes with horseradish peroxidase-conjugated streptavidin. Unbound reagents were washed and the membranes developed with the ECL system.

Statistical Analysis

The data are expressed as the mean ± SE. Groups of data were compared by Dunnett multiple comparison tests (SAS version 6.12; SAS Institute, Cary, NC). $P < .05$ was considered statistically significant.

Results

ED Amino Acids Ameliorate Murine IL-10$^{-/-}$ Cell Transfer Colitis Models

We previously reported that ED reduces colonic inflammation in murine IL-10$^{-/-}$ cell transfer colitis models.[23] ED was composed of 17.6% amino acids, 79.3% dextrin, 0.6% soybean oil, 0.5% vitamins, and 2.0% minerals (Table 1). We first analyzed the efficacy of a premixture of EDAAs on IL-10$^{-/-}$ cell transfer colitis models. EDAAs mixed in the standard formula improved colonic weight in a dose-dependent manner (Figure 1*A*). TNF-α

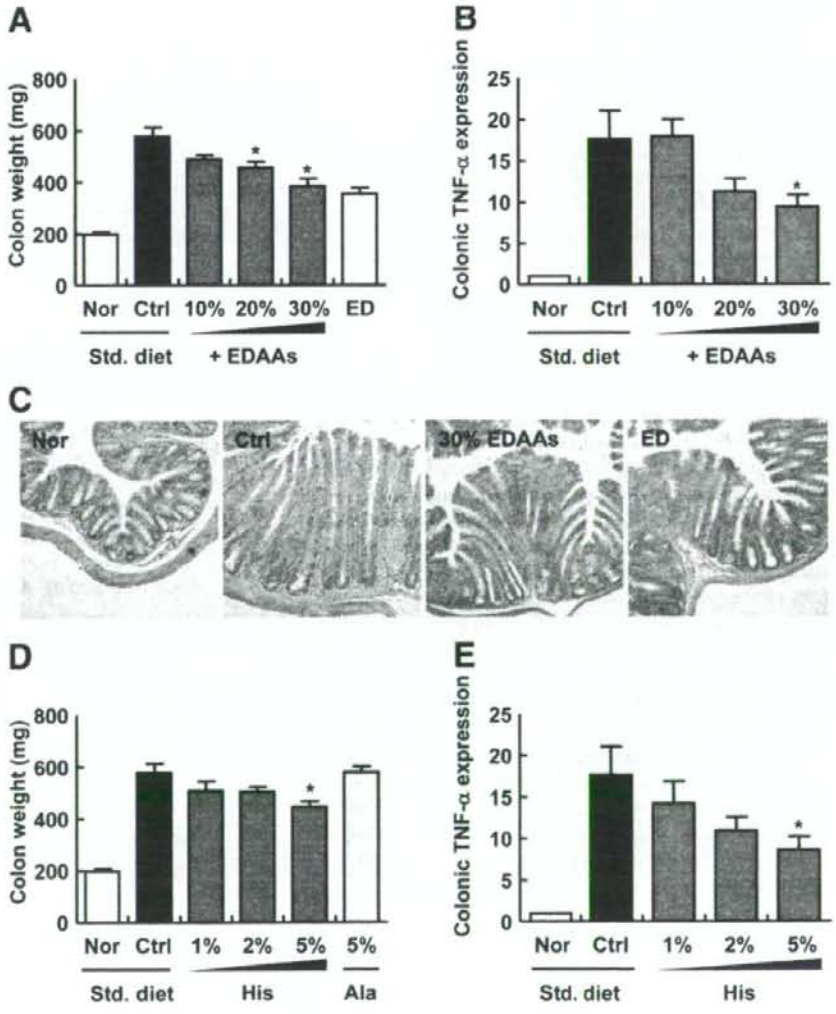

Figure 1. Dietary EDAAs or histidine ameliorate colon inflammation in IL-10$^{-/-}$ cell transfer colitis model. A conventional powder chow (*Std. diet*) containing 0%–30% EDAAs (*+EDAAs*) or 100% elemental diet (*ED*) was fed to SCID mice with experimental colitis induced by spleen/mesenteric lymph node cells from IL-10$^{-/-}$ mice with colitis (n = 6–8). (*A*) At 21 days, colon weight was measured. (*B*) Colon TNF-α mRNA expression was observed by quantitative RT-PCR. (*C*) H&E staining of distal colon at 21 days. Next, diet containing 1%, 2%, and 5% histidine (*His*) or 5% alanine (*Ala*) was fed to mice with experimental colitis (n = 6 or 7). (*D*) At 21 days, whole colon weight was measured. (*E*) Colon TNF-α mRNA expression was observed by quantitative RT-PCR. *Error bars* indicate standard error (SE). *Statistically significant differences compared with control (Ctrl) group ($P < .05$).

has been well characterized as an important inflammatory cytokine produced from inflammatory cells during colonic inflammation; therefore, we measured messenger RNA (mRNA) levels of TNF-α in the colon using RT-PCR. As shown in Figure 1*B*, TNF-α mRNA was increased in the colon 3 weeks after IL-10$^{-/-}$ cell transfer, reaching levels nearly 17-fold greater than control values. This increase of TNF-α mRNA transcription by IL-10$^{-/-}$ cells was significantly attenuated by dietary EDAAs in a dose-dependent manner (Figure 1*B*). Dietary uptake of EDAAs reduced mucosal thickness and cell infiltration (Figure 1*C*). Thus, the amino acids mixture found in ED also had a therapeutic effect on IL-10$^{-/-}$ cell transfer colitis models.

Histidine Ameliorates IL-10$^{-/-}$ Cell Transfer Colitis Models

To establish which EDAAs have anti-inflammatory potential, we investigated the effects of individual several amino acids, including histidine, alanine, glutamine, and so on, on IL-10$^{-/-}$ cell transfer colitis models. Among them, oral supplementation with 5% histidine, but not alanine and glutamine (data not shown), in standard formula ameliorated colonic inflammation (Figure 1*D*). Increases of TNF-α mRNA transcription were suppressed by dietary histidine in a dose-dependent manner (Figure 1*E*).

Effect of Amino Acids on LPS-Induced TNF-α Secretion From Inflamed Mouse Colon Tissue and LPMCs

We investigated the mechanisms involved in the antiinflammatory effects of histidine on IL-10$^{-/-}$ cell transfer colitis models. Immunohistochemistry revealed the presence of macrophages, a major source of TNF-α, infiltrating the colon lamina propria in this colitis model (Figure 2*A*). We hypothesize that histidine directly inhibits its TNF-α secretion from the intestinal macrophages. We thus measured LPS-stimulated production of TNF-α by colonic biopsy specimens of inflamed colon mucosa. Stimulated TNF-α was reduced by the presence of histidine, but not in that of lysine or alanine, in a concentration-dependent manner (Figure 2*B*). LPMCs isolated from IL-10$^{-/-}$ cell transfer colitis models fed by standard diet with 5% of dietary histidine showed reduced production of TNF-α (Figure 2*C*). Furthermore, dietary histidine showed an anti-inflammatory effect on the production of TNF-α from peritoneal macrophages (Figure 2*D*).

Histidine Suppresses the Production of Proinflammatory Cytokines by Macrophages

The anti-inflammatory effects of histidine were confirmed in primary mouse peritoneal macrophages. Histidine, but not lysine or alanine, inhibited the production of TNF-α and IL-6 by LPS-stimulated peritoneal macrophages in a concentration-dependent manner (Figure 3*A*). The viability of peritoneal macrophages was not affected by any of the amino acids studied at concentrations up to 25 mmol/L (Figure 3*B*). Histidine also inhibited mRNA transcription of both TNF-α and IL-6 (Figure 3*C* and *D*).

We assessed whether either L-histidine enantiomer D-histidine or the histidine metabolite carnosine (Figure 4*A*) also have anti-inflammatory activity in murine macrophages. Both D-histidine and carnosine scarcely suppressed the production of TNF-α and IL-6 (Figure 4*B* and *C*). Neither compound had any effect on cell viability (Figure 4*D*).

Antiinflammatory Effect of Histidine Is Independent of Histamine Synthesis

Intracellular histidine is enzymatically metabolized to histamine by HDC, which regulates histamine synthesis (Figure 4*A*). Previously, it has been reported that histamine affects cytokine production and alters the balance of Th-1/Th-2 immune responses.[24,25] Therefore, we investigated whether the anti-inflammatory effect of histidine was mediated through histamine synthesis using HDC$^{-/-}$ peritoneal macrophages. HDC mRNA was induced by LPS stimulation in peritoneal macrophages (Figure 5*A*). Although histamine was deficient in HDC$^{-/-}$ cells (Figure 5*A*), histidine showed an anti-inflammatory effect on the production of TNF-α and IL-6 by LPS-stimulated HDC-deficient peritoneal macrophages (Figure 5*B* and *C*). Thus, the anti-inflammatory effect of histidine was independent of histamine synthesis.

Profiling of LPS-Induced Soluble Inflammatory Factors in Macrophages Treated With Histidine

Cytokine array analysis of LPS-stimulated peritoneal macrophages showed that histidine suppressed the production of not only TNF-α and IL-6 but also macrophage inflammatory protein (MIP)-1α, MIP-1γ monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinase-1 (Figure 6*A*). By contrast, LPS-induced KC was not suppressed by histidine, as shown by both cytokine array (Figure 6*A*) and ELISA (Figure 6*B*).

Histidine Inhibits NF-κB Activation in Macrophages

The activation of transcription factor NF-κB is important for the production of proinflammatory cytokines by activated macrophages. NF-κB is usually held in an inactive state in the cytoplasm of unstimulated cells through interaction with IκB-α. After stimulation with LPS, IκB-α is degraded and releases NF-κB to be translocated to the nucleus, where NF-κB induces the expression of proinflammatory cytokines. NF-κB was rapidly activated when peritoneal macrophages were stimulated with LPS. The addition of 10 mmol/L histidine significantly inhibited LPS-induced IκB-α degradation (Figure 7*A*). p65 Nuclear translocation was also inhibited by histidine in a concentration-dependent manner (Figure 7*B*).

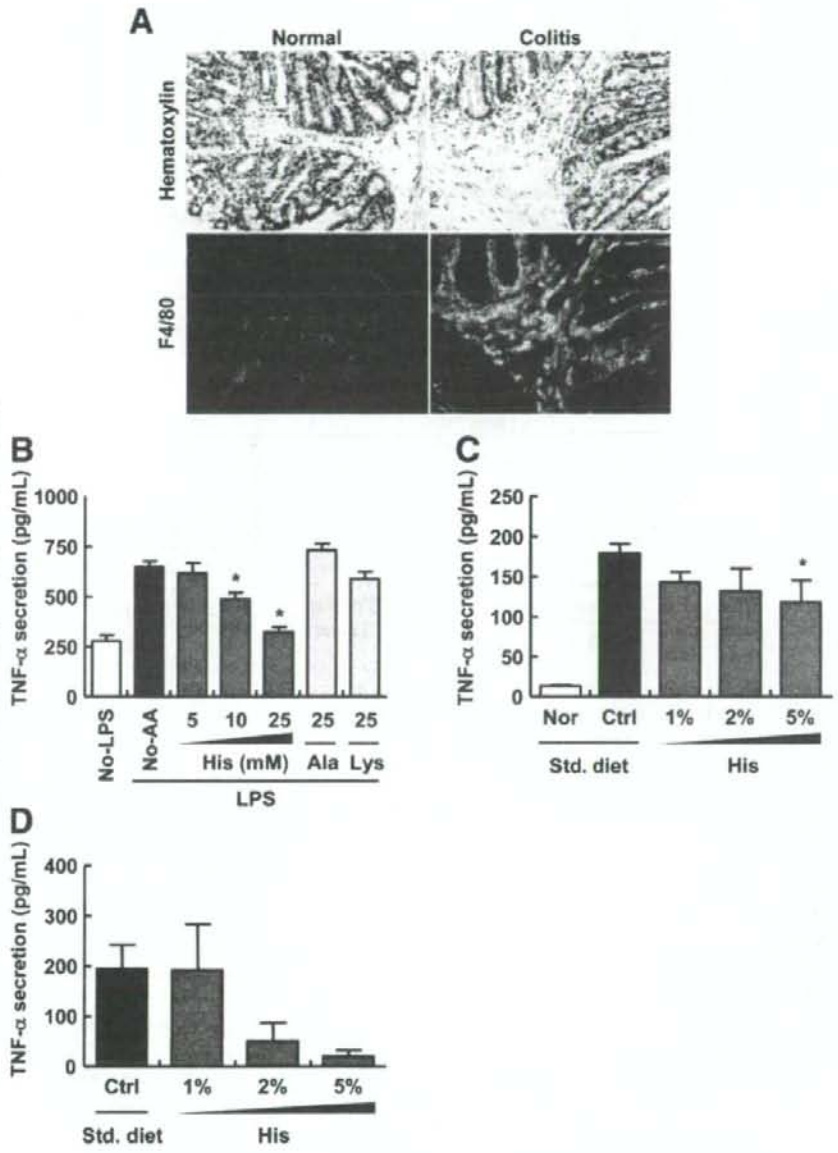

Figure 2. Histidine directly inhibits LPS-induced TNF-α secretion from inflamed mouse colon tissue, LPMCs, and peritoneal macrophages. (*A*) The number of macrophages was significantly increased in the inflamed colon. Sections of distal colon are stained with hematoxylin (*upper panels*), immunostained for F4/80 (*lower panels*). (*B*) Three-millimeter punch biopsy specimens were prepared from inflamed colon in IL-10$^{-/-}$ mice. Biopsy specimens were cultured for 6 hours in DMEM containing 1 mg/mL LPS, and TNF-α in the culture supernatants was measured (n = 12–18). (*C*) A conventional powder chow (*Std. diet*) containing 1%, 2%, and 5% histidine (*His*) was fed to mice with experimental colitis (n = 5). At 21 days, LPMCs from each colon were incubated for 16 hours with neither LPS nor amino acid. (*D*) A conventional powder chow (*Std. diet*) containing 1%, 2%, and 5% histidine (*His*) was fed to mice with experimental colitis (n = 4). After 24 hours, peritoneal macrophages were incubated for 16 hours. The culture supernatants were analyzed for TNF-α. *Error bars* indicate SE. *Statistically significant differences compared with nonamino acid or Ctrl group ($P < .05$).

Discussion

We demonstrated that orally administered histidine ameliorated intestinal inflammation in an IL-10$^{-/-}$ cell transfer colitis model. It has been reported that IL-10$^{-/-}$ cell transfer colitis models exhibit features similar to IBD, both pathophysiologically and pharmacologically.[18] Consistent with previous reports that show the efficacy of ED for CD,[10-13] we found that ED was effective in this colitis model. ED was composed of 17 amino acids, dextrin, bean oil, vitamins, and minerals (Table 1). Interestingly, amino acid components of ED also were efficacious to ameliorate inflammation in this model. These findings indicated that amino acids themselves have anti-inflammatory activity. Histidine, one of the amino acids in the EDAAs mixture, was shown to reduce colon inflammation in this model.

Histidine is one of the most common natural amino acids and belongs to the conditionally essential amino acids because only adults, not children, can produce an adequate amount of histidine by themselves. It has been

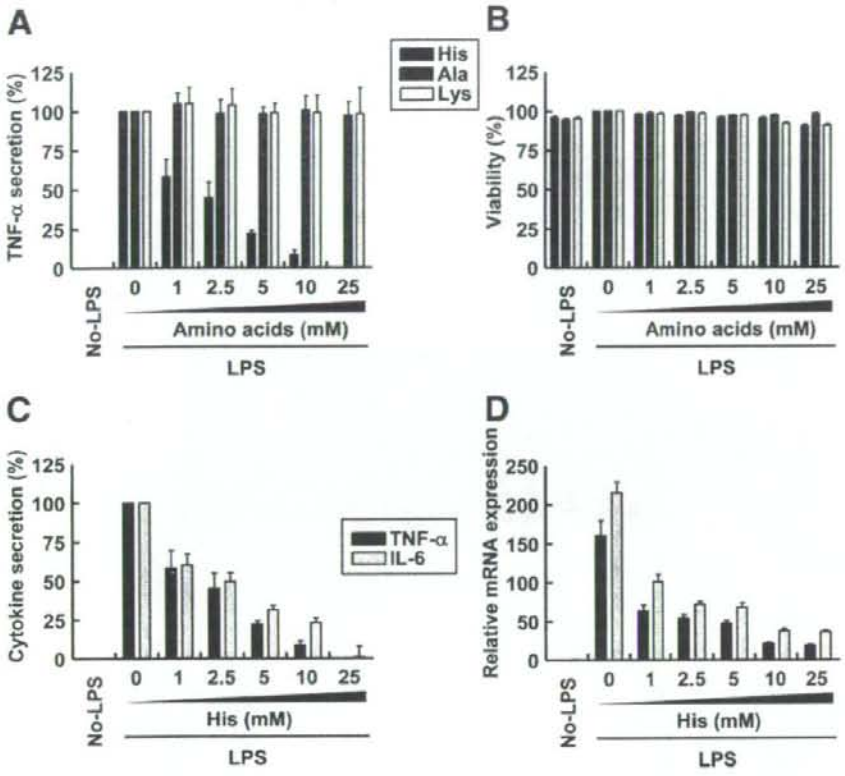

Figure 3. Histidine inhibits LPS-induced proinflammatory cytokine secretion from macrophages. (*A*) TG-PECs were incubated for 2 hours with 10 ng/mL LPS and histidine (*His*), alanine (*Ala*), or lysine (*Lys*). The culture supernatants were analyzed for TNF-α (*A*). Cell viability was determined by colorimetric assay (*B*). Suppressive effects of histidine on TNF-α and IL-6 were observed at the protein (*C*) and transcriptional (*D*) levels. Data are expressed as the mean ± SE of 6 well samples, and the results are representative of 3 experiments with similar results.

reported that histidine has the potentiality as a scavenger of the hydroxyl radical and the nonradical toxic oxygen species. Recently, it has been reported that histidine can inhibit the production of IL-8 by intestinal epithelial cell lines treated with TNF-α or H_2O_2.[26] It has been reported that several amino acids in addition to histidine are radical scavengers in epithelial injury, and their oral or rectal administration shows efficacy in dextran sodium sulphate or trinitrobenzene sulfonate colitis models.[16,17] Furthermore, inflammation in IL-$10^{-/-}$ cell transfer colitis models is characterized by mucosal thickness because of infiltration of many inflammatory cells and no epithelial injury. We observed a number of infiltrating F4/80-positive macrophages in the lamina propria in our colitis models. Therefore, we focused on the anti-inflammatory effects of histidine on macrophages. Indeed, dietary histidine inhibited proinflammatory cytokine secretion from LPMCs in the IL-$10^{-/-}$ cell transfer colitis model and peritoneal macrophages (Figure 2*C* and *D*). On the other hand, we first demonstrated in vitro that histidine, but not alanine or lysine, inhibited the production of proinflammatory cytokines, including TNF-α and IL-6, by LPS-stimulated macrophages. Although carnosine and anserine, dipeptide derivatives containing histidine synthesized via the carnosine synthase and the carnosine *N*-methyl transferase pathway, show antioxidative effects in muscle, brain, and other tissues,[27,28] they showed less inhibition of TNF-α and IL-6 production by LPS-stimulated macrophages. Thus, it is possible that these metabolites of histidine suppress production of proinflammatory cytokines intracellularly. However, intracellular level of histidine has no difference between L- and D-histidine-treated macrophages, and intracellular level of carnosine was equal to that of histidine (see supplementary Figure 1 online at www.gastrojournal.org). Therefore, these results clearly indicate that the antioxidant properties of histidine alone cannot explain its anti-inflammatory effect in macrophages. Transporters play important roles for transportation of amino acids or peptide in intestinal epithelial cells, whereas the functional role of these transporters in macrophages remains unknown. For example, one of the histidine transporters and neutral and basic amino acid transporter (NBAT) are expressed mainly in renal and intestinal epithelia.[29]

Another pathway of histidine metabolism is HDC, which regulates the histidine-histamine pathway. Elenkov et al have reported that histamine suppresses IL-12 and stimulates IL-10 production via H2 receptors in human monocytes.[25] Horvath et al have also reported that histamine inhibits LPS-induced transcription of interferon (IFN)-γ in PBMCs.[30] On the other hand, Vannier

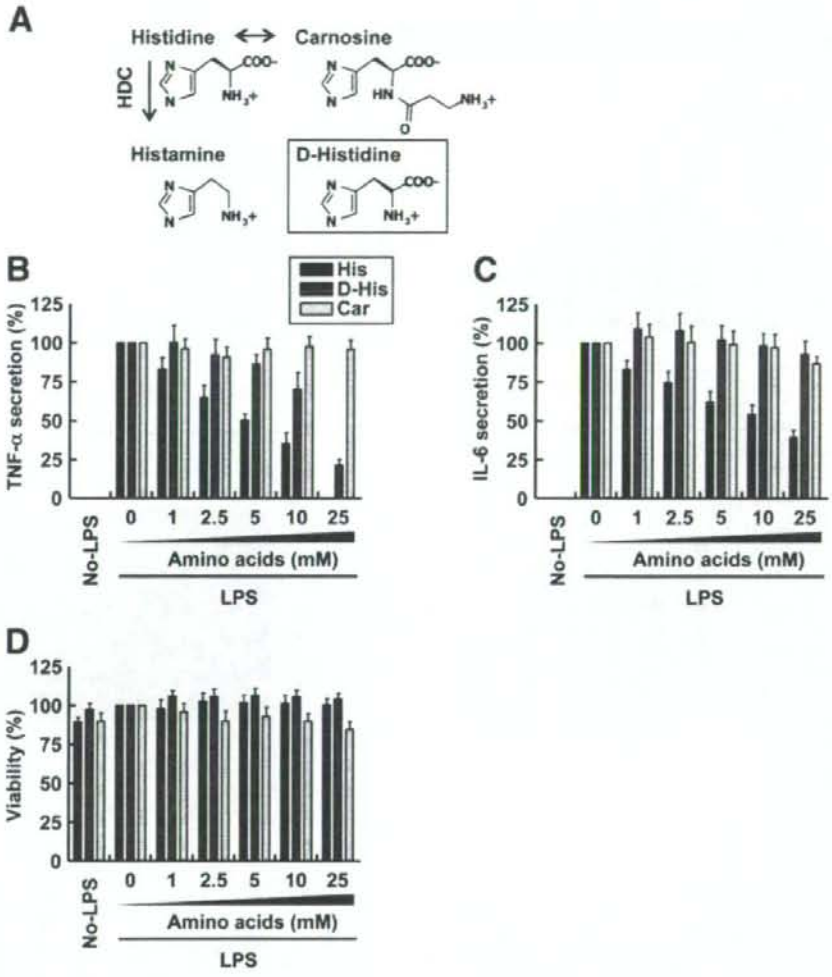

Figure 4. The anti-inflammatory effect of histidine is stronger than that of histidine-related compounds on macrophages. The major metabolic pathways of histidine are charted in (*A*). TG-PECs were incubated for 2 hours with 10 ng/mL LPS and L-histidine (*His*), D-histidine (*D-His*), or carnosine (*Car*). The culture supernatants were analyzed for TNF-α (*B*) and IL-6 (*C*). Cell viability was determined by colorimetric assay (*D*). Data are expressed as the mean ± SE of 6 well samples, and the results are representative of 3 experiments with similar results.

and Dinarello have reported that histamine enhances IL-1-induced IL-6 production via H2 receptors in PBMCs.[31] Experimental autoimmune encephalomyelitis, the most commonly used animal model for human multiple sclerosis, is generally thought to be a Th-1-mediated disease, similar to CD. Blockade of histamine with specific histamine receptor 1 (H1R) antagonists reduces the pathology associated with experimental autoimmune encephalomyelitis.[32] However, Musio et al reported that experimental autoimmune encephalomyelitis is significantly more severe in HDC null mice.[33] Histamine can mediate diverse biologic activities through 4 types of histamine receptors: H1Rs, H2Rs, H3Rs, and H4Rs. Histamine can promote Th-1 responses through H1Rs, whereas it can down-regulate both Th-1 and Th-2 responses through H2Rs.[34] Thus, the effect of histamine on the Th-1/Th-2 balance remains controversial. We isolated peritoneal macrophages from HDC null mice and assessed the effect of histidine on LPS-induced TNF-α production. Our results indicate that the inhibitory effect of histidine on LPS-induced TNF-α production by peritoneal macrophages is independent of histamine synthesis because histidine suppressed the production of TNF-α by HDC null peritoneal macrophages.

In the studies of LPS-stimulated peritoneal macrophages, histidine suppressed the production of various soluble inflammatory factors including cytokines (IL-6), tissue inhibitors of matrix metalloproteinase (TIMP-1), and chemokines (MIP-1α, MIP-1γ) but not KC. It has been reported that MIP-1α plays an important role in the development of murine colitis models and human IBD. Pender et al have reported that systemic administration of MIP-1α exacerbates murine trinitrobenzene sulfonate colitis.[35] In rat trinitrobenzene sulfonate colitis models,

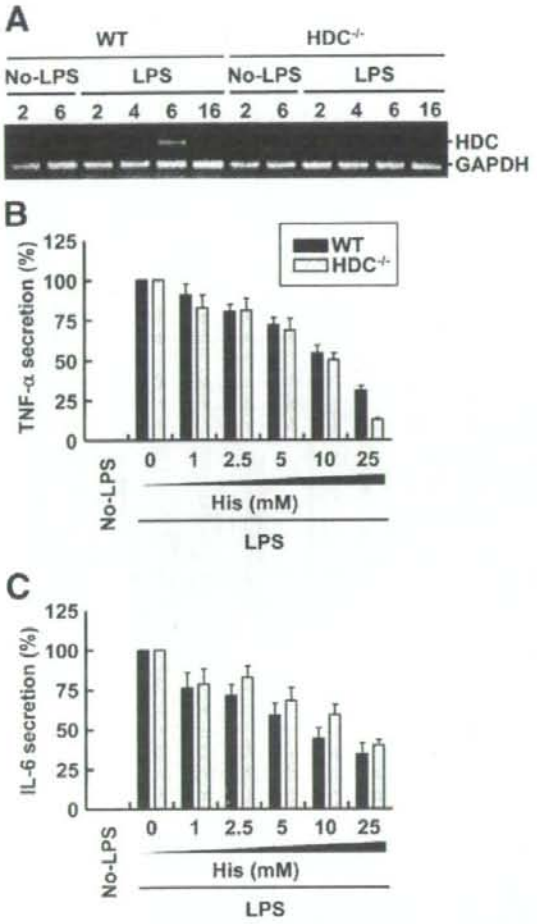

Figure 5. Anti-inflammatory effect of histidine is independent of histamine synthesis. (*A*) TG-PECs from wild-type (*WT*) or HDC$^{-/-}$ mice were incubated for 2, 4, 6, and 16 hours with LPS. HDC and GAPDH mRNA expression as a housekeeping control gene was detected. TG-PECs from WT or HDC$^{-/-}$ mice were incubated for 2 hours with 10 ng/mL LPS and histidine (*His*). The culture supernatants were analyzed for TNF-α (*B*) or IL-6 (*C*). Data are expressed as the mean ± SE of 6 well samples.

mucosal MIP-1α level is increased.[36] The suppressive effects of histidine on the production of proinflammatory cytokines in these studies were, at least partially, dependent on the NF-κB-signaling pathway. Histidine inhibited activation of NF-κB, as reflected by inhibition of both IκB-α degradation and p65 nuclear translocation, and it seemed that it resulted in the suppression of the production of proinflammatory cytokines because the activity of NF-κB controls the transcription of several kinds of proinflammatory cytokines including TNF-α. Although the chemokine KC also appears to be important in the development of colitis,[37] LPS-induced KC was not suppressed by histidine in our experiments. Consistent with these results, a previous report showed that KC produced by LPS-stimulated murine peritoneal macrophages is independent of NF-κB signaling.[38] Thus, our results demonstrate that histidine inhibits activation of NF-κB following the suppression of the production of proinflammatory cytokines. Histidine also inhibited peptideglycan, which is a Toll-like receptor ligand, and induced TNF-α production in peritoneal macrophages (see supplemental Figure 2*A* online at www.gastrojournal.org). Thus, histidine could inhibit NF-κB signaling at common downstream of toll-like receptors.

In mice, 5% dietary histidine increased plasma and tissue histidine to greater than 1 mmol/L at a concentration sufficient to exert acute inflammatory effects on TG-PECs in vitro (see supplemental Figure 3 online at www.gastrojournal.org). Finally, we examined the inhibitory effect of histidine on the production of proinflammatory cytokines by human monocytes. Histidine inhibited TNF-α secretion from LPS-stimulated, CD14-positive hu-

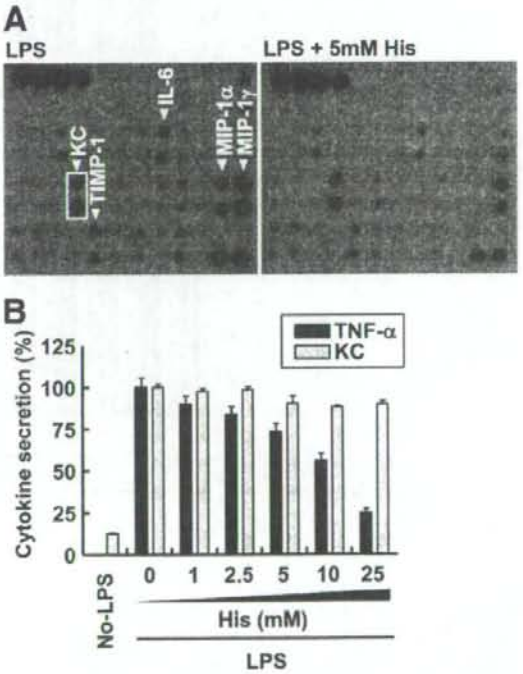

Figure 6. Profiling of LPS-induced soluble inflammatory factors in macrophages treated with histidine. (*A*) TG-PECs were incubated for 2 hours with 10 ng/mL LPS and 5 mmol/L histidine (*His*). Soluble inflammatory factors in the culture medium were analyzed using cytokine array membranes. Representative images were chosen from 2 independent experiments. (*B*) TG-PECs were incubated for 2 hours with 10 ng/mL LPS and histidine (*His*). Supernatants were collected and assessed for KC secretion by ELISA. Data are expressed as the mean ± SE of 6 well samples, and the results are representative of 2 experiments with similar results.

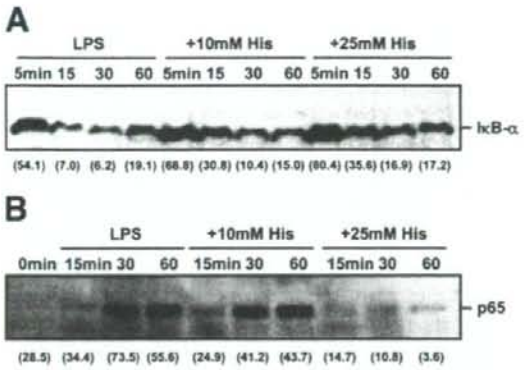

Figure 7. Histidine inhibits NF-κB activation in macrophages. Effect of histidine on LPS-induced degradation of IκB-α (*A*) and translocation of a subunit of NF-κB (*p65*) into the nucleus (*B*). Mouse peritoneal macrophages were treated with 10 ng/mL LPS with indicated concentration of histidine for 5, 15, 30, and 60 minutes. The expressions of IκB-α in total lysate and p65 in nuclear extracts were determined by immunoblotting analysis. The data by densitometry analysis are shown under the panels.

man monocytes in a concentration-dependent manner (see supplementary Figure 4 online at www.gastrojournal.org). It is therefore possible that orally administrated histidine may be clinically useful by reducing intestinal inflammation in human IBD. Intravenous administration may also inhibit mononuclear cell activation. Furthermore, as previously reported, other amino acids have been shown to contribute to the modulation of gut inflammation. It follows that because of their anti-inflammatory effects, amino acids may have potentiality as novel therapeutic agents for IBD. In conclusion, our results provide new insight into a therapeutic strategy for CD based on observations that amino acids, especially histidine, inhibit the production of proinflammatory cytokines from activated macrophages.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.09.062.

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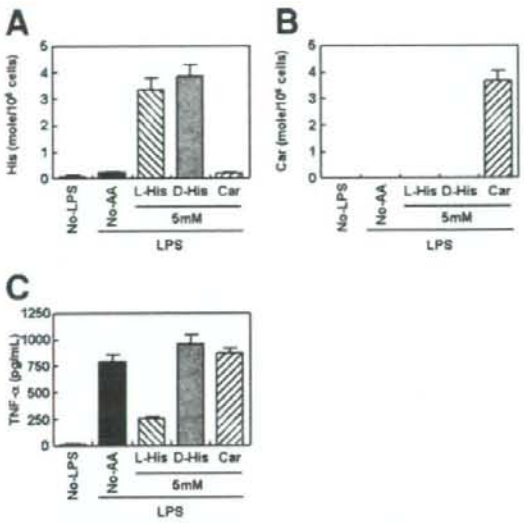
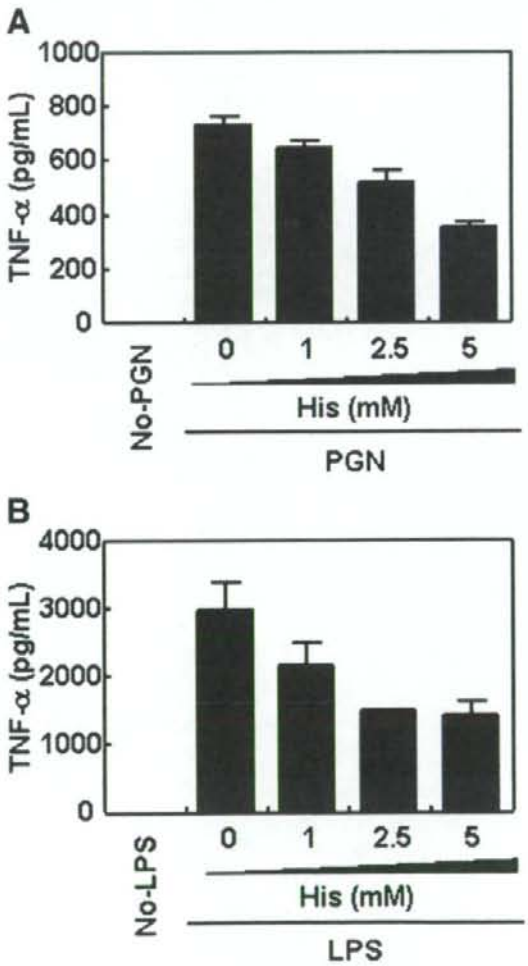
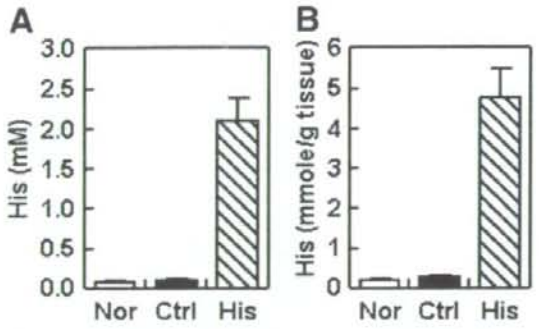


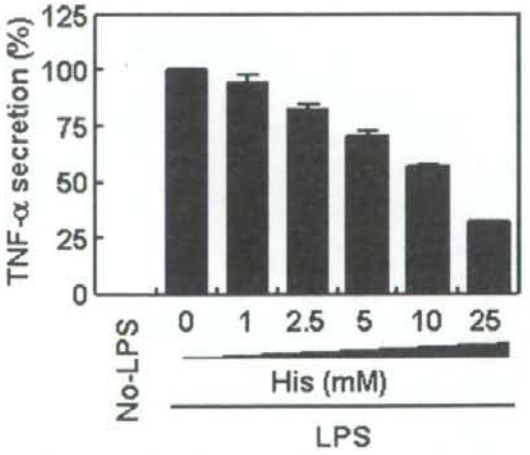
Supplementary Figure 1. The changes in the intracellular levels of histidine and histidine-related compounds. TG-PECs were incubated for 2 hours with 10 ng/mL LPS and 5 mmol/L L-histidine (*L-His*), 5 mmol/L D-histidine (*D-His*), or 5 mmol/L carnosine (*Car*). The intracellular levels of histidine (*A*) and carnosine (*B*) were determined to understand the transportation of histidine-related compounds into macrophages. The culture supernatants were analyzed for TNF-α (*C*). Data are expressed as the mean ± SE of 6 well samples, and the results are representative of 2 experiments with similar results.



Supplementary Figure 2. Histidine inhibits toll-like receptor (TLR) 2-ligand, peptideglycan-induced TNF-α secretion from macrophages. TG-PECs were incubated for 2 hours with histidine (*His*) and 10 μg/mL peptideglycan (*PGN*; *A*) or 10 ng/mL LPS (*B*). The culture supernatants were analyzed for TNF-α. Data are expressed as the mean ± SE of 3 well samples.



Supplementary Figure 3. Plasma and tissue concentration of histidine in the colitis model mice treated with histidine. A conventional powder chow (Std. diet) containing 5% histidine (*His*) was fed to mice with experimental colitis for 21 days (n = 8). For measurement of histidine concentration in plasma and tissue samples, we adopted precolumn derivatization to increase the ionization efficiency of the adducts before analysis by selected ion monitoring (SRM) mode reverse phase HPLC (Shimadzu Corporation, Kyoto, Japan) and triple-quadruple tandem mass spectrometry (Applied Biosystems). *Error bars* indicate SE.



Supplementary Figure 4. Histidine inhibits TNF-α secretion by human monocytes. Human monocytes were isolated from heparinized peripheral blood samples of healthy donors. Cells were cultured for 20 hours with LPS at a concentration of 10 ng/mL and histidine (*His*). Supernatants were collected and assessed for TNF-α secretion by ELISA. Data are expressed as the mean ± SE of 4 well samples, and the results are representative of 2 experiments with similar results.

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ORIGINAL ARTICLE

Enhanced Goblet Cell Hyperplasia in HDC Knockout Mice with Allergic Airway Inflammation

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ABSTRACT

Background: Histamine is known to have immunoregulatory roles in allergic reactions through histamine receptor 1 (H1R), H2R, H3R and H4R. However, its role in goblet cell hyperplasia in the airways of asthma patients is yet to be clarified.

Objective: This study was designed to examine the role of histamine in goblet cell hyperplasia using histamine-deficient mice (*Hdc-/-* mice) with allergic airway inflammation.

Methods: Wild-type and *Hdc-/-* C57BL/6 mice were sensitized with ovalbumin (OVA). After a 2-week exposure to OVA, goblet cell hyperplasia was evaluated. Cell differentials and cytokines in BALF were analyzed. The mRNA levels of MUC5AC and Gob-5 gene were determined quantitatively.

Results: The number of eosinophils in BALF increased in both the sensitized wild-type mice and *Hdc-/-* mice with OVA inhalation. In addition, the numbers of alveolar macrophages and lymphocytes in BALF increased significantly in the sensitized *Hdc-/-* mice with OVA inhalation compared to the wild-type mice under the same conditions. The concentrations of Interleukin-4 (IL-4), IL-5, IL-13, Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-2 in the BALF all increased significantly in both groups compared to those exposed to saline. In particular, the concentration of TNF-α in the *Hdc-/-* mice exposed to OVA was significantly higher than that in the wild-type mice under the same conditions. The mRNA levels of Gob-5 and MUC5AC, and the ratio of the goblet cells in the airway epithelium significantly increased in *Hdc-/-* mice exposed to OVA compared to wild-type mice.

Conclusions: These results suggested that histamine may play a regulatory role in goblet cell hyperplasia in allergic airway inflammation.

KEY WORDS

airway remodeling, asthma, Gob-5, MUC5AC, TNF-α

INTRODUCTION

Goblet cell hyperplasia and mucus overproduction are important features of bronchial asthma.[1,2] Although goblet cell hyperplasia with mucus hypersecretion has been reported to be associated with the development of airway hyperresponsiveness and the increase of severity and mortality in bronchial asthma[1-4], the mechanisms responsible for goblet cell hyperplasia are not completely understood.

Histamine is known to be a strong chemical mediator that stimulates secretion from goblet cells. Bryce *et al.* recently reported that the airway inflammatory response and goblet cell hyperplasia to allergic stimuli diminished in H1 receptor (H1R) knock-out mice, suggesting that histamine may be involved in the immunomodulation in airway allergic reaction including Th1 and Th2 cytokine production and goblet cell hy-

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perplasia.[5]

It has been reported that histamine can modulate T-cell-mediated immune responses.[6] Mast cells and basophils, which have been considered mainly to be effector cells for IgE-mediated reactions, can modulate the immune response through secreted histamine. Jutel *et al.* demonstrated, using H1R and histamine H2 receptor (H2R) knock-out mice, that histamine augmented TH1 response T lymphocytes through H1R and suppressed both TH1 and TH2 responses through H2R.[7] In addition, histamine H4 receptor (H4R) has recently been identified and an H4R inhibitor has been demonstrated to have a suppressive effect on allergic airway inflammation, including eosinophil and mast cell infiltration in murine asthma models.[8-11] In this context, histamine may have various roles in allergic airway inflammation through H1R, H2R and H4R in immune cells including T lymphocytes and dendritic cells.

To date, goblet cell hyperplasia has been thought to be induced after epithelial damage by numerous stimuli, such as endogenous oxidants induced by neutrophil elastase,[12] or exogenous oxidants produced by cigarette smoke[13] or diesel engine emissions.[14] On the other hand, it has been demonstrated that the TH2 lymphocyte-derived cytokines interleukin-4 (IL-4), IL-5, IL-9, and IL-13 induce goblet cell hyperplasia in animal models and *in vitro* studies.[15-19]

According to previous reports, several genes are up-regulated in association with goblet cell hyperplasia. Among them, MUC genes encode various mucin glycoproteins which are primary secretory proteins consisting of a family of genes (ie, MUC-1, MUC-2 and MUC4).[20,21] MUC5AC is the predominant MUC gene expressed in the airway.[21] It has been demonstrated that the level of MUC5AC gene expression increases in both animal models of asthma and tissue samples from patients with asthma.[22-24]

In addition, the overexpression of the Gob-5 gene has been reported in airway epithelia with exacerbated AHR, goblet cell hyperplasia, mucus overproduction, and eosinophil infiltration in a murine asthma model.[25] Gob-5 gene in the mouse corresponds to CLCA1 gene in humans, which regulates calcium activated chloride conductance. It has been shown to induce mucin gene expression (MUC5AC) in the human mucoepidermoid cell line NCI-H292 and its expression was demonstrated to increase in patients with asthma.[25-27]

Histamine-deficient mice are now available by disrupting *L*-histidine decarboxylase gene (HDC knockout mice: *Hdc*-/-).[28] Histamine exerts its various biological actions via H1, H2, H3 and H4. Due to the overlapping, and sometimes antagonistic function of the receptors in the presence of endogenous histamine, receptor blocking alone cannot achieve complete elimination of the histamine system. It is, therefore, reasonable to evaluate the total role of histamine with *Hdc*-/- mice.

An analysis of cytokine production and cell differentials in BALF in sensitized *Hdc*-/- mice with allergic airway inflammation has been previously reported, however, these investigated only the acute airway responses in murine asthma models with a short exposure to allergens.[29,30] There have been few data about airway remodeling that included goblet cell hyperplasia in histamine-deficient mice.

In this study, we designed an experimental asthma model with a relatively long duration of allergen exposure to *Hdc*-/- mice to elucidate the roles of histamine in airway remodeling, focusing especially on goblet cell hyperplasia.

METHODS

ANIMALS

HDC knockout (*Hdc*-/-) mice were generated by Ohtsu *et al.*[28]. In the present study, we used 6 to 8-week-old female *Hdc*-/- mice backcrossed for six generations with C57BL/6 mice, and used age/gender-matched C57BL/6N mice (Japan SLC, Shizuoka, Japan) as controls. The mice used for this study were generated by breeding homozygous *Hdc*-/- mice in the facilities of Tohoku University (Sendai, Japan). Both HDC-/- and wild-type mice were kept on a normal diet containing <0.3 mg of histamine/g of food. All experiments described in this study were performed according to the guidelines for the care and use of experimental animals as determined by the Japanese Association for Laboratory Animals Science in 1987.

IMMUNIZATION AND AEROSOLIZATION PROTOCOL

The mice were sensitized according to the methods described in a previous study.[31] In brief, mice were sensitized on days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 ml aluminum hydroxide-precipitated antigen containing 8 μg OVA (Sigma Chemical Co., St. Louis, MO) adsorbed overnight at 4℃ to 4 mg of aluminium hydroxide (Wako Chemical Co., Tokyo, Japan) in phosphate-buffered saline (PBS). Twelve days after the second immunization, the wild-type mice and *Hdc*-/- mice were divided into 3 groups respectively, each consisting of 6 animals. Each of the wild-type mice groups and one of the *Hdc*-/- mice groups were killed for analysis as controls without inhalation. To perform chronic exposure of OVA, 2 groups of each of the wild mice and *Hdc*-/- mice were placed in a plastic chamber (10 cm × 15 cm × 25 cm) and exposed to aerosolized OVA (5 mg/ml in 0.9% saline) for 1 hour every other day for 14 days. The other 2 groups of the wild-type mice and *Hdc*-/- mice were exposed only to 0.9% saline every other day for 14 days. The aerosolized OVA was produced by a Pulmo-Aide Compressor/Nebulizer

(Devilbiss) (Sunrise Medical HHG, Inc., Somerset, PA, USA) at a flow rate of 5–7 liter/minute.

COLLECTION AND MEASUREMENT OF SPECIMENS

After being exposed to aerosolized saline or OVA every other day for 2 weeks, each group of mice was killed on the 14th day, 24 hours after final inhalation, and bronchoalveolar lavage fluid (BALF) and lung tissues were collected. To collect BALF, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD, USA). The lungs were lavaged twice with 0.5 ml PBS, and up to 0.8 ml of the instilled fluid was consistently recovered. The recovered fluid was centrifuged (300 × *g* for 6 minutes) and the cells were resuspended in 0.5 ml PBS. The total number of cells was counted using an improved Neubauer hemocytometer chamber. An air-dried slide preparation was made from each sample containing 10,000 cells by cytospin (Cytocentrifuge, Sakura Seiki, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain. Differential counts of at least 500 cells were made according to standard morphologic criteria. The numbers of cells recovered per mouse were then expressed as the mean and standard error of the mean (SEM) for each treated group.

After centrifugation, supernatants were stored at –80℃ for the measurement of cytokines. After harvesting BALF, lungs were fixed with formaldehyde and were embedded in paraffin. These 3-μm-thick sections were stained with hematoxylin eosin (HE) and periodic acid-Schiff (PAS). In addition, other parts of the left lungs were stored at –80℃ for RNA extraction.

MEASUREMENT OF SERUM CONCENTRATION OF IgE

Serum IgE levels were determined using the commercially available ELISA kit (Yamasa, Chiba, Japan).

HISTOLOGICAL MEASUREMENT OF GOBLET CELL NUMBER

The 3 mm paraffin sections of the lungs described above were stained with PAS for evaluation of goblet cells. Goblet cell hyperplasia was determined by counting the number of PAS positive cells in more than 5 large (diameter >200 μm) and more than 10 small bronchi (diameter <150 μm) per lung under microscopy. The results were expressed as percentages of PAS positive cells per total epithelial cells. In addition, lung sections were stained with HE.

ISOLATION OF TOTAL RNA AND REAL-TIME QUANTITATIVE PCR

Total RNA from the whole lobes of the left lungs was obtained using ISOGEN (Wako Pure Chemicals, Osaka, Japan), quantified by spectrophotometry. The quality of the obtained RNA was confirmed by agarose gel electrophoresis.

To quantify the mRNA of Gob-5, MUC5AC, and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) expression in the murine lungs, quantitative PCR was carried out using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) as previously described.[32]

Oligonucleotide PCR primer pairs and fluorogenic probes for murine MUC5AC and Gob-5 were designed from the published sequences using Primer Express software (Perkin-Elmer) (sense primer: 5'-ACTGTTACTATGCGATGTGTAGCCA-3', antisense primer; 5'-GAGGAAACACATTGCACCGA-3'; Taqman probe: 5'-[FAM] ACTGCCACCTGTCACTGGGCGG [TAMRA]-3') for MUC5AC and (sense primer: 5'-AGGGCATCGTCATCGCC-3'; antisense primer: 5'-TCCTTTATGTGTTGAATGAGGGC-3'; Taqman probe: 5'-[FAM] AGACCACGACGTGCCGGAAGATG [TAMRA]-3') for Gob-5.[33] Primers and the labeled probe (VIC) for rodent GAPDH were purchased from Perkin-Elmer Applied Biosystems. 100 ng of RNA dissolved in 10 μl of water from each aliquot of murine lung tissue was denatured at 90℃ for 90 seconds. Each RNA sample (100 ng/10 μl of water) was mixed in 40 μl of buffer containing the following reagents for the one-step RT-PCR reaction: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 5 mM $MgCl_2$, 100 nM sense primer, 100 nM antisense primer, 0.3 mM deoxynucleoside triphosphate (Boehringer), 0.4 U/μl RNase inhibitor (Promega), 0.4 U/μl Moloney murine leukemia virus RT (Perkin Elmer), 0.0025 U/μl *Taq* Gold Polymerase (Perkin Elmer), and 100 nM Taqman probe, as described above. The fragment of mRNA for Gob-5, MUC5AC and GAPDH was reversely transcribed into cDNA (30 minutes at 48℃) and amplified by PCR for 40 cycles (15 seconds at 95℃ and 1 minute at 60℃). Whole reactions of the RT-PCR and detection of the fluorescence emission signal for every PCR cycle were performed at the same time in a single tube in a sequence detector (ABI 7700). The minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold (C), which is predictive of the quantity of an input target fragment.[34] The standard curve was obtained between the fluorescence emission signals and C by means of duplicated serial dilutions of each quantified cDNA fragment which contained the targeted sequences. The expression of MUC5AC and Gob-5 mRNA was normalized to the constitutive expression of GAPDH mRNA.

CYTOKINE ASSAY

Concentrations of IL-4, IL-5, Interferon-g (IFN-g), tumor necrosis factor-a (TNF-α) and IL-2 in BALF were determined with cytokine Bead Array inflammatory kits using flow cytometry according to the manufacturer's instructions (BD PharMingen, San Diego, CA,

Table 1 Total cell number and cell differential in BALF of the wild-type and *Hdc*-/- mice after saline and OVA inhalation

	C57BL/6: wild saline, 14th day	C57BL/6: wild OVA, 14th day	C57BL/6: *Hdc*-/- saline, 14th day	C57BL/6: *Hdc*-/- OVA, 14th day
Total cells ($\times 10^4$/ml)	2.29 ± 0.64 (n = 6)	28.2 ± 5.1 (n = 6)	3.60 ± 0.21 (n = 6)	62.1 ± 7.0* (n = 6)
Alveolar macrophages	2.16 ± 0.06 (94.2 ± 2.5%)	2.09 ± 0.56 (7.40 ± 2.0%)	3.43 ± 0.11 (95.2 ± 3.0%)	23.5 ± 3.73* (37.9 ± 6.0%)
Lymphocytes	0.13 ± 0.06 (5.75 ± 2.5%)	0.45 ± 0.25 (1.6 ± 0.9%)	0.15 ± 0.09 (4.20 ± 2.6%)	10.8 ± 4.04* (17.4 ± 6.5%)
Eosinophils	< 0.001 (< 0.1%)	25.7 ± 0.50 (91.0 ± 1.8%)	0.22 ± 0.01 (0.6 ± 0.4%)	27.8 ± 6.40 (44.8 ± 10.3%)
Neutrophils	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)
Epithelial cells	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)	< 0.01 (< 0.1%)

The absolute number of cells in BALF on 14th day after saline and OVA inhalation in mice sensitized with OVA was calculated. The numbers in parentheses indicated % in the total cells in BALF. BALF was collected as described in "Methods". Values were expressed as the means ± SEM of 6 mice. * $p < 0.01$ relative to the respective value of wild-type mice with OVA inhalation.

USA). The concentration of IL-13 in BALF was measured using ELISA (RayBio, Norcross, GA, USA).

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM. Multiple comparisons of mean data among the groups were analyzed by the Mann-Whitney U test. Probability values of less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

CELL DIFFERENTIALS IN BALF OF *Hdc*-/- MICE

Repetitive exposure to OVA for 2 weeks induced a marked increase of the total cell numbers in BALF in both wild-type and *Hdc*-/- mice sensitized with OVA, compared with those from the wild-type mice group and mice sensitized with OVA and exposed to saline (Table 1). The total cell numbers in BALF from the *Hdc*-/- mice sensitized with OVA and then exposed to OVA increased significantly more than in wild-type mice under the same conditions. The absolute numbers of alveolar macrophages, lymphocytes and eosinophils in *Hdc*-/- mice sensitized with and exposed to OVA increased significantly compared to mice sensitized with OVA and exposed to saline. In particular, the absolute numbers of alveolar macrophages and lymphocytes in *Hdc*-/- mice sensitized with and exposed to OVA were significantly higher than those of wild-type mice under the same condition. Eosinophils were the predominant cells in BALF from the wild-type mice exposed to OVA and the ratio of eosinophils in BALF was higher compared with that of *Hdc*-/- mice. However, there was no significant difference in the absolute cell numbers of eosinophils in these 2 groups.

CYTOKINES IN BALF OF *Hdc*-/- MICE

TNF-α, IL-4, IL-5, IL-13, INF-γ and IL-2 in BALF were measured. The concentrations of TNF-a in BALF of both wild (15.08 ± 0.58 pg/ml: mean ± SEM) and *Hdc*-/- mice (25.30 ± 4.23 pg/ml) exposed to OVA increased markedly compared to those of wild-type (2.28 ± 0.40 pg/ml) and *Hdc*-/- mice (2.27 ± 0.40 pg/ml) exposed to saline (Fig. 1A). The concentration of TNF-α in the *Hdc*-/- mice exposed to OVA was significantly higher than that of the wild-type mice under the same conditions.

The concentrations of IL-4 in BALF from both wild-type and *Hdc*-/- mice sensitized with and exposed to OVA increased significantly compared to wild and *Hdc*-/- mice sensitized with OVA and exposed to saline. The concentration of IL-4 in the *Hdc*-/- mice exposed to OVA was significantly lower than that of wild mice under the same conditions (Fig. 1B).

The concentrations of IL-2, IL-5, IL-13 and INF-γ in BALF from both wild-type and *Hdc*-/- mice sensitized with and exposed to OVA increased significantly compared with that of wild-type and *Hdc*-/- mice sensitized with OVA and exposed to saline. No statistically significant difference in these cytokines between the wild-type and *Hdc*-/- mice exposed to OVA (Fig. 1C, D, E, F).

SERUM IgE OF *Hdc*-/- MICE

The concentrations of serum IgE in both wild-type (2133.6 ± 370.3 ng/ml) and *Hdc*-/- mice (998.9 ± 254.0 ng/ml) exposed to OVA were markedly higher than those exposed to saline (wild-type exposed to saline: 416.1 ± 25.1 ng/ml, *Hdc*-/- mice exposed to saline: 371.7 ± 17.2 ng/ml) and without exposure (wild-type: 390.1 ± 32.5; *Hdc*-/-: 328.1 ± 14.8) (Fig. 2). The concentration of serum IgE in the *Hdc*-/- mice exposed to OVA was significantly lower than that of the wild-type mice under the same conditions.

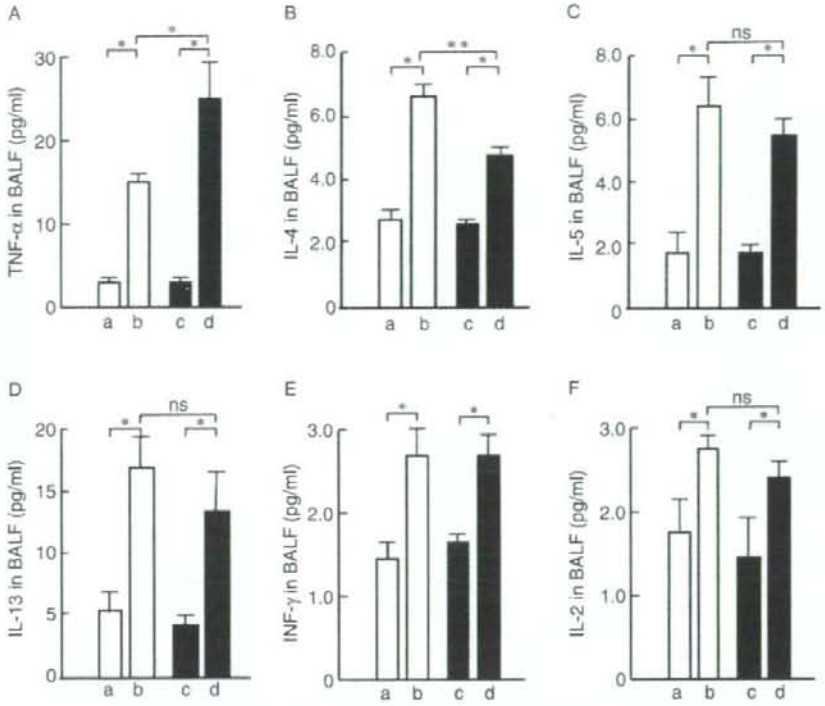

Fig. 1 **A, B, C, D, E, F**: Outlined column: wild-type mice; Solid column: *Hdc-/-* mice; Column **a** and **c**: saline inhalation; Column **b** and **d**: OVA inhalation, **A**: TNF-α, **B**: IL-4, **C**: IL-5, **D**: IL-13, **E**: INF-γ, **F**: IL-2 concentration in BALF. $*p < 0.01$, $**p < 0.05$.

Gob-5 GENE EXPRESSION IN THE LUNG OF *Hdc-/-* MICE

Levels of Gob-5 mRNA in lung tissue of both wild-type and *Hdc-/-* mice sensitized with and exposed to OVA increased significantly compared with those of wild-type and *Hdc-/-* mice sensitized with OVA and exposed to saline. The level of Gob-5 mRNA in the *Hdc-/-* mice exposed to OVA was significantly higher than that of wild-type mice under the same conditions (Fig. 3).

MUC5AC GENE EXPRESSION IN THE LUNG OF *Hdc-/-* MICE

Levels of MUC5AC mRNA in lung tissue of both wild-type and *Hdc-/-* mice sensitized with OVA and exposed to OVA increased markedly compared with those of wild and *Hdc-/-* mice sensitized with OVA exposed to saline. The level of MUC5AC mRNA in the *Hdc-/-* mice exposed to OVA was significantly higher than that of wild-type mice under the same conditions (Fig. 4).

GOBLET CELLS OF LARGE AND SMALL AIRWAYS

PAS staining demonstrated an increase in the number of goblet cells in the epithelium in large and small airways of both wild-type and *Hdc-/-* mice sensitized with OVA (Fig. 5A a–f, B a–f). Goblet cell hyperplasia was more prominent in the *Hdc-/-* mice exposed to OVA than in the wild-type mice under the same conditions (Fig. 5A c, A f, B c, B f). Repetitive saline inhalation induced a mild increase in the number of goblet cells in large and small airways of both wild-type and *Hdc-/-* mice (Fig. 5A b, A e, B b, B e).

QUANTIFICATION OF GOBLET CELLS IN LARGE AND SMALL AIRWAYS

The ratios of goblet cells in the epithelium in large and small airways, of both wild-type and *Hdc-/-* mice sensitized with and exposed to OVA, increased markedly compared with those of the wild-type and *Hdc-/-* mice sensitized with OVA and exposed to saline (Fig. 6A, B). The ratios of goblet cells in the epithelium in large and small airways of the *Hdc-/-* mice exposed to OVA were significantly higher than that of wild-type mice under the same condition.

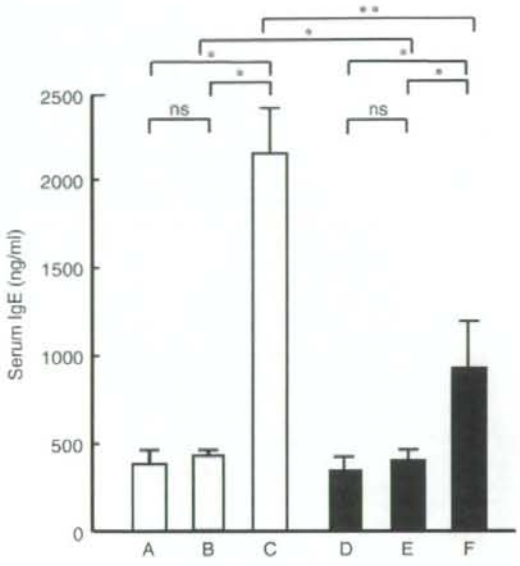

Fig. 2 IgE concentration in serum. Outlined column: wild-type mice; Solid column: *Hdc*-/- mice; Column **A** and **D**: before inhalation; Column **B** and **E**: saline inhalation; Column **C** and **F**: OVA inhalation. $*p < 0.01$, $**p < 0.05$, ns: not significant.

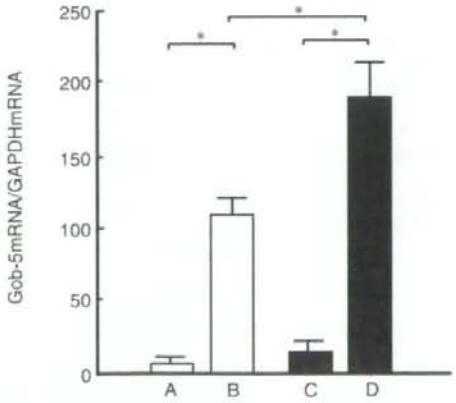

Fig. 3 Gob-5 gene expression in the lung. Outlined column: wild-type mice; Solid column: *Hdc*-/- mice; Column **A** and **C**: saline inhalation; Column **B** and **D**: OVA inhalation. $*p < 0.01$.

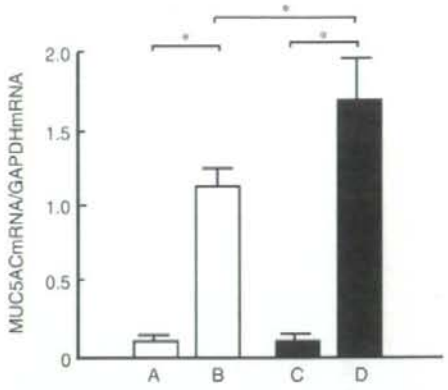

Fig. 4 MUC5AC gene expression in the lung. Outlined column: wild-type mice; Solid column: *Hdc*-/- mice; Column **A** and **C**: saline inhalation; Column **B** and **D**: OVA inhalation. $*p < 0.01$.

DISCUSSION

In this study, we demonstrated that goblet cell hyperplasia was enhanced in *Hdc*-/- mice sensitized with OVA after repetitive OVA exposure for 14 days. We also demonstrated a striking difference in differential cytological features in BALF between 2 murine asthma models with *Hdc*-/- mice, representing the acute allergic airway response with a short period of OVA exposure previously reported[29,30] and a relatively chronic allergic airway response shown in the present study. We found significant increases of alveolar macrophages and lymphocytes in BALF of *Hdc*-/- mice with repetitive exposure to OVA for 2 weeks compared with the wild-type mice under the same conditions. The concentration of TNF-a in BALF in the *Hdc*-/- mice after repetitive OVA exposure was significantly higher than that of the wild-type mice under the same conditions.

Histamine has been recognized as a chemical mediator playing a central role in allergic reactions including mucosal edema, mucous gland secretion, smooth muscle contraction, etc. Recent studies have demonstrated that histamine plays a critical role in immunomodulation by acting on different types of histamine receptors such as H1R, H2R and H4R on the surface of immune cells and inflammatory cells.[6-11] Using mutant mice lacking H1R and H2R, Jutel *et al.* demonstrated that histamine enhances TH1-type responses by triggering H1R, whereas both TH1- and TH2-type responses were negatively regulated by H2R. Indeed, the deletion of H1R resulted in suppression of IFN-γ, IL-4 and IL-13, and the deletion of H2R showed an up-regulation of both TH1 and TH2 cytokines (IL-4 and IL-13).[7] Bryce *et al.* recently demonstrated the interesting opposite effects of H1R inhibition on cultured T cells *in vitro* and the airway response with allergic inflammation *in vivo* using a murine asthma model.[5] In their report, allergen-stimulated splenic T cells from sensitized H1R knock-

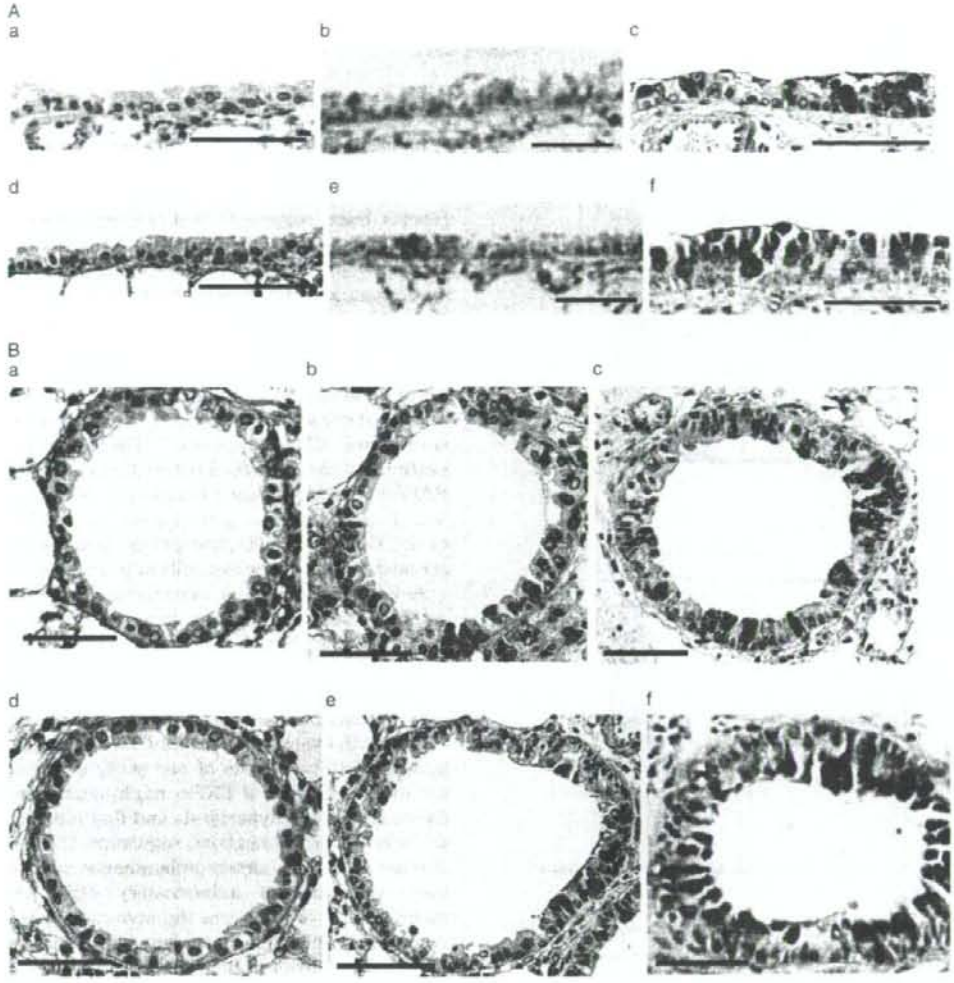

Fig. 5 **A**: Goblet cells in the large airways, **B**: Goblet cells in the small airways. The goblet cells in the epithelium were stained with PAS. **a**, **b** and **c**: wild-type mice; **d**, **e** and **f**: *Hdc*-/- mice; **a** and **d**: before inhalation; **b** and **e**: saline inhalation; **c** and **f**: OVA inhalation. black bars indicate 50μm.

out mice exhibited enhanced TH2 cytokine production. However, in contrast, allergen-challenged H1R knockout mice exhibited diminished lung TH2 cytokine mRNA levels, airway inflammation, goblet cell metaplasia, and airway hyperresponsiveness (AHR).

The present study demonstrated that the concentration of IL-4 in BALF was significantly lower in the *Hdc*-/- mice than in the wild-type mice after exposure to OVA, which was consistent with the experiment of the *Hdc*-/- mice by Kozma *et al.*,[30] despite differences in the duration of OVA exposure. The concentrations of IL-2, IL-5, IL-13 and INF-γ showed no significant differences between the *Hdc*-/- mice and the wild-type mice. The lower level of IL-4 in the *Hdc*-/- mice was consistent with the lower level of serum IgE in the *Hdc*-/- mice after the exposure to OVA. These changes may be explained at least in part by the suppressed recruitment of activated TH2 cells into the lung due to the blockade of H1R in T lymphocytes, as suggested by Bryce *et al.*[5]

H1R, H2R and H4R are expressed in monocytes, macrophages and dendritic cells, and histamine plays a regulatory role in expression of cytokines and adhesion molecules in these cells.[35] Mazzoni *et al.* demonstrated that histamine inhibited INF-a and released TNF-a from activated plasmacytoid dendritic cells

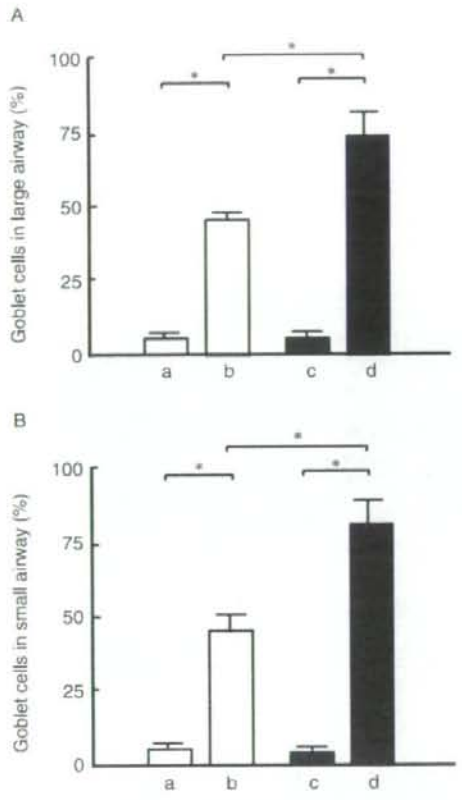

Fig. 6 **A**: Goblet cell ratio in the large airways, **B**: Goblet cell ratio in the small airways. Outlined column: wild-type mice; Solid column: *Hdc*-/- mice; Column **a** and **c**: saline inhalation, Column **b** and **d**: OVA inhalation. $*p < 0.01$.

through H2R.[36] Histamine also inhibited lipopolysaccharide-induced TNF-α production by down-regulating intercellular adhesion molecules (ICAM)-1 in human monocytes through H2R.[37] Alveolar macrophages are known to be a major source of TNF-α in the lung, and the increased production of TNF-α by alveolar macrophages in patients with asthma has been reported.[38,39] The present study demonstrated an increase in the TNF-α concentration in BALF in the *Hdc*-/- mice exposed to OVA compared to the wild-type mice under the same condition. This result suggested that histamine may play a role in suppressing TNF-α production through H2R in allergic airway inflammation in mice.

Busse *et al.* have recently demonstrated that chronic exposure of TNF-α to the airway induced goblet cell hyperplasia.[40] Taking these findings into consideration, we speculate that the depletion of histamine in the *Hdc*-/- mice induced the up-regulation of TNF-α production, resulting in the increase of goblet cells in the airways with allergic inflammation.

In association with the increase of goblet cells, higher levels of Gob-5 and MUC5ac mRNA were found in the *Hdc*-/- mice exposed to OVA. Previous reports have suggested that the expression of the Gob-5 gene, which corresponds to hCLCA1 in human beings, would be one of the first steps in mucus cell metaplasia and hyperplasia by inducing mucin gene expression.[25] MUC5ac gene, a member of the mucin gene family, has been shown to be expressed exclusively in the airway and associated with the hyperplasia of goblet cells. In addition to IL-4, IL-5 and IL-13, TNF-α was reported to increase the expression of the Gob-5 and MUC5ac genes.[41] These findings suggested that the increased production of TNF-α in the BALF of the *Hdc*-/- mice exposed to OVA may be, at least in part, involved in the increase of mRNA levels of the Gob-5 and MUC5ac genes, leading to the increased number of goblet cells in the airway.

Antigen challenge of sensitized mice or human asthmatic subjects results in increased TNF-α expression in BALF, peripheral blood, and tissue biopsy specimens.[42-46] TNF-α production is partly regulated by histamine via H2R in macrophages and dendritic cells, as described above. Although we had no direct evidence to evaluate the role of TNF-α in goblet cell hyperplasia, the results of our study suggested that the increased level of TNF-α might contribute to enhance goblet cell hyperplasia and that histamine may be involved in the negative regulation of TNF-α production in allergic airway inflammation *via* the histamine receptors of inflammatory cells, including macrophages. To confirm the above, further studies are needed, such as experiments using mice in which the TNF-α activity is blocked with antibody or gene disruption.

There was a discrepancy between the TNF-α levels and the macrophage counts. The concentration of TNF-α in the knock-out mice was twice that in the wild-type mice, and the number of alveolar macrophages in *Hdc*-/- mice was ten times that in the wild-type mice. We have no data to explain this, however, to answer this question, it would be necessary to evaluate the capacity of TNF-α production by alveolar macrophages in *Hdc*-/- mice and wild mice. It would also be necessary to evaluate the time course of the concentration of TNF-α and the number of alveolar macrophages to understand their relationship.

We demonstrated the increased number of lymphocytes in BALF of *Hdc*-/- mice. It has been reported by Jutel *et al.* that histamine stimulated the Th1 response through the H1 receptor. On the other hand, the activities of both Th1 and Th2 lymphocytes were inhibited by histamine through the H2 receptor.[7] Elimination of the inhibitory actions on lymphocytes by histamine through the H2 receptor in *Hdc*-/- mice may contribute to the increased number of lymphocytes in BALF.

The number of lymphocytes in the BALF of *Hdc-/-* mice was twenty times that of wild-type mice, although no significant difference in the concentration of IL-2 in the BALF was found between knock-out and wild-type mice. The number of cells and cytokine levels in BALF are not always concordant in the time courses of *in vivo* experiments. The level of IL-2 in BALF peaked within 1 week during exposure to OVA (unpublished data). However, in this study we examined the cytokine levels and the cell differentials in BALF only on the 14th day. To explain the discrepancy between the IL-2 level and the number of lymphocytes in BALF in the study, it would have been helpful to evaluate the time course of IL-2 concentration and the number of lymphocytes in BALF.

Although we designed a 2- week exposure program to evaluate the extent of goblet cell hyperplasia in the *Hdc-/-* mice, we did not examine airway hyperresponsiveness (AHR). However, we found no difference in AHR between the *Hdc-/-* and the wild mice in our preliminary study as was reported in the previous acute model (unpublished data).[29]

In the present study, we did not measure the anti-OVA specific IgE in serum. Although the levels of serum OVA-specific IgE and total IgE are not always consistent, the procedures for sensitization with OVA and exposure to OVA were directly associated with the increase of IgE in serum. Despite the fact that the levels of serum OVA-specific IgE were different from the level of total IgE in serum, the level of total IgE in serum reflected strongly the levels of serum OVA-specific IgE in our study.

Anti-histamine drugs available for therapy consist of H1 and H2 blockers. H1 blockers have not been recommended for therapy for asthma, but their anti-allergic actions have been recognized. According to the results of this study, the H2 blocker may have the possibility of stimulating goblet cell hyperplasia in patients with allergic asthma. It will be necessary to evaluate the effects of H2 blockers in a clinical study in the future.

In conclusion, we demonstrated enhanced goblet cell hyperplasia in the airway of OVA sensitized histamine-deficient mice. The results of our study suggested that histamine may play a significant role in goblet cell hyperplasia in the airway with allergic inflammation.

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