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Decreased expression of uteroglobin-related protein 1 in inflamed mouse airways is mediated by IL-9

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Chiba, Yoshihiko, Takashi Kusakabe, and Shioko Kimura. Decreased expression of uteroglobin-related protein 1 in inflamed mouse airways is mediated by IL-9. *Am J Physiol Lung Cell Mol Physiol* 287: L1193–L1198, 2004; doi:10.1152/ajplung.00263.2004.—Uteroglobin-related protein 1 (UGRP1) is a secretory protein, highly expressed in epithelial cells of airways. Although an involvement of UGRP1 in the pathogenesis of asthma has been suggested, its function in airways remains unclear. In the present study, a relationship between airway inflammation, UGRP1 expression, and interleukin-9 (IL-9), an asthma candidate gene, was evaluated by using a murine model of allergic bronchial asthma. A severe airway inflammation accompanied by airway eosinophilia and elevation of IL-9 in bronchoalveolar lavage (BAL) fluids was observed after ovalbumin (OVA) challenge to OVA-sensitized mice. In this animal model of airway inflammation, lung *Ugrp1* mRNA expression was greatly decreased compared with control mice. A significant inverse correlation between lung *Ugrp1* mRNA levels and IL-9 levels in BAL fluid was demonstrated by regression analysis ($r = 0.616$, $P = 0.023$). Immunohistochemical analysis revealed a distinct localization of UGRP1 in airway epithelial cells of control mice, whereas UGRP1 staining was patchy and faint in inflamed airways. Intranasal administration of IL-9 to naive mice decreased the level of *Ugrp1* expression in lungs. These findings suggest that UGRP1 is downregulated in inflamed airways, such as allergic asthmatics, and IL-9 might be an important mediator for modulating UGRP1 expression.

uteroglobin-related protein 1; interleukin-9; airway inflammation; allergic asthma

ALLERGIC BRONCHIAL ASTHMA is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness (11). The response to antigen in the airways involves infiltration of various types of inflammatory cells, such as eosinophils and T-helper 2 (Th2) subset of T lymphocytes (3, 4, 14). Although the exact mechanism(s) of the response has not been determined, there is increasing evidence that complex interaction among these cells leads to the production of inflammatory mediators, including cytokines and chemokines, which play a role in the pathogenesis of asthma (1, 4, 21).

Genetic mapping studies have demonstrated that one or more asthma susceptibility genes are located on chromosome 5q31-q33 in humans (8, 20). This chromosomal region contains a cytokine gene cluster that may potentially play a role in airway inflammation associated with atopic asthma. Interleukin (IL)-9, a Th2-type cytokine, is one of these cytokines and has been suggested as a candidate gene for asthma (10, 17). Studies with IL-9 transgenic mice have shown that overexpression of IL-9 in lungs results in a phenotype characteristic to human asthmatics that includes elevated IgE levels, mucus hypersecretion, airway eosinophilia, and

bronchial hyperresponsiveness (9, 24). Intratracheal administration of recombinant IL-9 in naive mice also causes airway eosinophilia (12). Moreover, treatment with anti-IL-9 antibody inhibits allergen-induced airway inflammation and hyperresponsiveness in mice (5). These findings suggest that IL-9 is an important mediator in the pathogenesis of allergic bronchial asthma. In fact, an increased expression of IL-9 has been reported in airways of atopic asthmatics (22).

Uteroglobin-related protein 1 (UGRP1) is a secretory protein of ~10 kDa that is highly expressed in epithelial cells of the trachea, bronchus, and bronchioles (18). The functional roles of UGRP1 in airway physiology are not understood. However, the following three lines of evidence suggest a possible involvement of UGRP1 in the pathogenesis of asthma: 1) high levels of expression in lungs, 2) amino acid sequence similarities to uteroglobin (also called Clara cell secretory protein; CCSP), which is known as a regulator of airway inflammation (16, 23), and 3) chromosomal location of the human *UGRP1* gene at 5q31-q32 (19), the region that has been assigned as one of asthma susceptibility loci (20). In addition, reduced expression of *Ugrp1* in airway inflammation induced by *Aspergillus fumigatus* antigen was demonstrated in mice (18). In this study, we show a possible involvement of IL-9 in the decreased airway UGRP1 expression in allergic airway inflammation in mice.

MATERIALS AND METHODS

Animals. 129Sv female mice were housed in a controlled environment with a 12:12-h light-dark cycle and had free access to food and water. Experiments were performed according to the Using Animals in Intramural Research Guidelines (National Institutes of Health Animal Research Advisory Committee, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee.

Ovalbumin antigen sensitization and challenge. Mice (6 wk old) were sensitized by an intraperitoneal injection of 10 μ g ovalbumin (OVA; Sigma Chemical, St. Louis, MO) adsorbed to 2.25 mg alum (ImjectAlum; Pierce, Rockford, IL). A booster injection of the same dose of alum-adsorbed OVA was given 5 days later. After the first immunization (12 days), mice were exposed for 30 min to 5 mg/ml OVA in saline (OVA challenge) aerosolized by a jet nebulizer (PARI LC Plus; PARI Respiratory Equipment, Monterey, CA) driven by an air compressor (PRONEB Ultra; PARI Respiratory Equipment) under conscious state in a Plexiglas chamber (220 $\times$ 230 mm, height: 140 mm). The "sensitized control" animals received the same immunization but inhaled saline only instead of OVA-saline.

Intranasal administration of IL-9. Mice (7 wk old) were anesthetized with 2.5% Avertin and allowed to breathe spontaneously. Sterile PBS or various amounts of recombinant mouse IL-9 (R&D Systems, Minneapolis, MN) were intranasally instilled in the trachea of each animal. After 24 h, total RNAs were prepared from whole lungs.

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Bronchoalveolar lavage. After antigen challenge (24 h), mice were killed, and blood was collected for measuring IgE and IgG levels in serum. The chest of each animal was opened, and a 20-gauge blunt needle was tied in the proximal trachea. Bronchoalveolar lavage (BAL) fluid was obtained by intratracheal instillation of 1 ml PBS (pH 7.5, room temperature) in the lung while it was kept located within the thoracic cavity. The lavage was reinfused in the lung two times before final collection. BAL cells were isolated by centrifugation at 500 *g* and resuspended in 500 μ l PBS. The resultant supernatant was stored at -80°C for cytokine analysis. A 100- μ l aliquot of BAL cell suspension was mixed in 100 μ l 0.4% trypan blue (GIBCO-BRL, Grand Island, NY), and cells were counted with a hemocytometer. To determine inflammatory cell types in BAL fluid, BAL cells were fixed by incubation with 10% formaldehyde for 10 min, washed by PBS, and resuspended in 400 μ l PBS. A 10- μ l aliquot of cell suspension was then mounted on slides and air-dried. Cell types were identified and counted by differential staining microscopy with Diff-Quik (Baxter Healthcare, Miami, FL) under $\times 400$ magnification. Eosinophils were defined as cells that had positive staining with typical morphology for eosinophils. Inflammatory cell populations were determined by first randomly counting 100 cells in duplicate and by multiplying total cell counts per milliliter BAL fluid by the percentages of each cell type.

RNA extraction and Northern blot analysis. Airway tissues below the main bronchi were removed immediately after BAL, quick-frozen in liquid nitrogen, and crushed to powders by using a mortar, and total RNA was isolated from each frozen sample with a one-step guanidium-phenol-chloroform extraction procedure using Tri-Zol Reagent (Invitrogen Life Technologies, Carlsbad, CA).

RNA (3.5 μ g/lane) was subjected to electrophoresis on a 1% agarose gel containing 0.7% formaldehyde and transferred to a GeneScreen Plus hybridization transfer membrane (NEN Life Science Products, Boston, MA). Membranes were prehybridized and hybridized in PerfectHyb Plus hybridization buffer (H-7033; Sigma) with mouse UGRP1 full-length cDNA as a probe that was labeled with [α - ^{32}P]dCTP (specific activity 3,000 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) using Ready-To-Go DNA labeling beads and ProbeQuant G-50 microcolumns (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was performed at 68°C for 14 h. Membranes were washed five times with $0.5\times$ saline-sodium citrate (SSC) containing 0.1% SDS at 68°C for 15 min before exposure to a PhosphorImager screen. Membranes were rehybridized with a full-length mouse β -actin or GAPDH cDNA probe as a normalization control after removing the UGRP1 probe by boiling in $0.1\times$ SSC containing 0.5% SDS two times. Expression signals were analyzed using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and image analysis was carried out using densitometry (Atto Densitograph Software for the Macintosh version 4.0; Atto). The ratio corresponding to *Ugrp1*/ β -actin or *Ugrp1*/GAPDH in each lane was calculated as an index for *Ugrp1* mRNA level.

Quantitation of IL-9 level in BAL fluid. IL-9 concentration was assessed in BAL fluids by ELISA. In brief, 96-well plates were coated overnight with monoclonal rat anti-mouse IL-9 antibody (clone no. D8402E8; 5 μ g/ml in 0.1 M Na_2HPO_4 , pH 9.0; BD Biosciences Pharmingen, San Diego, CA) at 4°C . Excess free antibodies were washed out with PBS-Tween (0.05% Tween 20 in PBS, pH 7.0) five times, and the wells were incubated with 200 μ l filtered blocking buffer (1% BSA in PBS) at room temperature for 2 h to block nonspecific protein binding sites. The plates were then washed three times with PBS-Tween, and 50 μ l of samples or standards (recombinant mouse IL-9; BD Biosciences Pharmingen) diluted in blocking buffer containing 0.05% Tween 20 were added to duplicate wells. Plates were incubated overnight at 4°C and washed five times with PBS-Tween. Biotinylated hamster anti-mouse IL-9 monoclonal antibody (clone no. D9302C12; 2 μ g/ml in blocking buffer containing 0.05% Tween 20; BD Biosciences Pharmingen) was then added (100 μ l/well) as a detection antibody and incubated at room temperature for 1 h. After five washes, the plates were incubated with streptavidin-horseradish peroxidase conjugate (1:1,000 dilution in blocking buffer

containing 0.05% Tween 20; BD Biosciences Pharmingen) at room temperature for 30 min, followed by five washes. Thereafter, 100 μ l/well of liquid substrate (product no. A3219; Sigma) were added and incubated at room temperature for 30 min under dark. An equal volume of stop solution (1% SDS) was finally added to stop the reaction, and the amount of substrate converted to product was detected as optical density at 405 nm. The limit of detection in this assay was <15.6 pg/ml.

Quantitation of IgE and IgG levels in serum. Serum IgE and IgG levels were measured by an ELISA system (Bethyl Laboratories) according to the manufacturer's instructions.

Immunohistochemistry. Airways below the main lung bronchi were fixed in 10% formaldehyde and embedded in Paraplast Plus paraffin. Sections (5 μ m) were obtained from blocks and mounted on silane-coated glass slides, deparaffinized with xylene and graded ethanol, and processed for immunohistochemistry. Immunostaining was performed with a rabbit polyclonal antibody against mouse UGRP1 as the first antibody by using a streptavidin-biotin immunoperoxidase method, as described previously (6) with minor modification. In brief, before immunostaining, rehydrated sections were pretreated by incubation in 0.5% Triton X-100-PBS for 10 min for permeabilization and were immersed in 0.3% hydrogen peroxide in 100% methanol for 30 min to remove endogenous peroxidase activity. The pretreated sections were washed with PBS and incubated in 5% skim milk in PBS for 1 h. The sections were then rinsed and incubated sequentially at room temperature in the following solutions: 1) first antibody (diluted 1:500 in PBS containing 1% skim milk) for 1 h, 2) biotinylated goat anti-rabbit IgG (diluted 1:50 in PBS containing 1% skim milk) for 30 min, and 3) avidin-biotinylated peroxidase complexes (Vector Laboratory, Burlingame, CA) in PBS for 30 min. The bound peroxidase activity was visualized by incubation with 0.7 mg/ml 3,3'-diaminobenzidine-0.02% H_2O_2 in 60 mM Tris buffer (pH 7.5). Sections were rinsed in PBS after each step of the immunostaining procedure. Finally, the sections were counterstained with Hematoxylin QS (Vector Laboratories), dehydrated, and mounted in permanent mounting medium.

The anti-mouse UGRP1 antibody used in the present study was prepared as previously described (18).

Statistical analyses. All data were expressed as means $\pm$ SE. Statistical significance of difference was determined by Dunnett's multiple analysis. A value of $P < 0.05$ was considered significant. Correlation between the levels of *Ugrp1* mRNA expression in airways and IL-9 in BAL fluids was analyzed by Fisher's regression analysis.

RESULTS

Airway inflammation after OVA challenge. Both sensitized control and antigen-challenged mice had high levels of serum IgE (516 ± 113 ng/ml, $n = 6$, and 539 ± 103 ng/ml, $n = 7$, respectively) and IgG ($1,490 \pm 8.3$ ng/ml, $n = 6$, and $1,483 \pm 14$ ng/ml, $n = 7$, respectively) without significant differences between groups. Total cell numbers in BAL fluids were significantly increased 24 h after OVA challenge ($68.7 \pm 16.8 \times 10^4/\text{ml}$, $n = 7$) compared with saline inhalation ($1.1 \pm 0.05 \times 10^4/\text{ml}$, $n = 6$; $P < 0.01$). The increase in total cells was associated with eosinophils, lymphocytes, and monocytes/macrophages (Fig. 1). Histological examination by staining lung sections with hematoxylin and eosin also demonstrated a marked infiltration of inflammatory cells in lung tissues of OVA-challenged mice (Fig. 2). Most of the infiltrated cells were eosinophils, as determined by Diff-Quik staining of lung sections (data not shown).

Change in airway UGRP1 expression after OVA challenge. Both Northern blot (Fig. 3) and immunohistochemical (Fig. 4) analyses demonstrated constitutive expression of UGRP1 in mouse airways. Hybridization of total RNA from mouse airways

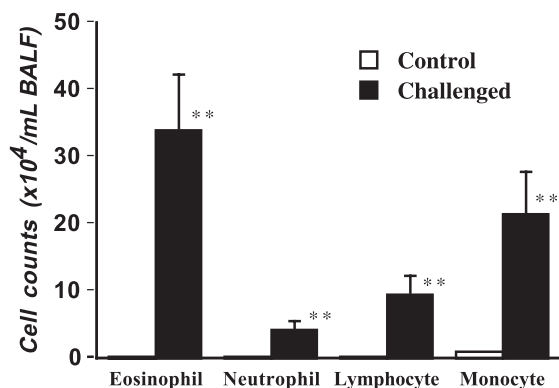


Fig. 1. Effects of ovalbumin (OVA) inhalation on cell counts in bronchoalveolar lavage fluid (BALF) in OVA-sensitized mice. The number of inflammatory cells in BALF was determined with Diff-Quik staining 24 h after saline (Control) or OVA (Challenge) inhalation. Each column represents the mean $\pm$ SE from 6 (Control) and 7 (Challenged) animals. ** $P < 0.01$ vs. sensitized control group.

with full-length mouse UGRP1 cDNA probe revealed a single 0.5-kb band, corresponding to *Ugrp1* mRNA (Fig. 3A). The expression level of *Ugrp1* mRNA in airways of OVA-challenged mice was significantly lower than that of the sensitized control group (Fig. 3B). Immunohistochemical staining of lung sections with anti-mouse UGRP1 antibodies revealed a distinct localization of UGRP1 in airway epithelial cells of sensitized control mice (Fig. 4, top), whereas UGRP1-positive staining was patchy and faint in airways of OVA-challenged mice (Fig. 4, bottom), indicating that downregulation of UGRP1 had occurred in antigen-induced airway inflammation. Neither *Ugrp1* mRNA expression nor UGRP1 immunostaining was affected by the sensitization procedure alone (data not shown).

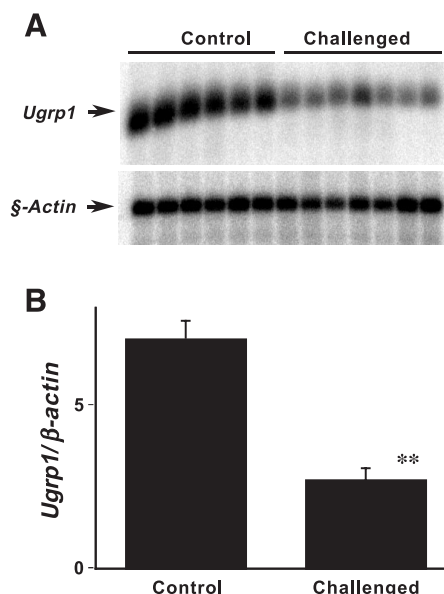
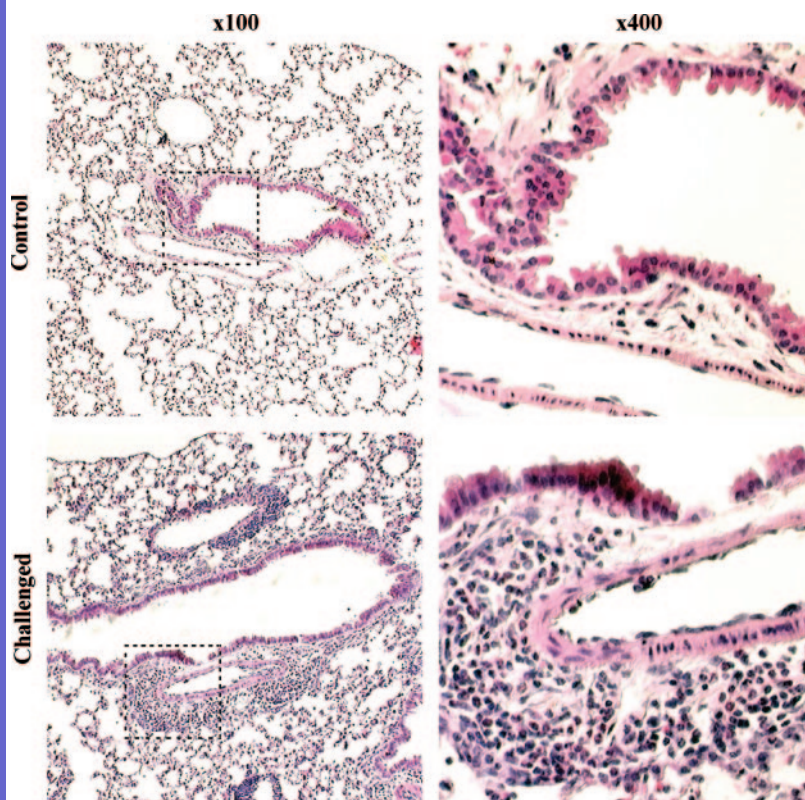


Fig. 3. Expression levels of uteroglobin-related protein 1 (*Ugrp1*) mRNAs in the lungs of sensitized control (Control) and ovalbumin-challenged (Challenged) mice. A: total RNAs (3.5 μ g/lane) from 6 (Control) and 7 (Challenged) individual lungs were analyzed by Northern hybridization using ³²P-labeled mouse UGRP1 cDNAs as a probe (top). After being stripped, the membrane was rehybridized with mouse β -actin probe (bottom). B: relative *Ugrp1* mRNA levels. Data represent means $\pm$ SE from 6 (Control) and 7 (Challenged) mice. *Ugrp1* mRNA expression is significantly decreased in lungs of the challenged group compared with that of control mice (** $P < 0.01$).

Fig. 2. Histological examinations of lung tissues 24 h after saline (Control; top) or OVA (Challenged; bottom) inhalation in OVA-sensitized mice. Sections of formalin-fixed lung tissues were stained with hematoxylin and eosin before examination by light microscopy. Original magnification: left, $\times 100$; right, $\times 400$.

Fig. 4. Immunohistochemical staining by polyclonal antibodies against UGRP1 in lungs of sensitized control (Control; *top*) and ovalbumin-challenged (Challenged; *bottom*) mice. Magnifications: $\times 40$ (*left*) and $\times 400$ (*right*). Distinct UGRP1-positive staining in the epithelia is observed in control mice, but only faint staining is found in the challenged group.

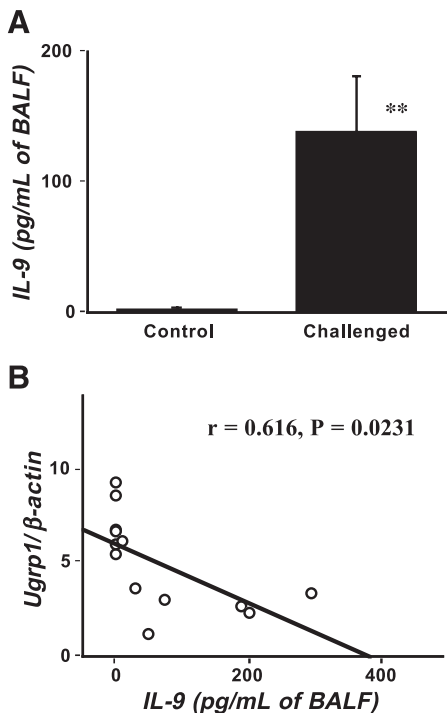
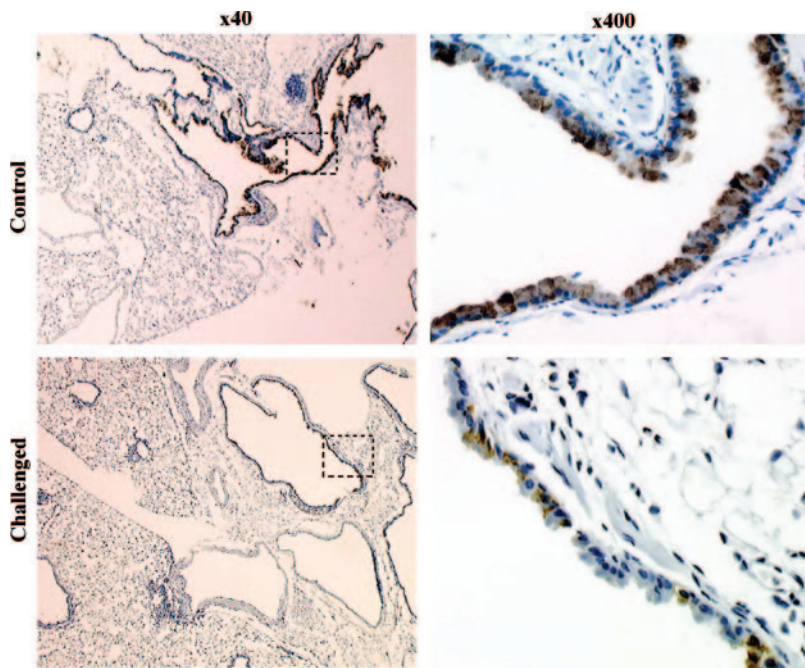


Fig. 5. A: interleukin-9 (IL-9) levels in BALF obtained from sensitized control (Control) and ovalbumin-challenged (Challenged) mice. IL-9 concentration in BALF was determined by ELISA, as described in MATERIALS AND METHODS. Each bar represents the mean $\pm$ SE from 6 (Control) and 7 (Challenged) mice. IL-9 level in BALF of challenged group is significantly greater than that of control mice (** $P < 0.01$). B: correlation between the levels of *Ugrp1* mRNA expression in airways and IL-9 in BALF. Relative airway *Ugrp1* mRNA levels after normalized to β -actin were regressed against IL-9 levels in BALF. A significant inverse correlation between the two parameters was observed ($r = 0.616$, $P = 0.0231$ by Fisher's regression analysis).

Correlation between the levels of *Ugrp1* mRNA in airways and IL-9 in BAL fluids. IL-9 level in BAL fluids of OVA-challenged mice was markedly and significantly increased compared with that of the sensitized control group (Fig. 5A). A regression analysis was performed to explore a potential relationship between the levels of *Ugrp1* mRNA in airways and IL-9 in BAL fluids. Relative airway *Ugrp1* mRNA levels decreased with increased IL-9 levels in BAL fluids (Fig. 5B), with a significant inverse correlation ($r = 0.616$, $P = 0.023$).

Effect of intranasal administration of IL-9 on lung *Ugrp1* mRNA expression. To demonstrate a possible direct correlation between IL-9 and the level of *Ugrp1* expression in mouse lungs, mice were intranasally administered IL-9, and lung mRNAs were subjected to Northern blot analysis (Fig. 6). The dose-dependent decrease of *Ugrp1* expression level was obtained, further demonstrating that *Ugrp1* expression is inversely correlated with IL-9 level.

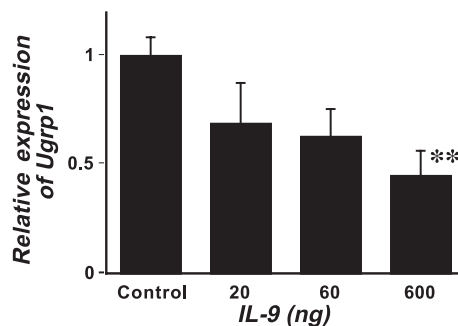


Fig. 6. Dose-dependent decrease of *Ugrp1* mRNA levels in lungs of mice intranasally administered IL-9. Mice received intranasal instillation of 0, 20, 60, and 600 ng IL-9, and RNAs were isolated 24 h later. Relative *Ugrp1* mRNA levels plotted against the amount of IL-9 administered. Values are means of those obtained from 3–6 mice. Note that a significant difference (** $P < 0.01$) was observed between 0 (Control) and 600 ng IL-9.



DISCUSSION

In the present study, airway inflammation characterized by an increase in cell numbers, especially eosinophils, was observed after OVA challenge in OVA-sensitized mice. In this animal model of allergic airway inflammation, a marked decrease in airway UGRP1 expression was found compared with control mice. Interestingly, a significant inverse correlation between *Ugrp1* mRNA levels in airway tissue and IL-9 level in BAL fluid was demonstrated by regression analysis. These findings suggest that IL-9 may be involved in the downregulation of UGRP1 observed in experimental allergic airway inflammation in mice. In fact, we were able to demonstrate that IL-9 has an ability to regulate UGRP1 expression in mice intranasally administered IL-9.

There is increasing evidence that IL-9 is an important mediator of the pathogenesis of allergic bronchial asthma. The human *IL-9* gene is located on chromosome 5q31-q33, a region close to where the *UGRP1* gene is located and where a major gene for asthma was assigned (8, 20). Indeed, *IL-9* was suggested as a possible asthma candidate gene (10, 17). Shimbara et al. (22) have shown that *IL-9* mRNA is dramatically increased in the airways of human asthmatics. The significant increase of IL-9 levels in BAL fluids after antigen challenge was reported using dogs that exhibit marked airway eosinophilic inflammation, as seen in the current study with mice (13). Moreover, neutralization of IL-9 by systemic administration of anti-IL-9 antibody can inhibit antigen-induced airway inflammation and hyperresponsiveness in mice (5).

The exact mechanism(s) for the effect of IL-9 on UGRP1 expression is not clear. However, our studies using intranasal administration of IL-9 to mice suggest, at least in part, a direct effect on epithelial cells. The biological effects of IL-9 are exerted via a functional IL-9 receptor complex, which consists of a ligand-specific α -chain and an IL-2 receptor γ -chain. Although the effects of IL-9 have mainly been described for hematopoietic cells, recent study revealed the presence of functional IL-9 receptors in airway epithelial cells (9, 13). Longphre et al. (13) demonstrated that IL-9 is able to stimulate mucin transcription via IL-9 receptors in airway epithelial cells, indicating that the IL-9 signaling pathway can modulate some transcriptional responses in epithelial cells. Furthermore, it has been reported that anti-IL-9 antibody treatment inhibits the release of IL-4, -5, and -13 in a murine model of allergic asthma (5). Indeed, these cytokines were also increased in the BAL fluid of OVA-challenged mice (personal communication). UGRP1 expression may be directly modulated by IL-9 signaling in airway epithelial cells and/or indirectly through other cytokines. The exact mechanism requires further studies.

UGRP1 has amino acid sequence similarity to the uteroglobin/CCSP that is also secreted by the epithelial cells in airways. Soluble phospholipase A₂ (sPLA₂) was suggested as a target protein for uteroglobin/CCSP (7) that can inhibit sPLA₂ activity, resulting in an anti-inflammatory effect. It is possible that UGRP1 is functionally related to uteroglobin/CCSP; however, to date, there is no evidence that UGRP1 is able to inhibit sPLA₂ activity. A recent study suggested that macrophage scavenger receptor with collagenous structure is a receptor for human UGRP1 (2), although the functional significance is not clear. Further studies are required to determine the exact role(s) of UGRP1 in airways.

In conclusion, our results clearly demonstrate that UGRP1 is downregulated in antigen-induced airway inflammation in mice. The increased IL-9 level in airways may, at least in part, be involved in the reduced UGRP1 expression in airway epithelial cells.

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