

A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma

Masao Toda, MD, PhD,^a Meri K. Tulic, PhD,^a Roy C. Levitt, MD,^b and
Qutayba Hamid, MD, PhD^a *Montreal, Quebec, Canada, and Plymouth Meeting, Pa*

Background: One of the cardinal features of airway remodeling in asthma is mucus gland hyperplasia and mucus overproduction and hypersecretion. Recently, a calcium-activated chloride channel, *HCLCA1*, was described that is upregulated by IL-9 and thought to regulate the expression of soluble gel-forming mucins, such as MUC5A/C, a critical component of mucus in the airways.

Objective: We sought to examine the expression of *HCLCA1* in bronchial biopsy specimens of asthmatic subjects compared with those of control subjects and to demonstrate its relationship with IL-9, IL-9 receptor (IL-9R), and markers of mucus production.

Methods: Bronchial biopsy specimens from asthmatic (n = 9) and control (n = 10) subjects were stained with periodic acid-Schiff to identify mucus glycoconjugates. IL-9- and IL-9R-positive cells were identified with immunocytochemistry, and *HCLCA1* expression was detected by means of in situ hybridization with cRNA probes.

Results: We demonstrate significant increases in IL-9 ($P < .001$) and IL-9R ($P < .05$) immunoreactivity, as well as increased expression of *HCLCA1* mRNA ($P < .001$), in the epithelium of asthmatic patients compared with that found in control subjects. There was also an increase in the number of mucus-producing cells in biopsy specimens from asthmatic subjects ($P < .001$). *HCLCA1* mRNA was strongly and selectively colocalized with periodic acid-Schiff and IL-9R-positive epithelial cells. In particular, a strong positive correlation was observed between *HCLCA1* mRNA expression and IL-9-positive ($r = 0.69$, $P < 0.01$) or IL-9R-positive ($r = 0.79$, $P < .01$) cells.

Conclusion: An upregulation of *HCLCA1* in the IL-9-responsive mucus-producing epithelium of asthmatic subjects compared with that seen in control subjects supports the hypothesis that this channel may be responsible, in part, for the overproduction of mucus in asthmatic subjects. These preliminary findings suggest the inhibition of *HCLCA1* may be an important new therapeutic approach to control mucus overproduction in chronic airway disorders. (*J Allergy Clin Immunol* 2002;109:246-50.)

Key words: IL-9, asthma, *HCLCA1*, mucus

One of the cardinal features of airway remodeling in asthma is mucus gland hyperplasia and mucus overproduction and hypersecretion. Mucus overproduction is generally thought to be responsible for small-airway obstruction and lung dysfunction that is closely linked to morbidity and mortality in asthma and other chronic obstructive lung diseases. A number of cytokines have been implicated in the regulation of mucus production in asthma, including IL-4, IL-13, and, recently, IL-9. Although IL-9 has been reported to be a dominant cytokine in MUC2 and MUC5A/C overproduction in vitro and ex vivo, its broader role in mucus production in asthma remains obscure.^{1,2}

A growing number of studies have demonstrated that IL-9 and its receptor (IL-9R) are strongly and broadly implicated in the pathogenesis of asthma in human subjects and in animal models of the disorder.^{3,4} Overexpression of IL-9 in the lungs of transgenic mice results in elevated IgE levels and an exaggerated T_H2-type allergic response characterized by enhanced tissue eosinophilia, bronchial hyperresponsiveness, mucus gland metaplasia, and mucus overproduction and hypersecretion.^{1,4-6} Longphre et al² recently described a dominant role for IL-9 in the regulation of MUC5A/C, a gel-forming soluble mucin and critical component of mucus in the airways. We have recently shown increased expression of IL-9 and IL-9R in bronchial biopsy specimens of asthmatic subjects compared with those of control subjects⁷ and have demonstrated a direct effect of IL-9 on inducing mucin production in human primary epithelial cell cultures in vitro.¹ Taken together, these results suggest that airway epithelial cells may be one major target in IL-9-related allergic inflammation. Nevertheless, the mechanism by which IL-9 may regulate soluble gel-forming mucin genes remains obscure.

Cl⁻ channels in epithelial cells are fundamental in the process of transepithelial electrolyte and fluid absorption or secretion and have been implicated in the regulation of mucus production.^{8,9} Recently, a new family of chloride channels has been discovered in epithelial cells that are Ca²⁺ dependent.^{9,10} To date, 3 types of Ca²⁺-dependent Cl⁻ channels (CLCAs) have been described, namely CLCA1, CLCA2, and CLCA3.^{8,10-12} In the mouse

From ^aMeakins-Christie Laboratories, Montreal, and ^bGenaera Pharmaceuticals, Plymouth Meeting.

Received for publication October 15, 2001; revised November 8, 2001; accepted for publication November 12, 2001.

Reprint requests: Qutayba Hamid, MD, PhD, Meakins-Christie Laboratories, 3626 St Urbain St, Montreal, Quebec H2X 2P2, Canada.

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0091-6749/2002 \$35.00 + 0 1/10/121555

doi:10.1067/mai.2002.121555

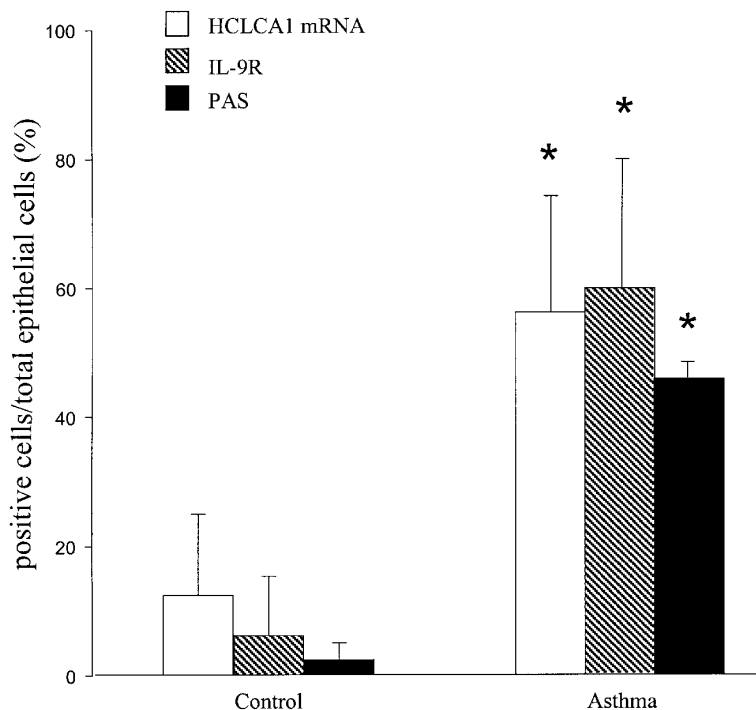


FIG 1. Expression of *HCLCA1* mRNA-, IL-9R-, and PAS-positive cells in bronchial epithelium of asthmatic subjects (n = 9) compared with that seen in healthy control subjects (n = 10). Results are expressed as a mean of positive cells per total epithelial cells $\pm$ SD. * $P < .001$ versus healthy control subjects.

Abbreviations used

- AB: Alcian blue
- CLCA: Ca^{2+} -dependent Cl^- channel
- FVC: Forced vital capacity
- IL-9R: IL-9 receptor
- PAS: Periodic acid-Schiff

MCLCA3, or *GOB5*, mRNA was difficult to detect in the lung but strongly expressed in the gut and the urogenital system of healthy mice.^{13,14} *MCLCA3* expression was increased dramatically in the lungs of IL-9 transgenic animals and after intratracheal IL-9 and $\text{T}_\text{H}2$ cytokine instillations.¹⁴ Interestingly, the ectopic expression of either *HCLCA1* or *MCLCA3* in the human mucoepidermoid cell line NCI-H292 resulted in the upregulation of *MUC5A/C* but not membrane-bound *MUC1*.^{14,15} Moreover, upregulation of *MCLCA3* in murine models of asthma is strongly associated with mucus overproduction.^{14,15} Nevertheless, the role of *HCLCA1* in asthma and the potential relationship with mucus overproduction in the airways remains unclear.

In the present study we set out to examine the expression of *HCLCA1* in lung biopsy specimens of asthmatic subjects compared with that in normal healthy subjects to test whether the expression of this gene is upregulated in asthma and specifically in IL-9-responsive mucus-producing cells.

METHODS

Patient profile

Nine asthmatic subjects (mean age, 39 ± 12 years) and 10 healthy nonatopic control subjects (mean age, 40 ± 13 years) were recruited. Asthma severity was assessed on the basis of the Aas scoring system.¹⁶ Subjects were excluded if they had a lower respiratory tract infection, an exacerbation of their asthma for up to 8 weeks before the study, or treatment with corticosteroids within 4 months before the study. All subjects were nonsmokers. Approval was obtained from the local ethics committee, and informed consent was obtained from all subjects before being recruited into this study.

Pulmonary function and methacholine inhalation tests

FEV_1 and forced vital capacity (FVC) were measured before medication administration and fiberoptic bronchoscopy. These parameters were used to confirm the presence of asthma. Patients were required to discontinue β_2 -agonists 12 hours before methacholine challenge. Differences in responses to methacholine were assessed with the provocative dose producing a 20% increase in response. Asthma was confirmed by decreased FEV_1 and FEV_1/FVC ratio, as well as a decreased provocative dose producing a 20% increase in response. The technique of fiberoptic bronchoscopy and the methods for processing of bronchial biopsy specimens have been previously described.¹⁷ Normal control subjects had no history of asthma or airway hyperresponsiveness and had not previously taken asthma medication.

Probe preparation

Sulfur 35 (S^{35})-labeled complementary RNA probe coding for the murine homolog of *HCLCA1* mRNA was prepared from cDNA,

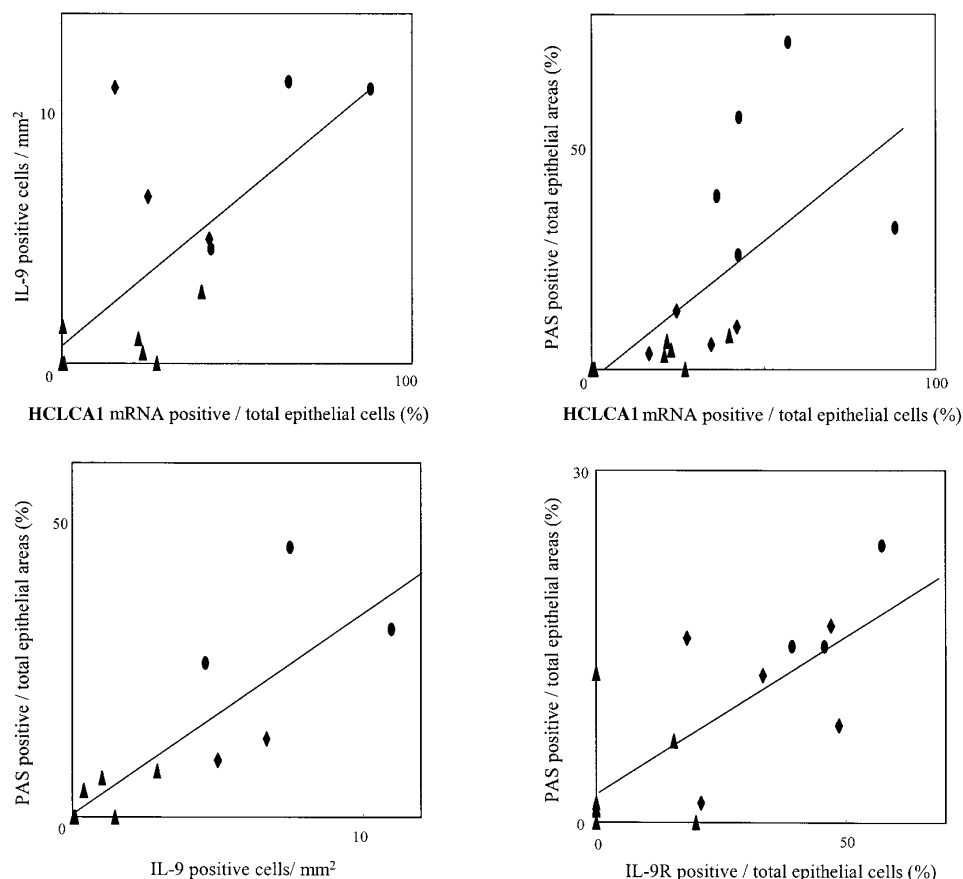


FIG 2. Correlation between *HCLCA1* mRNA- and IL-9-positive cells (A, $r = 0.69$) or PAS-positive cells (B, $r = 0.64$) in both asthmatic and control subjects. Also, correlation between PAS-positive epithelial cells and IL-9-positive cells (C, $r = 0.83$) or IL-9R-positive cells (D, $r = 0.78$) is shown. Results are expressed as a mean of positive cells per total epithelial cells $\pm$ SD.

as described previously.¹⁸ In brief, cDNA was inserted into expression vectors, linearized, and transcribed in vitro in the presence of S³⁵-UTP, T7, and SP6 polymerase in either direction to produce antisense (complementary) and sense probes (identical to mRNA).

In situ hybridization

Sections of bronchial tissue were processed for in situ hybridization to identify *HCLCA1* mRNA, according to the method of Hamid et al.^{19,20} Briefly, after permeabilization, sections were prehybridized with 50% formamide in 2 $\times$ standard sodium citrate for 15 minutes at 37°C. Hybridization was carried out overnight at 40°C with the hybridization mixture containing the appropriate S³⁵-labeled sense or antisense probe (0.75×10^6 cpm/slide). Posthybridization involved a series of washes in decreasing concentrations of saline-sodium citrate buffer at 42°C. Samples were washed with ribonuclease solution for 20 minutes at 42°C to remove any unbound RNA probes. Hybridization signal was detected with standard autoradiography.

Immunohistochemistry

Bronchial sections were fixed with 4% paraformaldehyde and then stained with periodic acid-Schiff (PAS) and Alcian blue (AB) to identify mucus glycoconjugates and mucus-producing goblet cells. Immunocytochemistry was used to detect T cells, eosinophils, and IL-9 and IL-9R immunoreactivity within the bronchial biopsy sections. Immunostaining was performed with specific antibodies to

eosinophils (anti-major basic protein, a gift from Dr R. Moqbel), T cells (anti-CD3; Becton-Dickinson, Mississauga, Canada), IL-9 (anti-IL-9; R&D Systems, Minneapolis, Minn), or IL-9R (anti-IL-9R; Santa Cruz Biotechnology, Santa Cruz, Calif) and the alkaline phosphatase-antialkaline phosphatase technique, as previously described.²¹ For negative controls, the primary antibody was replaced with an irrelevant isotype-matched control immunoglobulin or TRIS-buffered saline.

Combined in situ hybridization and histochemistry-immunocytochemistry

To study the association of the *HCLCA1* expression and IL-9, we simultaneously applied radiolabeled cRNA probe coding for *HCLCA1* mRNA with IL-9 receptor antibody, as previously described.²² For the colocalization of *HCLCA1* to mucus-producing cells, sections were first stained with PAS or AB and subsequently hybridized with S³⁵-labeled probe coding for *HCLCA1*.

Quantification

All slides were read blinded (coded), and positive cells were counted. Epithelial cells with positive PAS staining, positive IL-9R cells, or *HCLCA1* mRNA signals were expressed as a percentage of positive cells per total epithelial cells. IL-9-, CD3-, and major basic protein-immunoreactive cells were expressed as the number of positive cells per square millimeter of submucosa. The within-observer coefficient of variation for repeated measures was less than 5%.

Statistical analysis

The difference in the number of cells expressing *HCLCA1* mRNA, IL-9, or IL-9R or staining for mucin (PAS/AB) were compared with the nonparametric Kruskal-Wallis test. Statistically significant differences between groups were subsequently analyzed with the Mann-Whitney *U* test (Systat version 7.0; SPSS, Chicago, Ill). Correlation coefficients were calculated from the Pearson moment coefficient and were corrected for multiple comparisons by use of the Bonferroni correction factor. Results are expressed as means $\pm$ SD and were significant at a *P* value of less than .05.

RESULTS

Baseline description of asthmatic subjects compared with healthy control subjects

Asthmatic subjects had significantly increased numbers of major basic protein-immunoreactive eosinophils ($23.8 \pm 18.4/\text{mm}^2$ of submucosa vs $4.0 \pm 4.8/\text{mm}^2$ of submucosa, $P < 0.01$) and significantly increased number of CD3-immunoreactive T cells ($83.7 \pm 33.7/\text{mm}^2$ of submucosa vs $51.5 \pm 19.3/\text{mm}^2$ of submucosa, $P < .05$) in the submucosa compared with that seen in normal control subjects. IL-9 immunoreactivity was significantly higher in asthmatic subjects compared with that in healthy control subjects ($9.0 \pm 4.2/\text{mm}^2$ of submucosa vs $0.7 \pm 1.0/\text{mm}^2$ of submucosa, $P < .001$). FEV₁ and FVC measurements were similar between the groups; however, asthmatic subjects had a significantly lower FEV₁/FVC ratio ($72.8\% \pm 5.8\%$) compared with that seen in control subjects ($85.4\% \pm 7.0\%$, $P < .01$).

Expression of *HCLCA1* mRNA, IL-9R, and PAS staining in the airway epithelium

Positive expression of *HCLCA1* mRNA was detected in the airways epithelium of both asthmatic and control groups of patients (Fig 1). The percentage of *HCLCA1* mRNA-positive cells in asthmatic subjects ($55.4\% \pm 19.3\%$) was significantly higher compared with that found in control subjects ($14.9\% \pm 15.1\%$, $P < .001$). No positive hybridization signals were observed when the *HCLCA1* sense probe was used. IL-9R immunoreactivity was detected in both patient groups; however, the percentage of IL-9R-immunoreactive cells was significantly increased in the airway epithelium of asthmatic subjects ($55.2\% \pm 10.8\%$) compared with that in control subjects ($6.5\% \pm 9.9\%$, $P < .001$, Fig 1). PAS staining and the number of mucus-producing cells were significantly increased in the epithelium of asthmatic subjects compared with that seen in control subjects ($P < .001$, Fig 1). Similar results were obtained with the AB mucus stain.

Correlation of IL-9 and IL-9R with expression of *HCLCA1* mRNA in the epithelium

Positive correlation was observed between *HCLCA1* mRNA expression and IL-9-positive cells (correlation coefficient, $r = 0.69$; $P < .01$; Fig 2, A). A positive correlation between IL-9R expression in the epithelium and IL-9-positive cells was observed ($r = 0.66$, $P < .05$). A positive correlation was also observed between the expression of *HCLCA1* mRNA and IL-9R-immunoreactive cells (correlation coefficient, $r = 0.79$; $P < .01$).

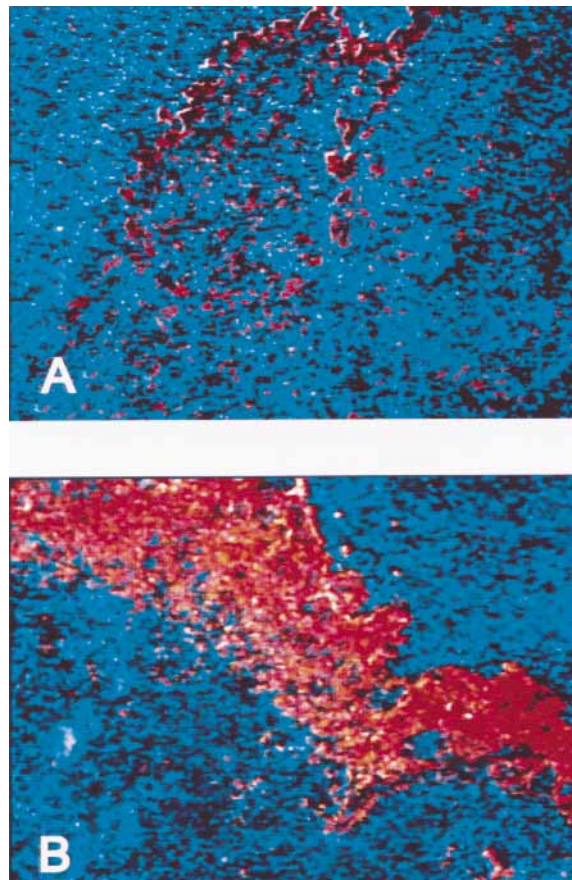


FIG 3. Colocalization of *HCLCA1* mRNA and IL-9R protein in bronchial tissue of asthmatic subjects (B) compared with that seen in normal control subjects (A). Simultaneous in situ hybridization and immunocytochemistry.

The percentage of mucus-containing PAS-positive epithelial cells was correlated with the percentage of *HCLCA1* mRNA-positive cells ($r = 0.64$, $P < .01$; Fig 2, B), with the number of IL-9-immunoreactive cells ($r = 0.83$, $P < .001$; Fig 2, C) and with the percentage of IL-9R-positive epithelial cells ($r = 0.78$, $P < .001$; Fig 2, D). Similar correlations were observed with AB staining.

Colocalization of IL-9R and *HCLCA1* mRNA expression

Colocalization of IL-9R with *HCLCA1* mRNA was seen in all groups studied. In asthmatic subjects $78.0\% \pm 22.9\%$ of all IL-9R-immunoreactive cells were also positive for *HCLCA1* mRNA, whereas only $55.3\% \pm 48.5\%$ of IL-9R-positive cells were positive for *HCLCA1* mRNA (Fig 3).

DISCUSSION

This is the first study that demonstrates the significantly increased expression of *HCLCA1* in asthmatic subjects and suggests that this channel is involved in regulating IL-9-dependent mucus gene expressions in asth-

ma. Although the phenomenon of mucus hypersecretion and inflammation have been documented as features of asthma for many years, little is known about their potential relationship. A number of cytokines, including IL-4, IL-13, and IL-9, have been shown, in human asthmatic subjects and animal models, to affect epithelial cells directly.²³⁻²⁵ These cytokines have recently been linked to upregulated expression of *HCLCA1*, and the role of this channel was described in the specific upregulation and increased production of gel-forming mucins in vitro.^{14,15} Although these data suggest a role for *HCLCA1* in asthma and specifically in the regulation of mucin production in the airways, this hypothesis has not been tested in human subjects.

We have clearly shown here, confirming our previous findings, that IL-9 and its receptor are upregulated on airway epithelial cells in asthmatic subjects compared with in control subjects.^{1,7} Our findings of increased expression of *HCLCA1* in these IL-9R-positive cells in asthmatic subjects compared with that in healthy control subjects are in agreement with the observations of Nakanishi et al¹⁵ and support the hypothesis that this channel may be involved in human asthma. Moreover, we were able to demonstrate low levels of *HCLCA1* in healthy subjects, which is consistent with our previous findings that this channel is specifically upregulated in disease states and not easily detected in healthy animals.¹⁴ Although we did not examine specific *MUC* genes, we have examined the translated products histochemically by means of PAS/AB staining and have demonstrated that *HCLCA1* is highly correlated with PAS staining and predominantly colocalized to PAS-positive epithelial cells. Furthermore, the tight positive correlation between the expression of *HCLCA1* mRNA and the number of IL-9- or IL-9R-immunoreactive cells and the colocalization of *HCLCA1* expression with IL-9R on mucin-bearing cells in lung biopsy specimens of asthmatic, but not healthy control, subjects strongly supports the conclusion that *HCLCA1* may regulate airway mucus production in asthma.

It is commonly believed that mucus overproduction in the small airways of subjects with asthma and, potentially, other chronic obstructive lung disorders leads to lung dysfunction and is linked to morbidity and mortality. Despite these observations, there are virtually no therapeutic approaches available to control the overproduction of mucus in asthmatic subjects. Nakanishi et al¹⁵ have demonstrated the potential clinical advantages of *HCLCA1* inhibition in animal models of asthma. These data collectively suggest that *HCLCA1* inhibition in asthma may be a useful new therapeutic strategy. Moreover, our preliminary data suggest that additional human investigations that further explore the role of *HCLCA1* in asthma and, potentially, other chronic obstructive lung disorders are warranted.

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