

Original article

Low levels of CC16 in nasal fluid of children with birch pollen-induced rhinitis

Background: Clara cell protein 16 (CC16; secretoglobin 1A1) is an anti-inflammatory protein mainly expressed in the epithelial cells in the airways.

Objective: To compare the levels of CC16 in nasal lavage (NAL) from children with intermittent allergic rhinitis and healthy controls and to study the effect of a local steroid.

Methods: Thirty schoolchildren with birch pollen allergy and 30 healthy controls from the same schools were included in the study. The NAL fluid was collected before the season, during the birch pollen season and, for the patients, after 1 week of treatment with a local steroid. Symptom scores were obtained on every occasion. CC16 and eosinophil cationic protein (ECP) were analyzed with enzyme-linked immunosorbent assay.

Results: The nasal fluid levels of CC16 were significantly lower in patients than in controls, before and during pollen season. Before the season, the median CC16 concentrations were 9.1 (range 1.1–117) µg/l in patients and 25.7 (6.1–110.2) µg/l in controls. During the season, the median CC16 concentrations in nasal fluid were 12.9 (2.3–89.7) µg/l in the allergic children and 22.0 (9.5–90.1) µg/l in the healthy controls ($P = 0.0005$). Symptom scores, nasal fluid eosinophils and ECP were higher in patients during the season. Treatment with a local steroid did not change the CC16 levels.

Conclusions: Nasal fluid CC16 levels were lower in children with birch pollen-induced allergic rhinitis than in healthy controls both before and during the pollen season. We speculate that reduction in anti-inflammatory activity by CC16 may contribute to the pathogenesis of allergic rhinitis.

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Intermittent allergic rhinitis (IAR) is believed to be dependent on an imbalance in T-helper (Th) cells 1 and 2 with a shift toward a Th2 response. Interestingly, recent studies reveal that the reduced expression of anti-inflammatory genes may also contribute to IAR (1, 2).

Clara cell protein 16 [CC16, secretoglobin 1a1 (SCGB 1a1)], also referred to as uteroglobin, is an anti-inflammatory protein that is primarily expressed in the airways, although small amounts of mRNA encoding for this protein have been found in several other organs (3). The protein is secreted constitutively at high levels by Clara cells in the bronchi and nasal epithelial cells (4, 5).

The anti-inflammatory effect of CC16 is thought to be dependent on the inhibition of the pro-inflammatory enzymes phospholipase A₂ (PLA₂) and transglutaminase (6). Both these enzymes play a key role in arachidonic acid release during acute allergic inflammation. High levels of PLA₂ are released at sites of allergic reactions and are found in the nasal lavage (NAL) fluids of patients with IAR (7). By contrast, Lindahl et al. demonstrated lower levels of CC16 in the NAL fluids of four children

with various forms of allergic rhinitis compared with healthy controls (4). In a recent study of nasal polyps, which are immunohistochemically similar to IAR, CC16 expression was also found to be lower than in healthy nasal mucosa (5). As CC16 displays potent anti-inflammatory activity and is abundantly expressed, it has been suggested that this protein may play a role in controlling inflammation in the airways (8).

In animal studies, CC16 expression has been described as being regulated by glucocorticosteroids (GC), both during development and in adulthood (9, 10). An *in vitro* study of primary Clara cells revealed the upregulation of CC16 after stimulation with GC (11). On the contrary, an animal study of acute lung inflammation in rats showed no effect on CC16 levels in bronchoalveolar lavage (BAL) after treatment with dexamethasone (12). Our objective in this study was to compare the levels of CC16 in the NAL fluids of schoolchildren with birch pollen-induced IAR and healthy controls. We also wanted to compare CC16 levels in the NAL fluids of allergic children before the pollen season, during the pollen season and after treatment with a local GC.

Methods

Patients

The study comprised 30 children (18 girls) with IAR, median age 13 years (range 10–15) and 30 nonallergic controls (19 girls), median age 15 years (range 12–16). Inclusion and exclusion criteria were the same as previously described (13). The diagnosis of birch pollen-induced allergic rhinitis was based on a positive history of seasonal allergic rhinitis, positive nasal provocation test with birch extract and a positive skin prick test (SPT) for birch. The controls were healthy children with no history of rhinitis, negative provocation test, and a negative SPT for birch.

Study design

Allergic children and healthy controls were seen before and during the pollen season. The allergic children were also seen after a period of treatment with a nasal steroid. The first contact with the children and sample collection took place in January and February 2003, when all the subjects were asymptomatic.

During the birch pollen season, April–May 2003, the second samples were collected. The pollen count was registered daily by the Department of Botany, Göteborg University. The allergic children were seen after few days of symptoms of allergic rhinitis and the healthy controls were called in during the same period. The allergic children were given treatment for 7–10 days with budesonide nasal spray; sodium cromoglycate eye drops were given if needed. The study was approved by the Ethics Committee of the Medical Faculty, Göteborg University.

Recovery of nasal lavage fluids

Nasal lavage fluids were obtained as previously described (13, 14). Sterile saline (5–8 ml) was delivered by aerosol into each nostril, while alternately clearing the other. The nasal fluids were allowed to return passively and were collected in a graduated test tube until 5 ml had been recovered. The collected fluid was kept on ice until it was centrifuged at 4°C. The supernatant was put in a freezer (–80°C) within 3 h. Before centrifugation, slides were prepared and stained according to the May–Grünwald–Giemsa method. The average of two slides and the percentage of each cell type, neutrophil, epithelial, and eosinophil were calculated.

Nasal symptom scores

Nasal secretion, itching, and blockage were scored from 0 to 3 (0 = no, 1 = mild, 2 = moderate, 3 = severe symptoms). The number of sneezes during the last hour were counted and transformed into a score (0 = 0 sneezes, 1 = 1–4 sneezes, 2 = 5–9 sneezes and 3 = 10 or more sneezes). A total symptom score was calculated by adding the four scores. The maximum score was 12. Nasal symptoms were recorded before recovering secretion fluids.

Nasal fluid mediator assays

Eosinophil cationic protein in nasal fluid was determined with enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (MBL, Nagoya, Japan).

Clara cell protein was determined with ELISA. Plates were coated with Mab 788 (kindly provided by Pharmacia Diagnostics AB, Uppsala, Sweden). Wells were washed with 0.1% Tween 20 in phosphate-buffered saline and then blocked with bovine serum albumin

(1%) in washing solution. After another wash, sample was added to each well, together with 6% diluent (6% PEG 6000; VWR, Stockholm, Sweden). Human recombinant CC16 (kindly provided by Claragen Inc., Bethesda, MD, USA) was used as standard. After incubation and a third wash, rabbit polyclonal antibody against human urine protein 1 (Dako, Glostrup, Denmark) was added. The plate was again incubated and washed, and horseradish peroxidase-conjugated goat antirabbit IgG antibody (Dako) was added. After another incubation and wash, the plate was developed with TMB (Neogen Corp., Lexington, MA, USA) during approximately 20 min in darkness. The reaction was then stopped with 1 M H₂SO₄.

Enzyme activity was measured at an absorbance of 450 nm with a 650 nm correction on a Vmax Kinetic microplate analyzer (Molecular Devices Corporation, Sunnyvale, CA, USA). Titration curves for standard and unknowns were fitted from a four-parameter logistic model using the SoftMaxTM software package (Molecular Devices Corporation).

Statistical analysis

The results are expressed as the median, inter-quartile range (IQR) with the first and third quartiles, and the range. Comparisons of the results from the allergic children and the healthy controls were performed using the Mann–Whitney *U*-test (15).

To investigate the change over time in the levels of CC16 in allergic children and healthy controls, respectively, the differences in concentration between two consecutive occasions (before the season and during the season) were calculated in each individual. The median, range, and IQR of the differences were calculated for each group.

To compare the overall CC16 levels between the groups of allergic children and healthy controls, a cut-off between high and low concentrations of CC16 was defined by the median concentration of the total data set (allergic children and healthy controls) before and during the season, respectively. These medians were used to identify high and low producers of CC16 on both occasions. Subjects who had levels below both the two median levels were considered to be overall low producers of CC16, while subjects with levels above the medians on both occasions were regarded as overall high producers of CC16. The proportion of allergic children and healthy controls defined as low producers was calculated in this way. The difference in proportions of low producers between allergic children and healthy controls and the 95% confidence interval (CI) of this proportion were calculated. The sign test with correction of continuity was used to analyze the hypothesis that there was no difference of proportions between the groups (16). A *P*-value < 0.05 was considered significant. Statistical analyses were performed on a personal computer using the SPSS software package, version 11 (SPSS Inc., Chicago, IL, USA).

Results

Pollen counts

Pollen counts were 0/m³ air for birch pollen (*Betula*) during the first sample period (January–February) and, during the sampling in the pollen season, the pollen counts varied from 6 to 1394/m³ air. The median for this period was 119/m³.

Symptoms

All the subjects were free from symptoms when they were first seen in January (*n* = 30 + 30). Two subjects (one

from each group) were lost to follow-up because of a change of school. Only one child in the group of allergic children was free of symptoms and received no treatment. One child was lost to follow-up after treatment because of illness ($n = 27$). None of the healthy controls had symptoms. The symptom score was significantly higher in the group of allergic children compared with the healthy controls during the pollen season (Table 1, Fig. 1A).

Nasal fluid eosinophils and eosinophil cationic protein

The percentage of eosinophils was significantly higher during the season in allergic children compared with controls (Table 1, Fig. 1B). Moreover, the amount of ECP was significantly higher in the group of allergic children during the season than in the healthy controls (Table 1, Fig. 1B).

Table 1. CC16, eosinophils, eosinophil cationic protein (ECP) and symptom score in the nasal fluids of children with allergic rhinitis and healthy controls before and during the birch pollen season and after treatment with budesonide (allergic children)

	Before season		During season		After treatment
	Healthy controls ($n = 30$)	Allergic children ($n = 30$)	Healthy controls ($n = 29$)	Allergic children ($n = 28$)	Allergic children ($n = 27$)
CC16 ($\mu\text{g/l}$)					
Median	25.7	9.2*	22.0	12.9*	12.5
IQR	12.2–52.4	4.4–25.1	13.5–47.7	6.8–20.9	4.0–21.3
Range	6.1–110.2	1.1–117.0	8.0–90.1	2.3–89.7	1.1–56.6
Eosinophils (%)					
Median	0	0	0	2†	0‡
IQR	0	0–1	0	0–8	0–1
Range	0–6	0–1	0–2	0–47	0–40
ECP ($\mu\text{g/l}$)					
Median	11.2	24.6	6.9	34.8§	9.9
IQR	3.2–28.7	8.8–36.0	2.5–17.5	7.5–79.2	1.3–62.2
Range	0.83–481.6	0.11–287.3	0–87.21	0.13–644.2	0–332.1
Symptom score					
Median	0	0	0	8†	3
IQR	0	0	0	5.5–9	1–5
Range	0	0–1	0–1	1–12	0–11

* $P = 0.0005$ compared with healthy controls (sign test with correction for continuity).

† $P < 0.0001$ and § $P = 0.002$ compared with healthy controls during the season (Mann–Whitney U -test).

‡ $P = 0.001$ compared with allergic children during season (Wilcoxon signed rank test).

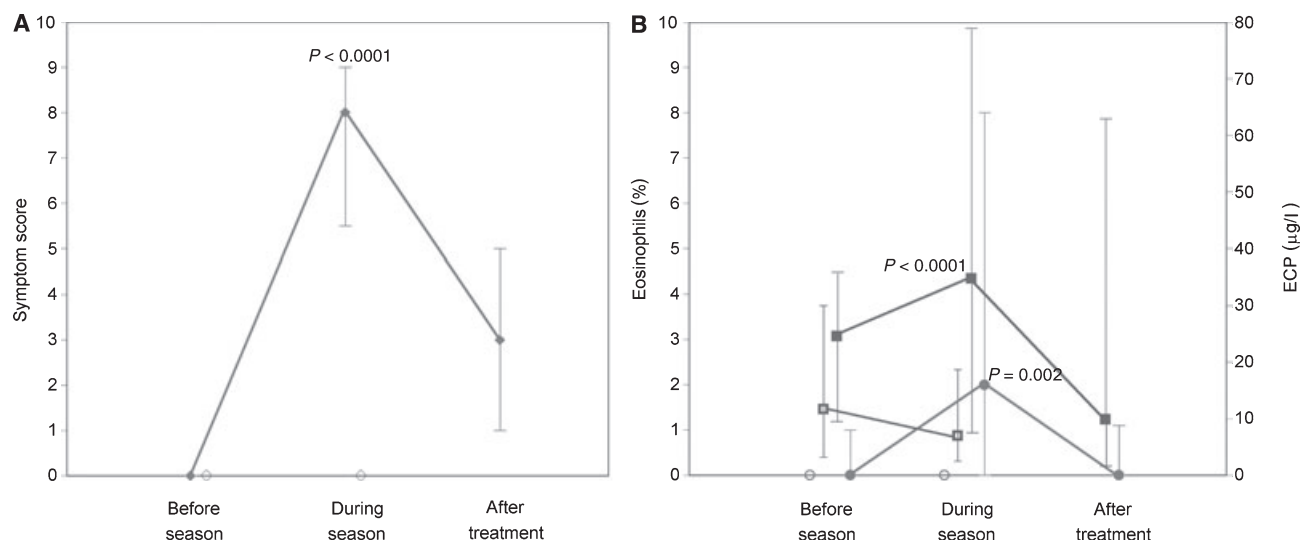


Figure 1. Medians of symptom scores (diamonds) (A) and percentage of eosinophils (circles) and medians of eosinophil cationic protein concentrations (squares) (B). Filled and open symbols represent allergic children and healthy controls, respectively. Error bars show inter-quartile range on each occasion. Significant difference between allergic children and healthy controls during season are indicated.

Nasal fluid CC16

The median change (IQR; range) for the two groups was very similar -1.5 (-6.8 – 2.7 ; -60.0 – 72.6), n.s., and -2.7 (-6.5 – 13.9 ; -57.8 – 99.2), n.s., for allergic children and healthy controls, respectively. Evaluations of the individual changes in CC16 levels over time showed only slight variations.

The allergic children were found to have significantly lower levels of CC16 than the controls, both before and during the pollen season (Table 1, Fig. 2). The proportion of allergic children regarded as low producers was 75% (15/20) and the proportion of controls regarded as low producers was 32% (7/22). There was, therefore, a higher proportion of low producers in the group of allergic children (the 95% CI for difference of proportions was 16–70%; $P = 0.0005$).

Discussion

This study reveals significantly lower CC16 levels in the NAL fluid of children with birch pollen-induced allergic rhinitis compared with healthy controls. This difference was found both before and during the season. No such differences were found in CC16 levels in the allergic children when comparing the levels before and during the season or after treatment with a local steroid.

Mediator concentrations in NAL fluid can be affected by the complex dynamics of fluid movements in the

nasal cavity. However, in this material, we demonstrate higher levels of ECP in the NAL fluid of the allergic children during the season compared with the healthy controls, which supports the validity of our findings (17).

The findings in this study also agree with the report by Lindahl et al. that revealed lower CC16 in the NAL fluid of four allergic children compared with healthy controls. Similar observations have been made in the BAL fluid (18) and serum (8) of patients with asthma. Clinical studies reveal that the number of CC16-positive epithelial cells was reduced in the small airways of patients with chronic asthma and the percentage of these epithelial cells was inversely correlated with the number of T cells and mast cells in the small airways (19). This supports the hypothesis that a reduction in CC16 contributes to allergic inflammation.

The enzyme PLA₂ is believed to be an important factor in the inflammatory reaction in allergic rhinitis (7). The anti-inflammatory effect of CC16 is believed to be the result of inhibition of this enzyme. PLA₂ plays a key role in the arachidonic-acid metabolism and thereby the synthesis of inflammatory mediators such as prostaglandins and leukotrienes.

In this study, there was no difference in CC16 levels in the NAL fluid of allergic children before and during the pollen season. To our knowledge, there are no previous data comparing CC16 concentrations in allergic children before and during active disease.

Recent data indicate that genetic variability could explain lower levels of CC16 and increased airway responsiveness in allergic individuals (20). Taken together with the known anti-inflammatory effects of CC16, these findings suggest that low levels of CC16 in our allergic children both before and during the season can contribute to the pathogenesis of IAR.

Experimental data are contradictory regarding the effects of GC on the expression of CC16 (9–12, 21, 22). To our knowledge, there are no studies investigating the effect of GC on CC16 levels in allergic inflammation. In these children with allergic rhinitis, we did not detect any increase in CC16 level after 7–10 days of treatment with a local steroid. It is possible that poor compliance could confound the results. On the contrary, the reduction in eosinophils and ECP in the GC-treated allergic children would argue against this. Alternatively, it is possible that a reduced capacity to produce CC16 because of gene polymorphisms, as discussed above, could explain why CC16 levels did not increase after GC treatment. To summarize, nasal fluid levels of CC16 are lower in children with birch pollen-induced allergic rhinitis than in healthy controls. No differences were found when comparing allergic children before and during the season or after treatment with GC. We speculate that a reduction in the production of the anti-inflammatory protein CC16 contributes to the pathogenesis of allergic rhinitis.

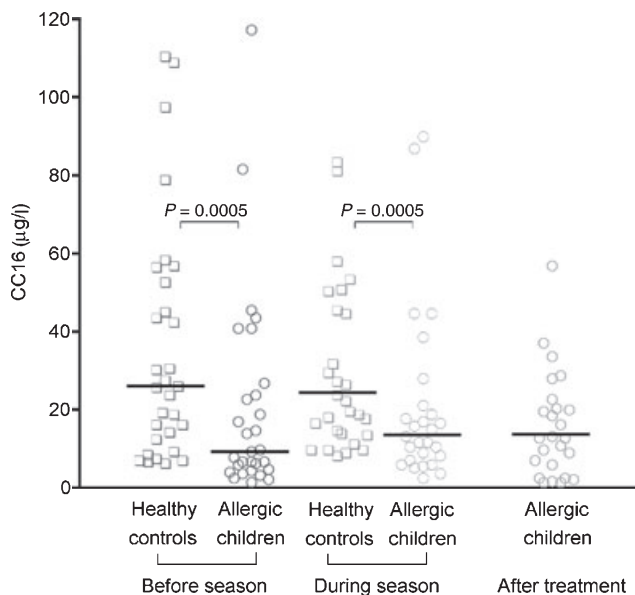


Figure 2. Concentration of CC16 in nasal lavage fluids of children with intermittent allergic rhinitis and healthy controls.

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References

- Scavuzzo MC, Rocchi V, Fattori B, Ambrogi F, Carpi A, Ruffoli R et al. Cytokine secretion in nasal mucus of normal subjects and patients with allergic rhinitis. *Biomed Pharmacother* 2003;**57**:366–371.
- Benson M, Carlsson B, Carlsson LM, Möstad P, Svensson PA, Cardell LO. DNA microarray analysis of transforming growth factor-beta and related transcripts in nasal biopsies from patients with allergic rhinitis. *Cytokine* 2002;**18**:20–25.
- Peri A, Cordella-Miele E, Miele L, Mukherjee AB. Tissue-specific expression of the gene coding for human Clara cell 10-kDa protein, a phospholipase A2-inhibitory protein. *J Clin Invest* 1993;**92**:2099–2109.
- Lindahl M, Svartz J, Tagesson C. Demonstration of different forms of the anti-inflammatory proteins lipocortin-1 and Clara cell protein-16 in human nasal and bronchoalveolar lavage fluids. *Electrophoresis* 1999;**20**:881–890.
- Benson M, Carlsson L, Adner M, Jernås M, Rudemo M, Sjögren A et al. Gene profiling reveals increased expression of uteroglobin and other anti-inflammatory genes in glucocorticoid-treated nasal polyps. *J Allergy Clin Immunol* 2004;**113**:1137–1143.
- Sohn J, Kim TI, Yoon YH, Kim JY, Kim SY. Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis. *J Clin Invest* 2003;**111**:121–128.
- Stadel JM, Hoyle K, Naclerio RM, Roshak A, Chilton FH. Characterization of phospholipase A2 from human nasal lavage. *Am J Respir Cell Mol Biol* 1994;**11**:108–113.
- Shijubo N, Itoh Y, Yamaguchi T, Sugaya F, Hirasawa M, Yamada T et al. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. *Lung* 1999;**177**:45–52.
- Nord M, Andersson O, Brönnegård M, Lund J. Rat lung polychlorinated biphenyl-binding protein: effect of glucocorticoids on the expression of the Clara cell-specific protein during fetal development. *Arch Biochem Biophys* 1992;**296**:302–307.
- Lombardero M, Nieto A. Glucocorticoid and development regulation of uteroglobin synthesis in rabbit lung. *Biochem J* 1981;**200**:487–494.
- Berg T, Cassel TN, Schwartz PE, Nord M. Glucocorticoids regulate the CCSP and CYP2B1 promoters via C/EBPbeta and delta in lung cells. *Biochem Biophys Res Commun* 2002;**293**:907–912.
- Arsalane K, Broeckaert F, Knoops B, Wiedig M, Tobeau G, Bernard A. Clara cell specific protein (CC16) expression after acute lung inflammation induced by intratracheal lipopolysaccharide administration. *Am J Respir Crit Care Med* 2000;**161**:1624–1630.
- Benson M, Strannegård I-L, Strannegård Ö, Wennergren G. Topical steroid treatment of allergic rhinitis decreases nasal fluid TH2 cytokines, eosinophils, eosinophil cationic protein, and IgE but has no significant effect on IFN-gamma, IL-1beta, TNF-alpha, or neutrophils. *J Allergy Clin Immunol* 2000;**106**:307–312.
- Kuna P, Lazarovich M, Kaplan AP. Chemokines in seasonal allergic rhinitis. *J Allergy Clin Immunol* 1996;**97**:104–112.
- Svensson E. Guidelines to statistical evaluation of data from rating scales and questionnaires. *J Rehabil Med* 2001;**33**:47–48.
- Altman DG. Statistical reviewing for medical journals. *Stat Med* 1998;**17**:2661–2674.
- Benson M, Strannegård I-L, Wennergren G, Strannegård Ö. Cytokines in nasal fluids from school children with seasonal allergic rhinitis. *Pediatr Allergy Immunol* 1997;**8**:143–149.
- Van Vyve T, Chanez P, Bernard A, Bousquet J, Godard P, Lauwerijs R et al. Protein content in bronchoalveolar lavage fluid of patients with asthma and control subjects. *J Allergy Clin Immunol* 1995;**95**:60–68.
- Shijubo N, Itoh Y, Yamaguchi T, Imada A, Hirasawa M, Yamada Y et al. Clara cell protein-positive epithelial cells are reduced in small airways of asthmatics. *Am J Respir Crit Care Med* 1999;**160**:930–933.
- Sengler C, Heinzmann A, Jerkic SP, Haider A, Sommerfeld C, Niggemann B et al. Clara cell protein 16 (CC16) gene polymorphism influences the degree of airway responsiveness in asthmatic children. *J Allergy Clin Immunol* 2003;**111**:515–519.
- Lopez DE, Haro MS, Nieto A. Glucocorticoids induce the expression of the uteroglobin gene in rabbit foetal lung explants cultured in vitro. *Biochem J* 1985;**225**:255–258.
- Fernandez-Renau D, Lombardero M, Nieto A. Glucocorticoid-dependent uteroglobin synthesis and uteroglobin mRNA levels in rabbit lung explants cultured in vitro. *Eur J Biochem* 1984;**144**:523–527.