

Complement activation in the nasal mucosa following nasal ragweed-allergen challenge

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The aim of this study was to explore complement activation in the nasal lavage following a nasal ragweed-allergen challenge. The study was carried out with 15 adolescents who were allergic to ragweed and with six non-allergic healthy volunteers. Following the baseline measurement after the symptoms were registered, subjects were given increasing doses of ragweed allergen. Lavage fluid was collected and tested for a complement-activation product (C3bBbP). The allergic patients responded to allergen provocation with an increase in C3bBbP formation compared to the initial lavage ($p=0.001$). The C3bBbP level remained low in the lavage fluids of the non-allergic controls. We found a strong correlation between the threshold dose that induced symptoms and the dose where the maximum complement activation was detected ($r=0.78$, $p=0.001$). Our findings indicate that in allergic patients nasal challenge with ragweed allergen induces a rise in complement activation in the nasal lavage fluid. These results highlight the role of the complement system in the allergic inflammation on the nasal mucosal surface.

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A possible role of complement activation in the development of allergic symptoms has been reported by several authors (1–6). Andersson et al. (7) reported the generation of two complement activation products (C3a des-arg and C5a des-arg) after nasal allergen (birch pollen and timothy grass pollen) challenge concomitantly with the development of nasal symptoms in patients with seasonal allergic rhinitis.

In our previous studies (8, 9), we found a significant, positive correlation between the extent of ragweed-induced C3bBbP (high-molecular-weight product of activation of the alternative complement pathway) generation in the sera and symptoms of allergic rhinitis in allergic adults. Similar results were obtained in ragweed-allergic adolescents (10). We have also demonstrated a strong positive correlation between skin reactivity and *in vitro* complement activation in ragweed-allergic patients (9).

The aim of the present study was to measure complement activation locally in the nasal lavage following nasal allergen challenge and to assess its relationship to allergic symptoms and the objective parameters of the nasal obstruction.

Materials and methods

Patients and controls

The study was performed in 15 adolescents (eight male, seven female), 14.3 ± 3.2 years of age (range 10–20 years), with positive skin-prick tests (SPTs) for the ragweed allergen and a history of allergic rhinitis in the ragweed season (from August to October in Hungary) treated at our out-patient department. The patients studied were without any symptoms of acute or allergic rhinitis and did not receive any therapy outside the pollen season. The nasal challenge was used to define the allergic

sensitization of the patients before considering the introduction of a specific immunotherapy. Six volunteers (18–24 years of age; two male, four female) with a negative SPT and a negative history for atopic diseases served as non-allergic controls. The examination was approved by the local ethics committee and performed after obtaining informed consent. The study outline, which was identical for patients and control subjects, is given in Fig. 1.

SPTs

In order to verify the allergic state of the patients, prescreening SPTs were performed with a standard set of the 20 most important inhalatory allergens (Soluprick; ALK/AS, Horsholm, Denmark) and negative and positive controls. After this SPT screening, all the patients were tested with Ambrosia mix (RSR 43, U/ml; Lofarma Allergeni, Milan, Italy). Histamine di-hydrochloride at 10 mg/ml was applied as a positive control. The wheal size was calculated as $[(D+d) \div 2]$, where D is the largest diameter and d is the mid-perpendicular diameter. The skin-prick index (SI) was calculated (11) as $[(D+d)_A \div 2]$ for the allergen wheal reaction divided by $[(D+d)_H \div 2]$ for the positive control reaction (where $_A$ =allergen and $_H$ =histamine). The following SI values were obtained: 1.52 ± 0.49 with Soluprick and 1.78 ± 0.31 with the Lofarma ragweed allergen. The patients showed high skin sensitivity with both allergens; a correlation exists between the two indices. Control subjects had negative SPT results with ragweed allergen.

Nasal challenge

Nasal provocation tests (12) were conducted in the morning, after a 30-min adaptation period in the same room where all the tests were carried out (Fig. 1.). Nasal temperature (T) was measured using a rhinothermometer, which did not influence the results of other measurements. Nasal inspiratory peak flow (NIPF) was measured using a Youlten peak-flow meter, and active posterior rhinomanometry was carried out in all the patients using a MES Rhinotest 500 rhinomanometer. The inspiratory and expiratory nasal airway resistances (R_{in} and R_{ex}) were calculated individually. We evaluated the flow curves reaching the cut-off value of 150 kPa. After the basic measurements of temperature, peak flow, and resistance, each nasal cavity was washed with 5–5 ml of saline solution to reduce the cell-free mediators on the nasal mucosal surface. The same parameters were measured again, followed by insufflation of 25 mg of lactose in each nasal

cavity (see Fig. 1). Allergen provocation was performed as follows: increasing concentrations of the ragweed allergen (2.5, 5, 10, 20, and 40 allergen units [AU], Allerkin Nasal Challenge test; Laboratorio Farmaceutico Lofarma, Milan, Italy) were used in alternating nasal cavities, with 15-min intervals between insufflation of each dose. The provocations by a standard dry powder insufflator (Lofarma) were followed by the same order of measurements after each challenge. In order to collect nasal fluid we used a previously described method (12). Lactose insufflation was used to establish the baseline. Lactose itself did not provoke symptoms in either of the groups and it was not accompanied with complement activation. The nasal lavage was also repeated after each dose of allergens. The lavage fluid was collected onto a flat dish and the volume was measured. In order to prevent inhalation of the allergen during insufflation, the patient had to vocalize a sound between K and Q (similar to coughing). After washing the nasal cavity with saline, the returned lavage fluid was kept cold (4°C) until the end of the procedure, then it was immediately cold-centrifuged at 210 g for 10 min. Each supernatant of the lavages was separated and stored at –80°C until they were analyzed separately.

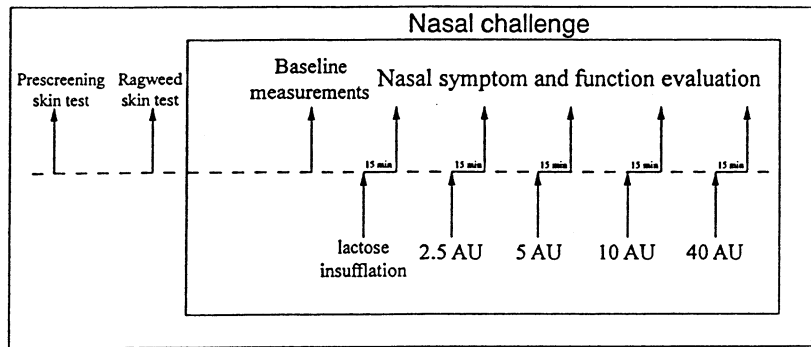
Symptom registration

Immediately before each lavage, the symptoms of blockage and itching were rated on the following four-point scale: 0=no symptoms, 1=mild symptoms, 2=moderate symptoms, 3=severe symptoms. A composite nasal symptom score was calculated, including the sum of blockage, itching scores, the number of sneezings, and drop count of the secretion. The lowest allergen dose that resulted in more than three drops of nasal secretion or at least three sneezes in 15 min was considered as the threshold dose. In most cases the provocation process was terminated when the symptoms reached the threshold level or when the inspiratory peak flow dropped to nearly zero. If the challenge was continued after reaching the threshold symptoms, further elevation in symptom score was observed, as illustrated in the case of a 14-year-old boy (Fig. 2). In that case the threshold level was 5 AU but on continuing the challenge with 10 AU, the symptoms became more severe.

C3bBbP measurement in the nasal lavage fluid

Generation of the alternative convertase, C3b-BbP, was measured using a sandwich enzyme-linked immunoassay (ELISA), as described by

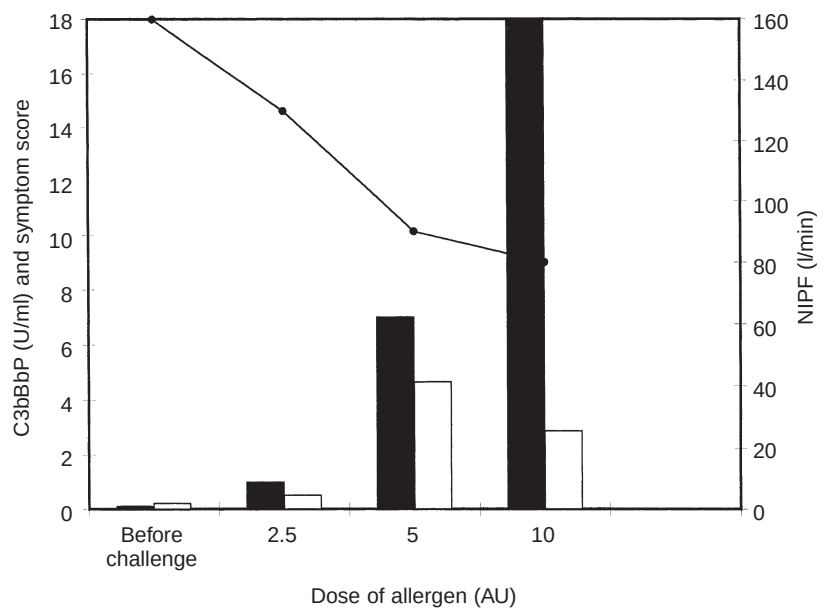
Fig. 1. Flow chart of the study protocol.



Zilow et al. (13). Microtiter plates (Maxisorp; Nunc A/S, Roskilde, Denmark) were coated with a 1:1000 dilution of goat anti-serum to human properdin (ATAB, Stillwater, MN, USA) in 0.1 M carbonate buffer, pH 9.5 (100 µl/well) at 4°C overnight and then washed twice with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-Tween). The plates were blocked with 200 µl of PBS containing 1% bovine serum albumin (BSA) (ICN, Costa Mesa, CA, USA) at room temperature (20–25°C) for 60 min. After washing twice, undiluted samples were applied to plates (100 µl/well) at room temperature for 60 min and washed five times with PBS-Tween. The calibration curve was prepared using seven dilutions (from 1:100 to 1:6,400) of normal human serum activated at 37°C for 60 min with zymosan (Sigma, St. Louis MO, USA; 1 mg/ml of serum). In the next step, 100 µl of a 1:500 dilution of biotin-labeled rabbit anti-serum to human C3c (DAKO, Glostrup, Denmark) in PBS was added

and incubated at room temperature. After 60 min, the washes (described above) were repeated. Bound human C3bBbP complexes were detected by using streptavidin-peroxidase conjugate (Jackson ImmunoResearch, West Grove, PA, USA), where 100 µl of a 1:2,000 dilution was added to each well and incubated for 60 min. The plates were washed five times and 100 µl of 0.4 mg/ml *o*-phenylene-diamine substrate (Sigma) was added to each well. The substrate was diluted in 0.1 M citrate buffer, pH 5.2, containing 4 µl/10 ml of 30% hydrogen peroxide solution. After ≈10 min, the color reaction was stopped by addition of 2 N H₂SO₄ solution (50 µl). Absorbance values were measured at 492 nm against a range of blank wells treated in the same manner but without addition of the C3bBbP sample. All the samples (in triplicate per plate) were assayed in at least three independent experiments. Intra-assay coefficients of variance (CVs) ranged from 1.5 to 2.5%, and inter-assay

Fig. 2. Changes in symptom scores (black bars), C3bBbP (white bars), and nasal inspiratory peak flow (NIPF) (•), during nasal allergen challenge in a 14-year-old boy. The threshold dose of the ragweed allergen was 5 allergy units (AU). Higher allergen doses caused higher symptom scores and further diminution in NIPF, but complement activation was highest at the threshold dose.



CVs ranged from 4 to 6%. C3bBbP levels were extrapolated from the standard curve and each concentration was expressed as the mean of the data. Concentrations are expressed in units/ml of sample substrate. One-thousand units corresponded to the content of 1 ml of undiluted zymosan-treated standard serum.

Statistical evaluation

Comparisons between controls and subjects were analyzed using the Mann–Whitney *U*-test. Response intensities were analyzed using the Wilcoxon matched pair test. The Spearman correlation coefficient served to measure the relationship between the threshold allergen dose that induced symptoms and the dose corresponding to maximum complement activation. Statistical analysis was performed by using GraphPad Prism, version 3.00 for Windows (GraphPad Software, San Diego, CA, USA; <http://www.graphpad.com>).

Results

Symptoms and changes in the airflow after allergen challenge

Symptoms and changes in the airflow after allergen challenge are summarized in Table 1. In the control group no significant effect was observed after insufflation of the vehicle or the allergen. In the allergic patients the vehicle did not induce symptoms, whereas insufflation of increasing doses of ragweed allergen induced an increasing number of sneezes and drop counts of nasal discharge. The nasal temperature in the control group showed no significant increase after the provocation, as opposed to the patient group where the allergen challenges induced a significant rise in temperature. It was noted that the baseline nasal temperatures of the patient group were slightly higher than in the control group, corresponding to the well-known observation that patients with allergic rhinitis have

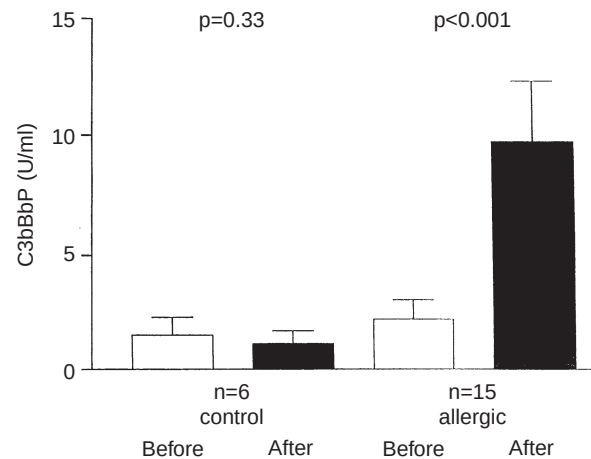


Fig. 3. Mean (and standard error of the mean [SEM]) C3bBbP level in the nasal lavage fluid before (white bars) and after (black bars) nasal ragweed challenge in control and allergic subjects. The C3bBbP level remained low in the lavage fluid of the control group. Allergic patients responded with a significant ($p=0.001$, Wilcoxon matched-pair test) increase in the C3bBbP level.

higher baseline nasal temperature values than the controls (14).

Both expiratory and inspiratory nasal resistance increased during the nasal challenge, but only the change in the inspiratory resistance was significant in allergic patients.

The non-allergic controls had the highest NIPF values, which showed no change after the challenge. In the group of allergic patients, a significant decrease in the NIPF was observed following the provocation process.

Complement activation product levels in nasal lavage fluids

Complement activation product levels in nasal lavage fluids after allergen provocation are shown in Fig. 3. In the control group we did not find any change from the initial low level of C3bBbP. Allergic patients, however, responded with a greater than four-fold rise (from 2.23 to 9.73 U/ml) in the C3bBbP levels measured in the nasal

Table 1. Comparison of clinical data recorded in ragweed provocation tests

	Allergic patients (n=15)			Controls (n=6)		
	Before provocation	After provocation at the threshold dose	p-value*	Before provocation	After provocation at the threshold dose	p-value*
Symptom score	0.00 (0)	12.2 (3.5)	<0.001	0.00 (0)	0.00 (0)	
Nasal temperature (°C)	33.10 (0.84)	35.34 (0.36)	0.03	30.50 (1.19)	32.25 (0.62)	0.33
NIPF (l/min)	102.5 (12.78)	53.75 (11.45)	0.001	137.90 (11.4)	128.30 (16.21)	0.41
Nasal resistance exp. (kPa/l/s)	0.36 (0.05)	0.66 (0.16)	0.33	0.23 (0.06)	0.41 (0.12)	0.66
Nasal resistance insp. (kPa/l/s)	0.30 (0.03)	0.52 (0.07)	0.03	0.19 (0.01)	0.26 (0.03)	0.67

*Wilcoxon matched-pair test.

exp., expiratory; insp., inspiratory; NIPF, nasal inspiratory peak flow.

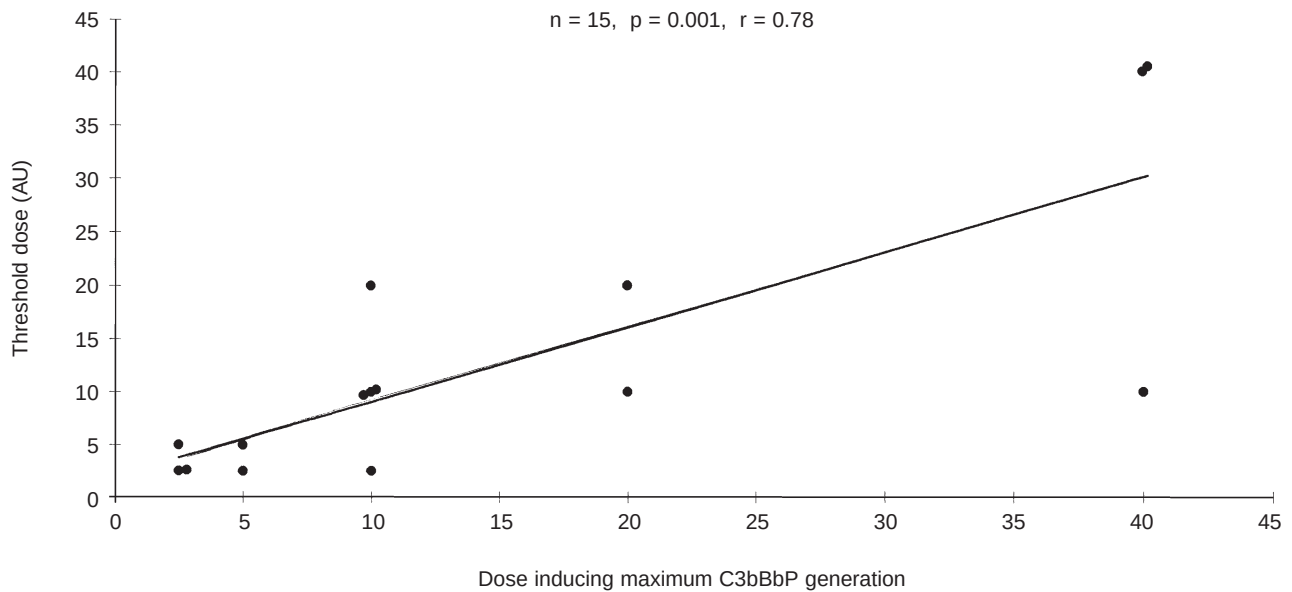


Fig. 4. Scatter plot showing the threshold dose that induced symptoms and the dose where the maximum complement (C3bBbP) activation was detected, in 15 ragweed allergic patients after nasal ragweed challenge.

lavage fluid after the nasal challenge with ragweed allergen ($p=0.001$).

Figure 4 shows a strong correlation between the threshold dose that induced symptoms and the dose where the maximum complement (C3bBbP) activation was detected in allergic patients ($r=0.78$, $p=0.001$).

Discussion

In this study we tried to model the pollen season with nasal challenge. The baseline measurements and the nasal challenge were carried out on the same day. The changes on the nasal mucosa following ragweed insufflation were evaluated with objective clinical parameters. The young adult controls were close in age to the adolescent patients.

The nasal challenge was started with an allergen dose that is appropriate for measuring nasal sensitivity in clinical practice (12). Earlier we observed that the symptoms and the registered objective parameters could worsen after the threshold level was exceeded (Fig. 2). The treatment of the nasal lavages fulfilled the requirements of the original work of Andersson et al. (7). Under the experimental conditions of this study, C3bBbP is not degraded until the end of the examination as a result of the stabilizing effect of propeptide.

In the present study we showed how the development of clinical symptoms and the objective signs of nasal mucosal inflammation are related to the formation and release into the

lavage fluid of a complement activation product, C3bBbP, in ragweed-allergic adolescents, in a nasal challenge setting. The same events, however, did not occur in the allergen-exposed mucosa of non-allergic subjects. These findings confirm and extend the previous observations of Andersson et al. (7) on a very rapid generation of C3a des-arg and C5a des-arg after a challenge with grass and birch allergens in patients allergic to these pollens. Here we demonstrated that the same local complement activation also occurs in patients allergic to ragweed, which is the main allergen in the late summer season in Hungary (15). The novel finding is the strong correlation between threshold dose and the dose where the maximum complement activation was detected, indicating that the maximum complement activation appears at the same time as the symptoms of allergic rhinitis. Moreover, the generation of C3bBbP, an activation product of the alternative complement pathway, indicates that the allergen challenge leads to direct activation of this pathway and/or an amplification of the classical pathway. Our present findings are in agreement with the previous results of several authors who found local or systemic complement activation in the mucosa of allergic patients after being in contact with allergens (3,5,7).

The involvement of complement in type I hypersensitivity reactions is under debate. According to our previously published hypothesis (10), exposure of the mucosa of the ragweed-allergic patients to ragweed pollen triggers both a type I hypersensitivity reaction and a local

generation of complement activation products. This hypothesis is based on the strong correlation between the amount of complement activation products generated upon incubation with ragweed allergen extract in the sera of ragweed-allergic patients and the symptom score registered in the same patients during the ragweed season (8–10). The complement activation products belong to the most efficient inflammatory mediators. A functional, active receptor for C3a is present in chronic myelogenous leukemia-derived basophil granulocytes (16). C3a, which is generated during complement activation, can lead to increased vascular permeability (17), markedly facilitates mucus formation in the lower parts of the respiratory tract (18), and induces production and release of interleukin-1 by cultured human monocytes (19). Fraser et al. (20), in the guinea-pig model, using trimellitic anhydride (a low-molecular-weight industrial compound that can cause asthma-like symptoms in humans), could directly prove the role of complement in the allergic response of the lung.

Our present findings indicate that in patients who are allergic to ragweed, provocation with ragweed allergen results in activation of the complement system and consequently in a rising level of complement activation products in the nasal mucosa. The lack of local complement activation in the non-allergic controls indicates that sensitization of subjects with specific allergen and triggering of the immunoglobulin E (IgE)-mediated type I hypersensitivity reaction by exposure to the allergen are prerequisites for local complement activation by the allergen. The most probable explanation for this observation is as follows. Exposure of the mucosa of allergic patients to specific allergens leads to the degranulation of mast cells and basophils, as well as to the release of histamine and other mediators in the mucosa. These mediators cause a markedly increased exudation from vessel walls, which results in a high concentration of plasma proteins, including complement components, on the mucosal surface. This process provides sufficient complement proteins for an efficient allergen-induced local activation of the complement pathways. As both IgE-mediated type I hypersensitivity reactions and complement activation are known to be very rapid processes, the whole process may take place within minutes, as found in the present study and by Andersson et al. (7).

Mast cells are known to carry receptors for the complement activation products, C3a and C5a. Therefore it is possible that complement activation may facilitate degranulation of the mast cells

and in this manner enhance the IgE-mediated type I hypersensitivity reaction. Experiments of Thomas et al. (21), on the augmentation of antigen-stimulated histamine release from human basophils by serum-treated zymosan particles, also seem to support this assumption.

Clearly, further studies are necessary to clarify the exact mechanism of interaction between the IgE-induced mediator formation and the complement activation products that are generated on the allergen-exposed mucosa of the allergic patients. The significance of the allergen-induced complement activation in the allergic inflammation, however, can be assessed by local application of complement-inhibitory drugs (22). If the role of complement activation in allergen-induced local inflammation is definitely demonstrated, these drugs may be appropriate for patient management.

In summary, the principal finding in this study was the demonstration of a strong correlation between the threshold dose that induced symptoms and the dose where the maximum complement activation was detected in the nasal lavage fluid after nasal ragweed allergen challenge in allergic adolescents. The results support the hypothesis of a role of the complement system in nasal allergic inflammation.

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