Complement Factors C3a and C5a Are Increased in Bronchoalveolar Lavage Fluid after Segmental Allergen Provocation in Subjects with Asthma

NORBERT KRUG, THOMAS TSCHERNIG, VEIT J. ERPENBECK, JENS M. HOHLFELD, and JÖRG KÖHL

Fraunhofer-Institute of Toxicology and Aerosol Research, Hannover, Germany; and Departments of Respiratory Medicine, Functional and Applied Anatomy, and Microbiology, Hannover Medical School, Hannover, Germany

Allergic asthma is thought to be the result of an inappropriate specific immune response against common environmental antigens. However, studies of animal asthma models have also linked the innate immune system, in particular complement factors C3a and C5, to murine airway hyperresponsiveness. Because the possible role of these anaphylatoxins in patients with asthma is not understood, we tested the hypothesis that C3a and C5a will increase in the bronchoalveolar lavage (BAL) fluid of patients with asthma after segmental allergen provocation. In a group of 15 subjects with mild asthma we found a significant upregulation of C3a and C5a 24 h after allergen challenge compared with baseline values ($p < 0.01$). In a control group of healthy volunteers the concentrations remained basically unchanged. Furthermore, we found a strong correlation between both anaphylatoxins and the number of eosinophils ($p < 0.01$) and, to a lesser degree, with the number of neutrophils ($p < 0.05$) in BAL fluid. These data suggest a contribution of anaphylatoxins C3a and C5a to the pathogenesis in asthma. However, the pathogenic role of these substances in relation to asthma remains to be elucidated, for example, by using anaphylatoxin receptor blockers as a possible new therapeutic principle.

Keywords: asthma; complement; C3a; C5a

Asthma is a chronic inflammatory disease of the bronchi that arises as a result of inappropriate acquired immunological responses to common environmental antigens in genetically susceptible individuals. It is thought to be mediated by $CD4^+$ T lymphocytes producing a type 2 cytokine profile, which leads to elevated specific IgE, eosinophilia and airway hyperresponsiveness (AHR). However, the hypothesis has been proposed that the complement system, which forms a central core of innate immune defense against mucosal bacteria, viruses, fungi, helminths, and other pathogens, might also play an important role in the pathogenesis of asthma: Humbles and coworkers (1) and Bautsch and coworkers (2) have shown that C3a receptor-deficient mice and guinea pigs are protected against bronchoconstriction and AHR seen after allergen challenge. Interestingly, there was no difference in eosinophilic airway inflammation, helper T cell type 2 (Th2) cytokine production, and IgE production between C3a receptor-deficient and wild-type animals, which demonstrates that airway inflammation and AHR are two independent features of asthma.

On the other hand, Karp and coworkers (3) have shown that C5 deficiency leads to increased susceptibility to allergen-induced AHR in mice, which might be explained by decreased interleukin 12 (IL-12) production in C5-deficient animals. These animal studies suggest that the complement proteins C5a and C3a have opposite effects. However, for the *in vivo* situation in patients with asthma only limited information about the role of these anaphylatoxins is available. Many features of bronchial asthma such as smooth muscle contraction; mucous secretion; and recruitment of eosinophils, mast cells, and neutrophils are consistent with the action of C3a and C5a.

Therefore, the aim of our study was to evaluate the local production of C3a and C5a at the site of allergic inflammation in bronchial asthma. For this purpose, the well-defined and standardized protocol of segmental allergen challenge followed by bronchoalveolar lavage (BAL) was used in asthmatic and control subjects. The samples were taken 24 h after the challenge, a time point when the late-phase inflammatory response was characterized by marked eosinophilia.

METHODS

Study Subjects

Fourteen patients with mild asthma (mean age, 25.5 ± 0.7 yr; 5 women and 9 men; mean FEV_1, 97.4 ± 4% of predicted value; geometric mean $PC_{20}FEV_1$, 3.4 mg of histamine per ml; geometric mean IgE, 160 kU/L) and 9 normal control subjects (mean age, 27.6 ± 1.4 yr; 2 women and 7 men; mean FEV_1, 102 ± 1% of predicted value; geometric mean $PC_{20}FEV_1$, 14.5 mg of histamine per ml; geometric mean IgE, 10 kU/L) participated in the study. All patients had mild allergic asthma according to the Heart, Lung, and Blood Institute of the National Institutes of Health (4) and each patient had elevated IgE levels and a positive skin prick test to one or more of eight common allergens. Most of them (n = 8) had seasonal asthma and were tested out of season. Patients were using only salbutamol when required for relief of symptoms. The normal control subjects had no history of allergic or other diseases, negative skin prick tests, normal IgE ($\leqslant$ 100 IU/ml), normal lung function tests, and no bronchial hyperresponsiveness ($PC_{20} > 8$ mg/ml). All study subjects were nonsmokers and no subject had acute bronchitis 4 wk before the investigations. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the studies, which were approved by the Ethics Committee of Hannover Medical School (Hannover, Germany).

Segmental Allergen Challenge

Segmental allergen challenge was performed as previously described (5, 6). Briefly, a baseline BAL was performed in the inferior lingular bronchus with five 20-ml volumes of sterile saline. As a control, 10 ml of saline solution was instilled into the superior lingular bronchus. Finally, 10 ml of allergen solution was instilled into the medial segment of the middle lobe. The allergen extract used for segmental allergen challenge was that which produced the largest wheal response on skin prick testing and the chosen concentration was one-tenth the dilution in saline that elicited a 3-mm-diameter skin wheal response. After 24 h, subjects (all patients with asthma and five control subjects) were rebronchoscoped and the superior lingular bronchus and the medial middle bronchus were lavaged with 100 ml of saline. A commercially available endotoxin detection assay (E-toxate, *Limulus* amebocyte lysate; Sigma, St. Louis, MO) was used to demonstrate that the instilled saline and allergen solutions were free of endotoxin.

(*Received in original form October 18, 2000; accepted in final form August 17, 2001*)

Supported by research grants from the Deutsche Forschungsgemeinschaft to N.K. (Kr 1405/2-1) and J.K. (KO 1245/1-1).

Correspondence and requests for reprints should be addressed to Norbert Krug, M.D., Department of Immunology, Allergology and Clinical Inhalation, Fraunhofer-Institute of Toxicology and Aerosol Research, Nikolai-Fuchs-Str. 1, 30623 Hannover, Germany. E-mail:Krug@ita.fraunhofer.de

Am J Respir Crit Care Med Vol 164. pp 1841–1843, 2001
DOI: 10.1164/rccm2010096
Internet address: www.atsjournals.org

Processing of BAL Fluid

BAL fluid samples were processed as described (5, 6). Briefly, cells were filtered through a 100-μm pore size filter and centrifuged, and the supernatant was stored at −80° C. The total count of nucleated cells was performed with a Neubauer hemocytometer. Differential cell counts were performed from Cytospin slides, with 300 cells per slide being counted.

Determination of C3a/C3adesArg and C5a/C5adesArg Concentrations in BAL Fluid

C3a/C3adesArg and C5a/C5adesArg concentrations were measured by the ABICAP assay (Biognosis, Jülich, Germany) as described (7). Briefly, C3a/C3 neoepitope-specific anti-human C3a/C3adesArg or anti-human C5a monoclonal antibodies (MAbs) were coupled to CNBr–Sepharose and packed into small polystyrol columns (ABICAP). The sample was then applied to the column, diluted 1:2 in phosphate-buffered saline (PBS). After antigen capture, the column was washed with PBS and a complex consisting of a biotinylated detection MAb and streptavidin–fluorescein isothiocyanate (FITC) was applied to the column. C3a/C3adesArg or C5a/C5adesArg concentrations were determined by eluting the immune complex comprising the corresponding anaphylatoxin, the biotinylated antibody, and the streptavidin–FITC and subsequently determining the fluorescence intensity.

Statistical Analysis

The Mann–Whitney U test was used for intergroup comparison between patients with asthma and control subjects. For comparison of paired data within the major study groups (baseline, saline, and allergen), significant variability was first established by using the Friedman nonparametric test. The Wilcoxon test was then used for the individual comparisons. Correlation coefficients were obtained by the Spearman rank-order method. Probability values of $p < 0.05$ were accepted as significant.

RESULTS

As shown previously (5, 6), there was no difference in the recovery of BAL fluid between patients with mild asthma and normal subjects before or 24 h after sham or allergen challenge. The number of neutrophils was increased in BAL fluid from patients with asthma and healthy subjects after saline or allergen challenge ($p < 0.05$), whereas the number of eosinophils increased only in the BAL fluid from patients with asthma after allergen challenge ($p < 0.01$).

At baseline, concentrations of C3a and C5a in BAL fluid were not different between patients with asthma and healthy control subjects. After allergen challenge in patients with asthma there were increased concentrations of C3a (median, 29.0 ng/ml [range, 3.9–854.0 ng/ml]) and C5a (16.4 [1.0–54.0] ng/ml) compared with baseline (C3a, 3.4 [1.9–13.5] ng/ml; C5a, 1.2 [0.5–3.7] ng/ml) and saline challenge (C3a, 3.9 [3.9–46.0] ng/ml; C5a, 3.6 [1.0–18.0] ng/ml) (Figures 1 and 2). The small increase in C3a and C5a levels after saline challenge was also significantly different from baseline. In healthy control subjects no change in the concentrations of C3a and C5a were detectable after either saline or allergen challenge.

In patients with asthma there was a significant correlation between the concentration of C3a ($R = 0.90$, $p < 0.01$) or C5a ($R = 0.88$, $p < 0.01$) and the number of eosinophils 24 h after allergen challenge. Furthermore, the number of neutrophils after allergen challenge was correlated to C3a ($R = 0.66$, $p < 0.05$) and C5a ($R = 0.71$, $p < 0.05$) levels and after saline challenge to C5a ($R = 0.68$, $p < 0.05$).

DISCUSSION

We investigated the production of the anaphylatoxins C3a and C5a in BAL fluid after segmental allergen challenge in allergic asthma. We found a significant upregulation of C3a and C5a 24 h after allergen, and a small, but statistically significant, increase after saline challenge. In a control group of healthy, nonatopic volunteers the concentrations remained basically unchanged. Furthermore, after allergen challenge, we found a strong correlation between both anaphylatoxins and the number of eosinophils and, to a lesser degree, with the number of neutrophils.

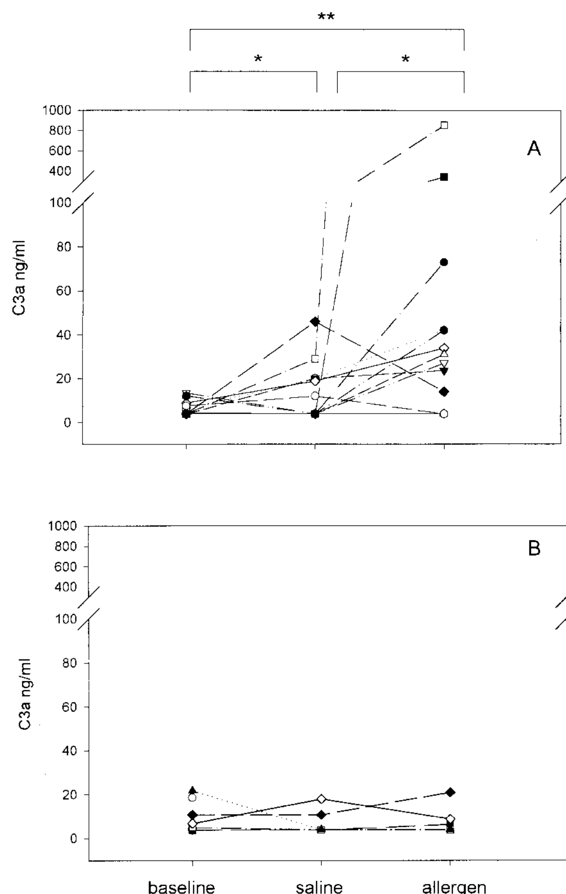

Figure 1. C3a levels in BAL fluids of (*A*) patients with asthma (n = 14) and (*B*) healthy control subjects (n = 9). Depicted are the data obtained from BAL samples before challenge and 24 h after segmental challenge with either saline or allergen. All healthy volunteers were lavaged before challenge. From five volunteers samples were taken after challenge with saline or allergen (*p < 0.05; **p < 0.01).

These data support the hypothesis proposed by animal studies (1–3) that, in addition to acquired immune responses, the innate immune system and in particular the complement system are involved in the pathogenesis of allergic asthma. Because we have not used a control group of nonasthmatic atopic subjects we cannot differentiate the role of the complement system in asthma versus atopy.

Limited data from other human *in vivo* studies exist, which further suggest the involvement of C3a and C5a in allergic asthma: C3a has been shown to be increased in BAL fluid of patients with stable asthma compared with healthy control subjects (8). In the study by Humbles and coworkers (1) C3a was elevated 4–6 h after segmental allergen challenge compared with sham challenge in patients with mild asthma, an early time point, which precedes the recruitment of eosinophils into the airways. In that study the median concentration of C3a at baseline was 17 times higher and after allergen challenge 4 times higher as compared with our results. Therefore,

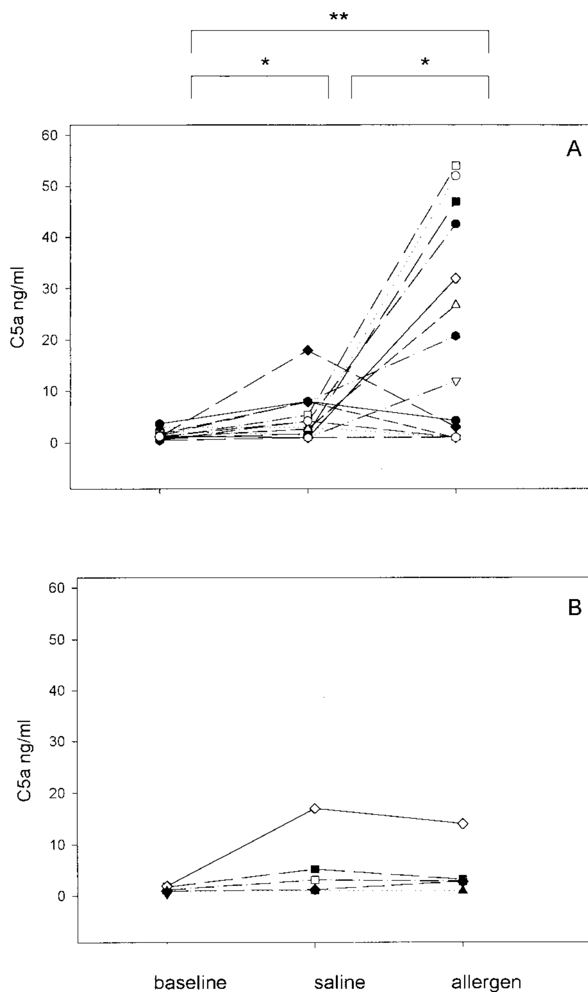

Figure 2. C5a levels in BAL fluids of (*A*) patients with asthma and (*B*) healthy control subjects. For further information *see* Figure 1.

the 2-fold increase 4–6 h after allergen challenge was less pronounced than the 8-fold increase at 24 h in our study. Regarding C5a, a study from Teran and coworkers (9) identified C5a in BAL fluid of patients with asthma as a major neutrophil chemotactic factor. The question remains whether the upregulation of anaphylatoxins in BAL fluid is the result of increased local production or of increased spillover from the systemic circulation. It has been clearly shown by other investigators that pulmonary alveolar Type II epithelial cells synthesize and secrete C3 and C5 (10). Because we determined high levels of anaphylatoxins in the BAL fluid of subjects without any signs of systemic inflammation a major contribution of local complement production is most likely.

Data from our own animal studies (2, 3) and results from Humbles and coworkers (1) primarily suggest opposite effects of C3a and C5a on AHR, for example, C3a increases AHR whereas C5a reduces AHR by release of IL-12 from macrophages (11). Here we found elevated levels of both C3a and C5a in human asthmatic airways after allergen challenge, suggesting an exacerbating effect of both anaphylatoxins. Our data fit with the results of two reports demonstrating that under some circumstances C5a may suppress IL-12 production (12, 13).

Strong correlation between elevated C3a and C5a concentrations and eosinophilia as well as neutrophilia suggests that eosinophilia might be induced by C3a and neutrophilia by C5a. However, definite proof concerning whether the anaphylatoxins C3a and C5a are causally related to the allergic inflammation in human asthma will not be possible before anaphylatoxin receptor blockers, which have been successfully used in mice (14, 15), are available for clinical use in patients with asthma. It has been demonstrated that receptors for C3a and C5a are expressed and upregulated on bronchial epithelial and smooth muscle cells in a murine asthma model (16).

References

1. Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y, Gerard NP, Gerard C. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 2000;406:998–1001.
2. Bautsch W, Hoymann HG, Zhang Q, Meier-Wiedenbach I, Raschke U, Ames RS, Sohns B, Flemme N, Meyer zu Vilsendorf A, Grove M, *et al.* Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. *J Immunol* 2000;165:5401–5405.
3. Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ, Kohl J, Wahl L, Kuperman D, Germer S, *et al.* Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 2000;1:221–226.
4. National Heart, Lung, and Blood Institute. International Consensus Report on diagnosis and treatment of asthma. NHLBI publication no. 92-3091. *Eur Respir J* 1992;5:601–641.
5. Hohlfeld JM, Ahlf K, Enhorning G, Balke K, Erpenbeck VJ, Petschallies J, Hoymann HG, Fabel H, Krug N. Dysfunction of pulmonary surfactant in asthmatics after segmental allergen challenge. *Am J Respir Crit Care Med* 1999;159:1803–1809.
6. Krug N, Cruikshank WW, Tschernig T, Erpenbeck VJ, Balke K, Hohlfeld JM, Center DM, Fabel H. Interleukin 16 and T-cell chemoattractant activity in bronchoalveolar lavage 24 hours after allergen challenge in asthma. *Am J Respir Crit Care Med* 2000;162:105–111.
7. Hartmann H, Lubbers B, Casaretto M, Bautsch W, Klos A, Kohl J. Rapid quantification of C3a and C5a using a combination of chromatographic and immunoassay procedures. *J Immunol Methods* 1993;166:35–44.
8. van de Graaf EA, Jansen HM, Bakker MM, Alberts C, Eeftinck Schattenkerk JK, Out TA. ELISA of complement C3a in bronchoalveolar lavage fluid. *J Immunol Methods* 1992;147:241–250.
9. Teran LM, Campos MG, Begishvilli BT, Schroder JM, Djukanovic R, Shute JK, Church MK, Holgate ST, Davies DE. Identification of neutrophil chemotactic factors in bronchoalveolar lavage fluid of asthmatic patients. *Clin Exp Allergy* 1997;27:396–405.
10. Strunk RC, Eidlen DM, Mason RJ. Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. *J Clin Invest* 1988;81:1419–1426.
11. Henson P. Complementing asthma. *Nat Immunol* 2000;1:190–192.
12. Wittmann M, Zwirner J, Larsson VA, Kirchhoff K, Begemann G, Kapp A, Gotze O, Werfel T. C5a suppresses the production of IL-12 by IFN-gamma-primed and lipopolysaccharide-challenged human monocytes. *J Immunol* 1999;162:6763–6769.
13. Braun MC, Lahey E, Kelsall BL. Selective suppression of IL-12 production by chemoattractants. *J Immunol* 2000;164:3009–3017.
14. Heller T, Hennecke M, Baumann U, Gessner JE, zu Vilsendorf AM, Baensch M, Boulay F, Kola A, Klos A, Bautsch W, *et al.* Selection of a C5a receptor antagonist from phage libraries attenuating the inflammatory response in immune complex disease and ischemia/reperfusion injury. *J Immunol* 1999;163:985–994.
15. Ames RS, Lee D, Foley JJ, Jurewicz AJ, Tornetta MA, Bautsch W, Settmacher B, Klos A, Erhard KF, Cousins RD, *et al.* Identification of a selective nonpeptide antagonist of the anaphylatoxin C3a receptor that demonstrates antiinflammatory activity in animal models. *J Immunol* 2001:166:6341–6348.
16. Drouin SM, Kildsgaard J, Haviland J, Zabner J, Jia HP, McCray PB Jr, Tack BF, Wetsel RA. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. *J Immunol* 2001;166:2025–2032.