

Expression of the anaphylatoxin receptors C3aR and C5aR is increased in fatal asthma

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Background: The mechanisms leading to death from asthma are not completely understood. Recent studies suggest the involvement of the anaphylatoxins C3a and C5a, generated during complement activation, and their receptors C3aR and C5aR in the pathogenesis of asthma.

Objective: The aim of our study was to investigate the expression of C3aR and C5aR in fatal asthma.

Methods: We analyzed lung tissue from 14 subjects who died of asthma (fatal asthma; FA) and 14 subjects who died of nonpulmonary causes (controls) and bronchial biopsy specimens from 16 subjects with mild intermittent asthma (MIA). C3aR and C5aR expression was evaluated by immunohistochemistry, and a semiquantitative analysis of the intensity of staining was performed according to a visual analogue scale (score, 0-3).

Results: C3aR was expressed on airway epithelium, smooth muscle, submucosal, and parenchymal vessels. C5aR was expressed on myeloid cells infiltrating the submucosa and on airway epithelium. Statistical analysis demonstrated higher expression of C3aR on submucosal vessels in FA compared with controls and MIA (median [minimum-maximum], controls, 0.24 [0-1.48]; MIA, 0.0 [0-1.00]; FA, 1.56 [0.13-3]; $P = .002$). C3aR was also increased on parenchymal vessels in FA (controls, 0.56 [0-2.00]; FA, 1.81 [0.5-3]; $P = .0004$). C5aR expression on airway epithelium was increased in FA compared with controls and MIA (controls, 1.25 [0.25-3]; MIA, 1.00 [0-2.00]; FA, 3.00 [1.13-3.00]; $P = .001$).

Conclusion: The results of our study suggest a role of complement in FA. (J Allergy Clin Immunol 2005;115:1148-54.)

Key words: Complement, C3aR, C5aR, fatal asthma, lung tissue, mild intermittent asthma, submucosal vessels, parenchymal vessels, epithelium

Abbreviations used

FA: Fatal asthma
LA: Large airway
MIA: Mild intermittent asthma
SA: Small airway

Despite improved knowledge of the natural history of the disease and the availability of new treatments, asthma mortality rates are still unacceptably high,¹ and the mechanisms leading to death are not completely understood. The most important determinants of fatal asthma (FA) include underestimation of disease severity, inadequate treatment with inhaled bronchodilator abuse in the absence of regular anti-inflammatory therapy, and poor socioeconomic status.²⁻⁴ Although severe asthma and previous near-fatal attacks are risk factors for subsequent fatal episodes, sudden death also occurs in milder forms of the disease.² Examination of tissue specimens from FA showed not only signs of activation of an allergic, IgE-mediated immune response with typical eosinophil infiltration, mast cell activation, and expression of T_H2-type cytokines,⁵ but also neutrophil infiltration⁶ and CD8⁺ cytotoxic T-cell activation.⁷ New insights in the pathologic mechanisms of asthma death are urgently needed to lead to new treatment strategies.

The complement system, a key element of innate immune defense against bacteria, viruses, fungi, and other pathogens, has been shown to be involved in the inflammatory reactions that characterize asthma. The anaphylatoxins C3a and C5a, generated during complement activation, can stimulate *in vitro* smooth muscle contraction, increase microvascular permeability,⁸ and induce activation and migration of several inflammatory cells, including neutrophils, eosinophils, and mast cells.⁹⁻¹² In animal models of asthma, complement activation has been demonstrated to modulate airway inflammation and hyper-responsiveness,¹³⁻¹⁵ whereas in patients with asthma, increased C3a and C5a levels have been detected in the bronchoalveolar lavage fluid and in induced sputum.¹⁶⁻¹⁸ On the basis of these data, it is suggested that C3a and C5a may contribute to the pathogenesis of asthma by 2 main mechanisms: their proinflammatory effects and their capability to regulate T_H2 polarization (see review¹⁹).

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The C3a and C5a anaphylatoxins bind to specific receptors, C3aR and C5aR, and the recently discovered orphan receptor C5L2.²⁰⁻²² The expression of C3aR and C5aR, formerly thought of as exclusive for myeloid cells, has now been demonstrated on nonmyeloid cells of the liver and brain and on isolated endothelial and epithelial cells.²³⁻²⁶ A role for the anaphylatoxin receptors in asthma is suggested by the finding that in different animal models, C3aR gene deletion results in attenuation of bronchoconstrictive and inflammatory responses.^{16,27,28} Similarly, blocking C5aR with a selective antagonist inhibits the late allergic response and suppresses tissue eosinophilia and neutrophilia induced by allergen challenge.²⁹ The presence of both receptors has recently been described in noninflammatory cells in normal human lung,⁸ but until now, no studies investigated their expression in the lungs of subjects with asthma.

On the basis of this background, we decided to investigate whether there was a different expression of the anaphylatoxin receptors, C3aR and C5aR, in lung tissue from patients with FA and mild intermittent asthma (MIA) and controls who died of nonpulmonary causes.

METHODS

Study population

Fatal asthma and control lung tissue was obtained from subjects undergoing autopsy in the years 1996 to 2000 at the Department of Pathology of Sao Paulo University, Brazil. Lung tissue from a subgroup of these patients was already used in other studies.^{30,31} Bronchial biopsies of MIA were collected at the Department of Pulmonology, Leiden University Medical Centre, The Netherlands, during another study.³²

Fourteen nonsmoking subjects who died of asthma were included in the study. All had a history of asthma (information provided by the next of kin before autopsy) and died during an acute attack. Further clinical data were obtained through interviews with the relatives of the deceased. For all of the included subjects, information about treatment, duration of disease, and previous hospitalization for asthma was obtained. Subjects were not included in the study when lung diseases other than asthma or any systemic diseases were reported. Pathology inclusion criteria were lung hyperinflation and hypersecretion and the following histological changes related to asthma: epithelial desquamation, thickening of the subepithelial reticular layer, thickening of the smooth muscle layer, hypertrophy of submucosal glands, and mucosal inflammation with or without eosinophils.

Fourteen nonsmoking subjects who died of nonpulmonary causes, with no clinical history or pathological indications of lung disease, were selected as a control group. Most of the subjects had myocardial infarction as the cause of death. Those subjects whose cause of death was known to activate systemically the complement cascade, such as sepsis or trauma,³³⁻³⁵ were not included in the study. Use of FA and control tissue was approved by the Board for Human Studies of the School of Medicine of the University of Sao Paulo. All of the FA and control subjects were Brazilians.

Sixteen nonsmoking subjects with MIA underwent bronchoscopy with bronchial biopsy. All subjects had a clinical history of asthma and documented airway responsiveness to methacholine (PC₂₀ to methacholine bromide <9.6 mg/mL).³⁶ Symptoms of asthma were stable and controlled by on-demand use of inhaled β_2 -agonists for the 6 months preceding bronchoscopy. The subjects had no history of recent exposure to allergens or upper respiratory tract infections.³²

The Hospital Medical Ethics Committee of the Leiden University Medical Center approved the study, and all participants gave written informed consent.

Tissue specimens

Blocks of lung tissue of FA and controls were collected between 6 and 12 hours after death randomly from central and peripheral lung compartments. Specimens were fixed in 4% formaldehyde for 24 hours and paraffin-embedded. Sections 5 μ m thick were cut and screened by using a hematoxylin-eosin staining to identify the presence of airways suitable for analysis. Large and small airways were classified on the basis of the presence/absence of cartilaginous plaques in their wall,³⁷ and airway basement membrane perimeter was measured in the transversally cut airways (short/long diameter ratio larger than 0.6) with the aid of the KS400 image analysis apparatus and KSRun Software (Version 3.0, 1997; Carl Zeiss Vision GmbH, Göttingen, Germany). Only large and middle-sized bronchi, with a perimeter larger than 6 mm, were used for comparison between resection and biopsy material.

Bronchial biopsy specimens were obtained from the segmental or subsegmental bronchi of the left upper lobe during fiberoptic bronchoscopy, as previously described.³²

Immunohistochemistry

The sections were deparaffinized for immunoperoxidase staining with xylene and 100% ethanol. Endogenous peroxide was blocked with 0.3% hydrogen peroxide in methanol for 10 minutes at room temperature. Antigen retrieval was performed when required. Sections were incubated for 1 hour at room temperature in a humid chamber with either mouse monoclonal antihuman C3aR antibody (clone 8H1, dilution of 1/600; BD Pharmingen, San Diego, Calif) or mouse monoclonal antihuman C5aR antibody (P12/1, dilution of 1/1250; a gift of Dr J. Zwirner, Göttingen, Germany). To control for possible aspecific binding, every sixth slide in each experiment was incubated with PBS/1% BSA. All of the sections were stained within 1 staining session using antibodies coming from 1 batch. After washing in PBS pH 7.5, slides were incubated for 30 minutes with antimouse horseradish peroxidase (DAKO Corp, Carpinteria, Calif). Tissue-bound immunoglobulin complexes were visualized by incubation with the Novared-EnVision system (DAKO Corp) and counterstained with hematoxylin (Klinipath, Duiven, The Netherlands). No evidence of aspecific binding was detected in the slides incubated with PBS.

Histological analysis and quantification

Tissue sections were mounted in Pertex (Histolab, Gothenburg, Sweden) and analyzed by light microscopy at 400 $\times$ magnification. Two different observers performed 2 measurements independently. The specimens were coded and the measurement made without knowledge of the clinical data. A semiquantitative analysis was performed on the intensity of the staining by using a visual analogue scale (0 = absent or very weak; 1 = weak; 2 = moderate; 3 = strong). To avoid possible biases caused by the selection of the fields to examine, we selected the airways suitable for analysis on the basis of a priori criteria (integrity of the epithelium for the analysis of epithelial staining, and intactness of the lamina propria in the whole airway circumference for the analysis of the smooth muscle and submucosal vessels), and in those airways, we analyzed all of the tissue available. In postmortem specimens, parenchymal staining was also evaluated, screening 10 to 20 fields covering all parenchymal tissue on the slide.

Data analysis

Statistical analysis was performed by using the SPSS for Windows software package version 11.0 (SPSS, Chicago, Ill). Group data were

TABLE I. Clinical characteristics of control subjects and subjects with MIA and FA

	Controls (n = 14)	MIA (n = 16)	FA (n = 14)
Sex (M/F)	4/10	6/10	5/9
Age, y*	48.5 ($\pm$ 4.4)	23.9 ($\pm$ 1.3)	38.2($\pm$ 4.9)
Duration of disease, y*	NA	ND	21 ($\pm$ 14.8)
Age of onset of disease†			
<10 y	NA	ND	57.1
10-20 y	NA	ND	21.4
>20 y	NA	ND	21.4
Oral and/or inhaled corticosteroids†	NA	0	28.5
Inhaled bronchodilators†	NA	100	100
Hospital admissions in last year†	NA	ND	28.5
Mechanical ventilation†	0	NA	0
Atopy†	ND	93.7	ND
FEV ₁ (% predicted)*	ND	105.4 ($\pm$ 3.6)	ND
Forced vital capacity (% predicted)*	ND	109.1 ($\pm$ 3.2)	ND
PC ₂₀ (mg/mL)*	ND	2.6 ($\pm$ 0.4)	ND

ND, Not determined; NA, not applicable.

Data are expressed as *mean value $\pm$ SEM and †percentage of group.

expressed as means ($\pm$ SEMs) when normally distributed or medians (minimum-maximum) when not normally distributed. The non-parametric Kruskal-Wallis test was performed to compare median expression of C3aR and C5aR in controls, MIA, and FA, and the Mann-Whitney *U* test was used post hoc for pairwise comparisons. Age differences in the 3 groups were calculated by 1-way ANOVA, and the Bonferroni post hoc correction was applied. The Student *t* test was used to compare airway sizes of controls and FA. Correlations were evaluated by using a nonparametric test (Spearman rank test). In all cases, differences at *P* values less than .05 were considered significant. To assess intraobserver and interobserver repeatability, intraclass correlation coefficient was calculated by reliability analysis in a 2-way mixed model.³⁸

RESULTS

Characteristics of study subjects

Characteristics of the study subjects are presented in Table I. Age was similarly distributed in the FA and control groups, whereas it was significantly different between FA and MIA (*P* = .038) and between controls and MIA (*P* = .000). In the FA group, 78.5% of the patients had early onset of the disease and a long history of asthma. There was no correlation between fatality and age of onset or duration of asthma. Only a small proportion of the subjects who died from asthma were using regular anti-inflammatory treatment. In the MIA group, all subjects but 1 were atopic; most of them were allergic to house dust mite.

C3aR expression in FA, MIA, and controls

C3aR expression was detected in epithelial cells, the smooth muscle layer, and submucosal and parenchymal

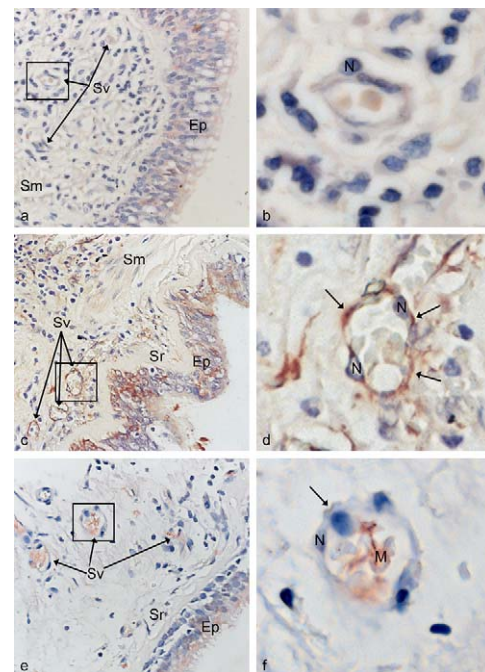


FIG 1. C3aR expression in large airways of a control patient (CTRL; A, B), a patient with FA (C, D), and a patient with MIA (E, F). A, Bronchial wall of a CTRL subject, with no staining of the submucosal vessels (arrows). B, Submucosal vessel in detail. C, Bronchial wall of a subject with FA, with intense staining of the submucosal vessels (arrows) and the epithelium. D, Submucosal vessel in detail. E, Bronchial biopsy of a subject with MIA, with weak or absent staining in the submucosal vessels (arrow). The epithelium is negative. F, Submucosal vessel in detail. E, Epithelium; M, myeloid cells; N, nucleus; Sm, smooth muscle layer; Sr, subepithelial reticular layer; Sv, submucosal vessels. The white squares in the left panels indicate the areas magnified in the right panels. Original magnification, 400 $\times$.

vessels (Figs 1 and 2). Cells infiltrating the submucosal layer, with the morphology of myeloid cells, were stained in a few subjects. We evaluated a mean number of 5 airways (2 large airways [LAs] and 3 small airways [SAs]) from each FA and 8 (3 LAs, 5 SAs) from the controls. LA perimeter range was 6.24 to 18.67 mm in FA (mean, 10.15 mm) and 7.37 to 13.75 mm in controls (mean, 7.37 mm), corresponding with airway generations 2 to 7.³⁹ There was not significant difference in the airway size of controls and FA. The expression of C3aR in the smooth muscle and epithelial layer was weak, with no differences among the 3 groups (data not shown). On the other hand, C3aR staining on submucosal vessels was significantly more intense in FA compared with MIA and controls (*P* = .002; Fig 3), whereas there were no differences between MIA and controls. A higher expression of C3aR was also detected in parenchymal vessels of FA compared with controls (*P* = .0004; Fig 3). In the FA and control groups, there were no significant differences in C3aR expression between large and small airways (data not shown). No correlation was found in the FA group between C3aR expression and duration or age of onset of the disease. C3aR expression was not correlated with age.

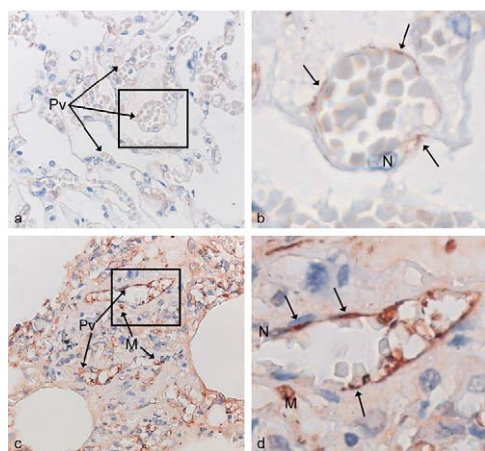


FIG 2. C3aR expression in parenchymal tissue from a control subject (CTRL; **A, B**) and FA (**C, D**). **A**, CTRL subject with weak or absent staining in the parenchymal vessels (small vessels and capillaries; arrows). **B**, Vessel in detail. **C**, Diffuse and intense staining in the parenchymal vessels in FA (arrows). Mild edema of the alveolar septa is present. **D**, Vessel in detail. N, Nucleus; M, myeloid cells; Pv, parenchymal vessels. The white squares in the left panels indicate the areas magnified in the right panels. Original magnification, 400 $\times$.

C5aR expression in human lung from FA, MIA, and controls

Staining for C5aR was present on myeloid cells infiltrating the submucosa and on airway epithelium in all groups (Fig 4). The staining pattern was granular. We evaluated a mean number of 4 airways (2 LAs, 2 SAs) from each FA and 6 (2 LAs, 4 SAs) from the controls. The expression of C5aR on epithelium was higher in FA compared with MIA and controls ($P = .001$; Fig 5). Post hoc analysis showed no differences between controls and MIA. C5aR expression was higher in large airways than in small airways ($P = .032$; data not shown) in FA subjects, but not in the control group. No correlation was found between C5aR expression and clinical characteristics of the FA subjects. There was no correlation between C3aR and C5aR expression in the FA group. C5aR expression was not correlated with age.

Intraobserver and interobserver repeatability

The intraclass correlation coefficient for intraobserver repeatability ranged from 0.92 to 0.97, showing a good agreement between the 2 measurements. The intraclass correlation coefficient for interobserver repeatability ranged from 0.90 to 0.91, showing a good agreement between the 2 observers.

DISCUSSION

Our study showed a higher expression of C3aR and C5aR in subjects who died of asthma compared with control subjects and with MIA. The higher expression of C3aR was detected in the submucosal and parenchymal

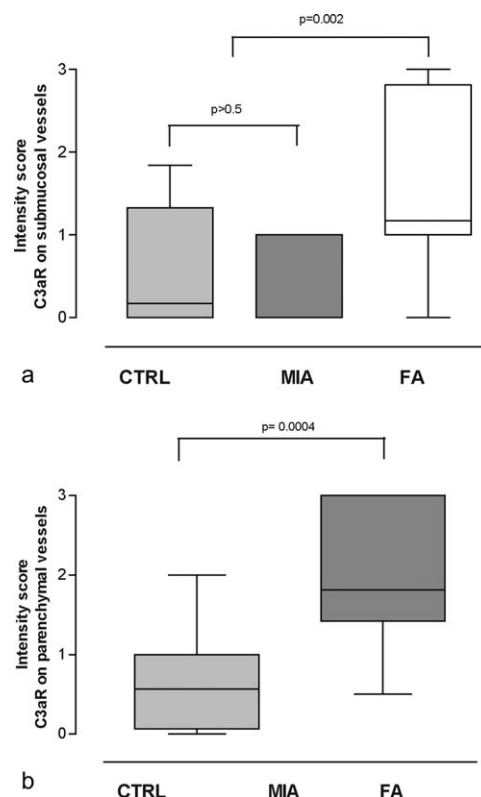


FIG 3. Box-whisker plots of C3aR in submucosal vessels of control (CTRL), MIA, and FA subjects (**A**) and in parenchymal vessels of CTRL and FA subjects (**B**).

vessels but not in epithelium and smooth muscle, whereas the higher C5aR expression was observed in the epithelium. Our results support recent studies demonstrating a role of innate immunity in the pathogenesis of asthma and extend them by suggesting that the patients who died of asthma may have been more susceptible to the effects of complement activation.

The high expression of C3aR and C5aR shown in our study appears to be a hallmark of FA, because in biopsies from patients with MIA, the intensity of staining of both receptors was low. Whether this indicates that receptor expression is related to the severity of the disease or to exacerbations or whether it is a marker of those exacerbations with a fatal outcome requires further investigation. A recent study from Nakano et al⁴⁰ showed that patients admitted to the hospital because of nonresolving asthma attacks had higher C3a plasma levels than patients immediately discharged after the attack and patients with stable chronic asthma. Furthermore, in other chronic inflammatory diseases such as central nervous system vasculitis, serial measurements of C3a and C5a have been shown to correlate with disease activity.⁴¹ Thus, it is likely that the high expression of C3aR and C5aR we demonstrated in FA signifies an increased sensitivity to the anaphylatoxins in a distinct group of patients. More studies on the kinetics of expression of the receptors, the signal transduction mechanisms, and their genetic

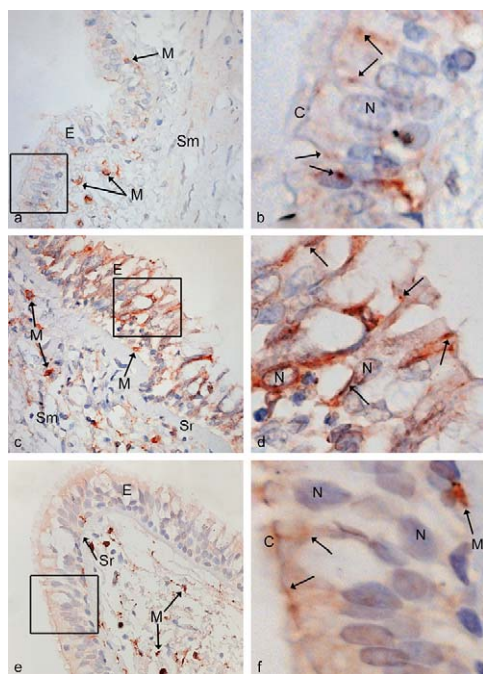


FIG 4. C5aR in large airways from control (CTRL; **A, B**) and FA (**C, D**) subjects, and in biopsies from MIA (**E, F**). **A**, Bronchial wall of a CTRL subject, with positive inflammatory cells infiltrating the submucosal and the epithelial layer (arrows). **B**, A detail of the epithelial layer, showing weakly positive granular staining. **C**, Bronchial wall of a FA subject, with intense epithelial staining for C5aR. Positive myeloid cells infiltrate the submucosal and epithelial layer (arrows). **D**, A detail of the epithelial layer, showing intense granular staining (arrows). **E**, Bronchial biopsy of a MIA subject, with positive myeloid cells infiltrating the submucosal and epithelial layer (arrows), and moderate epithelial staining. **F**, A detail of the epithelial layer. **C**, Cilia; **E**, epithelium; **M**, myeloid cells; **N**, nucleus; **Sm**, smooth muscle layer; **Sr**, subepithelial reticular layer. The white squares in the left panels indicate the areas magnified in the right panels. Original magnification, 400 $\times$.

variants⁴² are required to elucidate this point better and explore new potential therapeutic options.

How can a high expression of C3aR on vessels and of C5aR on airway epithelium contribute to the fatal outcome of the asthma attack? *In vitro*, triggering of C3aR on the surface of human endothelial cells induces expression of adhesion molecules, cytokines, and chemokines such as IL-8 and RANTES.^{43,44} This may facilitate migration of inflammatory cells into the lung tissue, one of the most important pathogenetic events in asthma. Indeed, leukocyte infiltration is a consistent finding in asthma and is more pronounced in FA.^{5,45} Thus, a higher C3aR expression on vessels can contribute to an increased and/or more rapid leukocyte recruitment in the airway of the subjects who died of asthma.

The finding that C3aR expression is higher on parenchymal vessels in patients with FA is intriguing. Even though asthma is a chronic disease of the airways, inflammatory processes have been described in the parenchymal tissue of subjects with asthma, such as alveolar eosinophilia and CD4⁺ T-lymphocyte infiltrate

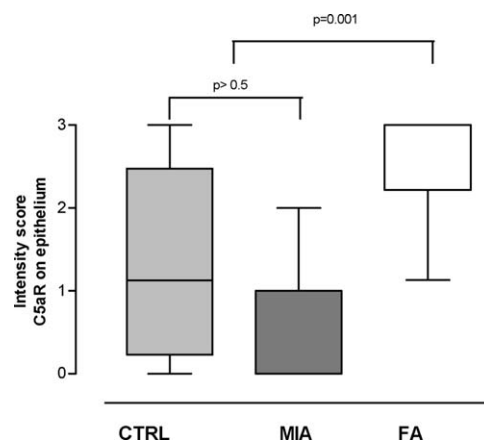


FIG 5. Box-whisker plots of C5aR in epithelium from large airways of controls (CTRL), MIA, and FA.

in nocturnal asthma⁴⁶ and increased expression of IL-5.⁴⁷ Furthermore, morphologic and inflammatory changes in the alveolar attachments were demonstrated in a group of subjects from the same population of patients with FA.^{31,48} Together with these results, our findings support the involvement of alveolar lung tissue in FA, but further studies are required on this topic.

We found higher expression of C5aR in the epithelium of subjects who died of asthma. C5aR mRNA is highly expressed in lung tissue in animal models of asthma and sepsis and is increased *in vitro* in endothelial cells by LPS, IFN- γ , and IL-6,⁴⁹ mediators involved in the inflammatory response to infections. The importance of infectious agents, mainly viruses, as triggers of asthma attacks is well known.⁵⁰⁻⁵² It has also been shown that allergens can directly activate the complement cascade *in vitro*,^{53,54} and recent studies demonstrated the involvement of the complement system in the airway response to ozone.⁵⁵ Indeed, air pollutants, particularly ozone, can induce or worsen asthma.^{56,57} Thus, a high expression of C5aR in the epithelium could play a role in the inflammatory response elicited by environmental stimuli such as infectious agents, allergens, and pollutants.

In our study, we have focused on the expression of specific complement receptors (C3aR and C5aR) in fatal asthma. On the basis of this study, it appears that complement activation, and the cellular response to complement activation, is part of the incompletely understood network of immunologic, inflammatory, and neural mechanisms that contribute to the pathogenesis of FA.

One limitation of our study, which is partly inherent to the study of FA, is that clinical data of the subjects who died of asthma were incomplete. Our study population had no regular documentation of respiratory function, and the estimation of their asthma severity is difficult. Data on atopy were not available for the population with FA. Nevertheless, the weak intensity of C3aR and C5aR staining in the atopic patients with MIA demonstrates that in baseline conditions, allergic inflammation alone is likely not sufficient to increase the expression of either of

the receptors. Future studies should elucidate the role of allergens as triggers of complement activation in asthma *in vivo*. The possibility of nonspecific changes in C3aR and C5aR expression induced by death *per se* is ruled out by the fact that a low expression of the receptors was detected in the group of subjects who died of non-pulmonary causes. Furthermore, it is important to note that none of the patients with FA underwent mechanical ventilation during the acute asthma episode, indicating that a possible activation through mechanical or physical factors is unlikely.

A possible methodological limitation of our study is the comparison of postmortem tissue from patients with FA and controls with bronchial biopsy tissue from patients with MIA. Only large and middle-sized bronchi (perimeter > 6 mm) from FA and controls were compared with MIA biopsies. Furthermore, the size range of LA from the postmortem tissue included the airway size sampled by bronchial biopsies, assuring that we compared airways of approximately the same size. It should be noted, however, that previous studies showed no differences in inflammation between airways of different sizes in FA, nonfatal asthma, and control subjects,⁵⁸ and we are not aware of information about a possible different expression of epithelial markers within large airways of different generations.

In conclusion, our data support an active role of complement in FA and suggest that the airway and parenchymal vessels and the airway epithelium might be the major sites of action of C3a and C5a during the fatal attacks. These findings lend further support to the notion that, in addition to adaptive immunity, the innate immune system plays a crucial role in asthma, and open the way to investigate new therapeutic options to prevent FA.

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