

Early activation of coagulation after allergen challenge in patients with allergic asthma

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Asthma is characterized by allergic airway inflammation which is associated with bronchial hyperresponsiveness and airway obstruction [1]. Recent evidence indicates that activation of coagulation within the airways in asthma may aggravate inflammation [2]. Asthma patients were found to have elevated concentrations of thrombin, thrombin-antithrombin complexes (TATc) and soluble tissue factor and reduced activated protein C (APC)/thrombin ratios in induced sputum [3,4]. However, knowledge on coagulation activation in the lower airways in asthma in humans is limited, especially with regard to the acute impact of an allergen challenge. We therefore determined activation of coagulation in the bronchoalveolar space and the acute effect of a segmental allergen challenge hereon in asthma patients as compared to healthy controls.

Our study population has been described previously [5]. In short, thirteen allergic asthmatic subjects and nine healthy volunteers were included. Patients had a positive skin prick test for house dust mite allergens, grass pollen or both. Patients had not experienced an exacerbation of asthma during at least 2 months and had not used bronchodilators for at least 8 h before the investigations. None of the subjects had experienced recent airway infection or used anti-inflammatory or anticoagulant drugs. The study was approved by the Internal Review Board of the Academic Medical Center Amsterdam and written informed consent was obtained from all participants. Intracutaneous dose-response series with house dust mite or grass pollen (ALK Abello, Nieuwegein, The Netherlands) were performed to determine the concentration that produced a 10 mm wheal response 15 min after injection. Asthmatic

subjects underwent an intrabronchial challenge with 1 mL of this allergen concentration – brought to a final volume of 5 mL with saline – whereas controls were challenged with the highest concentration applied in the patient group. Levels of lipopolysaccharide in the allergen solution were $< 1.3 \text{ pg mL}^{-1}$ in all subjects.

Bronchoscopy and bronchoalveolar lavage (BAL) were performed as described previously [6]. Directly preceding allergen challenge, a BAL of the lingula was performed. After this lavage, allergen was administered in the lateral or medial segment of the right middle lobe. Four hours after allergen challenge, a BAL was performed of the segment challenged with allergen. Total cell numbers in BAL fluid (BALF) were determined by manual counting. For differential cell counts, cells were centrifuged and stained with Romanovsky and Jenner-Giemsa. All other measurements were performed in cell free supernatants obtained after centrifugation of BALF at $500 \times g$ and 4°C . TATc, soluble tissue factor and soluble thrombomodulin were measured using commercially available ELISAs (TATc: Behringwerke AG, Marburg, Germany; soluble tissue factor: American Diagnostics, Greenwich, CT, USA; soluble thrombomodulin: Diagnostica Stago, Asnières-sur-Seine, France). Activated protein C (APC) was measured with an enzyme capture assay as described earlier [7]. Tumor necrosis factor (TNF)- α , interleukin (IL)- 1β , IL-2, IL-4, IL-8, IL-10 and interferon (IFN)- γ were measured by multiplex bead array (Luminex, Austin, TX, USA).

At baseline, asthma patients had elevated TATc and soluble tissue factor levels in BALF as compared to controls (Fig. 1A,B). After allergen challenge, asthma patients showed a substantial rise in TATc, whereas control subjects displayed only a small increase. Soluble tissue factor levels increased in both patients and controls. Since the activation of PC is mediated by cell-bound thrombomodulin and previous studies had shown that shedding of thrombomodulin during pulmonary inflammation impairs the activation of PC [8], we in addition to APC measured soluble thrombomodulin levels. At baseline, there were no significant differences in APC and soluble thrombomodulin levels between patients and controls (Fig. 1C,D). However, upon allergen challenge, APC levels markedly decreased in patients as compared to controls. Moreover, allergen challenge induced a rise in soluble thrombomodulin concentrations in patients only.

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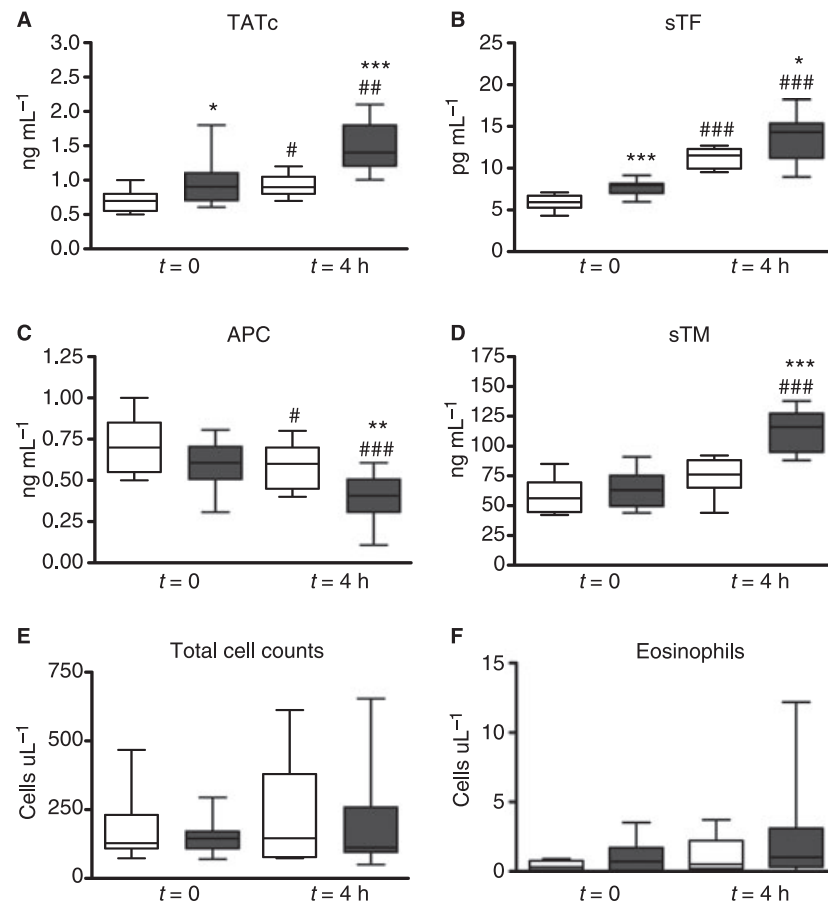


Fig. 1. Activation of coagulation and cell influx. Levels of (A) thrombin-antithrombin complexes (TATc), (B) soluble tissue factor (sTF), (C) activated protein C (APC) and (D) soluble thrombomodulin (sTM), (E) total cell counts and (F) eosinophils in bronchoalveolar lavage fluid obtained before and 4 h after an intrabronchial allergen challenge in controls (white) and asthma patients (grey). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. *, ** and *** indicate statistical significance as compared to controls ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, Mann-Whitney U Test) and #, ## and ### indicate statistical significance as compared to $t = 0$ ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, paired t test).

At baseline and after allergen challenge there were no differences in total cell counts (Fig. 1E) or differentials (not shown) in BALF between patients and controls. Notably, there were no differences in eosinophil count between patients and controls either at baseline or after allergen challenge (Fig. 1F). There were no differences in cytokine and chemokine levels between patients and controls at baseline (not shown). Upon allergen challenge, levels of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ increased modestly in both patients and controls, but in controls this was not significant. After allergen challenge, levels of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ were not different between patients and controls: $\text{TNF-}\alpha$ median 0.13 (interquartile range 0.12–3.5) vs. 0.13 (0.12–6.4) pg mL^{-1} ; $\text{IL-1}\beta$ 0.19 (0.06–0.63) vs. 0.13 (0.06–0.57) pg mL^{-1} . Levels of IL-6 and IL-8 increased in both patients and controls, but were not different between groups after challenge: IL-6 6.2 (5.3–21.5) vs. 8.3 (4.8–14.6) pg mL^{-1} ; IL-8 84 (37–529) vs. 68 (8.0–512) pg mL^{-1} . Levels of IL-2 , IL-4 , IL-10 and $\text{IFN-}\gamma$ were below detection in both groups.

Our findings indicate that airway inflammation is associated with a procoagulant state in the bronchoalveolar space in

asthma. This extends previous investigations in asthma patients: Gabazza *et al.* [3] reported increased concentrations of thrombin, TATc and soluble tissue factor in induced sputum in a study population comparable with our population. Terada *et al.* [9] found increased thrombin activity in BALF of asthma patients 48 h after a bronchial allergen challenge. Of note, in this study cell counts increased more than 4-fold and eosinophil counts increased to 44% of the total count, whereas we saw no increase in cell counts and no alterations in eosinophil counts. This difference most likely is due to the different sampling time after allergen challenge. Importantly, eosinophils express substantial amounts of tissue factor [10], the main inducer of coagulation in blood and the lung [11]. However, our results demonstrate pulmonary activation of coagulation in asthma and on allergen challenge independent of eosinophil influx.

APC has been implicated as an important regulator of coagulation and inflammation and earlier evidence has suggested insufficient generation of APC in the upper airways of asthma patients: Hataji *et al.* [4] reported decreased APC/thrombin and APC/PC ratios in induced sputum in asthma

patients. In accordance, mice with allergic airway inflammation demonstrated reduced APC/thrombin ratios in BALF [12]. In this model, inhalation of APC inhibited not only coagulation activation but also inflammation and airway hyperresponsiveness [12]. We here show that APC levels are reduced in BALF of asthma patients 4 h after a bronchial allergen challenge. Soluble thrombomodulin levels were increased only in patients, which may have contributed to a reduced capacity to generate APC. Reduced APC levels in turn could at least partially explain the observed rise in thrombin generation. Remarkably, inflammatory responses were not different between patients and controls.

Our study has limitations: We studied only patients with mild asthma. Moreover, we studied a small number of subjects and the effect of a bronchial allergen challenge was studied after only one time point. We can not exclude that the bronchoscopy and BAL procedure contributed to the inflammatory response. Conceivably the allergen itself contributed to the inflammatory response. Since controls were challenged with the highest allergen dose applied in patients they received a higher amount of allergen than most of the patients. This probably made them more susceptible to aspecific effects of the challenge, which could have masked a relatively larger inflammatory response in asthma patients to a comparable stimulus. Moreover, this could have influenced coagulation measurements, which would then have resulted in an underestimation of the differences found between patients and controls.

In conclusion, we show that coagulation is activated in the bronchoalveolar compartment in asthma patients and that intrabronchial allergen challenge aggravates coagulation activation and induces a decrease in APC concentrations in asthma patients as early as within 4 h. Although animal studies have suggested a pathogenetic role for activation of coagulation and downregulation of APC in asthma, it remains to be established in a larger study whether the procoagulant state as observed in our study contributes to disease and whether restoring the balance between coagulation and anticoagulation in asthma patients would impact on inflammation and disease activity.

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Disclosure of Conflict of interests

The authors state that they have no conflict of interest.

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