

Original article

Plasma protein profiles in early asthmatic responses to inhalation allergen challenge

Although mediators, such as lipids, cytokines, and chemokines, are related to the appearance of an IPR, there has been no reliable indicator to predict conditions for the appearance of an IPR.

In this study, we adopted a proteomic approach to investigate the pathogenesis at the level of the plasma proteins and to develop plasma markers to predict the appearance of an IPR following an inhalation challenge with *Dermatophagoides pteronyssinus* (D.p.). Sixteen mild asthmatics were recruited. Plasma was obtained before challenge and when a decline in forced expiratory volume in 1 s (FEV₁) values greater than 20% from the phosphate-buffered saline value was achieved during D.p. allergen challenge (positive responders), or at 60 min after the highest concentration of D.p. allergen was inhaled (negative responders).

After comparing normalized volumes of the spots in the two groups, differentially expressed spots were identified using intra-gel digestion and mass spectrometric analysis. Before D.p. antigen challenge, four spots of gamma fibrinogen and its isoforms were significantly decreased and two spots of complement C3 fragments were significantly increased in the positive responders compared to the negative responders. After D.p. antigen challenge, complement C3 fragment was persistently higher, while gamma fibrinogen was lower in the positive responders than in the negative responders. A validation study using Western blotting showed that gamma fibrinogen expression in the IPR-positive asthmatics was significantly decreased compared to the average of the IPR-negative asthmatic control group. These results indicate that alterations in the complement cascade and fibrinogen may predispose patients to the appearance of an immediate response to D.p. allergen challenge and may provide plasma markers to predict the appearance of an IPR.

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Allergic asthma is characterized by immunoglobulin E (IgE)-mediated sensitization following allergen exposure, leading to CD4⁺ lymphocyte responses and subsequent eosinophilic inflammation of the airways, resulting in enhanced bronchial reactivity and reversible airflow obstruction (1). Allergen inhalation in sensitized asthmatic patients results in an immediate-phase reaction (IPR), occurring within minutes and resolving in 30–60 min. A substantial number of patients also present with late-phase reactions (LPRs) that typically begin 3–4 h after allergen inhalation (2). Mechanisms behind airway narrowing in the IPR are primarily secondary to mast cell activation, while LPR is thought to depend on the infiltration of inflammatory cells, including eosinophils and T cells, into the bronchial wall (3, 4). However, even in subjects strongly sensitized to inhalant allergens, some are IPR-negative (5). Why some subjects do not react to allergen inhalation, even though they are sensitized to the allergen, is unclear. Several physiological

and immunological factors have been studied in relation to IPRs. Among them, nonspecific bronchial responsiveness is mostly related to the appearance of IPR, although the degree of antigen sensitization, indicated by total and specific IgE, skin test reactions, and the ability to cause basophil histamine release, are less related to the appearance of an IPR (6, 7).

The IPR is initiated by mast cells releasing preformed mediators from their granules, such as histamine, tryptase, and chymase (8). In addition, activated mast cells generate leukotriene C₄, prostaglandins, thromboxane, cytokines, and chemokines when stimulated with specific allergens. The changes in these mediators in the peripheral blood, bronchoalveolar lavage (BAL), and urine parallel the appearance of the IPR (9–12). Additionally, mediators, such as thrombin, and lung tissue kallikrein, from activated resident and recruited inflammatory cells, including eosinophils, platelets, alveolar macrophages, and neutrophils, have been demonstrated

in the peripheral blood, airway secretions, and urine in IPR following specific allergen challenge (13, 14). However, these mediators do not differ in amount between the IPR-positive and -negative groups before a specific allergen challenge. Thus, to our knowledge there is no reliable indicator in the peripheral blood or urine to predict conditions for the appearance of an IPR.

In this study, we adopted a proteomics approach to investigate the pathogenesis at the level of the plasma proteins and to develop plasma markers to predict the appearance of an IPR following *Dermatophagoides pteronyssinus* (D.p.) inhalation challenge, because house dust mites are globally the most important sources of indoor allergens responsible for sensitization and the development of asthma.

Material and methods

Subjects

We conducted proteome analyses on plasma obtained from 16 nonsmoking mildly asthmatic patients who had previously undergone a D.p. antigen provocation test at their initial visit to the outpatient clinic of Soonchunhyang University Hospital. Asthmatics had clinical symptoms and signs compatible with asthma and were in stable states of mild, intermittent asthma, according to the Global Initiative for Asthma (GINA) guidelines (15). Each patient showed airway reversibility, as documented by a positive bronchodilator response with a greater than 12% and 200 ml increase in forced expiratory volume in 1 s (FEV₁) and/or bronchial hyperresponsiveness with less than 10 mg/ml of methacholine inhalation. All patients were screened for the presence of atopy with a skin-prick test using 24 common inhalant allergen extracts (*Dermatophagoides*, *Alternaria*, *Aspergillus*, grasses, weeds, trees, cockroach, cat, dog; Bencard Co., England, UK). Specific IgE to D.p. antigen was measured using the CAP system (Pharmacia Diagnostics, Uppsala, Sweden) and is presented as specific IgE classes (1–5), according to UniCap specific IgE units (kU_A/l). Bronchial methacholine challenge was performed according to the 2-min tidal breathing method, as described previously (16). None of the patients had received house dust mite (HDM)-specific immunotherapy.

Bronchial allergen provocation tests

Allergen bronchoprovocation tests were conducted in subjects meeting the following criteria: (i) positive skin reaction to D.p. greater than or equal to that elicited by 10 mg/ml histamine and specific IgE to D.p. with a score greater than 2 (≥ 0.7 kU_A/l), and (ii) no asthmatic exacerbations or viral infections in the previous month. Inhaled steroids, theophylline, long-acting bronchodilators, or cromolines were prohibited in the previous month. Only short-acting inhaled beta-agonists were permitted, as needed, in the previous month. Short-acting bronchodilators were prohibited 24 h before testing. Patients sensitized to the other perennial (cat, dog, cockroach) and seasonal (pollen) allergens were excluded.

All subjects gave written informed consent before starting the study. The ethics committee of our hospital approved the study protocol.

The starting dose for allergen challenge was determined by the relationship between skin prick test thresholds of D.p. allergen, as

described by Cockcroft et al. (7). The allergen bronchoprovocation test was started between 08:00 and 09:00. Before starting the test, patients inhaled phosphate-buffered saline (PBS), and a change in FEV₁ values of less than 5% between baseline and postdiluent FEV₁ values was required to start the test. FEV₁ was measured with a dry rolling-seal spirometer (SensorMedics BV, Bithoven, the Netherlands). Series of ten-fold increasing doses of the D.p. allergen (Allergopharma, Hamburg, Germany) were inhaled in tidal breathing using a DeVilbiss 646 nebulizer (flow, 5.8 l/s) for 1 min in 10-min intervals until a fall in FEV₁ of 20% or greater (best of two measurements) relative to baseline FEV₁ occurred. After the final doses of allergen, which were equivalent concentrations showing the same wheal size to histamine (1 mg/ml) on skin prick test in each subject, FEV₁ was measured every 10 min until 60 min and at 90 min, then hourly for the following 8 h. Peripheral venous blood was drawn in a tube containing ethylenediamine tetraacetic acid (EDTA, 0.020 g/10 ml of blood) immediately before the allergen challenge and again when a fall in FEV₁ values greater than 20% from the PBS value was achieved (positive responder), or at 60 min after the highest concentration of allergen was inhaled (negative responder). Plasma was separated by centrifugation (1000 g). FUT-175 (BD Biosciences, Palo Alto, CA, USA) was added to stabilize plasma samples for complement measurements (final concentration of 50 µg/ml plasma), and samples were stored at –80°C until analysis.

Two-dimensional electrophoresis and image analysis

Immobiline DryStrips (24 cm, pH 3–10, pH 3–7; Amersham Biosciences, Uppsala, Sweden) were used for isoelectric focusing (IEF). The DryStrips were first rehydrated in 450 ml of rehydration buffer, containing 8 M urea, 2% CHAPS, 13 mM DTT, 1.2% IPG buffer, and a trace of bromophenol blue, and were mixed with each sample. IEF was carried out for a total of 146 kVh, using an IPGphor system (Amersham Biosciences), with 40 mg of plasma protein. Following IEF separation, the gel strips were equilibrated twice for 10 min with gentle shaking in equilibration buffer containing 50 mM Tris-HCl buffer, pH 8.8, 6 M urea, 20% glycerol, 0.1% SDS, and 1% DTT. In the second equilibration buffer, DTT was replaced with 2.5% iodoacetamide to remove excess DTT. After equilibration, the proteins were separated on 5–18.5% SDS-polyacrylamide gels, using the Ettan Dalt II system (Amersham Pharmacia Biotech, Inc., Sweden). At the end of each run, the gel was stained with Coomassie brilliant blue G-250. Digitized images of the Coomassie brilliant blue G-250-stained gels were analyzed using the two-dimensional electrophoresis (2-DE) gel analysis program IMAGEMASTER 2D (version 4.0; Amersham Pharmacia Biotech, Inc.). Coomassie-stained spots of 2-DE gel of 16 subjects were quantified on the basis of the normalized volume, i.e. the spot volume divided by the total sum of all spot volumes.

Intra-gel digestion and mass spectrometric analysis

Differentially expressed protein spots were excised from the gels, cut into smaller pieces, and digested with trypsin (Promega, Madison, WI, USA), as described previously (17). For MALDI-TOF MS analysis, the tryptic peptides were concentrated on POROS R2 columns (Applied Biosystems, Foster City, CA, USA). After successive column washings with 40% methanol, 100% acetonitrile, and 50 mM ammonium bicarbonate, the samples were applied to the R2 column and eluted in 2 ml of α -cyano-4-hydroxycinnamic acid, before MALDI-TOF analysis (18). The spectra for the protein samples were obtained using a Voyager DE PROMALDI-TOF spectrometer (Applied Biosystems). Searches of protein databases

were performed with the MSFit program (<http://prospec-tor.ucsf.edu/ucsfhtml3.4/msfit.htm>), using monoisotopic peaks. A mass tolerance within 50 ppm was allowed for the first analysis; the system was subsequently recalibrated at 20 ppm using the lists of proteins obtained from the initial analysis. The spectra were also internally calibrated, using trypsin autolysis products. Peptide matching and protein searches against the Swiss-Prot and NCBI databases were performed using the Mascot search (<http://www.matrixscience.com/>) and ProFound programs (<http://prowl.rockefeller.edu/>).

Western blot analysis

Proteins were fractionated by 15% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Inc.). Albumin was removed from plasma for detecting the three fibrinogen chains (alpha, beta, gamma). The proteins were blotted onto NC membranes. The membranes were incubated with blocking solution, containing a 1 : 500 dilution of goat anti-human fibrinogen polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then incubated with blocking solution containing a 1 : 5000 dilution of a horseradish peroxidase-conjugated polyclonal anti-goat IgG antibody. Enhanced chemiluminescence (ECL) detection of was performed, according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

Statistical analysis was performed with SPSS 8.0 (SPSS Inc., Chicago, IL, USA). The Mann-Whitney *U*-test (two-sample rank sum test) was applied to the comparison of spot intensities on the 2-D gel and the concentration of gamma fibrinogen between the groups. The change in the intensity following antigen challenge in the same subjects was analyzed using the Wilcoxon signed rank sum test. All data are expressed as mean $\pm$ SEM, and statistical significance was defined as $P < 0.05$.

Results

Characteristics of patients participating in the study

Patient characteristics are summarized in Table 1. There were no significant differences in terms of age, gender, initial FEV₁, or specific IgE to D.p. between the IPR-positive and -negative groups. PC20 methacholine was significantly lower in the IPR-positive group than in the -negative group, while eosinophil % and total IgE were significantly higher in the former than the latter.

Differential expression analysis and identification of plasma proteins between the IPR-positive and IPR-negative groups

To examine the differential expression of proteins between the IPR-positive and -negative groups, proteomic analysis was performed on plasma obtained immediately before the D.p. antigen challenge test, using high-resolution 2-DE. Average spot numbers were similar between the two groups (620 $\pm$ 35 vs 617 $\pm$ 28, $P < 0.05$; Fig. 1). At baseline, before challenge (Fig. 2), six spots differed in quantity, measured as normalized

intensity, between the two groups. The normalized intensities of spot numbers 1, 2, 3, and 4 were significantly lower ($P < 0.05$) in the IPR-positive than in the -negative group (Fig. 2A). In contrast, the intensities of spots 5 and 6 were higher in the IPR-positive than in the -negative group ($P < 0.05$). These spots were identified using MALDI-TOF MS (Table 2). Spots 1, 2, 3, and 4 were identified as gamma fibrinogen or fragments thereof. Spots 3 and 4 are likely isoforms of spots 1 and 2, respectively, because spots 3 and 4 had molecular weights of 48.1 kDa and spots 1 and 2 had molecular weights of 49.4 kDa. Further, spots 2 and 4 are likely post-translational modifications of spots 1 and 3, respectively, because spots 2 and 4 had pIs of 5.56 and spots 1 and 3 had pIs of 5.44. Spots 5 and 6 were identified as a complement C3 fragment and a complement C3d fragment, respectively.

Changes in the proteins after D.p. antigen challenge.

After D.p. antigen challenge, five spots differed in normalized intensity between the IPR-positive and -negative groups (Fig. 3). The normalized intensities of spots 6 (complement C3d fragment), 8, 9, and 10 were significantly higher in the IPR-positive than in the IPR-negative group ($P < 0.05$), while the normalized intensity of spot 4 (fibrinogen gamma) was lower in the IPR-positive patients ($P < 0.05$). Spots 8, 9, and 10 were identified as human serum albumin complexed with thyroxine, serum albumin, and transthyretin, respectively.

Changes in intensity before and after D.P. antigen challenge were also examined between the IPR-positive and -negative groups (Fig. 4). The normalized intensity of spot 5 (C3 fragment) significantly decreased in the IPR-positive group, compared to the intensity change in the -negative group ($P < 0.05$). In contrast, the intensities of spots 6 (complement C3d fragment), 7 (complement C4a fragment), 10 (transthyretin), and 11 significantly increased in the IPR-positive group, compared to those in the -negative group. Spot 11 was identified as apolipoprotein E3.

Table 1. Clinical characteristics of the study subjects

	Negative group	Positive group
No. (gender, M/F)	8 (3/5)	8 (4/4)
Age (years)	29.5 $\pm$ 12.6	28.9 $\pm$ 8.1
FEV ₁ , % predicted	99.2 $\pm$ 14.8	93.0 $\pm$ 12.8
Δ FEV ₁ (%)	4.8 $\pm$ 4.7	25.1 $\pm$ 10.6*
PC ₂₀ , methacholine (mg/ml)	9.7 (1.44–25)	0.53 (0.19–23)*
Total IgE (IU/ml)	90.2 (42.1–856)	319.5 (206–1175)*
Eosinophil (%)	8.1 $\pm$ 14.8	3.8 $\pm$ 1.3*
Specific IgE to D.p.†	3.6 $\pm$ 1.2	4.5 $\pm$ 1.8

Δ FEV₁ (%) represents the rate decrease of FEV₁ after the antigen provocation test. Values are mean $\pm$ SD. PC₂₀ and IgE concentration are median (range).

* $P < 0.05$ between the two groups.

†Specific IgE represented as class (1–6), according to UniCAP specific IgE units.

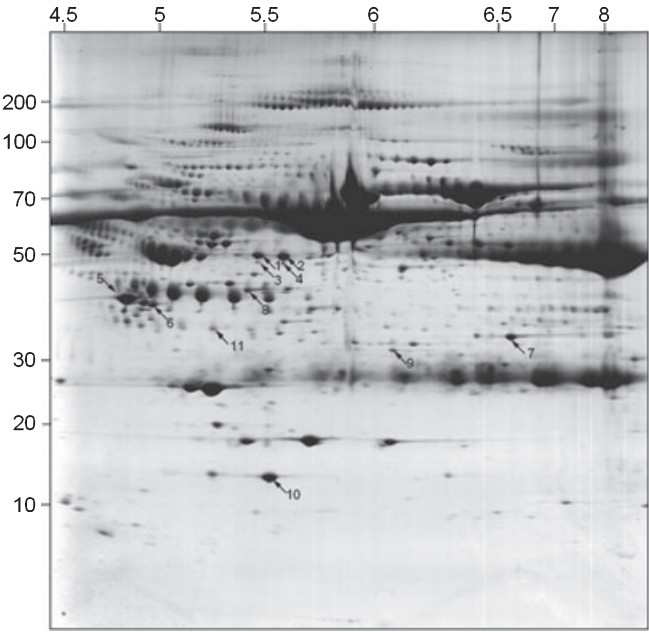


Figure 1. Photograph of 2-DE separation of plasma proteins. Plasma proteins (1 mg) were focused on a pH 3–10 IPG strip and then separated on an 8–18% gradient SDS-PAGE gel, stained, and visualized, as described in the Material and Methods. Protein spots of plasma collected before allergen provocation were picked and identified by MALDI-TOF MS. They are marked by the spot number.

Western blot analysis for fibrinogen

To validate the decreased normalized intensity of gamma fibrinogen on the 2-DE analysis of plasma from the IPR-positive group, compared to the -negative group before

Table 2. Proteins identified by MALDI-TOF MS or TOF/TOF MS/MS analysis

Number	Protein	Accession no.	Seq. coverage (%)	Theory-Mass (kDa)/pI
1	Fibrinogen gamma	223170	24	49.4/5.44
2	Fibrinogen gamma	223170	18	49.4/5.56
3	Fibrinogen fragment D	2781209	30	48.1/5.44
4	Fibrinogen Gamma 30 Kd C-terminal fragment	2098509	17	48.1/5.56
5	Complement component 3	40786791	32	40.9/4.84
6	Complement C3d fragment	3745750	31	40.2/4.97
7	Complement C4a fragment	14577919	27	31.7/6.54
8	Human serum albumin complexed with thyroxine	31615331	17	41.6/5.39
9	Serum albumin	23307793	16	30.7/6.1
10	Transthyretin	7770217	57	13.8/5.52
11	Apolipoprotein E3	4557325	33	35.3/5.24

the D.p. antigen challenge, levels of fibrinogen protein in plasma were analyzed by Western blotting with appropriate antibodies. Fibrinogen α , β , and γ chains were detected in the plasma of the IPR-positive and -negative groups (Fig. 5). There was no difference in the expression of fibrinogen α (95 kDa) and β chain (56 kDa) between the groups. However, gamma fibrinogen (49 kDa) expression in the IPR-positive asthmatics was significantly decreased, compared to the average of the IPR-negative asthmatic control group ($50.8 \pm 7.2\%$ vs $100 \pm 12.2\%$, $*P < 0.001$, Fig. 5B).

Discussion

Although sensitization to an allergen is not equivalent to the development of asthma, allergic asthma requires

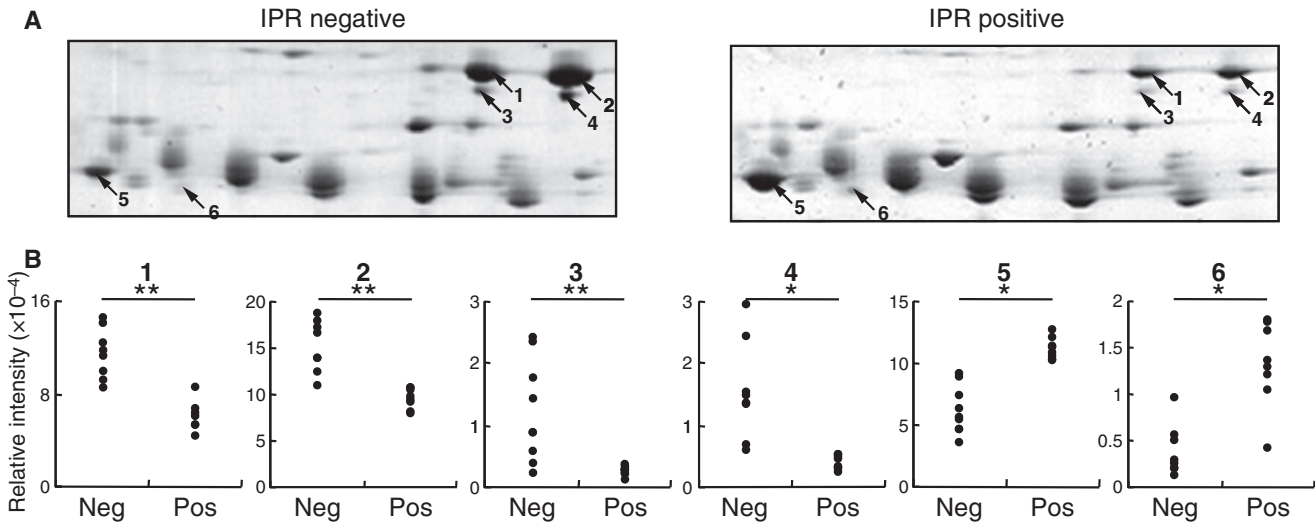


Figure 2. Six protein spots showing significantly different normalized intensities in plasma before D.p. antigen challenge between IPR-positive (Pos) and -negative (Neg) asthmatics ($P < 0.05$). Panel A: Segments of the 2-DE gel map. Arrows indicate the six protein spots that were differently expressed in the two groups. Panel B: Mean value of the normalized intensity of the six spots. The normalized intensity represents the spot intensity divided by the total sum of all spot intensities. For each spot, the normalized spot intensity was averaged and is expressed as the mean $\pm$ SE.

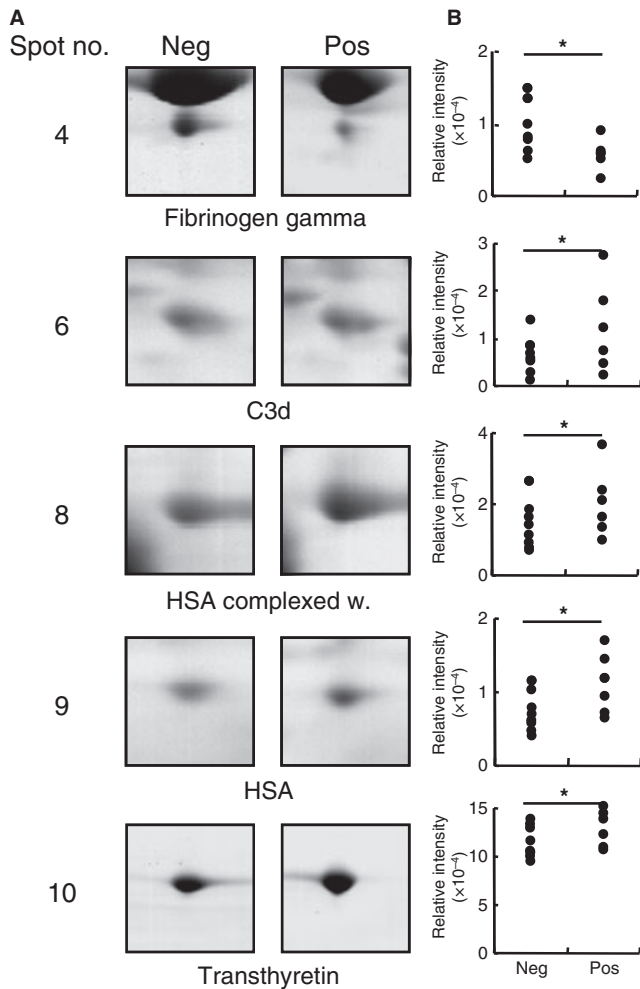


Figure 3. Five protein spots showing significantly different normalized intensities in plasma after D.p. antigen challenge between IPR-positive (Pos) and -negative (Neg) asthmatics ($P < 0.05$). Panel A: Segments of the 2-DE gel map. Panel B: Mean value of normalized intensity of the five spots. For each spot, normalized spot intensity was averaged and is expressed as the mean $\pm$ SE.

systemic or local sensitization to the allergen and the subsequent development of bronchial hyper-responsiveness (5). Approximately 50% of allergic children and young adults who are exposed to relevant allergens in their houses do not wheeze (19). Thus, identification of factors predisposing patients to bronchospasm in connection with inhaled allergens would be of great help in understanding the pathogenesis behind allergen-induced bronchospasm. Furthermore, proteins in plasma that are related to a predisposition to bronchospasm would be useful biomarkers for predicting the development of bronchospasm in allergen-sensitized persons. For this purpose, we adopted a proteomics approach. As an initial step, we screened the target proteins on a 2-D gel to differentiate IPR-positive from -negative asthmatics. At baseline, the expression levels of two complement

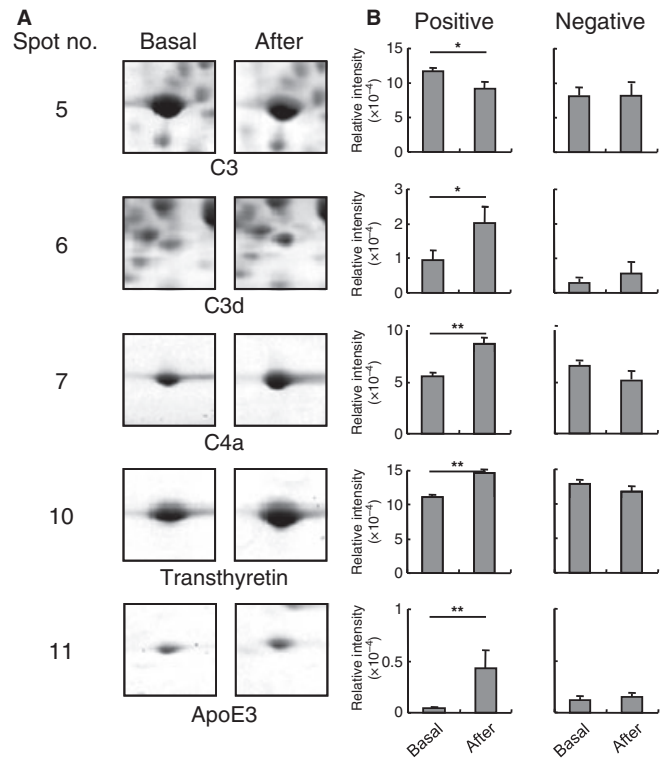


Figure 4. Changes in protein expression induced by D.p. antigen challenge in IPR-positive and -negative asthmatics. Panel A: Segments of the 2-DE gel map of five protein spots that were increased in IPR-positive asthmatics at basal and after D.p. antigen challenge. Panel B: Comparisons of protein expression changes by D.p. antigen challenge between IPR positive and negative groups. The levels of normalized intensities are expressed as the mean $\pm$ SE.

fragments (spots 5 and 6) were higher in the IPR-positive than in the -negative group. In contrast, the expression levels of gamma fibrinogen (spots 1, 2, 3, 4) were lower in IPR-positive responders. Gamma fibrinogen (spot 4) and complement C3d (spot 6) were differentially expressed at baseline and after D.p. challenge. This suggests that these differences between IPR-positive and -negative responders after D.p. challenge were caused by differences at baseline, rather than by the D.p. challenge. Additionally, IPR-positive asthmatics, but not IPR-negative asthmatics, showed decreased C3 (spot 5) and increased C3d fragment (spot 6) and C4a fragment (spot 7) following D.p. antigen challenges. This indicates that alterations in the complement cascade may predispose patients to the appearance of immediate responses to D.p. allergen challenge.

The complement cascade is associated with triggers evoking the development of asthma. Segmental allergen challenge of allergic asthmatics elevates C3a and C5a in BAL levels (20). In addition to allergens, ozone (21), RSV (Respiratory syncytial virus) infection (22), smoking (23), and particles (24) can result in activation of the comple-

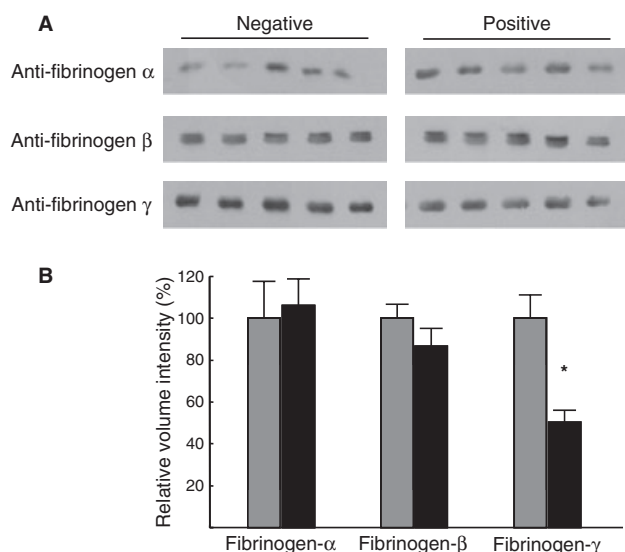


Figure 5. Fibrinogen chains in plasma of IPR-positive and -negative asthmatics. (A) Western blot of plasma proteins (40 µg) from IPR-positive and -negative asthmatics. (B) Quantitative analysis of gel bands for fibrinogen alpha (95 kDa), beta (56 kDa), and gamma chains (47 kDa). To calculate percent changes in densities in the IPR-positive group, the average of the control IPR-negative group was considered 100%, and the values in the IPR-positive group are expressed as a percentage of this average. The bars represent the mean $\pm$ SEM of density of gel bands and differences were assessed using Student's *t*-test (* $P < 0.001$).

ment cascade. We previously reported that plasma levels of C3a and C4a were higher in aspirin-sensitive individuals, and these levels correlated with changes in FEV₁ in aspirin-sensitive individuals after aspirin challenge (25). These data suggest that alterations in the complement cascade may not be limited to D.p. antigen challenge, but may be a general predisposing factor in the development of bronchospasm.

As an explanation for the mechanism behind C3 and C4 complement activation following the D.p. antigen provocation, proteases such as trypsin, thrombin, and elastase released from mast cells may cleave C5 and C3 in the airway wall via activation of IgE-mediated processes. Second, some macromolecular structures from dust mites may be recognized by the alternative pathway and fix the complement directly. Lastly, exogenous proteases derived from allergens, such as *Der p3* and *Der f3*, directly cleave C3 and C5 into their active fragments (26). However, the process involved remains unknown at present.

Another interesting finding is that the gamma fibrinogen chain and its isoforms were significantly decreased in IPR-positive responders, compared to IPR-negative responders (Fig. 4). The differences in the gamma fibrinogen chain in plasma in the two groups were confirmed by Western blot analysis. The expression level of the gamma fibrinogen chain in IPR-positive asthmatics was less than in the -negative group, while fibrinogen alpha

and beta chain levels were the same in both groups (Fig. 5).

Fibrinogen is the six-chain protein precursor to the main clot structural protein, fibrin. Fibrinogen is a dimer of three polypeptide chains termed α (or αA), β (or βB), and γ (27). While all three chains are essential for the normal function of fibrinogen, due to their intertwined three-dimensional structure (28), the gamma chain contains a number of sites that interact with other fibrin(o-gen) molecules, clotting factors, growth factors, and integrins (29). The gamma chain can bind to factor XIII zymogen and to thrombin. Fibrinogen interactions with integrins modulate the processes of platelet aggregation, inflammation, and wound healing. Interestingly, many of these processes involve the fibrinogen gamma chain (27). Fibrinogen gamma participates in inflammation by binding to leukocyte integrin $\alpha M\beta 2$ (CD11b/CD18, Mac-1, CR3). This binding interaction mediates the recruitment of phagocytes during inflammation (30). In addition to adhesion molecules, fibrinogen contains binding sites for several different growth factors and cytokines, including vascular endothelial growth factor (31), fibroblast growth factor-2 (32), and interleukin-1 β (33). Thus, these binding sites may serve as storage depots to concentrate growth factors and cytokines in inflammatory and repair processes. The γ chain arises from an alternative mRNA processing event (34), and differs only in its carboxyl terminus from the gamma A isoform. This isoform is often referred to as gamma A/ γ fibrinogen, and constitutes approximately 10% of an individual's fibrinogen, although the ratio can vary from 3% to 40% among individuals (35). The fibrinolysis rate of recombinant γ'/γ' fibrinogen is about 10-fold slower than recombinant $\gamma A/\gamma A$ fibrinogen, in the presence of factor XIIIa (36). This suggests that the function of fibrinogen may be dependent on the ratio of these isoforms. In the present study, spots 1, 2, 3, and 4 were identified as fibrinogen gamma. Spots 3 and 4 are likely isoforms of spots 1 and 2, respectively, because spots 3 and 4 had molecular weights of 48.1 kDa and spots 1 and 2 had molecular weights of 49.4 kDa (Fig. 2). Spots 2 and 4 are likely post-translational modifications of spots 1 and 3, respectively, because spots 2 and 4 had pIs of 5.56 and spots 1 and 3 had pIs of 5.44. All the spots were significantly decreased in the IPR-positive group compared to the IPR-negative group. The sum of these four isoforms in IPR-positive asthmatics was also lower than in the IPR-negative asthmatic controls (41.7% vs 100%, $P < 0.005$).

The role of platelet activation and fibrinolytic activity has been demonstrated in asthma. Activation of fibrinogen is the initial and essential step in platelet aggregation and fibrin clot formation (37). Platelets are activated during antigen-induced airway reactions in asthmatic subjects (38). Symptomatic asthmatics have increased concentrations of beta-thromboglobulin and platelet factor-4 in plasma and BAL fluid (39, 40). In experimen-

tal asthma models, platelet activation within the circulation is necessary for transmigration of eosinophils and lymphocytes into the airways (41). In our study, we did not evaluate changes in fibrinogen gamma in the LPR. However, intravascular platelet activation after bronchial allergen exposure occurs during the IPR independent of the pattern of asthmatic response, and the degree of platelet activation during the IPR may affect the development of prolonged inflammation during the LPR (42). It is unknown why IPR was associated with a decreased amount of fibrinogen gamma in our study. A possible mechanism is that the released mediators from mast cells may activate fibrinogen gamma in IPR positive subjects. Recent data suggest that mast cells and their products such as heparin and proteases are involved in the regulation of coagulation and fibrinogenolysis (43). IgE-mediated immediate hypersensitivity reactions have been known to result in local extravasation of fibrinogen and deposition of fibrin (44). IgE-mediated activation of mast cells may inactivate the fibrinogen gamma in IPR positive subjects subclinically before specific antigen challenge. In IPR-negative asthmatics, lung mast cells are not activated. As the results, relatively high concentration of plasma fibrinogen gamma blocks the tissue injury by blood clotting and inflammation by competing

local fibrinogen. However, in IPR-positive patients, fibrinogen gamma does not protect because its lower levels in plasma. However, the exact mechanism is remained unsolved to date. In addition, our result of the specific antigen challenge should be cautiously extended to the natural exacerbation of allergic asthma on exposure to allergens.

In conclusion, our study is the first to show differences in protein expression in the plasma of IPR-positive and -negative asthma patients at baseline and after D.p. antigen challenge. As a result, levels of fibrinogen gamma and complement components may be determinants for the IPR following D.p. antigen inhalation, possibly providing plasma markers to predict the likelihood of IPR.

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