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## T and B cell immune response to a 55-kDa endothelial cell-derived antigen in severe asthma\*

Current concepts on the pathogenesis of chronic asthma emphasize the role of several inflammatory cell populations and their respective mediators that interact in a complex network. However, beside inflammatory cells, lymphocytes are also present in asthmatic airways. Although little is known about their involvement in asthma, it has been suggested that lymphocytes may participate in the development of chronic inflammation either through lymphokine secretion or through antibody production. In this study, we describe circulating IgG auto-antibodies, directed against a common 55-kDa antigen shared by platelets and cultured endothelial cells, and found in 34 out of 97 asthmatic patients. Among epidemiological, clinical and biological characteristics of these asthmatic patients, the anti-55-kDa antigen antibodies are mainly restricted to patients with negative cutaneous prick tests ( $p = 0.0014$ ), and corticosteroid-dependent asthma ( $p = 0.0036$ ). These antibodies were also detected in a few patients with autoimmune disorders like systemic lupus erythematosus (3/30) or rheumatoid arthritis (2/36). Both platelet and endothelial cell antigens were cross-reactive, had an isoelectric point between 8.0 and 9.0, were unsensitive to reducing agents such as 2-mercaptoethanol, and were not present on either platelet or endothelial cell surface, as determined by immunostaining assay. [<sup>3</sup>H] Thymidine incorporation assay with peripheral blood mononuclear cells from patients in the presence or in the absence of 55-kDa antigen, purified from nitrocellulose sheets demonstrated a specific incorporation in 6 out of 13 patients with circulating anti-55-kDa antigen antibodies, with index values ranging from 12 to 3. Such a T cell reactivity has also been observed in 3 out of 17 patients without detectable serum anti-55-kDa antigen antibodies. Moreover, a significant correlation was found between index values of antigen-specific T cell reactivity and the forced expiratory volume in one second ( $r = 0.544$ ,  $p = 0.003$ ). Our data indicate that the detection of such antibodies allows to distinguish a subgroup of asthmatics in terms of severity and to suggest a relationship between clinical severity and T and B cell autoreactivity to the 55-kDa platelet/endothelial cell antigen.

### 1 Introduction

Asthma affects 5–10% of the population and there is convergent evidence from many countries that prevalence, severity and mortality have increased in recent years, despite improvement in asthma therapy [1–4]. Usually, in adults, asthma is operationally classified as "allergic" if elevated levels of IgE specific for aeroallergens exist and "nonallergic" if they do not exist. The current knowledge on the pathogenesis of allergic asthma emphasizes the role for inflammatory cells (*i.e.* mast cells, eosinophils, macrophages, and platelets) that may be triggered through an IgE-dependent pathway [5–10]. In nonallergic asthma (also called intrinsic or nonatopic asthma), the understanding of involved mechanisms is still more approximative. Although an IgE-dependent mechanism could not be excluded, there are a number of considerations which distinguish this

particular type of asthma from atopy: first, the occurrence of the disease in the 40–50-year-old age group; second, the usually normal total IgE levels and the lack of identifiable allergens; third, the feature of nonatopic asthma which is recognized to be more severe [11]. Moreover, the frequent requirement of anti-inflammatory drugs, such as corticosteroids, argues for a dominant inflammatory component in the pathogenesis of non-atopic asthma [11].

Pathological studies have also shown that the bronchial mucosa from asthmatics is the site of cell infiltration and inflammatory reaction. The number of activated T cells and eosinophils was highly increased and correlated with the severity of asthma [12–15]. T cell mediators such as soluble IL-2 receptor (sIL-2R) and IFN- $\gamma$  increased in the sera from patients with acute asthmatic attack and returned to baseline after 3 days of corticosteroid therapy [16, 17]. An unexpected finding was that T cell activation was much more strongly associated with nonatopic asthma. First, the percentage of peripheral IL-2R-positive CD4 T cells and the levels of serum sIL-2R were found to be higher in nonatopic patients with acute severe asthma than in atopics [17]. Second, elevated HLA-DR-positive CD4 and CD8 T cells were found only in blood of nonatopic asthmatics [18]. Third, sIL-2R levels correlated with blood eosinophilia and the lung function values (forced expiratory volume in 1 s, FEV<sub>1</sub>) in nonatopic asthmatics but not in atopics [19].

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Therefore, pathogenesis of nonatopic asthma appears to be more closely linked with T cells and T cell mediators. With the particular clinical features of nonatopic asthma in mind, one can hypothesize that this form of asthma may be the result of T cell hyper-reactivity against an unknown antigen contained in bronchial structures. **Because endothelial cells represent the first resident cell type in the bronchial tissues [20, 21], we searched for an endothelial cell-derived component, which might act as a target antigen for T and B cells in asthmatics.**

Here we describe, in a subgroup of asthmatics, a circulating autoantibody that recognizes a cross-reactive 55-kDa platelet and human endothelial cell antigen. The patients displaying these antibodies were mainly nonatopic asthmatics, and had a more severe asthma. Moreover, a statistical link was found between the presence of these autoantibodies and aspirin-sensitive asthma. An antigen-specific T cell proliferation was also demonstrated in some patients with circulating anti-55-kDa antigen antibodies. Together with our previous results, this study indicates the possible implication of an autoimmune process involving endothelial cells in a subset of patients with severe asthma.

## 2 Materials and methods

### 2.1 Sources of sera

The study group comprised 97 patients referred for asthma to the Department of Pneumology (Centre Hospitalier Régional et Universitaire, Lille, France). Asthma was diagnosed on the basis of a history of episodic or continuous

wheezing. To be included into this study all subjects experienced mild to severe asthma with an  $FEV_1$  of  $< 70\%$  of predictive values, with a reversibility of  $FEV_1$  of  $\geq 15\%$  after inhalation of two puffs of Beta-2 agonist. Medications required to control symptoms included theophylline, inhaled beta-2 agonists or inhaled steroids. Some patients required long-term oral steroid therapy. Patients with clinical and pathological features of allergic granulomatosis and angiitis were excluded from this study. Atopy was defined on the basis of positive skin prick tests to extracts of common aeroallergens (see Table 1 for more details). Aspirin-induced asthma was identified either on a typical clinical history, when available, or a bronchospastic adverse reaction after oral provocation [22]. Nonasthmatic controls included 30 patients with systemic lupus erythematosus (SLE; diagnosis of SLE was determined according to the criteria of the American Rheumatism Association [23]; sera were kindly provided by Prof. JP Dessaint, Department of Immunology, CHRU, Lille), 36 patients with rheumatoid arthritis and 10 patients with established diagnosis of temporal arteritis (sera provided by Prof. B. Devulder, Department of Internal Medicine, CHRU, Lille), 17 from chronic obstructive bronchitis and 28 healthy subjects including sera from 10 elderly subjects of 71–86 years old. All sera were aliquoted and stored at  $-20^\circ\text{C}$ .

### 2.2 Cell isolation

#### 2.2.1 Endothelial cells (EC)

EC were obtained from human umbilical veins, using previously described methods [24, 25] which were slightly

**Table 1.** Characteristics of the asthmatic population classified upon the detection of anti-55 kD antibodies<sup>a)</sup>

|                                          | Absence of anti-55-kDa antigen antibodies | Presence of anti-55-kDa antigen antibodies | Significance                  |
|------------------------------------------|-------------------------------------------|--------------------------------------------|-------------------------------|
| Number of patients                       | 65                                        | 34                                         |                               |
| Age (year $\pm$ SD)                      | 42 $\pm$ 14                               | 44 $\pm$ 13                                | ns                            |
| Sex ratio (F/M)                          | 13/29                                     | 21/13                                      | ns ( $\chi^2$ test)           |
| Duration of asthma (year $\pm$ SD)       | 12 $\pm$ 11                               | 14 $\pm$ 11                                | ns                            |
| $FEV_1$ (%) (mean $\pm$ SD)              | 73 $\pm$ 22                               | 64 $\pm$ 19                                | ns                            |
| Atopy (number of patients) <sup>b)</sup> | 38                                        | 19                                         | $p = 0.0014$ ( $\chi^2$ test) |
| Total IgE (KIU/L) <sup>c)</sup>          | 556 $\pm$ 709                             | 362 $\pm$ 455                              | ns                            |
| Steroid-dependent asthma <sup>d)</sup>   | 18                                        | 20                                         | $p = 0.0036$ ( $\chi^2$ test) |
| Number of patients                       | 18                                        | 20                                         |                               |
| Duration (years $\pm$ SD)                | 11 $\pm$ 10                               | 6 $\pm$ 3                                  | ns                            |
| Aspirin-sensitive asthma <sup>e)</sup>   | 8                                         | 10                                         | $p = 0.0043$ ( $\chi^2$ test) |

- The presence of these antibodies has been determined by immunoblots of both endothelial cell and platelet extracts with the sera from the population studied. The presence of such antibodies was recorded when a serum reacted with the 55-kDa antigen band from both endothelial cell and platelet extracts in two separate experiments. Some sera which reacted strongly with the 55-kDa antigen from both cell types were further used as internal positive controls. All patients were subjected to skin prick tests under standard method at the Department of Pneumology (Hôpital Calmette, Lille).
- Atopy was defined on the basis of at least two positive cutaneous reactions to a battery of common allergen extracts (wheel and flare  $\geq$  positive control histamine).
- Total IgE levels were measured with routinely well defined PRIST (Prof. JP Dessaint, Department of Immunology, Lille). Statistical analysis was performed with the  $\chi^2$  test and the Wilcoxon rank sum test for unpaired data as described in Sect. 2.11.
- Corticosteroid dependence was assumed when control of asthma required daily oral steroids for at least 1 year despite usual anti-asthma therapy and daily 2000  $\mu\text{g}$  inhaled beclomethasone.
- Diagnosis of aspirin sensitivity was established on either typical clinical or a bronchospastic adverse reaction after oral provocation.

modified here. Recovered EC were cultured in 35-mm-diameter tissue culture wells in RPMI 1640 supplemented with 20 % heat-inactivated fetal calf serum (FCS, Gibco BRL, France) 100 µg/ml heparin and 25 µg/ml endothelial cell growth supplement (ECGS; Sigma; St. Louis, MO). The medium was replaced twice a week and the cultures reached confluency within 5 to 7 days. EC were then detached by incubation with 5 mM EDTA in phosphate-buffered saline (PBS) for 10 min at 37°C, centrifuged at  $500 \times g$  for 10 min, resuspended in fresh medium and further cultured in 35-mm-diameter tissue culture wells. The culture reached confluency in 3–5 days. Only 2nd- to 4th-passage EC cultures were used in these studies. The purity of EC cultures was assessed by microscopical examination and the presence of FVIII-related antigen (assessed by immunofluorescence staining assay) was detected in more than 98 % of cells. Cultures contaminated with smooth muscle cells were excluded.

### 2.2.2 SV40-transfected endothelial cells (EC-pSV1)

EC-pSV1 were obtained by transfection of human EC with a plasmid derived from the early gene region of the simian virus SV40 [26]. This cell line grew in medium supplemented with 10 % FCS.

### 2.2.3 Fibroblasts

The human lung fibroblast cell line MRC5 was kindly provided by C. Lagrou (INSERM U 186, Lille, France). The cells were cultured in medium containing 10 % FCS.

### 2.2.4 Lymphocytes, neutrophils, and red blood cells

These cells were isolated from heparinized venous blood using Ficoll Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation [27]. Briefly, 40 ml of 1X diluted heparinized blood in PBS was overlaid on 10 ml of Ficoll Paque and centrifuged at  $500 \times g$  for 30 min, at 15°C. The pellet containing red cells and neutrophils was isolated. Most of the red cells were removed by sedimentation with 3 % dextran for 30 min. The red blood cells were resuspended in PBS pH 7.4. In the neutrophil fraction, the remaining red blood cells were removed by hypotonic lysis, and neutrophils were resuspended in PBS. The peripheral blood mononuclear cells (PBMC) were washed and resuspended in PBS.

### 2.2.5 Platelets

For platelet isolation, venous blood was collected on acid citrate dextrose anticoagulant. Platelet-rich plasma was obtained by centrifugation ( $120 \times g$  for 15 min) and the platelets were washed by repeated centrifugation ( $2000 \times g$  for 15 min) in PBS buffer containing  $10^{-2}$  M EDTA,

with PBS ( $\text{Ca}^{2+}$  free) and incubated for 5 min in PBS ( $\text{Ca}^{2+}$  free) at 37°C. The confluent monolayers were then easily detached by pipetting. All cell types were adjusted at  $2 \times 10^8$  cells/ml in lysis buffer (10 mM Tris-HCl, 7.5 mM  $\text{MgCl}_2$ , 1 mM phenylmethylsulfonyl fluoride, pH 6.8) except red cells ( $2 \times 10^9$  cells/ml) and platelets ( $4 \times 10^9$  cells/ml). Cells were lysed by addition of 0.5 % Nonidet P40 for 30 min in ice followed by clearance of nuclei by centrifugation at  $400 \times g$ . Aliquots of cell lysates were kept frozen at  $-70^\circ\text{C}$  until use.

### 2.4 SDS-PAGE and Western blotting

Cell samples, in reducing or nonreducing conditions (*i.e.* with or without 2-mercaptoethanol) were separated by discontinuous SDS-PAGE according to the technique of Laemmli [28] using a 10 % resolving gel pH 8.8 and a 5 % stacking gel pH 6.8. Following electrophoresis, proteins were transferred onto nitrocellulose (0.45 µm BA 85; Schleicher and Schuell, Dassel, FRG) [29]. After transfer, the nitrocellulose sheets were incubated for 30 min at room temperature in quenching buffer (5 % nonfat dry milk in PBS, pH 7.4) and washed with PBS. The nitrocellulose was probed with human sera overnight at 4°C. Bound human immunoglobulins were detected with either peroxidase-conjugated rabbit anti-human IgG, IgA, IgM diluted at 1:500 or peroxidase-conjugated rabbit anti-human IgE diluted at 1:250 (Tago, Burlingame, CA) in PBS for 3 h at room temperature. After a final washing step, the nitrocellulose strips were stained with horseradish peroxidase color development (Bio-Rad Laboratories).

To determine optimal quantities of antigen deposition onto the gels, preliminary experiments included gel electrophoresis followed by Coomassie blue staining of various amounts of cell samples. Based on the results, we used standard depositions of  $1 \times 10^6$  EC and  $5 \times 10^7$ – $6 \times 10^7$  platelets/cm acrylamide gel.

### 2.5 Two-dimensional immunoblotting

According to the experimental procedure described by O'Farrell [30], the samples of  $4 \times 10^7$  platelets and  $2 \times 10^6$  endothelial cells (experimentally defined as optimal depositions) were separated in first dimension by isoelectric focusing (IEF). First-dimensional polyacrylamide tube gel contained 9 M urea, and the pH gradient was formed with pH 3.5–10 ampholines (Pharmacia). Gels were prefocused at 600 V for 40 min and the samples were separated at a total of 10 000 V for 18 h. For the second migration, IEF gels were equilibrated with SDS equilibration buffer (62.5 mM Tris-HCl, 5 % 2-mercaptoethanol, 10 % glycerol, pH 6.8) for 5 min at room temperature. Proteins were separated in the second dimension in 10 % polyacrylamide gel, followed by electrophoretic transfer to nitrocellulose and immunoblotting with human sera according to the method described above.

### 2.6 Fractionation by HPLC

Cell detergent lysates from  $10^7$  EC-pSV1 were fractionated by reverse-phase HPLC on a Shimadzu liquid chromato-

### 2.3 Preparation of cell lysates

Cultured adherent cells (*i.e.* normal and SV40-transfected endothelial cells, and fibroblasts) were washed three times

graph (model LC6A) fitted with a UV spectrophotometer; detector Shimadzu model SPD6A (Touzart et Matignon, Vitry Sur Seine, France). We used a Vydac C4 silica, 7- $\mu$ m particle diameter, 30-nm pore size, packed in a stainless steel column, 150 mm  $\times$  4.6 mm i.d. (Touzart et Matignon). Chromatographic conditions were as follows: 15 min isocratic elution, 2% B, then linear 90-min gradient 2–50% B, then linear 45-min gradient 50–98% B, where solvent A was H<sub>2</sub>O containing 0.05% TFA, and B was 75% acetonitrile, 25% H<sub>2</sub>O containing 0.045% TFA. Flow rate, 500  $\mu$ l/min; column temperature, 25°C. Fractions were collected automatically (1 ml), and lyophilized. They were examined for the presence of the 55-kDa antigen by Western blotting.

## 2.7 Cell labelling

Cultured normal and transformed endothelial cells were labeled in 35-mm petri dishes with [<sup>35</sup>S]methionine (50  $\mu$ Ci/10<sup>6</sup> cells) for, respectively, 4 and 1 h at 37°C in RPMI medium containing 10% dialyzed FCS. The radioactivity was determined by the trichloroacetic acid-protein-precipitation assay. After cell lysis, [<sup>35</sup>S]methionine-labeled proteins were electrophoresed as described above, or precipitated by addition of methanol:acetic acid (100:1) overnight at –70°C. The precipitated proteins were centrifuged at 45 000 rpm for 60 min and resuspended in 10  $\mu$ l of sample buffer for 2-D gel electrophoresis.

## 2.8 Cell fractionation

Fractionation of the 55-kDa antigen was performed with three cycles of freeze-thawing at –20°C followed by centrifugation at 12 000 rpm for 15 min at 4°C in a Sigma centrifuge [31]. The supernatants, collected as the "cytosolic" fractions, and the pellets were analyzed in SDS-PAGE followed by immunoblotting, using a serum containing anti-55-kDa antigen antibodies as described above.

## 2.9 Immunofluorescence assay

Indirect immunofluorescence on EC and platelets was performed as previously described [32]. Briefly, cultured EC were washed three times with Hanks' balanced salt solution (HBSS) and incubated on an ice bath for 30 min with sera diluted at 1:20 (v:v) in HBSS. After three washes with HBSS, EC were incubated for another 30 min with 1:100 (v:v) fluorescein-labeled anti-human IgG (Tago). Cells were washed three times with Ca<sup>2+</sup>-free PBS and could then be easily detached by pipetting. Cell suspensions were analyzed on an Ortho System H50 Cytofluorograf (Ortho Diagnostics System, Westwood, MA). A similar procedure was used for immunostaining of platelets, except that incubation medium was PBS supplemented with 0.01 M EDTA and that platelets were washed by centrifugation at 2000  $\times$  g for 10 min.

## 2.10 Lymphocyte proliferation assay

We tested proliferative response of peripheral blood T lymphocytes to human endothelial cell proteins bound onto

nitrocellulose microparticles. Preparation of nitrocellulose particle suspensions was performed as previously [33, 34]. Briefly, areas from blotted nitrocellulose strips containing the band to be tested (identified from strips run in parallel for detection of patient's antibodies) were minced and suspended in 250  $\mu$ l dimethylsulfoxide (DMSO) at room temperature. Sterile nitrocellulose particles were precipitated by addition of 250  $\mu$ l carbonate/bicarbonate buffer (0.05 M, pH 9.6), centrifuged at 10 000  $\times$  g for 10 min, washed in RPMI 1640 and resuspended in 1 ml of the culture medium (RPMI 1640, 5% FCS). PBMC obtained as described above, were washed three times and suspended in culture medium. Cells were cultured in flat-bottom microculture plates at 250 000 cells/wells together with 100  $\mu$ l of the particle suspension, diluted 1:10 or 1:100 (v:v). Control wells contained nitrocellulose particles with unrelated major band protein (as determined by amido black staining of nitrocellulose sheets). Cell cultures were incubated for 4 days at 37°C and harvested onto filters after an overnight pulse of 18.5 kBq [<sup>3</sup>H] thymidine/well (CEA, Gif-sur-Yvette, France). [<sup>3</sup>H] Thymidine uptake was determined with a liquid scintillation spectrophotometer and the results from triplicate determination were expressed as proliferation index, calculated as the ratio of the thymidine incorporation by cells with the antigen to the thymidine incorporation by cells with unrelated control protein. In all the experiments, part of PBMC were pre-cultured with concanavalin A (Con A; 0.25  $\mu$ g/well) for 48 h in 35-mm petri dishes. Then, PBMC were washed with RPMI 1640 and tested for antigen-dependent proliferation 4 days later, as described above.

## 2.11 Statistical analysis

The  $\chi^2$  test was used to determine statistical significance between the sex ratio, the distribution of skin tests, the frequency of corticosteroid-dependent and drug-induced asthma. Differences between the mean ages and the IgE levels were analyzed with the unpaired Wilcoxon test. A *p* value of < 0.05 was considered significant.

# 3 Results

## 3.1 Detection of autoantibodies to the 55-kDa endothelial cell antigen

Antibodies directed against an EC component with molecular mass of 55 kDa were detected in a subset of patients with asthma (Fig. 1). In the group of patients with anti-55-kDa antigen antibodies, the isotype was defined as IgE in 2 cases, and as IgG isotype in the 32 others. No IgM or IgA isotypes were detected. It was not possible to distinguish a significant binding on fixed and permeabilized endothelial cells using indirect immunofluorescence test with anti-55-kDa antigen-positive sera. Pre-clearing of the positive sera on endothelial cells or platelets did not remove the anti-55-kDa antigen antibodies.

Among the patients with other autoimmune diseases, 6 exhibited a specific binding to the 55-kDa antigen (3 out of 30 patients with SLE, 2 out of 36 patients with rheumatoid arthritis, and 1 out of 17 sera from chronic obstructive bronchitis). All other patients were negative (temporal

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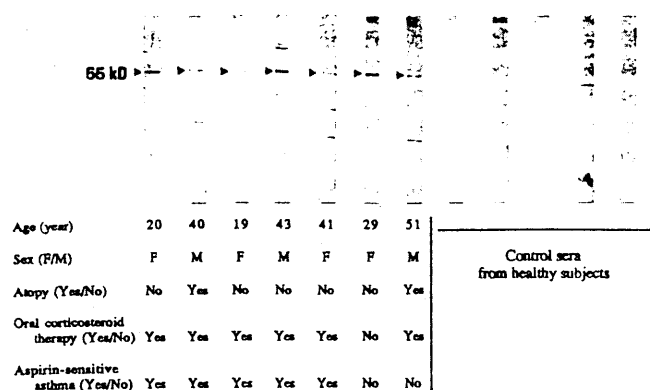


Figure 1. Binding of serum antibodies to the 55-kDa endothelial cell antigen in patients with asthma. The immunoblots were here revealed with anti-heavy gamma chain antibodies conjugated with peroxidase.

arteritis,  $n = 10$ ), and healthy subjects ( $n = 38$ , including elderly subjects).

The detection of anti-55-kDa antigen antibodies was evaluated according to the clinical, biological and therapeutic characteristics of the asthmatic population (Table 1): age, sex ratio, duration of asthma,  $FEV_1$ , and serum total IgE levels were found to be not statistically different in the two groups of asthmatics. By contrast, anti-55-kDa antigen antibodies were detected mainly in asthmatics with negative cutaneous prick tests ( $p = 0.0014$ ), in corticosteroid-dependent asthma ( $p = 0.0036$ ), and in aspirin-induced asthma ( $p = 0.0043$ ).

### 3.2 Cell specificity and antigenic community between platelet and EC 55-kDa antigens

Among the different human cell populations studied here, only platelets and SV40-transfected EC shared a 55-kDa antigen common to EC. No such antigen could be detected on fibroblasts (MRC5 cell line), neutrophils, mononuclear cells, red blood cells, or K562 cell line. The antigenic community was confirmed by absorption of positive sera onto nitrocellulose sheets containing the platelet-derived 55-kDa antigen band (identified from strips run in parallel for detection of patient's antibodies) compared with nitrocellulose sheets containing a non-relevant major platelet protein (as determined by amido black staining). Sera specifically pre-cleared with the 55-kDa platelet antigen, did not recognize the corresponding endothelial antigens (Fig. 2). The anti-55-kDa antigen antibodies recovered from nitrocellulose sheets by elution (0.1 M glycine-HCl, pH 4), were able to recognize EC-, SV40-transfected-EC-, and platelet-derived 55 kDa antigen without significant loss of specificity (Fig. 2).

### 3.3 Proliferative response to 55-kDa proteins

To detect the presence of reactive T lymphocytes, we used a [ $^3H$ ] thymidine incorporation assay with PBMC from patients in the presence or in the absence of 55-kDa antigen, resuspended from nitrocellulose sheets. Initial

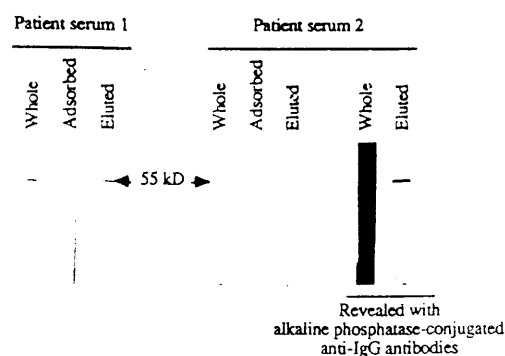


Figure 2. Antigenic community between platelet- and EC-derived 55-kDa antigen. Anti-55-kDa antigen antibodies were removed by incubation of positive sera [1 ml final diluted at 1:10 (v:v) in PBS in an Eppendorf microfuge tube] with a nitrocellulose band containing the 55-kDa platelet antigen. Following overnight incubation at 4°C, and removal of supernatants, the antibodies bound to nitrocellulose were eluted by 0.1 M glycine-HCl, pH 4 for 2 min, and then quickly neutralized with 1 M NaOH. The immunoreactivity of untreated (whole), adsorbed and eluted sera from two patients was then tested on EC, platelet (not shown), and SV40-transfected EC (not shown). Control of specificity was performed with nitrocellulose saturated with 5% non-fat milk diluted in PBS. These controls did not show any loss of antibody binding to the 55-kDa antigen and elution was negative (not shown). All the immunoblots were revealed with peroxidase-conjugated anti-heavy gamma chain antibodies. In some experiments, where indicated, these were replaced by alkaline phosphatase-conjugated antibodies.

results did not find any proliferative response with whole PBMC or with PBMC partly depleted of monocytes by adherence for 1 h in petri dishes before the assay. Because of the possible immunosuppressive effect of corticosteroids that patients received before the time of sampling, PBMC from patients and controls were stimulated by Con A for 48 h before [ $^3H$ ] thymidine assay.

The mode of action of Con A on the response of steroid-treated mononuclear cells is unknown; however, a selected effect of Con A on activated rather than resting T cells has been proposed as an indication that, in a putative T cell-mediated autoimmune disease, Con A acts preferentially on autoreactive T cells, which are thought to be in an activation state [34, 35]. Under these conditions we observed a specific [ $^3H$ ] thymidine incorporation in 6 out of 13 patients with circulating anti-55-kDa antigen antibodies, with index values ranging from 12 to 3 (Fig. 3A). However, this T cell reactivity has been also observed in 3 out of 17 patients without detectable serum anti-55-kDa antigen antibodies. Index values of antigen-specific T cell reactivity correlated with pulmonary function test values (*i.e.*  $FEV_1$ :  $r = 0.544$ ,  $p = 0.003$ ; Fig. 3B).

### 3.4 Characterization of the 55-kDa antigen

The stimulating idea of a putative B and T cell autoreactivity against an EC derived antigen in a subset of asthmatics led to the necessity of characterizing the properties of this antigen to obtain an efficiently enriched antigenic fraction.

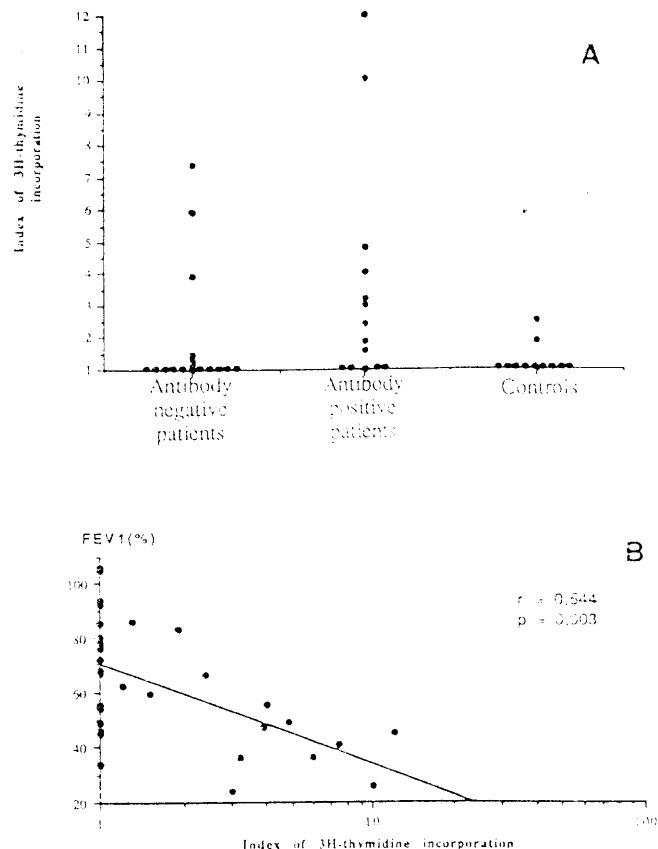


**Figure 3.** 55-kDa antigen-specific [ $^3\text{H}$ ]thymidine incorporation by T cells. **A** Antigen-specific T cell response in antibody-positive and antibody-negative groups of asthmatic patients. The antibody-positive patients ( $n = 14$ ) were almost all corticosteroid-dependent ( $13/14$ ) and severe asthmatics ( $\% \text{FEV}_1 \pm \text{SD}: 60 \pm 24$ ). The antibody-negative patients included 17 asthmatics in whom clinical features were well cross matched (in age, sex ratio, duration of asthma, mean  $\text{FEV}_1$ , and level of treatment) with the antibody-positive patients. Controls comprised 4 healthy subjects and 7 atopic asthmatics as defined in Sect. 2. **B** Correlation between  $\text{FEV}_1$  expressed in percentage of the predictive value, and indexes of antigen-specific [ $^3\text{H}$ ]thymidine incorporation.  $\text{FEV}_1(\%)$  was measured at the time of blood sampling. Statistical analysis was performed with the Spearman correlation test.

The 55-kDa antigen was not expressed on the surface of either platelets or EC (as determined by negative immunostaining and by the absence of antibody depletion by incubation of whole cells with sera; data not shown). On the other hand, the immunoblots of fractionated EC and platelets lysed by three cycles of freeze-thawing demonstrated that the 55-kDa antigen was recovered in the pellets of both cell types but not in the "cytosolic" fractions. The electrophoretic mobility of platelet and EC 55-kDa antigens remained unchanged with or without 2-mercaptoethanol. The electrophoretic migration of these antigens, analyzed by 2-D immunoblots of platelets and EC, showed that the 55-kDa antigens had an identical mobility, whatever the cell origin, with an isoelectric point between 8.0 and 9.0 (data not shown).

The 2-D gel analysis of [ $^{35}\text{S}$ ]Methionine-labeled normal and SV40-transfected EC showed that the 55-kDa antigens incorporated methionine only poorly at the basal state (as

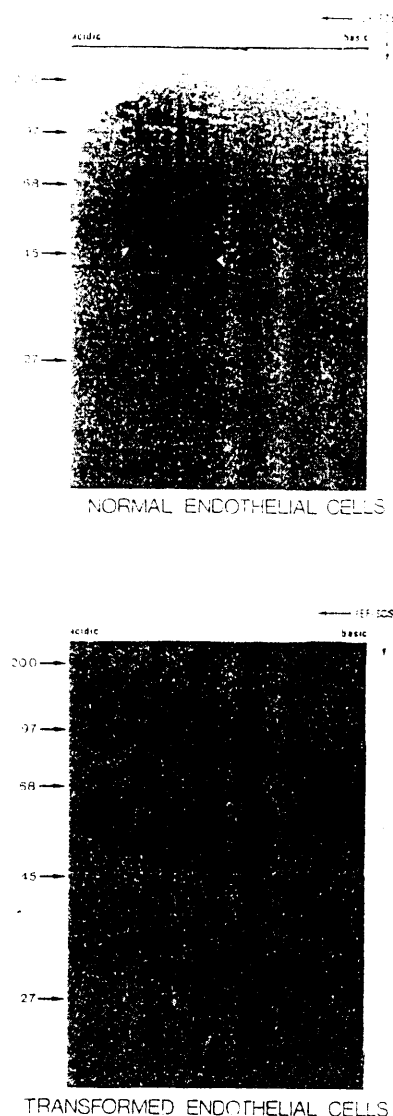


**Figure 4.** Two-dimensional analysis of [ $^{35}\text{S}$ ]methionine-labeled EC. Autoradiographies of 2-D immunoblots with 4-h-pulsed normal EC (**A**), and 2-h-pulsed SV40-transfected EC (**B**). Labeled EC ( $5 \times 10^6$ ) were 2-D electrophoresed and transferred onto nitrocellulose sheets. The immunoblotting was performed with sera from patients containing anti-55-kDa antigen antibodies as described in Sect. 2.5. Landmarks were made in each corner of the nitrocellulose sheets with a pen containing [ $^{35}\text{S}$ ]methionine and then autoradiographed. The 55-kDa antigen has been identified by superposition of immunoblotted and autoradiographed spots (arrowheads indicate the 55-kDa antigen in **A** and **B**).

determined by the comparison of 2-D immunoblots and their respective autoradiographies, Fig. 4), and silver-stained gels indicated that the 55-kDa antigen of platelet and of EC origin is a minor protein (not shown). However, the rate of synthesis and expression of the 55-kDa antigen was higher in SV40-transfected than in normal EC.

Reverse-phase HPLC fractionation of SV40-transfected EC detergent lysates resulted in a complex pattern of protein-containing peaks. After lyophilization, the collected fractions were examined for the presence of the 55-kDa antigen by Western blotting. This antigen was recovered totally in two fractions (Fig. 5A,B). However, no

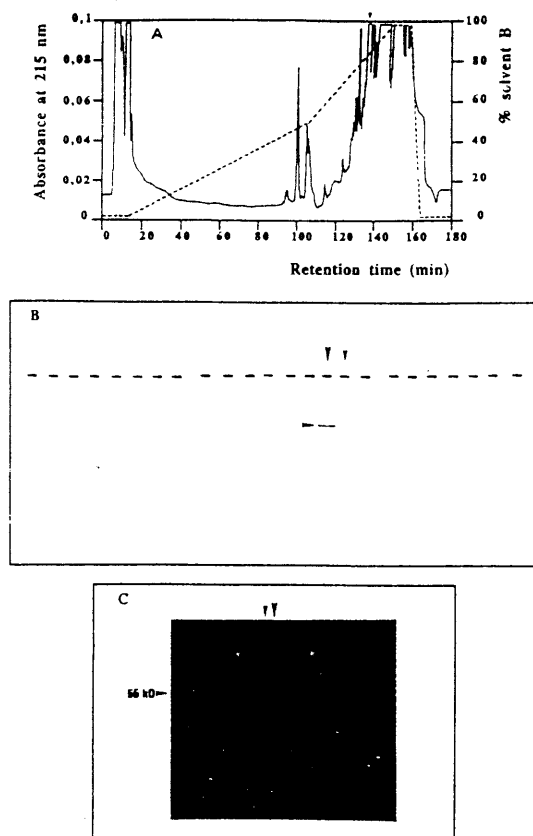


**Figure 5.** Characterization of the 55-kDa antigen by reverse-phase HPLC, SDS-PAGE electrophoresis, and Western blotting. (A) Extracts from  $10^7$  SV40-transfected endothelial cells were fractionated by reverse-phase HPLC (model LC6A) fitted with a UV spectrophotometer detector (model SPD6A). Chromatographic conditions: column Vydac C4, 7- $\mu$ m particle diameter 30-nm pore size, 150 mm  $\times$  4.5 mm. Solvent A: 0.05% TFA (v:v) in  $H_2O$ ; solvent B 75% acetonitrile, 0.045% TFA in  $H_2O$ ; flow rate: 500  $\mu$ l/min; UV detection 215 nm, 0.1 absorbance units (full scale). (B) Immunoblots of the reverse-phase-HPLC-collected fractions. (C) Coomassie blue-stained gel from half of the collected fractions. The 55-kDa antigen-containing fractions are designed with arrowheads in the A, B, and C.

corresponding band was detectable in the corresponding Coomassie blue-stained gel (Fig. 5C). An identical pattern of results was obtained with normal EC and platelet extracts. Silver nitrate-stained gels showed only a weakly stained band (data not shown), which confirmed that this antigen is a weakly expressed endothelial and platelet component.

#### 4 Discussion

Our results demonstrate the presence of circulating antibodies to a 55-kDa component common to platelets and EC in 34 from 97 patients suffering from bronchial asthma. Such antibodies were absent in healthy controls. Although a few patients (3/30) with SLE and 2/36 with rheumatoid arthritis, had such circulating autoantibodies, no anti-nuclear antibodies were detected in the whole of the asthmatic population tested. When the presence of anti-55-kDa antigen antibodies was analyzed according to the clinical and biological characteristics of the asthmatic

patients, neither the age, the sex ratio, the duration of asthma, nor the total serum IgE levels were discriminant. However, the subset of patients with circulating anti-55-kDa antigen antibodies was characterized (1) by an higher frequency of nonatopic asthma, as defined by the negative cutaneous tests ( $p = 0.0014$ ), and (2) by a more severe asthma, based on the frequent requirement of long-term oral steroid therapy ( $p = 0.0036$ ). The duration of oral steroid therapy was slightly shorter in the group of asthmatics displaying anti-55-kDa antigen antibodies than in the negative group, indicating that steroid therapy did not significantly interfere in the detection of these antibodies. Whatever the explanation may be, detection of such antibodies allows the classification of a subgroup of asthmatics in terms of severity.

Mechanisms by which the anti-55-kDa antigen autoantibodies can act to generate asthma remain, at the present time, speculative. The absence of cell surface expression both on EC and platelets from normal individuals is not in favor of an antibody, a complement- or a cell-dependent cytotoxicity. Another hypothesis might imply a possible platelet abnormality in aspirin-sensitive asthma [36]; we did not find any abnormal surface expression of this 55-kDa antigen on platelets from two patients with established aspirin-sensitive asthma by comparison with platelets from normal individuals. One can hypothesize that the anti-55-kDa antigen antibodies might form immune complexes when platelets and EC become activated or damaged releasing the 55-kDa molecule. However, the role of such immune complexes in the pathogenesis of some kinds of asthma remains highly speculative since the nature and functions of the 55-kDa molecule in its putative released form is presently under investigation. Previous anecdotal investigations have already indicated a possible role for these anti-55-kDa antigen antibodies in asthma. In one patient with nonatopic and cortico-steroid-dependent asthma associated with aspirin sensitivity and circulating anti-55-kDa antigen antibodies, plasma exchanges simultaneously induced a fall in these antibodies and clinical improvement. When clinical relapse occurred, it was associated with an increase of these same antibodies [37]. This relationship between clinical and biological data suggested a link between clinical severity and B cell autoreactivity to the 55-kDa platelet/EC antigen.

The demonstration of autoantibodies against a same structure shared by platelets and EC raises the question of the origin of this antibody response and its putative role in asthma. Cell mediated-immunity has been suggested in the pathogenesis of asthma: CD4-positive T lymphocytes expressed activation markers, such as IL-2R, class II major histocompatibility antigens, and very late activation antigen (VLA-1) during acute severe asthma and the percentage of activated cells decreased with treatment and clinical improvement [16, 17]. On the other hand, it has been recently demonstrated that the T cell reactivity to soluble nuclear antigens was clearly correlated with the disease activity in patients with autoimmune diseases such as SLE [38]. In the present study, analysis of T cell proliferation induced by purified antigen preparations indicated that among patients with severe asthma, some of them had a detectable reactive T cell response. The T cell proliferation was found to be specific but was only observed in a co-stimulatory system using Con A, as described elsewhere

[34, 35]. This may be due to the corticosteroid therapy or, more probably, to the low amount of the relevant 55-kDa antigen within the microparticles, as indicated by our data on the characterization of the 55-kDa antigen. The magnitude of antigen-specific T cell response was found to be linked to the pulmonary function values, namely to FEV<sub>1</sub>, as already seen in other studies [16, 17]. Unexpectedly, the antigen-specific T cell response was also found in some patients with undetectable anti-55-kDa antigen antibodies. At present there is no clear explanation for this apparent discrepancy. The discrepancy may be linked to the lack of sensitivity of the method used for the detection of anti-55-kDa antigen antibodies. Also, if one cannot exclude a nonspecific effect of the antigen-containing microparticles on T cells, the low number of antibody-negative patients and controls (3/28), in whom blood T cells have responded to antigen, would suggest that an antigen-specific T cell response could exist in a restricted subset of patients with severe asthma (without detectable autoantibodies). The discrepancy may also reflect the respective magnitude of T and B cell immune response to the 55-kDa antigen which may vary in some patients among whom T cell response appears as predominant. Antibodies to the 55-kDa antigen are, in our experimental conditions, still undetectable at the time of the blood sampling. These results have established a link between an immune response to the 55-kDa platelet/EC antigen and a subset of severe asthmatics. However, additional investigations are necessary to establish the role of this abnormal immune response in the development of chronic and severe asthma. The molecular cloning of this antigen is presently in progress and will provide helpful probes for investigating the significance of these findings.

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