

Quantitative analysis of peripheral allergen-specific B lymphocytes in asthma

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We have recently shown that the number of peripheral allergen-specific T cells can, in part, predict, together with methacholine hyperresponsiveness, the bronchial response to inhaled allergen in asthmatic patients. This study was designed to explore the role of blood B cells committed to produce allergen-specific IgE in asthma. Twenty-three asthmatic patients sensitized to *Dermatophagoides pteronyssinus* and 11 control subjects were studied. Peripheral blood B cells, committed to produce allergen-specific IgE, were enumerated by limiting dilution microcultures of Epstein-Barr virus (EBV)-transformed B cells. An allergen inhalation challenge was performed in all asthmatic subjects. No difference was found in the frequency of B cells committed to produce allergen-specific IgE either between asthmatic patients and controls or between asthmatic patients with or without late-phase bronchial response to allergen. No correlation was found between the frequency of B cells committed to produce allergen-specific IgE and the bronchial response to the allergen inhalation challenge. We conclude that, in quantitative terms, peripheral allergen-specific B cells are not as relevant as T cells to the development of the asthmatic response in the model of provoked asthma.

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Bronchial asthma is characterized by an increased airway responsiveness to different stimuli (1). In allergic asthma, the inhalation of the relevant allergen triggers an IgE-dependent reaction that is supposed to play a major pathogenetic role in the cascade of events leading to narrowing of the bronchial lumen (4, 15). T cells, in particular those belonging to the Th2-like subset, are involved in the immune response to the allergen (16). Moreover, the bronchial response to the sensitizing allergen is partly determined by the frequency of specific T cells (3). Whether a relationship also exists between the allergen-specific B-cell repertoire (i.e., the number of blood B cells committed to produce allergen-specific IgE) and the bronchial responsiveness to allergen has not been investigated so far.

In the present study, we analyzed, by limiting dilution cultures, the peripheral blood B cells from atopic asthmatic patients sensitized to *Dermatophagoides pteronyssinus* and from controls. We found that the frequency of blood B cells committed to produce allergen-specific IgE was not abnormally elevated in atopic patients with asthma and did not correlate with the severity of the bronchial response to the allergen inhalation.

Material and methods

Subjects

Twenty-three outpatients with a history of perennial asthma for the last 2 years or more were studied. Their characteristics are presented in Table 1. Allergic sensitization to *D. pteronyssinus* was demonstrated in all asthmatic subjects by skin prick test and radioallergosorbent test (RAST). All of them had high specific IgE levels (Table 1). The criteria for inclusion of asthmatic subjects in the study were the following: not to have suffered from respiratory infections for the previous 4 weeks, to have a forced expiratory volume in 1 s (FEV₁) greater than 70% of predicted values (17), and not to take any antiasthmatic treatment other than inhaled β_2 -stimulants on an "as necessary" basis. The drugs, if any, were discontinued at least 12 h before bronchial challenge.

Eleven healthy subjects with no history of allergic disease or asthma, total serum IgE levels < 100 IU/ml, negative skin prick tests, and negative RAST for aeroallergens served as controls. All subjects were informed of the nature and scope of the study and gave written consent.

Table 1. Subject characteristics

Groups	Age	Sex F/M	FEV ₁ (% pred)	Pd ₁₅ MCh µg	Pd ₁₅ All AU	Max LAR (% control)	RAST (PRU)
Controls	31±2	4/7	99±2	>5000	nd	nd	<0.35
Isolated EAR	21±1	0/7	100±5	331	144	4±1.5	15.2±1.5*
EAR and LAR	20±3	1/15	96±11	63**	28**	30±2	15.2±1.1*

EAR: early-phase asthmatic response; LAR: late-phase asthmatic response; Pd₁₅: dose causing 15% FEV₁ fall; max LAR: maximum FEV₁ fall 3–8 h after allergen challenge; MCh: methacholine; All: allergen; AU: arbitrary units; nd: not determined; PRU: Pharmacia RAST units. Pd₁₅ values are geometric means; other values are mean±SE.

* Significantly different from controls ($P<0.05$).

** Significantly different from EAR group ($P<0.01$).

Allergen extract

The allergen extract we used for both B-cell assays and bronchial challenge was obtained from Pharmacia (lot N. II 31217), and was developed for tissue culture. A preliminary 12% SDS–PAGE, performed according to Laemmli (11), showed, in reducing conditions, three main bands corresponding to about 27, 29, and 40 kDa at silver stain analysis (12) run with proper mol. mass controls, including lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), and bovine serum albumin (69 kDa) (not shown). Multiple faint bands were also visible. The amount of *Der p* I content in this extract was quantified by the enzyme-linked immunosorbent test (ELISA) with a *Der p* I-specific monoclonal antibody, as described by Chapman et al. (7), and corresponded to 32% of the dry weight of this extract (courtesy of Dr Falagiani, Lofarma Allergeni, Milan, Italy). The allergen extract was also tested for its bacterial endotoxin content by the *Limulus* amoebocytes gelification test. The assay was performed, upon reconstitution of the allergen extract at 1 mg/ml in culture medium, according to the manufacturer's instructions (PBI International, Milan, Italy), yielding 0.020 endotoxin units/ml, the same amount found in the medium alone.

Bronchial challenges

Forced expiratory maneuvers were recorded by a wedge spirometer (Vitalograph®, Buckingham, UK), and the highest FEV₁ from three technically acceptable maneuvers was retained for analysis. Aerosols were delivered by an ampule dosimeter device (MEFAR, Brescia, Italy), according to a method previously described (5).

For methacholine challenge, scalar dilutions of 1, 10, and 50 mg/ml were prepared, and incremental doses were obtained by changing the methacholine concentration, number of breaths, or both. The challenge was started with a 10-µg dose and continued with twofold increments until FEV₁ measured 1 min

after inhalation, was ≤80% of control, or up to a maximum dose of 5000 µg.

For allergen challenge, scalar dilutions of *D. pteronyssinus* reconstituted from predosed (arbitrary units, AU) dried allergen (Pharmacia, Uppsala, Sweden) were prepared. Challenge was started from a dose of 4 AU and continued with twofold increments until FEV₁ was ≤80% of control, or up to a maximum dose of 500 AU. The twofold increments of allergen dose were obtained by inhaling doubled allergen concentrations for 70 quiet breaths. The amount of solution inhaled at each step was 0.5 ml. Fifteen minutes was allowed between allergen inhalation and FEV₁ measurements. For detection of the occurrence of the late-phase asthmatic reaction (LAR), FEV₁ was measured after challenge at 30 and 60 min, and then hourly for 8 h. LAR was defined as a FEV₁ fall ≤15% during the 3–8 h period after the initial early-phase asthmatic reaction (EAR). LAR were stopped with β₂-stimulants and steroid treatment, if required by the patients or when the FEV₁ fall was >35% of base-line values.

Methacholine challenge always preceded the allergen challenge by 24 h.

Asthmatic subjects were subdivided into two groups according to the pattern of bronchospastic response to the inhaled allergen, i.e., whether subjects exhibited an isolated EAR or a dual (EAR and LAR) response.

Immortalization and limiting dilution cultures of B cells

Blood was drawn immediately before methacholine challenge. Peripheral blood mononuclear cells (PBMC) were isolated from 20–40 ml heparinized blood on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) and washed twice with RPMI-1640 (Flow Laboratories) containing penicillin (100 µg/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM). Cells were cultured in this medium supplemented with 5% fetal bovine serum, which will be referred to as "complete medium". Epstein-Barr virus (EBV) was obtained from the supernatant of

the B95-8 marmoset cell line (American Type Culture Collection, Rockville, MD, USA). PBMC ($1-10 \times 10^6$ per ml) were incubated for 2 h at 37°C in a 5% CO_2 , humidified atmosphere to immortalize B cells (2). Cells were subsequently seeded at scalar doses (2.0, 1.0, 0.5, 0.25, 0.125, 0.062, 0.031, and 0.015×10^4 per well) in 96-well U-bottom plates (Costar, Cambridge, MA, USA). Complete medium was used, to which cyclosporin A (500 ng/ml) (Sandoz, Basel, Switzerland) was added to prevent the cytotoxic effect of donor T cells (specific for EBV antigens expressed by infected B cells) or other lymphokine-dependent influence on B-cell differentiation and proliferation (2). For improvement of test sensitivity for very low precursor frequencies, two 96-well plates for each cell dose were prepared. Autologous, 1500-rad irradiated PBMC (10^5 per well) were added as feeders. Three weeks later, 90% of the supernatant volume was removed in order to discard antibodies produced in the context of the initial polyclonal activation induced by EBV, and replaced with fresh medium. Seven weeks later, the number of wells displaying foci of growing B cells, as assessed by phase-contrast inverted microscopy, was determined in each single plate, in order to calculate the immortalization rate (see below). The plates were then frozen at -70°C until analysis.

Detection and enumeration of B cells producing allergen-specific IgE

Supernatants of microcultures were firstly tested for total IgE content by a sandwich ELISA method. Plates were coated with a mouse monoclonal antibody against human IgE (Clone GE1, Sigma, St Louis, MO, USA). Samples were diluted 1:2 in saline before the test. Alkaline phosphatase-conjugated, affinity-purified, polyclonal goat antibodies against human IgE (Sigma), were used as a probe, at a 1:1000 dilution, and the test was developed with the appropriate substrate. The specificity of this antibody was previously tested with ELISA plate wells coated with purified human IgM, IgG, and IgA, 200 ng/ml (Sigma) or with human IgE, 50 ng/ml (Pharmacia). No detectable activity was observed in wells coated with any immunoprotein other than IgE after addition of substrate. For determination of total IgE content, wells were enumerated as positive when optical density was scored as 3 SD above the mean values displayed by supernatants of eight microcultures containing feeder only, as determined in each single experiment. The detection limit was 0.1 U/ml, as assessed by a standard with known IgE content (Pharmacia).

Microcultures were subsequently analyzed for *D. pteronyssinus*-specific IgE content. Plates were coated with 30 $\mu\text{g/ml}$ of the purified allergen,

and the above indicated alkaline phosphatase-conjugated, affinity-purified polyclonal antibody against human IgE was used as a probe. Samples were diluted 1:2 in saline before the test. Supernatants scoring 3 SD above the mean value of optical density of eight control microcultures (feeder only) were considered to be positive. The results obtained with this assay were comparable with those obtained with a commercially available ELISA (Pharmacia) in both inter- and intra-assay variation.

Statistical analysis

The results of limiting dilution experiments (frequency of immortalization of B cells and B-cell frequencies) were analyzed according to the method of maximum likelihood (9). A TI-59 calculator (Texas Instruments, USA), fitted with the statistics module, was used for calculation. This program evaluates the goodness of fit and lists the χ^2 and P values, allowing statistically unacceptable experiments to be discarded. Results were log-transformed. The unpaired Student's t -test was used to compare the frequencies of allergen-specific B cells between groups of subjects. Simple and multiple regression analysis was used to assess the correlation between allergen Pd_{15} (provocative dose causing 15% fall of FEV_1) and the following independent variables: methacholine Pd_{15} and total and specific IgE-producing B cells. Data are shown as mean values $\pm$ standard error of the mean (SEM), or of the geometric mean (GM) when indicated.

Results

Base-line values

Physiologic data, RAST results, and bronchial response to inhalation challenge are shown in Table 1. In subjects with EAR and LAR, both methacholine Pd_{15} (63 μg) and allergen Pd_{15} (28 AU) were significantly lower ($P < 0.01$, for both) than in subjects with isolated EAR. The maximum late FEV_1 fall in subjects with EAR and LAR ($30 \pm 2\%$) was significantly ($P < 0.0001$) larger than in subjects with EAR alone ($4 \pm 1.5\%$).

Frequencies of total and allergen-specific, IgE-producing B cells

Immortalization frequency in the different experiments ranged from 0.016 to 0.006 plated PBMC. In asthmatic patients, the frequency of B cells committed to produce total IgE upon EBV immortalization was not significantly different from controls ($P > 0.4$) (Table 2). Moreover, in asthmatic patients, the frequency of B cells committed to produce specific IgE against *D. pteronyssinus* was not different from con-

Table 2. Frequency of blood B cells committed to produce total and allergen-specific IgE in control subjects and asthmatic patients with isolated EAR or EAR and LAR

Subject no.	Total IgE-producing B cells $\times 10^{-4}$	Allergen-specific, IgE-producing B cells $\times 10^{-4}$
Controls		
1	223.0	130.0
2	198.0	<2
3	7.8	53.0
4	156.0	<2
5	8.7	<2
6	30.0	120.0
7	137.0	<2
8	13.0	89.0
9	40.0	<2
10	50.0	10.0
11	12.5	<2
Geometric mean	41.4	9.3
Patients with isolated EAR		
12	180.0	106.0
13	10.0	<2
14	165.0	13.0
15	148.0	<2
16	11.9	98.0
17	45.0	72.0
18	60.0	72.0
Geometric mean	54.4	13.4
Patients with EAR and LAR		
19	235.0	15.0
20	193.0	85.0
21	211.0	141.0
22	6.4	13.0
23	72.0	38.0
24	40.0	<2
25	9.5	<2
26	45.0	56.0
27	194.0	<2
28	19.0	<2
29	175.0	7.2
30	137.0	<2
31	14.6	115.0
32	166.0	108.0
33	157.0	<2
34	125.0	87.0
Geometric mean	69.7	14.1

trols ($P > 0.3$) (Table 2). This difference was not significant even when only subjects with EAR and LAR were compared with controls ($P > 0.5$) (Table 2).

B-cell frequency data were also corrected according to the immortalization frequency obtained in single experiments (not shown), but this did not affect the results of the statistical analysis.

Relationship between bronchial responsiveness and frequency of IgE-producing B cells

In asthmatic patients, methacholine Pd_{15} correlated neither with the frequency of total IgE-producing B cells ($r = 0.003$; $P > 0.9$) nor with the frequency of

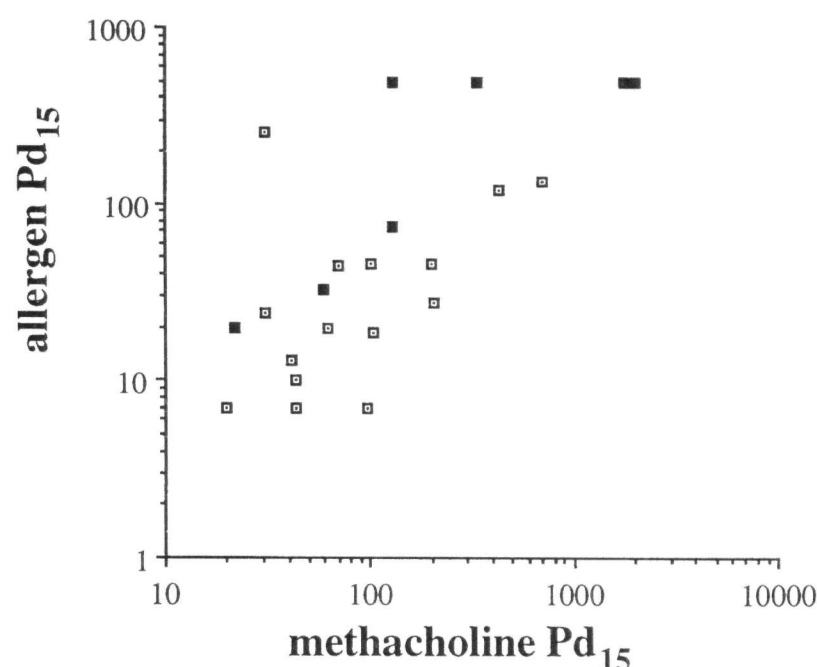


Fig. 1. Relationship between allergen provocative dose causing 15% fall of FEV_1 (Pd_{15}) and methacholine provocative dose causing 15% fall of FEV_1 in patients with isolated (full squares) or dual reaction. Allergen and methacholine doses were calculated by interpolation of dose-response curves.

allergen-specific, IgE-producing B cells ($r = 0.06$; $P > 0.7$). Therefore, these variables were considered to be independent.

Allergen Pd_{15} significantly correlated with methacholine Pd_{15} ($r = 0.61$; $P < 0.005$) (Fig. 1) but with neither the frequency of total IgE-producing B cells ($r = 0.09$; $P > 0.6$) (not shown) nor with the frequency of allergen-specific, IgE-producing B cells ($r = -0.05$; $P > 0.8$).

Moreover, no correlation was found between specific IgE levels in serum and frequency of allergen-specific, IgE-producing B cells ($r = 0.05$; $P > 0.7$). In nine patients with EAR and LAR, the severity of the symptoms required treatment.

Discussion

In atopic asthma, allergen-specific IgE levels are increased both in serum and on the surface of airway epithelium (1). In addition, it has been shown that allergen-specific IgE bound to the relevant allergen can trigger release of cells carrying on their surface the high-affinity receptors for IgE, i.e., mast cells and basophils (18–22). The IgE-allergen complexes mediate cell activation, which results in the release of various mediators able to cause bronchial inflammatory changes and smooth-muscle contraction (18, 22). These observations led to the hypothesis that the allergen-induced, IgE-dependent reaction plays a major pathogenic role in allergic bronchial asthma.

The control of IgE synthesis by B cells is probably dependent on T cells with a definite pattern of lymphokine secretion (4). B cells participate in the last step of the allergic reaction by differentiating into

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