

An increased frequency of IgE-producing B cell precursors contributes to the elevated levels of plasma IgE in atopic subjects

M. T. KASAIAN, C. H. MEYER, A. K. NAULT and J. F. BOND

ImmuLogic Pharmaceutical Corporation, Waltham, MA, USA

Summary

Background The production of specific IgE, which underlies the allergic response, may be a normal correlate of the immune response to a certain class of antigen (allergens), or could represent a unique response driven by regulatory signals that are absent in non-allergic individuals. If atopic subjects do possess a regulatory environment favoring IgE production, they may display not only allergen-specific IgE, but also higher levels of total IgE and higher frequencies of IgE-producing B lymphocytes.

Objective To address the contribution of antibody-producing cell number to the circulating IgE titre in atopic vs non-atopic subjects.

Methods Frequency determination by limiting dilution of EBV transformants and Poisson distribution analysis. Titres of total and allergen-specific IgM, IgG, and IgE by specific ELISA.

Results In contrast to findings reported by others, the atopic subjects had a significantly higher frequency of IgE-producing B cells than non-atopics (0.79% of total Ig-producing cells, as compared with 0.17% for the control group; $P < 0.01$), suggesting that one factor contributing to the high plasma IgE titres in atopic subjects is the high frequency of B lymphocytes with the potential to produce IgE. Although only the atopic subjects produced allergen-specific IgE, the frequency of specific IgE-producing B cells was undetectable in both groups.

Conclusion Atopic subjects have higher frequencies of IgE-producing B cell precursors than non-atopics. A correlation exists between IgE-producing B cell frequency and levels of circulating IgE.

Keywords: IgE, B cells, precursor frequency, house dust mite, ragweed

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Introduction

The production of allergen-specific IgE initiates hypersensitivity reactions through binding to the high affinity IgE receptor on mast cells and basophils. The cross-linking of receptor-bound IgE with allergen triggers degranulation with the release of histamine and other mediators, resulting in an immediate inflammatory response (weal-and-flare), followed, in some cases, by

the allergic late phase. Mediator release by mast cells and basophils is directly responsible for producing potentially severe allergic symptoms [1–3], and is under strict regulatory control, a key element of which is the control of IgE metabolism. IgE is present at lower levels than IgM, IgG, or IgA in the circulation, and has a more rapid *in vivo* half-life [4]. In allergic subjects, however, relatively high levels of circulating IgE are common, including not only IgE directed against the allergen, but also, frequently, higher total IgE levels (of unknown specificity). Elevated serum IgE levels are also found in patients with hyper-IgE syndrome and in patients with

Correspondence: M. T. Kasasian, ImmuLogic Pharmaceutical Corporation, 610 Lincoln Street, Waltham, MA 02154, USA.

parasitic helminth infections. In those conditions, IgE titres correlate with high frequencies of B cell precursors committed to IgE production, suggesting that expansion of IgE-producing B lymphocytes is a major mechanism whereby high levels of IgE are generated [5,6].

An increased frequency of IgE-producing B lymphocytes may also account for the elevated IgE levels in allergy. In this report, we use EBV² transformation of peripheral blood B lymphocytes followed by limiting dilution analysis to investigate whether atopic subjects have a higher frequency of IgE-producing B cell precursors than non-atopic subjects, and to assess the relationship between plasma IgE levels and the frequency of IgE-producing B cell precursors in the peripheral blood.

Materials and methods

Patients and allergens

Plasma samples were obtained from volunteer subjects with skin-prick test reactivity of 2+, 3+, or 4+ (weal diameter of 5–8 mm, 8–10 mm, and >10 mm, respectively) to cat, house dust mite (HDM; *Dermatophagoides pteronyssinus*), or ragweed (RW; *Ambrosia artemisiifolia*). Non-atopics did not react to the following allergens by skin-prick test: cat, house dust mite (*D. pteronyssinus* or *D. farinae*), ragweed, mix grass, ryegrass, timothy grass, and trees. Allergic subjects were not receiving immunotherapy and were not taking medication to treat their allergies. The allergens *Der p*I and *Der p*II were purified from *D. pteronyssinus* extract by affinity chromatography, using the monoclonal antibodies 4C1 [7] and 6D6 [8], respectively. *Amb a*I was purified from short ragweed pollen as described [9].

Analysis of plasma antibody levels

For determination of total IgM and IgG levels in plasma, ELISA plates (Costar) were coated with goat-anti-human Ig(M+G+A) (Organon Teknika-Cappel) in 0.05 M carbonate:bicarbonate buffer, pH 9.6. The plates were washed with PBS containing 0.05% Tween-20 (PBS-Tween), then blocked with 0.5% gelatin containing 1 mM polyvinylpyrrolidone (gelatin-PVP). Standards of purified human IgM or IgG (Sigma Chemical Co., St Louis, MO, USA), or human plasma samples diluted in PBS-Tween were added and incubated overnight at 4°C. The plates were treated with either biotinylated goat-anti-human IgM or IgG (Caltag Laboratories, San Francisco, CA, USA), followed by incubation with streptavidin conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Binding

was detected using the substrate TMB (3,3',5,5'-tetramethylbenzidine) (Kirkegaard and Perry Labs, Inc., Gaithersburg, MD, USA), and the products were read in a plate reader at 450 nm. For determination of antigen-specific IgM, ELISA plates were coated with purified *Amb a*I (2.5 µg/mL) or a mixture of purified *Der p*I and *Der p*II (2.5 µg/mL each) in PBS, and blocked with PBS containing 0.05% human serum albumin (ICN, Irvine, CA, USA). For the antigen-specific IgG assay *Amb a*I- or *Der p* (I+II)-coated plates were blocked with gelatin-PVP, and plasma samples were diluted in gelatin-PVP. For determination of total IgE levels, ELISA plates were coated with polyclonal goat-anti-human IgE (Kirkegaard and Perry Labs), blocked with gelatin-PVP, then incubated with plasma or with human myeloma IgE standard (clone 795; Biodesign Int., Kennebunk, ME, USA), in PBS-Tween. IgE was detected using biotinylated goat-anti-human IgE (Kirkegaard and Perry Labs). For all assays, background signals on uncoated plates were subtracted from signals on plates coated with specific allergen. The limits of sensitivity of the ELISAs were 100 pg/mL for total IgE, 2.5 ng/mL for total IgM, and 250 pg/mL for total IgG. Determination of antibody levels in culture supernatants was performed as for plasma samples, except that the blocking step was omitted.

Allergen-specific IgM was further analysed by competitive inhibition ELISA. Serial dilutions of the mixture of *Der p*I and *Der p*II (5 µg/mL–0.15 µg/mL each) or *Amb a*I (from 5 µg/mL to 0.15 µg/mL) were incubated with plasma diluted in PBS-Tween containing 1% bovine serum albumin (BSA). After overnight incubation at room temperature with soluble antigen, the plasma samples were transferred to ELISA plates and developed as for allergen-specific IgM.

Lymphocyte isolation, EBV transformation, and limiting dilution analysis

The mononuclear cell fraction was isolated by Ficoll centrifugation (LSM; Organon Teknika-Cappel) from 60 mL peripheral blood donated by six pairs of non-atopic subjects. (One non-atopic subject was later found to have non-allergic rhinitis, and was dropped from analysis). The lymphocyte fraction was depleted of T cells by rosette formation with sheep red blood cells (Colorado Serum Co., Denver, CO, USA) treated with 2-aminoethylisothiuronium bromide (AET, Sigma) [10]. T cell depletions were over 95% efficient in each case. A sample of the T-depleted fraction was stained with PE-labelled MoAb to CD20 (Coulter, Hialeah, FL, USA), and the percentage of B cells (CD20⁺) was found to be 15–25% for all subjects by flow cytometry (FACScan; Becton-Dickinson, Mountain View, CA,

USA). The T-depleted lymphocytes were incubated with EBV (supernatant of the infected B95-8 marmoset cell line) for 2 h, 37°C, then plated at limiting dilution in 96-well U-bottom microtiter plates (Costar), containing 200 000/well irradiated PBMC as feeders. Forty-eight wells were plated at each of 12 twofold cell dilutions, ranging from 2×10^5 to 48 cells per well. After 3 weeks, the culture supernatants were assayed for total and antigen specific IgM, IgG, and IgE by ELISA. The fraction of negative wells was plotted as a function of the number of cells originally plated per well. In accordance with Poisson distribution analysis [11], frequency was determined as the cell concentration at which 37% of the wells contained no detectable antibody, as predicted by linear regression analysis (Statview SE + Graphics, Abacus Concepts, Inc., Berkely, CA, USA). For each plot, r^2 values were >0.9 .

Results

Total plasma IgM, IgG, and IgE levels in atopic and non-atopic subjects

Plasma samples from 12 atopic subjects (3+ or 4+ skin-test reactivity to cat, RW, or HDM allergens) and 12 non-atopic subjects (absence of skin-test reactivity to a panel of allergens) were assayed for levels of total IgM, IgG, and IgE using isotype-specific ELISAs. Antibody levels were quantitated by comparison to standard

purified human immunoglobulins of known concentration. The atopics had higher levels of plasma IgE than the non-atopics, and the difference was statistically significant (Table 1). The patient groups did not, however, differ significantly in their plasma levels of total IgM or IgG (Table 1).

Titres of allergen-specific IgM, IgG, and IgE in atopic and non-atopic subjects

Titres of IgM, IgG, and IgE directed against a mixture of the major HDM allergens, *Der p I* + *Der p II*, or the major RW allergen, *Amb a I*, were determined in plasma samples from the 12 atopic and 12 non-atopic subjects (Table 2). Only those subjects with mite allergy had IgE to the HDM allergens. With the exception of patients #1080 and #1194, all RW-allergic subjects had detectable IgE to *Amb a I* (Table 2).

All atopic and non-atopic subjects tested had detectable plasma levels of HDM-specific IgM and IgG (Table 2). The majority of subjects in both groups had IgM to *Amb a I*, but only half of the ragweed-allergic subjects in this small patient set had IgG to this allergen (Patients #440, #146, #153, and #1080). Patients #147, #1206, #1102, and #1194, who are ragweed-allergic, did not have detectable levels of specific IgG. In contrast, patients #9 and #1813, who are non-allergic, did display IgG specific for *Amb a I*. In general, there were lower levels of both IgM and IgG to *Amb a I* than to the mite

Table 1. Levels of total plasma IgE, IgM, and IgG in atopic and non-atopic subjects*

Subject	Atopic			Subject	Non-atopic		
	Total IgE ($\mu\text{g/mL}$)	Total IgM (mg/mL)	Total IgG (mg/mL)		Total IgE ($\mu\text{g/mL}$)	Total IgM (mg/mL)	Total IgG (mg/mL)
288	0.3	1.8	4.5	1127	0.02	6.0	6.0
147	0.9	3.0	4.5	165	0.07	3.0	6.0
440	1.8	9.0	2.0	255	0.3	6.0	6.0
146	1.3	1.6	3.0	256	0.02	4.0	6.0
1309	0.6	1.8	6.0	249	0.05	0.5	3.0
153	1.6	3.0	2.7	9	0.3	3.0	6.0
1194	0.6	3.0	1.8	1813	0.03	8.0	2.4
1345	3.0	7.0	4.5	1846	0.2	4.2	7.5
1102	5.0	4.0	3.5	1724	0.009	12.0	9.0
1222	0.6	9.0	4.5	1331	0.4	4.5	1.8
1080	0.045	7.0	6.0	1333	0.1	5.0	2.8
1256	0.15	3.0	6.0	1340	0.007	7.5	2.4
Mean	1.32	4.43	4.08	Mean	0.13†	5.31	4.91
SD	1.43	2.78	1.50	SD	0.15	2.94	2.33

* Values determined by ELISA and comparison to standards of known concentration. Results are reproducible to within a factor of two.

† $P < 0.001$ vs atopic by Mann-Whitney *U*-test.

Table 2. Titre of allergen-specific IgE, IgM, and IgG in plasma of atopic and non-atopic subjects*

Subject	Skin test reactivity†	IgE		IgM†		IgG	
		Der p I/II	Amb a I	Der p I/II	Amb a I	Der p I/II	Amb a I
288	3+ cat	< 4§	< 4	350	40	900	< 4
147	4+ RW#	< 4	100	350	100	400	< 4
440	4+ RW, 4+ HDM¶	90	100	450	60	900	100
146	3+ RW, 2+ cat	< 4	10	100	< 4	160	5
1309	4+ HDM	300	< 4	400	75	500	< 4
153	4+ RW, 4+ HDM	100	30	200	150	300	30
1194	3+ RW, 2+ HDM	35	< 4	450	30	700	< 4
1345	4+ HDM	20	60	800	75	2000	< 4
1102	3+ RW	< 4	30	400	100	400	< 4
1222	2+ RW, 4+ HDM	60	< 4	650	75	80	< 4
1080	4+ RW	< 4	< 4	150	20	110	15
1256	4+ HDM, 2+ cat	45	< 4	400	40	300	< 4
1127		< 4	< 4	400	25	300	< 4
165		< 4	< 4	550	100	2000	< 4
255		< 4	< 4	1010	100	1100	< 4
256		< 4	< 4	400	40	450	< 4
249		< 4	< 4	110	40	40	< 4
9		< 4	< 4	100	60	90	50
1846		< 4	< 4	200	100	80	< 4
1724		< 4	< 4	150	20	200	< 4
1331		< 4	< 4	250	30	1100	< 4
1333		< 4	< 4	400	< 4	800	< 4
1340		< 4	< 4	100	< 4	500	< 4
1813		< 4	< 4	300	400	300	80

* Titre determined as plasma dilution required to achieve half maximum response (absorbance at 450 nm) in specific ELISA.

† Specificity of the IgM reactivity was confirmed for each subject by competitive inhibition ELISA.

‡ Results of skin-prick test.

§ No specific ELISA signal (absorbance at 450 nm) at 1:4 plasma dilution.

Ragweed (*A. artemisiifolia*).

¶ House dust mite (*D. pteronyssinus*).

allergens. Because of the difficulty of assaying specific IgM in plasma, the specificity of the IgM reactivities to mite allergens and to *Amb a I* were confirmed in each case by competitive inhibition ELISA (data not shown).

Frequency of total IgM, IgG, and IgE-producing B lymphocytes in atopic and non-atopic subjects

The frequencies of total IgM, IgG, and IgE-producing cell precursors were determined using PBMC from six of the atopic and five of the non-atopic subjects, and calculated according to Poisson distribution analysis. For one non-atopic subject (#165), the frequency of total IgE-producing B lymphocytes was below the limits of detection of the assay (< 1/800 000 plated cells). The frequency of cells committed to the production of allergen-specific IgM could also be calculated, but the frequencies of cells producing allergen-specific IgE and IgG were undetect-

able by these methods. In order to compare frequencies among the 11 patient samples, which were prepared on different days, contained different percentages of B cells, and had possible variations in the efficiency of EBV transformation, the frequency of cells committed to the production of either IgM, IgG, IgE, or allergen-specific IgM was expressed as a percentage of total Ig-producing cells. Atopic and non-atopic subjects display a statistically significant difference ($P < 0.01$, Mann-Whitney *U*-test) in the frequency of cells committed to production of total IgE, but not in the frequency of those producing total IgM, total IgG, or allergen-specific IgM (Table 3).

Correlation of plasma IgE levels with total IgE-producing B cell precursor frequency

The frequencies of IgE-producing B lymphocytes from the six atopic and five non-atopic subjects analysed

Table 3. Frequencies of Ig-producing cell precursors in atopic and non-atopic subjects

	Allergy	Total IgE	Total IgM	Total IgG	Specific IgM
Atopic subjects					
288	Cat	0.370*	59.2	40.4	
1080	RW	0.608	80.0	19.4	0.391†
1309	HDM	0.539	87.6	11.9	1.24†
153	RW, HDM	1.22	77.9	20.9	0.409‡, 0.199†
1194	RW, HDM	0.840	91.3	7.9	1.38†
1355	HDM	1.16	49.3	49.5	1.33†
Average		0.790	74.2	25.0	0.825
SD		0.345	16.5	16.4	8.546
Non-atopic subjects					
165		n.d.§	68.0	32.0	
1127		0.119	55.5	44.4	0.101‡
249		0.043	88.6	11.4	2.81†
9		0.322	86.4	13.2	1.40‡, 0.897†
256		0.200	93.6	6.2	0.503†
Average		0.171#	78.4	21.4	1.142
SD		0.119	16.1	16.1	1.05

* Number of IgE producing cells per 100 Ig-producing cells.

† Cells producing IgM to *Der p* (I + II).

‡ Cells producing IgM to *Amb a* I.

§ ND, not detectable.

$P < 0.01$ by Mann-Whitney *U*-test.

above (Table 3) were compared with the total plasma IgE levels in the same subjects (Table 1). When values for the 11 subjects were compared by simple linear regression analysis, there was a positive correlation between these two parameters ($r^2 = 0.689$; $P < 0.002$) (Fig. 1), which was strengthened by deleting data points from the atopic subjects with the highest (#1345) and lowest (#1080) IgE from the analysis ($r^2 = 0.909$; $P < 0.0001$). Although the number of patients included in this analysis was small, the data are consistent with an association between the IgE-producing B cell precursor frequency and the amount of IgE produced *in vivo*.

Discussion

To assess the contribution of antibody-producing cell frequency to the high circulating IgE levels associated with allergic disease, we compared the frequencies of B cells committed to production of IgE in atopic and non-atopic subjects. Atopic subjects were found to have higher frequencies of total IgE-producing B cells, higher levels of total plasma IgE, and detectable titres of allergen-specific IgE. There was a positive correlation between plasma IgE levels and the frequency of IgE-producing B cell precursors, suggesting that one factor contributing to elevated plasma IgE levels in atopic

subjects is a higher frequency of B cells with the potential to produce IgE. Allergen-specific IgE-producing B cells could not be detected in either group, however. Evaluation of the frequency of B cells that maintain plasma levels of allergen-specific IgE must await the development of more sensitive methods.

We found a statistically significant difference in the frequency of total IgE-producing B cell precursors in atopic vs non-atopic subjects, in disagreement with the conclusions of Martinez-Maza *et al.* [12] which is, to our knowledge, the only other study in which these frequencies were compared directly. The reasons for this disagreement are not readily apparent, as both studies utilized limiting dilution analysis of T cell-depleted, EBV-transformed PBMC. The major difference may be related to the patient pools. The non-atopes utilized by Martinez-Maza *et al.* were described only as negative for pollen-specific IgE by radioallergosorbent test (RAST) [12], whereas those utilized here were skin-test negative for a panel of allergens. Thus, the control group in this study was more stringently identified as non-atopic.

Our findings of IgE-production by 1/300–1/3000 Ig-secreting cells in non-atopes are consistent with results of other studies in which limiting dilution analysis of EBV transformants was used to determine precursor frequency. Lanzavecchia and Parodi [13], Martinez-Maza

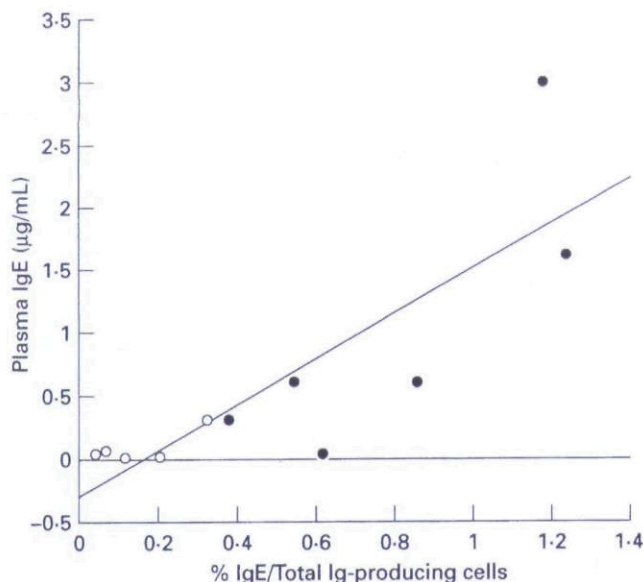


Fig. 1. Correlation between plasma IgE levels and IgE-producing B cell precursor frequency. The total plasma IgE levels and IgE-producing B cell precursor frequency were compared using linear regression analysis. Data are included for all six atopic subjects (●) and five non-atopic subjects (○). $r^2 = 0.689$; $P < 0.002$.

et al. [12], and Mackenzie and Dosch [14] all estimated a frequency of IgE-producers between 1/800 and 1/6000 B lymphocytes in healthy subjects. Stein *et al.* [15] found the IgE-committed B cell subset to comprise 0.1–1% of all EBV-responsive B lymphocytes, in excellent agreement with our results. These estimates are all much greater than those of King *et al.* who utilized a spot ELISA without polyclonal B cell activation to arrive at a frequency of 0.42 IgE-producing cells/100 000 B lymphocytes [5], a result which may be related to the resting state of most circulating B lymphocytes. EBV transformation, which induces production of all Ig classes, allows analysis of (resting) precursor B cell frequencies [11,16,17]. King *et al.* have raised the criticism that EBV may introduce a bias in that any IL4 produced by T cells in the culture would, in the presence of EBV, induce an isotype switch to IgE in a fraction of B lymphocytes [5]. In the present study, however, T lymphocytes were depleted prior to EBV transformation, making such an *in vitro* class switch less likely. Basophils, which comprised < 2% of the cells in our cultures, would retain the ability to secrete IL-4, but as the cultures were maintained in the absence of allergen, lymphokines, or other agents required to provoke IL4 secretion by these cells [18], they are an unlikely source.

In addition to IgE, we quantitated plasma levels of allergen-specific IgM and IgG. Levels of *Der p*-specific

IgM and IgG and *Amb a*I-specific IgM were comparable in atopic and control subjects. *Amb a*I-specific IgG, however, was undetectable in half of the ragweed-allergic subjects and in all but two of the non-atopes. The higher antibody titres to HDM allergens may be related to antigen exposure levels. HDM allergens can be found at levels of up to 100 µg/g of dust [19,20], a virtually unavoidable environmental allergen. In contrast, exposure to ragweed, a seasonal allergen, may not be great enough to elicit high levels of specific plasma IgM or IgG, even in RW-allergic subjects. Although some researchers have found evidence of higher specific IgG titres in atopic subjects, the demonstration of allergen-specific antibodies in both atopes and non-atopes is in agreement with a number of studies [20–23]. Thus, neither allergen-specific IgM nor IgG is associated with allergy, and neither correlates with the presence or titre of allergen-specific IgE.

The factors influencing the progression of a normal immune response involving IgM and IgG to an allergic response involving IgE are unknown, as are the properties of allergens that induce this transition. Although Burastero *et al.* [24] reported that atopic subjects had significantly higher frequencies of *Der p*-specific T cells than did healthy subjects, both O'Hehir *et al.* [25] and Wierenga *et al.* [26] have cloned T cells specific for the HDM allergens from both atopic and non-atopic donors. Only those clones isolated from allergic subjects, however, could be induced by HDM allergens to provide help for IgE synthesis by autologous B lymphocytes [25–27]. These studies suggest that although both atopic and non-atopic subjects mount an immune response to allergens, T lymphocytes of atopic subjects respond differently than those of non-atopes, producing a panel of lymphokines (high IL4, low IFN γ) that favours the production of IgE [28]. Because IL4 enhances IgE production by directing isotype switch recombination to the C ϵ locus [29], such a mechanism would result in an increased frequency of IgE-producing B cell precursors, as was observed here. The specific genetic, regulatory, or environmental differences that underlie the distinct T cell responses to allergens in atopic and non-atopic subjects remain to be elucidated.

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