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The majority of allergen-specific IgE in the blood of allergic patients does not originate from blood-derived B cells or plasma cells

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Abstract

Background The production of allergen-specific IgE antibodies is a hallmark of IgE-mediated allergy but the contribution of blood cells to allergen-specific IgE production in allergic patients has not been studied in detail.

Objective Aim of this study was the characterization of IgE-producing cells in the blood of allergic patients and the determination of the amount of IgE antibodies which are produced by these cells in relation to total amounts of circulating specific IgE.

Methods Peripheral blood mononuclear cells (PBMCs) were isolated from allergic patients and cell populations were purified or depleted using magnetically labelled antibodies directed against specific cell surface markers (CD19, CD20, CD22, CD27, CD38, CD126, CD138, CD203c). Allergen-specific IgE was measured in serum samples and cell culture supernatants by quantitative ImmunoCAP measurements and by ELISA using purified recombinant allergens. IgE transcripts were detected using RT-PCR with primers specific for human IgE.

Results We found that allergen-specific IgE levels in PBMC supernatants correlated strongly with specific serum IgE but represented less than 1% of circulating IgE. Depletion of basophils resulted in substantial reduction of allergen-specific IgE levels in PBMC culture supernatants suggesting that an important source of allergen-specific IgE in PBMC supernatants could be IgE derived from the surface of basophils. Newly synthesized IgE was derived from CD138+ plasma cells, but not from B and B memory cells, and accounted for only approximately 0.2% of circulating IgE in blood.

Conclusion and Clinical Relevance Our finding that the majority of allergen-specific IgE in the peripheral blood is not derived from IgE-secreting cells in the blood suggests local IgE production in tissues as a major source for allergen-specific IgE and possible target for therapeutic intervention.

Keywords basophils, CD138+ plasma cells, IgE synthesis, specific-IgE levels

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Introduction

Production of IgE antibodies to otherwise harmless environmental antigens (i.e. allergens) represents the hallmark of type I allergy [1]. Allergen-induced cross-linking of IgE antibodies bound to the surface of effector cells (i.e. mast cells and basophils) leads to their degranulation and to the release of histamine

and other inflammatory mediators, thus causing the symptoms of the immediate-type allergic reaction [2]. Furthermore, allergen uptake and presentation to T cells can be enhanced by IgE via the high and the low affinity IgE receptor (FcεRI and CD23) on the surface of dendritic cells, monocytes and B cells [3–5]. The importance of IgE antibodies has also been underlined by the successful use of non-anaphylactic anti-IgE antibodies for the treatment of allergic patients [6].

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Serological diagnosis of allergy relies mainly on the measurement of IgE antibodies in allergic patients' blood [7]. IgE is the least abundant immunoglobulin class and the half-life of IgE is rather short [8]. Therefore, a continuous IgE production is necessary to maintain the levels of allergen-specific IgE. In this context it has been demonstrated that mucosal allergen contact leads to a strong increase of allergen-specific IgE in the blood within 2–4 weeks [9, 10]. However, the major source and site of allergen-specific IgE production remain a matter of debate. It has been unambiguously shown that the peripheral blood of allergic patients contains cells producing allergen-specific IgE antibodies [11]. cDNAs coding for allergen-specific antibodies have been isolated from allergic patients' PBMCs and could be isolated by combinatorial cloning [12]. In addition, the presence of IgE-secreting plasma cells in the blood of allergic patients' was demonstrated by intracellular IgE and CD138 surface staining in a flow-cytometric approach [13]. On the other hand, there is increasing evidence for local IgE production in the various target tissues of allergen contact and allergic inflammation [14–18]. In the present study we characterized the cells producing allergen-specific IgE in the peripheral blood and weighed their contribution to allergen-specific IgE in the blood.

Materials and methods

Patients

In total, nine allergic patients (ID1–ID9) and two non-atopic subjects (ID10 and ID11) were included in the study. For cell separation experiments, we analyzed allergen-specific IgE antibody levels in sera and supernatants from cultured PBMCs of patients allergic to birch pollen, timothy grass pollen, ash pollen, cat or house dust mite (ID1–ID8). Patients' sensitization profiles were determined using allergen chip analysis (ISAC Immuno Solid-phase Allergen Chip; VBC-Genomics, Vienna, Austria). The study was approved by the ethical committee of the Medical University of Vienna and blood was taken after informed consent had been given. Total IgE levels and IgE levels specific for recombinant (r) Bet v 1, rDer p 2, rOle e 1, rFel d 1, rPhl p 1 and rPhl p 5 were quantified using the ImmunoCAP System (Phadia Diagnostics, Uppsala, Sweden). In addition, semi-quantitative ELISAs were used for measuring IgE levels for rBet v 1, rDer p 2, rPhl p 1 and rPhl p 5, as described [19].

Reagents

Recombinant allergens, rBet v 1, rPhl p 1, rPhl p 2, rPhl p 5 were obtained from Biomay, Austria. rDer p

2, rOle e 1 and rFel d 1 were expressed in *Escherichia coli* and purified as described [20–22]. The protein synthesis inhibitor cycloheximide was purchased from Sigma-Aldrich (St Louis, MO, USA). ^{32} P-ATP was purchased from Perkin Elmer (Waltham, MA, USA) and T4 polynucleotide kinase from Promega (Madison, WI, USA).

Isolation and cultivation of peripheral blood mononuclear cells (PBMCs)

The PBMCs were isolated from heparinized blood samples of allergic patients using Ficoll density gradient separation (Amersham Biosciences AB, Uppsala, Sweden) [11]. Cells were washed three times in phosphate buffered saline and counted under the light microscope using a counting chamber. Dead cells were excluded by Trypan Blue staining. PBMCs or PBMC fractions prepared as described in 'magnetic cell separation' were cultured in duplicates at 37°C/5% CO₂ in RPMI 1640 containing L-glutamine (BioWhittaker, Verviers, Belgium), supplemented with 10% FCS (PAA Laboratories, Pasching, Austria) and 50 µg/mL gentamycin (Sigma, St. Louis). In indicated experiments, cycloheximide was added at day 0 of culture at a concentration between 25 and 100 µg/mL. In other experiments, PBMCs were cultured for 1 week in the presence of rBet v 1, rOle e 1, rDer p 2, rFel d 1, rPhl p 1 or rPhl p 5 at 250 ng/mL. Furthermore, the stability of IgE was assessed in PBMC cultures by adding sera from two allergic patients to PBMC cultures and quantification of IgE in cultures by ImmunoCAP measurements on day 0 and 7. Supernatants were taken after 1 week of culture and stored at –20°C until analysis.

Magnetic cell separation and flow cytometry

Magnetic cell separations using MicroBead-labelled antibodies directed against cell surface antigens were performed according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Direct bead conjugates were used to deplete or enrich cells expressing CD3, CD14, CD19, CD20, CD22, CD27 and CD138 surface antigens from the PBMCs. CD38 cell separations were performed using the CD38 MicroBeads Kit which includes a biotin-labelled anti-CD38 antibody and anti-biotin bead conjugates. CD126 and CD203c cell separations were achieved by staining total PBMCs with a phycoerythrin (PE)-conjugated mouse anti-human CD126 (BD Pharmingen, San Diego, CA, USA) antibody or anti-human CD203c-biotin (Miltenyi Biotec) and a secondary labelling step with an anti-PE MicroBead or anti-biotin conjugate from Miltenyi Biotec. Depletion of 'various antigens' (CD2, CD14, CD16,

CD36, CD43, CD235a) was achieved by a basophil isolation kit II from Miltenyi Biotec. Each cell separation experiment except for CD14 was performed in at least two different patients.

To verify successful cell separation, total PBMCs, depleted PBMCs and positively enriched PBMCs were analysed for surface marker expression by flow cytometry (Beckman Coulter, Fullerton, CA, USA). PE- or FITC-conjugated anti-human CD19, CD22, CD27, CD38, CD138 mouse monoclonal antibodies (mAbs) were purchased from BD Bioscience. Anti-human CD203c-PE was purchased from Immunotech (Marseille, France). Anti-human CD20-FITC was purchased from Miltenyi Biotec. Cells were gated according to forward and side-ward scatter properties. Dead cells were stained using 7-AAD (Beckman Coulter, France) and excluded from analysis. A minimum of 10 000 cells was counted.

Isolation of RNA and RT-PCR

Total RNA was purified using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was extracted from 5×10^6 cells and eluted in 50 μ L RNase-free water. Samples were stored at -80°C until RT-PCR was performed. The quality of isolated RNA samples was checked on an ethidium bromide-stained 1% agarose gel. The amount of nucleic acids was quantified spectrophotometrically (A260 nm/A280 nm).

Synthesis of cDNA and PCR reactions were carried out using the Qiagen OneStep RT-PCR Kit. RNA template of 0.01 μ g per reaction were used together with 25 pmol of oligonucleotide primers [11, 12] specific for the constant region of the epsilon chain (GAG AGG AAT TCG CTA CTA GTT TTG TTG TCG ACC CAG TCT GTG) and 25 pmol of oligonucleotide primers specific for the variable heavy chain region of the different VH gene families (VH1: GGA ATT CAC TCC CAG GTG CAG CTG CTC GAG TCT GG, VH2: GGA ATT CGT CCT GTC CCA GGT CAA CTT ACT CGA GTC TGG, VH3: GGA ATT CGT CCA GGT GGA GGT GCA GCT GCT CGA GTC TGG, VH4: GGA ATT CGT CCT GTC CCA GGT GCA GCT GCT CGA GTC GGG, VH5: GGA ATT CGT CTG TGC CGA GGT GCA GCT GCT CGA GCT CGG, VH6: GGA ATT CGT CCT GTC ACA GGT ACA GCT GCT CGA GTC AGG) (VBC Biotech, Vienna, Austria). In pilot experiments it was tested which VH family primers (VH1-VH6) gave IgE-encoding cDNA products. These primer pairs were used for RT-PCR reactions as follows: One cycle of 30 min at 50°C for reverse transcription and 15 min at 95°C , 40 cycles of 20 s denaturation at 94°C , 30 s annealing at 59°C , and 60 s elongation at 72°C , followed by one cycle of 10 min final extension. Amplicons were visualized on an ethidium

bromide-stained Criterion TBE Gel (5% Acrylamide; Bio-Rad, Hercules, CA). DNA ladder and sample buffer were purchased from Fermentas Life Sciences (St. Leon-Rot, Germany). As negative control PCR, reactions were carried out without RNA template. RT-PCRs with GAPDH primers spanning exon 8 and exon 9 (Biomol Research Laboratories Inc., PA, USA) were performed as a positive control.

Southern blot hybridization was performed as previously described [23]. Briefly, 8 μ L aliquots of each RT-PCR reaction were separated on a 1.5% agarose gel and transferred onto a nitrocellulose membrane. The presence of IgE-encoding cDNA was confirmed by hybridization with a ^{32}P -labelled oligonucleotide specific for C ϵ 1 [23].

Semi-quantitative measurement of allergen-specific IgE by ELISA

ELISA plates were coated with 3 μ g/mL recombinant allergens and blocked with 1% BSA. A total of 100 μ L aliquots of PBMCs supernatant/well were added and incubated overnight at 4°C on a shaker. Specific IgE was detected using an alkaline phosphatase-conjugated mouse mAb to human IgE (555859; BD Pharmingen). Para-nitrophenylphosphate substrate tablets (N9389; Sigma-Aldrich) were used for the enzymatic colour reaction. OD levels corresponding to IgE levels were read at 405 nm in the slope of the curve exactly 2 h after substrate addition. To allow a comparison of OD and thus IgE levels in cultures, supernatants from different cultures were analysed on the same plate. Each of the experiments was performed in duplicates with an error of less than 10%.

Quantitative measurements of allergen-specific IgE levels and statistical analysis

Using the Ficoll gradient we typically obtained 1.5×10^6 PBMCs/mL blood, consisting of 60–80% lymphocytes, approximately 10% monocytes and 1–5% granulocytes. In the cell culture experiments 15×10^6 cells were cultured in 3 mL medium in duplicate wells using 6-well plates. Thus, cultured PBMCs were concentrated approximately 3.3-fold compared to blood. Cell lysis in triplicate cultures was performed in the presence of a protease inhibitor (1 mM PMSF; Sigma) using an Ultraturrax on ice (IKA, Heidelberg, Germany).

To estimate the percentage of allergen-specific serum IgE which is derived from cultured PBMCs, we determined allergen-specific IgE levels by quantitative CAP analysis for five patients in the serum and in the supernatants of the cultured PBMCs. IgE levels were then

correlated using the Pearson correlation coefficient for statistical evaluation.

Allergen-specific IgE levels in cultured PBMC with or without depletion for CD126, CD38 and CD203c-positive cells or for cells positive for the other markers

displayed in Table 1 (CD138, CD27, CD19, CD20, CD22, CD3, CD14, CD2, CD16, CD36, CD43, CD235a) were compared using *t*-test and *P* values for the reduction of IgE levels after depletion were calculated.

Table 1. Allergen-specific IgE levels in supernatants of total peripheral blood mononuclear cells (PBMCs) cultures and PBMC cultures depleted of cell populations of allergic patients (1–8) expressing certain surface markers (CD19, CD20, CD22, CD27, CD138, CD126, CD38, CD3, CD14, CD203c, CD2, CD16, CD36, CD43, CD235a). Displayed are mean OD values ($A_{405\text{ nm}}$) corresponding to allergen-specific IgE levels of duplicate experiments with a variation of less than 10%.

Cell populations	Surface Antigen	Patient ID	Allergen	Total PBMCs ($A_{405\text{ nm}}$)	Depleted PBMCs ($A_{405\text{ nm}}$)	Date of blood sampling
Plasma cells	CD126	1	Bet v 1	0.228	0.038	18 May 2007
			Bet v 1	0.329	0.058	28 June 2007
			Bet v 1	0.192	0.000	1 February 2008
		2	Phl p 1	0.251	0.042	16 January 2008
			Phl p 5	0.355	0.071	16 January 2008
		3	Bet v 1	0.090	0.009	14 December 2008
			Bet v 1	0.305	0.052	4 June 2007
			Bet v 1	0.659	0.052	13 February 2008
		4	Der p 2	0.224	0.113	22 October 2007
			Der p 2	0.137	0.010	1 February 2008
			Der p 2	0.360	0.136	30 October 2008
		5	Phl p 1	0.458	0.109	30 November 2007
			Phl p 1	0.181	0.027	6 February 2008
		6	Phl p 5	0.292	0.094	5 November 2007
			Phl p 1	0.234	0.074	5 November 2007
			Phl p 2	0.110	0.032	5 November 2007
			Phl p 5	0.616	0.024	8 February 2008
			Phl p 1	0.176	0.014	8 February 2008
		7	Bet v 1	0.095	0.000	27 November 2007
			Bet v 1	0.130	0.000	8 February 2008
		8	Der p 2	0.083	0.000	11 January 2008
			Phl p 1	0.074	0.021	11 January 2008
	CD138	1	Bet v 1	0.418	0.335	26 September 2007
		3	Bet v 1	0.557	0.309	8 August 2007
	CD38	1	Bet v 1	0.436	0.004	20 March 2009
		3	Bet v 1	0.104	0.089	21 September 2006
		4	Der p 2	0.231	0.000	4 April 2009
Memory cells	CD27	1	Bet v 1	0.341	0.793	5 November 2007
		3	Bet v 1	0.109	0.191	14 November 2006
B cells	CD19	1	Bet v 1	0.329	0.398	28 June 2007
		3	Bet v 1	0.305	0.226	5 June 2007
	CD20	1	Bet v 1	0.341	0.322	5 November 2007
		3	Bet v 1	0.109	0.141	14 November 2006
	CD22	1	Bet v 1	0.418	0.435	26 September 2007
		3	Bet v 1	0.135	0.071	14 December 2006
T cells	CD3	1	Bet v 1	0.228	0.159	18 May 2007
			Bet v 1	0.213	0.268	11 July 2007
		3	Bet v 1	0.557	0.387	8 August 2007
			Bet v 1	0.516	0.204	17 April 2007
Monocytes	CD14	1	Bet v 1	0.474	0.531	6 August 2008
Basophils	CD203c	1	Bet v 1	0.436	0.066	20 March 2009
		4	Bet v 1	0.231	0.076	4 April 2009
Various	CD2, CD14, CD16, CD36, CD43, CD235a	1	Bet v 1	0.329	0.411	28 June 2007
		7	Bet v 1	0.136	0.103	1 October 2007

Results

Basophils represent a major source of allergen-specific IgE in cultured PBMCs

The PBMCs from patients ($n = 8$) allergic to the major birch pollen allergen, Bet v 1, the major grass pollen allergen, Phl p 1 and the major house dust mite allergen, Der p 2, were isolated. Then, cell depletion experiments using magnetically labelled antibody conjugates directed against surface antigens expressed on B cells (CD19, CD20 and CD22), memory B cells (CD27) and plasma cells (CD138) were performed. The depletion of CD138-positive cells led to a small but distinct (approximately 20%) decrease of specific IgE levels in cell culture supernatants as compared to total PBMCs (Table 1). It is unlikely, that this decrease was due to degradation of IgE in culture supernatants as IgE was shown to be stable in supernatants of cultured PBMCs from allergic patients for 1 week (Supplementary Table 1). Depletion of B cells and memory cells from cultures did not cause a decrease of allergen-specific IgE levels as compared to non-depleted PBMCs (Table 1). Removal of memory cells using anti-CD27 even appeared to increase allergen-specific IgE levels in the supernatants (Table 1). Successful depletion was verified for each experiment by flow cytometry and representative data from one patient are shown in supplementary Fig. 1. Each of the experiments was repeated at least once with different patients, yielding similar results (Table 1).

In a next step, we used CD38 and CD126 as target antigens for magnetic cell separation since they are known to be expressed on plasma cells. Depletion of CD126 and CD38-expressing cells strongly reduced allergen-specific IgE levels in cell culture supernatants (Fig. 1A) whereas high levels of specific IgE could be found in the cell populations enriched for these markers. However, CD38 and CD126 are also expressed on other cell populations including basophils. When we performed depletion experiments using the basophil-specific surface marker CD203c, we observed a strong reduction of allergen-specific IgE levels in supernatants (Fig. 1A). The OD levels corresponding to allergen-specific IgE levels decreased from OD = 0.436 (total PBMCs) to OD = 0.066 (basophil-depleted cultures) or from 0.231 to 0.076 respectively (Table 1). Depletion of other cell populations bearing the high affinity IgE receptor such as monocytes (CD14 positive) or neutrophils (CD16 positive) did not have an effect on allergen-specific IgE levels.

Allergen-specific IgE levels in serum or supernatants from PBMC of non-allergic subjects were not detectable and therefore could not be analysed in our study as well as in earlier studies [11, 24]. The majority of specific IgE that can be measured in supernatants of cul-

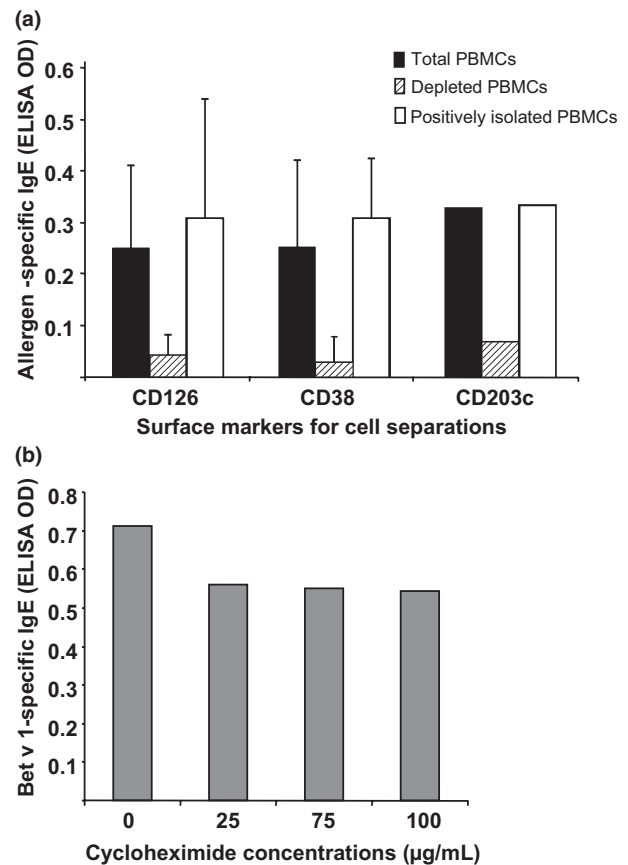


Fig. 1. Allergen-specific IgE levels in peripheral blood mononuclear cells (PBMCs) culture supernatants from allergic patients. (a) Mean allergen-specific IgE levels $\pm$ SD (y-axis) in total PBMCs, or PBMCs that were depleted of or enriched for cell populations expressing CD126 ($n = 8$ patients), CD38 ($n = 3$ patients) or CD203c (basophils) ($n = 2$ patients) (x-axis). Values for the graph are derived from data shown in table 1. (b) Bet v 1-specific IgE levels (y-axis) were measured in untreated PBMCs or in PBMCs treated with different concentrations of cycloheximide (x-axis). Shown are mean OD values corresponding to IgE levels of duplicate experiments with a variation of less than 10%.

tured PBMCs is thus derived from basophils. To investigate the proportion of produced allergen-specific IgE, we cultured PBMCs without and with addition of different concentrations of the protein synthesis inhibitor cycloheximide (Fig. 1B). Inhibition of protein synthesis decreased IgE-specific OD levels only by 23% (total PBMCs, untreated: OD = 0.711; PBMCs plus 100 µg/mL cycloheximide: OD = 0.544).

We next investigated whether or not allergen-stimulation can increase allergen-specific IgE production in cultured PBMCs from allergic patients. PBMCs from five patients were stimulated with two different unrelated allergens, designated allergen 1 and 2. Upon stimulation with allergen 1, allergen 1-specific IgE could not be detected anymore in supernatants of stimulated cultures whereas allergen 2-specific were at levels similar to

unstimulated cultures and *vice versa* (Supplementary Table 2). We were thus not able to induce profound increases of allergen-specific IgE levels by addition of allergens to the cultures.

These experiments indicate that *de novo* IgE production accounts for a small part of the IgE levels detected in PBMC culture supernatants. In summary, the cell selection and cycloheximide experiments suggest that more than 70% of allergen-specific IgE detected in cultured PBMCs is derived from basophils and less than 30% is newly synthesized, most likely by plasma cells as defined by anti-CD138 (Table 1 and Fig. 2).

IgE-encoding mRNA in the peripheral blood is derived from circulating CD138+ plasma cells

To elucidate whether or not IgE from CD138 positive plasma cells results from *de novo* production involving mRNA synthesis, we isolated RNA from CD138-depleted and CD138-enriched PBMCs and performed RT-PCR experiments using primer pairs specific for the constant and variable heavy chain DNA of IgE. The IgE-encoding PCR product of 650 bp could not be amplified from total PBMCs and the depleted cell population (Fig. 2A; lanes 2, 3 and 4) whereas it accumulated in the CD138-enriched fraction (Fig. 2A; lane 5). Lane 1 in Fig. 2A contains an IgE-encoding PCR product from a control IgE RT-PCR which migrates at 650 bp (positive control).

To ensure that the RNA preparations were intact, cDNAs coding for the housekeeping gene GAPDH were amplified and could be detected as 240 bp product spanning exons 8 and 9 in each of the samples (GAPDH, positive control; Fig. 2B). Hybridization with a ^{32} P-labelled oligonucleotide specific for C ϵ 1 confirmed

the specificity of the PCR product (Supplementary Fig. 2).

Allergen-specific IgE levels in PBMC supernatants correlate with serum IgE levels but represent less than 1% of free serum IgE in the blood

Using the ImmunoCAP system, a quantitative IgE detection system, we measured IgE antibody levels specific for Bet v 1, Phl p 1 and Der p 2 in serum and PBMC supernatants from five allergic patients. The analysis revealed that birch pollen, grass pollen or house dust mite allergen-specific IgE levels in serum samples and supernatants from cultured PBMCs were in a relation of 99 : 1 and highly correlated ($R = 0.96$; $P = 0.002$) (Fig. 3). Based on the quantitative IgE measurements (Fig. 3) it can be safely estimated that less than 1% of free serum IgE is derived from circulating PBMCs. Considering that on average approximately 1.5×10^6 PBMCs were isolated from 1 mL of peripheral blood in our experiments and that IgE levels were measured in cultures containing PBMCs which were 3.3-fold concentrated compared to blood indeed less than 1% of serum IgE can be obtained from circulating PBMCs (Fig. 4). Results from the measurements of allergen-specific IgE in cultures which had been enriched or depleted for basophils or plasma cells suggest that less than 30% of allergen-specific IgE found in the culture supernatants is derived from plasma cells (Table 1) whereas the majority is derived from basophils (Table 1 and Fig. 1A).

To determine the proportion of allergen-specific IgE which might be associated with cells and hence possibly does not appear in the culture supernatants,

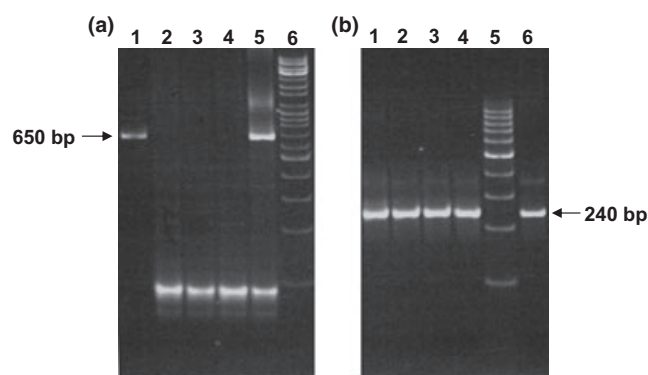


Fig. 2. RT-PCR products representing (A) IgE cDNA (650 bp, arrow) or (B) GAPDH cDNA (240 bp, arrow) derived from allergic patient's total peripheral blood mononuclear cells (PBMCs) or PBMCs depleted of or enriched for CD138-positive plasma cells. Lanes are as follows: (A) 1, IgE control product; 2, Total PBMCs; 3 and 4, CD138-depleted sample 1 and 2; 5, CD138-enriched; 6, DNA ladder mix; (B) 1, Total PBMCs; 2 and 3, CD138-depleted sample 1 and 2; 4, CD138-enriched; 5, 100 bp ladder; 6, GAPDH control product.

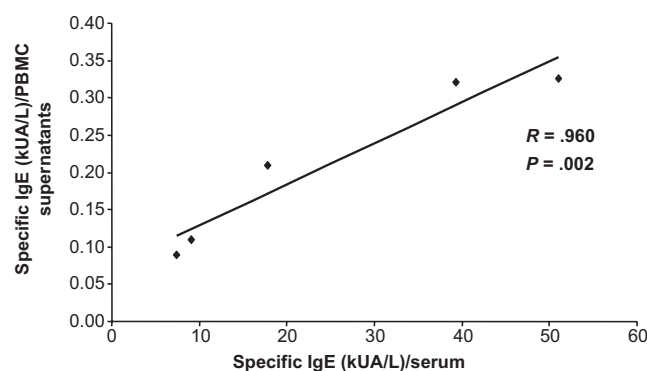


Fig. 3. Correlations between allergen-specific IgE levels in peripheral blood mononuclear cells (PBMCs) supernatants and sera. IgE levels specific for rBet v 1 ($n = 2$), rDer p 2 ($n = 1$), rPhl p 1 ($n = 1$) and rPhl p 5 ($n = 1$) were measured by ImmunoCAP (kUA/L) in serum samples (x-axis) or in PBMC supernatants (15×10^6 cells/3 mL; y-axis) from birch pollen, house dust mite and grass pollen allergic patients ($n = 5$). Pearson correlation coefficient (R) and P value are indicated and a trend line is displayed.

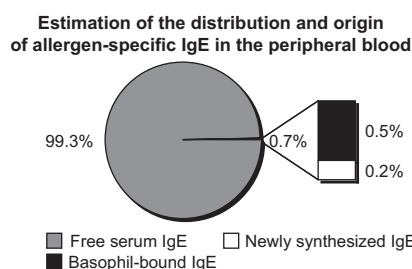


Fig. 4. Percentages of allergen-specific IgE levels in the peripheral blood of allergic patients. Approximately 0.7% is derived from peripheral blood mononuclear cells, of which 0.5% represents IgE most probably derived from the surface of basophils and approximately 0.2% is newly synthesized by circulating plasma cells. The remaining 99.3% represent free serum IgE from another origin.

we analysed IgE levels in cell lysates of two patients and compared them with those in the culture supernatants. ImmunoCAP measurements showed that IgE levels did not increase in a relevant manner when cells were lysed in the culture medium. Total IgE levels in the cell lysates were similar to those in supernatants (Lysate: 5.1 ± 0.58 kUA/L; supernatant: 4.96 ± 0.28 kUA/L). Similar findings were made for allergen-specific IgE levels (Lysate: 0.46 ± 0.06 kUA/L; supernatant: 0.43 ± 0.08 kUA/L).

Discussion

In this study we investigated the contribution of IgE-producing cells in the blood to the amount of IgE that circulates in the blood of allergic patients. Several studies have demonstrated that PBMCs from allergic patients contain cells which produce allergen-specific IgE [11, 24] and these antibodies have been characterized regarding sequence and structure [12, 25–27]. In a series of cell separation experiments performed by negative depletion and positive selection we could locate IgE-producing cells in the fraction of CD126- and CD38-positive cells which contain plasma cells but not in the B cell-containing fractions (i.e. CD19, CD20, CD22 positive cells). The CD126- and CD38-positive fractions contain plasma cells and we therefore hypothesized that plasma cells represent the main source of allergen-specific IgE production. However, when we depleted CD138+ plasma cells only a small (approximately 20%) reduction of allergen-specific IgE was obtained. Since the CD126- and CD38-positive fraction also contains basophils we specifically depleted basophils using anti-CD203c antibodies and obtained an approximately 80% reduction of IgE production. It is conceivable that also other cells bearing IgE on their surface (e.g. neutrophils, monocytes, dendritic cells) may contribute to IgE levels in PBMC cultures. However, depletion of neutrophils by CD16, monocytes by

CD14 and B cells did not result in a relevant reduction of allergen-specific IgE.

In our experiments the supernatants analyzed for IgE levels were always derived from 15×10^6 PBMCs regardless of which population was depleted. For depletion of larger populations such as CD38 (ca. 40% of PBMCs) or CD27 (ca. 60% of PBMCs) positive cells, a change in cell ratios of the other populations may in principle have effects on IgE levels. However, the most profound changes in IgE levels of PBMC cultures were observed when we depleted basophils and plasma cells which account for less than 1% of PBMCs.

The finding that the majority of IgE in PBMC cultures was not derived from plasma cells was confirmed by inhibition of protein synthesis using cycloheximide which caused an approximately 20% reduction of allergen-specific IgE production. We thus estimated that only approximately 20% of allergen-specific IgE which is present in PBMC culture supernatants is derived from CD138-positive plasma cells which is in agreement with data obtained by Horst et al. for circulating plasma cells [13]. Using RT-PCR experiments and IgE-specific primers together with hybridization experiments we could demonstrate that CD138-positive plasma cells indeed transcribe IgE-encoding mRNA. The lack of IgE-encoding mRNA in the total PBMC fraction of the same patient can be explained by the low frequency of allergen-specific plasma cells in the blood. As 5×10^6 PBMCs were used for preparation of mRNA and the frequency of allergen-specific plasma cells is 3×10^{-7} to 7×10^{-6} depending on the atopic status of the patient [13], it is thus possible that the total PBMC sample did not contain enough IgE-producing plasma cells to yield a positive RT-PCR result.

Next we compared allergen-specific IgE levels in sera and PBMC culture supernatants from allergic patients and found that less than 1% of IgE in the blood is derived from blood cells and only approximately 0.2% is newly produced by CD138-positive blood plasma cells. Nevertheless, there was an extremely high correlation of allergen-specific IgE levels in the culture supernatants and sera. The latter finding indicates that the CD138-positive plasma cells in blood are in equilibrium with cells producing the majority of IgE which circulates in allergic patients.

There are several studies indicating that allergen-specific IgE is produced in target organs of allergy such as the nasal mucosa, the respiratory tract and the gut [14–18]. Furthermore, it has been shown that allergy can be transferred by bone marrow transplantation [28–30]. It is therefore possible that the majority of allergen-specific IgE is produced either locally in target organs of allergy and/or in the bone marrow from where a small percentage of plasma cells migrates into the peripheral blood and continues to produce allergen-specific IgE.

One possible scenario may be that IgE-producing B cells in the target organs of allergy (e.g. nasal, bronchial and intestinal mucosa) become activated upon allergen contact and then give rise to plasma cells producing allergen-specific IgE antibodies which appear only transiently in the blood but quickly home to other sites such as the bone marrow as has been described for plasma cells which develop in response to immunization [31]. The observation that anti-CD20 (i.e. rituximab) treatment has only modest effects on IgE production in allergic patients [32, 33] would be in accordance with the latter hypothesis. In fact, rituximab eliminates effectively B cells from the blood but only partially affects B cells in tissues and has no effects on plasma cells.

We think that our results are important for the investigation of mechanisms of IgE production in allergic patients and in particular for the development of new therapeutic strategies which may target IgE-producing

cells because they provide another piece of information regarding the possible sites of IgE production in allergic patients. Since the peripheral blood contains only a minor fraction of IgE-producing CD138-positive plasma cells, therapeutic strategies targeting allergen-specific IgE production [34, 35] will have to focus on the major sites of IgE production in local tissues and/or bone marrow.

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Conflict of interest: None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effectiveness of cell separation. PBMCs from allergic patients were depleted of or enriched for CD20, CD27, CD38, CD126 or CD203c using magnetic separation, stained with the respective antibodies and subjected to flow cytometry. Gates indicate the percentage of positive staining for the respective antibodies in total PBMCs (upper panels), depleted (middle panels) or enriched populations (lower panels). Blots are representative for cell preparations from 8 allergic patients.

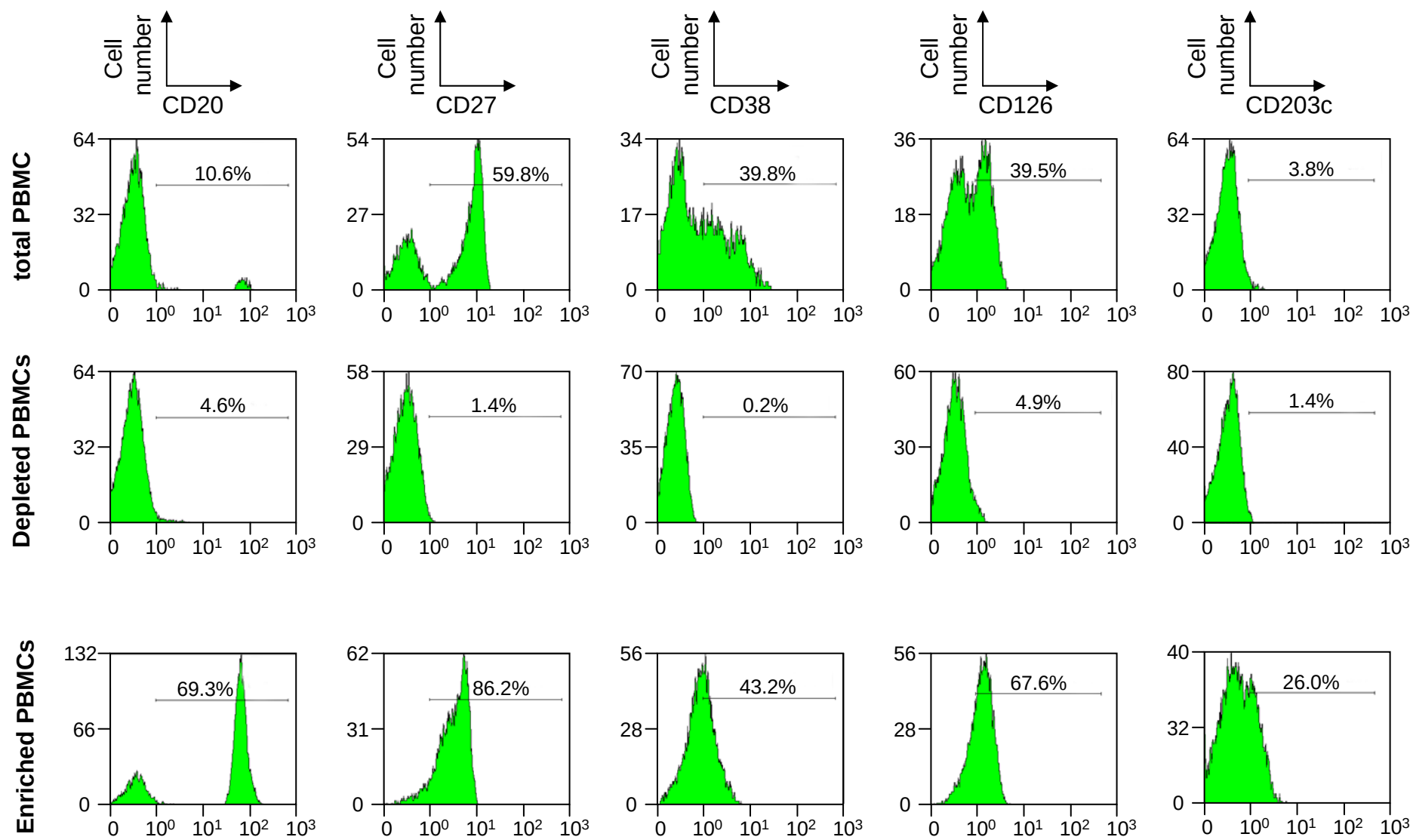
Figure S2. Southern blot for IgE. Nitrocellulose-blotted IgE heavy chain-encoding cDNAs were hybridized with a ³²P-labelled oligonucleotide probe specific for Cε1. Lanes are as follows: (A) 1: Total PBMCs, 2 and 3: CD138-depleted sample 1 and 2, 4: CD138-enriched, M: DNA ladder mix.

Table S1. IgE is stable for 1 week in supernatants of PBMC cultures. PBMCs from three patients (ID9–11)

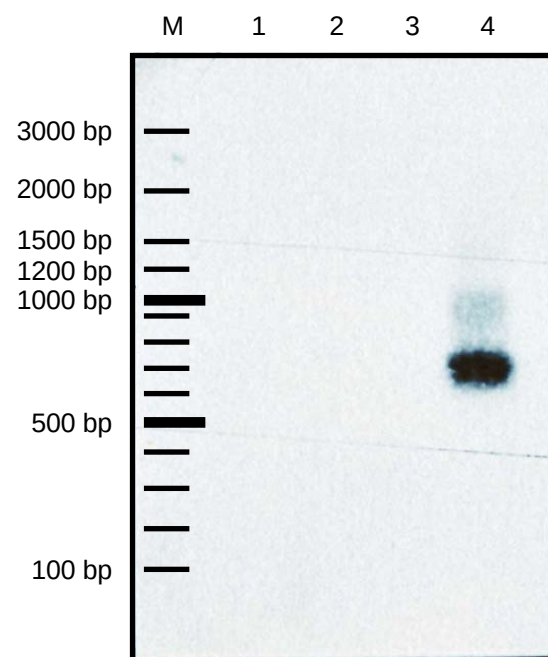
were cultured in medium containing 20% serum from allergic patients. Total IgE levels were measured by ImmunoCAP (kUA/L) in PBMC supernatants (15 × 10⁶ cells/3 ml) on day 0 and day 7 of culture.

Table S2. Allergen-specific IgE levels in supernatants of allergen-stimulated PBMC cultures from 5 allergic patients. PBMC cultures were either left unstimulated or stimulated for 1 week with two different allergens (250 ng/mL) to which the patient was sensitized to. Allergen-specific IgE levels were measured by ultrasensitive ImmunoCAP measurements (kUA/L) in PBMC supernatants (15 × 10⁶ cells/3 ml) on day 7 of culture.

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Supplementary Fig 1



Supplementary Fig 2

Patient ID	Total IgE day 0 (kUA/L)	Total IgE day 7 (kUA/L)
9	57.27	51.67
10	3.44	4.58
11	36.47	35.47

Supplementary Table 1

	Patient ID	Allergen 1	Allergen 2	Allergen 1-specific IgE (kU/L)			Allergen 2-specific IgE (kU/L)		
<i>Stimulation</i>				<i>Medium</i>	<i>Medium plus Allergen 1</i>	<i>Medium plus Allergen 2</i>	<i>Medium</i>	<i>Medium plus Allergen 1</i>	<i>Medium plus Allergen 2</i>
	2	Bet v 1	Phl p 5	0.07	0.00	0.07	0.05	0.05	0.01
	3	Bet v 1	Ole e 1	0.36	0.01	0.4	0.01	0.02	0.00
	5	Bet v 1	Phl p 1	0.04	0.00	0.04	0.07	0.09	0.01
	6	Phl p 1	Ole e 1	0.01	0.00	0.01	0.06	0.05	0.02
	8	Bet v 1	Fel d 1	0.04	0.00	0.04	0.26	0.27	0.04

Supplementary Table 2