

Frequency and Activity of IgE-secreting Peripheral Blood B-cells in Atopic Eczema

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Increased immunoglobulin (Ig) E-levels are frequently found in the sera of patients with atopic eczema. To further understand the mechanisms underlying this increase of IgE, like enlarged number of IgE-producing cells, enhanced activity of IgE-producing cells or altered IgE metabolism, we analyzed the frequency and activity of IgE-producing B-lymphocytes within peripheral blood mononuclear cells of patients with atopic eczema.

By use of a sensitive solid phase enzyme-linked immunospot assay (ELISPOT), IgE-secreting cells could be visualized within peripheral blood mononuclear cells of 14 individuals (12 patients with atopic eczema, one patient with impetigo contagiosa, one patient with allergic contact dermatitis) at a frequency of 10–1,120 IgE-secreting cells/10⁵ B-lymphocytes. The number of IgE-secreting cells was markedly correlated with the corresponding serum IgE-level ($r=0.97$). Secretory activity was also reflected by *in vitro* IgE production, which was assessed in parallel 7-day cultures and found to be related with actual serum IgE-levels. The enhanced serum IgE-levels in patients with atopic eczema seem to result primarily from an increased number of circulating IgE-secreting B-cells, which may also be found, however, in patients with elevated serum IgE-levels, showing different skin diseases. **Key words:** ELISPOT assay; IgE-production; B-lymphocytes

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Despite the heterogeneity of clinical manifestations, patients with atopic eczema (AE) exhibit typical alterations in skin physiology and in cellular and humoral immunity (1–4). A major characteristic immunological abnormality is enhanced production of IgE, of which a substantial fraction is of known specificity for environmental antigens. A variety of activating signals, with a central role of TH2-type like CD4⁺ T-lymphocytes, have been identified and described in the two-step model of induction of B-cell switching to IgE production (5). However, accumulated IgE is often barely measurable in human sera or human cell culture supernatants (6, 7), and quantitation, if feasible, does not permit one to draw conclusions with regard to the number of IgE-secreting cells or their present secretory activity. To visualize IgE-secreting cells at the single cell level, we established a more sensitive, modified filter-plaque assay (ELISPOT assay), based on the original technique developed in 1983 by Czerkinsky et al. (8) and Sedgwick & Holt (9). The aim of the present study in patients with AE was to assess the relation between serum IgE-levels and the frequency and activity of IgE-secreting cells within the respective peripheral blood mononuclear cells (PBMC).

MATERIAL AND METHODS

Patients

Four healthy donors with serum IgE-levels below 10 kU/l (range 0–8 kU/l) and negative history for atopic disposition served as controls. In addition, 14 individuals (12 patients with AE, one patient with impetigo contagiosa, one patient with allergic contact dermatitis; 7 females, 7 males, aged 15–52 years) volunteered to take part in the study after having given their informed consent (Table I). Blood samples were taken prior to systemic treatment.

Preparation and culture of PBMC

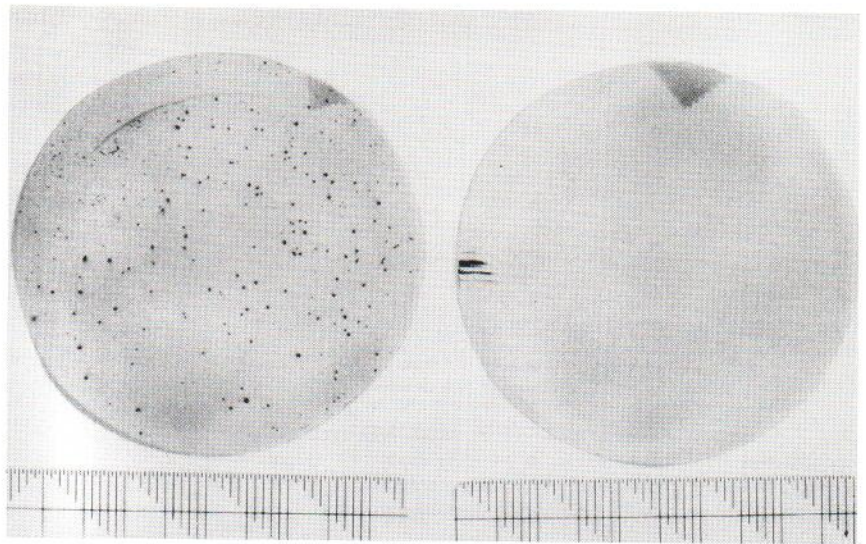
Heparinized venous peripheral blood (40 ml, diluted 1:1 with PBS) was centrifuged over Ficoll-Hypaque® (720xg, 20 min, 20°C). Cells from the interphase were washed twice with PBS-1%FCS and resuspended at 1 x 10⁶/ml in complete medium consisting of RPMI 1640 (Seromed, Berlin, Germany) supplemented with 10% FCS (GIBCO/BRL, Eggenstein, Germany), 2 mmol/l L-glutamine (Seromed), 2 mmol/l non-essential aminoacids (GIBCO/BRL) and 10 U/ml of penicillin/streptomycin (GIBCO/BRL). The percentage of the B-cell fraction was determined by immunostaining with phycoerythrin-labelled anti-CD19 antibody (Becton-Dickinson, Ermbadegen, Belgium) and flow cytometry using FACScan (Becton-Dickinson). Subsequently, cell suspensions were divided for ELISPOT assay and parallel 1-week *in vitro* culture to assess spontaneous IgE-antibody formation. For the latter, cells (1 x 10⁶/ml, 1 ml/well) were kept 7 days in duplicate cultures on 24-well culture plates (Greiner, Trichenhausen, Germany) in the presence or absence of the protein synthesis inhibitor cycloheximide (Sigma, Deisenhofen, Germany; 100 µg/ml) to distinguish between preformed and newly synthesized IgE. U266-IgE-myeloma cells (kindly provided by L. K. Poulsen, Copenhagen, Denmark) were used in some control

Table I. Patient characteristics

M: male; F: female; AE: atopic eczema; ARC: allergic rhinoconjunctivitis; ABA: allergic bronchial asthma; EH: eczema herpeticatum; IC: impetigo contagiosa; ACE: allergic contact eczema; skin testing if performed: Prick+: positive prick testing to common aeroallergens; ECT+: positive epicutaneous test reactions to potential contact allergens.

Age, y/Sex	Disease	Other	Serum-IgE (U/ml)
48/ M	AE	Bronchitis	937
52/ M	AE		1640
33/ F	AE		2960
31/ M	AE, ARC	Prick+	894
31/ M	AE, ARC	Prick+, ECT+	18100
26/ F	AE	Prick+	528
15/ F	AE, ARC	Acne, Prick+	687
37/ M	AE, ABA		29300
20/ F	AE, EH		475
25/ F	AE	ECT+	5990
25/ M	AE, EH		4940
21/ F	AE	Prick+, ECT+	548
21/ M	IC		5100
44/ F	ACE		1700

Fig. 1. Detection of IgE-secretion at the single cell level. *Left*: specific spot formation visualized from a patient's peripheral blood mononuclear cells. *Right*: same experiment in the presence of the protein synthesis inhibitor cycloheximide.



cultures. Cell culture supernatants were assessed by ELISA for the content of IgE.

IgE-determination

Serum IgE-levels were measured by a commercially available ELISA (CAP-system, Pharmacia, Uppsala, Sweden). Enzyme immunoassay for quantitation of IgE in the cell culture supernatants was performed on 96-well ELISA plates (Dynatech, Denkendorf, Germany) coated with polyclonal rabbit anti-human IgE-antibody (Dakopatts, Hamburg, Germany). Mouse monoclonal anti-IgE antibody E5595 (ATCC, Rockville, USA) was biotinylated (biotinamidocaproate-N-hydroxysuccinimide ester, Sigma) to serve as detecting antibody. Visualisation for colorimetric evaluation was performed with streptavidine-alkaline-phosphatase (AV-ALP) (Calbiochem La Jolla USA) and PNPP-substrate (Sigma).

ELISPOT assay

Nitrocellulose membrane disks (0.45 µm pore size, 50 mm diameter; Schleicher & Schüll, Dassel, Germany) were placed into tissue culture Petri plates (Greiner), coated at 5°C overnight with 5 ml of PBS containing polyclonal anti-IgE antibody (5 µg/ml) and after three washes with PBS blocked for 2 h at 37°C with complete culture medium. Subsequently, 5×10^6 freshly isolated PBMC (5 ml of the 1×10^6 cells/ml containing cell suspension) were layered onto the nitrocellulose disks and incubated overnight at 37°C in a humidified CO₂ incubator. Parallel cultures containing 100 µg/ml cycloheximide (Sigma) served as controls. After removal of the cells by repeated (4x) washing, secreted

and bound IgE-antibody was visualized by sequential 2-h incubation with biotinylated anti IgE mab and AV-ALP. The colouring reaction was performed by adding the substrate BCIP (5 mg/ml)/NBT (1 mg/ml) (Sigma) at pH 9.5. Blue "spots" visible at naked eye appeared within minutes (Fig. 1). The spots were counted by use of a magnifying lens and – after subtracting possible unspecific signals as assessed in parallel cycloheximide controls – expressed as IgE-B-cells/ 10^5 B-cells.

To exclude adherence of PBMC to the nitrocellulose membranes as a disturbing factor in the ELISPOT assay, PBMC (5×10^6 /disk) of 2 patients with AE and 2 controls were layered and kept overnight onto nitrocellulose disks pretreated with FCS-containing culture medium or disks coated with anti-IgE-antibody and identical blocking procedure. After 4x washing to remove the PBMC, one set of both types of nitrocellulose disks was examined for adherent cells through epiluminescent light microscopy independently by two investigators unaware of the experimental design. The other set of both types of disks was further processed by use of biotinylated anti-CD19-antibody (Biermann, Bad Neuheim, Germany) to reveal B-lymphocytes – if present – or by use of the biotinylated anti-IgE-antibody to detect captured IgE-antibody. The colouring reaction was as described above.

RESULTS

Visualisation of IgE-forming cells

In contrast to the control subjects, where no IgE-secreting cells could be detected, the binding of IgE-antibodies secreted from cells within the PBMC of the 14 patients was localized in distinct areas corresponding to blue spots visible without magnification, after being layered onto the nitrocellulose disc. Upon addition of cycloheximide in parallel cultures, no specific spots could be seen, thus pointing to the "de novo" Ig-synthesis (Fig. 1) in either PBMC and U266 control cultures. The spots obtained with PBMC of the patients did not always show the same size, probably as a consequence of different degrees of secretory activity.

The additional control experiments with PBMC from each 2 control subjects and 2 patients with AE did not reveal adherent cells on the nitrocellulose disks by epiluminescent light microscopy. The ELISPOT assay performed in parallel with B-cell-specific biotinylated anti-CD19-antibody or biotinylated anti-IgE-antibody gave negative results for all 4 individuals with regard to screening for the presence of B-cells on the disks, but showed spot signals for the 2 patients with AE upon use of disks

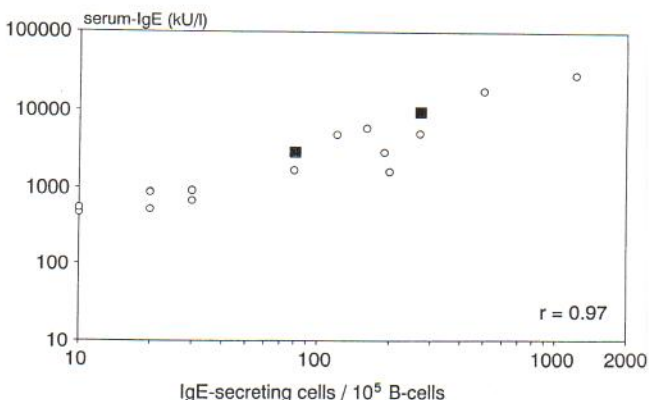


Fig. 2. Correlation between serum IgE-levels (kU/l) and numbers of ELISPOT-signals (expressed as IgE-secreting cells/ 10^5 B-cells) within PBMC of 14 individuals with elevated serum IgE-levels.

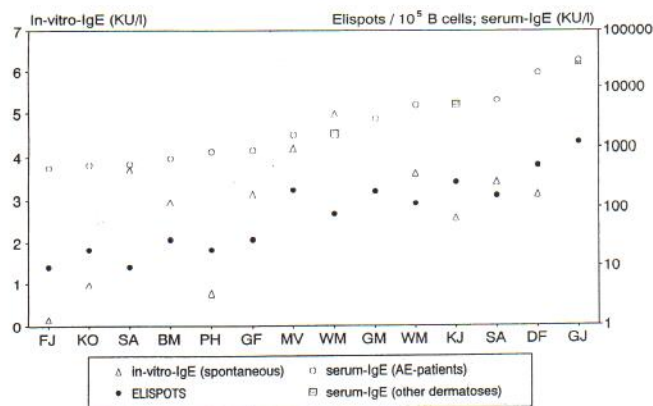


Fig. 3. Correlation between ELISPOTS, serum IgE-level and corresponding in vitro IgE-secretion in 14 individuals (12 patients with atopic eczema, 1 patient with impetigo contagiosa, 1 patient with allergic contact dermatitis) with elevated serum IgE-levels.

coated with anti-IgE-antibody and biotinylated anti-IgE-detecting antibody.

Correlation between serum IgE-levels and ELISPOT signals

By relating the amount of total serum IgE-levels to the number of IgE-secreting B-cells/ 10^5 B-cells, a highly significant correlation could be found ($r=0.97$, correlation by linear regression) (Fig. 2). The 2 patients with impetigo contagiosa or allergic contact dermatitis, respectively, also stayed within this relation.

In vitro IgE production

In the 14 patients, spontaneously secreted IgE could be detected in all supernatants from cell culture performed in parallel with the immunospot assay. To distinguish newly synthesized IgE from preformed IgE, we examined supernatants from cycloheximide-treated parallel cultures; these mostly had IgE-levels under the detection limit. The patients with the highest serum IgE-levels also showed higher in vitro IgE-levels. But in a few cases, despite relatively high serum IgE-levels, lower actual spot-forming activity and in vitro IgE-secretion seemed to reflect actual IgE production better (Fig. 3).

DISCUSSION

IgE-secreting cells were detected in the peripheral blood of all the 14 patients with elevated serum IgE-levels. The frequency of IgE-secreting B-lymphocytes within the PBMC of the 14 subjects correlated closely to the amount of corresponding serum IgE-levels. These findings are in accordance with reports of other investigators (10, 11) and indicate that the elevated serum IgE-levels result in the first line from an increased number of IgE-producing B-cells. This is also reflected by the actual ongoing in vitro IgE production. However, the sometimes visible varying strength of specific spot formation points to possible variations in the secretory activity of single cells.

In our study nitrocellulose disks were used to achieve greater efficiency of anti-IgE-antibody coating to enhance capturing of IgE secreted from PBMC from individuals with elevated serum IgE-levels. Möller & Borrebäck indicated that the protein-binding capacity of nitrocellulose discs can be up to 100-fold to that

on polystyrene wells (12). As previously reported, these adsorption properties of nitrocellulose membranes can also be used to increase the sensitivity for the detection of antigen-specific IgE secreted at the single cell level (13). In the present study, the best "fingerprint" of spot-forming cells was obtained after over-night incubation. Furthermore, the formation of specific spots could be completely prevented when the protein synthesis was inhibited by addition of cycloheximide, thus ruling out the possibility of cytophilic/preformed antibodies being responsible for the plaque formation.

These findings may have several implications for the interpretation of the encountered elevated serum IgE-levels in patients with AE. The close relation between serum IgE-level and the frequency of IgE-secreting cells points to the accumulation of IgE-antibody in the blood as a result of intravasal production rather than an overhang/"wash-out" phenomenon from peripheral tissues. Indeed, B-lymphocytes and plasma cells are only rarely, if ever, encountered in the cellular infiltrates of AE lesions (14). The preferential expansion of IL-4- and IL-5-producing (allergen-specific) T-cells in patients with AE (15, 16) may influence B-cell switching to IgE production not only in the skin-draining lymph nodes, but also in distant compartments based on the amplification by spread in lymphatic or blood circulation. Besides some dermal IgE-production, IgE antibody in the circulatory compartment is in part redistributed throughout the body in free/complexed form or bound on the surface of various cell types like basophils to reach for example the skin. This would further contribute to the amplification system (e.g. represented by facilitated allergen-presentation through IgE on Langerhans' cells/monocytes, preferential inflammatory infiltrate etc.) for local inflammation. However, there remains the question up to which degree an altered rate of IgE-metabolism (17) or compartmentalization (18) contributes to the final serum IgE-level. For this purpose, the detection of IgE-formation at the single cell level offers an additional approach to the study of IgE-regulatory mechanisms.

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