

Original article

Characterization of specific IgE response *in vitro* against protein and drug allergens using atopic and normal donors

Background: As the incidence of allergy to different compounds increases in society, the need to understand and characterize specific IgE responses becomes obvious. Different cell culture systems have been evaluated for their ability to support such IgE secretion.

Methods: One system employed human peripheral lymphocytes (PBL) from normal donors stimulated with anti-CD3 activated T cells with or without the presence of allergens like benzylpenicillin (BP) and *Phleum pratense* (PP). Secretion of IgE was analyzed in ELISA and compared to the IgG response to the nonallergenic antigen tetanus toxoid (TT). Another system employed stimulation of T and B cells with a heterotope, consisting of a T helper cell epitope derived from TT, and a B cell allergen epitope derived from BP. The specific IgE secretion was compared, using lymphocytes from normal as well as BP-allergic donors.

Results: Anti-CD3 stimulated T cells supported BP-specific IgE secretion in six of 11 normal donors. This response was inhibited in four donors and enhanced in two donors by the addition of the BP-allergen to the culture. In contrast, addition of the protein allergen (PP) or antigen (TT) to the same culture system inhibited both IgE and IgG synthesis in all experiments. Cells from the majority (10/16) of the BP-allergic donors failed to produce BP-specific IgE *in vitro*, when cultured in the presence of allergen.

Conclusions: An allergen specific immune response is readily generated *in vitro*. The differential response against benzylpenicillin between different donor categories most probably reflects the level of pre-exposure to this allergen *in vivo*.

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Key words: allergenicity; benzylpenicillin; grasspollen; IgE; *in vitro*.

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Accepted for publication 23 October 2001

IgE-mediated allergic reactions to a wide range of drugs are relatively rare but nevertheless represent a serious clinical problem (1, 2). Currently there are no tests that are predictive either of the capacity of novel compounds to induce IgE-mediated allergic reactions, or of the susceptibility of individuals to experience allergic reactions. Nevertheless, it is clear that the future direction of predictive drug allergy testing is not with animal tests (that are a poor indicator of human responses) but with the use of *in vitro* human cell culture systems that model the human *in vivo* immune response.

Designing cell culture systems resulting in specific *in vitro* IgE-responses against primary, as well as recall antigens, could have a potential to pave the way for a predictive allergenicity test strictly based on human cells. The increased knowledge available today on interactions between T and B lymphocytes and antigen presenting cells in terms of cell surface molecule interaction and secretion of soluble mediators, allows us to manipulate the cells to differentiate and secrete different antibody isotypes *in vitro*. Switching of

antibody isotype depends upon a combination of signals including receptor–ligand interactions such as those between CD40 and CD40L (3), and cytokines such as IL-4, IL-13, TGF- β and IFN- γ that strongly influence which isotype is secreted (4–9). Allergic responses of the immediate type are caused by inappropriate production of IgE antibodies that sensitize mast cells and basophils to release histamine and other inflammatory mediators upon activation by allergen.

In this study we examined the possibility of developing a human cell culture system in which IgE responses to common allergens could be induced under defined experimental conditions. In the first system, human peripheral lymphocytes (PBL) from normal donors were stimulated with anti-CD3 activated T cells and the secretion of specific IgE antibodies from the cells stimulated with or without the allergen was analyzed in ELISA. In this system we selected the allergen β -lactam antibiotic benzylpenicillin (BP) that induces IgE responses in some individuals and occa-

sional anaphylaxis (1, 2, 10, 11) and a protein allergen, the common environmental grass pollen *Phleum pratense* (PP). We compared the *in vitro* IgE responses to these allergens with the previously well-characterized *in vitro* IgG response to the nonallergenic antigen TT, tetanus toxoid (12).

The drug BP contains a β -lactam ring that is known to bind covalently with serum and cellular proteins to generate a number of chemical determinants including the major benzylpenicilloyl moiety (13–15). In the present study we synthesized a heterotope peptide (BP-peptide) incorporating a T helper cell epitope from TT and a sequence from human serum albumin incorporating the BP hapten for use as a nonprotein allergen in so-called *in vitro* immunization, using cells from normal and atopic donors.

We found that specific IgE responses could be induced *in vitro* by both drug and protein allergens in both cell culture systems. Interestingly, the pattern of IgE responses from normal B cell donors in the anti-CD3 system showed that the drug allergen (BP-peptide) behaved differently compared to the protein allergen PP and the recall antigen TT. This data shows that it could be possible to predict pre-exposure to a protein grass pollen allergen, whereas for allergenic drugs this is a more complex issue requiring further *in vitro* investigations.

This study also contributes by identifying some of the parameters influencing secretion of specific IgE antibodies and points out some factors to be considered in designing an *in vitro* test.

Material and methods

Antibodies, antigens and cytokines

The S2C6 anti-CD40 mAb was a gift from Dr S. Paulie (Stockholm University). The G28-5 anti-CD40 mAb and the anti-CD3 mAb 64.1 were kindly supplied by Dr J. Gordon (Birmingham, UK) and E. Vitteta (Dallas, USA), respectively. The pollen allergen was *Phleum pratense* (PP), purchased from Pharmacia Upjohn, Sweden. Tetanus toxoid (TT) was purchased from the Swedish Institute for Infectious Disease Control (Stockholm, Sweden). A synthetic peptide region of TT (pTT, QYIKANSKTEL, amino acids 830–844) was kindly provided by Elias Krambovitis (Institute of Molecular Biology and Biotechnology, Greece). IL-4 was obtained from Genzyme (Cambridge, MA) or R & D Systems Ltd, Oxford, UK. IFN- γ was obtained from Genzyme (Cambridge, MA) and IL-2 from Sandoz Research Institute, Vienna, Austria.

Peptide synthesis and haptentation

The peptide QYIKANSKTELDEGKASSAKQR incorporating at the N-terminus the TT sequence QYIKANSKTEL and at the C-terminus the human serum albumin (aa 211–221) sequence DEGKASSAKQR was synthesized by the solid phase method (Milligen 9050 automated synthesiser). All α -amino groups were fmoc (9-fluorenylmethyloxycarbonyl) protected and all reactive side chains boc (t-butyloxycarbonyl) protected with the exception of the lysine in position 19 that was aloc (allyloxycarbonyl) protected on the side chain. To selectively haptentate the lysine residue in position 19, the peptide on the resin was reduced with tetrakis (triphenylphosphine groups attached to an atom of palladium) to remove the aloc side chain, and the resin then suspended in 10 mg/ml of benzylpenicillin (Sigma) in 0.1 M carbonate buffer pH 10.5 for 24 h at 37°C. These conditions had previously been established as optimal for BP haptentation of HSA. The resin was then washed twice in methanol and once in dichloromethane before cleavage of the peptide from the resin in 95% trifluoroacetic acid (TFA). The peptide was precipitated and washed five times in diethylether, dissolved in water and then freeze-dried. Peptides were purified by reverse phase HPLC (Phenomenex Jupiter column, C18 bonded, 10 micron particle size, 250 $\times$ 21.2 mm) using 0.01% TFA in water as the equilibration solvent and 0.01% TFA in acetonitrile as the mobile phase eluted at 4 ml/min. The peptide, that eluted at 34–38 min, was analyzed by positive ion electrospray mass spectrometry (Quadrupole). The major peak of the transformed m/z spectrograph had a molecular mass of 2883 corresponding to a predicted value from the amino acid sequence of 2882 + one proton. Subsidiary peaks at 2866 and 2925 were seen. The haptentated peptide (referred to as BP-peptide) was shown to be BP-positive by dot blotting on Hybond-P membrane (Amersham Pharmacia Biotech) probed with rabbit anti-BP anti-serum.

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Cell cultures

PBL in buffy coats from normal donors (University Hospital Blood Bank, Lund) and from whole blood samples obtained from BP-allergic patients attending the Allergy Unit, Hospital Carlos Haya, Malaga were isolated by density centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). The cells were then washed three times in phosphate buffered saline (PBS) pH 7.2. PBL were treated with leucine-leucyl methyl ester (LeuLeu-OMe) (Bachem Feinchemikalien AG, Budendorf, Switzerland) as described (16) to remove cells suppressing the Ig response. Briefly, washed PBL were incubated with freshly prepared 0.25 mM LeuLeu-OMe in RPMI 1640, containing 2% fetal calf serum (FCS) for 15 min at room temperature and then washed three times at 4°C in RPMI 1640 medium containing 2% FCS.

PBL were suspended in RPMI-1640 medium, supplemented with 4 mM L-glutamine, nonessential amino acids, 50 mg/ml gentamycin (Biological Industries, Haemek, Israel), 10% heat inactivated human serum (University Hospital Blood Bank, Lund, Sweden) or fetal calf serum (Gibco, Grand Island, NY). Serum or plasma samples from all blood donors were tested by ELISA for antibodies against TT.

CD3 stimulation culture

LeuLeu-OMe treated PBL (300 000 cells/well) were seeded in 96 well flat bottom tissue culture plates (No 3599; Costar, Cambridge, MA) in 200 μ l RPMI-1640 supplemented with 4 mM L-glutamine, 1% (v/v) nonessential amino acids, gentamycin (50 μ g/ml) and 10% FCS. The wells were precoated with 50 ng of purified mAb 64.1 (anti-CD3 monoclonal antibody) in 50 μ l PBS for 1 h at 37°C and then washed twice with PBS as described (12). The cultures were incubated for 7 days at 37°C without or with 20 ng/ml BP-peptide, 25 ng/ml PP or 1 or 25 ng/ml pTT. At the end of the culture period the supernatant medium was removed and analyzed for specific immunoglobulin secretion by ELISA.

In vitro immunization

A primary immunization was performed by seeding 300 000 LeuLeu-OMe treated human PBL/well and culturing the cells for 7 days in medium containing 10% human serum, 50 μ M β -mercaptoethanol, 40 ng IL-2/ml, 25% T cell replacing factor (TRF) as previously described (17, 18) with 20 ng/ml BP-peptide. In some experiments TRF was replaced by IFN- γ and IL-2 or IL-4 alone. T helper cells were generated for secondary immunization cultures by activating the LeuLeu-OMe treated cell population. Briefly, 1×10^6 cells/ml

together with 1×10^6 /ml feeder cells (irradiated autologous PBL) were activated in tissue culture flasks using 50 mM 2-mercaptoethanol and 10 μ M pTT for 7 days (17, 18). On day 3 fresh complete culture medium was added to the cultures.

Primary immunized cells as well as the activated T cells were harvested and centrifuged through 40% Ficoll-Paque, on day 7. After one wash, the proportion of B and T cell was determined by FACS analysis. Cells (0.5×10^6 /ml) from the primary immunization were cocultured with T cells (0.2×10^6 /ml) from the antigen-specific T cell activation on a monolayer of irradiated CD32-transfected fibroblasts for a secondary immunization period. The cells were cultured for 4 days in medium supplemented with 5% human serum, 50 mM 2-mercaptoethanol, 7 nM BP-peptide, 0.5 μ g/ml anti-CD40 antibody and 100 U IL-4/ml. After simplification of the culture system unhaptenated 26-mer TT-HSA peptide was consistently included as control. Supernatants were collected from non-EBV transformed cells at day 7 and analyzed for IgE by radioallergosorbent assay (RAST) or ELISA. In some cases, cells from the *in vitro* immunization were infected with EBV and cultured for 10–20 days as described previously (17, 18). The culture supernatants from the cells were removed and analyzed for specific-IgE by ELISA.

Antibody analysis

ELISA was performed by coating plates (Life Technologies, Sweden) with 0.1 μ g PP/well or 0.1 μ g BP-HSA/well, 1 μ g human serum albumin/well or 0.05 μ g rat antihuman IgE/well (Biosource) in 50 mM sodium carbonate buffer pH 9.5, overnight at 4°C. Blocking was performed for 1 h in 37°C with 200 μ l SDB: 10 mM sodium phosphate buffer, pH 8.0 with 0.5 M NaCl and 0.05% Tween 20. SDB was supplemented with 1% milk powder. Coated plates were incubated for 1–2 h in 37°C with culture supernatants, diluted in SDB with 1% milk powder. After washing, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat antihuman-IgE (1:1000) (KPL, MD, USA). The same procedure was then used for detection of TT specific IgG antibodies, as described previously (12), coating the wells with TT and using HRP-conjugated goat antihuman-IgG for detection. To investigate the specificity of the IgE assay, any cross-reactivity with other isotypes was tested by adding monoclonal IgG, IgM and IgA to serum containing BP specific IgE. These experiments showed a cross reactivity with other isotypes of <5% at a 100-fold excess of monoclonal antibodies.

A standard RAST used in the clinical laboratory (Carlos Haya Hospital) was used to detect specific IgE in patient sera and in cultured cells obtained from the same patients. Cyanogen bromide activated cellulose discs, as described previously (19), were conjugated with 10 mg/ml poly L-lysine (PLL) 3KD (Sigma), and 10 mg/ml of BP or AX (amoxicillin) (Beecham). The RAST assays were carried out as previously described (19) using either 30 μ l of human serum or supernatant from the cultures and 30 μ l of I^{125} radiolabeled anti-IgE (Pharmacia Diagnostics, Uppsala, Sweden). Results obtained with BP-PLL were expressed as percentage of maximum-labeled uptake after subtracting the uptake of the respective carrier single disc.

The nomenclature used in this manuscript adhere to the EAACI recommendations (20).

Results

Allergen/antigen specific IgE and IgG antibody responses of B cells stimulated with anti-CD3 activated T cells.

Peripheral blood lymphocytes from normal donors were cultured for seven days together with T cells polyclonally activated by anti-CD3 antibodies. Under these conditions bystander B cells are activated to produce antibodies in a polyclonal manner, which is

clearly illustrated in Fig. 1. Anti-CD3 stimulation of PBL led to IgG antibody production to TT by cells from three out of three donors (Fig. 1A) and IgE antibody production to PP by cells from three out of three donors (Fig. 1B) and to BP-peptide by cells from six of 11 donors (Fig. 1C). When TT antigen (25 ng/ml) was added to the cultures for the full seven day period, specific IgG antibody responses to TT were abrogated in cells from three out of three donors (Fig. 1A). A similar phenomenon was seen when PP (25 ng/ml) was added to cultures; the allergen completely inhibited production of anti-PP IgE (Fig. 1B). In contrast, addition of BP-peptide (20 ng/ml) to cell cultures resulted in a different response pattern. The frequency of IgE positive wells was diminished by the allergen in cells from four of

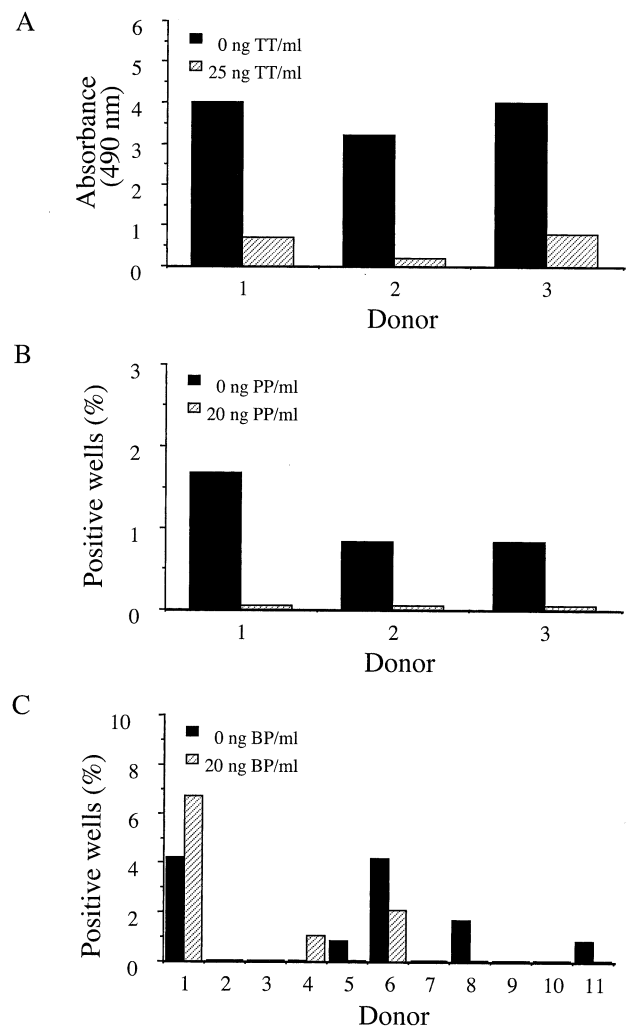


Figure 1. Effect of antigen on specific IgE and IgG responses. Anti-CD3 stimulated PBL were cultured with soluble antigen: (A) tetanus toxoid, TT; (B) *Phleum pratense*, PP; and (C) BP-peptide. After seven days of culture, the supernatants were measured for specific IgG (TT) or IgE (PP, BP) secretion by ELISA. Results are shown as (A) mean values of triplicate cultures from three representative experiments (B and C) percent of positive wells.

11 donors, whereas the allergen enhanced IgE anti-PP production by cells from two of 11 donors (Fig. 1C). Furthermore, in five of 11 donors no specific IgE response could be detected, whether or not BP-peptide was added to the cultures. The antigen-induced decrease in antibody production to TT and PP, and in some cases BP, was not due to absorption of produced antibodies by the antigen since removal of antigen by washing and a subsequent re-culture still resulted in a decreased response (12) (data not shown). The decrease in specific antibody response was rather an elimination of antigen-specific memory B cells, as previously shown by frequency analysis of antigen-specific B cells (12).

Benzylpenicillin specific IgE response of B cells *in vitro* immunized with the heterotope

We have previously described a human lymphocyte culture system in which the production of specific antibodies can be induced by stimulation *in vitro* with a heterotope peptide incorporating a recall T helper cell epitope derived from tetanus toxoid (TT) and a B cell epitope (17, 18). A primary/secondary *in vitro* challenge with this heterotope peptide antigen resulted in isotype switching of antigen-specific B cells from IgM to IgG production (18). In this study, a specific *in vitro* IgE response to benzylpenicillin was measured after stimulating cells from normal donors with a heterotope (TT- BP hapten). However, this approach is technically rather complicated and before comparing the IgE response from normal and atopic donors we simplified this system.

We specifically addressed four parameters that create a quicker *in vitro* test. First, we examined whether the time-consuming EBV transformation of *in vitro* immunized B cells was necessary to measure specific and total IgE. Thus, the IgE secretion from EBV transformed B cells (on day 21–31) was compared with secretion from cells that were taken directly from the *in vitro* culture on day 11. The results (Fig. 2A) show that EBV infection of *in vitro* immunized cells was not necessary for detection of allergen specific IgE *in vitro*, thus shortening the time of analysis considerable. Secondly, since the capacity of various anti-CD40 antibodies to stimulate B cells differs according to their epitope specificity on the CD40 molecule, different anti-CD40 antibodies (G28-5 and S2C6) were compared during the secondary immunization. It is clear from Fig. 2B that G28-5 stimulated, to a much higher degree, the secretion of specific IgE in the absence of exogenous IL-4. In the presence of exogenous IL-4 the production of IgE anti-BP antibodies was not influenced by the G28-5 antibody. IL-4 was however, required for specific IgE responses in the presence of the S2C6 anti-CD40 antibody.

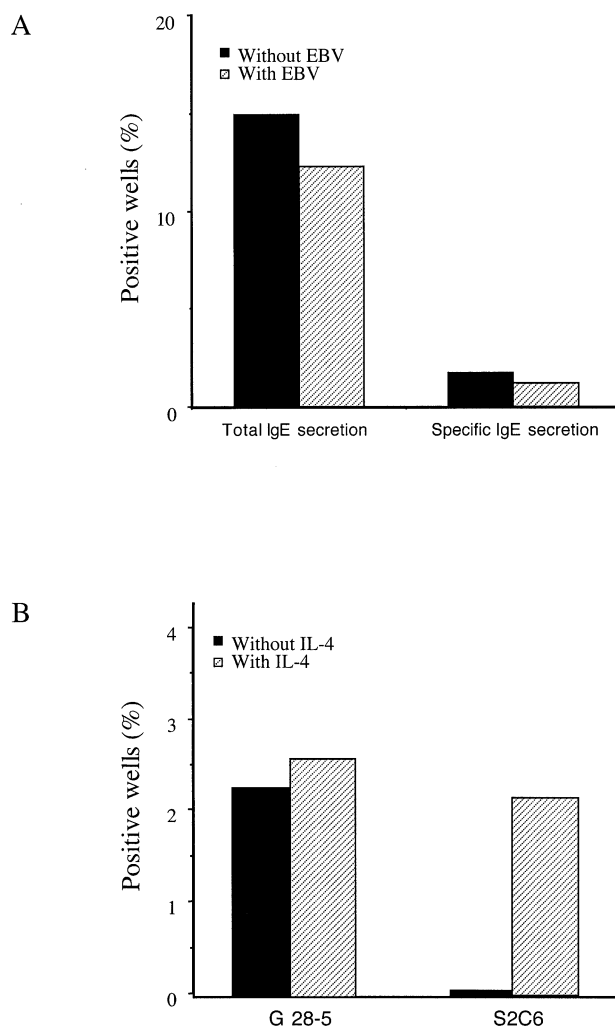


Figure 2. (A) EBV transformation does not increase detection level for total and specific IgE from supernatants derived from the *in vitro* immunization test. Results are shown as percent of positive wells in four representative experiments. (B) Two anti-CD40 antibodies have different capacity to induce specific IgE secretion. Human PBL were cultured in the *in vitro* immunization test and two different anti-CD40 antibodies were used for stimulation during the secondary period in the presence or absence of IL-4. Specific IgE secretion from the cultures was analyzed by ELISA. Results are shown as percent of positive wells in three representative experiments.

Thirdly, we tested whether the T cell replacement factors (TRF), used in the primary stimulation period of the original *in vitro* immunization protocol (17, 18), could be replaced by recombinant factors. Analysis of a number of TRF preparations showed the presence of Th1 cytokines like IFN- γ (0.4–2.8 ng/ml) and IL-2 (0.8–5.7 ng/ml), but no IL-4 or IL-5 (data not shown). Consequently, in the present series of experiments, the IgE-promoting effects of TRF were compared with those of the Th1 cytokines IFN- γ plus IL-2 or the Th2 cytokine IL-4. Table 1 shows the reactivity index (RI) of five antigen-specific

Table 1. Effect of cytokines on Reactivity Index (RI) for specific IgE production against a drug allergen *in vitro**

Culture condition	RI > 5	RI > 25
TRF	5/5	0/5
IFN- γ + IL-2	5/5	2/5
IL-4	5/5	4/5

* The presence of specific IgE antibodies in supernatants was analyzed by ELISA after 11 days. Wells were considered positive if the OD was higher than mean value of the medium control + 10 $\times$ standard deviation of the medium control.

antibodies selected from 500 wells, using the different culture conditions. Values for RI were calculated using the following equation:

$$RI = \frac{\text{absorbance (TT-HSA-BP)} - \text{absorbance (medium control)}}{\text{absorbance (HSA)} - \text{absorbance (medium control against HSA)}}$$

No RI value above 25 was recorded with the original TFR, whereas the combination of IFN- γ plus IL-2 increased the RI value to > 25 in two out of five wells. The frequency of IgE secreting clones with RI > 25 was, however, highest in cultures in which only IL-4 was present. Fourthly, Table 2 shows that secretion of BP-specific IgE was detected equally after both primary (seven day culture) and secondary (re-stimulation for four days after the primary culture period) stimulation. The number of wells containing B cells secreting antigen-specific IgE antibodies was similar to what has been observed earlier, using a peptide that induced IgG switch (18) and demonstrated that this *in vitro* immunization could be used to analyze allergen specific IgE responses. Since the secretion of IgE after the primary and secondary immunization was similar (Table 2), we concluded that a primary immunization (7 day stimulation period) was sufficient and that IL-4 was the additive that best supported specific activation.

Table 2. Comparison of specific IgE secretion during primary and secondary immunization

Donor	Specific IgE secretion *			
	Primary immunization	%	Secondary immunization	%
1	1/108	0.92	3/108	2.8
2	2/78	2.5	1/54	1.9
3	0/162	0	1/108	0.92
4	1/108	0.92	1/108	0.92
5	2/54	3.7	0/54	0
6	6/162	3.7	1/216	0.46
7	1/162	0.6	1/54	1.9
8	1/162	0.6	0/54	0
9	0/162	0	n.d.	

* Secretion of specific IgE after primary and secondary immunization was analyzed by ELISA. Wells were considered positive if the OD was higher than the mean value of the medium control + 10 $\times$ standard deviation of the medium control. Results are shown as percentage positive wells of total wells measured

The resulting culture system was consequently easier and quicker to perform for evaluation of allergen specific IgE secretion and was the method of choice when testing cells from normal and atopic donors.

To investigate the response patterns of cells from normal blood donors and atopic patients we then performed a comparative study between the BP-specific IgE responses of lymphocytes obtained from normal blood donors and from patients with known IgE-mediated allergy to BP. The subjects were allergic to BP or AX or both, according to their clinical history. All of them were positive to AX and/or BP in skin test and RAST although, at the moment of the study, only six cases remained positive as shown in Table 3. Normal blood donors did not have detectable levels of IgE antibodies to BP or AX (data not shown). Lymphocytes from the penicillin-allergic and the normal donors were primary immunized *in vitro* for seven days with BP-peptide (20 ng/ml) or unhaptenated control peptide. Cells from six of 16 normal donors responded to the BP-peptide by producing more anti-BP IgE antibodies compared to cells stimulated with control unhaptenated peptide. Cells from an equal number of donors (six of 16) produced less IgE when stimulated with BP-peptide compared with the unhaptenated peptide. Cells from four of 16 donors failed to produce anti-BP IgE antibodies when cultured with either the haptenated or unhaptenated peptide (Fig. 3A). Cells from the penicillin-allergic donors showed a quite different pattern of responsiveness in that 10 out of 16 failed to produce anti-BP IgE antibodies after culture with either the BP-haptenated or unhaptenated peptide, despite exhibiting the same viability compared to cells from normal donors. Cells from three of 16 allergic donors produced more anti-BP IgE antibodies when

Table 3. Serum values of BP and AX specific IgE antibodies in allergic donors

Patient number	BP	AX
1	—	0.7
2	2.25	—
3	0.06	—
4	—	0.4
5	2.4	0.2
6	—	0.38
7	1.9	—
8	—	0.7
9	—	—
10	3.7	2.1
11	—	0.16
12	—	0.18
13	12.8	—
14	3.7	—
15	—	0.28
16	3.9	6.7

Sera from allergic patients was tested for benzylpenicillin (BP) and amoxicillin (AX) specific antibodies by RAST assay before the cells were subjected to primary immunization. The values are percentages of radiolabeled uptake. Results are considered as positive when the values are equal or greater than two.

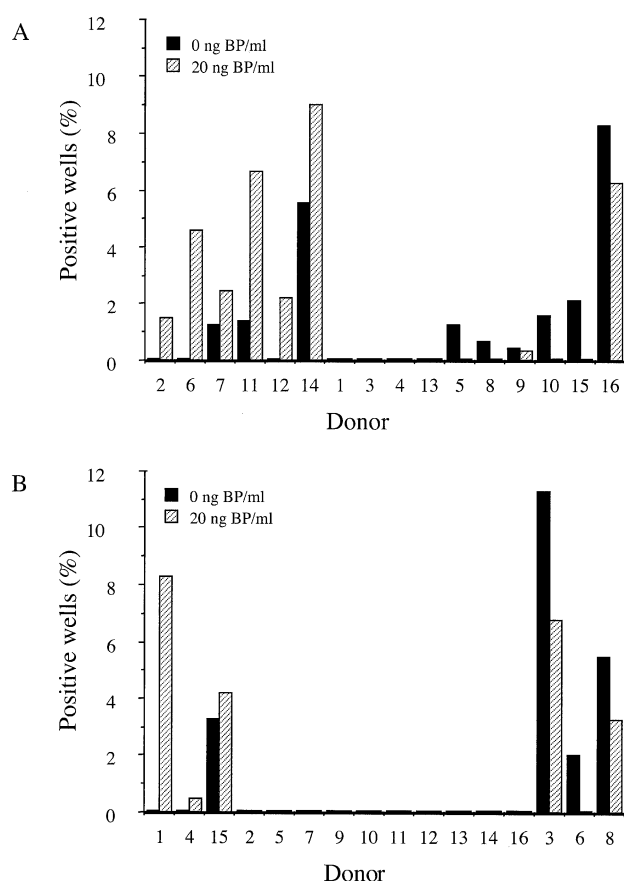


Figure 3. Comparison of drug-specific IgE secretion after a primary immunization with BP-peptide vs unhaptenated peptide, using cells from either normal or atopic donors. Results are shown as cases where number of positive wells increases, decreases or is equal when BP-haptenated peptide is added to the cultures as compared to the response induced by unhaptenated peptide. Secretion of specific IgE was analyzed by ELISA for cells from normal donors (A) and by RAST for cells from allergic donors (B). Wells in ELISA were considered positive if the OD was higher than the mean value of the medium control + $10 \times$ standard deviation of the medium control and positive results in RAST were values higher than one.

cultured with the BP-peptide compared to the unhaptenated peptide, and the same proportion (three of 16) produced less anti-BP IgE antibodies in response to the BP-peptide compared to the unhaptenated peptide (Fig. 3B).

Discussion

Immunotoxic reactions induced by drugs such as penicillins, cephalosporins, sulfonamides, insulin and streptokinases are often caused by specific IgE responses (1, 2). The reactions range from those which are relatively mild to those that are life threatening. Currently no model exists which provides predictive information on IgE-mediated hypersensitivity reactions. *In vitro* testing may be of value in predicting the risk of allergic reactivity on re-challenge

as well as the allergenicity of novel compounds in terms of their capacity to induce antibody, and particularly IgE, responses. Different human *in vitro* systems leading to antibody production by B cells have previously been reported. One of these systems is based on using anti-CD3 activated T cells that induce proliferation and differentiation of resting B cells into Ig secreting cells (12). T cells activated polyclonally with anti-CD3 antibodies provide help via CD40L and lymphokines, as described previously (21–23). We have recently demonstrated an effect of anti-CD3 stimulation of T cells on antigen-specific antibody responses *in vitro*, using cells from tetanus toxoid vaccinated donors (12). Our data (12) then showed that addition of TT to cell cultures containing TT-specific memory B cells, resulted in a decrease in anti-TT IgG production (12). In the present study this effect of TT antigen on IgG production was confirmed, and furthermore the same effect on IgE memory cells was seen when adding the pollen allergen PP to the same anti-CD3 stimulated lymphocyte culture (Fig. 1B). Given this common behaviour of antigens and protein allergens upon re-challenge, we can conclude that the anti-CD3 lymphocyte system could have the potential to be predictive of previous allergen exposure. Analysis of B cell responses to the BP-peptide in the anti-CD3 system resulted, however, in a different behaviour as both increased as well as decreased BP allergen-specific IgE responses by lymphocytes from different normal donors were observed. This may reflect differences in duration since last exposure to BP, which could affect the frequency of BP-specific B cells in the periphery or the sensitivity of BP-specific B cells to signalling via the B cell receptor. Therefore, data from the present study seems to indicate that the anti-CD3 system could give an indication of previous protein allergen exposure and possibly also previous drug exposure.

It is known that isotype switching and differentiation of B cells to plasma and memory cells occurs in the germinal centres of lymphoid organs (24). We have recently described an attempt to mimic the germinal center reaction leading to isotype switch using a two step *in vitro* immunization system (18). By using this system with BP, we could detect secretion of allergen-specific IgE antibodies already after primary immunization. Our results show that IgE is induced more readily as compared to antigen-specific IgG (18), possibly reflecting the mere fact that the majority of blood donors have previously been exposed to penicillin. This is also supported by the fact that when a viral peptide was used for *in vitro* immunization of sero-negative blood donors no IgG could be detected after the primary immunization period (18, 25).

Cytokines have been shown to influence the immune response by binding to receptors on B and T cells. Since cytokines have an important role in influencing the immune response by activating T and B cells, we also

evaluated the effect of different cytokines in this system. It is known that IL-2 is produced early in the T cell activation process and will bind to the IL-2 receptor inducing an autocrine proliferation (26). IFN- γ is produced by Th1 cells and could have an inhibitory effect on switch to certain isotypes (27), while IL-4 is produced by Th2 cells (28) and has been shown to be important for IgE switching (29). In order to produce IgE antibodies, B cells need to make a cognate interaction with a Th2 cell (30). We have observed in our earlier studies on *in vitro* B cell immunization, a change of the cytokine profile during the culture period, i.e. a rapid T helper cell polarization *in vitro* (31). The majority of the cells were of Th2 phenotype after primary immunization but were rapidly polarized to Th1 cells after the secondary stimulation period that could explain the synthesis of IgE already after the primary immunization step. The positive effect of IL-4 on the number of IgE secreting B cells in our present *in vitro* system formed the basis for replacement of previously undefined T cell derived factors (TRF) (32).

Beta-lactam antibiotics form immunogenic conjugates with many serum and surface proteins and can induce IgE responses in allergic patients (13–15). We applied the simplified *in vitro* immunization system to cells from penicillin-allergic donors to analyze if a previous reaction *in vivo* influenced the secretion of specific IgE antibodies *in vitro*. All of the allergic donors had experienced immediate allergic reactions to penicillin and were initially anti-BP and/or anti-AX IgE antibody positive by RAST although at the moment of the study specific IgE antibodies were only positive in six cases. Addition of BP-peptide to the cultures caused a heterogeneous anti-BP IgE antibody response: cells from some BP-allergic donors (three out of 16) showed an increase in anti-BP IgE antibody secretion whereas others (three of 16) showed a decrease. Cells from the majority (10 of 16) of the BP-allergic donors did not respond at all, which was a significant difference compared to cells from normal donors, most of which produced anti-BP IgE antibody when stimulated with

the BP-peptide. A possible explanation for this observed difference between healthy donors and atopic patients may be that the circulating memory B cells derived from BP-allergic donors require a different set of signals. Another possibility is that the proportion of specific memory B cells located at effector sites compared to the periphery varies and that we are partly looking at a primary response to the drug *in vitro*. Serum levels of anti-BP/AX IgE antibodies from the patient donors did not relate to the specific IgE response obtained *in vitro*, indicating a complex relationship between allergen specific IgE antibodies in serum and clinical syndromes (33). From these results we can conclude that the *in vitro* immunization system is not predictive of allergenicity of haptened drugs in its present form.

An *in vitro* assay offers numerous advantages such as ethical, speed, increased sensitivity and control of test conditions. Since an allergic response *in vivo* is a complex reaction it might be difficult to cover the diverse functions that may be affected in an *in vitro* assay. In the present investigation we tried to analyze different culture conditions that induce allergen specific IgE and clearly demonstrated that such specific secretion is easily detected in human B cells, a consideration implying that a predictive *in vitro* test should be based on rather few cellular signals. However, a consequence of using human peripheral blood lymphocytes in systems that predict allergenicity, is that memory cells influence the response, as the donors might have been pre-exposed to many of the common allergens. In conclusion, the *in vitro* systems described here provide information to form a basis for testing pre-exposure and possibly also to a more predictive test for allergenicity although further investigations are clearly needed.

Acknowledgments

This investigation was supported by grants from the European Commission (BIO4-CT96-0246), Trygg-Hansas forskningsfond and Vårdalstiftelsen.

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