

# Allergens, IgE, mediators, inflammatory mechanisms

## Induction and differential regulation of bee venom phospholipase A<sub>2</sub>-specific human IgE and IgG<sub>4</sub> antibodies in vitro requires allergen-specific and nonspecific activation of T and B cells

Cezmi A. Akdis, MD,<sup>a</sup> Thorsten Blesken, IngFH,<sup>a</sup> Mübeccel Akdis, MD,<sup>a</sup>  
Sefik S. Alkan, PhD,<sup>b</sup> Brunello Wüthrich, MD,<sup>c</sup> Christoph H. Heusser, PhD,<sup>b</sup>  
and Kurt Blaser, PhD<sup>a</sup> Davos, Basel, and Zurich, Switzerland

Investigations on the mechanisms of IgE regulation in vitro have been conducted thus far in systems that allow the synthesis of total rather than specific IgE. To study the regulatory prerequisites of antigen-specific IgE antibody production, we have established a culture system that allows the generation of bee venom phospholipase A<sub>2</sub>-specific IgE and IgG<sub>4</sub> antibodies. Allergen-specific IgE was induced by simultaneously activating T cells and B cells specifically with allergen and polyclonally with anti-CD2 and soluble CD40 ligand in the presence of IL-4. Additional stimulation of T cells through the CD2 activation pathway by two different anti-CD2 monoclonal antibodies enhanced both the allergen-specific and the total IgE and IgG<sub>4</sub> responses. An optimal amount of allergen (0.1 ng/ml) resulted in the induction of both allergen-specific IgE and IgG<sub>4</sub> antibodies. Higher antigen doses reduced allergen-specific antibodies and enhanced total isotype production. This differential regulation of allergen-specific and total isotypes reflects different allergen dose-dependent mechanisms in specific and polyclonal activation of T and B cells. Although both isotypes require IL-4 for initial induction, opposite regulatory effects by T cells were observed for IgE and IgG<sub>4</sub> antibody expression. In peripheral blood mononuclear cell cultures stimulated with soluble CD40 ligand, IL-4, and phospholipase A<sub>2</sub>, stimulation of T cells with higher amounts of anti-CD2 enhanced IgG<sub>4</sub> in parallel to increased IL-2 and interferon- $\gamma$  secretion but inhibited IgE synthesis. These results provide evidence for differential regulation of allergen-specific and total IgE and IgG<sub>4</sub> by antigen concentration and demonstrate the pivotal role of T cells controlling the synthesis of the IgE and IgG<sub>4</sub> antibody isotypes. (*J Allergy Clin Immunol* 1997;99:345-53.)

**Key words:** Allergen-specific IgE, isotype regulation, bee venom phospholipase A<sub>2</sub>, IgE antibody, IgG<sub>4</sub> antibody

Although allergen-specific IgE antibodies mediate type I hypersensitivity, the production of specific IgG<sub>4</sub> antibodies is claimed to represent normal, protective immunity to the allergen.<sup>1-3</sup> Although knowledge of the molecular and cellular regulatory basis of human IgE synthesis has increased during recent years, the mechanisms involved in the control of allergen-specific IgE production need further elucidation.

The major difficulties in studying the molecular and cellular mechanisms of allergen-specific IgE synthesis are the low frequency of specific IgE-producing cells in the circulation<sup>4</sup> and the accurate determination of the low levels of in vitro synthesized specific IgE antibodies. Although a variety of in vitro systems have been designed to study the induction and isotype switch requirements of IgE,<sup>5-8</sup> de novo induced antigen-specific IgE responses in human beings remain to be investigated. So far, the induction of allergen-specific IgE antibodies could only be monitored by enumeration of secreting cells in ELISA spot assay.<sup>9</sup>

To establish an allergen-specific IgE response in vitro, bee venom phospholipase A<sub>2</sub> (PLA) was used. PLA, a 14 to 18 kd glycoprotein consisting of 134 amino acids,<sup>10</sup> represents the major allergen in bee venom and can induce both allergy and immunity to bee sting.<sup>11, 12</sup> The initial response to bee sting elicits low-affinity anti-PLA IgG<sub>1</sub> antibodies,<sup>13, 14</sup> whereas a protective immune response with high-affinity anti-PLA IgG<sub>4</sub> antibodies is generated after repeated exposure.<sup>13</sup> In contrast, in individuals allergic to bee sting, anti-PLA IgE antibodies develop.<sup>11</sup> Successful bee venom immunotherapy usually results in a decline of PLA-specific IgE to IgG<sub>4</sub> antibody ratio.<sup>13, 15-17</sup> These observations suggest a reciprocal regulation of specific IgE and IgG<sub>4</sub> antibodies, although the switch of both isotypes depends on IL-4.<sup>18</sup>

The essential role of regulatory T cells in total IgE and IgG<sub>4</sub> synthesis has been clearly demonstrated. Selected

From the <sup>a</sup>Swiss Institute of Allergy and Asthma Research, Davos; <sup>b</sup>Ciba Geigy Ltd., Basel; and <sup>c</sup>Allergy Unit, Department of Dermatology, University Hospital, Zurich.

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Reprint requests: Cezmi A. Akdis, MD, Swiss Institute of Allergy and Asthma Research, Obere Strasse 22, CH-7270 Davos Platz, Switzerland.

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*Abbreviations used*

|                 |                                        |
|-----------------|----------------------------------------|
| FACS:           | Fluorescence-activated cell sorter     |
| IFN- $\gamma$ : | Interferon- $\gamma$                   |
| mAb:            | Monoclonal antibody                    |
| PBMCs:          | Peripheral blood mononuclear cells     |
| PHA:            | Phytohemagglutinin                     |
| PLA:            | Bee venom phospholipase A <sub>2</sub> |
| PMA:            | Phorbol myristate acetate              |
| sCD40L:         | Soluble CD40 ligand                    |
| TT:             | Tetanus toxoid                         |

T-cell clones<sup>3, 7, 8</sup> and their supernatants,<sup>7, 19</sup> together with membranes of activated T cells or ligands expressed on the surface of activated T cells, can induce IgE production by B cells.<sup>8, 19-21</sup> In this study we have investigated the cellular and molecular requirements for allergen-specific IgE and IgG<sub>4</sub> antibody production and regulation of these two isotypes *in vitro*.

**METHODS****Study populations**

Twelve individuals allergic to bee venom (mean age, 29 years), who had systemic immediate-type reactions to bee stings, were studied. All patients showed positive intracutaneous test responses to honeybee venom (Pharmalgen venom; ALK, Horsholm, Denmark) at a concentration of less than 10<sup>-4</sup> gm/L and had more than 2 KU/L bee venom-specific IgE antibodies as estimated by CAP immunoassay (Pharmacia, Uppsala, Sweden). Three healthy donors (mean age, 31 years), who were not sensitized to PLA and/or bee venom and showed no IgE reactivity to other allergens, were included as control subjects.

**Reagents**

The following recombinant cytokines and monoclonal antibodies (mAbs) were kindly provided as indicated. IL-4 was from Ciba Geigy (Basel, Switzerland). Anti-CD2 (4B2 and 6G4) mAbs were from the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).<sup>22</sup> Anti-CD3 was produced by clone CRL 8001 obtained from ATCC (Rockville, Md.). Phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) were from Sigma Chemical Company (St. Louis, Mo.). PLA was from Boehringer (Mannheim, Germany). The cell toxicity of PLA was assessed by viability and proliferation assays of peripheral blood mononuclear cells (PBMCs), Epstein-Barr virus-transformed B-cell lines and cytotoxic lymphoid line cells. It was not toxic at concentrations of less than 50  $\mu$ g/ml as used in our experiments. Soluble CD40 ligand (sCD40L) (originally generated and kindly provided by Dr. P. Lane; msCD40L-mCD8- $\alpha$  fusion protein; Institute for Immunology, Basel, Switzerland) was produced by culturing the transfectant cell line 8-40-1<sup>23</sup> and standardized by its maximal IgE-inducing activity in a 10-day culture of PBMCs, co-stimulated with 500 U/ml IL-4, as described for anti-CD40 mAb.<sup>8</sup> The untransfected cell line J558L was obtained from Dr. M. Reth (University of Freiburg, Germany) and used as a control.

**Induction of immunoglobulin synthesis**

PBMCs from peripheral venous blood were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation,

washed three times, and resuspended in RPMI-1640 medium supplemented with 1 mmol/L sodium pyruvate, 1% minimum essential medium with nonessential amino acids and vitamins, 2 mmol/L L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ mol/L 2-mercaptoethanol (all from Gibco, Basel, Switzerland), 40  $\mu$ g/ml human transferrin, 4  $\mu$ g/ml bovine insulin (Sigma Chemical Co.), and 10% heat-inactivated fetal calf serum (Sera-Lab, Sussex, England). B cells were purified from PBMCs by using the magnet-activated cell separation system (MACS; Miltenyi Biotech, Marburg, Germany) and anti-CD19 antibodies after depletion of monocytes by anti-CD14.<sup>24</sup> The purity of cells was greater than 95% as determined by fluorescence-activated cell sorter (FACS) analysis with fluorescein isothiocyanate-labeled anti-CD20 (Immunotech, Paris, France). PBMCs ( $2.5 \times 10^6$ ) were cultured in 5 ml of medium in 6-well plates (Costar, Cambridge, Mass.) and stimulated with IL-4 (500 U/ml), 25% of sCD40L containing 8-40-1 cell supernatant, a mixture of the two anti-CD2 mAbs 4B2 and 6G4, and the PLA antigen. Purified B cells ( $5 \times 10^5$ /ml) were cultured in 24-well plates and stimulated with sCD40L and IL-4.

**Measurement of cytokines**

The solid-phase ELISA for interferon (IFN)- $\gamma$  was described previously.<sup>25</sup> Briefly, 96-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with mAb 43-11 to IFN- $\gamma$  (0.5  $\mu$ g/well) and specifically developed with biotinylated mAb 45-15 (0.1  $\mu$ g/ml) and ExtrAvidin-Peroxidase (Sigma Chemical Co.). The sensitivity of the IFN- $\gamma$  ELISA was 20 pg/ml. The IL-2 activity was measured by tritiated thymidine uptake of the IL-2-dependent cytotoxic lymphoid line cells as described by Gillis et al.<sup>26</sup>

**Measurement of immunoglobulin isotypes**

Supernatants were measured for their total and anti-PLA IgE and IgG<sub>4</sub> antibody content by ELISA as described previously.<sup>27, 28</sup> Briefly, for PLA-specific antibody detection, ELISA plates (Nunc, Maxisorb) were coated with 1  $\mu$ g/well PLA. Biotinylated anti- $\epsilon$  mAb 6-7 (Ciba-Geigy, Basel, Switzerland) and ExtrAvidin-Peroxidase were used to detect anti-PLA IgE. Anti- $\gamma$ 4 mAb RJ4 (Oxoid, Basingstoke, U.K.) and peroxidase-labeled anti-mouse immunoglobulin antibody (Tago, Burlingame, Calif.) were used to develop the anti-PLA IgG<sub>4</sub> ELISA. PLA-specific IgG<sub>4</sub>/ $\lambda$  human mAb BVA2<sup>29</sup> was used as standard for anti-PLA IgG<sub>4</sub>; and serum from a patient allergic to bee venom reacting only with PLA, which showed 72 KU/L in bee venom RAST (Pharmacia), was used as anti-PLA IgE standard.<sup>2, 3</sup> One unit corresponds to 2.15 ng of specific IgE. These assays detected 0.1 ng/ml anti-PLA IgE and 0.1 ng/ml anti-PLA IgG<sub>4</sub>. For total IgE determination, anti-IgE mAb 14-41 (Ciba-Geigy) was used for coating, and biotinylated mAb 6-7 was used for detection. The detection limit of the IgE assay was 0.2 ng/ml human IgE standard (Behring, Marburg, Germany). Mouse anti- $\gamma$ 4 mAb RJ4 and peroxidase-conjugated anti-IgG antibody (Sigma Chemical Co.) were used to quantify IgG<sub>4</sub>.<sup>3</sup> The detection limit of total IgG<sub>4</sub> was 0.6 ng/ml World Health Organization reference serum 67-97. For detection of tetanus toxoid (TT)-specific IgG, 80 U/ml TT (Berna, Bern, Switzerland), diluted 1:500, was used in phosphate-buffered saline for coating. The anti-tetanus standard serum (Tetuman, Berna) and samples were incubated for 2 hours at room temperature. Peroxidase-conjugated anti-IgG antibody was used for development. In all immunoglobulin isotype-specific ELISAs, o-phenylenediamine HCl was used as substrate. Optical density was

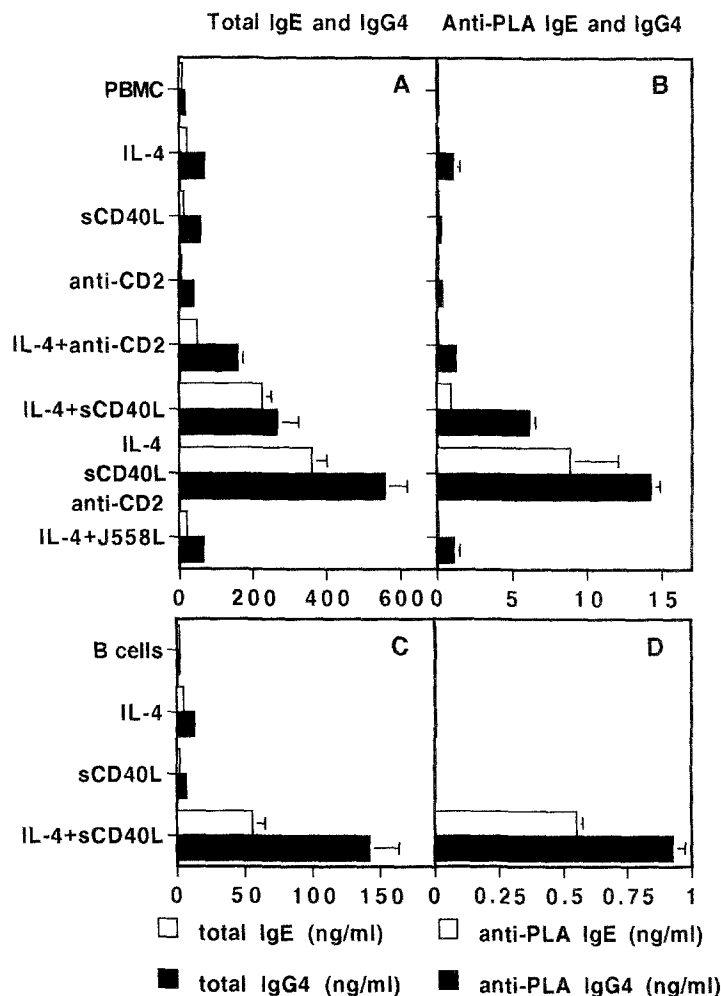


**FIG. 1.** In vitro production of PLA-specific and total IgE and IgG<sub>4</sub>. **A and B**, PBMCs of subjects allergic to bee venom were stimulated with IL-4, sCD40L, and a combination of two anti-CD2 mAbs (0.1  $\mu$ g/ml each). PLA (0.1 ng/ml) was present in all conditions. Total IgE (open bars) and IgG<sub>4</sub> (black bars) (**A**) and anti-PLA IgE (open bars) and anti-PLA IgG<sub>4</sub> (black bars) (**B**) are shown from one of nine experiments (mean of duplicate cultures  $\pm$  SEM). **C and D**, Purified B cells from a patient allergic to bee venom were stimulated with IL-4 and sCD40L. Total IgE and IgG<sub>4</sub> (**C**) and anti-PLA IgE and anti-PLA IgG<sub>4</sub> (**D**) are shown from one of three experiments.

measured at 490 nm in an ELISA reader (Molecular Devices, Menlo Park, Calif.) after stopping the reaction with 0.5N H<sub>2</sub>SO<sub>4</sub>.

#### Determination of cell proliferation

Proliferative responses were measured on day 5 of culture. One hundred-microliter aliquots from  $2.5 \times 10^6$ /5 ml/6-well plate PBMC cultures were transferred to 96-well flat-bottom culture plates in triplicate (Costar) and pulsed with 1  $\mu$ Ci/well tritiated thymidine. The plates were harvested 20 hours later, and radioactivity was measured in a Pharmacia Wallac Beta Plate Reader (Turku, Finland).

#### Statistical interpretation

Data are presented as means  $\pm$  SEM. Statistical analysis was performed by using Student's paired *t* test for comparing changes in total and specific immunoglobulin levels between paired stimulation conditions.

## RESULTS

### In vitro production of IgE and IgG<sub>4</sub> anti-PLA antibodies

Primary investigations to induce in vitro synthesis of allergen-specific IgE and IgG<sub>4</sub> antibodies showed that the stimulation of PBMC or B-cell cultures with PLA alone or together with either IL-4, sCD40L, or anti-CD2 was not sufficient to induce anti-PLA IgE. As shown in Fig. 1, IL-4 and sCD40L were required together to induce detectable amounts of anti-PLA IgE and IgG<sub>4</sub> in  $2.5 \times 10^6$  PBMCs/5 ml (Fig. 1, *B*) or in  $5 \times 10^5$  B cells/ml (Fig. 1, *D*) in 12 days. Additional stimulation of T cells in the PBMC culture with a mixture of two different anti-CD2 mAbs enhanced both total and specific IgE and IgG<sub>4</sub>, as shown in Fig. 1, *A* and *B*. PLA was present at 0.1 ng/ml in all culture conditions. No PLA-specific

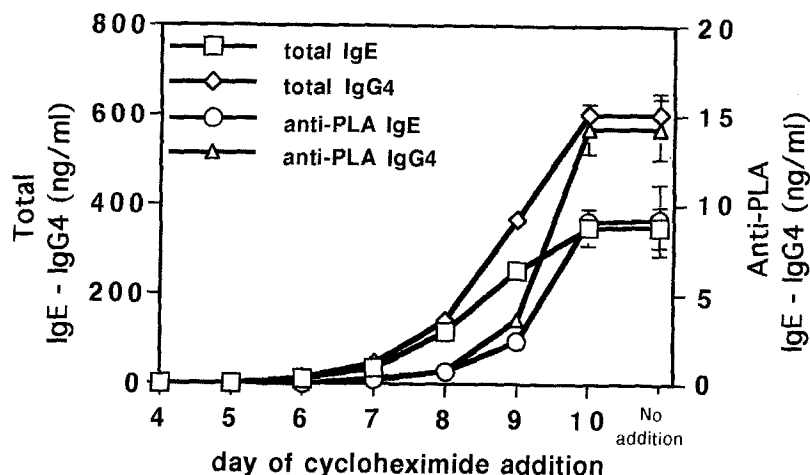


**FIG. 2.** Inhibition of antibody synthesis by cycloheximide. Cycloheximide (10  $\mu$ mol/L) was added each day from day 1 to day 10 to anti-CD2-, IL-4-, sCD40L-, and PLA-stimulated PBMCs. Total and specific immunoglobulins were determined after 12 days. Means of duplicates  $\pm$  SEM are shown.

antibodies were obtained with J558L control supernatant instead of sCD40L and with cells from control subjects not sensitized to bee venom whose sera were negative for PLA-specific IgE.

To determine whether allergen-specific antibodies were newly synthesized in the culture, cycloheximide was added to PBMCs stimulated with anti-CD2, IL-4, sCD40L, and PLA (Fig. 2). Total inhibition of immunoglobulin synthesis was achieved by the addition of 10  $\mu$ mol/L cycloheximide each day until day 5. The addition of cycloheximide on days 6 to 9 partially inhibited specific and total IgE and IgG<sub>4</sub> synthesis and had no significant effect when added on day 10.

#### Antigen dose-dependent differential regulation of PLA-specific and total IgE and IgG<sub>4</sub>

Detectable amounts of anti-PLA antibodies could be induced without PLA (Fig. 3) in anti-CD2/sCD40L/IL-4 co-stimulated PBMCs. However, optimal amounts of the PLA allergen in the culture substantially increased the production of anti-PLA antibodies. Thus the production of specific and total IgE is determined by the dose of allergen. Although high allergen doses ( $10^2$  to  $10^4$  ng/ml) induced IgE and IgG<sub>4</sub> secretion, PLA-specific antibody production was inhibited at those concentrations of allergen. The optimal amount of antigen to induce anti-PLA IgE and IgG<sub>4</sub> was found at 0.1 ng/ml, which is about  $10^5$  times lower than the optimal amount for total IgE enhancement. The levels of PLA-specific IgE and IgG<sub>4</sub> induced by 0.1 ng/ml PLA varied among seven different donors with bee venom allergy in the range of 2.8-fold to 5.5-fold for IgE and 2.0-fold to 5.3-fold for IgG<sub>4</sub> ( $p < 0.001$  for both). Inhibition of PLA-specific antibodies by high doses of antigen in the culture could potentially result from blocking anti-PLA IgE and IgG<sub>4</sub> detection by excessive PLA. This was excluded by washing cultures on day 7 and re-culturing without PLA. In these experiments the same levels of specific IgE anti-

bodies were found in similar dose-response curves with and without washing, with full inhibition at high antigen doses (data not shown). As a further control, TT-specific IgG production was not affected by PLA. We then analyzed proliferation in cultures in response to various concentrations of PLA. Increased doses of PLA induced proliferation in IL-4/anti-CD2/sCD40L-stimulated cultures. As assessed by counting the cells in each well and FACS analysis by anti-CD3 and anti-CD20, the proliferating cells in high antigen concentrations were T cells but not B cells (Fig. 2, C). These high antigen concentrations, which stimulate T cells for proliferation, were the same doses that induced total IgE and IgG<sub>4</sub> production but inhibited specific IgE and IgG<sub>4</sub> production.

#### Differential regulation of total and anti-PLA IgE and IgG<sub>4</sub> by T-cell activation through CD2

Different conditions of T-cell activation for production of total and anti-PLA IgE and IgG<sub>4</sub> were investigated by stimulation with various concentrations of anti-CD2 mAbs in IL-4-, sCD40L-, and PLA-supplemented PBMC cultures. As shown in Fig. 4, increasing amounts of anti-CD2 resulted in significantly increased IL-2 and IFN- $\gamma$  secretion and higher production of total and PLA-specific IgG<sub>4</sub> antibodies. In contrast to IgG<sub>4</sub>, total and anti-PLA IgE formation were inhibited. Increasing concentrations of anti-CD2 mAb from 0.1  $\mu$ g/ml to 2.5  $\mu$ g/ml decreased total IgE levels from  $361 \pm 41$  ng/ml (mean  $\pm$  SEM) to  $263 \pm 39$  ng/ml ( $p < 0.01$ ) by 27% (11% to 41%, range of three experiments), whereas anti-PLA IgE decreased by 95% (86% to 99%) from  $8.86 \pm 0.11$  ng/ml to  $0.46 \pm 0.02$  ng/ml ( $p < 0.001$ ). In contrast, the same changes in anti-CD2 concentrations enhanced the total IgG<sub>4</sub> by 147% (118% to 182%) from  $557 \pm 61$  ng/ml to  $821 \pm 91$  ng/ml ( $p < 0.05$ ) and anti-PLA IgG<sub>4</sub> by 672% (429% to 913%) from  $2.1 \pm 0.30$  ng/ml to  $14.2 \pm 1.50$  ng/ml ( $p < 0.001$ ). Thus modification of

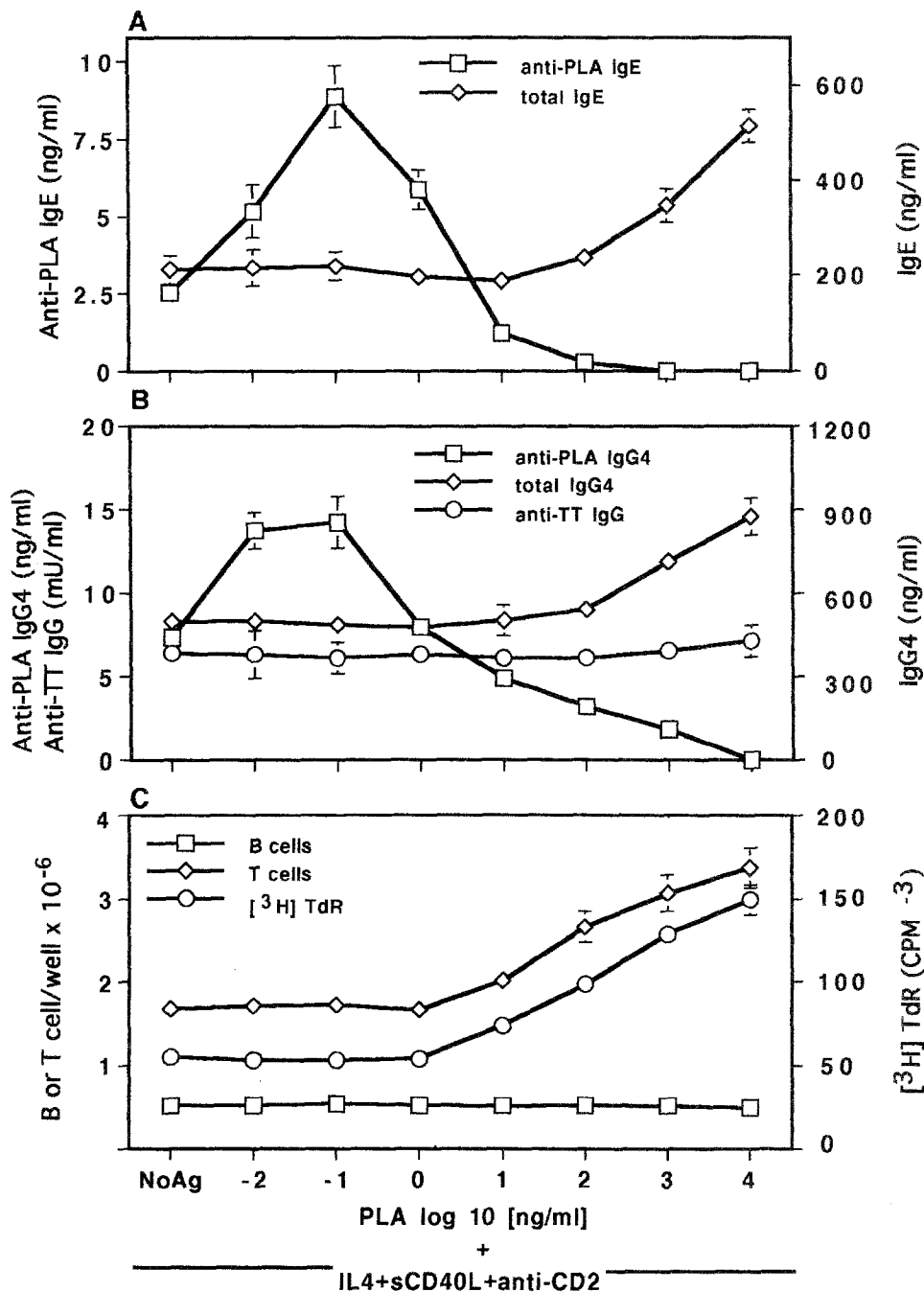


**FIG. 3.** Effect of allergen doses on production of PLA-specific and total IgE and IgG<sub>4</sub>. PBMCs from a patient allergic to bee venom were stimulated with IL-4, sCD40L, and anti-CD2 mAb in the presence of different concentrations of PLA. Anti-PLA and total IgE (A) anti-PLA and total IgG<sub>4</sub> and anti-TT IgG (B) were determined on day 12. Estimation of B cell and T cell absolute numbers in cultures was made on day 7 by trypan blue dye exclusion and FACS analysis by staining for CD20 (B cells) and CD3 (T cells), respectively. Tritiated thymidine (<sup>3</sup>H] TdR) incorporation was measured on day 6 (C) (representative experiment of seven). Means of duplicates ± SEM are shown.

T-cell activation through CD2 influenced PLA-specific antibody formation more than the total isotypes, demonstrating a higher sensitivity of specific antibody synthesis to additional T-cell activation signals. In addition, other conditions of T-cell activation were

studied. Stimulation of PBMCs with anti-CD3 (0.1, 5 µg/ml), PHA (0.04 to 5 µg/ml), and PMA (0.08 to 50 ng/ml) in the presence of sCD40L and IL-4 resulted in high IL-2 and IFN-γ secretion. These cytokines, which are known to display inhibitory effects on IgE synthe-

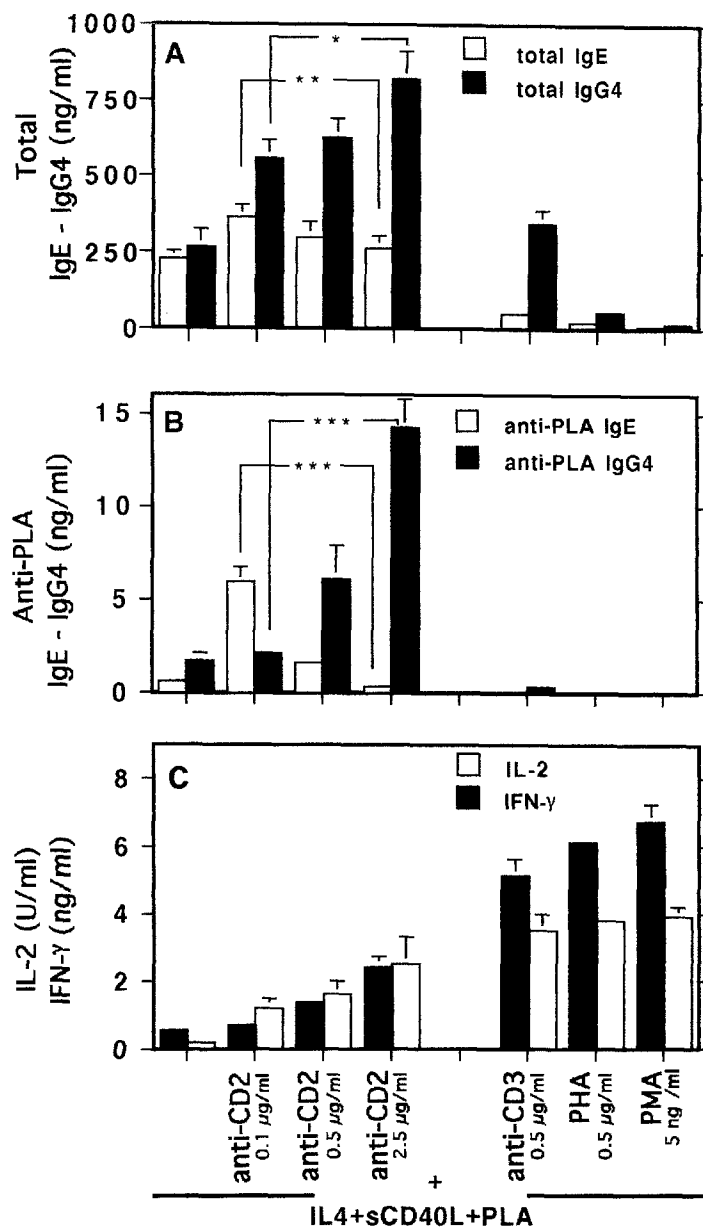


**FIG. 4.** Differential regulation of total and anti-PLA IgE compared with IgG<sub>4</sub> by increasing T-cell activation. PBMCs from a patient allergic to bee venom were stimulated with IL-4, sCD40L, and different concentrations (0.1 to 2.5 μg/ml) of two anti-CD2 mAbs in the presence of 0.1 ng/ml PLA. Similarly, anti-CD3 (0.5 μg/ml), PHA (0.5 μg/ml), and PMA (5 ng/ml) were used instead of anti-CD2 for stimulation. In various concentrations anti-CD3, PHA, and PMA were inhibitory for total and anti-PLA IgE as a result of enhanced IL-2 and IFN-γ production. One of three representative experiments is shown. Results show mean of duplicates ± SEM. Statistical significance is expressed as: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

sis, inhibited the production of total and anti-PLA immunoglobulins.

## DISCUSSION

Although basic cellular and molecular requirements for the induction and synthesis of nonspecific IgE have been extensively investigated, the mechanisms involved

in the regulation of allergen-specific IgE synthesis have not yet been elucidated in human systems. By culturing higher numbers of PBMCs or isolated B cells from bee venom-sensitized donors, we established an in vitro system that allows sufficiently high production of allergen-specific IgE and IgG<sub>4</sub> antibodies. We analyzed the effects of allergen dose and of different T-cell and B-cell

activators on the production of total and specific IgE and IgG<sub>4</sub>.

As previously shown for total IgE, the induction of anti-PLA IgE and IgG<sub>4</sub> in PBMCs or purified B cells also requires both stimulation with IL-4 and activation through CD40.<sup>8, 30, 31</sup> The two cytokines IL-4 and IL-13 are known to induce IgE messenger RNA transcription.<sup>7, 32</sup> A second signal is required for the deletional switch leading to the expression of  $\epsilon$  mRNA and class switch for IgE protein synthesis. CD40L expressed on the T-cell surface has a pivotal role in B-cell activation through CD40.<sup>33</sup> This interaction is required in T cell–B cell collaboration for antibody production to T-cell-dependent antigens,<sup>8, 30, 31</sup> for isotype switching,<sup>8, 34</sup> and for the generation of memory B cells and germinal center cells.<sup>35</sup> Moreover, activation of CD40 was shown to increase B-cell responsiveness to IL-4 and the promotion of IgE and IgG<sub>4</sub> formation.<sup>8, 36–39</sup> Furthermore, interaction of CD40L with CD40 was shown to prevent B cell apoptosis.<sup>40</sup> In our study, stimulation with sCD40L made it possible to generate PLA-specific antibodies in vitro and to enhance the total IgE and IgG<sub>4</sub> production. This is in accordance with previous reports on their co-regulation in vitro, and PBMCs from both atopic and normal donors have been shown to produce both total IgE and IgG<sub>4</sub> on IL-4 stimulation.<sup>3, 18, 41</sup> Furthermore, the addition of either anti-CD40 mAb or activated allergen-specific CD4<sup>+</sup> T-cell clones to purified B cells, in the presence of IL-4, was shown to induce both IgE and IgG<sub>4</sub> switching.<sup>8</sup>

The role of T cells in the induction of specific IgE and IgG<sub>4</sub> was further investigated with different T-cell activators. The finding that optimal stimulation by two anti-CD2 mAbs with different epitope specificity, in addition to IL-4 and sCD40L, enhanced both total and PLA-specific IgE antibodies suggests that accessory molecules other than CD40 may play a role in B-cell activation for IgE production.<sup>21, 42, 43</sup> Activation through CD2 and CD28 was shown to induce long-term autocrine proliferation of CD4<sup>+</sup> T cells,<sup>44</sup> and anti-CD2 mAbs were shown to induce T-cell proliferation and cytokine production.<sup>45, 46</sup> Interestingly, differential cytokine synthesis can be induced by mAbs recognizing different epitopes on the CD2 molecule.<sup>47</sup> In our study, stimulation of T cells by increasing doses of anti-CD2 mAb mixture inhibited total and specific IgE and simultaneously enhanced secretion of IgG<sub>4</sub>. The increase in the production of both anti-PLA and total IgG<sub>4</sub> correlated with IL-2 and IFN- $\gamma$  secretion. Because a constant amount of IL-4 was added at the beginning of culture, increasing IgG<sub>4</sub> antibody secretion most likely resulted from a shift in IL-4 to IFN- $\gamma$  ratio in cultures toward a T<sub>H0</sub> pattern. On the other hand, stimulation of PBMCs by anti-CD3, PHA, or PMA resulted in higher levels of IFN- $\gamma$  and IL-2 and inhibition of IgE and IgG<sub>4</sub> production. Several investigators have demonstrated that IgE can be induced in purified B cells if they have been co-cultured with anti-CD3-activated T-cell clones.<sup>6, 48, 49</sup> However, anti-CD3 stimulation of PBMCs was shown to

inhibit IgE production as a result of enhanced IFN- $\gamma$  production.<sup>6, 50</sup> In our in vitro system, anti-PLA IgE and anti-PLA IgG<sub>4</sub> synthesis appeared to be more sensitive to changes in T-cell stimulation than total IgE and IgG<sub>4</sub>. Differential cytokine formation by T cells and changes in surface molecule expression by increasing anti-CD2 stimulation may be encountered for the more pronounced shift from PLA-specific IgE to IgG<sub>4</sub>. It appears that a qualitatively distinct status of T-cell activation is required for optimal allergen-specific antibody production. Moreover, optimal conditions for total IgE synthesis were shown to be suboptimal for IgG<sub>4</sub> antibody formation, further demonstrating counterregulatory mechanisms on the specific IgE and IgG<sub>4</sub> synthesis.

It has been demonstrated that the in vitro expressed cytokine pattern of PLA-specific T-cell clones directly depends on the concentration of allergen in the culture.<sup>3, 51, 52</sup> and that ratios of IL-4 to IFN- $\gamma$  decline with increasing allergen doses.<sup>51, 52</sup> Similarly, we have observed a distinct effect by the allergen dose on the allergen-specific IgE response. Doses of 0.01 to 1 ng/ml, which are too low for significant T-cell proliferative stimulation in vitro, substantially enhanced PLA-specific IgE and IgG<sub>4</sub> production. On the other hand, the formation of PLA-specific antibodies was inhibited at high allergen concentrations at which specific T-cell proliferation was induced. Because neutralization of specific IgE by antigen excess could be excluded, it can be concluded that low amounts of allergen may be processed and presented preferentially by specific B cells and that CD40L further activates their antigen-presenting cell function and polyclonal activation.<sup>53, 54</sup> High concentrations of allergen, at which the induction of specific IgE is suppressed, result in polyclonal activation of B cells. Such effects have been demonstrated in murine systems in which high levels of polyclonal B-cell activation led to suppression of specific antibody formation.<sup>55, 56</sup> Similar to our findings, the optimal concentrations for induction of specific antibody synthesis were several orders of magnitude lower than the concentrations required for polyclonal activation.<sup>55, 56</sup> The same mechanisms may play a dominant role at high concentrations of PLA, which induce T-cell proliferation and bystander activation of B cells, which finally lead to enhanced production of total IgE and IgG<sub>4</sub> isotypes.

Antigen-driven in vitro systems have more physiologic significance for the study of multifactorial mechanisms of regulation of immune responses in human beings than nonspecific, polyclonal activation. Here we describe a culture system that enables the induction of allergen-specific IgE antibodies from human PBMCs and demonstrate that the allergen dose-dependent regulation of specific IgE and IgG<sub>4</sub> antibodies follows mechanisms different from those of total IgE and IgG<sub>4</sub> production.

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