The Role of Interleukin-4 in the Regulation of Sequential Isotype Switch from Immunoglobulin G1 to Immunoglobulin E Antibody Production

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The immunoglobulin (Ig)E immune response against protein antigens is profoundly influenced by the antigen dose used for immunization. Whereas immunization of CBA/J mice with low antigen doses results in the production of large amounts of IgE antibody, priming with high antigen doses leads to only marginal IgE antibody production in animals. However, in vitro restimulation of spleen cells from mice primed with high antigen doses leads to considerable activation of IgE-producing B cells, which suggests that B cells primed for IgE antibody production do exist among spleen cells. We investigated the modalities of activation of these memory B cells. The data presented here reveal that the anamnestic IgE immune response in vitro is strictly dependent on the presence of IgG1-expressing B cells, which differentiate after a sequential isotype switch into IgE-producing plasma cells with the help of primed CD4+ T cells. The induction of IgE-producing plasma cells requires a cognate interaction between B cells and CD4+ T cells. Interleukin (IL)-4 seems not to be involved in this process, since IgE production in vitro is resistant to suppression by anti-IL-4 monoclonal antibody. Finally, we show that IgG1-expressing B cells represent a relevant memory cell population in vivo also, but in contrast to the in vitro situation the final differentiation into IgE-producing plasma cells is dependent on IL-4.

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INTRODUCTION

Excessive production of antibody of the immunoglobulin (Ig)E class plays a central role in the pathogenesis of atopic disorders such as asthma or type I allergy [1]. It is known that type I allergic symptoms are caused by a small number of B cells, which secrete allergen-specific IgE antibodies that are bound to high-affinity Fcε receptors on mast cells and basophils [2]. Subsequent cross-linking with allergens triggers the release of histamine and other inflammatory mediators [3]. Therefore, considerable attention has been given to the regulation of IgE antibody synthesis.

According to a well-established model, class switching to IgE during primary immune responses requires two signals, one delivered by the cytokine interleukin (IL)-4 and the other by a contact-dependent interaction between B- and T cells [4, 5]. Whereas IL-4 regulates the critical molecular event of IgE switching, namely the induction of germ-line ε transcripts [6, 7], interactions between cell-surface molecules on B- and T cells provide costimulatory signals, that induce DNA switch recombination. CD40 on B lymphocytes and the ligand on T lymphocytes, CD154 (CD40L), seem to play a prominent role in the stimulation of IgE secretion [8, 9]. For anamnestic IgE antibody immune responses the situation is much more puzzling. In particular, the requirement for IL-4 in the generation of secondary IgE production is controversial [10–12]. Although there have been numerous in vitro studies on polyclonal murine IgE synthesis after stimulation with IL-4 and lipopolysaccharide (LPS) [13–15], relatively few investigations of parameters affecting antigen-specific secondary IgE antibody production in vitro have been published [16–22]. We developed a culture system that permits examination of the
modalities for activation of B cells primed for IgE synthesis (Be memory cells). Although mice primed with high doses of protein antigen-like phospholipase A2 or keyhole limpet haemocyanin (KLH) lack circulating IgE antibody as well as IgE-secreting plasma cells in situ, they harbour a population of Be memory cells that, in vitro, can be stimulated in the presence of antigen to produce large amounts of IgE antibody [23, 24].

The aim of this study was to investigate the parameters that influence the outcome of the anamnestic secondary IgE antibody response with special consideration to the dependence of IL-4.

MATERIALS AND METHODS

Mice and immunization protocol. CBA/J mice were purchased from Charles River Wiga (Sulzfeld, Germany). IgG1-deficient ΔS5'γ1 mice [25] were obtained originally from the Institute for Genetics, University of Cologne, Germany, and bred in the animal facilities at Münster. IL-4-deficient BALB/c-IL-4" mice [26] and BALB/c] control mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were 8–12 weeks old at the beginning of the experiments. Mice were immunized every 2 weeks by i.p. injections of low doses (0.1 μg/injection; K01 mice) or high doses (100 μg/injection; K100 mice) of KLH (Sigma, Munich, Germany) adsorbed to 2 mg Al(OH)3 (Serva, Heidelberg, Germany).

Magnetic cell sorting. B cells, total T cells, CD4+ and CD8+ T cells were purified from spleen cells by magnetic separation using the MiniMACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany). According to the manufacturer’s instructions spleen cells were suspended in 80 μL ice-cold separation buffer [phosphate-buffered saline (PBS) with 5 mM EDTA and 0.5% bovine serum albumin (BSA)] per 107 cells. A 20-μl aliquot of antibody-conjugated microbeads per 107 cells was added and incubated for 30 min at 4°C. The following microbeads were used: anti-CD43 microbeads (Miltenyi Biotec) for negative isolation of resting B cells, anti-CD90 microbeads (Miltenyi Biotec) for positive isolation of total T cells, anti-CD4 and anti-CD8 microbeads (Miltenyi Biotec) for positive isolation of the respective T-cell subset. The labelled cell suspension was pipetted on top of a separation column (Type MS: Miltenyi Biotec), which had been washed three times with separation buffer and placed in the MiniMACS separation unit. The suspension was allowed to pass through the column. In case of negative selection of CD43- B cells the effluent was collected as a B-cell fraction and washed three times with medium. In case of positive selection of CD90+, CD4+ or CD8+ T cells the effluent was discarded and the columns were washed twice with 500 μL separation buffer. Subsequently, columns were removed from the separator and magnetically labelled cells were flushed out with 1 mL separation buffer using a plunger. The respective T-cell fraction was washed three times with medium. The purity of the various cell fractions was assessed by flow cytometry analysis using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone 145-2C11), phycoerythrin (PE)-conjugated anti-CD19 (clone ID3), FITC-conjugated anti-CD4 (clone RM4-5) and PE-conjugated anti-CD8 (clone 53-6.7) (all purchased from Pharmingen, Hamburg, Germany) and analysed according to standard procedures using Lysis II software.

Cell culture conditions. Spleen cell cultures or T/B-cell cocultures were set up on 24-well tissue culture plates (Greiner, Nürtingen, Germany) with the indicated cell numbers in 1 mL Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS, Sigma) and KLH as antigens at a concentration of 1 μg/ml. Antigen was replaced by fresh medium on day 3 as described previously [23]. In some experiments cocultures of B cells and CD4+ T cells were incubated with anti-IL-4 monoclonal antibody (MoAb) 1B11 [27] or rIL-4 (Pharmingen) in various concentrations. In double chamber experiments physical contact between cocultured B cells and CD4+ T cells was prevented by cultivation of cells in different compartments of tissue culture inserts (Nunc, Wiesbaden, Germany) separated by a 0.4-μm polycarbonate membrane. In all experiments the frequency of KLH-specific IgE and IgG1 antibody forming cells (AFC) was determined by spot enzyme-linked immunosorbant assay (spot-ELISA) on day 6 of the culture period.

Efficiency of anti-IL-4 MoAb 11B11. The anti-IL-4 MoAb 11B11 was used to neutralize IL-4 activity in T/B-cell cocultures. Its efficiency was determined by inhibition of the IL-4-dependent proliferation of CTLL-2 cells [28]. For that reason CTLL-2 cells (1 × 105/well) were cultured on 96-well tissue culture plates (Greiner, Nürtingen, Germany) in a volume of 100 μL DMEM containing 10% FCS with rIL-4 (5 and 25 U/ml) and various concentrations of anti-IL-4 MoAb 11B11. After a culture period of 2 days CTLL-2 proliferation was measured by [3H]Tdr incorporation over 18 h.

ELISA for determination of KLH-specific antibody. The quantification of KLH-specific antibody of different isotypes (IgE, IgG1, IgG2a, IgM) in sera of primed mice was performed by ELISA as described previously [23].

Spot-ELISA for determination of KLH-specific AFC. The determination of the frequency of IgE or IgG1 AFC among spleen cells ex vivo or in T/B-cell cocultures in vitro was performed by spot-ELISA as described previously [23].

Adoptive transfer. B cells were purified from spleen cells of K100 or naive control mice by magnetic separation as described. B cells (1 × 106) were transferred via tail vein into naive recipients. On the following day animals were immunized with a single injection of 100 μg KLH. After 10 days mice were bled and the sera analysed for KLH-specific antibody. Twelve days after immunization mice were killed, spleens were removed and the frequency of KLH-specific AFC among spleen cells was determined ex vivo.

RESULTS

Antigen dose-dependent regulation of IgE antibody production

The in vivo IgE antibody response against protein antigens, such as KLH, is profoundly influenced by the antigen dose used for immunization. As shown previously [23, 24] repeated i.p. injections of low doses of KLH (0.1 μg; K01 mice) in CBA/J mice induce IgG1 antibody and high titres of IgE antibody, whereas priming with high doses of KLH (100 μg; K100 mice) leads to high IgG1 antibody production, but virtually no IgE antibody titres in the immune sera of CBA/J mice (Fig. 1A). This phenomenon is not limited to a certain haplotype of mice since immunization of animals from different inbred strains and with different genetic backgrounds and opposite T helper (Th1/Th2) prevalences (BALB/c, C57BL/6) [29] in principle reveals the same characteristics of antigen dose-dependent regulation of IgE antibody production (Fig. 2).

The absence of IgE-producing B cells in K100 CBA/J mice was confirmed by the determination ex vivo of AFC by spot-ELISA.

Only marginal numbers of IgE AFC could be detected in the spleen (Fig. 1B) or other lymphoid tissues, such as bone marrow, lymph nodes and peritoneum (data not shown), of K100 CBA/J mice, whereas substantial numbers of IgE AFC were demonstrated in K01 CBA/J mice. In contrast the cultivation and antigenic stimulation of spleen cells from K100 CBA/J mice leads to a considerable expansion of IgE-producing plasma cells in vitro, whereas this anamnestic IgE immune response is lacking for the most part in K01 CBA/J mice (Fig. 1C). However, the ratio of IgE AFC to IgG1 AFC among cultured spleen cells from both immunization groups is $<1:100$.

In vitro expansion of IgE-producing B cells requires help of primed CD4+$^+$ T cells

The appearance of IgE AFC at high frequencies among cultured spleen cells isolated from K100 mice, which show virtually no IgE immune response in vivo, demands an explanation. In order to trace the requirements for activation of B$e$ memory cells spleen cells of immunized CBA/J mice were separated by magnetic activated cell sorting (MACS). CD90$^+$ whole T cells, CD4$^+$ and CD8$^+$ T cells were enriched by positive separation, which results in a population of $>$95% pure T cells and $<$5% contaminating B cells (Fig. 3). B cells were collected as untouched cells by negative separation via the surface marker CD43, which is expressed on the majority of haemopoietic cells [including T cells, granulocytes, natural killer (NK) cells, monocytes, macrophages, pro-B cells, plasma cells and B-1 cells], but not on mature resting conventional B cells [30, 31]. This isolation

Fig. 1. Antigen dose-dependent regulation of IgE antibody production. Groups of three CBA/J mice were immunized repeatedly with 0.1 $\mu$g KLH (K01 mice) or 100 $\mu$g KLH (K100 mice). Antibody titres in the immune sera were determined around day 70 of the immunization period by ELISA (A). AFC frequencies were determined ex vivo 2 weeks after the last injection of KLH by spot-ELISA as described in Materials and Methods (B). At this time $4 \times 10^6$ spleen cells were cultured in 24-well tissue culture plates in 1 ml DMEM/FCS with 1 $\mu$g KLH/ml as the antigen. The number of AFC among cultured spleen cells in vitro was determined on day 6 by spot-ELISA (C). Antibody isotypes: IgE (solid bars), IgG1 (open bars). The figures represent the data of three independent experiments and their mean values.

Fig. 2. Reciprocal regulation of IgE antibody and IgG1 antibody production in vivo occurs in inbred strains with opposite Th1/Th2 prevalence. Groups of four mice of different inbred strains (CBA/J, BALB/c, C57BL/6) were immunized repeatedly with 0.1 $\mu$g KLH (K01 mice) or 100 $\mu$g KLH (K100 mice). IgE antibody titres (solid bars) and IgG1 antibody titres (open bars) in the immune sera were determined on day 65 of the immunization period by ELISA. Data are presented as mean $\pm$ SEM.
procedure usually yields a B-cell fraction with a purity of >98% B cells and <1% remaining T cells (Fig. 3).

The expansion of IgE AFC after restimulation of spleen cell bulk cultures can also be observed in cocultures of B- and T cells from mice primed with KLH (Fig. 4A). The activation of Bε memory cells only occurs in the presence of primed T Cells, unprimed T cells isolated from naïve mice are not able to induce proliferation of IgE AFC. Increasing numbers of primed T cells in the cocultures lead to enlarged populations of IgE AFC (Fig. 4B). In accordance with the situation described for whole spleen cell cultures the anamnestic IgE immune response in both experiments is much more augmented in K100 than in K01 cocultures. Furthermore secondary IgE antibody production is strictly dependent on the presence of the nominal antigen KLH during the initial culture period (Fig. 4A). The cultivation of primed T cells with naïve B cells reveals only marginal amounts of antigen specific IgE AFC, probably due to the presence of residual Bε memory cells in the T-cell fraction.

To trace which T-cell subset is responsible for the induction of IgE antibody production CD4⁺ and CD8⁺ T cells were isolated from K100 mice and cocultured with primed B cells. Figure 5 shows that CD4⁺ T cells, but not CD8⁺ T cells, are

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**Fig. 3.** Separation of B- and T cells from spleen cells using magnetic cell sorting. Flow cytometric analysis of spleen cells (left), B cells (middle) and T cells (right) after isolation with MACS as described in Materials and Methods. Cells were stained with FITC-conjugated anti-CD3 and PE-conjugated anti-CD19. The plots are representative of all experiments.

**Fig. 4.** In vitro expansion of IgE AFC requires help from primed T cells. B- and T cells were isolated from spleen cells from naïve, K01 and K100 CBA/J mice as described in Materials and Methods. B- and T cells were cocultured with the indicated cell numbers in 24-well tissue culture plates in 1 ml DMEM/FCS with 1 µg KLH/ml as the antigen. The number of IgE AFC was determined on day 6 by spot-ELISA. Results are presented as the mean of triplicates ± SEM. The data are representative of three independent experiments.

**Fig. 5.** CD4⁺ T cells induce in vitro expansion of IgE AFC. B cells, total T cells, CD4⁺ and CD8⁺ T cells were isolated from spleen cells from K100 mice as described in Materials and Methods. B cells (3×10⁶) and 1×10⁶ cells from the respective T-cell subset were cocultured in 24-well tissue culture plates in 1 ml DMEM/FCS with 1 µg KLH/ml as the antigen. The number of IgE AFC was determined on day 6 by spot-ELISA. Results are presented as the mean of triplicates ± SEM. The data are representative of three independent experiments.
able to cause the expansion of IgE AFC. CD4⁺ T cells induce 2.7-fold more IgE AFC than do equal numbers of whole T cells. Because 46% of the total T cells were CD4⁺ positive as determined by FACS analysis (data not shown) the proliferation of IgE AFC elicited by this fraction can be attributed to the effect of CD4⁺ T cells.

_B cells from K01 and K100 mice differ in their capacity to develop into IgE AFC_

The fact that the anamnestic IgE immune response is much more distinctive in cocultures of K100 mice can be explained by two possible reasons: either CD4⁺ T cells from K01 and K100 mice differ in their ability to induce activation of Bε memory cells or B cells from K100 mice show a markedly increased potential for differentiation into IgE AFC. To address this question, B cells and CD4⁺ T cells from K01 and K100 mice were cocultured vice versa (Fig. 6). It turns out that the magnitude of the expansion of IgE antibody-secreting plasma cells is dependent exclusively on the status of the B-cell population with K100 B cells producing 10 times the number of IgE AFC than B cells from K01 mice. In comparison, CD4⁺ T cells from K01 mice were just as effective in eliciting an _in vitro_ IgE antibody response as CD4⁺ T cells from K100 mice. Consequently, B cells from K01 mice have only a very limited capacity to differentiate _in vitro_ into IgE-producing plasma cells, whereas Bε memory cells from K100 mice can be activated under the given culture conditions.

_IgG1-expressing B cells are a prerequisite for the induction of IgE AFC in vitro_

For spleen cell cultures it was shown that IgE AFC were generated via sequential isotype switch with IgG1-expressing B cells as an intermediate Bε memory cell pool [24]. To extend these data, B cells and CD4⁺ T cells from KLH-primed Δ5'Sy1 mice and BALB/c control mice were

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**Fig. 6.** B cells from K01 and K100 mice differ in their capacity to develop into IgE AFC. B cells and CD4⁺ T cells were isolated from spleen cells from naïve, K01 and K100 CBA/J mice as described in Materials and Methods. B cells (3 x 10⁶) and 5 x 10⁵ CD4⁺ T cells were cocultured in 24-well tissue culture plates in 1 ml DMEM/FCS with 1 μg KLH/ml as the antigen. The number of IgE AFC was determined on day 6 by spot-ELISA. Results are presented as the mean of triplicates ± SEM. The data are representative of two independent experiments.

**Fig. 7.** IgG1-expressing B cells are a prerequisite for the induction of IgE AFC in vitro. B cells and CD4⁺ T cells were isolated from spleen cells from K100 BALB/c control mice and K100 Δ5'Sy1 mice as described in Materials and Methods. B cells (3 x 10⁶) and 5 x 10⁵ CD4⁺ T cells were cocultured in 24-well tissue culture plates in 1 ml DMEM/FCS with 1 μg KLH/ml as the antigen. The number of IgE AFC was determined on day 6 by spot-ELISA. Results are presented as the mean of triplicates ± SEM. The data are representative of three independent experiments.

isolated and cocultured vice versa (Fig. 7). In confirmation of the previous experiments a large population of IgG1 AFC, as well as IgE, AFC appears after antigenic restimulation of lymphocytes from BALB/c mice, whereas in cultures from Δ5’Sγ1 mice an increased frequency of IgG2a AFC, but almost no IgG1 AFC and only marginal numbers of IgE AFC, can be demonstrated. Indeed the status of the B-cell population is decisive for the outcome of the secondary IgE antibody immune response in vitro. CD4+ T cells from BALB/c mice also fail to induce IgG1 or IgE AFC among B cells from Δ5’Sγ1 mice, whereas IgG2a AFC are generated in a comparable quantity. In contrast, CD4+ T cells from Δ5’Sγ1 mice are almost as effective in activating Bε memory cells among B cells from BALB/c mice as are the respective CD4+ T cells from BALB/c mice. The above experiments require the presence of KLH, cocultivation in the absence of antigen prevents the appearance of AFC (data not shown). Consequently, the data presented show that the existence and activation of IgG1-expressing B cells are crucial prerequisites for the expansion of IgE AFC in vitro.

In vitro expansion of IgE AFC requires cognate interaction with CD4+ T cells

It could be shown that KLH-primed CD4+ T cells produce large amounts of IL-4 after stimulation in vitro [32], a cytokine which is known to be a strong inducer of antibody isotype switch to IgE. To elucidate whether the activation of Be memory cells and the strong increase in the number of IgE AFC in this in vitro system is dependent on the activity of IL-4, cocultures of purified CD4+ T cells and B cells from K100 mice were stimulated with KLH and incubated with anti-IL-4 MoAb or exogenously added rIL-4. Figure 8A shows that the concentrations of anti-IL-4 MoAb used in this experiment are able to inhibit in a CTLL-2 proliferation assay any biological activity of IL-4 in quantities higher than those typically produced by KLH-primed spleen cell cultures (data not shown). Thus, the level of anti-IL-4 MoAb is thought to be sufficient to neutralize the endogenous IL-4 produced in vitro. It turns out that the neutralization of blocking anti-IL-4 MoAb throughout the culture period does not lead to a significant decrease in the number of IgE AFC (Fig. 8B). Similarly, the IgG1 anti-KLH response is not affected by neutralizing anti-IL-4 MoAb (data not shown). The addition of rIL-4 has no enhancing effect on the anamnestic immune response (Fig. 8B).

The requirement for physical contact between B cells and CD4+ T cells in the generation of IgE AFC was investigated by using Transwell cultures. B cells and CD4+ T cells were either cocultured in the same chamber or separated by a 0.4 μm membrane in double chambers to allow diffusion of soluble cytokines but prevent direct cell contact. The number of IgE AFC in both compartments was determined after a culture period of 6 days. Considerable induction of IgE-producing plasma cells can only be observed when B cells and CD4+ T cells are placed in the same chamber so that they are able to come into close contact (Table 1). However, physical separation prevents the activation of Be memory cells and consequently the appearance of IgE AFC. Again, the expansion of IgE AFC is strictly dependent on the presence of antigen in the culture.

In vivo relevance of IL-4-independent sequential isotype switch to IgE

It is questionable whether IgG1-expressing B cells can represent in vivo a relevant Be memory cell population, that can be induced.
Table 1. Physical contact between B cells and CD4\(^+\) T cells is required for expansion of IgE AFC

<table>
<thead>
<tr>
<th>Culture conditions(^a)</th>
<th>KLH-specific IgE AFC/culture(^b)</th>
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<tbody>
<tr>
<td></td>
<td>Upper chamber</td>
</tr>
<tr>
<td>-</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>B(_{naive}) + CD4(^+) T</td>
</tr>
<tr>
<td>medium</td>
<td>B(_{primed}) + CD4(^+) T</td>
</tr>
<tr>
<td>B(_{primed}) + CD4(^+) T</td>
<td>4320 ± 640</td>
</tr>
<tr>
<td>B(_{primed}) + CD4(^+) T</td>
<td>4160 ± 640</td>
</tr>
</tbody>
</table>

\(^a\)B cells and CD4\(^+\) T cells were isolated from spleen cells from naïve and K100 CBA/J mice as described in Materials and Methods. CD4\(^+\) T cells (5 \(\times\) 10\(^5\)) were cocultured with 3 \(\times\) 10\(^5\) B cells either in the same compartment of double chamber wells to ensure physical contact or in different compartments separated by a 0.4-μm microporous membrane. Cultures were incubated with or without 1 μg KLH/ml. The number of IgE AFC in the upper as well as in the lower chamber was determined on day 6 by spot-ELISA. Data are representative of three independent experiments. Results are presented as mean of triplicates ± SEM. In vivo

\(^b\)KLH-specific IgE AFC/culture was monitored in their sera and the numbers of AFC were determined \(ex vivo\). Therefore, B cells from K100 BALB/c mice were highly purified (CD4\(^+\) T cells: < 0.5%) and adoptively transferred into naïve recipients. K100 BALB/c donor mice \(in vivo\) exhibited the typical characteristics of antibody formation after repeated immunization with high antigen doses with very low IgE antibody titres and enhanced IgG1 antibody production (data not shown).

B cells from K100 BALB/c mice or naïve BALB/c control mice were transferred either into wild-type BALB/c mice or into IL-4-deficient recipients with BALB/c background. Subsequently animals of both groups received a single injection of 100 μg KLH in order to challenge the transferred B cells. Ten days after immunization, production of KLH-specific antibodies was monitored in their sera and the numbers of AFC were determined \(ex vivo\). The transfer of unprimed B cells into IL-4 producing wild-type BALB/c mice has no effect on the elicitation of the antibody immune response: IgG1 and IgG2b antibody titres are low and comparable with those from animals that had not received any lymphocytes (Table 2). In contrast, the application of KLH-primed B cells into wild-type mice leads to a remarkable increase in antibody titres. At the same time, the numbers of IgG1 AFC and IgE AFC are greatly increased in comparison with control mice.

IL-4-deficient BALB/c mice produce IgG1 antibodies, even though reduced to approximately half the amount of IL-4-expressing mice, but IgE antibodies are almost absent in the sera of these animals (Table 2). Whereas the transfer of naïve B cells into IL-4-deficient mice has no significant consequences on the outcome of the antibody immune response, the transfer of primed B cells again augments the IgG1 level in the sera of these mice and leads to a strong increase in the number of IgG1 AFC among spleen cells \(ex vivo\). However, in contrast to IL-4-expressing animals, neither IgE antibody titres nor IgG2b AFC can be detected in IL-4-deficient mice. Therefore, primed B cells with high IgG1 antibody expression but marginal IgE antibody production possibly represent a B cell memory cell pool that can be forced to substantial IgE antibody formation \(in vivo\). In contrast to the seemingly IL-4-independent induction of IgE antibody production \(in vitro\), the sequential isotype switch from IgG1 to IgE, as monitored in the transfer experiments, seems to be dependent on the activity of IL-4.

**DISCUSSION**

Antigen dose-dependent induction (low doses of KLH) or suppression (high doses of KLH) of IgE antibody production in CBA/J mice (Fig. 1) is not due to opposite polarization towards Th1 and Th2 cell subsets in the two immunization groups because CD4\(^+\) T cells from K01 mice, as well as K100 mice, release predominantly the Th2 cytokines IL-4 and IL-10 after \(in vitro\) restimulation [32]. These data are supported by the observation that inbred strains with opposite Th1/Th2 prevalence (BALB/c, C57BL/6) [29] reveal the same characteristics of antigen dose-dependent regulation of IgE antibody production (Fig. 2).

In contrast to the \(in vivo\) situation, the anamnestic IgE antibody immune response after restimulation of spleen cells \(in vitro\) is much more pronounced in cultures from K100 mice. Parallel to an increase in the number of IgG1 AFC, a large population of IgE AFC can be detected among K100 spleen cells after a culture period of 6 days. However, in relation to the number of IgG1 AFC, the frequency of B cells proceeding to IgE formation (1–2%) is comparable in both immunization groups (Fig. 1C). This frequency is in the range of, or even higher than, those published previously in other studies dealing with secondary antigen-specific IgE responses \(in vitro\) [16, 17, 22, 33].

To further investigate the generation of IgE AFC \(in vitro\), B cells and T-cell subsets were highly purified from K01 and K100 mice and cocultures were set up. The data show that the expansion of IgE AFC was strictly dependent on the help of antigen primed CD4\(^+\) T cells (Figs 4, 5), which have to be activated in the cocultures by incubation with the nominal antigen.
In vivo relevance of IL-4-independent sequential isotype switch to IgE

Table 2. In vitro relevance of IL-4-independent sequential isotype switch to IgE

<table>
<thead>
<tr>
<th>KLV-specific AFC ex vivo x 10⁶ spleen cells</th>
<th>IgE</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c naïve</td>
<td>&lt;1</td>
<td>0.1</td>
<td>1.2</td>
<td>3</td>
</tr>
<tr>
<td>BALB/c IL-4+</td>
<td>1</td>
<td>2.2</td>
<td>6.4</td>
<td>0.2</td>
</tr>
<tr>
<td>BALB/c IL-4−</td>
<td>1</td>
<td>2.2</td>
<td>6.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* B cells were isolated from spleen cells of naïve or K01 BALB/c mice by magnetic separation as described in Materials and Methods. B cells (1 × 10⁷) were transferred via tail vein into naïve or K01 BALB/c mice as recipients. Each group consisted of four mice. On the following day animals were immunized with a single injection of 100 μg KLH. After 10 days mice were bled and the sera were analysed for KLH-specific antibodies. Twelve days after the immunization mice were killed, spleens removed and the frequency of KLH-specific AFC among spleen cells was determined as described in Material and Methods.²Concentrations of serum antibodies are given in μg/ml for IgE and in arbitrary units for IgM.

As shown for whole spleen cell cultures, cocultures from K100 mice produce many times the number of IgE AFC than do K01 cocultures. Because CD4⁺ T cells from K01 and K100 mice, as well as CD4⁺ T cells from IgG1-deficient ΔS1ΔS1 and wild-type mice, have comparable capacities to induce IgE AFC (Figs 6, 7), the decisive prerequisite for the expansion of IgE AFC in vitro is the state of the B cell. Previous experiments have revealed that after depletion or inhibition of IgG1 positive B cells in vitro IgE production is almost completely lacking [24]. The interpretation that not IgE positive memory B cells but primarily the presence of IgG1-expressing precursor B cells is decisive for the outcome of the secondary IgE antibody immune response is underlined by the fact that the differentiation into IgE AFC is inhibited for the most part in B cells from ΔS1ΔS1 mice (Fig. 7). As a result of activation by primed CD4⁺ T cells IgG1-positive B cells expand, and up to a certain ratio develop after a sequential isotype switch into IgE AFC [15, 21, 34–36]. The process of sequential isotype switch would explain the simultaneous expression of intracellular or surface IgG1 and IgE on B cells observed by others [15, 37, 38]. Consequently, the limited capacity of B cells from K01 mice to generate IgE AFC after cultivation is because of the low number of IgG1-expressing intermediates, whereas the augmented expression of IgG1 antibody in mice primed with high doses of KLH is the prerequisite for the increased appearance of IgE AFC in K100 cultures.

The induction of IgE AFC depends on a cognate interaction between B cells and CD4⁺ T cells. The cultivation of the two populations in double chambers prevents the expansion of IgE AFC (Table 1). Only when B cells and CD4⁺ T cells are placed in the same compartment, so that they are able to come into physical contact, and when antigen is present in the cultures can a substantial release of IgE antibodies be determined. For that reason we can rule out the possibility that the stimulation of B cell memory cells is mediated solely by the action of soluble lymphokines released by CD4⁺ T cells, but rather depends necessarily on a cognate interaction between B- and T cells. Prominent candidates for the study of cell-surface molecules that mediate the activation of B cells and induction of IgE synthesis are CD154/CD40, CD28/B-7 and CD21/CD23, which have all been shown to be involved in the regulation of IgE antibody production [8, 9, 38–40]. At least one report [41] suggested that an isotype switch to IgE may be possible in an IL-4-independent way via the CD40 signalling pathway.

The outstanding role of IL-4 in the induction of primary IgE antibody responses in polyclonally activated B cells has been documented extensively in different murine and human model systems. However, several reports have been published which show that certain secondary antigen-specific IgE responses in vitro are either completely or at least partially resistant to suppression by the addition of blocking anti-IL-4 MoAb, and therefore seem to be independent of the activity of IL-4 [16, 18–20, 23, 42]. Restimulation of B cells and CD4⁺ T cells from KLH-primed animals in the presence of saturating amounts of neutralizing anti-IL-4 MoAb did not significantly decrease the expansion of IgE AFC (Fig. 8). This observation may be
interpreted as follows, namely that IL-4 does not play an essential role in the induction of memory IgE antibody responses in vitro. The requirement for IL-4 to differentiate into IgE-producing plasma cells seems to be less stringent for IgG1-expressing B cells. However, we cannot completely exclude the possibility that in the situation of a tight T–B-cell cognate interaction IL-4 is transferred from CD4+ T cells to B cells at the site of their contact in such a manner that IL-4 may not be accessible to inhibition by anti-IL-4 MoAb.

In previous experiments, IL-4-independent sequential isotype switching to IgE could only be demonstrated in vitro. To investigate whether IgG1-positive B lymphocytes possess relevance as B memory cells in vivo, highly purified B cells from K100 mice were transferred into naïve IL-4-competent mice and subsequently challenged by a single injection of KLH. Levels of IgG1 and IgE antibody in the sera of the recipients as well as the numbers of IgG1 and IgE AFC are increased considerably in comparison with control animals (Table 2). Apparently, IgE-producing plasma cells have been derived from the population of IgG1-expressing B cells, since donor mice show almost no IgE immune response, but outstanding IgG1 production. The generation of IgE AFC via a direct isotype switch from IgM-positive B cells [43, 44] does not seem to be likely because IgM antibody titres are not raised in mice that had received K100 B cells (Table 2). Likewise, the transfer of K100 B cells into IL-4-knock-out mice and the subsequent immunization with KLH causes a strong increase in IgG1 formation. However, in contrast to IL-4-positive wild-type animals, IL-4-deficient mice exhibit only marginal IgG1 antibody titres and no IgE AFC can be detected ex vivo. The data suggest that in vivo IgG1-expressing B cells may actually represent a memory cell population for IgE production as well. In contrast to the in vitro situation, the outcome of the anamnestic IgE immune response in situ seems to be dependent on the capability of lymphocytes to produce IL-4.

The discrepancy concerning the IL-4 dependence of sequential isotype switch to IgE may be explained in that, under culture conditions, IgG1-positive B cells and activated CD4+ T cells are located close to each other. As a result of the tight interaction between B- and T cells in vitro, signals mediated by the ligation of cell-surface molecules on the B cell may be sufficient to activate B memory cells and induce the isotype switch to IgE. However, transferred B cells in vivo proliferate and differentiate into IgG1 AFC after stimulation with antigen but because of less intense physical interaction between B cells and CD4+ T cells in situ the threshold for the induction of the isotype switch and subsequent IgE antibody synthesis can only be overcome by a strong additional signal, delivered by IL-4. Alternatively, the inability of IL-4-deficient mice to stimulate B memory cells might be explained by a failure of CD4+ T cells to provide sufficient helper functions on the basis of altered expression of activating cell-surface molecules.

Despite the fact that in the described transfer experiments the secondary IgE immune response appears to be IL-4 dependent, further attempts have been made to reveal conditions under which, in the mouse, IgG1-expressing B cells can be forced into IgE production without participation of IL-4. In the past, a few in vivo studies on IL-4-independent IgE synthesis in different murine models have been published [12, 42, 45–47], but none have considered IgG1-positive B cells as B memory cells. Recently, some reports have confirmed a role for mouse IL-13 in the development of IL-4-independent IgE responses [47, 48]. However, the possibility that IL-13 is the IgE-inducing factor in T–B-cell cocultures is not very likely because soluble mediators, such as IL-13, could be excluded as IgE inducers in the course of the double chamber experiments.

For several reasons it is tempting to speculate that IL-4-independent sequential isotype switch to IgE also has significance in humans, particularly in connection with atopic diseases. A sequential isotype switch from IgG-expressing intermediates to IgE has been demonstrated for human B lymphocytes in vitro as well as in vivo [49–52]. It has been shown that in patients with atopic dermatitis, or after infection with Schistosoma mansoni, the frequency of B cells that had undergone a sequential isotype switch was considerable [52]. Peripheral blood mononuclear cells (PBMC) from atopic donors in vitro produce spontaneous IgE without addition of IL-4, PBMC from nonatopic subjects failed to synthesize detectable levels of IgE [53, 54]. Cultivation with a neutralizing MoAb to human IL-4 did not inhibit spontaneous IgE synthesis, whereas the IgE response induced by exogenously added IL-4 was suppressed completely [55]. Similarly, spontaneous IgE release in cultures of PBMC from patients with hyper-IgE syndrome occurs without the contribution of endogenous IL-4 [56]. In marked contrast to the total IgE synthesis, allergen-specific IgE was secreted spontaneously in cultures of PBMC from patients with allergy and was not increased by addition of IL-4 or reduced by treatment with a neutralizing anti-IL-4 MoAb [57]. From these data, and the results obtained from the murine model, it is tempting to speculate that in situations of excessive formation of IgE antibody, such as atopic diseases and allergy, IgG-expressing B lymphocytes represent a pool of memory cells for IgE antibody synthesis. This B memory cell population is no longer responsive to IL-4-mediated signals and differentiates terminally to IgE AFC after sequential isotype switch. Thus, the question of whether the generation of IgE antibody-producing plasma cells via sequential isotype switch is dependent on IL-4 plays an important role in the effectiveness of therapeutic approaches based on attempts to inhibit or antagonize the activity of IL-4.

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