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Antigen dose-dependent predominance of either direct or sequential switch in IgE antibody responses

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SUMMARY

Priming of CBA/J mice with minute doses of protein antigens (Ag) leads to high IgE antibody (Ab) titres in the immune sera of these animals. In contrast priming with large doses elicits only a marginal production of IgE Ab. *In vitro* restimulation of spleen cells from animals primed with large doses and lacking *in vivo* IgE Ab leads to a burst of IgE Ab-forming cells. This *in vitro* anamnestic response is lacking in mice primed with minute doses of Ag. In order to trace the cellular basis of the *in vitro* IgE memory response we have extended the analysis of the distribution of Ab isotypes to Ag-primed IgG1-deficient $\Delta 5'S\gamma 1$ mice. The data presented here must be interpreted as followed. Priming of mice with minute doses of Ag leads to a direct switch from IgM to IgE Ab expression in both strains. These animals have high IgE Ab titres without establishing an IgE memory. The direct switch was verified by polymerase chain reaction and Southern blot analysis of switch circle DNA isolated from Ag-specific B cells of CBA/J mice primed with minute doses of Ag. In contrast to immunization with minute doses, priming with large doses of Ag fails to induce *in vivo* IgE Ab production in CBA/J and $\Delta 5'S\gamma 1$ mice but establishes a Bε memory in CBA/J mice which involves IgG1-bearing intermediate B cells. *In vivo* these Bε memory cells do not enter the status of IgE Ab-producing cells. *In vitro* they can be released from this anergy and presumed suppression and develop in an anamnestic response into a large population of IgE Ab-forming B cells. This increase in the number of IgE Ab-producing cells after restimulation *in vitro* is lacking in $\Delta 5'S\gamma 1$ mice, apparently because of their inability to generate IgG1-expressing precursor cells. The notion of a sequential switch and an IgG1 intermediate Bε memory status is also supported by depletion and inhibition experiments. Elimination of IgG1-expressing B cells in CBA/J mice primed with high doses of Ag prevents the IgE Ab burst after *in vitro* challenge with Ag. The data further suggest that the two switch pathways are not mutually exclusive and that the Ag dose can decide which pathway is preferentially used.

INTRODUCTION

Although antibodies (Ab) of the IgE class represent only a minor immunoglobulin class in the blood circulation they are of great importance in immunity against parasites and in disease association, like allergic reactions of the immediate type.[1] Analysis of parameters of the IgE Ab response gave insight into the regulation of immunoglobulin class switching and underlined the importance of lymphokines, like interleukin-4 (IL-4), as external switch signals.[2–4] Since the constant region gene for the ε chain lies downstream of the genes for the μ chain with γ chain genes as intermediates, two differentiation pathways are possible for expression of the IgE isotype. The direct switch envisages a direct transition within one cell from IgM to IgE expression without intermediate stages.[5–7] The sequential switch model proposes that IgE-producing cells originate from intermediate precursors between IgM and IgE expression.[8–12] B cells programmed for an IgG1 response are favourite candidates.

Priming of CBA/J mice with different doses of antigen (Ag) has a profound effect on the ratio of IgE versus IgG Ab appearing upon immunization.[13] Repeated injections of minute doses induce IgG and high titres of IgE Ab. Large doses of Ag induce a high IgG but a very low IgE Ab titre.[14,15] This low IgE Ab titre reflects a genuine lack of IgE Ab formation and is not just due to masking the IgE response by IgM or IgG Ab competing for common epitopes on the antigen.[16] We have recently found that although mice primed with large doses of Ag lack IgE Ab production and IgE Ab-forming cells *in vivo* they harbour a population of B cells which are programmed for an IgE Ab response (subsequently called Bε memory cells).[17] These cells *in vitro* can be released from unresponsiveness in the presence of Ag and produce large amounts of IgE Ab. Such Bε memory cells do not exist in mice primed with minute doses of Ag and producing high titres of IgE Ab.

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In this paper we investigate the possible origin of the Bε memory cells in large dose-primed mice and the reasons for their absence in animals primed with minute doses. This would lead to an understanding of the physiological compartment of an IgE memory.

MATERIALS AND METHODS

Mice and immunization protocol

CBA/J male mice were purchased from Charles River Wiga (Sulzfeld, Germany). The mutant mouse strain Δ5′Sγ1 was from a breeding nucleus of the Institute for Genetics, University of Cologne, Germany, and was expanded in the animal facilities in Münster. Animals of this strain exhibit a selective IgG1 agammaglobulinaemia due to deletion of a switch region control element.[18] Animals were 8 to 12 weeks old at the beginning of the experiments. For each experiment, groups of three mice were immunized in intervals of 2 weeks by intraperitoneal injection of minute doses (0·1 μg/injection) or large doses (100 μg/injection) of keyhole limpet haemocyanin (KLH) (Sigma, München, Germany) adsorbed to 2 mg $Al(OH)_3$ (Serva, Heidelberg, Germany). Sera were taken and analysed for KLH-specific Ab. For cell culture experiments spleen cells were collected and pooled 2 weeks after the last immunization. The data presented in Fig. 1 and 2 are from three independent experiments. Means ±SEM have been determined.

Cell culture conditions

Spleen cells (4×10^6/well) from immunized mice were cultured in 24-well tissue culture plates (Greiner, Nürtingen, Germany) in a volume of 1 ml Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS) (Boehringer Mannheim, Mannheim, Germany) and KLH as Ag at a concentration of 1 μg/ml. Ag was replaced from the cultures on day 3 of the culture period by changing 600 μl of the culture supernatant against fresh medium without Ag. The plates were centrifuged (50 ***g***, 10 min) and the medium was changed once more. Subsequently this procedure was repeated five times. On various times of the culture period the level of KLH-specific IgE and IgG Ab in aliquots of the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA) and the frequency of KLH-specific IgE and IgG Ab-producing cells was estimated by spot-ELISA.

Isotype-specific inhibition of B-cell subpopulations by Ab directed against cell surface immunoglobulin

Spleen cell cultures from K100 mice were set up as described. To inhibit B cells expressing a particular surface immunoglobulin, isotype-specific Ab (affinity-purified goat anti-mouse IgG1 or anti-mouse IgG2a; Bibby Dunn, Asbach, Germany) or control Ab (goat IgG standard; Bibby Dunn) were added to the cultures after extensive dialysis against phosphate-buffered saline (PBS; pH 7·4) at a concentration of 50 μg/ml. On day 6 of the culture period the medium was changed as described. The frequencies of KLH-specific IgE, IgG1 and IgG2a Ab-producing cells were determined by spot-ELISA at day 8 of the culture period.

Depletion of surface immunoglobulin-positive cells

IgG1- and IgG2a-positive cells were removed from spleen cell suspension from K100 mice by magnetic separation with the MiniMACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany). For this reason spleen cells were suspended in 80 μl ice-cold separation buffer [PBS with 5 mM ethylene diamine tetraacetic acid (EDTA) and 0·5% bovine serum albumin (BSA)] per each 10^7 cells. Twenty microlitres of Ab–labelled microbeads (rat anti-mouse IgG1 microbeads or rat anti-mouse IgG2a/b microbeads; Miltenyi Biotec) per 10^7 cells were added and incubated for 30 min at 4°. The labelled cell suspension was then 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. In case of depletion, a G26-needle (Terumo, Leuven, Belgium) was attached for flow regulation. The suspension was allowed to pass through the column. The effluent was collected as negative fraction and washed three times with medium. Cells were cultured as described. The frequencies of IgE-, IgG1- and IgG2a-producing cells as well as the levels of KLH-specific Ab in the culture supernatants were determined on subsequent days and compared to cultures which had not been separated with the MiniMACS system.

ELISA for KLH-specific Ab

The level of KLH-specific IgE Ab in the sera of mice and in the supernatant of spleen cell cultures was determined by ELISA. Assays were set up in triplicates. All reagents except the blocking buffer were used in volumes of 100 μl/well. Incubation time for all reagents was 1 hr at room temperature. After every incubation step ELISA plates were washed three times with PBS (pH 7·4) containing 0·1% Tween-20 (PBS–Tween). MaxiSorp ELISA plates (Nunc, Wiesbaden, Germany) were incubated overnight with KLH (5 μg/ml) in coating buffer (0·1 M Na_2CO_3, 0·1 M $NaHCO_3$, pH 9·6). After coating, 200 μl of PBS containing 1% BSA (Sigma, München, Germany) were added to each well to block any residual protein-binding sites. In the next step sera or culture supernatants were serially twofold diluted in PBS-Tween. Then the plates were incubated with the rat anti-mouse IgE mAb 4B3-39[19] at a concentration of 1 μg/ml in PBS–Tween. The isotype specificity of this mAb has been documented extensively.[19] Biotinylated polyclonal mouse anti-rat IgG Ab (Dianova, Hamburg, Germany) and subsequently extravidin-peroxidase (Sigma) were added in a 1:1000 dilution in PBS–Tween. The ELISA was developed by addition of *o*-phenylenediamine (Sigma) (1 mg/ml) and 30% H_2O_2 (1 μl/ml) in citrate buffer (0·2 M NaH_2PO_4, 0·1 M sodium citrate, pH 5·0). The reaction was stopped with 1 M H_2SO_4. The absorption was measured in a microplate reader (Dynatech, Denkendorf, Germany) at 490 nm.

The procedure for the determination of IgG Ab was the same as for IgE Ab, except for the addition of biotinylated goat anti-mouse IgG Ab (Dianova) instead of rat anti-mouse IgE and biotinylated anti-rat IgG Ab. For the determination of different IgG subclasses the following Ab were used: biotinylated goat anti-mouse IgG1, RPN 1180 (Amersham, Braunschweig, Germany); biotinylated goat anti-mouse IgG2a, RPN 1181 (Amersham); biotinylated sheep anti-mouse IgG2b, AB275 (The Binding Site, Heidelberg, Germany); biotinylated sheep anti-mouse IgG3, AB276 (The Binding Site).

For the quantification of Ab the anti-phosphorylcholine mAb 12-3 (IgE),[20] 56-1 (IgG1)[21] and 55-1 (IgG2a)[21] were used as standards. Phosphorylcholine linked to BSA[22] was

used as Ag. Although differences in the avidity of polyclonal anti-KLH Ab and monoclonal anti-phosphorylcholine Ab might represent a constant error factor in the actual quantification of Ab, this does not affect the conclusions drawn from the experiments.

KLH-specific spot-ELISA

The determination of the frequency of IgE or IgG Ab-producing cells (spot-forming cells, SFC) among spleen cells *ex vivo* or after cultivation *in vitro* was performed using a spot-ELISA.[23] Assays were set up in triplicate. After every incubation step the plates were washed three times with PBS–Tween. Nylon filter (Biodyne B membrane) bottomed 96-well Silent Monitor plates (Pall, Portsmouth, UK) were incubated overnight at 4° with 20 μg KLH/ml in coating buffer. Then the filters were blocked with 200 μl of 10% (w/v) non-fat dry milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, 1 M HCl, pH 7·6). In the next step cell suspensions were added and serially twofold diluted. Cells were incubated for 4 h at 37° in a humidified atmosphere containing 10% CO_2. According to the ELISA protocol, plates were treated with rat anti-mouse IgE Ab (5 μg/ml) and biotinylated mouse anti-rat IgG Ab (1:2000) for the determination of IgE SFC or biotinylated anti-mouse Ab specific for the determination of SFC of the different IgG subclasses (see ELISA protocol), followed by an incubation step with extravidin peroxidase (1:1000). All reagents were used in volumes of 100 μl/well. Incubation time for all reagents was 1 hr at 37°. The substrate 3-amino-9-ethylcarbazole (Sigma) was prepared by dissolving 5 mg in 0·5 ml dimethylformamide and subsequent addition of 15 ml 0·1 M sodium acetate buffer (pH 5·0). To remove aggregates the solution was filtered through a 0·45 μm-membrane. Prior to use, 7 μl of 30% H_2O_2 were added. Fifty microlitres of substrate solution were added to each well. After 1–5 min at room temperature macroscopic red spots became visible. The substrate was removed by flicking the plates. The spots could be counted in a dissecting microscope using 4–10 × magnification. Appearance of nonspecific spots was determined by comparison with filters which were not coated with KLH; they amounted to less than 5% of total spots.

Enrichment of Ag-specific B cells

Ag-specific B cells were enriched from spleen cell suspensions by a modified panning technique. Six-well polysterene tissue culture plates (Greiner) were incubated overnight at 4° with 1·7 ml per well of coating buffer containing 20 μg KLH/ml. Each well was washed with 15 ml Earle's balanced salt solution (EBSS) and blocked for 1 hr at 4° with 1·7 ml EBSS containing 10% FCS. After washing the wells with EBSS twice, spleen cells (8×10^6 cells in 1·7 ml) were incubated on Ag-coated wells for 1 hr at room temperature. Subsequently the unattached cells in the supernatant were removed carefully by two washes with EBSS. The adhering cells were recovered by flushing the wells vigorously with 2 ml EBSS using a Pasteur pipette. This suspension was used as enriched Ag-specific B-cell fraction and contained 80–85% B cells as determined by fluorescence-activated cell sorter (FACS) analysis with a phycoerythrin-labelled rat anti-mouse B220 mAb (Pharmingen, Hamburg, Germany).

Isolation of circular and genomic DNA

Circular DNA was extracted from B cells as described by Griffin *et al.*[24] with slight modifications. Briefly, enriched B cells were washed twice with EBSS and lysed with 1 ml lysis buffer [1% sodium dodecyl sulphate (SDS), 50 mM NaCl, 2 mM EDTA, pH 12·4] per 10^7 cells. High MW DNA was sheared by vortexing followed by a 30-min incubation at 30°. It was separated from circular DNA and RNA by phenol–chloroform extraction. RNA was digested for 1 hr at room temperature with DNase-free RNase (Pharmacia Biotec., Uppsala, Sweden) and removed by a second phenol–chloroform extraction. The circular DNA was precipitated in 70% ethanol, washed and stored at 4° in TE-buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7·5) until usage as polymerase chain reaction (PCR) templates for amplification of switch circle fragments.

Genomic DNA from the IgM-producing hybridoma 7D7.3.4[25] was used as a positive control in PCR analysis and was isolated and purified from 10^7 cells with the Rapid® Genomic DNA Isolation Kit (Pharmacia Biotec.) according to the manufacturer's instructions. The content of DNA in the samples was estimated by DNA DipStick® test (Invitrogen, San Diego, CA).

PCR amplification

PCR was performed in 50 ml vol/reaction with 50 mM KCl, 20 mM Tris–HCl (pH 8·4), 1·5 mM $MgCl_2$, 2 mM dNTP, 0·4 μM of each primer and 2·5 U of *Taq* polymerase. Aliquots of circular DNA from K01 and K100 mice were used as DNA templates; genomic DNA was used as a positive control. For amplification of DNA 40 cycles (denaturation at 94° for 1 min, annealing at 74° for 2 min and extension at 72° for 30 seconds) were conducted, followed by a final extension step at 72° for 7 min. To amplify selectively the circular DNA derived from a direct isotype switch to ε and thus containing the complete Sγ1 switch region, primers specific for sequences of this region were synthesized (Biometra, Göttingen, Germany): primer 1, 5′-GGA GAG CTG GGA ATA GCT ATG TGG G-3′ (corresponding to position 122 to 146 of Sγ1[26]); primer 2, 5′-CCA GCC CCA TTT ATC CCA GCT ACC C-3′ (complementary to position 512 to 536 of Sγ1[26]) (Fig. 1). The resulting amplification product has a length of 415 base pairs (bp).

Southern blot analysis

After electrophoretical separation on a 1·5% agarose gel PCR samples were transferred to a nylon membrane (Boehringer Mannheim). For the detection of Sγ1 DNA sequences a specific oligonucleotide probe (Biometra) was used: 5′-GAC ACA GGG GAG CTG AGG AAC CTA GTA CTA-3′ (corresponding to position 211 to 240 of Sγ1[26]). The probe was labelled with digoxygenin at the 3′-end according to standard protocols.[27] Hybridization was performed for 6 hr at 55°. Sγ1–specific sequences were detected by chemiluminescence staining according to standard protocols.[28] Reagents for oligonucleotide labelling and detection were purchased from Boehringer Mannheim.

RESULTS

Priming of mice with 0·1 μg (minute doses) of KLH (K01) leads to high IgE Ab titres detectable in the immune sera of

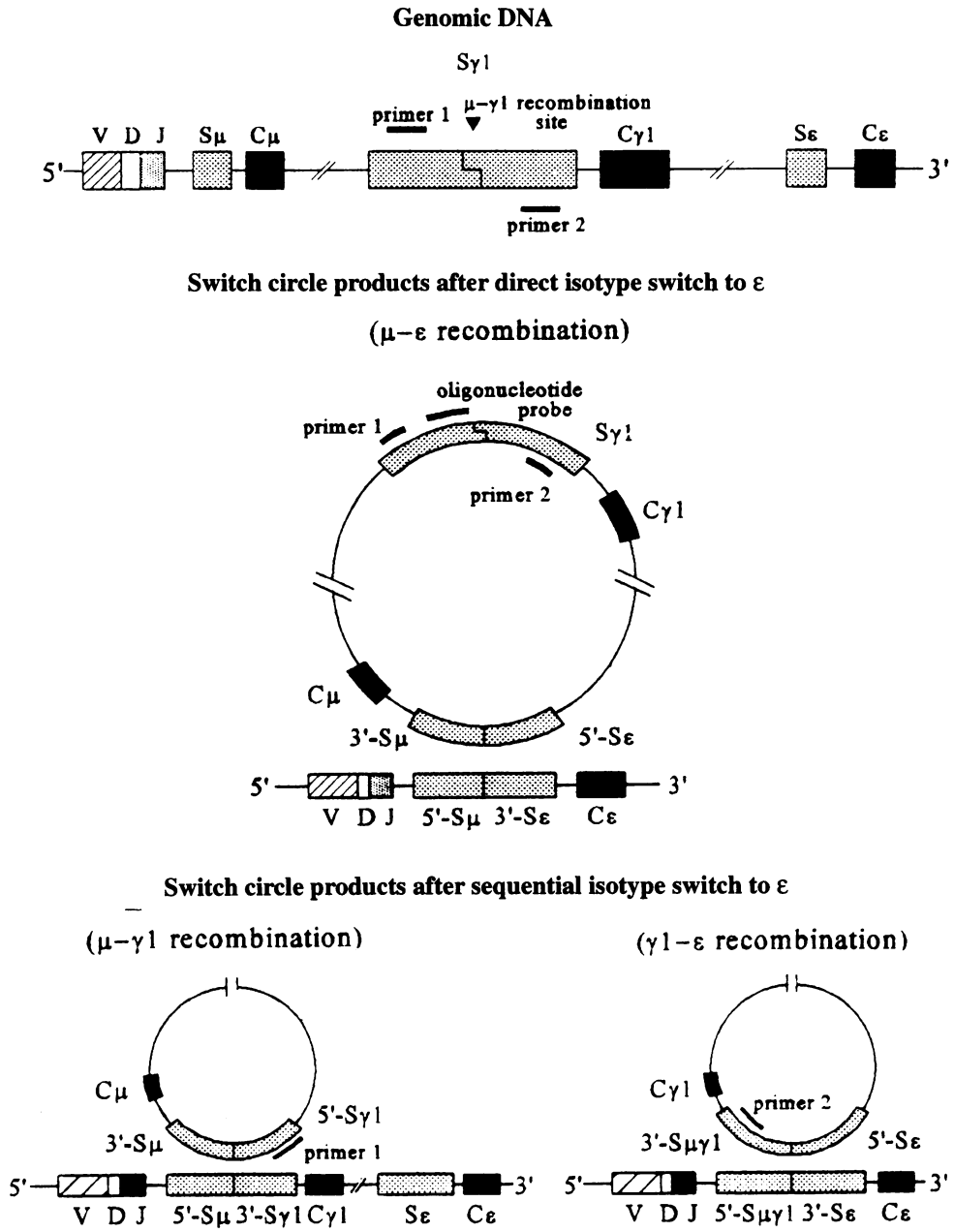

Figure 1. Schematic diagram of switch circle products after direct or sequential isotype switch to IgE. Shaded boxes represent switch regions (S regions), filled boxes represent C_H genes. Recombination sites are indicated by vertical lines.

these mice. In contrast, priming with 100 μg (large doses) of KLH (K100) elicits only a marginal IgE response. The absence of IgE Ab is a true lack of IgE Ab production. Technical problems intrinsic to IgE Ab determinations, like masking by competing IgG or IgM Ab, have been excluded.[17] The basic feature of this response has also been documented for several other protein antigens like bee venom phospholipase A_2 and ovalbumin (OVA).[14] After cultivation and restimulation with Ag *in vitro*, spleen cells from K01 mice develop only a marginal secondary IgE Ab response. In contrast spleen cells from K100 mice show a vigorous anamnestic IgE Ab response measurable both in an increase in the frequency of IgE Ab-forming cells in culture and in high IgE Ab titres in culture supernatants.

In order to trace the origin of this *in vitro* IgE memory response in K100 mice we compared primed CBA/J and Δ5′Sγ1 mice with regard to the *in vivo* and *in vitro* humoral immune response. Analysis of the isotype distribution of primed mice leads to the following results. In contrast to high IgG1 Ab production in CBA/J mice and due to the deletion of a switch region control element the serum IgG1 Ab titres are very low in Δ5′Sγ1 mice. They amount to around 1% of the IgG1 Ab production in CBA/J mice (Fig. 2 and 3). The lack of IgG1 production is compensated by the production of other IgG subclasses, especially IgG2a and IgG3 (Table 1). This compensation is also reflected in the *ex vivo* spot-ELISA data and in the *in vitro* anamnestic response.

Priming of CBA/J and Δ5′Sγ1 mice with minute doses leads to IgE Ab titres in the immune sera which are high and

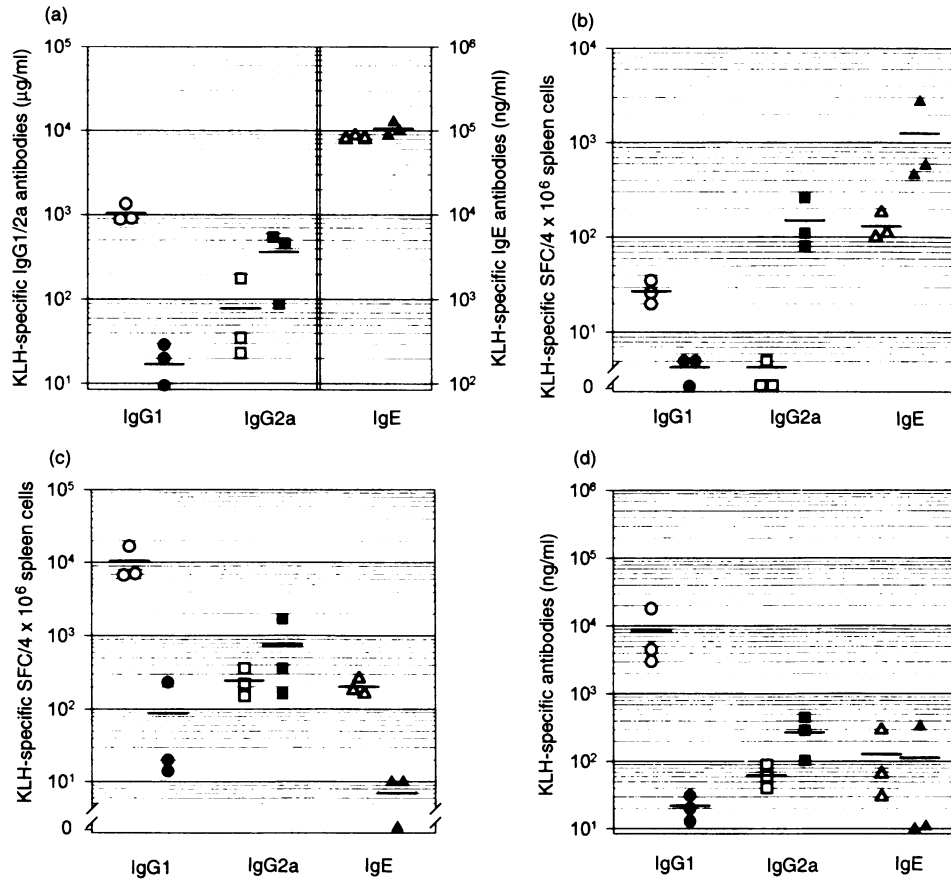

Figure 2. Distribution of immunoglobulin isotypes in the *in vivo* and *in vitro* humoral immune response of CBA/J and Δ5′Sγ1 mice repeatedly immunized with 0·1 μg KLH (K01 mice). Figures represent the data of three independent experiments and their mean. Each value in (a), (b), (c) and (d) indicates the mean of triplicates in the respective assay. For each experiment groups of three CBA/J mice (open symbols) and Δ5′Sγ1 mice (closed symbols) were primed with minute doses of antigen as described in the Materials and Methods. Ab titres in the immune sera were determined around day 70 of the immunization period (a). Ab-producing cells were determined *ex vivo* 2 weeks after the last injection of KLH by spot-ELISA (b). At this time 4×10^6 spleen cells were cultured in 24-well tissue culture plates in a volume of 1 ml DMEM/FCS with 1 μg KLH/ml as antigen. The number of SFC among cultured spleen cells was determined on day 6 by spot-ELISA (c). Ab titres in the culture supernatants were measured on day 12 to 14 of the culture period (d).

comparable in both strains (Fig. 2a). *Ex vivo* IgE Ab SFC are demonstrated in both strains but their number is five to ten times higher in Δ5′Sγ1 mice (Fig. 2b). When spleen cells from K01 mice were tested for an anamnestic response no memory expression was found *in vitro* (Fig. 2c and d). It is noteworthy that in Δ5′Sγ1 mice with high numbers of IgE Ab SFC *ex vivo* the *in vitro* IgE Ab response remains at the base level given by the CBA/J mice, underlining the complete lack of IgE memory expression in K01 mice.

K100 mice show the following features (Fig. 3). In both CBA/J and Δ5′Sγ1 animals serum IgE Ab and *ex vivo* IgE Ab SFC are low or virtually lacking (Fig. 3a and b). However, one finds a high *in vitro* anamnestic IgE response in spleen cell cultures from CBA/J mice as measured by the appearance of IgE Ab SFC and IgE Ab in culture supernatants (Fig. 3c and d). This anamnestic IgE response is totally lacking in Δ5′Sγ1 mice.

To clarify whether IgG1-expressing B cells are essential for the expansion of IgE-producing cells, also, in cultures of K100 CBA/J mice, two different sets of experiments were performed. Depletion of surface IgG1-positive B cells prior to cultivation leads to an extensive reduction of the frequency of IgE-secreting B cells (Fig. 4a) and to a strongly decreased production of KLH-specific IgE Ab (Fig. 4b) in such cultures. In contrast, elimination of IgG2a-expressing B cells has no inhibitory effect on the outcome of the anamnestic IgE response. A similar suppression of IgE production can be achieved by co-cultivation of spleen cells from K100 mice with isotype-specific Ab against cell surface immunoglobulin. The IgE Ab response *in vitro* can be reduced by anti-IgG1 Ab but not by anti-IgG2a Ab (Fig. 5).

To determine whether IgE-producing B cells from K01 mice have passed through a direct or sequential isotype switch, circular DNA from enriched B cells was analysed by PCR amplification and Southern blot hybridization of the amplification products. Switch circles produced after direct switch by rearrangement of the Sμ and Sε switch regions contain the complete IgG1 switch region (Sγ1) and thus can be amplified with Sγ1-specific primers and proved by a Sγ1-specific oligonucleotide probe (Fig. 1). Figure 6 shows that PCR products of circular DNA from purified B cells from K01 CBA/J mice can be detected by hybridization with this probe. In contrast,

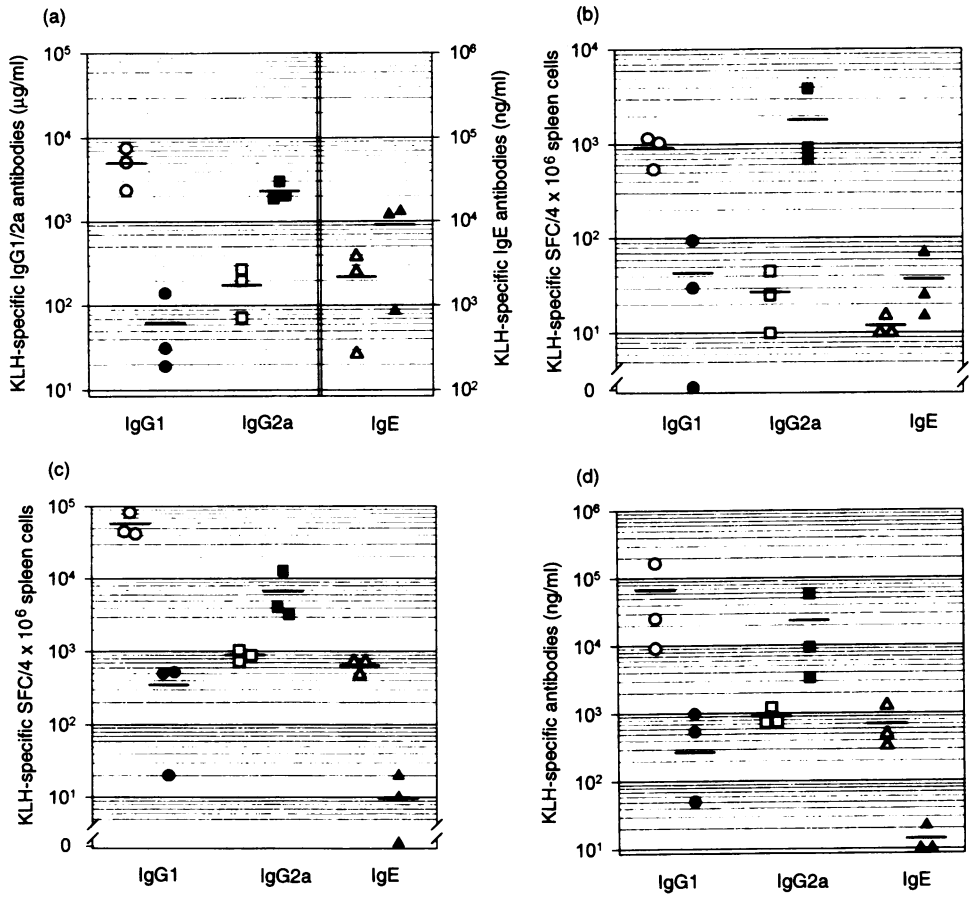

Figure 3. Distribution of immunoglobulin isotypes in the *in vivo* and *in vitro* humoral immune response of CBA/J and Δ5′Sγ1 mice repeatedly immunized with 100 μg KLH (K100 mice). Figures represent the data of three independent experiments and their mean. Each value in (a), (b), (c) and (d) indicates the mean of triplicates in the respective assay. For each experiment groups of three CBA/J mice (open symbols) and Δ5′Sγ1 mice (closed symbols) were primed with large doses of antigen as described in the Materials and Methods. Ab titres in the immune sera were determined around day 70 of the immunization period (a). Ab-producing cells were determined *ex vivo* 2 weeks after the last injection of KLH by spot-ELISA (b). At this time 4×10^6 spleen cells were cultured in 24-well tissue culture plates in a volume of 1 ml DMEM/FCS with 1 μg KLH/ml as antigen. The number of SFC among cultured spleen cells was determined on day 8 by spot-ELISA (c). Ab titres in the culture supernatants were measured on day 12 to 14 of the culture period (d).

the expression of switch circles containing the complete Sγ1 region is very low or totally lacking among circular DNA extracted from B cells from K100 mice. These data support by molecular analysis the notion that B cells in K01 CBA/J mice switch directly from IgM to IgE antibody formation.

DISCUSSION

CBA/J and Δ5′Sγ1 mice primed with minute doses of KLH (K01 mice) or large doses of KLH (K100 mice) show the following features with regard to the IgE Ab response. K01 mice from both strains have equally high IgE Ab titres and harbour a large number of IgE Ab SFC but lack *in vitro* memory expression.

K100 mice from both strains have low IgE Ab titres in their immune sera and only a marginal number of IgE Ab SFC *ex vivo*, but behave differently with regard to *in vitro* Bε memory expression. The IgE burst is only found in spleen cell cultures of CBA/J mice but fails to appear in those of Δ5′Sγ1 mice. These findings as well as the IgG1 depletion and inhibition experiments are pertinent for the understanding of the ontogeny of Bε cells. Two possible pathways have been discussed. One implies a direct switch from IgM-expressing B cells to IgE-expressing and producing B cells.[5–7] The other possibility suggests a sequential switch from IgM with an intermediate IgG1-expressing differentiation stage before the B cell enters the Bε status.[8–12] For both differentiation pathways experimental evidences exist. The best evidence for a direct switch is the fact that Δ5′Sγ1 mice can have an unimpaired IgE response.[5] On the other hand a sequential switch is suggested from the analysis of the IgE response of BALB/c mice against phosphorylcholine, where the majority of IgE Ab fail to carry the germ-line T15 idiotype, indicating a hypermutating intermediate on the path from IgM to IgE expressing B cells.[10] The data presented in this communication suggest that the two switch programmes are not mutually exclusive but are individually determined by the antigen dose. Priming with minute doses of Ag favours a direct switch without establishing a Bε cell memory. This direct switch has been demonstrated

Table 1. Distribution of IgG isotypes in the *in vivo* and *in vitro* humoral immune response of K01 and K100 CBA/J and Δ5′Sγ1 mice*

	K01		K100	
Isotypes	CBA/J	Δ5′Sγ1	CBA/J	Δ5′Sγ1
SFC *ex vivo*				
IgG1	25	0	1040	0
IgG2a	0	80	25	920
IgG2b	0	40	20	150
IgG3	0	80	0	360
Serum Ab				
IgG1	901	3	2327	19
IgG2a	23	88	72	2004
IgG2b	1	3	4	35
IgG3	<1	19	<1	137
SFC *in vitro*				
IgG1	7040	20	41 600	20
IgG2a	150	160	1040	3200
IgG2b	280	80	1040	1280
IgG3	0	1120	20	4800
Ab in culture supernatants				
IgG1	4337	29	9109	50
IgG2a	61	103	747	9363
IgG2b	10	16	76	284
IgG3	<1	90	<1	1654

*CBA/J and Δ5′Sγ1 mice were immunized as described in the Materials and Methods. Ab titres in the immune sera and *ex vivo* SFC were determined 2 weeks after the fifth immunization and spleen cell cultures were set up at this time. The number of SFC/4×10^6 spleen cells was determined on day 5 (K01) and day 7 (K100). Ab titres in the culture supernatants were measured on day 18 (K01) and day 12 (K100). Concentrations of serum Ab are given in μg/ml for IgG1 and IgG2a and in arbitrary units for IgG2b and IgG3. Concentrations of Ab in supernatants are given in ng/ml for IgG1 and IgG2a in arbitrary units $\times 10^{-3}$ for IgG2b and IgG3.

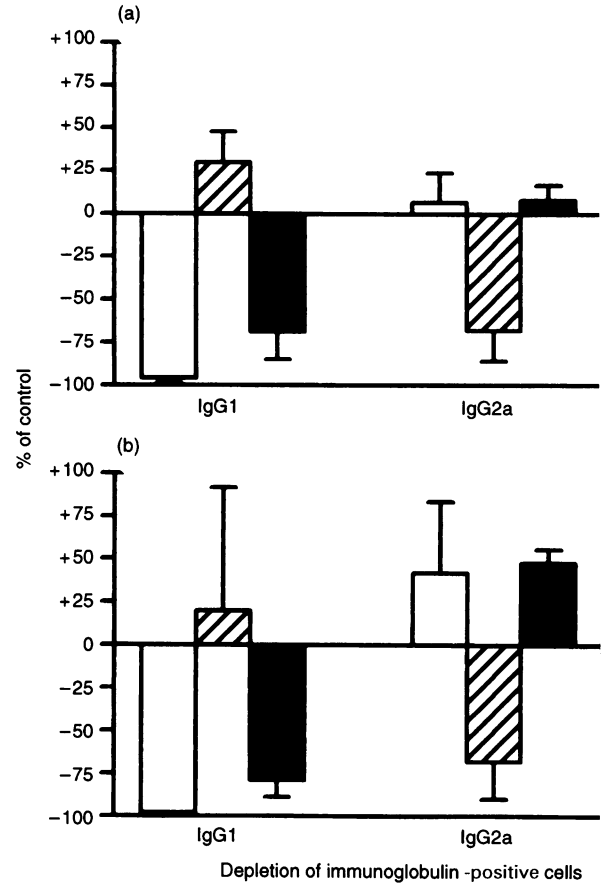

Figure 4. Isotype-specific depletion of IgG1-positive cells inhibits the expansion of IgE-producing cells and the production of IgE *in vitro*. IgG1- and IgG2a-positive cells were removed from spleen cell suspensions from K100 mice by magnetic separation as described in the Materials and Methods. Following depletion, 4×10^6 negative cells were cultured in a volume of 1 ml DMEM/FCS with 1 μg KLH/ml. The frequencies of Ab-producing cells (a) as well as the levels of KLH-specific Ab in the culture supernatants (b) were determined on subsequent days (IgG1, open bars; IgG2a, hatched bars; IgE, solid bars) and compared to control cultures which were not separated with the MiniMACS system. Figures represent the mean of three independent experiments ±SEM.

in this communication by two different experimental approaches: Δ5′Sγ1 mice are able to mount a high IgE antibody response which would be impossible with a sequential switch. Furthermore, switch circles from K01 mice contain a complete Sγ1 switch region as predicted with a direct switch.[8,11] In contrast, exposure to large doses favours a sequential switch; but *in vivo* the B cells are silenced on their way to IgE expression and fail to proceed into IgE Ab-forming plasma cells.

At the moment there is only a little evidence about the mechanism of this Bε cell anergy, except for the finding that transfer of lymphocytes from CBA/J mice primed with large doses into naive recipients conditions these animals such that they cannot mount a high titre IgE response even upon immunization with minute doses of Ag.[14]

The data presented in this communication are relevant in evaluating the process of IgE memory formation. In K01 CBA/J mice the ratio of *ex vivo* IgE/IgG1 SFC is a factor of 5. The *in vitro* ratio after restimulation drops to a factor of 0·02 due to the *in vitro* expansion of IgG1-producing B cells and due to complete lack of expansion in the IgE pool. In the K100 CBA/J mice, in addition to the anamnestic expansion of the IgG1 pool *in vitro*, there is also a strong 50–100-fold net increase in the IgE response compared to the frequency of IgE Ab SFC *ex vivo*. The data suggest that there is no significant memory cell population among IgE-producing B cells originating from the direct switch.[29,30] In contrast the memory for IgE expression[31,32] is associated with the sequential switch program and probably intimately associated with intermediate IgG1-bearing B cells.

The Ag dose dependency of the two pathways of IgE expression probably reflects a differential activation of Th1 and Th2 cells.[33–35] Minute doses favour a direct switch through Th2 activation and IL-4 production, without establishing a memory pool,[36,37] whereas large doses favour Th1 activity with lack of IgE formation *in vivo*.[15,38] However, this Th1 response might lead to accumulation of a Bε memory pool. These cells, when released from the presumed suppression *in vitro*, would then burst into an IgE Ab response.[17] Apart from

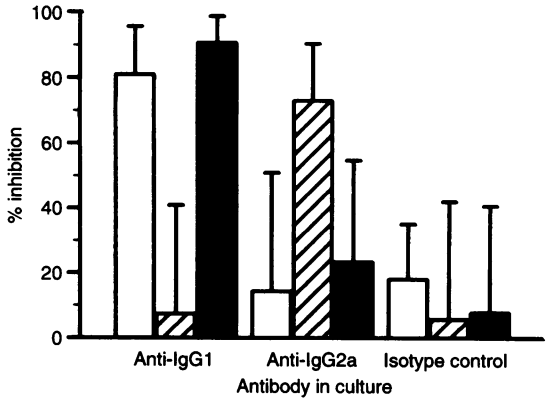

Figure 5. Isotype-specific inhibition of IgG1-positive cells by anti-IgG1 Ab blocks the expansion of IgE-producing cells *in vitro*. Spleen cells (4×10^6/ml) of K100 mice were cultured in 24-well tissue plates in a volume of 1 ml DMEM/FCS with 1 μg KLH/ml and isotype-specific Ab (goat anti-mouse IgG1 or anti-mouse IgG2a) or control Ab (goat IgG) at a concentration of 50 μg/ml. The frequencies of KLH-specific IgG1 (open bars), IgG2a (hatched bars) and IgE (solid bars) Ab-producing cells were determined by spot-ELISA at day 8 of the culture period and compared to control cultures which were not incubated with Ab. Figures represent the inhibition of the respective isotypes as mean of three independent experiments $\pm$SEM.

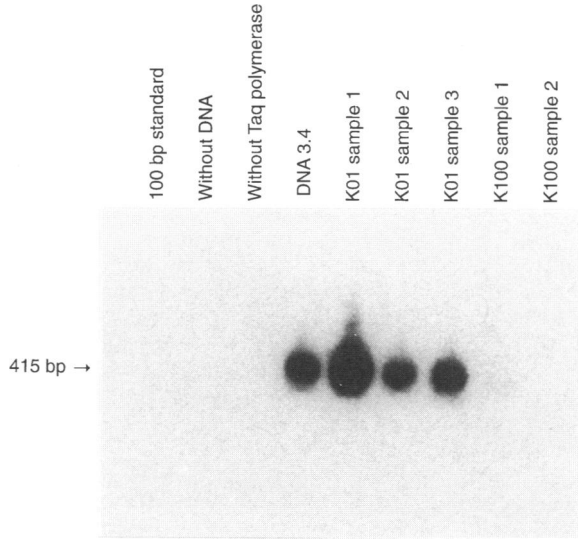

Figure 6. PCR and Southern blot analysis of circular DNA isolated from purified B cells from K01 and K100 CBA/J mice 3 days after the last immunization. Circular DNA was amplified by PCR with Sγ1-specific primers and amplification products were blotted and hybridized with a Sγ1-specific oligonucleotide probe as described in the Materials and Methods. Southern blots of the following samples are shown: lane 1, standard; lane 2, negative control without DNA; lane 3, negative control without *Taq* polymerase; lane 4, genomic DNA 7D7.3.4; lanes 5–7, circular DNA from K01 CBA/J mice (three independent experiments); lanes 8 and 9: circular DNA from K100 CBA/J mice (two independent experiments).

the implication of T-cell-mediated regulation of Ab class expression the experimental system described here provides a tool to investigate up- and down-regulation of IgE Ab formation in culture by exogenous mediators.

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