

## **Frequency of Immunoglobulin E Class Switching Is Autonomously Determined and Independent of Prior Switching to Other Classes**

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### **Summary**

Both, in humans and in mice, a major fraction of immunoglobulin E (IgE)-expressing B lymphocytes develops by sequential Ig class switching from IgM via IgG to IgE. This sequential class switch might have functional implications for the frequency and repertoire of IgE<sup>+</sup> cells. Here we show that in mutant mice, in which sequential switching to IgE via IgG1 is blocked, the frequency of cells switching to IgE is not affected. Thus, sequential class switching to IgE merely reflects the simultaneous accessibility of two acceptor switch regions for switch recombination, induced by one cytokine, but with markedly distinct efficiency. Analysis of switch recombination on both IgH alleles of switched cells shows that the low frequency of switching to IgE is an inherent feature of the S<sub>ε</sub> switch region and its control elements.

As an antigen receptor of mast cells and basophils, IgE plays a key role in type I atopic immune disorders. IgE is produced by plasma cells, which are derived from IgM-expressing, activated B lymphocytes that have been instructed by IL-4 to undergo Ig class switching to IgE (1). Despite recent advances in understanding the control of isotype switching (2), it is still not clear why IgE serum titers in vivo range orders of magnitude below titers of other classes (3), and why, under saturating concentrations of IL-4 in vitro, the frequency of IgE switching reaches at most 10% of the frequency of switching to IgG1, which is also induced by IL-4 (4, 5).

Ig class switching occurs by a directed loop-out-and-deletion recombination between two highly repetitive switch regions (S<sub>n</sub>), resulting in hybrids that are composed of 5' and 3' parts of the donor and acceptor regions, respectively (6–8). These hybrid switch regions can serve as donor regions in further switch recombinations (9, 10). Although all murine switch regions have been shown to be involved in sequential switch recombination, only for S<sub>ε</sub> sequential switching via S<sub>γ</sub>1 appears to be dominant over direct switching. The frequent observation of S<sub>γ</sub>1 sequences in the chromosomal (5) and excised circular products (11) of IgE switch recombinations, and the fact that in vitro, the appearance of IgE<sup>+</sup> cells in LPS/IL-4 cultures can be largely suppressed by anti-IgG1 antibodies (5, 12), led to the suggestion that most, if not all, IgE<sup>+</sup> cells are generated by sequential switching. To assess the functional importance of sequential class switching to IgE, we have analyzed class switching to IgE in the mutant mouse strains Δ5'S<sub>γ</sub>1 and neoΔ5'S<sub>γ</sub>1 (13), in which sequential switching to IgE via IgG1 is blocked, because of

the impaired ability to switch to IgG1. We have determined whether in these mice switching to IgE is disabled, enhanced, or unchanged.

### **Materials and Methods**

**Mice.** Animals were bred in the mouse facility of the Institute for Genetics. Two mutant mouse strains, neoΔ5'S<sub>γ</sub>1 (IgH<sup>neoΔ5'S<sub>γ</sub>1/neoΔ5'S<sub>γ</sub>1</sup>), in which the control region for S<sub>γ</sub>1 switch recombination is replaced by a *neo* gene, and Δ5'S<sub>γ</sub>1 (IgH<sup>Δ5'S<sub>γ</sub>1/Δ5'S<sub>γ</sub>1</sup>), in which this region is deleted, were used in this study. Both strains exhibit the same phenotype (13). Heterozygous and homozygous mutant animals were on (129/ola × CB.20) F<sub>1</sub> and F<sub>2</sub> background, respectively.

**Immunization.** 6-wk-old homozygous neoΔ5'S<sub>γ</sub>1 and allotype-matched (C57Bl/6 × CB.20) F<sub>2</sub> control mice were infected subcutaneously with 500 third-stage *Nippostrongylus brasiliensis* larvae (kindly provided by M. Harder, Beyer A.G., Mannheim, Germany). Serum was obtained before and 13 d after infection to determine the antibody titer. To elicit primary (1°), secondary (2°), and tertiary (3°) immune responses to 4-hydroxy-3-nitrophenyl acetate (NP), homozygous and heterozygous neoΔ5'S<sub>γ</sub>1 mutant mice were immunized with 100 μg i.p. of alum-precipitated NP<sub>6</sub>-OVA on days 1, 53, and 116. Serum antibody titers were determined before immunization and on days 6 (IgM), 14 (1°), 59 (2°), and 122 (3°). Serum titers of specific antibodies were determined by isotype-specific ELISA as described elsewhere (14). For determination of NP-specific IgE, serially diluted sera were applied to plastic plates coated with the IgE-specific mAb 95.3 (10 μg/ml) (15). NP-specific IgE was detected with 4-hydroxy-5-iodo-3-nitrophenyl-acetyl (NIP)<sub>15</sub>-BSA, biotinylated NP-specific mAb (N1G9, 16), alkaline phosphatase-conjugated streptavidin, and 4-nitrophenyl phosphate disodium salt as substrate. Dilutions and washing were carried out with PBS containing 0.5% BSA, 0.02% Na<sub>2</sub>N<sub>3</sub>, and 0.05% Tween 20.

**Cell Culture.** Spleen cells were cultured in 3 ml medium at a

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concentration of  $5 \times 10^5$ /ml in 6-well plates, and in 50 ml at  $10^6$ /ml in 250-ml bottles (Costar Corp., Cambridge, MA) with RPMI 1640 containing 40  $\mu$ g/ml bacterial LPS (serotype 055 B5; Sigma Chemical Co., St. Louis, MO) (5). Supernatant of a NIH 3T3 cell line stably transfected with an IL-4 cDNA expression vector (gift of W. Müller, University of Cologne) was added to a final concentration of 10% as a source of recombinant IL-4. This amount had been shown to be sufficient to induce switching to IgE at a maximum frequency (5).

**Immunofluorescence and Flow Cytometry.** Frequencies of in vitro-generated B cell blasts expressing the various isotypes were determined by cytoplasmic staining of cells with fluorochrome-conjugated, isotype-specific antibodies (Southern Biotechnology Associates, Birmingham, AL; Nordic, Tilburg, Netherlands) and analysis by fluorescence microscopy, as described before (7). Flow cytometric analysis and cell sorting were performed on a modified FACS<sup>®</sup> 440 combined with the electronic console of FACStar Plus<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA). Cells were harvested on day 5 of culture, purified on a Ficoll gradient, fixed in 70% methanol, washed, and stained with the IgM-specific, PE-conjugated mAb R33-24-12 (gift of Rudi Grützmán, University of Cologne) and FITC-conjugated goat anti-mouse IgE (Nordic). Gates and windows for analysis and sorting were set according to light scatter and red and green fluorescence.  $1.3\text{--}2.7 \times 10^6$  deflected cells were collected for preparation of genomic DNA.

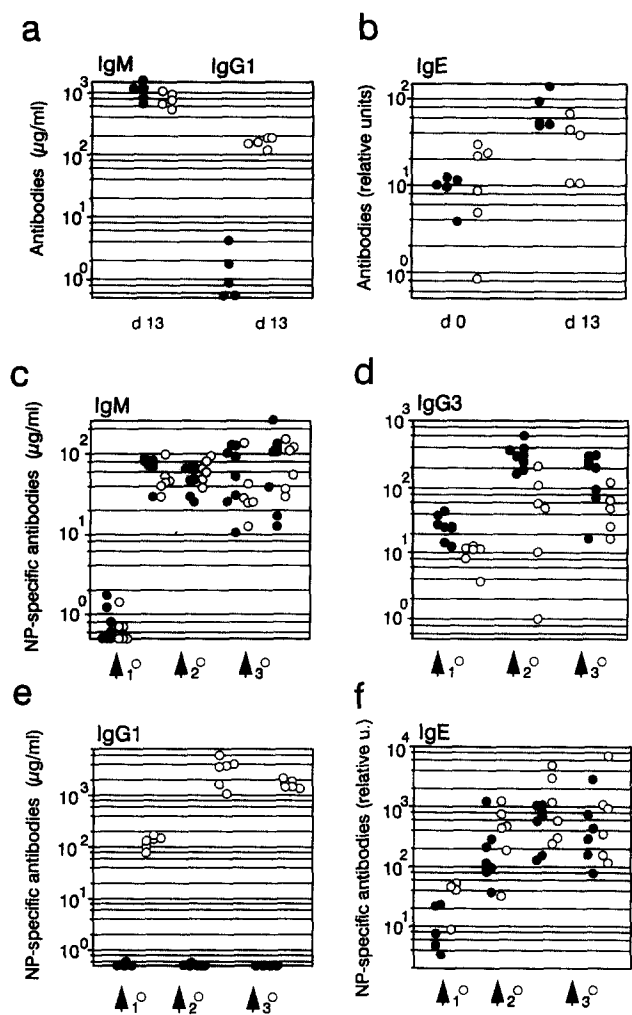
**Restriction Analysis.** Rearrangements of individual switch regions were determined by restriction analysis on a Southern blot as described elsewhere (17). We employed the following probes (see in Fig. 2): S $\mu$  (from pM2-20) (18), a 1.7-kb HindIII fragment; 3'C $\delta$  (derived from phage CH28-257.3) (19), a 1.4-kb KpnI fragment downstream of C $\delta$ ; 5'S $\gamma$ 1 (from p $\gamma$ 1EH10.0) (20), a 590-bp SspI/NsiI fragment that lies 5' of the targeted deletion in  $\Delta$ 5'S $\gamma$ 1 mice; C $\gamma$ 1 (from pG1A), a 3.5-kb EcoRI/BamHI fragment (5); S $\epsilon$ , a 1.3-kb HindIII/KpnI genomic fragment carrying the 3' half of S $\epsilon$  of the BALB/c IgH locus (5); C $\alpha$  (from pAB $\alpha$ -1, C $\alpha$ ) a cDNA derived from the cell line HOPC-2020 (Bothwell, A., unpublished data), a 500-bp PstI fragment carrying  $\alpha$ 3; IL-4, a 900-bp BamHI/SalI genomic IL-4 fragment (14). The intensities of the hybridization signals were determined by scanning with a chromoscan 3 (Joyce, Loebel and Co., Ltd., Gateshead, UK).

## Results and Discussion

**IgE Immune Responses.** To assess the effect of impaired IgG1 class switching on switching to IgE in vivo, we compared the polyclonal IgE responses of neo $\Delta$ 5'S $\gamma$ 1 and wild-type mice to infection with *N. brasiliensis*. The titers of total serum IgE of homozygous neo $\Delta$ 5'S $\gamma$ 1 mice on day 13 after infection were similar to those in wild-type mice, whereas their titers of serum IgG1 were at least 60-fold reduced (Fig. 1, *a* and *b*).

An antigen-specific IgE response was analyzed in homozygous and heterozygous neo $\Delta$ 5'S $\gamma$ 1 mice, primed, and boosted with NP $\epsilon$ -OVA (Fig. 1, *c-f*). In homozygous neo $\Delta$ 5'S $\gamma$ 1 mice, titers of NP-specific IgG1 were below the level of detection (60 ng/ml), whereas sera of heterozygous mice contained from 100  $\mu$ g/ml to >1 mg/ml NP-specific IgG1 (Fig. 1 *e*). In both, homo- and heterozygous mice, however, titers of NP-specific IgE reached identical levels. Thus, expression of IgE in vivo is not impaired in mice that cannot perform class switching to IgG1.

**IgE Switching In Vitro.** Equal titers of IgE in vivo, how-



**Figure 1.** IgE response of  $\Delta$ 5'S $\gamma$ 1- and wild-type mice. Serum concentrations of Ig isotypes of Nb-infected neo $\Delta$ 5'S $\gamma$ 1 mice (●) and controls (C57Bl/6  $\times$  CB20) F<sub>2</sub> (○) (*a* and *b*) and homozygous (●) and heterozygous (○) mutant neo $\Delta$ 5'S $\gamma$ 1 mice immunized with NP $\epsilon$ -OVA (*c-f*). The values indicate titers of individual mice.

ever, do not exclude differences in switch frequency, which can be compensated by antigenic selection. Therefore, we also analyzed IgE class switching in vitro. Splenic B cells from homozygous  $\Delta$ 5'S $\gamma$ 1 and BALB/c control mice were activated in vitro with LPS in the presence of IL-4. The frequencies of cells expressing the various Ig isotypes on day 5 of culture are given in Table 1. Virtually no IgG1-expressing (<0.1%), but 4.6% ( $\pm$  1.1%) IgE-expressing homozygous  $\Delta$ 5'S $\gamma$ 1 cells were induced. The frequency of IgE<sup>+</sup> cells was even higher than in cultures of wild-type cells ( $2.0 \pm 0.5\%$ ), suggesting that sequential switching to IgE via IgG1, if it has any effect at all, may reduce the frequency of switching to IgE, rather than enhance it. However, the overall frequency of IgE<sup>+</sup>  $\Delta$ 5'S $\gamma$ 1 cells still remained low (<5%) compared with that of IgG1-expressing wild-type cells (29%).

**Analysis of IgE Switch Rearrangements.** In  $\Delta$ 5'S $\gamma$ 1 mice, B cells do not necessarily have to switch directly to IgE. Sequential switching may involve switch regions other than

**Table 1.** *In Vitro* Switch Frequencies of  $\Delta 5'S\gamma 1$ - and Wild-type Cells

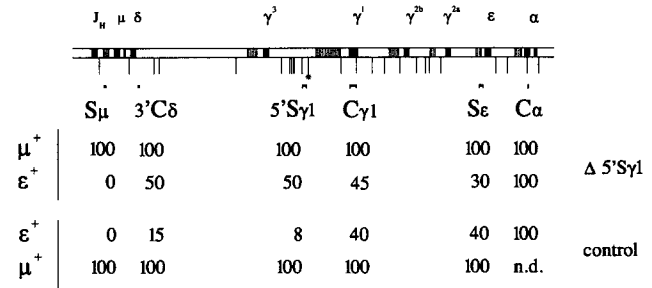
Isotypes	BALB/c		$\Delta 5'S\gamma 1$	
$\kappa$	70.1	( $\pm$ 22.2)	58.2	( $\pm$ 10.1)
$\mu$	37.5	( $\pm$ 16.6)	47.2	( $\pm$ 5.9)
$\gamma$ 3	0.49	( $\pm$ 0.22)	3.29	( $\pm$ 0.45)
$\gamma$ 2b	3.32	( $\pm$ 1.06)	2.46	( $\pm$ 0.78)
$\gamma$ 1	29.0	( $\pm$ 7.1)	0.05	( $\pm$ 0.08)
$\epsilon$	1.97	( $\pm$ 0.53)	4.55	( $\pm$ 1.12)

Numbers indicate percentages of total living cells on day 5 of culture that express the indicated isotype as determined by immunofluorescence and fluorescence microscopy (mean value and standard deviation of six independent LPS/IL-4 cultures).

$S\gamma 1$ , e.g.,  $S\gamma 3$ , in light of the elevated frequencies of  $IgG3^+$  cells in LPS/IL-4 cultures (Table 1), and specific  $IgG3$  titers after NP<sub>6</sub>-OVA immunization (Fig. 1 d). To test this possibility, we analyzed switch rearrangements on the inactive IgH alleles of  $IgE^+$  cells from normal and  $\Delta 5'S\gamma 1$  mice. Since both IgH alleles of a cell are subjected to the same Ig class switch instructions, the inactive allele of a given switched cell is usually found recombined either to the same switch region (7) or to one representing the intermediate of a sequential switch program (5). Thus, the inactive allele can be used to monitor the switch instructions and history of cells expressing a given isotype.

After 5 d of culture with LPS and IL-4, cells from  $\Delta 5'S\gamma 1$  and control mice, stained for IgM and IgE in the cytoplasm, were isolated by fluorescence-activated cell sorting to a purity of 98% for  $IgM^+$  cells and 95 and 99% for  $IgE^+$   $\Delta 5'S\gamma 1$  and control cells, respectively. DNA extracted from the sorted cells was analyzed for switch recombination by restriction analysis on a Southern blot. The extent of rearrangement and deletion could be inferred from the relative numbers of switch ( $S_H$ ) and constant region gene segments ( $C_H$ ), respectively, retained in germline configuration (7) (Fig. 2).

Since it had been shown that  $IgM^+$  cells from LPS cultures rarely show rearrangements of  $S_H$  regions (5, 7, 17), the relative intensities of the hybridization signals of  $IgM^+$  wild-type cells were considered to be 100% of the  $S_H/C_H$  fragments in germline configuration. In  $\Delta 5'S\gamma 1$   $IgM$  cells, all  $S_H/C_H$  sequences were still in germline configuration when compared with  $IgM$  wild-type cells. In  $IgE^+$  cells, 50% of the IgH alleles were expected to show deletions spanning the region from  $C\delta$  to  $S\epsilon$ , because of IgE class switch recombination on the active allele. DNA rearrangement of the inactive alleles results in a >50% reduction of signal intensities of germline fragments. In  $IgE^+$  wild-type cells, 70% of the inactive alleles showed a deletion of  $C\delta$  and 84% showed rearrangement of  $S\gamma 1$ . Thus, 70–84% of the cells switched to  $S\gamma 1$  on their inactive IgH allele, reflecting the simultaneous instruction of individual cells to switch to  $IgG1$



**Figure 2.** Characterization of switch rearrangements of IgM- and IgE-expressing cells on day 5 of LPS + IL-4 culture of splenic B cells from homozygous mutant  $\Delta 5'S\gamma 1$ - and wild-type 129/sw mice. DNA configuration of IgH loci of cells sorted for cytoplasmic expression of IgM or IgE. DNA was digested with (vertical lines) EcoRI. (Asterisk) An EcoRI site introduced by the  $\Delta 5'S\gamma 1$  mutation. After gel electrophoresis and blotting, the filter was sequentially hybridized to the probes indicated. Band intensities were quantified relative to the hybridization signal obtained with an IL-4 probe for the same lane, and normalized to the corresponding signal from  $IgM^+$  cells of control mice.

and IgE (5). Only 20% lost the  $C\gamma 1$  gene from the inactive IgH allele and continued switching to  $S\epsilon$ . For the inactive IgH alleles of  $IgE^+$   $\Delta 5'S\gamma 1$  cells, no deletion of the  $C\delta$  gene and the sequence 5' of the mutated  $5'S\gamma 1$  region, and only a marginal signal reduction of  $C\gamma 1$  is detectable, i.e., essentially no switch recombination occurred.

**Intra Switch Region Rearrangements.** Since  $C\delta$  and  $C\gamma 1$  were not deleted from the inactive IgH alleles of  $IgE^+$   $\Delta 5'S\gamma 1$  cells, no switch recombination occurred on these alleles. However, none of the  $S\mu$  and only about 60% of the  $S\epsilon$  regions were in germline configuration, indicating recombinations within those switch regions. The different extent of deletions suggests a lower accessibility of  $S\epsilon$  to switch recombinase. Intra Switch region recombinations have been observed in case of activation of a switch region in the absence of reaction partners accessible to switch recombinase (17, 21). In  $\Delta 5'S\gamma 1$  cells, both  $S\mu$  and  $S\epsilon$  are accessible, but they recombine with themselves and not with each other.  $S\epsilon$  appears to be a relatively good substrate for switch recombinase, but an inferior partner for recombination with  $S\mu$ .

The lack of switch recombination on inactive IgH alleles of  $IgE^+$  cells shows that class switching to IgE is independent of prior switch recombination to any other class. The overall low IgE switch frequency is an autonomously determined intrinsic feature of  $S\epsilon$  and its control elements. The apparent dominance of sequential switch recombination over direct switch recombination in the generation of IgE-expressing cells is due to the parallel activation of  $S\gamma 1$  and  $S\epsilon$  by IL-4,  $S\gamma 1$  being more accessible to switch recombination with  $S\mu$ . By competing for switch recombinase, an accessible  $S\gamma 1$  switch region may even contribute to limiting the frequency of switching to IgE, rather than to enhancing it. The high threshold for and low frequency of switching to IgE may contribute to the generation of an efficient IgE repertoire for mast cells and basophils (22). The excess of IgG antibodies with an overlapping repertoire generated in the same immune response (23, 24) may further increase the threshold for IgE-mediated reactions by masking the antigen.

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