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+ 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 frequency of switching to IgE is an inherent feature of the Se switch region and its control elements.

Materials and Methods

Mice. Animals were bred in the mouse facility of the Institute for Genetics. Two mutant mouse strains, neoΔ'S'yl (IgHΔ'S'yl/Δ'S'yl/neoΔ'S'yl), in which the control region for S'y1 switch recombination is replaced by a neo gene, and Δ'S'y1(S'y1/Δ'S'y1), 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 × C57.B1/6) F1 and F2 background, respectively.

Immunization. 6-wk-old homozygous neoΔ'S'y1 and allotype-matched (C57Bl/6 × C57.B1) F2 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 neoΔ'S'y1 mutant mice were immunized with 100 μg i.p. of alum-precipitated NP6-OVA on days 1, 53, and 116. Serum antibody titers were determined before immunization and on days 6 (IgM), 14 (1°), and 122 (3°) immune responses to 4-hydroxy-3-nitrophenyl acetate.

Cell Culture. Spleen cells were cultured in 3 ml medium at a...
concentration of $5 \times 10^5$ units/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® 440 combined with the electronic console of FACStar Plus® (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 Rudolf Grützmann, 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. 1.3–2.7 x 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): Sp (from pM2-20) (18), a 1.7-kb HindIII fragment; 3’C8 (derived from phage CH28-257.3) (19), a 1.4-kb KpnI fragment downstream of C8; 5’Sy1 (from p7IEH10.0) (20), a 590-bp SspI/SalI fragment that lies 3’ of the targeted deletion in Δ5’Sy1 mice; Cy1 (from pG1A), a 3.5-kb EcoRI/BamHI fragment (5); Se, a 1.3-kb HindIII/KpnI genomic fragment carrying the 3’ half of Se of the BALB/c IgH locus (5); Cc (from pAB1-1, Cc) a cDNA derived from the cell line HOPE-2020 (Bothwell, A., unpublished data), a 500-bp PstI fragment carrying ccc3; IL-4, a 900-bp BamHI/Sall genomic IL-4 fragment (14). The intensities of the hybridization signals were determined by scanning with a chromoscan 3 (Joyce, Loebl 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 neoΔ5’Sy1 and wild-type mice to infection with *N. brasiliensis*. The titers of total serum IgE of homozygous neoΔ5’Sy1 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 neoΔ5’Sy1 mice, primed, and boosted with NP6-OVA (Fig. 1, c–f). In homozygous neoΔ5’Sy1 mice, titers of NP-specific IgG1 were below the level of detection (60 ng/ml), whereas sera of heterozygous mice contained from 100 μg/ml to >1 mg/ml NP-specific IgG1 (Fig. 1 e). In both, homo- and heterozygous mice, however, titers of NP-specific IgE achieved 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, however, 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 Δ5’Sy1 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% ± 1.1% IgE-expressing homozygous (O) and heterozygous (●) mutant neoΔ5’Sy1 mice immunized with NP6-OVA (●). The values indicate titers of individual mice.

**Analysis of IgE Switch Rearrangements.** In Δ5’Sy1 mice, B cells do not necessarily have to switch directly to IgE. Sequential switching may involve switch regions other than...
both IgH alleles of a cell are subjected to the same class ability, we analyzed switch rearrangements on the inactive IgH cells in region (7) or to one representing the intermediate of a sequence between C6 and Se, because of IgE class switch recombination on the active allele. DNA rearrangement of IgH loci of cells sorted for cytoplasmic expression of IgM or IgE. DNA was digested with restriction analysis on a Southern blot. The extent of rearrangement and deletion could be inferred from the relative intensities of the hybridization signals of IgM + wild-type cells from LPS/IL-4 cultures.

Since it had been shown that IgM + cells from LPS cultures rarely show rearrangements of Sp regions (5, 7, 17), the relative intensities of the hybridization signals of IgM + wild-type cells were considered to be 100% of the Sp/Cn fragments in germline configuration. In Δ5'Sy1 IgM cells, all Sp/Cn 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 Cδ to Se, 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 Cδ and 84% showed rearrangement of Sy1. Thus, 70–84% of the cells switched to Sy1 on their inactive IgH allele, reflecting the simultaneous instruction of individual cells to switch to IgG1 and IgE (5). Only 20% lost the Cy1 gene from the inactive IgH allele and continued switching to Se. For the inactive IgH alleles of IgE + Δ5'Sy1 cells, no deletion of the Cδ gene and the sequence 5' of the mutated 5'Sy1 region, and only a marginal signal reduction of Cy1 is detectable, i.e., essentially no switch recombination occurred.

Intra Switch Region Rearrangements. Since Cδ and Cy1 were not deleted from the inactive IgH alleles of IgE + Δ5'Sy1 cells, no switch recombination occurred on these alleles. However, none of the Sp and only about 60% of the Se regions were in germline configuration, indicating recombinations within those switch regions. The different extent of deletions suggests a lower accessibility of Se 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 Δ5'Sy1 cells, both Sp and Se are accessible, but they recombine with themselves and not with each other. Se appears to be a relatively good substrate for switch recombinase, but an inferior partner for recombination with Sp.

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 Se 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 Sy1 and Se by IL-4, Sy1 being more accessible to switch recombinase with Sp. By competing for switch recombinase, an accessible Sy1 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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