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Sequential Switching from μ to ε Via γ4 in Human B Cells Stimulated with IL-4 and Hydrocortisone[1]

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Abstract. The molecular events leading to IgE synthesis in human B cells stimulated with IL-4 and hydrocortisone were analyzed. IL-4, but not hydrocortisone, induced Cε germ line transcription. However, hydrocortisone increased the levels of IL-4-induced germ line Cε transcripts by twofold and delivered the signal required for transcription of mature Cε mRNA. Nested primer polymerase chain reaction of high m.w. DNA revealed deletional switch recombination occurring in B cells sorted for lack of expression of surface IgE and stimulated with both IL-4 and hydrocortisone, but not in B cells stimulated with IL-4 alone or hydrocortisone alone. DNA sequence analysis of 10 switch fragments revealed direct joining of Sμ to Sε in eight fragments, one of which exhibited an 876-bp deletion in Sμ. The ninth fragment contained a 50-bp insertion at the Sμ/Sε junction, which was likely to be derived from Sγ4. The sequence of the 10th fragment was consistent with either a 17-bp insertion at the Sμ/Sε junction derived from Sγ4 or with a complex 38-bp deletion within Sε. Mapping of the switch junction sites showed "hot spots" for recombination within Sμ but not within Sε. These findings indicate that hydrocortisone induces Sμ-Sε deletional switch recombination in IL-4-treated B cells, and support a model of sequential isotype switching from IgM to IgE via IgG4. *Journal of Immunology,* 1993, 151: 4528.

Induction of IgE synthesis in human B cells requires two signals (1). The first signal is delivered by the cytokine IL-4 and results in the expression of ε-GLT[3], but not in IgE isotype switching (2, 3). A second signal is required for isotype switching and for the synthesis and secretion of IgE. This second signal can be delivered by T cells (4–6), EBV (3, 7), engagement of CD40 (6, 8–10), and hydrocortisone (11–13). We have recently shown that IgE isotype switching induced by EBV and anti-CD40 mAb in the presence of IL-4 is accompanied by DNA deletional switch recombination (14, 15). Analysis of switch fragments isolated from B cells stimulated with anti-CD40 mAb plus IL-4 revealed direct joining of the μ S region (Sμ) to Sε (15) without the deletion or duplication of events within Sμ, which were found in B cells stimulated with IL-4 and EBV (14).

The signal delivered by hydrocortisone differs from the signals delivered by EBV and anti-CD40 mAb in that it bypasses cell surface receptor signaling (16–18). In this study, we analyzed the molecular events involved in the induction of IgE isotype switching in human B cells stimulated with hydrocortisone and IL-4. We show that hydrocortisone enhanced the level of IL-4-induced ε-GLT. More importantly, we show that deletional switch recombination underlies IgE class switching induced by hydrocortisone and IL-4 in B cells sorted for lack of sIgE expression. Sequencing of 10 switch fragments revealed direct joining of Sμ to Sε in eight fragments. The ninth fragment contained a 50-bp insertion at the Sμ/Sε junction, which was derived from Sγ4. The sequence of the 10th fragment was consistent with either a 17-bp insertion located at the Sμ/Sε junction and derived from Sγ4 or with a complex 38-bp deletion within Sε. These findings indicate that hydrocortisone in-

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[3] Abbreviations used in this paper: GLT, germ line transcripts; S region, switch region; sIgE, surface IgE; PCR, polymerase chain reaction.

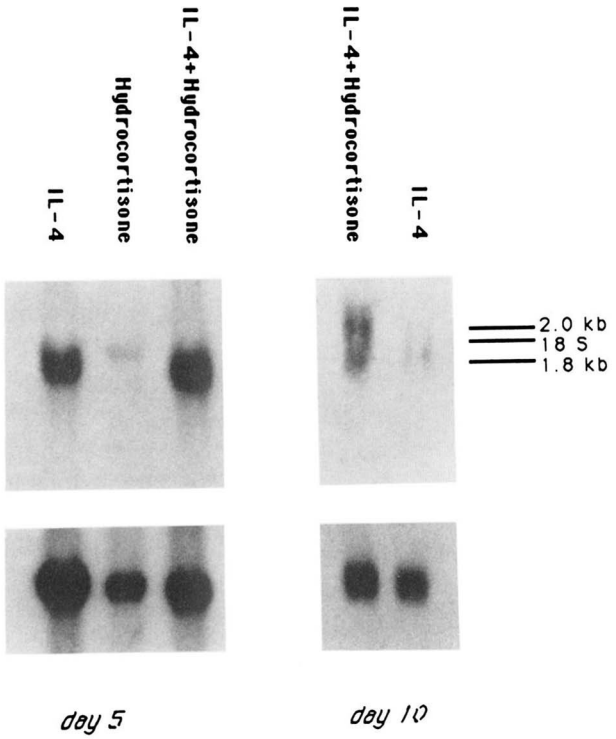

FIGURE 1. Induction of ϵ-GLT and mature Cϵ transcripts in normal human B cells stimulated with rIL-4 and hydrocortisone. Total RNA (10 μg) from normal peripheral blood B cells stimulated with rIL-4 (200 U/ml) and/or hydrocortisone (10^{-6} M) for 5 and 10 days was subjected to electrophoresis on a 1% formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized to [^{32}P]-labeled 0.88-kb *Hin*fI (top) or actin (bottom) probes. Top, exposed for 3 days; *bottom*, exposed for 1 day.

duces Sμ-Sϵ deletional switch recombination in IL-4-treated B cells. They also support a sequential model of isotype switching from IgM to IgE via IgG4.

Materials and Methods

B cell preparations

Highly purified human peripheral blood B cells from nonatopic subjects and B cells sorted for lack of expression of sIgE (sIgE-B cells) were isolated and cultured as previously described (3, 8).

Northern blot analysis was performed as previously described (3) using a [^{32}P]-labeled 0.88-kb *Hinf*I fragment, which spans the first two exons of Cϵ (3) and a cDNA β-actin probe. Densitometric analysis was performed with an LKB Ultrascan XL Densitometer (Bromma, Sweden).

PCR primers/amplification, cloning, and sequencing of switch fragments

Nested primer PCR runs for switch fragments were performed on high m.w. DNA isolated from cultured B cells as described (14, 15). Four PCR primers were used: S6 (5′-CTGCAGACTCAGAAGGGAGGGGATGCTCCG-3′), S4 (3′-ACTGATCCAAGACAGGAGTGTGGCGGATGT-5′), S7 (5′-GAGGGTGGTAATGATTGGTAATGCTTTGGA-′), and S9 (3′-GAACCCTGAGGTCCGGTCCCCGCTTCCCGG-5′). The locations of these primers in the Sμ and Sϵ regions are shown in Figure 4. The first round of PCR was performed using primers S6/S4. The second round of PCR was performed on a 5-μl aliquot of the first round PCR mixture with primers S7/S9. PCR amplification and purification of the amplified fragments were conducted as previously described (14, 15). Purified fragments were cloned into pGem 3zf(+) using the *Escherichia coli* strain JM 109. dsDNA sequencing was performed on purified constructs using Sequenase Version 2.0 (United States Biochemical, Cleveland, OH).

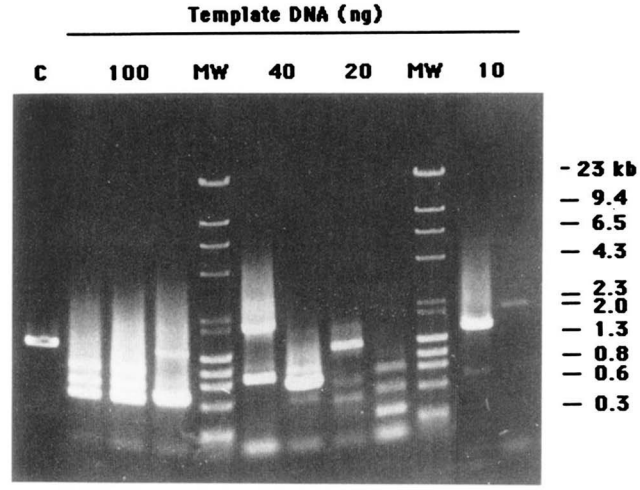

FIGURE 2. Nested primer PCR amplification of switch fragments. Serially diluted aliquots of DNA from B cells stimulated with hydrocortisone and IL-4 were amplified by nested primer PCR. The quantities of serially diluted template DNA used in the first round of PCR are noted above the gel; the second or nested round of PCR used 10% of the original PCR reaction mixture as DNA template. Final PCR products were subjected to agarose gel electrophoresis. In the control lane (C), primers specific for the human IL-1β promoter (−1323/+72) yielded the expected 1.4-kb fragment.

Results and Discussion

Role of hydrocortisone in Cϵ transcription

Highly purified human B cells were incubated with hydrocortisone (10^{-6} M), rIL-4 (200 U/ml), or both for 5 days, and their RNA was analyzed by Northern blotting with the 0.88-kb *Hinf*I probe, which hybridizes to 1.8-kb ϵ-GLT (3). A representative experiment is shown in Figure 1. As previously shown (3), a 1.8-kb ϵ-GLT mRNA was detected in B cells stimulated with IL-4 alone. Hydrocortisone by itself did not induce ϵ-GLT mRNA but slightly enhanced ϵ-GLT accumulation: in two experiments, there was a twofold increase, as assessed by densitometric analysis of ϵ-GLT bands versus actin mRNA. Examination of mRNA from B

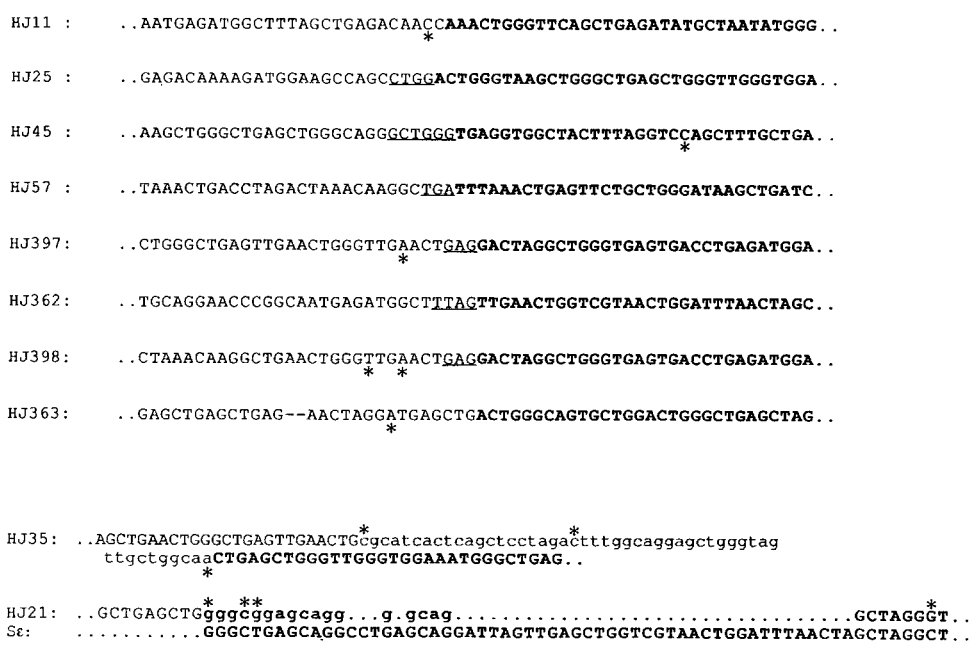

FIGURE 3. Sequence of the switch junction in the cloned fragments. The key indicates nucleotides that originate from Sμ (capital letters), Sε (bold letters), Sγ4 (lower case letters), or from any of the above S regions (underlined letters). Nucleotides noted by an asterisk (*) represent differences from the published S sequences; these differences could represent either a DNA polymorphism, a PCR-generated error, or a point mutation occurred during the switch event. The two hyphens in the HJ363 sequence represent an 876-bp deletion in the Sμ region.

cells cultured for 10 days with hydrocortisone plus IL-4 but not with hydrocortisone or IL-4 alone revealed the presence of an additional 2.0-kb band corresponding to mature Cε transcripts (Fig. 1), as previously reported for B cells stimulated with anti-CD40 and IL-4 (15). Hydrocortisone, and to a lesser extent anti-CD40 mAb, induce vigorous IgE synthesis in the nanogram range (8, 13). Because CD40 engagement results in a marked (7-fold) enhancement in ε-GLT accumulation compared with hydrocortisone, but in less vigorous IgE synthesis (8, 13, 15), the present data suggest that induction of high-rate IgE synthesis by hydrocortisone might reflect effect(s) on step(s) other than ε-GLT.

PCR amplification, cloning, and sequencing of switch fragments from B cells cultured with hydrocortisone and IL-4

We next examined whether IgE isotype switching induced by hydrocortisone in sIgE-B cells was accompanied by deletional switch recombination. Nested primer PCR (14, 15) was conducted to amplify switch fragments containing recombined Sμ/Sε regions. As shown in one such amplification (Fig. 2), distinct fragments were obtained because more diluted high m.w. DNA from sIgE-B cells stimulated with both IL-4 and hydrocortisone for 10 days was used in the first round of PCR. Single distinct fragments were amplified when 10 to 20 ng of template DNA was used. At template DNA concentrations of less than 10 ng, amplified fragments were rarely detected (data not shown). In contrast, no fragments were amplified by this technique from DNA of sIgE-B cells cultured with hydrocortisone alone or IL-4 alone, or from DNA of control sIgE-B cells cultured without either stimulus (data not shown). The detection of Sμ/Sε switch fragments only in IgE-secreting B cell cultures stimulated with both IL-4 and hydrocortisone suggests that at least some of the fragments represent recombination events on the productive chromosome. It is possible, however, that some of the switch fragments represent recombination events on the chromosome in which a nonproductive VDJ rearrangement has occurred (19–21).

Ten switch fragments amplified from DNA isolated from B cell cultures stimulated with both IL-4 and hydrocortisone for 10 days were purified, cloned, and sequenced. The sequences of these fragments at the Sμ/Sε junction are shown in Figure 3. In 8 of 10 fragments (HJ11, HJ25, HJ45, HJ57, HJ397, HJ362, HJ398, and HJ363), the sequence shows direct joining of Sμ to Sε. In one of these eight fragments (HJ36), there was an 876-bp deletion (bp 438–1313) in the Sμ region. Deletions in Sμ have been previously reported in U266 and in two of six Sμ/Sε switch fragments isolated from human B cells stimulated with EBV plus IL4 (14). In the ninth fragment (HJ35), there was a 50-bp insertion at the junction between Sμ and Sε. With the exception of three nucleotides, this 50-bp segment

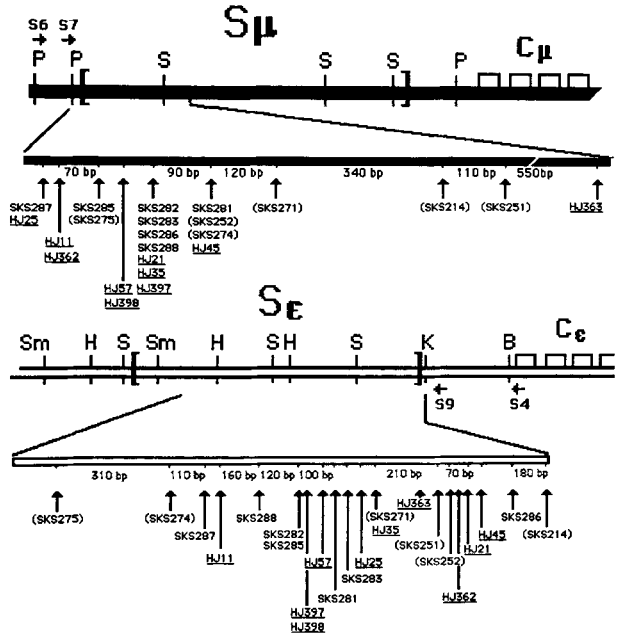

FIGURE 4. Switch recombination sites for the sequenced switch fragments. The positions of the PCR primers upstream from Sμ (S6, S7) and downstream of Sε (S4, S9) are shown. Switch fragments from B cells stimulated with hydrocortisone and IL-4 are underlined. Switch fragments from IgE-secreting EBV-transformed B cells and from U266 IgE myeloma cells are shown in parentheses, whereas switch fragments from B cells stimulated with anti-CD40 mAb and IL-4 are designated in plain characters. Approximate distances between recombination sites are noted (rounded off to the nearest 10 bp). B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sac*I; Sm, *Sma*I.

showed complete homology to a 50-bp segment contained within Sγ4 (22). Two of the three mismatched bases were located, respectively, at the 5′ and 3′-end of the insertion, raising the possibility that these two bases were added at the junction between Sμ and Sγ4 and between Sγ4 and Sε. Thus, the segment derived from Sγ4 is likely to span 48 bp, encompassing nucleotides 3408 to 3455 of the Sγ4 sequence. The sequence of the 10th switch fragment (HJ21) was consistent with a 17-bp insertion at the switch junction derived from Sγ4. Except for three mismatches, one of which is at the 5′-end, the sequence of this 17-bp segment is identical to a segment that encompasses nucleotides 3023 to 3039 in Sγ4. We cannot, however, rule out the possibility that this 17-bp insertion derives from Sγ1, Sγ2, or Sγ3, because the sequence of these S regions is not known. Alternatively, the sequence of the 10th switch fragment could also be consistent with direct Sμ/Sε joining and deletion within Sε. This deletion would start 13-bp downstream of the Sμ/Sε junction and extend over 43 bp. The 5-bp sequence in the place of the deleted segment may represent an insertion. Given the homology between four nucleotides of this 5-bp sequence and a four nucleotide stretch in the Sε-deleted segment (aligned in Fig. 3), a complex deletion involving 38 bp in two discontinuous segments of Sε may have taken place.

The presence of an Sγ4-derived insertion at the Sμ/Sε junction in 1 and possibly 2 of the 10 sequenced fragments suggests that some B cells might have undergone sequential isotype switching from IgM to IgG4 to IgE. This is consistent with the capacity of IL-4 to induce isotype switching to IgG4 (23) as well as to IgE and may underlie the finding that single B cells can give rise to clones that secrete IgM, IgG4, and IgE (6). Evidence for sequential switching to IgE has been obtained in B cells from mice infected with *Nippostrongylus brasiliensis*, as demonstrated by the isolation of ε circles, which were recombinant between Cγ1 and Cε (24). One of the ε circles contained a short segment of Sμ sequence, possibly a trace of the previous switch.

All 12 Sμ/Sε switch fragments previously derived from B cells stimulated with anti-CD40 mAb and IL-4 or with EBV and IL-4 exhibited direct Sμ/Sε joining (14, 15). However, the presence of direct Sμ/Sε joining does not rule out the possibility that sequential switching might have previously occurred. A B cell that first switches to IgG4 and then to IgE would loose Sγ4-derived sequences if the second switch recombination event again involves the Sμ rather than the Sγ4 region. Thus, our finding of 1 in 10 (10%) switch junctions showing evidence of sequential switching is a minimal estimate. Alternatively, nonsequential IgE switching in B cells that receive EBV or anti-CD40 mAb as second signals might reflect a different mode of activation of the recombination machinery compared with hydrocortisone. While this manuscript was being revised, three groups independently showed sequential switching from IgM to IgG to IgE in both mouse and human B cells (25–27). In mice, the switch intermediate was γ1 (25, 26), whereas the exact isotype of the intermediate γ-chain switch in humans was not ascertained (27). The minimal estimate of the percentage of sequential Sμ→Sγ1→Sε switching in murine B cells was 30% (25). It was also shown that sequential switching has occurred in human B cells derived from hyper-IgE patients who have undergone IgE isotype switching in vivo (27).

The maps of the 10 switch fragments isolated from B cells stimulated with hydrocortisone plus IL-4 are shown in Figure 4. Figure 4 also shows the maps of seven switch fragments isolated from normal B cells stimulated with anti-CD40 mAb plus IL-4, five switch fragments isolated from IgE-secreting EBV-transformed B cell lines, and the switch fragment derived from the IgE-secreting plasmacytoma, U266 (14, 15). For 22 of 23 Sμ/Sε fragments, the recombination sites in Sμ were clustered within 900 bp at the 5′-end of the 4502-bp Sμ region. Nineteen of 23 recombination events occurred at six sites within a 300-bp

stretch. In fragment HJ363, which exhibited an 876-bp deletion in the Sμ region, switching occurred 1453 bp from the 5′-end of the Sμ region. In contrast to the sites for Sμ recombination, the sites for Sε recombination were scattered throughout the smaller 2739-bp Sε region. These data suggest that there may be "hot spots" for recombination within Sμ, whereas recombination sites within Sε are less specific. Alternatively, the predilection of the Sμ recombination site in IgE B cells for the 5′-region of Sμ may be explained by sequential switching and/or by internal deletions in Sμ that precede switching to all isotypes. In murine B cells treated with LPS and IL-4 the Sμ recombination sites in Sμ/Sγ junctions are dispersed throughout the Sμ region (28), whereas in murine B cells stimulated with LPS and transforming growth factor β the Sμ recombination sites in Sμ/Sα junctions are clustered in the 3′-half of the Sμ region (29). The different findings from different models can only be reconciled after a better understanding of the molecular mechanisms underlying the switching process is achieved.

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