

Independent Regulation of DNA Recombination and Immunoglobulin (Ig) Secretion during Isotype Switching to IgG1 and IgE

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Summary

Induction of switch recombination to the $\gamma 1$ and ϵ immunoglobulin (Ig) heavy chain loci was examined in B cells preactivated with anti-Ig (B lymphoblasts). In B lymphoblasts cultured with interleukin 4 (IL-4), IL-5 induced the accumulation of $S\mu$ - $S\gamma 1$ rearrangements, but not ϵ recombination. Thus, IL-5 facilitates switch recombination directed to the $\gamma 1$ heavy chain locus by IL-4, but additional signals are required to drive rearrangements to ϵ . Lipopolysaccharide (LPS), in the presence of IL-4, induced the accumulation of both $S\mu$ - $S\gamma 1$ and $S\mu$ - $S\epsilon$ rearrangements, and cells treated with LPS exhibited 40–50-fold more $S\mu$ - $S\gamma 1$ rearrangements than cells cultured with IL-5. Induction of switch recombination was not always associated with secretion of the respective Ig isotype, since concentrations of IL-4 that were sufficient to direct switch recombination to $\gamma 1$ and ϵ in blasts treated with LPS failed to elicit secretion of IgG1 and IgE. These results demonstrate differential requirements for switch recombination to the $\gamma 1$ and ϵ loci, as well as independent regulation of Ig gene rearrangement and secretion of each isotype.

Cytokines produced by activated T cells influence the pattern of Ig isotype expression in B lymphocytes (for a review see reference 1). For example, IL-4 stimulates the production of IgG1 and IgE in murine B lymphocytes and induces IgG4 and IgE secretion from human B cells (2–7). IL-4 stimulates transcription of the unrearranged $\gamma 1$ and ϵ heavy chain loci resulting in the accumulation of germline $\gamma 1$ and ϵ transcripts (8–12). Thus, IL-4 may direct switch recombination to $\gamma 1$ and ϵ heavy chain loci by regulating switch region accessibility to a recombinase, a putative enzyme complex that mediates DNA rearrangement within the Ig heavy chain locus. Although IL-4 alone is sufficient to induce accumulation of germline transcripts in B cells, signals in addition to IL-4 are required for expression of IgG and IgE isotypes (1). In murine B cells, LPS provides a T cell-independent signal for isotype switching (2–5). B lymphocytes cultured with LPS and IL-4 exhibit $S\mu$ - $S\gamma 1$ rearrangements on both the active and dormant alleles (13, 14), suggesting that LPS promotes switch recombination directed to the $\gamma 1$ heavy chain locus by IL-4. However, in B cells activated with anti-Ig, LPS is a poor stimulus for accumulation of $\gamma 1$ and ϵ heavy chain mRNA and secretion of IgG1 and IgE (15). Therefore, LPS does not provide all the signals necessary for robust production of these proteins.

T cells provide costimulatory signals for isotype switching by direct physical contact with B lymphocytes and by the secretion of cytokines such as IL-5 (1, 16). In conjunction with IL-4, IL-5 promotes secretion of IgG1 and IgE from

B cells activated with anti-Ig (15), and IgG1 production from cells cultured with CD4⁺ T cell clones (17) or with membranes isolated from activated T cells (18). In contrast to LPS, IL-5 induces marked accumulation of $\gamma 1$ and ϵ heavy chain mRNA (15, 19). Thus, IL-5 acts in concert with IL-4 to promote expression of the $\gamma 1$ and ϵ heavy chains, but it is not clear whether IL-5 facilitates switch recombination to targeted IgH loci or acts subsequent to IgH rearrangement.

To clarify the mechanism(s) by which IL-5 promotes expression of IgG1 and IgE, we have directly examined switch recombination to the $\gamma 1$ and ϵ heavy chain loci in anti-Ig-activated B cell blasts (B lymphoblasts). Although LPS (with moderate concentrations of IL-4) failed to stimulate secretion of IgG1 and IgE, we show that LPS induced marked accumulation of $S\mu$ - $S\gamma 1$ and $S\mu$ - $S\epsilon$ rearrangements, indicating that switch recombination to $\gamma 1$ and ϵ can be dissociated from secretion of IgG1 and IgE. Conversely, IL-5 stimulated marked secretion of IgG1, but was less effective than LPS as a stimulus for recombination to $\gamma 1$ and failed to induce ϵ recombination. These results demonstrate that IL-5 regulates recombination to Ig loci targeted by IL-4 as well as expression of the rearranged $\gamma 1$ and ϵ heavy chain loci subsequent to recombination.

Materials and Methods

Mice. Female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA) and used at 10–14 wk of age.

Reagents. Affinity-purified antibodies were obtained from Jack-

son ImmunoResearch Laboratories, Inc. (West Grove, PA; goat anti-mouse IgM plus IgD, goat anti-mouse IgG, Fc fragment), Fisher Biotech (Pittsburgh, PA; biotinylated goat anti-mouse IgG1, mouse IgG1- κ standard), and Pharmingen (San Diego, CA; monoclonal rat anti-mouse Thy-1.2 and rat anti-mouse-CD4, biotinylated monoclonal rat anti-mouse IgE, mouse IgE- κ standard). ELISA substrate *p*-nitrophenyl phosphate and *Salmonella typhosa* LPS were purchased from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase-conjugated avidin was obtained from Fisher Biotech. Recombinant murine IL-4 and rIL-5 were prepared using the Baculovirus expression system and the activity of Sf9 cell supernatants was determined as described (15).

Cell Culture. Spleen cell suspensions were enriched for B lymphocytes by incubation with anti-Thy 1.2 and anti-CD4 followed by lysis with baby rabbit serum (Pel-Freeze Biologicals, Rogers, AR). High density B cells (1.081–1.086 g/ml) were isolated on discontinuous Percoll density gradients as described (20). Low density B cell blasts (anti-Ig blasts or B lymphoblasts) were prepared by culturing high density B cells with anti-Ig-Sepharose (0.5 ml of 10% Sepharose/10 ml RPMI 1640, 5% FCS, 5 μ g/ml gentamicin, and 50 μ M 2-ME) at 1–1.5 \times 10⁶ cells/ml for 2 d. At the end of the 2-d primary culture with anti-Ig, B lymphoblasts were either recultured (along with Sepharose beads) in 96-well microtiter plates at 2 \times 10⁵/ml (Ig secretion) or 4 \times 10⁵/ml (MTT assay) with lymphokines and LPS, or secondary stimuli were added directly to bulk cultures with anti-Ig-Sepharose for experiments requiring isolation of genomic DNA.

Proliferation and Ig Secretion Assay. B lymphoblast growth and viability was assessed in microcultures using the MTT ([3], 4,5-dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide) assay of Mosmann (21). Levels of IgG1 and IgE in culture supernatants were determined by solid phase ELISA as described (15) except that biotinylated rat anti-mouse IgE was used as the secondary antibody in the IgE ELISA and NP-40 was not included in the ELISA buffer.

Isolation of Genomic DNA. B lymphoblasts (0.5–1 \times 10⁷) cultured with LPS or lymphokines for 2–4 d were washed with PBS and lysed by resuspension in 0.6 ml of TNES buffer (1% SDS, 0.1 M EDTA, pH 8.0, 50 mM Tris, pH 8.0, 0.1 M NaCl). Cell lysates were digested with proteinase K (200 μ g) for 16 h at 55°C, followed by digestion with 20 μ g/ml RNase A for 1–2 h at 37°C. Genomic DNA was sequentially extracted with an equal volume of phenol, phenol/chloroform (50% vol/vol) followed by chloroform (100%), and then precipitated with an equal volume of isopropanol followed by a single wash with 80% ethanol. Genomic DNA was quantitated by UV spectrophotometry (1 OD A₂₆₀ = 50 μ g/ml).

Inverse PCR Assay. Genomic DNA (20 μ g) was digested with an initial aliquot of XbaI (Boehringer Mannheim, Indianapolis, IN) (2–3 U/ μ g) for 2–4 h at 37°C, after which a second aliquot was added and allowed to digest overnight at 37°C. After the overnight incubation, a third aliquot of XbaI (2–3 U/ μ g) was added for an additional 2–4 h. The XbaI enzyme was inactivated at 65°C for 15 min, and protein was removed from digested DNA by either phenol/chloroform (50:50% vol/vol), followed by chloroform extraction, or by centrifugation through polyvinylidene fluoride filters (Integrated Separation Systems, Natick, MA). The XbaI digests were precipitated with ethanol and quantitated by UV spectrophotometry.

XbaI-digested DNA (2 μ g/ml) was circularized by incubation with 0.02 Weiss U/ μ l T4 DNA ligase (Boehringer Mannheim) overnight at 16°C. Aliquots from ligation reactions containing 10–150 ng of DNA were placed directly into PCR reaction tubes and denatured by heating at 94°C for 30 min and then held at

4°C while a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 8.8, at 25°C, 1.5 mM MgCl₂, 1% Triton X-100, 200 μ M each dNTP, 2 μ M each primer, and 2.5 U *Taq* DNA Polymerase (Promega Corp., Madison, WI or Boehringer Mannheim) was added to a final volume of 50 μ l and overlaid with mineral oil (Sigma Chemical Co.). Amplification was performed using a DNA thermal cycler (Perkin Elmer Cetus; Norwalk, CT) and the following cycling parameters: 20–40 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min. For detection of S μ -Se rearrangements, the following modifications were employed: (a) the annealing temperature was increased to 68°C; (b) the total reaction volume was increased to 100 μ l; (c) the PCR reaction mixture was added to denatured DNA samples held at 80°C; and (d) 2 U/100 μ l of Perfect Match™ Polymerase Enhancer (Stratagene, La Jolla, CA) was included in the reaction mixture.

Primers for PCR. DNA oligomers were purchased from Operon Technol., Inc. (Alameda, CA). The S μ (γ 1), 5'-TTC ACA CAG AGC ATG TGG ACT GGC T-3', and the S μ (ϵ), 5'-CTG AAG TAG AGA CAG CAT CAG TAC C-3' oligomers correspond to nucleotides 3970–3946 or 4502–4478, respectively, of the antisense strand of the GenBank code MUSIGCD07 (sequences are available from EMBL/GenBank/DBJ under accession numbers J00440, J00480, and V01524). These oligomers prime synthesis of the antisense strand across the XbaI site (position 3868) immediately 5' to the S μ region. The Sy1 oligomer 5'-GAG AGC AGG GTC TCC TGG GTA GG-3', which corresponds to nucleotides 8893–8915 of the MUSIGHANB sequence (accession number M12389), primes synthesis of the sense strand across the XbaI site located 3' of the Sy1 region. The Sy1 primer anneals to a sequence located 51 nucleotides upstream of the EcoRI site within the 3' portion of the Sy1 region (22). The S ϵ Xba4 primer, 5'-GAT GAA AGT AAG CTG AGT TGG GCA G-3', corresponds to nucleotides 2382–2406 of the MUSIGHAHX sequence (accession numbers M57385 and X53677). The S ϵ Xba4 primer anneals to sequence immediately 5' to the XbaI cleavage site at nucleotide 2408. The 5' globin-XbaI oligomer, 5'-GGT TGA GCA TTA GAT CTG TCT ACA G-3' (nucleotides 14876–14852 of the MUSBGCXD sequence; accession numbers M36857, X14061, M27616, and M36856) primes the antisense strand 201 bases 3' to the XbaI site at position 14675. The 3' globin-XbaI oligomer, 5'-ATC TCT TGT CAA GGC TGG TGA TCT T-3' (nucleotides 17205–17229 of the MUSBGCXD sequence), primes the sense strand 388 bases 5' to the XbaI site at nucleotide 17593.

Southern Analysis. Products of PCR amplification were electrophoresed through 1% agarose gels. DNA in agarose gels was denatured by incubation in 1.5 M NaCl, 0.5 M NaOH for 30 min with agitation, and neutralized by incubation in 1.5 M NaCl, 0.5 M Tris, pH 6.0, for an additional 30 min. DNA was then transferred to nylon membranes (Stratagene) (23), cross-linked to the filters using the UV Stratalinker™ 2400 (Stratagene), and hybridized to ³²P-labeled DNA probes (see below). Hybridizations were carried out at 68°C for 1–4 h using QuikHyb™ solution (Stratagene), and filters were washed according to the protocol recommended by the manufacturer. After air drying, filters were exposed to Kodak X-OMAT AR film for 1–6 h at –70°C with an enhancer screen. The migration of HaeIII-digested PhiX174 molecular weight markers (New England Biolabs Inc., Beverly, MA) were used to verify the length of PCR products.

DNA Probes. The β -globin probe was composed of a 336-bp PCR product amplified using 3' globin-XbaI oligomer and the 3' globin primer 5'-GCA TTG TCT CAT CTC TTG ACA GGT C-3' (nucleotides 17541–17517 of GenBank code MUSBGCXD), ligated

to a 463-bp product amplified using the 5' globin primer, 5'-TGA TGT CCA GCA TTC TGT ACA CCC-3' (nucleotides 14413-14436 of GenBank code MUSBGXD), and the 5' globin-XbaI primer. The PCR-generated globin fragments described above were joined by PCR amplification to form a 799-bp β -globin probe using the 3' and 5' globin-XbaI primers. In some experiments, the products of either the 5' globin or the 3' globin primers were used as DNA probes. A ~500-bp fragment amplified from EcoRI-digested and circularized MOPC21 DNA using the $S\mu(\gamma 1)$ and $S\gamma 1$ primers was used to probe PCR products amplified from $S\mu$ - $S\gamma 1$ rearrangements. The $S\mu$ - $S\epsilon$ probe consisted of a 349-bp fragment generated by PCR amplification using $S\mu(\gamma 1)$ and the $S\epsilon$ XbaI primer, 5'-GCT GAG TCA TAC TAC AGT GCA CTG A-3' corresponding to nucleotides 468-493 of the Ige-7 clone (24). The β -globin $S\mu$ - $S\gamma 1$, and $S\mu$ - $S\epsilon$ probes were cloned into the pCR™ II TA cloning vector (Invitrogen, San Diego, CA). Insert DNA was released from the vector by digestion with KpnI and ApaI, or PCR amplified from vector DNA using appropriate primers. DNA fragments in low melt agarose were ^{32}P -labeled using 3,000 Ci/mmol α - ^{32}P dCTP (NEN/Dupont, Wilmington, DE) and the Prime-it II Random Primer Kit (Stratagene) according to protocols provided by the manufacturer.

Results

Differential Effects of IL-5 and LPS on B Lymphoblast Proliferation and Ig Isotype Production. The capacity of IL-5 and LPS to stimulate proliferation as well as IgG1 and IgE production by IL-4-treated blasts was compared by culturing B lymphoblasts with IL-4 (20–2,000 U/ml) and either LPS or IL-5. B lymphoblasts proliferated extensively when cultured with LPS or LPS plus IL-4 (Fig. 1) but were apparently unresponsive to IL-5 in isolation. The combination of IL-4 and IL-5 caused limited B lymphoblast proliferation (determined by the number of cells recovered at the end of 2- and 3-d cultures, data not shown) and enhanced long-term viability of cells in culture as measured by the MTT assay (Fig. 1).

Previous experiments indicated that secretion of IgG1 and IgE were differentially regulated (15). This is illustrated in Fig. 1 where culture of B lymphoblasts with optimal concentrations of IL-4 and IL-5 resulted in marked secretion of IgG1 but no detectable IgE even at supraoptimal concentrations of IL-4 (2,000 U/ml). Inclusion of LPS in cultures with IL-4 promoted both IgG1 and IgE secretion, but high concentrations of IL-4 (2,000 U/ml) were required to elicit the secretion of either isotype in the absence of IL-5. Virtually no IgG1 or IgE secretion was observed in cultures with LPS, IL-4, or IL-5 alone. These results confirm previous observations (15) demonstrating that LPS and IL-5 can each promote IL-4-dependent IgG1 production, but only LPS appears capable of inducing IgE production.

A Semi-quantitative Assay for Switch Recombination Based on Inverse PCR. To better understand the mechanisms by which LPS and IL-5 regulate Ig isotype expression, we developed a semi-quantitative assay for $S\mu$ - $S\gamma 1$ and $S\mu$ - $S\epsilon$ rearrangements based on the technique reported by Chu et al. (25). $S\mu$ - $S\gamma 1$ recombination juxtaposes two XbaI sites flanking the $S\mu$ and the $S\gamma 1$ regions (Fig. 2). Similarly, ϵ recombination juxtaposes the XbaI 5' to $S\mu$ with an XbaI site within the 3' portion of the $S\epsilon$ region (26). Since this XbaI site occurs within

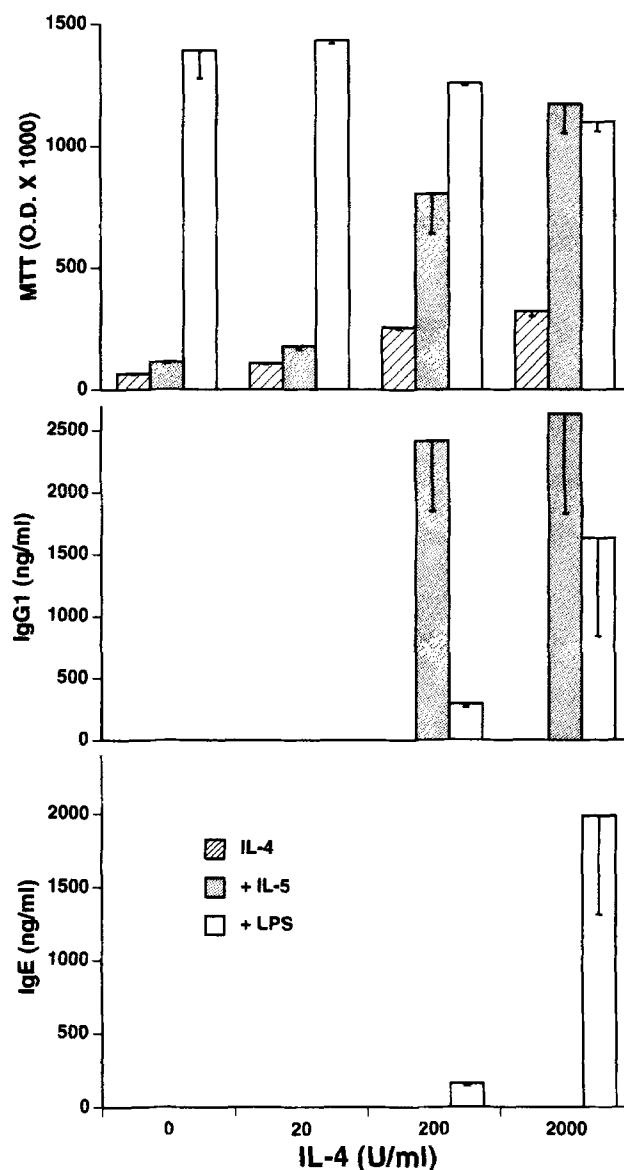


Figure 1. B lymphoblasts were cultured at 4×10^5 /ml (MTT assay) or 2×10^5 /ml (Ig secretion) with the indicated combinations of IL-4, IL-5 (50 U/ml), and LPS (20 $\mu\text{g}/\text{ml}$). B lymphoblast proliferation and viability was assessed by the MTT assay on the fourth day of culture. The quantity of secreted IgG1 and IgE in culture supernatants was determined by ELISA on day 5 and 7, respectively. Results presented are the mean and standard deviation of triplicate wells from a representative experiment.

the $S\epsilon$ region, rearrangements downstream of this site are not detected with this technique. Products of $S\mu$ - $S\gamma 1$ and $S\mu$ - $S\epsilon$ recombination were detected by circularization of XbaI-digested genomic DNA, and subsequent PCR amplification across the religated XbaI sites flanking the respective rearrangements. Since this PCR strategy does not amplify across the site of recombination, the 835- and 661-bp products will be generated irrespective of the site of recombination within $S\mu$ and the $S\gamma 1$ or $S\epsilon$ regions, but will not be generated in the absence of DNA recombination.

As an internal control for the efficiency of the XbaI digestion and ligation reactions, a segment of the β -globin gene

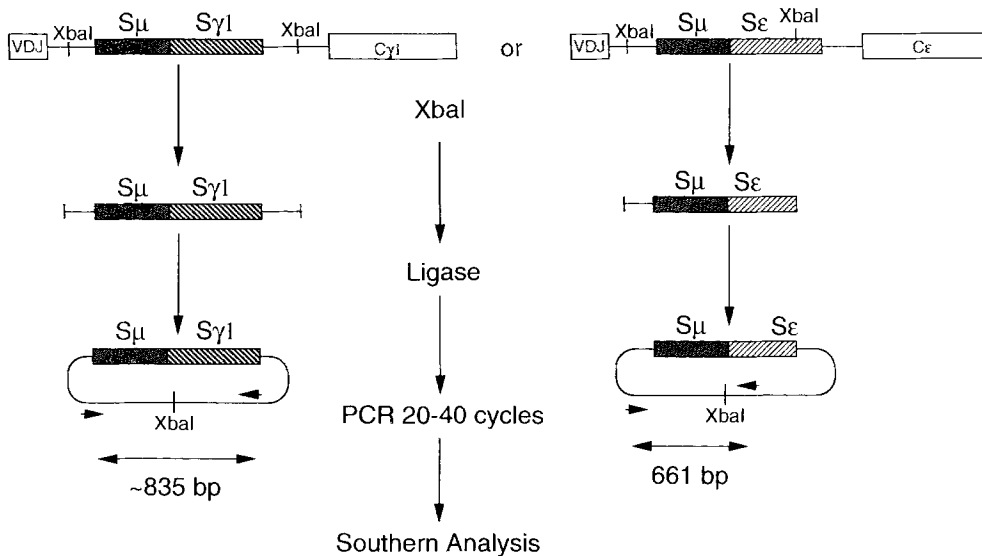


Figure 2. Strategy for detection of $S\mu$ - $S\gamma 1$ or $S\mu$ - $S\epsilon$ rearrangements. $S\mu$ - $S\gamma 1$ recombination juxtaposes an XbaI cleavage site immediately upstream of the $S\mu$ region with an XbaI site downstream of the $S\gamma 1$ region, whereas ϵ recombination juxtaposes the XbaI site 5' of $S\mu$ with an XbaI site in the downstream portion of the $S\epsilon$ region. Recombination to $\gamma 1$ or ϵ is detected by first digesting genomic DNA with XbaI, and then circularizing the resulting XbaI fragments with DNA ligase. PCR using the $S\mu(\gamma 1)$ and $S\gamma 1$ primers produces a 835-bp fragment from amplification of circularized $S\mu$ - $S\gamma 1$ rearrangements. Similarly the $S\mu(\epsilon)$ and $S\epsilon$ Xba4 primers yield a 661-bp product from circularized $S\mu$ - $S\epsilon$ rearrangements. PCR-amplified products are detected by Southern blot analysis using PCR-generated probes.

complex was amplified from XbaI-digested and -circularized DNA using β -globin-specific primers. All cells should provide equivalent target DNA for the β -globin primers, and a 589-bp product should be amplified independent of DNA rearrangements within the Ig heavy chain locus. In the experiment shown in Fig. 3, genomic DNA was isolated from B lymphoblasts after 3 or 4 d of culture, digested with XbaI, then circularized as described in Materials and Methods. Aliquots from each ligation were placed directly into PCR reactions containing β -globin primers. A semi-quantitative assessment of the amount of target DNA was achieved by removing 10 μ l aliquots from PCR reactions in three-cycle intervals. Since PCR amplification is a geometric progression, the amount of PCR product should increase eightfold with each three-cycle interval. Specific PCR products were detected by Southern analysis using PCR generated probes (see Materials and Methods). Amplification of the globin gene segment was detected after 23 cycles in each DNA sample, and product synthesis was comparable in each reaction through

26 cycles indicating that each DNA sample contained a comparable amount of target DNA (Fig. 3).

The sensitivity of the inverse PCR assay for detecting $S\mu$ - $S\gamma 1$ and $S\mu$ - $S\epsilon$ rearrangements was estimated by combining hybridoma cells with known rearrangements to either $\gamma 1$ (MOPC 21, an IgG1-producing myeloma) or ϵ (H1E cells, an IgE-producing hybridoma) with WEHI-231 cells (an IgM-expressing B lymphoma) at ratios ranging from 1:10 to 1:10,000. Genomic DNA was isolated from 10^7 total cells as described in Materials and Methods. Amplification was performed with the appropriate primer pair over 23–28 cycles and rearrangements detected by Southern analysis of PCR products. Under these conditions rearrangements to either the $\gamma 1$ or ϵ loci could be detected at cell ratios of 1 in 10,000, and appeared linear at ratios of 1:10 to 1:1,000 (data not shown).

IL-5 Induces Switch Recombination to the $\gamma 1$ Heavy Chain Locus. The ability of LPS or IL-5 to promote IL-4-directed recombination to $\gamma 1$ was evaluated by culturing B lympho-

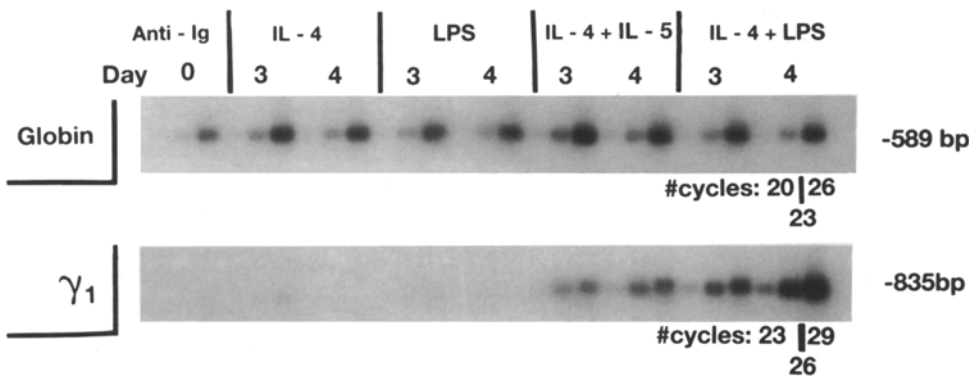


Figure 3. IL-5 induces $S\mu$ - $S\gamma 1$ rearrangements in B lymphoblasts cultured with IL-4. Genomic DNA was isolated from B lymphoblasts on day 0 or after an additional 3 or 4 d of culture with the indicated combinations of IL-4 (200 U/ml), IL-5 (50 U/ml), and LPS (20 μ g/ml). The DNA was digested with XbaI, and circularized by ligation. 5-(10 ng, globin amplification) or 25- μ l (50 ng, $S\mu$ - $S\gamma 1$ rearrangement) aliquots were taken directly from ligation reactions and subjected to PCR amplification using either the β -globin primers

or the $S\mu(\gamma 1)$ and $S\gamma 1$ primers. 10- μ l aliquots were removed from PCR reaction after the indicated number of cycles. PCR products were detected by Southern blot analysis.

blasts with IL-4 alone or in combination with LPS or IL-5 for 3 and 4 d. The appearance of $S\mu$ - $S\gamma$ 1 rearrangements in the DNA samples was examined by PCR amplification using the $S\mu(\gamma$ 1) and $S\gamma$ 1 primers. Although IL-4 is not by itself mitogenic for B lymphoblasts (27) it maintained the viability of blasts in culture for several days in the absence of other added cytokines. This observation allowed analysis of the effects of IL-4 alone on DNA recombination. In three independent experiments, no $S\mu$ - $S\gamma$ 1 rearrangements were detected in B lymphoblasts cultured for up to 4 d with IL-4 alone, suggesting that IL-4 cannot drive recombination to the γ 1 heavy chain locus. In contrast, γ 1 recombination was detected in DNA isolated from B lymphoblasts cultured with IL-5 and IL-4 for 3 and 4 d. Thus, IL-5 can promote switch recombination that is directed to the γ 1 heavy chain locus by IL-4. Similar findings were recently reported by Mandler et al. (28).

Consistent with previous reports (13, 14, 25, 29), LPS also induced marked accumulation of $S\mu$ - $S\gamma$ 1 rearrangements. Switch recombination to γ 1 was readily detectable in DNA isolated from cells stimulated with LPS and IL-4 (200 U/ml) after 3 d of culture, and the apparent number of $S\mu$ - $S\gamma$ 1 rearrangements increased after 4 d. In fact, $S\mu$ - $S\gamma$ 1 rearrangements were observed as early as 2 d after addition of LPS and IL-4 (Fig. 4).

LPS Is More Effective than IL-5 as a Stimulus for Switch Recombination to γ 1. The results presented in Fig. 3 suggest that B lymphoblasts cultured with LPS contained more $S\mu$ - $S\gamma$ 1 rearrangements than blasts cultured with IL-5. This observation contrasts with data presented in Fig. 1 demonstrating that IL-5 is more effective than LPS as a stimulus for IgG1 secretion. To further explore this observation, we focused on examination of $S\mu$ - $S\gamma$ 1 rearrangements at earlier time points to reduce the possibility of selective outgrowth of switched cells by LPS. B lymphoblasts were cultured with IL-4 and optimal concentrations of either LPS or IL-5, genomic DNA was isolated after 2 and 3 d of culture, and $S\mu$ - $S\gamma$ 1 rearrangements were examined by inverse PCR. As shown in Fig. 4,

$S\mu$ - $S\gamma$ 1 rearrangements were detected after only 20 cycles of PCR in DNA isolated from blasts treated with LPS and IL-4. In contrast, detection of γ 1 recombination in DNA from B lymphoblasts cultured with IL-5 required an additional six cycles. Under ideal conditions, six cycles of PCR amplifies the amount of PCR product 64-fold. Therefore, blasts cultured with LPS and IL-4 appear to contain at least 40–50-fold more γ 1 rearrangements than blasts cultured with IL-5. These results suggest that B lymphoblasts treated with LPS undergo switch recombination to γ 1 at a higher frequency than cells treated with IL-5.

LPS but Not IL-5 Induces Accumulation of $S\mu$ - $S\epsilon$ Rearrangements. In a previous study we were unable to detect germline ϵ transcripts or secretion of IgE in B lymphoblasts stimulated with IL-4 and IL-5 (15, Fig. 1). Accumulation of germline ϵ transcripts and secretion of IgE could be induced by inclusion of LPS in cultures with IL-4 (15). These results suggested that in the absence of LPS, IL-4 did not target the ϵ heavy chain locus for rearrangement. We examined this further by analyzing the ability of IL-5 or LPS to induce $S\mu$ - $S\epsilon$ rearrangements in B lymphoblasts cultured with IL-4. In three independent experiments, IL-5 and high concentrations of IL-4 (2,000 U/ml) did not promote accumulation of $S\mu$ - $S\epsilon$ rearrangements (Fig. 5). In contrast, LPS (plus IL-4) stimulated marked accumulation of $S\mu$ - $S\epsilon$ rearrangements that were detectable after 3 d of culture, and the apparent number of recombination events increased after 4 d. Induction of ϵ recombination was dependent on IL-4 as LPS alone had no effect. These results demonstrate that LPS facilitates isotype switching to IgE by promoting ϵ recombination.

As shown in Fig. 1, exceptionally high concentrations of IL-4 are required to elicit IgG1 and IgE secretion from B lymphoblasts cultured with LPS. Therefore, it was intriguing to find that moderate concentrations of IL-4 (200 U/ml), which did not promote IgG1 and IgE secretion, were sufficient to mediate γ 1 and ϵ recombination with LPS. Increasing the IL-4 concentration 10-fold consistently increased the apparent

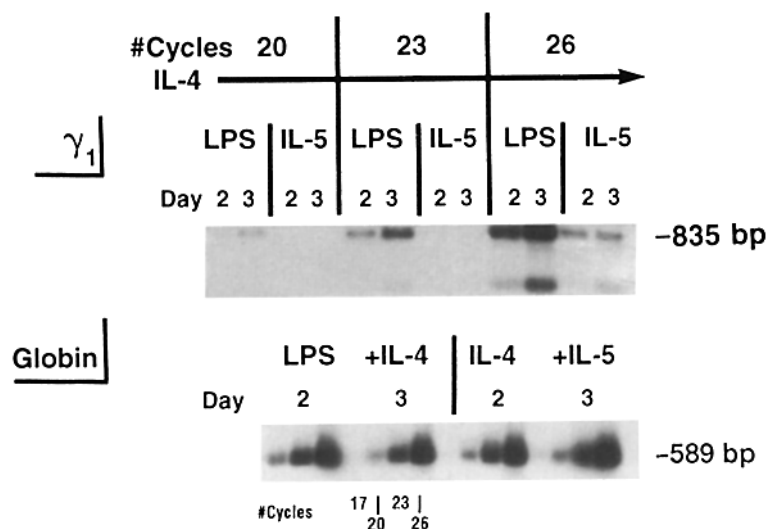


Figure 4. LPS is more effective than IL-5 as a stimulus for γ 1 recombination. Genomic DNA was isolated from B lymphoblasts cultured as in Fig. 3 for 2 and 3 d. PCR amplification of XbaI cut-circularized DNA and detection of PCR product was carried out as in Fig. 3 except that aliquots were removed from reactions after 20, 23, and 26 cycles. Occasionally, amplification using the $S\mu(\gamma$ 1)- $S\gamma$ 1 primers yielded the anticipated 835-bp product as well as a second smaller band. The second band may result from annealing of the $S\gamma$ 1 primer at a site 3' to the intended target sequence. Amplification of the β -globin sequences was equivalent for each DNA sample.

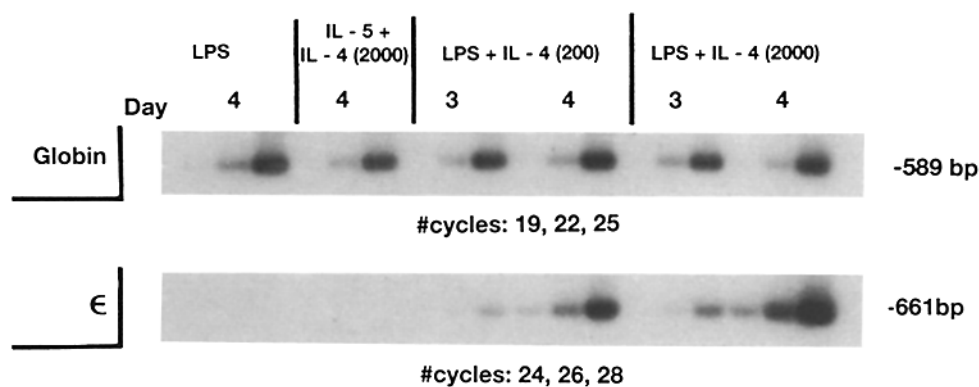


Figure 5. LPS, but not IL-5, induces $S\mu$ -Se rearrangement in B lymphoblasts stimulated with IL-4. B lymphoblasts were cultured as in Fig. 3, except that 2,000 U/ml IL-4 was included in cultures with IL-5 and some cultures with LPS. 75- μ l aliquots containing 150 ng of DNA from ligation reactions were subjected to PCR amplification using the $S\mu(\epsilon)$ and SeXBa4 primers. 20- μ l aliquots were removed from PCR reactions after the indicated number of cycles, and the amount of PCR product was determined by Southern blot analysis.

number of $S\mu$ -Se rearrangements, but it seems unlikely that this increase in ϵ recombination explains the marked stimulation of IgE secretion observed with high concentrations of IL-4. These results clearly demonstrate that induction of switch recombination to the $\gamma 1$ and ϵ loci can be dissociated from secretion of the respective isotype.

Discussion

The current model for IL-4-directed switching to IgG1 and IgE suggests that IL-4 targets the $\gamma 1$ and ϵ heavy chain loci for recombination by regulating accessibility to a switch recombinase. Accessibility is manifested by transcription of the unrearranged loci and by accumulation of germline transcripts (1). However, IL-4 alone is not sufficient to promote expression of IgG1 or IgE (1), or DNA rearrangement to either loci (Figs. 3 and 5). Results presented here (Figs. 3 and 4) and elsewhere (28) indicate that IL-5 induces the accumulation of $S\mu$ -S $\gamma 1$ rearrangements in B cells treated with anti-Ig and IL-4. In addition to inducing $\gamma 1$ recombination, IL-5 promotes the accumulation of $\gamma 1$ heavy chain mRNA and secretion of IgG1 (15–19). These results indicate that IL-5 provides signals that are required for completion of isotype switching to IgG1, but the mechanism by which IL-5 promotes accumulation of $S\mu$ -S $\gamma 1$ rearrangements has not been established. It appears unlikely that IL-5 promotes selective outgrowth of cells that have already switched to IgG. Therefore, we favor a model in which IL-5 induces the expression or activity of components of the putative switch recombinase. Direct examination of this hypothesis awaits identification and characterization of molecular components that catalyze rearrangements within the Ig heavy chain locus.

Optimal stimulation of switch recombination requires signals in addition to those provided by IL-5. Thus, IL-5 failed to induce ϵ recombination and LPS was more effective than IL-5 as a stimulus for $S\mu$ -S $\gamma 1$ rearrangement. Although LPS

was also a more effective stimulus for B lymphoblast proliferation than IL-5, the greater number of $S\mu$ -S $\gamma 1$ rearrangements in cultures with LPS is probably not due to selective outgrowth of switched cells. Recombination to $\gamma 1$ could be detected after only 2 d of culture with LPS and IL-4 (Fig. 4), an insufficient time for substantial expansion of a small subset of cells. Instead, LPS may be a superior stimulus for recombination because of its ability to induce expression of germline transcripts from unrearranged $\gamma 1$ and ϵ heavy chain loci. We and others have demonstrated that LPS promotes the accumulation of germline transcripts induced by IL-4 (8, 12, 15), whereas IL-5 does not (19, and our unpublished observations). The effect of LPS on accumulation of germline ϵ transcripts is due, at least in part, to an increased rate of transcription through the ϵ heavy chain locus (30). LPS may regulate the expression or activity of *trans*-acting factors that alter the chromatin state of the ϵ locus by binding to promoter and/or enhancer elements, which in turn may facilitate germline transcription as well as recombination.

It is noteworthy that the appearance of $S\mu$ -S $\gamma 1$ or $S\mu$ -Se rearrangements is not always accompanied by secretion of IgG1 and IgE. For example, concentrations of IL-4 that with LPS caused readily detectable $\gamma 1$ and ϵ recombination (Figs. 3–5), did not elicit secretion of either isotype (Fig. 1) or substantial accumulation of VDJ heavy chain mRNA (15). This suggests that additional factors are necessary for transcription of rearranged heavy chain alleles or mRNA stabilization. It is clear that IL-5 can stimulate the production or activity of such factors since it markedly enhances the appearance of $\gamma 1$ and ϵ mRNA in cells that have undergone rearrangements initiated by IL-4 and LPS (15, and Figs. 3 and 4). Although IL-5 can signal recombination to $\gamma 1$ (28, and Fig. 3), our data suggest that the primary role of IL-5 in isotype switching may be to promote expression of the $\gamma 1$ and ϵ heavy chain loci subsequent to recombination.

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References

1. Purkerson, J.M., and P.C. Isakson. 1992. A two-signal model for the regulation of immunoglobulin isotype switching. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:3245.
2. Isakson, P.C., E. Pure, E.S. Vitetta, and P. Krammer. 1982. T cell-derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* 155:734.
3. Noma, Y., P. Sideras, T. Naito, S. Bergstedt-Lindquist, C. Azuma, E. Severinson, T. Tanabe, T. Kinashi, F. Matsuda, Y. Yaoita, and T. Honjo. 1986. Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. *Nature (Lond.)* 319:640.
4. Coffman, R.L., J. Ohara, M.W. Bond, J. Carty, A. Zlotnik, and W.E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4538.
5. Snapper, C.M., F.D. Finkelman, and W.E. Paul. 1988. Differential regulation of IgG1 and IgE synthesis by interleukin 4. *J. Exp. Med.* 167:183.
6. Pene, J., F. Rousset, F. Briere, I. Chretien, J-Y. Bonnefoy, H. Spits, T. Yokota, N. Arai, K.-I. Arai, J. Blanchereau, and J.E. De Vries. 1988. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E_2 . *Proc. Natl. Acad. Sci. USA.* 85:6880.
7. Lundgren, M., U. Persson, P. Larsson, C. Magnusson, C.I.E. Smith, L. Hammarstrom, and E. Severinson. 1989. Interleukin 4 induces synthesis of IgE and IgG₄ in human B cells. *Eur. J. Immunol.* 19:1311.
8. Berton, M.T., J.W. Uhr, and E.S. Vitetta. 1989. Synthesis of germ-line $\gamma 1$ immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon γ . *Proc. Natl. Acad. Sci. USA.* 86:2829.
9. Esser, C., and A. Radbruch. 1989. Rapid induction of transcription of unrearranged S $\gamma 1$ switch regions in activated murine B cells by interleukin 4. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:483.
10. Rothman, P., S. Lutzker, W. Cook, R. Coffman, and F.W. Alt. 1988. Mitogen plus interleukin 4 induction of C ϵ transcripts in B lymphoid cells. *J. Exp. Med.* 168:2385.
11. Stavnezer, J., G. Radcliffe, Y. Lin, J. Nietupski, L. Berggren, R. Sitia, and E. Severinson. 1988. Immunoglobulin heavy chain switching may be directed by prior induction of transcripts from constant region genes. *Proc. Natl. Acad. Sci. USA.* 85:7704.
12. Gerondakis, S. 1990. Structure and expression of murine germ-line immunoglobulin ϵ heavy chain transcripts induced by interleukin 4. *Proc. Natl. Acad. Sci. USA.* 87:1581.
13. Radbruch, A., W. Muller, and K. Rajewsky. 1986. Class switch recombination is IgG₁ specific on active and inactive IgH loci of IgG₁-secreting B-cell blasts. *Proc. Natl. Acad. Sci. USA.* 83:3954.
14. Kepron, M.R., Y. Chen, J.W. Uhr, and E.S. Vitetta. 1989. IL-4 induces the specific rearrangement of $\gamma 1$ genes on the expressed and unexpressed chromosomes of lipopolysaccharide-activated normal murine B cells. *J. Immunol.* 143:334.
15. Purkerson, J.M., and P.C. Isakson. 1992. Interleukin 5 (IL-5) provides a signal that is required in addition to IL-4 for isotype switching to (Ig) G1 and IgE. *J. Exp. Med.* 175:973.
16. Noelle, R.J., and E.C. Snow. 1991. T helper cell-dependent B cell activation. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:2770.
17. DeKruyff, R.H., T.R. Mosmann, and D.T. Umetsu. 1990. Induction of antibody synthesis by CD4⁺ T cells: IL-5 is essential for induction of antigen-specific antibody responses by T_H2 but not T_H1 clones. *Eur. J. Immunol.* 20:2219.
18. Noelle, R.J., J. Daum, W.C. Bartlett, J. McCann, and D.M. Shepherd. 1991. Cognate interactions between helper T cells and B cells. V. Reconstitution of T helper cell function using purified plasma membranes from activated Th1 and Th2 helper cells and lymphokines. *J. Immunol.* 146:1118.
19. Noelle, R.J., D.M. Shepherd, and H.P. Fell. 1992. Cognate interaction between T helper cells and B cells. VII. Role of contact and lymphokines in the expression of germline and mature $\gamma 1$ transcripts. *J. Immunol.* 149:1164.
20. Birkeland, M.L., L. Simpson, P.C. Isakson, and E. Pure. 1987. T-independent and T-dependent steps in the murine B cell response to antiimmunoglobulin. *J. Exp. Med.* 166:506.
21. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55.
22. Mowatt, M.R., and D.W. Dunnick. 1986. DNA sequence of the murine $\gamma 1$ switch segment reveals novel structural elements. *J. Immunol.* 136:2674.
23. Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.
24. Nikaido, T., Y. Yamawaki-Kataoka, and T. Honjo. 1982. Nucleotide sequences of switch regions of immunoglobulin C ϵ and C γ genes and their comparison. *J. Biol. Chem.* 257:7322.
25. Chu, C.C., W.E. Paul, and E.E. Max. 1992. Quantitation of immunoglobulin μ - $\gamma 1$ heavy chain switch region recombination by a digestion-circularization polymerase chain reaction method. *Proc. Natl. Acad. Sci. USA.* 89:6978.
26. Scappino, L.A., C. Chu, and C.A. Gritzmacher. 1991. Extended nucleotide sequence of the switch region of the murine gene encoding immunoglobulin E. *Gene (Amst.)* 99:295.
27. Simpson, L., I. McNiece, M. Newberg, J. Schetz, K.R. Lynch, P. Quesenberry, and P.C. Isakson. 1989. Detection and characterization of a B cell stimulatory factor (BSF-TC) derived from a bone marrow stromal cell line. *J. Immunol.* 142:3894.
28. Mandler, R., C.C. Chu, W.E. Paul, E.E. Max, and C.M. Snapper. 1993. Interleukin 5 induces S μ -S $\gamma 1$ rearrangement in B cells activated with dextran-anti-IgD antibodies and interleukin 4: a three component model for Ig class switching. *J. Exp. Med.* 178:1577.
29. Chu, C.C., E.E. Max, and W.E. Paul. 1993. DNA rearrangement can account for in vitro switching to IgG1. *J. Exp. Med.* 178:1381.
30. Rothman, P., S.C. Li, B. Gorham, L. Glimcher, F.W. Alt, and M. Boothby. 1991. Identification of a conserved lipopolysaccharide-plus-interleukin-4-responsiveness element located at the promoter of germline ϵ transcripts. *Mol. Cell. Biol.* 11:5551.