Interleukin 5 Induces Sμ-Sγ1 DNA Rearrangement in B Cells Activated With Dextran-anti-IgD Antibodies and Interleukin 4: A Three Component Model for Ig Class Switching

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Summary

The cellular signals required for induction of immunoglobulin (Ig) class switching are only partially understood. Two processes that are considered to be necessary for such induction are DNA synthesis and germline constant heavy (C_H) gene transcription. We now show that an additional signal, as mediated by interleukin 5 (IL-5), is also required. To induce proliferation of resting B cells, but not Ig secretion, we utilized anti-IgD antibodies conjugated to dextran (αδ-dex). The addition of IL-4, a well-established switch factor for the IgG1 subclass, to αδ-dex-activated cell cultures failed to induce IgG1 secretion or mIgG1$^+$ cells unless IL-5 was also present. While IL-4 stimulated an increase in germline γ1 RNA in αδ-dex-activated cells, this effect could neither be induced nor enhanced by IL-5. By contrast, IL-5 strongly enhanced steady-state levels of productive γ1 RNA induced by αδ-dex and IL-4, suggesting that IL-5 stimulated IgG1 switch rearrangement. To test this possibility we measured switch (S)μ-Sγ1 DNA recombination events using a newly developed assay, digestion circularization polymerase chain reaction (DC-PCR). We demonstrated that IL-5 was necessary for induction of Sμ-Sγ1 DNA rearrangement in αδ-dex plus IL-4-activated cells but that it had little effect on rearrangement in the absence of IL-4. Our data strongly suggest, therefore, a three-component model for induction of Ig class switching. This model includes germline C_H gene transcription, DNA synthesis, and a third component that is necessary for recombination.

Ig class switching to the expression of a particular isotype constant heavy (C_H)[1] gene is preceded by transcriptional activity at that locus. It is hypothesized that as this activity is taking place the C_H locus becomes accessible to factors that mediate switch rearrangement. This hypothesis, known as the accessibility model (1, 2), is based on extensive studies of cytokines such as IL-4 (3–5), IFN-γ (6, 7), and TGF-β (8, 9). These cytokines, which promote switching to specific Ig isotypes, are capable of upregulating the steady-state levels of specific germline C_H RNA in a rapid and selective manner. The corresponding C_H genes subsequently undergo rearrangement. More recent data support this notion by demonstrating that both IL-4 (10) and TGF-β (11), switch factors for IgE and IgA, respectively, could induce an increase in the rate of transcription of the $C_H\epsilon$ and $C_H\alpha$ genes in tumor lines that subsequently switched to these respective Ig isotypes.

In addition to transcriptional activation of C_H genes, Ig class switching appears to require DNA synthesis. Thus, DNA synthesis inhibitors, such as thymidine, hydroxyurea, and bromodeoxyuridine, were found to selectively inhibit IgG, as opposed to IgM production by mitogen-stimulated B cells (reviewed in 12). Similarly the reversible DNA synthesis inhibitor, aphidicolin, abrogated switching from IgM to IgG1 in LPS + IL-4–activated B cells (13). Computer modeling based on cell cycle kinetics of LPS-activated B cells suggested that switch rearrangement occurred during the first S phase after LPS induction (14), although this has not been directly confirmed. Further evidence suggesting an association between DNA replication and Ig class switching came from molecular genetic studies of clonal progeny of I.29 B cell lymphoma cells that had switched from the expression of IgM to IgA (15, 16). Cytokines by themselves fail to drive resting

[1] *Abbreviations used in this paper:* C_H, constant heavy; DC, digested circularized; DC-PCR, digestion circularization polymerase chain reaction; αδ-dex, dextran-conjugated anti-IgD antibodies; m, membrane; nAChRe, nicotinic acetylcholine receptor; S, switch region.

B cells into the S phase. Therefore, these studies may explain, in part, why cytokine switch factors must act in concert with a B cell activator, such as LPS, T cells, or an antigen-receptor cross-linker, in order to induce resting B cells to undergo Ig class switching.

We have developed an in vitro model for studying polyclonal B cell responses to T cell independent type II (TI-2) antigens. Thus, anti-IgD monoclonal antibodies were conjugated to a high molecular weight dextran ($\alpha\delta$-dex) in order to simulate the repeating epitope nature of polysaccharide antigens (17). When activated with $\alpha\delta$-dex, resting B cells proliferate but do not secrete Ig unless additional stimuli are present (18). We recently demonstrated that IL-5 induced $\alpha\delta$-dex-activated B cells to secrete predominantly IgM. If IL-4 was also present large amounts of IgG1 were secreted, in addition to IgM (19). However, IL-4 failed to induce Ig secretion of any class in B cells activated with $\alpha\delta$-dex unless IL-5 was also present. This suggested that IL-5 acted as a maturation factor that promoted B cell Ig secretion in a manner analogous to that previously described by others (20, 21). However, in this report, we demonstrate an additional effect of IL-5 in this system. IL-5 was required for induction of switch region (S)μ-Sγ1 rearrangement by $\alpha\delta$-dex plus IL-4-activated cells, and hence for the generation of mIgG1$^+$ cells. Our findings strongly suggest a three-component model for Ig class switching that includes C_H gene activation, DNA synthesis, and a third component necessary for recombination.

Materials and Methods

Mice. Female BALB/c mice were obtained from the National Institute of Health, Small Animals Division (Bethesda, MD) and were used between 6–8 wk-of-age. The experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare Publication No. 78-23 (National Institutes of Health).

Reagents. Dextran-conjugated anti-IgD antibodies ($\alpha\delta$-dex) were a kind gift from Drs. James J. Mond and Andrew Lees (Uniformed Services University of the Health Sciences, Bethesda, MD) and were prepared as follows (17): monoclonal mouse IgG2b (*b* allotype), anti-mouse IgD (*a* allotype) antibody (Hδ^a/1) was purified from ascitic fluids and was coupled to a high molecular weight dextran (2×10^6 kd) at an antibody to dextran ratio of 6:1. The concentration of dextran-conjugated antibodies that is noted in the text reflects only the anti-Ig antibody concentration and not that of the entire dextran conjugate. Neutralizing monoclonal rat IgG2b anti-mouse IL4 antibody, BVD4.1D11.2 (22) was a gift from Dr. F. D. Finkelman (Uniformed Services University of the Health Sciences). FITC-labeled monoclonal rat IgG1 anti-mouse IgG1 was obtained from Zymed Laboratories (South San Francisco, CA). Monoclonal rat IgG2b anti-mouse FcγRII (2.4G2) (23) was purified from ascites. Recombinant murine IL-4 produced in *Escherichia coli* was a generous gift from Dr. A. D. Levine (Monsanto Corporate Research, St. Louis, MO). Recombinant murine IL-5 produced in the baculovirus system was a gift from Dr. R. Hodes (National Institutes of Health, Bethesda, MD). In all experiments the following concentrations of reagents were used: $\alpha\delta$-dex (3 ng/ml), rIL-4 (10,000 U/ml), and rIL-5 (150 U/ml). Percoll and Ficoll-Hypaque were obtained from Pharmacia Inc. (Piscataway, NJ).

Culture Medium. RPMI 1640 (Biofluids Inc., Rockville, MD) was supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY), L-glutamine (2 mM), 2-mercaptoethanol (0.05 mM), penicillin (50 μg/ml), and streptomycin (50 μg/ml) and was used routinely for culturing B cells.

Preparation and Culture of B Cells. An enriched population of B lymphocytes was obtained from splenocytes by elimination of T cells utilizing monoclonal rat IgM anti-Thy 1 (HO13-4), rat IgG2b anti-CD4 (GK1.5), and rat IgG2b anti-CD8 (2.43), followed by incubation with monoclonal mouse anti-rat Igκ (MAR 18.5) and guinea pig complement (Gibco Laboratories). Small, high density B cells were obtained by discontinuous Percoll gradient centrifugation by collecting cells that equilibrated between the 60 and 70% Percoll fractions (density of 1.081–1.086 g/ml). Cells were cultured at a concentration of 1.5×10^5 cell/ml in 75 cm^2 tissue culture flasks (Costar Corp., Cambridge, MA), 25 cm^2 flasks, or 96-well flat-bottom microtiter plates (Corning Inc., Corning, NY).

FACS® Analysis and Cell Sorting. For quantitation of mIgG1$^+$ cells, cultured B cells were harvested and dead cells were removed by Ficoll centrifugation. Cells were washed and then resuspended in cold HBSS without phenol red (BioWhittaker Inc., Walkersville, MD) +3% FBS. Cells were incubated for 15 min with rat IgG2b anti-FcγRII (2.4G2) [final concentration 5 μg/ml] in order to prevent cytophilic binding of the FITC-labeled rat IgG1 anti-mouse IgG1 which was subsequently added at a final concentration of 10 μg/ml for an additional 30 min. Fluorescence analysis was carried out on 15,000 viable cells utilizing a FACScan® (Becton Dickinson and Co., Mountain View, CA) set for logarithmic amplification. Viable cells were identified on the basis of their characteristic forward and side scatter profiles and their exclusion of propidium iodide (Sigma Chemical Co., St. Louis, MO). mIgG1$^-$ B cells were obtained by electronic cell sorting utilizing an EPICS Elite cytometer (Coulter Corp, Hialeah, FL). Sorted cells were reanalyzed immediately upon their isolation and were found to be >99% mIgG1$^-$.

Quantitation of Secreted IgG1 Concentrations. IgG1 concentrations in culture supernatants were measured by an ELISA as described (24). Briefly, 96-well flat-bottom ELISA plates (Immulon 2, Dynatech Laboratories Inc., Chantilly, VA) were coated with polyclonal goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL), followed by incubation with samples and standards, and then by incubation with affinity-purified polyclonal goat anti-mouse IgG1 antibodies conjugated to alkaline phosphatase (Southern Biotechnology Associates). A fluorescent product was generated by cleavage of 4-methylumbelliferyl phosphate (Sigma Chemical Co.) by specifically bound alkaline phosphatase-conjugated antibody. Fluorescence was measured on a FluoroFAST 96 fluorometer (3M Co., Mountain View, CA). The fluorescence units were compared to a standard curve using known amounts of mouse myeloma IgG1. This assay can detect as little as 1 ng IgG1/ml and is specific for IgG1 as described (24).

Northern Blot Analysis. Total RNA was extracted according to a standard protocol, using RNAzol A (Tel-Test, Friendswood, TX) and chloroform:isoamyl alcohol (Sigma Chemical Co.). Purified RNA samples (20 μg each) were separated by gel electrophoresis on a 1% formaldehyde-containing agarose gel, transferred by blotting onto a Nytran membrane (Schleicher & Schuell, Keene, NH) and fixed by 3 min of UV cross-linking. Membrane-bound RNA was analyzed first for the presence of germline γ1 and then for total γ1 transcripts by hybridization with ^{32}P-labeled Iγ1 and $C_H\gamma$1 probes, respectively. The Iγ1 cDNA probe is a 2-kb BamHI

fragment located at the I region 5′ to Sγ1 and was a kind gift from Dr. W. A. Dunnick (University of Michigan, Ann Arbor, MI). The $C_H\gamma 1$ probe is a 500-bp Pst-1 fragment from the 5′ end of a $C_H\gamma 1$ cDNA and was a kind gift from Dr. Alfred Bothwell (Yale University, New Haven, CT). After each hybridization the blot was exposed to XAR-5 x-ray film (Eastman Kodak, Rochester, NY). Before using a fresh cDNA probe, the previously hybridized probe was removed with 0.1% SDS solution at 90°C for 7 min.

Purification of Genomic DNA. Genomic DNA was extracted from cultured B using a high-salt extraction protocol with some modifications (28). Briefly, the cell lysate was prepared in 1% SDS buffer, pH 9.0, and treated with 0.5 mg/ml proteinase K (Sigma Chemical Co.); precipitated debris was removed by centrifugation in 1.5 M NaCl. Genomic DNA was extracted from the supernatant by mixing and precipitating in 95% ethanol. It was then collected, washed with 70% ethanol, air dried, and dissolved in water.

Digestion Circularization Polymerase Chain Reaction (DC-PCR). DC-PCR is a recently described technique for detecting and quantitating μ-γ1 switch rearrangement at the DNA level (29). In this method chromosomal DNA is digested with EcoRI and the fragments are circularized (see Fig. 1). If Sμ-Sγ1 rearrangements are present in the genomic DNA, this process will yield circularized EcoRI fragments containing 5′ Sμ and 3′ Sγ1 joined together. The fragments are detected by amplifying across the newly created EcoRI site using PCR primers derived from the 5′ Sμ and the 3′ Sγ1 loci.

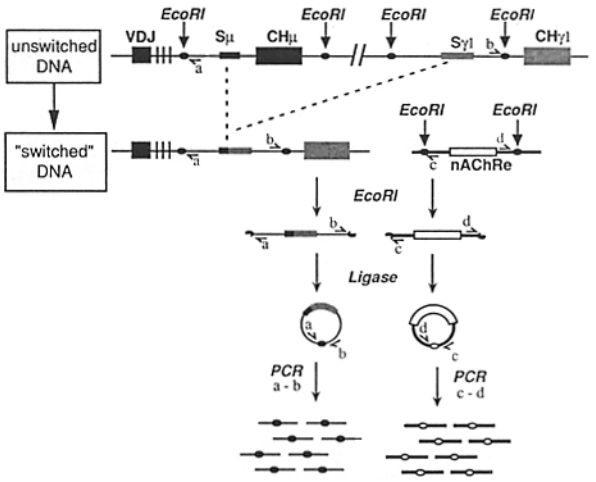

Figure 1. Schematic representation of the digestion circularization polymerase chain reaction. Nonrearranged chromosomal DNA is shown in the upper bar, depicting the relationships between the constant heavy gene for IgG1 ($C_H\gamma 1$), the IgG1 switch region (Sγ1), $C_H\mu$, and Sμ. The sequences designated *a* and *b*, which are 5′ to Sμ and 3′ to Sγ1, respectively, are ~100 kb apart. Therefore, upon EcoR1 digestion of nonrearranged DNA, *a* and *b* would be located on two separate fragments. However, if the IgG1 locus has been rearranged, as shown in the lower bar, *a* and *b* would be on the same fragment. After EcoR1 digestion the fragments are circularized by T4 ligase. A new EcoR1 site is thus created, joining 3′Sγ1 and 5′Sμ. The digested, circularized DNA is then subjected to PCR, using primers complementary to *a* and *b*. If Sμ-Sγ1 recombinations are present on the genomic DNA sample, they yield a PCR product of a distinct size (219 bp), whereas the nonrearranged IgG1 locus will not give rise to this PCR product. In a similar fashion another circularized fragment, which contains a portion of the acetylcholine receptor (nAChRe), is created and amplified. Unlike IgG1, the nAChRe gene is not subject to rearrangements and is present equally in DNA from B cells that have or have not undergone IgG1 switch recombination. It therefore serves as a quantitative control for the efficiency of DC template generation.

If no Sμ-Sγ1 rearrangement has occurred, these two primer targets will be located on different circularized EcoRI fragments and no PCR product will be formed.

DNA oligonucleotide primers were prepared on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) and purified through NAP-5 columns (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) under conditions suggested by the manufacturer. The 5′ Sμ primer (5′ GGC CGG TCG ACG GAG ACC AAT AAT CAG AGG GAA G 3′) and 3′ Sγ1 primer (5′ GCG CCA TCG ATG GAG AGC AGG GTC TCC TGG GTA GG 3′) generate a 219-bp PCR product from digested and circularized genomic DNA templates if Sμ-Sγ1 rearrangement has occurred.

DC-PCR was performed as described previously (29). Briefly, DNA samples (2 μg/100 μl) were digested by EcoR1 (Boehringer Mannheim Corp., Indianapolis, IN), diluted 10-fold and ligated with T4 DNA ligase (Boehringer Mannheim). After ligation, the DNA samples were dialyzed against distilled H_2O through filters for 10 min (0.05 μM VM filters; Millipore Corp., Bedford, MA). PCR was then performed on 5 ng of ligated DNA in 20 μl of PCR buffer (2.0 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl), 0.5 μM 5′ μ primer, 0.5 μM 3′ γ1 primer, 200 μM of each dNTP (Pharmacia Inc.), 0.1 μL α-[^{32}P]dCTP (3,000 Ci/mmol; Amersham Corporation, Arlington Heights, IL) and 1.5 U Taq DNA polymerase (Boehringer Mannheim Corp.). Amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT) under the following conditions: 94°C for 6 min; 5 cycles at 94°C for 1 min; 65°C for 1 min; and 72°C for 2 min; 30 cycles at 94°C for 1 min; 68°C for 1 min; and 72°C for 2 min; 72°C for 7 min. The PCR products were resolved on 8% PAGE and the gels were dried on a gel drier (Bio-Rad Laboratories, Richmond, CA) and autoradiographed. Quantitation of the PCR products was based on scanning densitometry of these gels (Computing Densitometers; Molecular Dynamics Inc., Sunnyvale, CA).

To monitor the efficiency of the amplification in each PCR tube, we added an exogenous Sμ-Sγ1 plasmid as an internal control. This plasmid, p4AP, was constructed so that it can be amplified with the same primers as the genomic DNA but generate a slightly larger (265-bp) PCR product (29). For quantitation, plasmid p4AP was titered into the digested circularized (DC) genomic DNA at 667, 222, 74, and 25 copies per 5 ng genomic DNA template.

To control for variations in the preparation of digested and circularized template DNA we measured the production of DC template DNA from the nicotinic acetylcholine receptor (nAChRe) gene. Since it is unaffected by the switch process, the nAChRe gene should be present in equal quantities and yield similar amounts of amplified PCR products from each sample of digested, circularized genomic DNA (29). With appropriate primers (5′-GGC CGG TCG ACA GGC GCG CAC TGA CAC TAA G and 5′-GCG CCA TCG ATG GAC TGC TGT GGG TTT CAC CCA G) the circularized nAChRe-containing EcoRI fragment from genomic DNA generates a 753-bp product. To monitor the nAChRe amplification efficiency in each PCR tube we introduced an internal control that was a plasmid construct (p2AO) (29) designed to generate a 490-bp PCR product with the same nAChRe primers. We quantitated the endogenous DC nAChRe template by a competitive substrate method using the p2AO construct (29, 30). This plasmid was titrated (6,000, 2,000, 667, and 222 copies) into 5 ng of digested, circularized genomic DNA. The resulting DNA mixtures were amplified as previously described.

In the quantitative analysis of the autoradiograph, scanning measurements were adjusted for the amount of radioactive cytosine incorporated per molecule of each PCR product. Thus, for Sμ-Sγ1 the ratio of cytosine content between genomic and p4AP PCR prod-

ucts is 89:108. For the nAChRe the corresponding ratio is 342:238. After determining an estimate of nAChRe and Sμ-Sγ1 copy numbers by DC-PCR the recombination index, i.e., number of Sμ-Sγ1 rearrangements per 100 diploid genomes in a sample is calculated by the following equation: (Sμ-Sγ1 copies/nAChRe copies/2)/1.89 × 100. The factor 2 accounts for a diploid genome and 1.89 is a normalization factor derived from measurements performed utilizing an IgG1-expressing cell line with known nAChRe and Sμ-Sγ1 copy numbers as described previously (29). This index can reach, theoretically, a value of 200% if both chromosomes have rearranged in 100% of the cells.

Results

IL-5 Induces IgG1 Secretion in Resting B Cells Activated with αδ-dex Plus IL-4. We previously demonstrated that resting B cells activated with αδ-dex or αδ-dex plus IL-4 secreted little, if any IgG1 (19). The addition of IL-5 to αδ-dex-activated cells led to a consistent, though modest, increase in IgG1 secretion, whereas the combination of IL-4 plus IL-5 stimulated a large IgG1 secretory response in αδ-dex-activated cells. We confirmed this data and further observed that these effects occurred to a similar extent using mIgG1$^-$ B cells, obtained by electronic cell sorting. (Table 1). The modest induction of IgG1 secretion observed in B cells activated with αδ-dex plus IL-5 was not due to the presence of endogenous IL-4. This was evidenced by the fact that the addition of neutralizing anti–IL-4 monoclonal antibody to cultures of αδ-dex plus IL-5-activated cells did not reduce the concentration of secreted IgG1 (data not shown). Further, direct measurement of IL-4 concentrations in αδ-dex plus IL-5-stimulated cultures, utilizing the IL-4 indicator T cell line, CT.4S (31), indicated an IL-4 concentration of <5 U/ml, an amount which is insufficient to induce IgG1 secretion in this system (data not shown). However, the addition of IL-4 typically induced 10–30× more IgG1 secretion than seen in cells stimulated with αδ-dex plus IL-5 only indicating the latter effect was of little significance.

Table 1. *Both IL-4 and IL-5 Are Required for IgG1 Secretion in αδ-Dex-activated B cells*

Treatment	IgG1 secretion	
	mIgG1$^-$	Unsorted cells
	ng/ml	
αδ-dex	<1.25	<1.25
αδ-dex+IL-4	38 ± 2.3	20 ± 4.0
αδ-dex+IL-5	175 ± 10.5	200 ± 3.9
αδ-dex+IL-4+IL-5	5,625 ± 500	2,500 ± 200

Resting B cells were stained with FITC-labeled monoclonal rat IgG1 anti-mouse IgG1 antibody and >99% mIgG1$^-$ cells were obtained by electronic cell sorting. Sorted, mIgG1$^-$ and unsorted B cells at 1.5 × 10^5 cells/ml were then stimulated with αδ-dex (3 ng/ml), IL-4 (10,000 U/ml), and/or IL-5 (150 U/ml) for 6 d. IgG1 concentrations in the culture supernatants were measured by ELISA. Values represent the mean of triplicate cultures ± SEM.

IL-5 Is Required for Induction of mIgG1$^+$ Cells in αδ-dex Plus IL-4–activated B Cell Cultures. The requirement for IL-5 for induction of secretory IgG1 could simply reflect its known ability to stimulate Ig secretion (20, 21) whereas IL-4, a known switch factor for IgG1 (32, 33), might be sufficient for optimal induction of IgG1 class switching by αδ-dex-activated cells. Alternatively, αδ-dex plus IL-4 might not be sufficient for induction of IgG1 class switching and may require the additional presence of IL-5. To distinguish between these possibilities, we first tested which combination of stimuli was required for optimal induction of mIgG1$^+$ cells as assessed by flow cytometric analysis. Thus, B cells were stimulated for 4 d with αδ-dex, with or without IL-4 and/or IL-5. In three separate experiments it was observed that activation of resting B cells with αδ-dex alone resulted in the generation of few, if any, mIgG1$^+$ cells (Fig. 2). IL-4 failed to induce mIgG1$^+$ cells in αδ-dex-activated cultures. IL-5, in the absence of IL-4, induced only a modest, ~2-fold, increase in mIgG1$^+$ cells in αδ-dex-activated cultures, but in combination with IL-4 an increase of ~10-fold was observed. This suggested that IL-5 was required to induce substantial IgG1 class switching in αδ-dex plus IL-4-activated cells.

IL-5 Exerts Little, If Any, Effect on the Accumulation of Germline γ1 RNA Transcripts in B Cells Activated with αδ-dex or αδ-dex Plus IL-4. To further assess the role of IL-5 in the induction of mIgG1$^+$ cells in αδ-dex plus IL-4-stimulated cell cultures, we tested whether IL-5 was required for, or upregulated, $C_H\gamma1$ gene activation. Transcriptional activation is believed to make the C_H gene accessible to the switch recombination machinery. Such activation is manifested by the appearance of specific germline C_H transcripts which have been shown to invariably precede switch recombination and expression of that C_H gene (1, 2). Thus, total RNA was extracted from B cells following stimulation with αδ-dex, with or without IL-4 and/or IL-5 for 4 d. The RNA samples were subjected

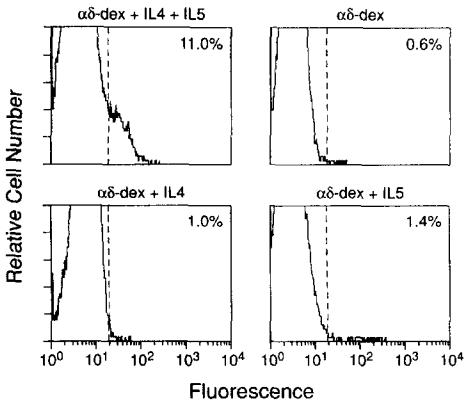

Figure 2. IL-5 is required for induction of mIgG1$^+$ cells in αδ-dex + IL-4-activated B cell cultures. Resting B cells were cultured at 1.5 × 10^5 cells/ml with αδ-dex (3 ng/ml), IL-4 (10,000 U/ml), and/or IL-5 (150 U/ml) for 4 d. The cells were then stained with FITC-labeled monoclonal rat IgG1 anti-mouse IgG1 and 15,000 cells from each treatment group were analyzed by FACS® for the expression of mIgG1. The percentage of mIgG1$^+$ cells in each sample is given in the upper right corner of each panel.

to Northern blot analysis using Iγ1- and $C_H\gamma1$-specific cDNA probes. Germline γ1 transcription, which occurs before Sμ-Sγ1 rearrangement (3, 5, 25–27), initiates within the Iγ1 region, yielding an RNA that contains both Iγ1 and $C_H\gamma1$. After rearrangement has occurred, the Iγ1 region is deleted and γ1 transcripts include VDJ joined to $C_H\gamma1$. The Iγ1 cDNA probe, which is a 2-kb BamH1 fragment located at the I region 5′ to Sγ1 is, therefore, specific for germline γ1 RNA only. By contrast, the $C_H\gamma1$ probe, which is a 500-bp Pst-1 fragment from the 5′ end of a $C_H\gamma1$ cDNA, detects both germline (Iγ1-$C_H\gamma1$) and rearranged (VDJ-$C_H\gamma1$) γ1. These probes bind to both the membrane (~3.2 kb) and secretory (~1.7 kb) form of γ1 transcripts (Fig. 3) (34). B cells activated with αδ-dex alone contained relatively low steady-state levels of germline membrane and secretory γ1 RNA (Fig. 3). The addition of IL-4 to αδ-dex-activated cultures led to a strong increase in both membrane and secretory germline γ1 RNA, consistent with reports by others (5, 25, 26). By contrast, IL-5 did not significantly increase germline γ1 transcripts in αδ-dex-activated cells nor did it augment germline γ1 expression in the presence of IL-4. Similar results were obtained when RNA was extracted 2 d after initiation of culture (data not shown). However, IL-5 significantly increased γ1 RNA, which is detected by the $C_H\gamma1$ probe, in B cells stimulated for 4 d with αδ-dex plus IL-4 (Fig. 3). The $C_H\gamma1$ probe reveals a doublet in the 3.2-kb range; the upper band (see arrow) of this doublet probably corresponds to the membrane form of the rearranged (productive) γ1 transcript. These results indicate that both IL-4 and IL-5 are required for optimal induction of productive γ1 RNA. The ability of IL-5 to increase total, but not germline, γ1 RNA in αδ-dex plus IL-4-activated B cells suggested that it may play a key role in mediating switch rearrangement of the $C_H\gamma1$ gene. This would be consistent with the results shown above that indicate that IL-5 was required for induction of $mIgG1^+$ cells.

IL-5 Induces Sμ-Sγ1 DNA Rearrangement in αδ-dex Plus IL-4-activated Cells. To determine directly whether IL-5 promoted Sμ-Sγ1 recombination in αδ-dex plus IL-4-activated cells, we used a recently developed assay, termed digestion circularization polymerase chain reaction (DC-PCR). This method allows for the detection and quantitation of specific C_H DNA rearrangement events in B cells that have undergone an Ig isotype switch (29) (see Materials and Methods and Fig. 1). Thus, resting B cells were stimulated for 4 d with αδ-dex, with or without IL-4 and/or IL-5; genomic DNA was then extracted and processed for the detection of Sμ-Sγ1 recombination. The amounts of PCR product amplified from the internal control plasmid p4AP were similar in all the DNA samples (Fig. 4), indicating that the PCR amplification proceeded equally well in each sample. However, these samples show different levels of genomic Sμ-Sγ1 rearrangement. Such rearrangements were hardly detectable in B cells stimulated with αδ-dex alone (Fig. 4). The addition of IL-4 to αδ-dex-activated cultures, which was already shown to induce germline γ1 RNA transcripts, nonetheless failed to significantly induce Sμ-Sγ1 rearrangements. The combination of IL-4 plus IL-5 was required for optimal induction of Sμ-Sγ1 switching. By calculating the ratio of p4AP/genomic Sμ-Sγ1 PCR products and adjusting for cytosine content, a semi-quantitative comparison was obtained for these samples. According to these ratios, IL-5, in the absence of IL-4, stimulated an increase in Sμ-Sγ1 rearrangement that was consistent but too small to quantitate. However, in cultures with IL-4 and αδ-dex, the stimulation by IL-5 was ~15-fold. Similar results were obtained with sort-purified B cells that were $mIgG1^-$ at the initiation of culture (data not shown). Previous data indicated that unstimulated resting B cells contained undetectable Sμ-Sγ1 rearrangements as measured by DC-PCR (29). Taken together,

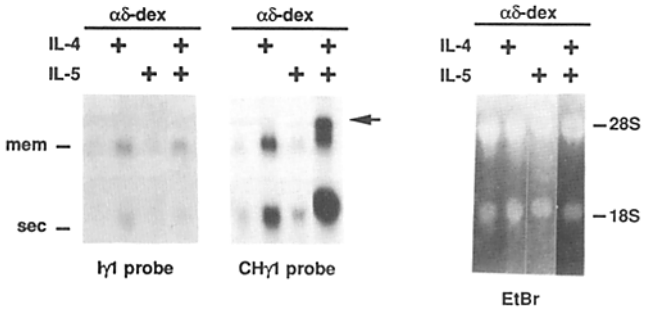

Figure 3. IL-5 increases steady-state levels of total IgG1 RNA transcripts but not the level of germline IgG1 RNA in αδ-dex+IL-4–activated B cells. Resting B cells at 1.5 × 10^5 cells/ml were stimulated for 4 d by αδ-dex (3 ng/ml), IL-4 (10,000 U/ml), and/or IL-5 (150 U/ml) as indicated. Total RNA was extracted and subjected to Northern blot analysis. Germline IgG1 transcripts were detected by hybridization with an Iγ1 cDNA probe, which is specific for the I region 5′ to Sγ1. Total IgG1 transcript levels were detected by hybridization with a $C_H\gamma1$ cDNA probe, which is specific for the 5′ region of $C_H\gamma1$. The membrane and secretory forms are ~3.2 and ~1.7 kb, respectively. A picture of an ethidium bromide-stained blot is included to demonstrate essentially equal quantities of transferred RNA in each lane. The 18s and 28s ribosomal bands are visualized.

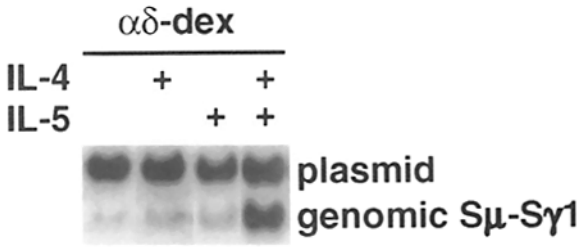

Figure 4. IL-5 induces Sμ-Sγ1 recombination in αδ-dex+IL-4-activated B cells. Resting B cells at 1.5 × 10^5 cells/ml were stimulated for 4 d with αδ-dex (3 ng/ml), IL-4 (10,000 U/ml), and/or IL-5 (150 U/ml). The cultures were harvested and genomic DNA was extracted, digested with EcoR1, and circularized with ligase. To each sample, p4AP plasmid DNA was added at 74 copies per 5 ng of genomic template DNA. The mixtures were subjected to DC-PCR amplification with 5′Sμ and 3′Sγ1 primers. A PCR product at the position of the band labeled genomic Sμ-Sγ1 is expected to be amplified only from genomic DNA template molecules that underwent a Sμ-Sγ1 rearrangement in culture (see Materials and Methods). PCR products were separated on 8% PAGE and the gels were then dried and autoradiographed. The figure depicts the 219 and 265 bp PCR products, amplified from the genomic 5′Sμ-3′Sγ1 sequence and p4AP, respectively, as obtained from each DNA sample.

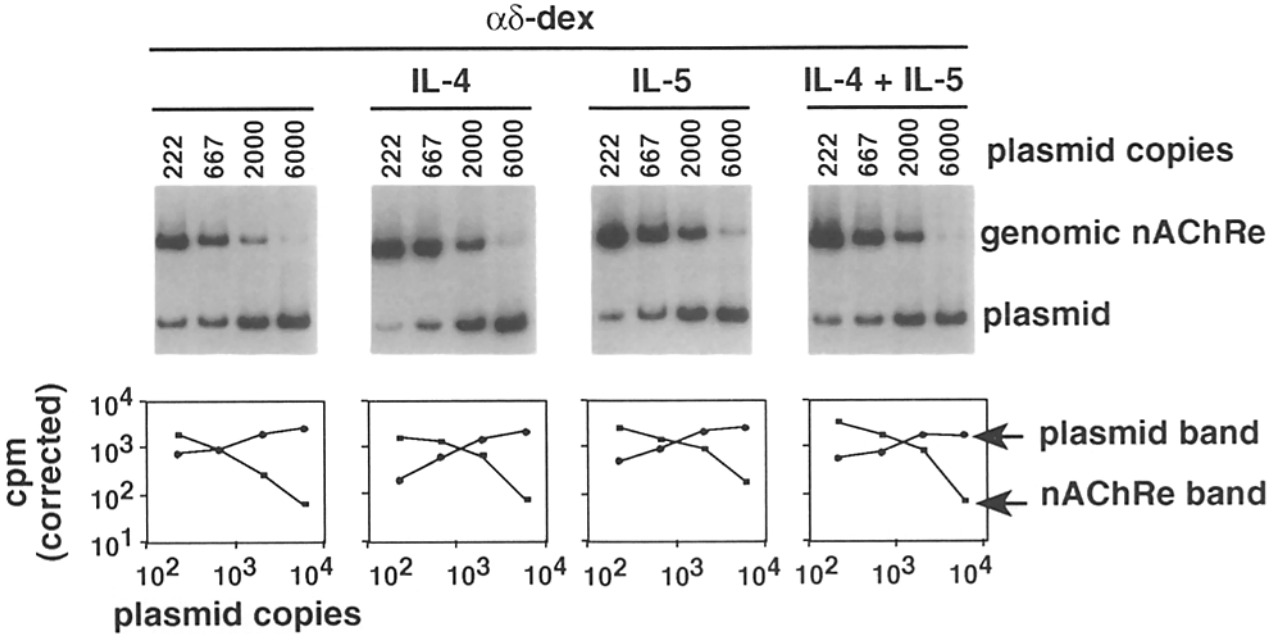

Figure 5. Genomic DNA samples from αδ-dex, IL-4, and/or IL-5-activated B cells contain similar quantities of digested, circularized nAChRe gene templates. Genomic DNA was extracted from B cells after 4 d of stimulation with αδ-dex, IL-4, and/or IL-5 and was processed for DC-PCR as described for Fig. 4. The samples were then tested for the amount of nAChRe DC template copy number by amplifying across the predicted religated EcoRI site (see Materials and Methods). The 5-ng digested, circularized genomic DNA samples were mixed with titrated amounts of the p2AO plasmid corresponding to 6,000, 2,000, 667, and 222 copies. The DNA mixtures were then amplified and the PCR products were resolved on 8% PAGE. The figure shows autoradiographs of the gels, with PCR products of endogenous nAChRe genomic template and the p2AO plasmid (753 bp and 490 bp, respectively) for each DNA sample. Scanning densitometry of these bands gave quantitative values for each titration. Titration curves derived from these values are shown in the lower panels after adjustment for relative cytosine content of the two products (see text). nAChRe gene copy numbers calculated from these data were as follows: αδ-dex alone, 630; αδ-dex + IL-4, 930; αδ-dex + IL-5, 960; and αδ-dex + IL-4 + IL-5, 910.

the results strongly suggested that IL-5-stimulated Sμ-Sγ1 switch recombination in these αδ-dex plus IL-4-stimulated cultures.

To ensure that these differences were not due to quantitative variations in the efficiency of digestion and circularization in the preparation of DNA templates, we amplified the DC product of the endogenous nAChRe gene, from the same DNA samples. Varying amounts of the modified nAChRe plasmid construct, p2AO, were added to each sample to allow quantitation. The plots of band intensities of the amplified products from p2AO and endogenous nAChRe template intersect at a point where the number of copies of each template in the mixture is equal and thus the amount of endogenous nAChRe template can be determined from the extrapolated number of plasmid copies added at this equivalence point (see Fig. 5). Such titrations showed that the number of DC templates derived from the genomic nAChRe gene were indeed very similar in all four culture conditions as reflected by a range of nAChRe gene copies between 630 and 960 among the different samples (Fig. 5). Therefore, the differences in the content of genomic Sμ-Sγ1 amplification products seen in the same four DNA samples in Fig. 4 cannot be due to differences in efficiencies of digestion and circularization, and must reflect differences in the amount of Sμ-Sγ1 switched DNA in the samples.

In an analogous manner we quantitated the number of Sμ-Sγ1 DNA rearrangement events in B cells activated with αδ-dex, IL-4, and IL-5. The calculated number of copies of Sμ-Sγ1 was 71 compared to 910 for nAChRe (Fig. 6). Thus, the recombination index was 8% (see Materials and Methods). This could represent as little as 4% of B cells each switching to IgG1 on both the expressed and nonexpressed chromosomes or as many as 8% of cells each undergoing an IgG1 class switch on only one chromosome. In the DNA samples from the other three experimental groups, Sμ-Sγ1 rearrangement events were ≤1% of total γ1 gene copy number, too low to obtain accurate quantitation of Sμ-Sγ1 rearrangement events by plasmid titration.

Discussion

The molecular requirements for induction of Ig class switching include both DNA synthesis, as occurs in cells entering S phase, and transcriptional activation of specific C_H genes. Those C_H genes are targeted for subsequent rearrangement presumably by being accessible to factors that mediate the Ig class switch. In our system, resting B cells are induced to enter the cell cycle and proliferate in response to αδ-dex stimulation, whereas transcriptional activation of the C_Hγ1 gene appears to be accomplished through the action of IL-4. We demonstrate that while the signals mediated by αδ-dex plus IL-4 were necessary, they were not

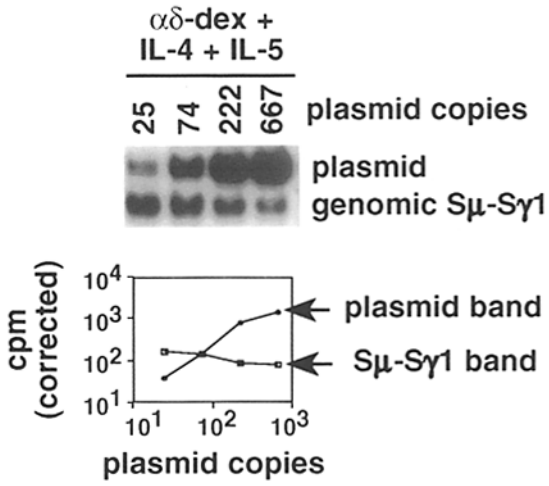

Figure 6. Quantitation of Sμ-Sγ1 recombination events in B cells activated by αδ-dex + IL-4 + IL-5. Genomic DNA was extracted from B cells activated with αδ-dex plus IL-4 and IL-5 for 4 d. It was processed for DC-PCR, as described for Fig. 4. Aliquots (5 ng) were then mixed with either 200, 75, 25, or 8 copies of p4AP plasmid, which carries a modified Sμ-Sγ1 template. The figure shows the DC-PCR products of endogenous and p4AP templates in this sample (219 and 265 bp, respectively). Band intensity levels were measured by scanning densitometry and these values were plotted, after adjustment for relative cytosine content of the two products (see text) against the known titrated amounts of p4AP in each DNA mixture. The titration curve is shown in the lower panel. The copy number of Sμ-Sγ1 rearrangements calculated from these data was 71. Copies of the C_H locus based on the nAChRε results for the same DNA samples is estimated to be 910 (see Fig. 5 and text), which indicates ~8% of $C_H\gamma1$ genes have undergone rearrangement in the αδ-dex +IL-5 + IL-4 activated B cells.

sufficient to drive substantial Sμ-Sγ1 rearrangement and hence IgG1 class switching. A third signal, which could be delivered by IL-5, was necessary for switching as demonstrated here by utilizing the newly developed technique of DC-PCR.

The ability of IL-5 to stimulate IgG1 class switching in αδ-dex plus IL-4-activated cells does not appear to be at the level of DNA synthesis or $C_H\gamma1$ activation. IL-5 stimulates less than twofold enhancements in [^{3}H]thymidine incorporation in αδ-dex plus IL-4–activated cells (data not shown). Further, we observed no significant effect of IL-5 on accumulation of germline γ1 RNA transcripts. Thus, our data strongly suggest that IL-5 induces or activates some pivotal component of the recombination machinery that is distinct from those processes induced by αδ-dex or IL-4. All three components appear to be necessary for substantial switching to occur. On this basis, we propose a three component model for Ig class switching: (*a*) transcriptional C_H gene activation; (*b*) DNA synthesis; and (*c*) a third process necessary for recombination.

DC-PCR analysis indicated that IL-5 could induce a low level of Sμ-Sγ1 rearrangement in αδ-dex-activated cells in the absence of detectable IL-4. The observation that the αδ-dex-activated cell population already contained low levels of germline γ1 RNA indicated that some degree of $C_H\gamma1$ gene activation had already occurred in this population, even in the absence of IL-4. This could allow for a modest stimulation of IgG1 class switching and IgG1 secretion in αδ-dex-activated cells in response to IL-5 alone. However, IL-4 was necessary for optimal IL-5-mediated IgG1 class switching. This is consistent with the ability of IL-4 to induce transcription of the $C_H\gamma1$ gene thus targeting the gene for switch recombination by making it accessible to the recombination machinery. Although resting B cells have been reported to lack detectable expression of germline γ1 RNA transcripts, we are currently testing whether αδ-dex, indeed, stimulates some degree of IL-4-independent transcriptional activation of the $C_H\gamma1$ gene as has been suggested for activated T cell membranes (35).

Our work both confirms and extends analogous work by Purkerson and Isakson (36). Their cellular system consisted of Sepharose anti-IgM-activated B cell blasts that were stimulated with LPS and IL-4. In this system IL-5 was required for stimulating productive, but not germline, γ1 and ε RNA as well as IgG1 and IgE secretion. Their data suggested a possible role for IL-5 in promoting IgG1 and IgE class switching although DNA switch rearrangement was not directly assessed in their study. Our data also extend previous studies indicating that cellular proliferation and germline C_H gene transcription were necessary but insufficient for the induction of Ig isotype switching. Thus, human B cells simultaneously stimulated with Epstein-Barr virus (EBV) and IL-4 switched to the expression of IgE (37). However IL-4 failed to stimulate IgE secretion or productive $C_H\epsilon$ RNA by proliferating B cells previously transformed by EBV, despite inducing large amounts of germline $C_H\epsilon$ RNA (38). A third signal was required for stimulating IgE expression, which was delivered by activated helper T cells.

IL-5 was originally defined both by its ability to promote B cell growth (leading to its earlier designation as B cell growth factor [BCGF]-2) (39) and by its role in stimulating maturation of B cells into antibody-producing cells (designated B cell differentiation factor [BCDF]-μ) (20, 21). IL-5, which stimulates antibody secretion in an Ig isotype-independent manner, has been shown to upregulate IgM secretion, at least in part, by favoring RNA processing to the $C_H\mu$ secretory form over the membrane form (39). This is in contrast to the mechanism of induction of Ig synthesis by LPS that increases the transcriptional rate of the $C_H\mu$ gene (40). Consistent with the notion that IL-5 acts as a maturation factor, we have previously shown that IL-5 is required for induction of IgM secretion in resting B cells activated with αδ-dex or αδ-dex plus IL-4 (18, 19). However, the data shown here indicate an additional role for IL-5 in promoting C_H gene recombination. Several observations strongly suggest that IL-5 promoted $C_H\gamma1$ DNA rearrangement during the course of culture, as opposed to selectively expanding already existing mIgG1$^+$ cells: (*a*) resting B cells have no detectable rearranged $C_H\gamma1$ DNA at initiation of culture (29), (*b*) IL-5 can stimulate $C_H\gamma1$ switch recombination by αδ-dex plus IL-4-activated cells which are mIgG1$^-$ at initiation of culture, and (*c*) αδ-dex, which drives proliferation in this system, binds only to cells expressing mIgD, and not to cells that have already switched to another Ig isotype and have lost mIgD expression.

Although we show that IL-5 induced Sμ-Sγ1 DNA recombination in αδ-dex plus IL-4-activated B cells, we suggest that IL-5 has a more general, Ig isotype-nonrestricted, effect, in promoting a key event in recombination. Thus, in a previous report we demonstrated that IL-5 selectively stimulated IgG3 secretion and the generation of mIgG3$^+$ cells in αδ-dex plus IFN-γ-activated cell cultures (6). In this system IL-5 had only a modest effect in upregulating the selective IFN-γ-induction of germline $C_H\gamma3$ RNA transcripts, and was slightly inhibitory for DNA synthesis. Furthermore, IL-5 has been shown to be essential for the optimal induction of both IgG1 and IgE secretion by B cells stimulated by activated T cell membranes plus IL-4 (41; Snapper, C. M., and R. J. Noelle, unpublished observations), although its effect on the generation of mIgG1$^+$ and mIgE$^+$ cells has not been reported. Finally, IL-5 was required for induction of both productive γ1 and ε RNA in anti-IgM–activated B cell blasts stimulated with LPS plus IL-4 (36). Confirmation of a more general ability of IL-5 to stimulate switch recombination will await further studies utilizing DC-PCR to measure C_H gene rearrangement for other isotypes.

Although the molecular mechanism underlying the C_H recombination event is unknown, one can speculate on several possible mechanisms by which IL-5 might mediate Sμ-Sγ1 recombination. The cytokine might induce or activate: (*a*) an enzymatic component of the recombinase machinery, (*b*) a component necessary for assembly of the recombinase complex or its binding to the DNA, and/or (*c*) a structural change in the chromatin (distinct from that associated with IL-4 induction of γ1 gene transcription) that is necessary for recombination. The mechanism of IL-5 action may overlap several of these possibilities.

LPS is another agent that stimulates switch recombination in an Ig isotype nonrestrictive fashion. Thus, LPS, which by itself stimulates IgG3 class switching, is also a competent costimulus for IL-4 induction of IgG1 (32, 33) and IgE (42), for IFN-γ induction of IgG2a (43), and for TGF-β induction of IgG2b (9) and IgA class switching (44, 45). Indeed, utilizing DC-PCR it has been shown that Sμ-Sγ1 rearrangement is induced in LPS plus IL-4-activated cells (29). It has been suggested that LPS promotes Ig class switching, in part, by inducing a protein (designated LR1) which binds to a consensus sequence found in a number of Ig switch regions (46). This suggests that LPS may induce recombinase activity when stimulating resting B cells. This effect of LPS is unlikely to be mediated by endogenous IL-5 since we observed that a neutralizing anti-IL-5 monoclonal antibody (TRFK-5) (47), in quantities sufficient to completely inhibit IL-5-induced IgG1 secretion by αδ-dex plus IL-4-activated B cells, did not inhibit LPS plus IL-4-induced IgG1 production (data not shown). Nevertheless, it remains to be determined whether LPS and IL-5 act through a final common pathway to engage the switch recombination machinery.

In conclusion we have demonstrated that IL-4 and IL-5 are synergistic for induction of Sμ-Sγ1 recombination in murine B cells activated with αδ-dex. IL-4 is distinct from IL-5 in that it induces $I\gamma1C_H\gamma1$ germline transcripts, an effect widely regarded as necessary for targeting the $C_H\gamma1$ gene for recombination. The precise mechanism by which these two cytokines mediate switch recombination remain to be determined.

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