

# Interleukin 5 (IL-5) Provides a Signal That Is Required in Addition to IL-4 for Isotype Switching to Immunoglobulin (Ig) G1 and IgE

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## Summary

We have examined the contributions of Interleukin 4 (IL-4), IL-5, and other stimuli to the expression of Immunoglobulin G1 (IgG1) and IgE in murine B lymphoblasts activated with anti-Ig. The combination of IL-4 and -5 induced B lymphoblasts to proliferate and to secrete IgM and IgG1. However, an additional stimulus was required along with IL-4 and -5 for induction of IgE secretion. This stimulus was provided by lipopolysaccharides (LPS) or cytokines produced by TC-1 or EL4 cells. In the absence of IL-5, exceptionally high concentrations of IL-4 (>1,000 U/ml) were required to elicit IgG1 and IgE secretion from B lymphoblasts cultured with either LPS or TC-1-conditioned media (CM). To investigate regulation of expression of  $\gamma$ 1 and  $\epsilon$  genes by IL-4, -5, and LPS, the requirements for induction of  $\gamma$ 1 and  $\epsilon$  germline and productive transcripts were examined. Germline  $\gamma$ 1, but not  $\epsilon$ , transcripts were detected in RNA from B lymphoblasts treated with IL-4 and -5 for 48 h. In contrast, both germline  $\gamma$ 1 and  $\epsilon$  transcripts could be detected in B lymphoblasts cultured with IL-4 and LPS, and steady state levels of germline  $\gamma$ 1 transcripts were four- to sevenfold higher in blasts cultured with LPS and IL-4, compared with blasts cultured with IL-4 and -5. LPS enhanced steady state levels of germline transcripts induced by IL-4, but LPS did not promote substantial accumulation of productive  $\gamma$ 1 and  $\epsilon$  transcripts. In contrast, IL-5 did not affect steady state levels of germline transcripts stimulated by IL-4, but did markedly increase levels of productive  $\gamma$ 1 and  $\epsilon$  transcripts. Thus, lymphokines regulate two distinct events in isotype switching: induction of germline transcripts (IL-4), and production of VDJ-C $\gamma$ 1 and VDJ-C $\epsilon$  mRNA (IL-5), which leads to secretion of IgG1 and IgE.

It is now well established that IL-4 regulates Ig isotype switching in B lymphocytes. IL-4 induces secretion of IgG1 and IgE, and suppresses secretion of IgM, IgG3, and IgG2b, from murine B cells stimulated with LPS (1-4). In addition, several groups have demonstrated that induction of IgE secretion from murine and human B cells by activated CD4<sup>+</sup> T cells is dependent on IL-4 (5-10). IL-4 also induces IgG4 and IgE secretion from human peripheral blood lymphocytes (11) and from purified human B cells transformed by EBV (12), or stimulated with anti-CD40 (13, 14) or hydrocortisone (15, 16). The important role of IL-4 in the production of IgG1 and IgE in vivo has been confirmed in several studies (17-21).

IL-4 induces alterations in the chromatin structure of the S $\gamma$ 1 region (22, 23) and the accumulation of germline  $\gamma$ 1 and  $\epsilon$  transcripts (24-27). These results led to the suggestion that IL-4 promotes switching to IgG1 and IgE by regulating the accessibility of S $\gamma$ 1 and S $\epsilon$  regions to a switch recombinase (26-28). Although IL-4 alone is sufficient to stimulate transcription of the unrearranged  $\gamma$ 1 and  $\epsilon$  H chain loci, a second signal is required for induction of switch recombina-

tion to the  $\gamma$ 1 and  $\epsilon$  H chain loci, synthesis of productive  $\gamma$ 1 and  $\epsilon$  transcripts, and secretion of the respective Ig isotypes. This second signal can be provided to murine and human B cells by noncognate interaction with activated T cells (9, 10, 29), by LPS in murine B cells (4, 30), by EBV transformation of human B cells (31), and by crosslinking the CD40 antigen on human B cells (32).

In several experimental systems, IL-5 has been shown to enhance secretion of IgG1 and IgE from B cells stimulated with IL-4. IL-5 and -2 promote secretion of IgG1 and IgE from B cells or B lymphoblasts stimulated with LPS and IL-4 (33, 34) and from B lymphoblasts treated with a mixture of T cell-derived lymphokines (35). IL-5 enhances secretion of IgE from human PBL stimulated with IL-4 (36, 37), and recent studies suggest that IL-5 plays an important role in induction of IgG1 and IgE secretion by murine T<sub>H</sub>2 clones (29, 38). Although numerous studies indicate a role for IL-5 in isotype switching, the mechanism by which IL-5 promotes expression of IgG1 and IgE is not understood.

We have used anti-Ig activated B lymphoblasts as a model

system to study lymphokine regulation of isotype switching. Resting, murine B cells exposed to insoluble anti-Ig for 48 h are quantitatively converted to low density B lymphoblasts (39). Reculture of anti-Ig activated B cells (B lymphoblasts) with T cell supernatants containing IL-4 and -5 results in secretion of IgM and IgG1 (40). In this study, we examined whether IL-5 could provide the second signal that is required, in addition to IL-4, for isotype switching to IgG1 and IgE. We found that the combination of IL-4 and -5 stimulated proliferation, IgM secretion, and isotype switching to IgG1, but an additional stimulus (e.g., LPS) was required for secretion of IgE. An investigation of the regulation of  $\gamma 1$  and  $\epsilon$  gene expression revealed that LPS enhanced accumulation of germline transcripts induced by IL-4, and -5 specifically enhanced the accumulation of productive  $\gamma 1$  and  $\epsilon$  transcripts. Taken together, these data suggest that IL-5 provides an essential second signal for isotype switching by B lymphoblasts by either regulating transcription of the rearranged  $\gamma 1$  and  $\epsilon$  H chain, or inducing switch recombination in B lymphoblasts stimulated with IL-4.

## Materials and Methods

### Mice

Female BALB/c mice were obtained (Cumberland Farms, Clinton, TN) and used at 8–12 wk of age.

### Reagents

Affinity-purified antibodies were obtained from Jackson Immunoresearch Laboratories, West Grove, PA (goat anti-mouse IgM plus IgG, anti- $\mu$ ), and from Southern Biotechnology Associates of Birmingham, AL (goat anti-IgG1 and goat anti- $\kappa$ ). Ig isotype standards were obtained from Pharmingen, San Diego, CA (IgE,  $\kappa$ ), and Southern Biotechnology Associates (IgM and IgG1). Polyclonal anti-IgE antibody was obtained from The Binding Site, Inc., San Diego, CA. Rat monoclonal anti-IgE was prepared from EM95 culture supernatants by precipitation with 45%  $\text{NH}_4\text{SO}_4$ .

### Lymphokines

Recombinant baculovirus containing either murine IL-4 or -5 cDNA were generously provided by Drs. William Paul, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), and Warren Strober, NIAID, NIH, respectively. rIL-4 and -5 were prepared using the baculovirus expression system as described (41). IL-4 activity in Sf9 cell supernatants was quantitated by comparison of activity on the HT-2 cell line with purified recombinant mouse IL-4 (7–14 U/ng), generously provided by Drs. Robert Kastelein, and Nobuyuki Harada of DNAX, Palo Alto, CA. IL-5 activity in Sf9 cell supernatants was assessed on BCL1 cells. 1 U of IL-5 was defined as the amount of IL-5 required to induce half-maximal stimulation of IgM secretion from BCL1 cells. Conditioned media derived from HeLa cells transfected with the IL-5 cDNA (generously provided by T. Honjo) was also used as a source of rIL-5. EL4 Sn was prepared and depleted of IL-4 as described (33).

### B Cell Preparations

B cells were prepared by treating spleen cells with monoclonal anti-Thy-1 and anti-L3T4 followed by lysis with baby rabbit serum (Pel-Freez Biologicals, Rogers, AR). High density B cells (1.081–

1.086 g/ml) were isolated on discontinuous Percoll density gradients, as described (42).

### Cell Culture

High density B cells were cultured with anti-Ig-Sepharose (0.5 ml of 10% Sepharose/10 ml of RPMI 1640, 5% FCS, 5  $\mu\text{g}/\text{ml}$  gentamicin, and 50  $\mu\text{M}$  2-ME) at  $1\text{--}1.5 \times 10^6$  cells/ml for 48 h. Low density B cell blasts (anti-Ig blasts) were isolated by centrifugation (400 g for 10 min) over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). B lymphoblasts were then washed and recultured in 96-well microtiter plates at  $2\text{--}4 \times 10^5$  cells/ml with lymphokines (IL-2, -4, and -5), or at  $0.5\text{--}1 \times 10^5$  cells/ml with LPS (20  $\mu\text{g}/\text{ml}$ )  $\pm$  lymphokines. All additions in microtiter plates were made in triplicate. In experiments requiring isolation of total RNA, 20–40-ml secondary cultures were carried out in 75-cm<sup>2</sup> culture flasks.

### MTT 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 with minor modifications (43). After incubation with substrate (500  $\mu\text{g}/\text{ml}$ ) for 2–3 h, unconverted substrate and culture medium was aspirated from the wells. Product was solubilized with 100  $\mu\text{l}$  isopropanol and the absorbance at 570 nm was measured using an ELISA plate reader (Molecular Devices Corp., Menlo Park, CA).

### Isolation of Total Cellular RNA and RNA Blot Analysis

Total cellular RNA was isolated by the method of Chirgwin et al. (44) with minor modifications. Total RNA was quantitated by UV spectrophotometry (1 OD  $A_{260}$  = 40  $\mu\text{g}/\text{ml}$ ). 10–20  $\mu\text{g}$  of RNA was fractionated on 1% agarose gels containing 0.22 M formaldehyde and then transferred to nitrocellulose filters. Ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ) staining of 28S and 18S rRNA was used to confirm spectrophotometric quantitation of RNA, and to assess the apparent efficiency of transfer to nitrocellulose. RNA blots were hybridized to either gel-purified fragments or whole plasmid that had been nick-translated with [<sup>32</sup>P]deoxycytidine triphosphate. Filters were washed twice for 30 min at ambient temperature in 2 $\times$  SSC/0.1% SDS, then twice for 30 min at 65°C in 0.1 $\times$  SSC/0.1% SDS. After air drying, filters were exposed to Kodak XAR-5 film for 2–5 d at –70°C with enhancer screen.

### Nuclease Protection Assay

RNase protection assays were performed according to the method of Melton et al. (45). Total RNA was hybridized with  $1\text{--}1.5 \times 10^5$  cpm of antisense RNA probe (see below) in 80% formamide/40 mM Pipes, pH 6.4/0.4 M NaCl/1 mM EDTA for 16–18 h at 45°C. RNA:RNA hybrids were digested with RNase A and RNase T1 for 1 h at 30°C, as described (45). Protected fragments were fractionated on 6% polyacrylamide/8 M urea sequencing gels. Gels were dried and exposed to Kodak SB5 diagnostic film for 24–48 h with intensifying screen at –70°C. Band intensities were quantitated by scanning densitometry (Ephortec™; Joyce Loebel Densitometer, Vickers Instrument Co., Malden, MA). Size markers were derived from HinfI-digested pBR322 labeled with <sup>32</sup>P, using Klenow polymerase and  $\alpha$ -[<sup>32</sup>P]dATP.

### Probes

**RNA Probes.** pI $\gamma 1$ /PCR3, a pGEM-4Z plasmid vector (Promega Biotec, Madison, WI) containing a 419-bp cDNA fragment

of the germline  $\gamma 1$  transcript was generously provided by Dr. M. Berton (University of Texas Southwestern Medical Center, Dallas, TX). The p $\gamma 1$  insert encompasses 285 bp of the I $\gamma 1$  exon and 134 bp of the C $\gamma 1$  exon of C $\gamma 1$ . The plasmid p $\gamma 1$  was linearized with EcoRI and used as a template to transcribe antisense RNA probe using T7 polymerase (Riboprobe Gemini II system; Promega Biotec), according to the method of Melton et al. (45). For preparation of control sense RNA, probe p $\gamma 1$  was linearized with BamHI and transcribed using SP6 polymerase.

**S $\gamma 1$  Probe.** Germline  $\gamma 1$  transcripts were detected using nick-translated pS $\gamma 1$ B.X plasmid. pS $\gamma 1$ B.X contains a 1.85-kb BamHI fragment from p $\gamma 1$ /EH10.0 (46) subcloned in the antisense orientation into the BamHI site of plasmid vector pGEM-3Zf(+) (Promega Biotec). The BamHI fragment of pS $\gamma 1$ B.X, which contains the I $\gamma 1$  exon (47), encompasses the 5' portion of S $\gamma 1$  and 5' flanking sequences. The S $\gamma 1$  probe did not detect transcripts in RNA isolated from MOPC 21 (an IgG1 producing myeloma), demonstrating that it does not hybridize to mRNA for the  $\gamma 1$  H chain.

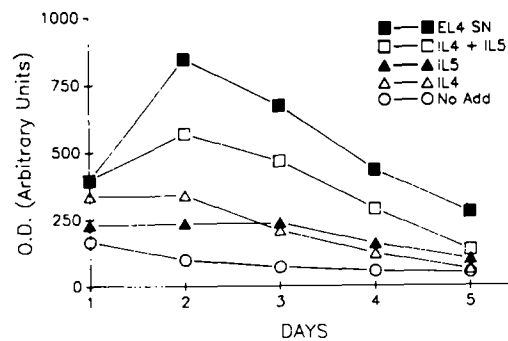
**C $\epsilon$  Probe.** Germline and productive  $\epsilon$  transcripts were detected using a nick-translated 797 bp AvaI/PstI fragment from p $\epsilon$  (48). The 797 bp AvaI/PstI fragment encompasses the CH1 exon and most of the IVS I of the  $\epsilon$  H chain gene. The pS $\gamma 1$ B.X and p $\epsilon$  plasmids were generously provided by Dr. M. Berton.

#### Assay for Ig Isotypes

Culture supernatant was assayed for Ig isotypes by solid phase RIA (IgG1) or ELISA (IgM and IgE). For IgE ELISA, plates were coated with a monoclonal anti-IgE, EM95. Primary incubation with IgE standards or sample was followed by a secondary incubation with biotinylated polyclonal anti-IgE (The Binding Site Inc.). Alkaline phosphatase-conjugated avidin (Zymed Laboratories, Inc., South San Francisco, CA) was then added, and plates were developed with p-nitrophenylphosphate (2 mg/ml) (Sigma Chemical Co., St. Louis, MO) in 0.1 M NaCO<sub>3</sub> pH 10, 2 mM MgCl<sub>2</sub>. All ELISA incubations were performed in 1% FCS/PBS/10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>/0.1% NP-40.

## Results

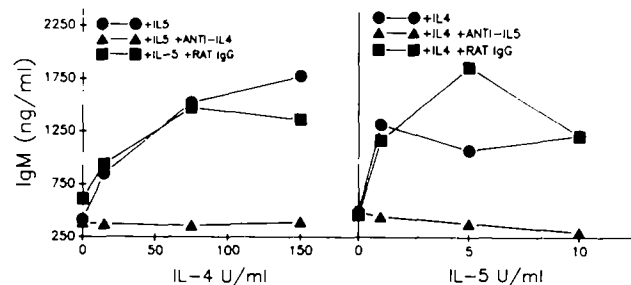
**The Combination of IL-4 and -5 Induces Proliferation and Maturation to IgM Secretion by B Lymphoblasts.** In the absence of an added stimulus, B lymphoblasts die within 24–48 h after removal of anti-Ig and initiation of secondary culture (39 and Fig. 1). In contrast, B lymphoblasts cultured with EL4 Sn or LPS undergo rapid and extensive proliferation (42). We first wished to define the lymphokines responsible for the mitogenic activity of EL4 Sn. Initial studies suggested that combinations of IL-2, -4, and -5 failed to mimic the activity of EL4 Sn on B lymphoblasts (49), but these studies were hampered by the lack of IL-5 preparations with high specific activity. Therefore, we have reexamined the ability of IL-4 and -5 to promote the growth and maturation of B lymphoblasts. B lymphoblasts were cultured with IL-5 and -4, alone or in combination, and B lymphoblast growth and viability was assessed at 24-h intervals by the MTT assay. Although IL-4 maintained B lymphoblast viability for 24–48 h, neither IL-4 nor -5 induced substantial B lymphoblast proliferation. In contrast, the combination of IL-4 and -5 induced marked B lymphoblast proliferation. In cultures with IL-4 and -5,



**Figure 1.** The combination of IL-4 and -5 promotes B lymphoblast proliferation. Anti-Ig-activated B lymphoblasts ( $2-4 \times 10^5$ /ml) were cultured in microtiter plates with the indicated combinations of IL-4 (150 U/ml) and -5 (100 U/ml) for 1–5 d. B lymphoblast growth and viability were assessed by the MTT assay. Results presented are the mean of triplicate wells from a representative experiment.

viability of B lymphoblasts peaked on day 2 or 3 and slowly declined thereafter. B lymphoblast proliferation in response to IL-4 and -5, as measured by the MTT assay, was lower in magnitude than the response to either LPS or EL4 Sn, and inclusion of IL-2 in cultures with IL-4 and -5 did not further enhance proliferation of B lymphoblasts (not shown).

We next assessed the ability of IL-4 and -5 to induce B lymphoblast maturation to Ig secretion. As shown in Fig. 2, culture of B lymphoblasts with both IL-4 and -5 resulted in marked secretion of IgM. Optimal induction of IgM secretion was dependent on both lymphokines, as addition of either monoclonal anti-IL-4 (10  $\mu$ g/ml) or anti-IL-5 (20  $\mu$ g/ml), but not control rat IgG, inhibited this response. IL-5 alone caused small increases in IgM secretion, and IL-4 consistently and markedly enhanced secretion of IgM in conjunction with IL-5. In contrast, IL-4 alone, even at concentrations in excess of 1,000 U/ml, failed to stimulate IgM secretion (not shown). IgM secretion was maximal at 50–150 U/ml IL-4 (with IL-5 at 100 U/ml), or 5–10 U/ml IL-5 (with 600 U/ml IL-4). As with B lymphoblast growth, inclusion of IL-2 (100 U/ml)

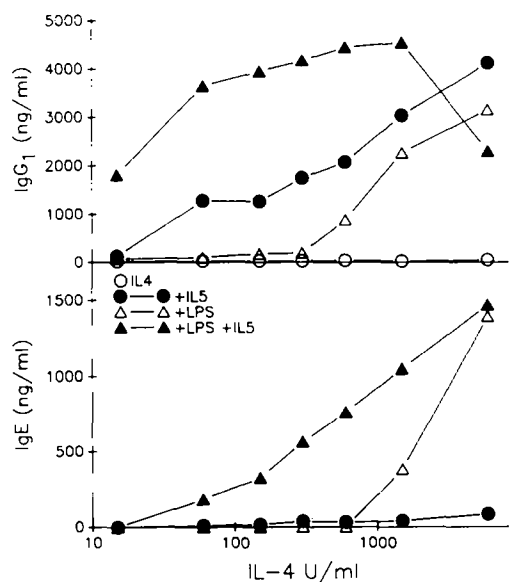


**Figure 2.** Induction of IgM secretion from B lymphoblasts requires both IL-4 and -5. B lymphoblasts ( $2-4 \times 10^5$ /ml) were cultured with IL-4 and/or -5 in the presence or absence of monoclonal anti-IL-4 and monoclonal anti-IL-5 for 3 d. The amount of secreted IgM in culture supernatants was determined by ELISA. (Left) 100 U/ml IL-5, 10  $\mu$ g/ml monoclonal anti-IL-4; (right) 600 U/ml IL-4, 20  $\mu$ g/ml monoclonal anti-IL-5.

had virtually no effect either alone or in combination with IL-4 and -5 (data not shown). These data demonstrate that the combination of IL-4 and -5 is sufficient to induce B lymphoblast proliferation and maturation to Ig secretion.

**IL-4 and -5 Induce Secretion of IgG1, but Not IgE from B Lymphoblasts.** Since the combination of IL-4 and -5 was sufficient to promote maturation of B lymphoblasts, the ability of IL-4 and -5 to induce isotype switching to IgG1 and IgE was examined. As shown in Fig. 3, culture of B lymphoblasts with both IL-4 (15–3,000 U/ml) and IL-5 (100 U/ml) resulted in substantial IgG1 secretion, but either lymphokine alone was ineffective. In four independent experiments, secretion of IgG1 was half-maximal between 50 and 150 U/ml IL-4, and maximal levels of IgG1 secreted in these cultures were comparable with those observed in cultures containing LPS. These results demonstrate that IL-5 induces secretion of IgG1 from B lymphoblasts stimulated with IL-4.

Previous studies demonstrated that LPS and IL-4 (up to 50 U/ml) failed to induce IgG1 secretion from B lymphoblasts (33). However, exceptionally high concentrations of IL-4 are required for induction of IgE secretion by B cells stimulated with LPS (4). Therefore, the ability of LPS and high concentrations of IL-4 to stimulate IgG1 secretion in the absence of IL-5 was examined. As shown in Fig. 3, marked secretion of IgG1 in cultures with LPS was not observed until IL-4 concentrations approached 1,000 U/ml. Inclusion of IL-5 in cultures with LPS and IL-4 shifted the concentration response curve to IL-4 100-fold to the left. Thus, consistent with our previous results, IL-5 promotes secretion of IgG1 from B lymphoblasts treated with LPS and IL-4 (33). However, induction of IgG1 secretion from B lymphoblasts cultured with LPS was observed in the absence of IL-5 when exceptionally high concentrations of IL-4 were employed.



**Figure 3.** Stimulation of IgG1 and IgE secretion from B lymphoblasts. B lymphoblasts were cultured with LPS (20  $\mu$ g/ml), IL-4, or -5 (100 U/ml) alone, or in the indicated combinations for 5 d. Levels of secreted IgG1 and IgE were determined by RIA and ELISA, respectively.

In contrast to what was observed with IgG1 secretion, virtually no secreted IgE was detected in cultures of B lymphoblasts with IL-4 and -5, even at very high concentrations of IL-4, whereas marked secretion of IgE was observed in cultures where LPS was included along with IL-4 and -5 (Fig. 3, bottom). Inclusion of IL-2 along with IL-4 and -5 failed to induce IgE secretion (not shown). Induction of IgE secretion by LPS and IL-4 required IL-4 concentrations of 1,000 U/ml or greater, and addition of IL-5 to these cultures shifted the concentration response 10-fold to the left.

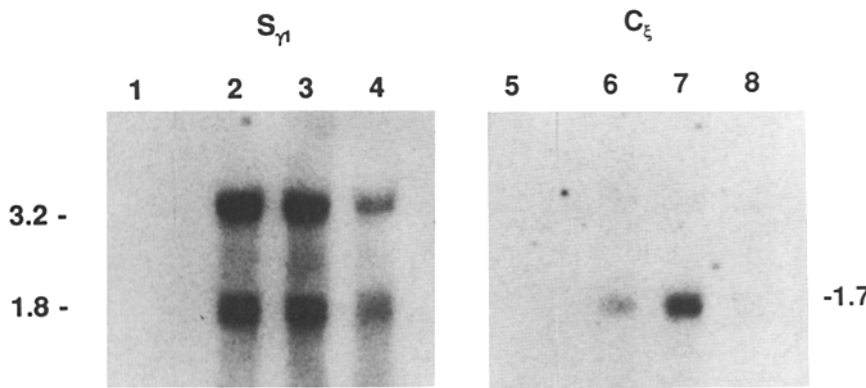
The preceding data suggest that an additional signal(s), which can be provided by LPS, is required for expression of IgE in B lymphoblasts cultured with IL-4 and -5. Whether a cytokine could also provide this other signal was examined. As shown in Table 1, culture of B lymphoblasts with EL4 Sn (which contains cytokines in addition to IL-4, -5, and -2) was sufficient to induce secretion of IgE. This result suggests that a cytokine(s) present in EL4 Sn can provide the additional signal required for expression of IgE. The observation that a stromal cell-derived cytokine is mitogenic for B lymphoblasts (49), prompted us to examine whether this cytokine could also support IL-4-mediated induction of IgE secretion. B lymphoblasts cultured with conditioned medium derived from TC-1 cells proliferate and secrete IgM (49), but do not secrete IgG1 and IgE (Table 1). Similar to results with LPS, marked induction of IgG1 and IgE secretion from B lymphoblasts cultured with TC-1 CM required IL-4 concentrations in excess of 1,000 U/ml. Inclusion of IL-5 in cultures with TC-1 CM and IL-4 (150 U/ml) promoted IgG1 and IgE secretion. Taken together, these data suggest that a cytokine(s) produced by TC-1 or EL4 cells provides a signal(s) that may play an important role in switching to IgE.

**LPS Promotes Accumulation of Germ-line Transcripts Induced By IL-4.** The next set of experiments were designed to in-

**Table 1.** A Cytokine Promotes IL-4-mediated Secretion of IgE

Exp.	Stimulus	IgG1		IgE	
		ng/ml	ng/ml	ng/ml	ng/ml
A	(D)EL4	ND	ND	15	15
	(D)EL4 + IL-4 (100 U/ml)	ND	ND	379	379
B	IL-4 (1,500 U/ml)	<10	<10	<10	<10
	IL-5	340	340	31	31
	IL-4 (1,500 U/ml) + IL-5	3,467	3,467	85	85
	TC-1 CM	73	73	<10	<10
	TC-1 + IL-4 (150 U/ml)	273	273	<10	<10
	TC-1 + IL-4 (150 U/ml) + IL-5	3,863	3,863	370	370
	TC-1 + IL-4 (1,500 U/ml)	2,725	2,725	567	567

B lymphoblasts ( $10^5$ /ml) were cultured with the indicated combinations of TC-1 CM (1% vol/vol), IL-4 and -5 (100 U/ml) for 6 d. The amounts of secreted IgG1 and IgE in culture supernatants were determined by RIA and ELISA, respectively. Results presented are the mean of triplicate wells. (D) EL4, IL-4 depleted EL4 Sn.



**Figure 4.** LPS promotes accumulation of germline transcripts induced by IL-4. Total cellular RNA was isolated from B lymphoblasts cultured with LPS for 48 h (lanes 1 and 5), LPS and 1,500 U/ml IL-4 for 24 (lanes 2 and 6) or 48 h (lanes 3 and 7), or IL-4 (1,500 U/ml) and -5 (100 U/ml) for 48 h (lanes 4 and 8). Steady state levels of germline  $\gamma 1$  and  $\epsilon$  transcripts were assessed by Northern blot analysis using the  $S\gamma 1$  and  $C\epsilon$  probes, respectively.

investigate the mechanisms by which: (a) LPS induces secretion of IgE in B lymphoblasts stimulated with IL-4 and -5; and (b) IL-5 promotes secretion of IgG1 and IgE from B lymphoblasts stimulated with IL-4. To investigate the molecular basis for the effects of LPS and IL-5 on isotype switching, we examined induction of germline  $\gamma 1$  and  $\epsilon$  transcripts in B lymphoblasts. Total RNA was isolated from B lymphoblasts 1-4 d after initiation of secondary culture with the indicated combinations of IL-4, -5, and LPS. In some experiments, steady state levels of germline  $\gamma 1$  transcripts were measured by Northern blot analysis using a  $S\gamma 1$  probe. The  $S\gamma 1$  probe detected two species of germline  $\gamma 1$  transcripts of 3.2 and 1.8 kb, that differ at the 3' end due to differential splicing of membrane exons and length of the 3' untranslated region (Fig. 4). Since germline and productive  $\gamma 1$  transcripts are equivalent in size (24), steady state levels of germline and productive transcripts were determined independently with the  $I\gamma 1$  probe in a nuclease protection assay (see Materials and Methods). The germline  $\gamma 1$  transcript is detected as a 419 bp protected fragment, and a 134-bp protected fragment corresponds to the productive  $\gamma 1$  transcript (Fig. 5). Steady state levels of germline (1.7 kb) and productive (1.9 kb)  $\epsilon$  transcripts were assessed independently by Northern blot analysis using a  $C\epsilon$  probe.

As shown in Fig. 4, germline  $\gamma 1$  transcripts were detected in RNA from blasts cultured for 24 or 48 h with LPS and IL-4 (1,500 U/ml). Marked induction of germline  $\gamma 1$  transcripts was also observed in B lymphoblasts cultured with LPS and 150 U/ml IL-4 for 48 h (Fig. 5). Steady state levels of germline  $\gamma 1$  transcripts decreased slightly after 3-4 d of culture. Thus, concentrations of IL-4 (e.g., 150 U/ml) that fail to induce secretion of IgG1 are sufficient to induce accumulation of germline  $\gamma 1$  transcripts. Although germline  $\gamma 1$  transcripts were also detected in RNA isolated from blasts cultured with IL-4 and -5 (Fig. 4 and 5), or IL-4 alone for 24 h (not shown), the steady state levels of germline  $\gamma 1$  transcripts in blasts cultured with IL-4 and -5 were four- to sevenfold lower than the levels in blasts cultured with LPS and IL-4.

In contrast, germline  $\epsilon$  transcripts were not detectable in blasts cultured with IL-4 and -5, in the absence of LPS (Fig. 4). Induction of germline  $\epsilon$  transcripts was observed in B

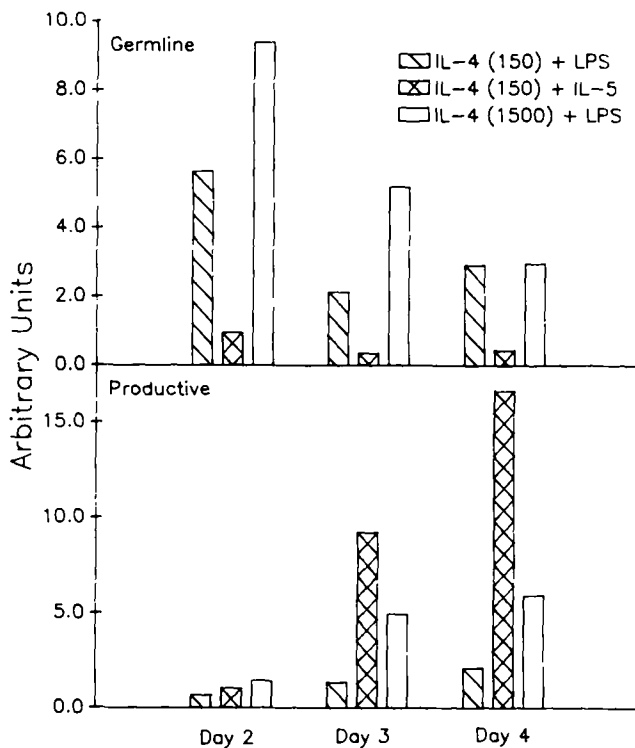
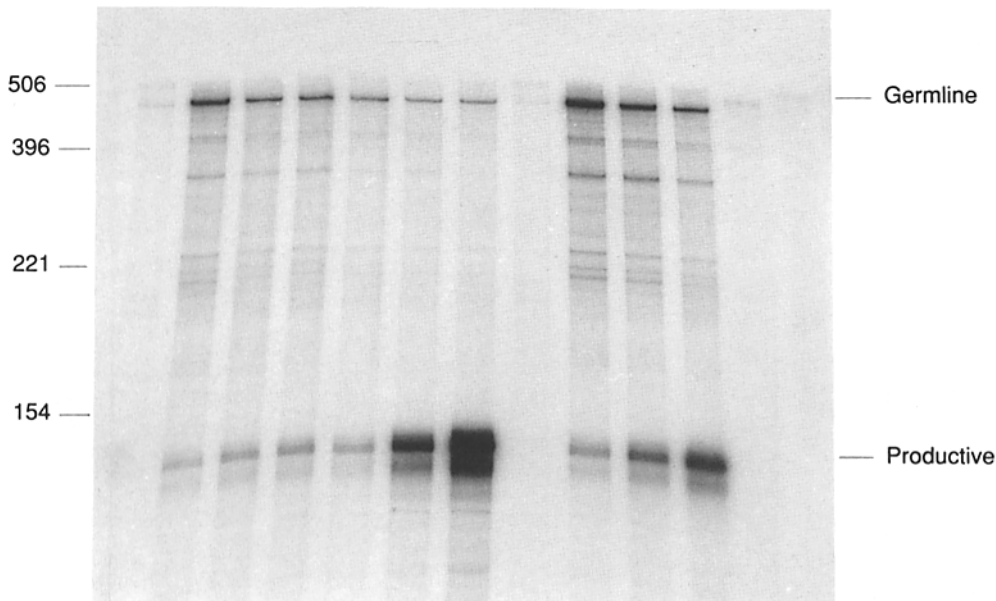
lymphoblasts cultured with LPS and IL-4 for 24 h, and steady state levels increased after 48 h of culture. Neither  $\gamma 1$  nor  $\epsilon$  transcripts were detected in RNA isolated from B lymphoblasts cultured with LPS alone (Figs. 4-6) or LPS and IL-5 (Fig. 5), demonstrating that induction of these transcripts was dependent on IL-4. Consistent with the results of others (24, 50), these data demonstrate that LPS promotes accumulation of germline transcripts induced by IL-4.

Whereas LPS enhanced accumulation of germline transcripts, LPS was relatively ineffective at promoting accumulation of productive  $\gamma 1$  and  $\epsilon$  transcripts. As shown in Fig. 5, low levels of productive  $\gamma 1$  transcripts were detected in RNA isolated from B lymphoblasts cultured with LPS and IL-4 after 2 or 3 d of culture, and the level of these transcripts increased slightly through 4 d of culture. Virtually no productive  $\epsilon$  transcripts were observed in B lymphoblasts cultured with LPS and IL-4 (150 U/ml) (Fig. 6). The failure to detect high steady state levels of productive transcripts under these conditions is consistent with the relative ineffectiveness with which LPS induces secretion of IgG1 and IgE from B lymphoblasts cultured with moderate concentrations of IL-4 (150-300 U/ml).

*High Concentrations of IL-4 Promote Accumulation of Productive  $\gamma 1$  and  $\epsilon$  Transcripts in B Lymphoblasts.* As shown in Fig. 3, marked secretion of IgG1 and IgE was observed in the absence of IL-5 when B lymphoblasts were exposed to LPS and IL-4 concentrations of 1,000 U/ml or more. Therefore it was interesting to determine whether high concentrations of IL-4 promoted accumulation of productive transcripts. As shown in Figs. 5 and 6 C, IL-4 (1,500 U/ml) induced accumulation of productive  $\gamma 1$  and  $\epsilon$  transcripts in B lymphoblasts cultured with LPS. In the presence of LPS, high concentrations of IL-4 (1,500 U/ml) induced three- to fivefold greater increases in steady state levels of productive  $\gamma 1$  transcripts than were observed with 150 U/ml IL-4. Thus, though IL-4 at 150 U/ml was sufficient to induce expression of germline  $\gamma 1$  and  $\epsilon$  transcripts, 10-fold higher concentrations of IL-4 were required for marked accumulation of the respective productive transcripts and secretion of IgG1 and IgE in the absence of IL-5.

*IL-5 Induces Accumulation of Productive  $\gamma 1$  and  $\epsilon$  Transcripts.*

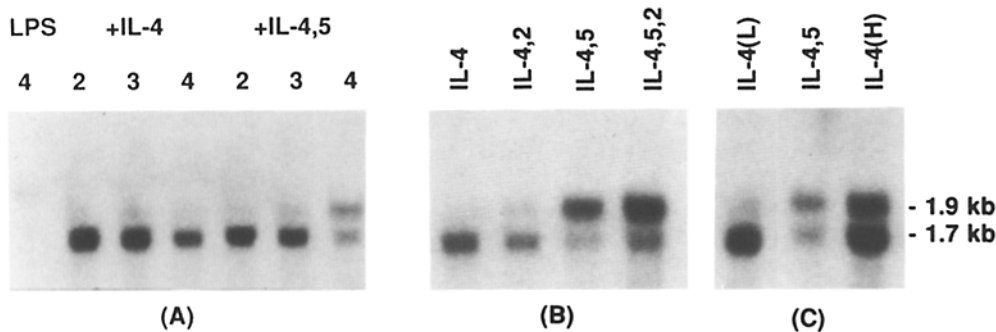
LPS	+	+	+	+	-	-	-	+	+	+	+	+	S.p.
IL-4	-	150	150	150	150	150	150	-	1500	1500	1500	1500	A.S.P.
IL-5	+	-	-	-	+	+	+	-	-	-	-	-	
DAY	3	2	3	4	2	3	4	3	2	3	4	4	



**Figure 5.** IL-5 promotes accumulation of productive  $\gamma 1$  transcripts. B lymphoblasts were cultured with LPS (20  $\mu\text{g}/\text{ml}$ ) and IL-4 (150, 1,500 U/ml), or IL-4 (150 U/ml) and -5 (50 U/ml). Total cellular RNA was isolated after 2, 3, and 4 d of culture. 5  $\mu\text{g}$  of total cellular RNA/condition was analyzed by the nuclease protection using the I $\gamma 1$  RNA probe. (Top) Autoradiogram. (Bottom) Band intensities were quantitated by scanning densitometry. SP = sense probe; ASP = antisense probe. (-)RNA = total RNA not included in hybridization mixture.

Inclusion of IL-5 (50 U/ml) in cultures with IL-4 resulted in marked induction of steady state levels of productive  $\gamma 1$  transcripts that was detectable after 3 d of culture, increased through day 4 (Fig. 5), and correlated with the appearance of secreted IgG1 in supernatants of parallel cultures (not

shown). IL-5 also enhanced steady state levels of both productive  $\gamma 1$  transcripts in B lymphoblasts treated with IL-4 (150 U/ml) and LPS. In three independent experiments, accumulation of  $\gamma 1$  productive transcripts was increased by IL-5 (50–100 U/ml) 4–10-fold over the levels stimulated by LPS.



**Figure 6.** IL-5 and high concentrations of IL-4 induce accumulation of productive  $\epsilon$  transcripts. B lymphoblasts were cultured with LPS and IL-4, with or without IL-5/IL-2. Total cellular RNA was isolated from blasts in each condition after either 2, 3, or 4 d (A), or 4 d of culture (B and C). 20  $\mu$ g of total cellular RNA/condition was analyzed by Northern blot analysis using a  $C_{\epsilon}$ -specific probe. IL-4(L) = 150 U/ml, IL-4(H) = 1,500 U/ml, IL-5 = 100 U/ml, IL-2 = 100 U/ml.

These results demonstrate that IL-5 promotes expression of productive  $\gamma 1$  transcripts, and that this effect of IL-5 is independent of costimulation with LPS.

IL-5 also stimulated accumulation of productive  $\epsilon$  transcripts in B lymphoblasts treated with LPS and IL-4 (150 U/ml). Accumulation of productive  $\epsilon$  transcripts by IL-5 was not observed before day 4 (Fig. 6 A), and coincided with the appearance of secreted IgE in culture supernatants (not shown). Induction of the 1.9-kb transcript by IL-5 was accompanied by an apparent decrease in the level of germline 1.7-kb transcripts, resulting in a marked increase in the apparent ratio of productive (1.9-kb)/germline (1.7-kb) transcripts (Fig. 6, A and B). In contrast, IL-2 did not enhance the accumulation of productive  $\epsilon$  transcripts in B lymphoblasts cultured with LPS and IL-4 (Fig. 6 B). Taken together, these data suggest that IL-5 promotes secretion of IgG1 and IgE by inducing accumulation of productive  $\gamma 1$  and  $\epsilon$  transcripts.

## Discussion

IL-4 induces transcription of the unrearranged  $\gamma 1$  and  $\epsilon$  H chain loci resulting in the accumulation of germline  $\gamma 1$  and  $\epsilon$  transcripts in B lymphocytes. However, IL-4 alone is not sufficient to induce switch recombination, accumulation of productive transcripts, or secretion of IgE (7–10). Results presented here demonstrate that IL-5 can provide a second signal necessary for isotype switching to IgG1 and IgE. In murine B lymphoblasts we found that IL-5 with IL-4 was both necessary and sufficient to elicit IgG1 secretion, and that IL-5 promoted IL-4 dependent IgG1 and IgE secretion from B lymphoblasts treated with LPS. This indicates that IL-5 can substitute for interaction with T cells by providing a second stimulus required for switching to IgG1 in B lymphoblasts. However, this effect of IL-5 is dependent on an initial B cell activation stimulus, such as crosslinking surface Ig, or contact with plasma membranes isolated from activated T cells (51), since resting B cells do not respond to IL-5 (42, 52). Furthermore, cognate interaction with T cells induces both IgG1 and IgE secretion (5, 6) but IL-5, in conjunction with IL-4, was not sufficient to induce IgE secretion from B lymphoblasts. Thus, IL-5 does not completely replace signals provided by contact with CD4<sup>+</sup> T cells.

Data presented in Figs. 5 and 6 suggest possible mecha-

nisms by which IL-5 promotes expression of IgG1 and IgE. A trivial explanation for the effects of IL-5 on Ig isotype expression was that IL-5 promoted maturation or growth of cells precommitted to IgG1 and IgE by IL-4. However, the observation that IL-5 induced accumulation of productive  $\gamma 1$  and  $\epsilon$  transcripts, and that (in the absence of LPS) steady state levels of productive  $\gamma 1$  transcripts were consistently increased by more than 10-fold over a period of 48 h, is inconsistent with the notion that IL-5 simply promotes maturation or selective expansion of “switched” cells. Alternatively, IL-5 may promote IgG1 and IgE secretion by enhancing transcription of the rearranged  $\gamma 1$  and  $\epsilon$  heavy loci (53–55), regulating RNA polymerase termination leading to preferential accumulation of  $\gamma 1$ , and  $\epsilon$ , mRNA (56), or increasing stability of the VDJ-C $\gamma 1$  and VDJ-C $\epsilon$  mRNA. However, results presented here are also consistent with the supposition that IL-5 stimulates accumulation of productive transcripts and secretion of IgG1 and IgE by inducing switch recombination to the  $\gamma 1$  and  $\epsilon$  H chain loci.

In contrast to IL-5, LPS was an ineffective second signal for isotype switching in B lymphoblasts. B lymphoblasts cultured with LPS and IL-4 (up to 300 U/ml) expressed low levels of productive transcripts and secreted little or no IgG1 and IgE. However, the combination of IL-4 (150 U/ml) and LPS was sufficient to induce marked accumulation of germline  $\gamma 1$  and  $\epsilon$  transcripts (Figs. 4 and 5), demonstrating that B lymphoblasts are capable of responding to LPS and IL-4. Although LPS stimulation was a relatively ineffective second signal for isotype switching in B lymphoblasts, LPS does contribute to lymphokine regulation of Ig gene expression. LPS promoted IgE secretion from B lymphoblasts cultured with IL-4 and -5 (Fig. 3), and increased the steady state levels of germline transcripts induced by IL-4 (Figs. 4 and 5). It has been suggested that steady state levels of germline transcripts correlate with the propensity of B cells to switch to the respective Ig isotypes (27). Thus, it is interesting to speculate that LPS increases the frequency of switching to IgE by promoting accumulation of germline transcripts, which in turn, may play a functional role in switch recombination.

Surprisingly, exceptionally high concentrations of IL-4 (>1,000 U/ml) induced marked accumulation of productive transcripts and secretion of IgG1 and IgE (Figs. 3, 5, and 6). Since high concentrations of IL-4 alone fail to induce Ig

secretion from B lymphoblasts, a mitogenic stimulus (e.g., LPS) is required in addition to high concentrations of IL-4 for stimulation of IgG1 and IgE secretion. Similar findings were reported by Snapper et al. (4) who showed that high concentrations of IL-4 were required for stimulation of IgE secretion from LPS-treated B cells. Concentrations of IL-4 (150–300 U/ml) that fail to elicit secretion of IgG1 and IgE are equivalent to 0.5–1 nM, which is 10–100-fold in excess of the reported  $K_d$  (10–100 pM) of the high-affinity receptor, and therefore should be sufficient to saturate this receptor (57). It is possible that the effects of high IL-4 concentrations are not mediated by interactions with the 140-kD IL-4 receptor (58). Fernandez-Botran et al. recently reported that, at high concentrations, IL-4 interacts with a second cell surface molecule that may be distinct from the 140-kD IL-4 receptor (59). It is interesting to speculate that IL-4 binds with low affinity to an uncharacterized cell surface molecule, and that this interaction produces additional signals that supplant the requirement for IL-5.

Another important finding of this study was that, although IL-4 and -5 were sufficient to induce switching to IgG1, an additional stimulus was required for induction of IgE secre-

tion. It is important to note that even exceptionally high concentrations of IL-4 (in excess of 1,000 U/ml) failed to induce IgE secretion from B lymphoblasts cultured with IL-5. Related findings were recently reported by Snapper et al. (60), who showed that induction of IgE secretion by lymphokines was dependent on the efficiency of surface Ig crosslinking (60). Thus, production of IgE in B lymphoblasts required three distinct signals: (a) an isotype-specific switch signal provided by IL-4; (b) a nonisotype-specific maturation and/or switch signal provided by IL-5; and (c) an additional signal(s) that can be provided by LPS. This additional stimulus was also provided by conditioned medium derived from EL4 or TC-1 cells (Table 1). As noted above, LPS may enhance switching to IgE by increasing the steady state levels of germline  $\epsilon$  transcripts induced by IL-4. Although we have not demonstrated that the cytokine(s) produced by EL4 or TC-1 cells regulates steady state levels of germline transcripts induced by IL-4, it seems likely that the cytokine(s) promotes switching to IgE by a similar mechanism. Nonetheless, these results suggest that in a T cell-driven response, other cytokines may act in concert with IL-4 and -5 to induce switching to IgE.

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