Allergy and Allergic Diseases

The New Mechanisms and Therapeutics

ALLERGY AND ALLERGIC DISEASES

THE NEW MECHANISMS AND THERAPEUTICS

Edited by

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To my father of blessed memory, a student day and night.

PREFACE

Allergy and Allergic Diseases has been organized to provide an up-to-date, clinically relevant compilation of one of the most exciting areas of investigation in medicine today–allergic disease, especially as it pertains to the skin, airways, and bowel. With the dramatic rise in the incidence of various allergic disorders worldwide, and the coming of age of the discipline of Clinical Immunology and Allergy, the interface between basic and clinical science in this arena demands highlighting in this comprehensive new synthesis. It is with the hope of filling this evident need that *Allergy and Allergic Diseases: The New Mechanisms and Therapeutics* has been put together.

The book's content is divided into both basic and clinical sections, with emphasis on various components of the immune and inflammatory response as they relate to the development of allergic disease. Topics span the range from molecular biology to clinical symptomatology, with an effort to make this of interest to as broad a constituency as possible. This book will therefore be of substantial interest to specialists in Clinical Immunology and Allergy, scientists studying the cellular and molecular biology of inflammation and immunity, as well as internists, teachers, developers of medical school curricula, and members of industry focused on drug discovery and therapeutics. Indeed, a separate section has been added to deal with some specific issues in this latter field.

Wherever possible, figures and schematic drawings of mechanisms have been added to chapters; of necessity, some areas might be covered in more than one chapter, and we expect that this minor redundancy will result in clearer, deeper understanding among readers.

Our hope in creating *Allergy and Allergic Diseases* is that the general principles enunciated, as well as the specific lines of investigation discussed in the basic science section, will result in a work having genuine and lasting value as a standard reference resource for years to come.

Finally, this book could not have come together without the patient and indefatigable assistance of Lynne Larocque.

Judah A. Denburg

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I EPIDEMIOLOGY/IGE

1 Molecular Mechanisms of Isotype Switching to IgE

Donata Vercelli, MD

CONTENTS

THE TWO-SIGNAL MODEL FOR THE INDUCTION OF IgE SYNTHESIS

During an immune response, B-lymphocytes express different immunoglobulin (Ig) heavy-chain isotypes sharing the same variable region. This phenomenon (isotype switching) allows a single B-cell clone to produce antibodies with the same fine specificity, but different effector functions. In order to switch to a particular isotype, a B-cell needs to receive two signals: signal 1 is cytokine-dependent, results in the activation of transcription at a specific region of the Ig locus, and determines isotype specificity. Signal 2 activates the recombination machinery, and leads to DNA switch recombination.

The two signals required for switching to the IgE isotype are delivered to B-cells by T-cells through a complex series of interactions. Allergen-specific B-cells capture the antigen via their surface Ig molecules, internalize it, and process it into peptides, which are then presented to T-cells in the context of major histocompatibility complex (MHC) class II molecules. Recognition of the antigen/MHC class II complex by the T-cell receptor leads to two crucial events: the secretion of lymphokines (interleukin [IL]-4 or IL-13) that provide signal 1 for IgE induction, and the expression of CD40 ligand (CD40L). Notably, CD40L is absent on resting T-cells, and it is the expression of this molecule following activation that renders T-cells fully competent to induce switching to IgE. Engagement of CD40 on B-cells by its ligand on T-cells delivers signal 2, and triggers switch recombination to IgE. Amplification circuits involving costimulatory molecules, particularly the CD28/B7 pair, then induce high-rate lymphokine secretion and IgE synthesis. In this chapter, the author discusses how the molecular events triggered in B-cells by these signals lead to IgE isotype switching.

MOLECULAR EVENTS IN THE INDUCTION OF IgE SYNTHESIS

Analysis at the DNA and RNA level provides the key to understanding the complex cell–cell interactions required to trigger IgE switching. Indeed, each of the main steps in T-/B-cell interactions described corresponds to a critical event that must occur at the gene level in order for switching to proceed. Signal 1, i.e., the cytokine IL-4 or IL-13, induces ε germline transcription, whereas CD40 engagement activates the switch recombination machinery (Fig. 1).

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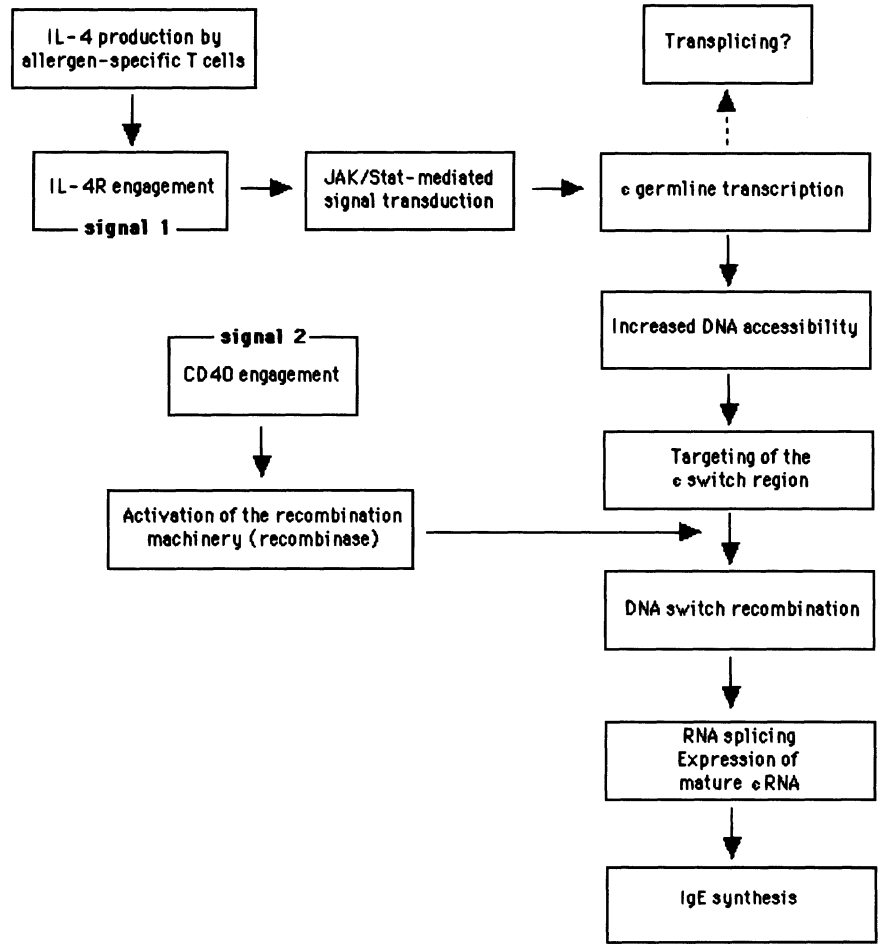

Fig. 1. Molecular events in isotype switching to IgE. IL-4 or IL-13 induce ε germline transcription and make the Sε region accessible for switch recombination. CD40 engagement activates the recombination machinery, and results in Sμ/Sε switch recombination. Intervening sequences are deleted as switch circles.

Signal 1 Induces Germline Transcription

Isotype switching results from a DNA recombination event that juxtaposes different downstream C_H genes to the expressed VDJ gene. Switching is not a random event, but is directed by cytokines in conjunction with the regulation of B-cell proliferation and differentiation *(1)*. Molecular analysis has shown that induction of isotype switching to a particular C_H gene invariably correlates with the transcriptional activation of the same gene in germline configuration. Several murine and human germline transcripts have been cloned, and share structural similarities *(2)*. The germline transcripts initiate from non-TATAA promoters a few kilobases upstream of the switch (S) region, and proceed through short exons (I_H exons) that are spliced to the first exon of the C_H gene. The region containing the germline promoter and the I_H exon is deleted during switch recombination.

Germline transcripts are unable to encode any mature protein of significant length, because the I_H exon contains multiple stop codons in all three reading frames. Therefore, these tran-

scripts are also referred to as "sterile." Alternatively, germline transcripts are referred to as "truncated," because the I exon is usually 200–300 basepairs (bp) shorter than the VDJ exon present in mature transcripts. Although there is no significant conservation of the sequences of germline transcripts of different isotypes, their overall structure is conserved *(2)*.

Germline transcription is thought to direct switching by modulating the accessibility of a particular S region to a common recombinase. The importance of germline transcription in the regulation of isotype switching has recently been shown by gene knockout experiments. Deletion of the Iγ1 *(3)* or Iγ2b *(4)* exons and their promoter resulted in the inhibition of class switching to the corresponding genes, even though the corresponding S regions were not affected by the mutation. These results suggest that transcription in the S region is necessary to target the appropriate S region for recombination and switching. However, it is apparently not sufficient: replacement of Iε with a B-cell-specific promoter cassette containing the murine Eμ intronic enhancer and a V_H promoter, without the Iε splice donor site, resulted in only marginal switch recombination to IgE, at about 1% of the frequency induced by IL-4 *(5)*. In contrast, replacement of all known IL-4-inducible control elements in the Sγ1 region with the heterologous human metallothionein II_A promoter did not impair switch recombination to IgG1, provided that a 114-bp sequence that contains the Iγ1 splice donor site was included in the construct, thus allowing for the induction of artificial, but processed, germline transcripts *(6)*. These data indicate that artificial induction of structurally conserved, spliced germline transcripts can target switch recombination, whereas transcription in the S region as such cannot. Spliced switch transcripts (or the process of splicing) may thus have a functional role in switch recombination *(6)*. The most intriguing speculation is that germline transcripts are part of the switch recombinase machinery, providing the specificity to target distinct S regions. Alternatively, the 114-bp region that contains the Iγ1 splice donor site may also contain regulatory elements for γ1 switching unidentified to date.

Interestingly, the Iμ germline promoter is constitutively active in B-cells before and after the Sμ region sequence has undergone a primary recombination event *(7)*. Because Cμ is deleted upon switch recombination, the Iμ-containing germline transcript generated after a primary switching event is a hybrid that includes Iμ correctly spliced to the now-juxtaposed downstream C_H exon (i.e., Iμ-Cγ2b) *(7)*. These findings are consistent with a model in which simultaneous germline expression of both the 5′ and 3′ genes involved in a given switch recombination event is needed to target them for the process. The specificity of primary or sequential switching events comes from cytokine-induced germline transcription of the involved 3′ C_H gene. Gene targeting experiments are required to understand whether continuous transcription through Iμ is required for either primary and/or sequential switching.

Germline transcripts have also been proposed to play a major role in interchromosomal RNA *trans*-splicing, whereby transcripts from rearranged VDJ exons are joined to C_H germline transcripts *(8)*. This process, which would result in heavy chain class switching in the absence of deletional DNA rearrangement in the C_H locus, has recently been shown to underlie multiple Ig isotype expression in the TG.SA transgenic mouse model *(9)*. Splenic B-cells from TG.SA mice expressed the transgenic human IgM and endogenous murine IgG simultaneously after stimulation with lipopolysaccharide (LPS) and IL-4. The B-cells bearing double isotypes expressed several mRNA species, i.e., transgenic human VDJ properly spliced to the endogenous mouse Cγ genes (*trans*-mRNA), together with the transgenic human μ RNA and germline transcripts derived from the mouse Cγ gene. No rearrangement of the transgene was detected *(10)*. It is conceivable that B-cells expressing multiple Ig isotypes might be intermediates for class switching. *Trans*-splicing involving germline transcripts might therefore be of general importance for class switching, although the role played by this mechanism in physiologic conditions is not yet clear.

ε *Germline Transcription Is Regulated By Nuclear Factors*

Nuclear factors specifically bind to relatively short (10–20 bp) DNA sequences, functionally defined as responsive elements (RE). The general paradigm for weak promoters, such as the I_H promoters, is that all slots for nuclear transcription factors need to be filled, in order for the gene to "fire." This implies a level of tight combinatorial control. Thus, the activation function of different transcription factors can operate at different limiting steps in the initiation reaction.

Because transcription through the I_H exon and the S region seems to be required to target the appropriate S region for recombination and switching, the induction of germline transcripts is a key step in determining the isotype specificity of the switching event. Different cytokines specifically induce different nuclear factors that activate transcription at the appropriate germline promoter. The specificity in the induction of transcription factors is essential for the specificity of cytokine-induced germline transcripts and isotype switching.

Expression of ε germline transcripts is regulated at the transcriptional level by nuclear factors that bind to the Iε promoter and adjacent regions. The requirements for the induction of ε germline transcripts seem to differ between mice and humans. Two signals, IL-4 (or IL-13) and LPS, are required for ε germline transcription in most murine B-cell lines, whereas the cytokine alone is sufficient in humans. A number of transcription factors have been found to bind to the ε germline promoter (Fig. 1); their roles, individually and with respect to each other, have not yet been fully characterized (Table 1).

A major IL-4 RE has been shown to bind a complex formed by a member of the C/EBP family, NF-kB p50, and the IL-4-inducible factor Stat6 (signal transducer and activator of transcription) (NF-IL-4, IL-4 NAF: *see below*) *(11)*. It has been proposed that these factors may have to interact physically in order to induce ε germline transcripts. The importance of NF-kB for the induction of murine germline transcripts has recently been confirmed by the finding that expression of germline transcripts for several isotypes, including IgE, and switching to the same isotypes, is severely impaired in NF-kB p50 knockout (KO) mice *(12)*. NF-kB seems to be essential for human ε germline transcription, as well (M. Woisetschläger, personal communication).

Stat6 *(13,14)* belongs to the newly identified family of signal transducers and activators of transcription (STAT) *(15)*. Binding of IL-4 to its receptor leads to activation by tyrosine phosphorylation of two receptor-associated cytoplasmic tyrosine kinases, Janus kinase (JAK)-3 and, to a lesser extent, JAK-1 *(16)*. These kinases are believed to rapidly induce tyrosine phosphorylation of Stat6, a latent cytoplasmic factor. The phosphorylated Stat6 homodimerizes, translocates to the nucleus, and binds to the promoter of a number of genes, contributing to the activation of transcription *(13)*. Stat6 preferentially binds dyad symmetric half-sites separated by 4 bp (TTCNNNNGAA). DNA binding specificity is localized to a region of 180 amino acids at the N-terminal side of the putative SH3 domain *(17)*. The discovery of JAKs and Stats has provided an explanation for the apparent paradox that the IL-4R, like the receptors for a number of other cytokines, lacks kinase domains, and yet couples ligand binding to tyrosine phosphorylation. Stat6 is not B-cell-specific, and is induced by IL-4 in monocytes, where it participates in the transcriptional regulation of the IL-4-inducible CD23b promoter, and possibly of the FcγR1 promoter *(18)*. Stat6 binding sites have been identified in the promoters of a number of other IL-4-responsive genes, such as Cε *(11,19,20)*, Cγ1, MHC class II *(18,20)*. The presence of homologous RE in the promoter of different genes underlies the concerted regulation of these genes by a single cytokine. Thus, the concerted modulation in the expression of ε germline transcription, CD23, and MHC class II in B-cells stimulated with IL-4 is mediated by IL-4 RE located in the promoters of these genes.

The B-lineage-specific B-cell-specific activator protein (BSAP) is essential for the IL-4-dependent induction of ε germline transcription. Deletion of the BSAP binding site has been

Table 1
Regulatory Elements in the ε Germline Promoter

Factor	*Function*
BSAP	Positive regulator of ε germline transcription
(B-cell lineage-specific	Positive regulator of the CD19 promoter
activator protein)	Repressor of the Ig 3′α enhancer and XBP 1 promoter
	Required for B-cell proliferation
	B-cell-specific
	Constitutively expressed
Stat6	Latent cytoplasmic factor
(signal transducer and	Induced by IL-4
activator of	Translocates to the nucleus upon phosphorylation and dimerization
transcription)	Positive regulator of IL-4-inducible genes (CD23b, MHC class II, FcγR1)
	Required for IL-4-dependent ε germline transcription and IgE switching
	Repressor of ε germline transcription at baseline
	Non-B-cell-specific
NF-κB	Involved in B-cell proliferation/differentiation
(nuclear factor-κB)	Required for germline transcription and class switching
	Non-B-cell-specific
	Constitutively expressed

shown to severely impair the induction of ε germline promoter activity by IL-4 + LPS in murine B-cells *(21)*, and by IL-4 alone in human B-cells *(22)*. BSAP is the mammalian homolog of the sea urchin DNA binding protein TSAP (tissue-specific transcription activator protein), a regulator of late histone genes, and belongs to the Pax gene family of homeodomain transcription factors. It contains at the N-terminus a paired domain, which is necessary and sufficient for DNA binding, even as a monomer. BSAP is expressed in B-lymphocytes (from pro-B to mature B, but not in terminally differentiated plasma cells), the developing central nervous system (CNS), and adult testis *(23)*. BSAP KO mice show a complete block of early B-cell differentiation (at the $B220^+/sIgM^-/CD43^+$ stage: pro–pre-BI cells) and alterations in the morphogenesis of the posterior midbrain. The role of BSAP in regulating transcription seems to be quite important, and certainly quite peculiar. BSAP interacts with DNA sequences of different composition: binding sites for BSAP have been identified in the promoter of a number of different B-cell-related genes, such as CD19, λ5, V_{preB1}, XBP 1, the tyrosine kinase *blk*, and the intronic regions of a number of Ig C_H genes (μ, γ1 γ2a, ε, α) *(24,25)*. Interestingly, although BSAP binding increases transcription from various promoters (e.g., CD19), binding of BSAP to sites in the Ig 3′ α enhancer and XBP 1 promoter has a negative effect *(25,26)*. Negative regulation of enhancer activity seems to be due to the ability of BSAP to suppress binding of NF-αP, a protein that positively controls enhancer activity and heavy chain transcription *(26)*. Interestingly, BSAP is upregulated by proliferative stimuli (mitogens, cross-linking of sIgD or CD40) *(27)*, and downregulated by OX40 ligand cross-linking *(28)*. Indeed, recent evidence suggests that BSAP may confer CD40 responsiveness to the ε germline promoter, and may thus be involved in CD40-dependent upregulation of IL-4-induced ε germline transcription *(22,29)*.

The minimal set of elements in the human ε germline promoter required to confer full IL-4 inducibility to a heterologous promoter has not been determined. In the mouse, a region containing the binding sites for Stat6 and a C/EBP factor seems to be sufficient to transfer

IL-4 inducibility to a minimal c-*fos* promoter *(11)*. Interestingly, the Stat6 binding site in the murine *(11)* and human ε germline promoter *(19)* shares a peculiar functional property with the site that binds the nonhistone chromosomal protein high mobility group (HMG-I)(Y) *(30)* in the mouse. Deletion or mutation of either site results in the loss of IL-4 inducibility, but in a marked increase in basal promoter activity. Thus, these elements seem to have a bifunctional activity, i.e., they are required for IL-4-induced promoter activation, but they repress the activity of the promoter in the absence of IL-4. These findings may suggest that expression of ε germline transcripts and IgE in resting B-cells is low in part because the germline promoter is kept in a state of repression that requires derepression through specific pathways.

Signal 2 Upregulates Germline Transcription and Induces DNA Switch Recombination

Although IL-4 (signal 1) is by itself sufficient for the initiation of transcription through the ε locus, switching and expression of mature Cε transcripts containing VDJ spliced to Cε1-4 require signal 2, i.e., engagement of CD40 by CD40L. The role of signal 2 in the induction of IgE switching is likely to be quite complex. In addition to triggering DNA recombination (see below), CD40/CD40L interactions are also critically involved in upregulating IL-4-induced ε germline transcription *(31,32)*. This transcriptional effect of CD40 engagement may be crucial for switching because optimal transcription through the S region may be required to target recombination. Thus, CD40 may control key events not only at the DNA, but also at the RNA level.

The DNA modifications that follow the delivery of the second signal for switching to IgE have only recently been fully characterized. According to the classical model switching occurs via loop-out and deletional recombination between highly repetitive S regions *(33)*. This was challenged in the late 1980s, at least for the IgE isotype. Indeed, it was reported that IgE could be produced by B-cells in which the immunoglobulin locus was retained in germline configuration *(34,35)*. Although that finding was probably the result of *trans*-splicing (*see* Signal 1 Induces Germline Transcription), it became important to characterize the molecular mechanisms that underlie the expression of mature VDJ-Cε mRNA in normal human B-cells. This issue was investigated using a polymerase chain reaction (PCR) that allows for amplification, cloning, and sequencing of either chimeric Sμ/Sε switch fragments (composed of the 5′ Sμ joined to the 3′ portion of the targeted Sε region) or switch circles, their reciprocal products. DNA sequencing of Sμ/Sε switch fragments amplified from IgE-producing B-cell cultures formally proved that deletional switch recombination had occurred. Most of the switch fragments represented direct joining of Sμ to Sε *(31,36,37)*. Interestingly, some switch fragments contained insertions at the Sμ/Sε junction that were derived from Sγ4 *(37)*. This finding suggested that some B-cells had undergone sequential isotype switching from IgM to IgG4 to IgE. Indeed, IL-4 has been shown to induce isotype switching to IgG4 *(38)*, as well as to IgE, and single B-cells can give rise to clones that secrete IgG4 and IgE *(39)*. More recently, analysis of switch circles has indicated that sequential switching from μ to ε can occur through γ1, as well as γ4 *(40,41)*.

Sequential switching and direct switching coexist. Sequencing of switch circles generated in B-cells triggered to switch to IgE by IL-4 and anti-CD40 mAb showed the presence of μ–γ–ε switching, and of sequential events even more complex (μ–α1–γ–ε). However, μ–ε circles representing direct switching events were also found, at high frequency *(42)*. Likewise, sequence analysis of Sμ/Sε switch fragments from B-cells of atopic dermatitis patients showed a predominance of direct Sμ/Sε joining *(43)*.

A chimeric Sμ/Sγ1 region resulting from a switching event between μ and γ1 can undergo a secondary recombination between the very 5′ end of Sμ and the very 3′ end of Sγ1. The secondary recombination essentially removes all the tandemly repeated S region sequences from

the corresponding chromosome *(44)*. The mechanisms responsible for secondary recombination events in previously rearranged, chimeric S regions are still unknown. In particular, it is unclear whether secondary recombination requires retargeting of the recombinase. This would be problematic because of the deletion of the region encompassing the I_H exon and the germline promoter. Secondary recombination may, however, represent a mechanism to prevent continued switching to downstream isotypes, and ensure isotype stabilization of switched B-cells. On secondary recombination, in fact, the S sequences retained by the active Ig gene may be insufficient to serve as a substrate for further S/S recombination.

Whether sequential switching is an obligate step in vivo was investigated by examining switching to IgE in mutant mice that lacked the Sγ1 region *(3)* and were therefore unable to support sequential switching via IgG1. In these mice, the frequency of switching to IgE was not affected *(45)*. These results indicated that sequential switching may merely reflect the simultaneous accessibility of two acceptor S regions for switch recombination induced by one cytokine. The apparent dominance of sequential switching observed in the generation of murine IgE-expressing cells following IL-4 stimulation may be due to the parallel activation of Sγ1 and Sε by IL-4, Sγ1 being intrinsically more accessible to recombination with Sμ *(3,45)*. Thus, the overall low frequency of IgE switching is an autonomously determined intrinsic feature of Sε and its control elements. This may explain why, in the presence of saturating concentrations of IL-4 in vitro, the frequency of IgE switching reaches at most 10% of the frequency of switching to IgG1, which is also induced by IL-4.

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