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Allelic exclusion of immunoglobulin gene rearrangement and expression: why and how?

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Since the discovery of the allelic exclusion of immunoglobulin (Ig) gene expression by Pervis in the 1960s [J. Exp. Med. 122 (1965) 853], much attention has been focused on its mechanism. Much less attention has been paid, however, to the question of why B cells demonstrate such unusual genetic regulation of antigen receptor gene expression. A large body of literature implicates the Ig gene products as feedback regulators of their own genetic rearrangement [Adv. Immunol. 78 (2001) 169; Science 236 (1987) 816]. While a role for Ig gene products in the regulation of V(D)J recombination is beyond debate, it is extremely unlikely that such a feedback mechanism would be fast enough to avoid occasional near-simultaneous rearrangement of allelic loci leading to dual receptor gene expression. This review will suggest an hypothesis to answer the 'why bother' aspect of allelic exclusion and then go on to propose a mechanism, distinct from feedback regulation, which may contribute to the allelic exclusion of Ig gene expression.

Key words: allelic exclusion / transcriptional enhancer / V(D)J recombination / chromatin / accessibility

What is the advantage to the organism of an allelically-excluded B cell repertoire?

B cells undergo selection at multiple developmental stages based on the specificity of its Ig-containing BCR. During the 'pre-antigenic' stages of development, this selection consists of proliferative expansion of Ig heavy-chain expressing cells at the early pre-B cell stage, negative selection or receptor editing of self-specific immature B cells, possible positive selection of certain B cell subsets (B-1 cells in particular), and the BCR-dependent survival of mature, peripheral B cells.[4–6] After antigen exposure, individual B cells undergo clonal expansion and affinity maturation based on BCR specificity. Many investigators have argued that the expression of multiple antigenic specificities by an individual B cell would somehow interfere with these various processes leading to 'inefficient' B cell development and selection, or under some circumstances, autoimmunity. While the specter of a clonally-selected B cell secreting both useful and 'bystander' Ig is esthetically disturbing, it is by no means certain that such a situation would necessarily hamper the immune response.

At the pre-BCR stage, developing B cells undergo surveillance for the production of a heavy-chain which is competent to pair with light-chain.[6] There is no obvious way in which the generation of two functional heavy-chains would debilitate this process. Somewhat more problematic is the immature B cell stage of development. Various theoretical arguments have been made regarding the frequency of self-specificity generated by combinatorial joining within the immature B cell population. Were this frequency high enough, the expression of multiple distinct immunoglobulins in a single B cell might lead to a situation where the vast majority of B cells would express at least one self-specific receptor. Cell loss at the immature to mature B cell transition in the setting of allelic exclusion approximates 90%.[7] In the setting of dual receptor expression, the likelihood of negative selection might approach 99%, making it likely that very few dual receptor-expressing B cells would survive. In addition, a more prevalent stimulus for receptor editing might result in an unacceptably high frequency of chromosomal translocation and consequent lymphoid malignancy. All this said, cell loss during T cell development may be as high as 99%, lessening the validity

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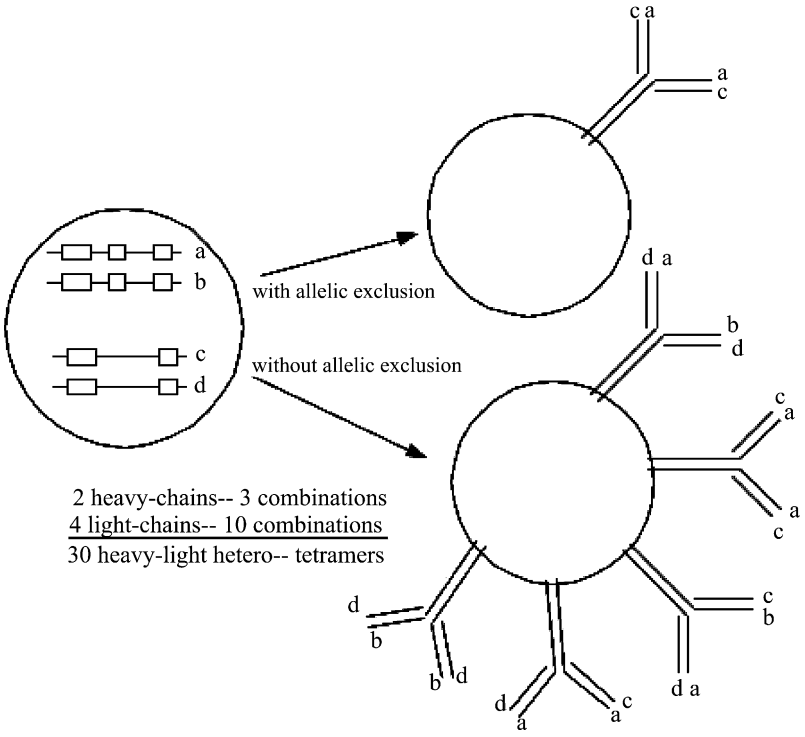

Figure 1. The consequences of expressing multiple Ig heavy and light chains in the same B cell. The diagram shows a B cell with two heavy-chain alleles (a and b) and two light-chain alleles (c and d). With allelic exclusion, that cell can produce only a single species of antibody containing two identical antigen binding domains. Without allelic exclusion, that same B cell could produce two different heavy chains and as many as four different light chains (two kappa and two lambda; only the two kappa chains shown in the diagram for simplicity). Presuming that the various heavy and light chains will associate with one another in an independent fashion, 30 different hetero-tetramers are possible, the majority of which will contain two different antigen binding domains.

of any 'efficiency' argument for allelic exclusion in B cells.

We would like to propose that the predominant force resulting in the allelic exclusion of Ig gene expression may be the biologically ineffective nature of bi-specific antibodies. Unlike TCR, immunoglobulin is bivalent and recognizes soluble antigen. This antigen, a capsular polysaccharide for example, is often repeated multiple times on the surface of a microbe. IgM expressed by the naïve B cell repertoire specific for a particular antigen has only a modest affinity—K_d values on the order of 10^{-5} to 10^{-6}. In this setting, the multivalent nature of Ig makes an enormous contribution to antibody avidity and allows for stable binding of antigen. This is true for IgM both as a component of the BCR and as a soluble, pentameric (decavalent) molecule. In the complete absence of allelic exclusion, B cells could express as many as 30 distinct IgM molecules, the majority of which will be univalent for any particular idotype (Figure 1). In this setting, the ability of the BCR to specifically trigger B cell activation would profoundly diminished. One of the predominant effector functions of IgM is its ability to activate the complement system. Complement activation via the classical pathway depends upon adjacent Fc regions associated with an antigenic surface. Decavalent IgM pentamers, despite the modest affinity of each idotype–epitope interaction, binds with great avidity to repetitive antigens on bacterial surfaces and efficiently activates complement. In the absence of allelic exclusion, each IgM pentamer is likely to have no more than one or two pathogen-specific antigen-binding sites resulting in only very inefficient binding and activation of complement-mediated effector functions. These arguments apply to other Ig isotypes as well. Thus, we think it is likely that the reason why allelic exclusion exists is to assure the expression of effective multivalent antibodies.

Feedback regulation cannot by itself explain allelic exclusion

Most models for the mechanism of allelic exclusion in B cells involve a feedback signal sent by either the pre-BCR or the BCR resulting in the inactivation of accessibility at the allelic heavy- or light-chain locus, or the inactivation of the recombinase itself (reviewed in 8). It is very unlikely, however, that such a mechanism can explain allelic exclusion in its entirety. This is because a level of recombinase activity sufficient to assure that recombination occurs on one substrate allele in most cells should also result in recognition of the second allele a significant fraction of the time. Feedback regulation models do not account for the near-simultaneous rearrangement of allelic loci. It is possible, of course, that recombinase activity is limiting in developing B cells, making it unlikely that multiple events would occur within the same cell. If this were the case, however, we'd expect to observe many developing cells lacking any rearrangements. Extensive analyses of primary developing B cells and of Abelson virus transformed cell lines have shown that pro-B cells invariably contain D-to-JH rearrangements on *both* heavy-chain alleles.[9,10] The observed frequency of receptor editing in the Ig light-chain loci also suggests that a feedback inhibition mechanism cannot explain allelic exclusion.[11] Immature B cells expressing a receptor with self-specificity display continued recombinase activity and further light-chain locus rearrangement. The frequency of such events has been estimated to be as high as 25%.[11] If all light-chain alleles within a cell were equally accessible to the recombinase, cells expressing two light chains should result from such editing. This is not observed. In a previously reported study, our lab demonstrated that the recombinase has a strong preference for activity on the previously rearranged light-chain allele.[12]

A role for mono-allelic activation of recombinase accessibility?

Compelling data supports the hypothesis that recombinase activity is regulated by template accessibility.[13,14] The 'accessibility hypothesis', first proposed by Yancopoulos and Alt, was based on the correlated observation of germline Ig gene transcripts and *de novo* Ig gene rearrangements.[15] This correlation has been observed with other rearranging loci as well. We showed a number of years ago that treatments which increase germline transcription amongst a population of pre-B cells increase the frequency of the corresponding gene rearrangement event.[16] More recently, the dependence of V(D)J recombination on transcriptional enhancers has been demonstrated using increasingly elegant gene-targeting approaches (reviewed in 13). Biochemical studies performed in our own lab have directly demonstrated a role for chromatin structure in targeting the V(D)J recombinase during lymphoid development.[17]

All of the studies noted above examined the behavior of cells or nuclei in bulk assays. In other words, the detection of increased germline transcript levels in these assays might be due to a modest increase in the rate of transcription of both alleles in all cells, or a profound increase in a small fraction alleles in the population of cells. Pioneering work from the labs of Weintraub, Groudine, and Martin has provided experimental support for the notion that under certain circumstances, the activation of a locus in a population of cells may be probabilistic rather than determinative.[18] These workers analyzed clones of transfected cells expressing a marker gene, β-galactosidase, which could be scored by a flow cytometric assay. When the transfected construct lacked a transcriptional enhancer, transfected cells rarely expressed β-galactosidase. In the presence of an enhancer, however, a significant fraction of cells expressed the marker protein. Interestingly, in cells which did express the reporter, the level of expression was unaffected by the presence of the enhancer. When these and other investigators debilitated certain enhancers by introducing mutations in various factor-binding sites, they found that the frequency of actively transcribing cells decreased, but in the cells which did express the marker gene, the level of expression remained relatively constant.[19,20]

The notion that enhancers may effect the probability of transcriptional activation rather than simply the rate of transcription has profound implications for the regulation of V(D)J recombination. As noted above, in several experimental systems, transcriptional enhancers were shown to be essential for recombinase accessibility. We propose that during early B cell development, the levels of various transcription factors are set such that the likelihood of locus activation by the Ig HC or Ig LC enhancers is quite low, perhaps 10% or less. If this were the case, the likelihood that any given cell would have two simultaneously active loci would be about 1%. Since the V(D)J recombination reaction is blind to translational reading frame, only one-third of rearrangements are productive, leading to an estimated maximal frequency of cells expressing two in-frame gene rearrangements at a given locus of

no more than 0.3%. This number agrees with recent estimates of dual light-chain expression.[11]

As an initial test of this stochastic activation hypothesis, we examined the chromatin structure of transcriptional enhancers associated with the Igκ locus in two transformed cell lines, 220-8 and P815. 220-8 is an Abelson virus-transformed pro-B cell line with non-productive V(D)J rearrangements on both of its heavy-chain alleles and no rearrangement at either of its Igκ light-chain alleles. P815 is a mastocytoma lacking in any gene rearrangement. The murine Igκ locus contains at least two transcriptional enhancers, the so-called intronic (Eκi) and 3′ (Eκ3′) enhancers (Figure 2). In order to assess the fraction of the time that Eκi and Eκ3′ were active, we applied a restriction enzyme accessibility assay. Boyes and Felsenfeld's have observed that restriction sites adjacent to transcriptional regulatory elements can be recognized and cleaved by the appropriate enzyme in nuclei purified from cells in which the enhancer is active.[19] When the enhancer is inactive, this group finds the restriction site to be inaccessible. This assay is conceptually similar to the more commonly applied 'DNase hypersensitivity assay'. Its advantage over DNase hypersensitivity, however, is the fact that the digestion can be done under saturating conditions, allowing one to quantify the fraction of alleles in a population of nuclei which contain accessible enhancer sequences.

To investigate the structure of Eκ3′, we purified nuclei from either 220-8 (grown in the absence or

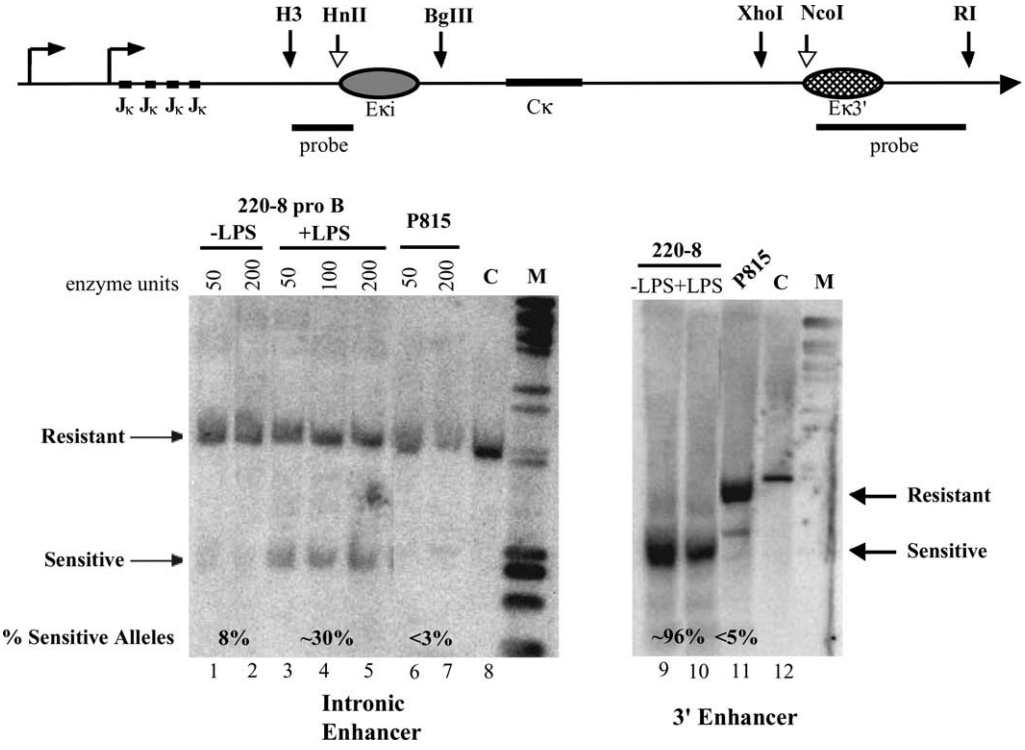

Figure 2. The Igκ locus intronic enhancer, Eκi, is active on only a fraction of the κ alleles in a population of transformed pro-B cells. Top: A map of the germline murine Igκ locus with the four functional Jκ gene segments and the Cκ exon represented as black filled rectangles and the intronic (Eκi) and 3′ (Eκ3′) enhancers represented as filled ovals. Bent arrows indicate the positions of the two germline κ transcript promoters and vertical arrows show the positions of relevant restriction sites. Bottom: Southern blot analysis of genomic DNA. 220-8 pro-B cell nuclei (from cells grown in the absence or presence of LPS) or P815 mastocytoma cell nuclei were digested with either *Hinc*II (left panel) or *Nco*I (right panel). Genomic DNA was then purified from these nuclei and subsequently digested with either *Hin*dIII and *Bgl*II (left) or *Xho*I and *Eco*RI (right). Southern blots of these multiply-digested DNA samples were then hybridized with the indicated probes and visualized by phosphorimager. Percentages indicated at the bottom of each set of lanes denote the fraction of accessible alleles within each sample of nuclei. Enzyme units refers to the amount of *Hinc*II used in the digestion of nuclei analyzed in lanes 1–7. Lanes 8 and 12 are DNA samples never exposed to digestion with either *Hinc*II or *Nco*I. M indicates DNA molecular weight marker lanes.

presence of LPS, a known inducer of germline κ locus transcription[16]) or P815 and digested them *in vitro* with *Nco*I. We then purified total DNA from the digested nuclei and further restricted it with *Xho*I and *Eco*RI. The resultant DNA fragments were then analyzed by agarose gel electrophoresis and Southern blot hybridization with a probe as indicated (Figure 2). As shown in lane 11, less than 5% of the Eκ3′ DNA sequences were accessible to *Nco*I digestion in P815 nuclei, while greater than 95% of such sequences were cleaved in 220-8 nuclei (Figure 2, lanes 9–11). We interpret this to reveal that nearly all κ loci in pre-B cells contain a protein complex bound to Eκ3′ and promoting its enzyme accessibility. When we performed an analogous experiment using *Hinc*II in an attempt to digest nuclei at a site adjacent to Eκi, we observed an interestingly different result (Figure 2, lanes 1–8). The *Hinc*II site adjacent to Eκi in nuclei isolated from 220-8 cells grown in the absence of LPS was cleaved in only 8% of κ alleles. After LPS treatment, this fraction increased to about 30 %, but in neither case was a majority of alleles accessible to restriction digestion. Furthermore, we found that the fraction of cleaved alleles was independent of the amount of enzyme added, suggesting that we were operating under saturating conditions. In contrast, only a very small fraction of κ alleles could be cleaved by *Hinc*II at Eκi in P815 nuclei. The fact that even under saturating conditions the intronic enhancer was accessible only a small fraction of the time is consistent with the stochastic activation of this critical regulatory element.

We propose that transcription factor association with Eκ3′ occurs with B lineage commitment. In a previous study, we reported that various sites within Eκ3′ were bound with protein in both pro-B and pre-B cells as assessed by *in vivo* footprinting.[21] In contrast, only a fraction of the time does Eκi associate with its cognate transcription factors, leading to a situation where only a fraction of κ alleles are fully active and accessible to cleavage by the V(D)J recombinase. As described above, depending upon the frequency of Eκi activation, only rarely would an individual pre-B cell contain two accessible κ alleles, thus, helping enforce allelic exclusion.

According to this model, and consistent with our data, the active and inactive states of individual alleles within a population of cells need not be static. It is possible that active alleles can become quiescent and inactive alleles can activate within the context of a population of alleles with an overall low frequency of active alleles. The would account for cells which contain two rearranged κ alleles were they to have rearranged at different times during the pre-B cell stage.

Epigenetic marks such as DNA methylation may contribute to the differential regulation of the two κ alleles within an individual cell. An elegant study presented by Mostoslavsky *et al.* showed that the region surrounding Eκi was heavily modified with CpG methylation in non-lymphoid cells and in early pro-B cells, but that about 10% of κ alleles become demethylated at the pre-B cell stage.[22] This frequency of demethylated κ alleles is quite similar to the frequency of restriction enzyme accessibility we observe at the *Hinc*II site adjacent to Eκi in primary pre-B cell nuclei isolated from bone marrow (data not shown). Thus, mono-allelic demethylation, perhaps influenced by enhancer activity, may contribute to the stochastic activation of κ loci for V(D)J recombination during B cell development.

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