

One Cytokine, Two Isotypes

A Trojan Horse, Pandora's Box, and an Evolving Paradigm

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You can't do it all. This is probably the reason why for a long time researchers interested in IgE regulation paid little attention to other immunoglobulin (Ig) isotypes and to the conditions required for their production. The discovery that isotype switching is directed by cytokines (reviewed in References 1 and 2), and that interleukin 4 (IL-4) is specifically responsible for the induction of switching to IgE (3), spurred a vast effort to try and understand the cellular and molecular mechanisms underlying the effects of this cytokine. In article after article (ours included) the leitmotif in the studies of IL-4-dependent IgE regulation was the specificity of the effects of this cytokine, that is, how IL-4 secures the production of IgE, and only IgE. One cytokine, one isotype. Molecular studies of the IgE system made a major contribution to the identification of germline transcription as a key step in determining the isotype specificity of switching. The expression of germline transcripts reflects the cytokine-dependent activation of transcription from an I_H region located upstream of the switch region subsequently targeted by recombination (4, 5). The germline proceed from the I_H region through the switch region itself and then all the way through the Ig constant heavy chain (C_H) gene expressed as a result of switching. The germline transcripts produced after splicing contain the I_H region directly joined to the C_H transcript (reviewed in Reference 1). It is by now generally accepted that processed germline transcripts and/or the mechanism of germline transcription are required to increase the accessibility of chromatin at a specific switch region (the one through which transcription is activated by the cytokine), which thus becomes the preferred target for the recombination machinery (6). Even though this model awaits direct confirmation (7), it is strongly supported by an overwhelming amount of data from different experimental systems, first and foremost mice in which genes essential for germline transcription have been deleted by homologous recombination (8–14).

B CELLS SWITCH SEQUENTIALLY TO IgE THROUGH $\gamma 4$

The regulation of ϵ germline transcription in human B lymphocytes has been worked out in some detail. We know where $I\epsilon$ and its promoter are (15, 16), we understand some of the crucial signals (IL-4, CD40 cross-linking) required to activate ϵ transcription (17), and we have identified a number of transcription factors (STAT-6, NF- κ B, and B cell-specific activator protein [BSAP] among others) that bind the promoter and get it to fire (S. Monticelli, L. De Monte, and D. Vercelli, unpublished data, 1999). We also know that the effects of IL-4 and CD40 cross-linking on the ϵ promoter are unusual in that IL-4 by itself induces a relatively modest activation and CD40

cross-linking almost none, but these two signals synergize quite impressively (18). This synergism reflects the stepwise build-up of a transcription activating complex that requires a strict cooperation between factors recruited through independent pathways.

In looking systematically at the effects of IL-4 on human B cells, it had been pointed out early on that this cytokine induces not only IgE, but also IgG₄ (19). A sophisticated blend of cell sorting and cloning showed that simultaneous production of IgM, IgG₄, and IgE in various combinations could be easily detected in B cell clones stimulated by IL-4 and activated CD4⁺ T cell clones. This observation was taken to suggest that during clonal expansion switching might occur in successive steps from IgM to IgG₄ and IgE (20). However, despite these sparkles of interest, IgG₄ remained the underdog among human Ig subclasses. Why bother about an isotype that normally represents only 4% of total IgG (21)?

Actually, there were at least two cases in which IgG₄ antibodies were thought to be major players in immune reactions. Parasite immunologists had known for some time that IgE-mediated hypersensitivity reactions occur rarely in patients with chronic helminth infections, even though basophils and mast cells in these patients are sensitized with anti-parasite IgE and are exposed, often continuously, to parasite antigens. The inhibition of allergic reactivity in chronic helminth infections was known to be due to serum "blocking antibodies" found predominantly in the IgG₄ subclass (22). IgG₄ antibodies are unusually predominant among specific antifilarial antibodies, representing 50–95% of the total IgG response (23). Indeed, depletion of IgG₄ by adsorption on anti-IgG₄ affinity columns specifically removed the blocking activity from the sera of microfilaremic patients. Blocking activity results from a direct competition for allergen binding between IgG₄ in the serum and IgE bound to high-affinity Fc ϵ receptors on cells (24). IgG₄ antibodies do not fix complement, are functionally monovalent, and bind weakly to Fc γ receptors (21, 25). Thus, antigen binding by IgG₄, unlike IgE, is likely to have no or minimal harmful consequences.

IgG₄ antibodies with blocking activity have also been detected in patients receiving immunotherapy for insect venom and house dust mite hypersensitivity (reviewed in Reference 26). It is interesting that IL-10, a cytokine reported to play a crucial role in the induction of peripheral tolerance associated with successful immunotherapy, suppresses allergen-specific IgE but enhances allergen-specific IgG₄ (27). However, a thorough understanding of the role of IgG₄ in immunity was still prevented by serious methodological problems (IgG₄-specific monoclonal antibodies became available only in the mid-1980s) (28) and perhaps even more by the somewhat magical nature of the IgE/IgG₄ relationship.

This state of affairs came to an end with the cloning and sequencing of switch fragments amplified from sIgE-negative B cells stimulated to switch to IgE by a combination of hydrocortisone and IL-4 (29). Most switch fragments contained sequences derived from the switch (S) μ region directly joined to S ϵ sequences and were therefore likely to be the product of di-

Supported by Telethon-Italy (grant E.661).

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Am J Respir Crit Care Med Vol 162, pp 586–590, 2000
Internet address: www.atsjournals.org

rect switching from μ to ε . Several clones, however, contained insertions between the S_μ and S_ε sequences. Close examination revealed that these insertions originated from $S_{\gamma 4}$ (30). The presence of $S_{\gamma 4}$ -derived fragments between S_μ - and S_ε -derived sequences had to mean that those composite switch regions were the result of sequential switching from IgM to IgG₄ to IgE. This interpretation was soon confirmed by data obtained through a specular approach, that is, by sequencing switch circles excised from B cells that had undergone isotype switching to IgE (31). With the demonstration that human B lymphocytes switch sequentially from IgM to IgG₄ to IgE, most loose ends were finally tied up. It was almost a truth of reason that IgG₄ and IgE could compete for the allergen if, and only if, the two antibody isotypes had a parallel specificity of antigen recognition (24). The model of sequential switching within one and the same B cell clone provided a molecular solution for this old puzzle. However, sequential switching also created a new problem: the necessity to understand how this process is actually regulated at the level at which the isotype choice is made, that is, germline transcription. Indeed, if the models generally accepted for switching are correct, it would be predicted that IL-4-dependent sequential switching to IgG₄ and IgE should go hand in hand with the IL-4-dependent induction of both $\gamma 4$ and ε germline transcription. But this is exactly where the problem arose: As discussed above, much was known about the regulation of ε germline transcription, but the state of knowledge about IgG₄ resembled a black hole. A number of groups investigated $\gamma 4$ germline transcription (32, 33) but ran into trouble because of the tremendous homology between the loci for the four IgG subclasses. After a long quest, $\gamma 4$ germline transcripts were finally cloned and characterized in our laboratory (34), and shown to contain $I\gamma 4$ spliced to the 5' portion of $C\gamma 4$ by using the splice donor site shared by $\gamma 1$ and $\gamma 3$ germline transcripts. Sequence analysis located the human $I\gamma 4$ exon ~ 0.6 kb upstream of the $S_{\gamma 4}$ region.

Four major transcription start sites located 547–583 bp upstream of the $I\gamma 4$ splice donor site were identified by primer extension analysis. A *HindIII*–*NaeI* region (–413/+466) had the highest activity in reporter assays. Transcription was induced 4.6-fold by IL-4, 2.1-fold by CD40 engagement, and 14.5-fold by a combination of the two signals. Thus CD40 cross-linking and IL-4 act synergistically (35). We are now in the process of systematically analyzing the promoter for the presence of transcription factor-binding sites. Most importantly, we have confirmed that IL-4 induces both ε and $\gamma 4$ germline transcripts in a B cell population, thus reiterating the role of germline transcription in choosing the isotype even when switching occurs sequentially. One cytokine, two isotypes.

HOW SOLVING ONE PROBLEM (SEQUENTIAL SWITCHING) LED TO ANOTHER

Sequential switching has certain implications. Mechanistically, once one accepts that such a process can occur, one has to wonder whether in fact it does not occur all the time. Indeed, finding direct S_μ/S_ε switch junctions does not rule out a previous sequential event that left no genomic trace. Functionally, it would be helpful to know how this process is regulated, because it is clear that there are polarized situations in which the IL-4-dependent response is essentially IgE (such as allergy) and other situations in which the IgG₄ response is dominant (such as helminth infections). Sequential switching leads to the production of two classes of Ig with antagonistic functions, and it is conceivable that the balance between the two is what determines the clinical features. It will be important to understand how the balance is controlled.

But there is yet another, more intriguing implication of IL-4-dependent sequential switching to IgG₄ and IgE. To see it, we need to think about similarities among DNA sequences, and what they mean in functional terms—a boring exercise, but fruitful and unexpectedly revealing in a case like this one. At first glance, nothing exciting comes from aligning the sequences of the 5' regulatory regions in the ε and $\gamma 4$ germline promoters: The sequences are not really homologous. However, an educated observer will immediately notice that, on the basis of what we can tell at this preliminary stage of our dissection of the $\gamma 4$ promoter, cassettes corresponding to binding sites for crucial transcription factors are preserved in both promoters. In fact, mutations of these sites have similar functional consequences.

Per se, this is not particularly surprising: We already knew that $\gamma 4$ and ε are both IL-4 driven. However, finding IL-4-regulated sites in the $\gamma 4$ promoter, and finding that they support $\gamma 4$ germline transcription, has momentous consequences for the issue of isotype specificity because (thanks to the extraordinary degree of homology within the γ locus) these same sites can also be found in the regulatory regions 5' to the $I\gamma 1$, $I\gamma 2$, and $I\gamma 3$ exons. In fact, these four regions in the γ locus are practically identical, and it would be difficult to conceive how one could be activated without the others also being triggered. The transcription factors involved in the formation of a competent initiation complex would have no way to decide whether to dock on one γ regulatory region rather than on the next one. Indeed, they don't. When we analyze RNA from B cell lines stimulated with IL-4, using a panel of reverse transcriptase-polymerase chain reactions (RT-PCRs) specific for ε and $\gamma 1$, $\gamma 2$, $\gamma 3$, and $\gamma 4$ germline transcripts, we see that the B cells express not only ε and $\gamma 4$ germline transcripts, as expected, but also all of the other γ germline transcripts. These results are real: When the amplified products are cloned and sequenced, they contain diagnostic nucleotides that allow for the unambiguous identification of the products.

At this stage the identity of the Trojan horse and the meaning of Pandora's box should be clear. An IgG isotype (IgG₄) that, in addition to IgE, can be induced by IL-4 is a Trojan horse that smuggles seeds of destabilization into a formidable fortress. Once this notion is accepted as true (as it should), the problem with the other IgG isotypes arises immediately and unavoidably, dictated by the homology between the γ loci. Pandora's box is open, and the one cytokine–two isotypes paradigm evolves and revolves around a crucial hinge: the IL-4 dependency of IgG₄ induction. Difficult as it may seem, our focus will have to shift from the mechanisms that determine the isotype specificity of switching to those that constrain it. We need to find new levels of control, and we don't expect them to be easy to unravel.

Before embarking on a search for mechanisms of constraint and control, we must formally prove that to postulate such mechanisms is wise—nay, necessary. The most persuasive evidence would probably come from DNA hypersensitivity studies demonstrating that sites hypersensitive to digestion with DNase I or restriction enzymes are simultaneously generated at multiple loci in single cells after IL-4 stimulation. Such studies are technically demanding but they can (and should) be done. If these studies will confirm that one cytokine (IL-4) can open up chromatin much more promiscuously than initially postulated, the main problem will become the identification of the mechanisms that constrain specificity distally to germline transcription. We know that such mechanisms are operative and not necessarily beneficial: Because antigen-induced responses *in vivo* (first and foremost, allergy) are often dominated by specific Ig isotypes, some degree of control must

pitch in to push the response in a certain direction rather than keeping it going across the board. In this evolving scenario, germline transcription remains an essential, in fact a necessary, step. We know that no switching occurs if germline transcription is blocked, and the role of this process in contributing to the determination of the isotype specificity of switching is by now undisputed. The point is, germline transcription, although necessary, does not seem to be sufficient.

Which other levels of control may account for the expression of Ig isotypes as we see it *in vivo*? One potential step available for control is obviously DNA switch recombination, through mechanisms that, although predicted on chromatin accessibility and therefore on germline transcription, may involve other factors related to architectural constraints and/or long-range interactions between functionally distinct DNA domains. Another level amenable to regulation may be the alternative splicing of RNA that generates membrane-bound or secreted Ig molecules. This step has not attracted great attention so far, perhaps because it has always been perceived as a rather automatic (and therefore uninteresting) process, but there are indications that it might be regulated (36). The possibility of control at the level of secretion should also be considered. It is not at all obvious that all Ig classes are assembled and released by the B cell with the same ease, speed, and efficiency.

CONCLUSION

In closing, it is impossible to resist the temptation to link the issue of specificity constraints to the problem of allergic sensitization. If the scenario sketched above is correct, triggering IL-4 production evokes a complex response that would not necessarily lead to allergy, because it would not necessarily be IgE and IgE only. Thus, producing IL-4 in response to an antigen would not be enough to make an individual allergic. The question would, rather, be whether IL-4 is backed up by other signals (or by the lack of other signals) that constrain the response to the IgE isotype, presumably after IL-4-dependent germline transcription has occurred. The nature of the antigen and the genetic background of the responding individual may have much to do with this post-germline transcription regulatory step. In this scenario, an allergen would be an antigen that keeps the response tightly confined to the IgE isotype, either actively (because it delivers signals to this effect) or passively (because it does not trigger the concomitant signals necessary for the isotype choice to become broader). The second possibility is more likely, because there are no allergens that induce IgE and IgE only in all individuals. Allergen = antigen-X: Debunking this equation will keep us busy for a number of years to come.

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DISCUSSION

Aarden: You showed that IL-4 gives germline transcription from $\gamma 1$ until $\gamma 4$. Nevertheless, if there is no IL-4 (and, in man, maybe IL-13), you do not get IgE production. There must be other factors also involved at the transcriptional level that are also not specific for IgE production. So, maybe you could argue that IL-4 is essential for the IgE (and maybe the $\gamma 1$) production, and that you only need other factors that cooperate with that.

Vercelli: It is possible. *In vitro* models are reductionist models, where you have a control over what you put in and what you read out. Unless it turns out that adding IL-4 induces the release of whatever cytokine you need to do this additional job, then I do not have a way to say.

Aarden: I agree, but you could still argue that IL-4 is the determining factor for the IgE production.

Vercelli: Absolutely. What I am trying to argue, however, is that in a situation like allergy, having predominantly IgE does not follow directly from having IL-4. Just that you must have something else that channels the response into IgE, because naturally IL-4 would not do that.

Aalberse: The emphasis is now beyond the switch rather than before . . .

Vercelli: Not beyond the switch, beyond germline transcription.

Aalberse: What Dr. Lamers stressed, and what I did not think through before, is that survival of the B cell is crucial. Some B cells may not make it. There may be signals that kill cells soon after the opening up of the DNA. Could it be that IgG_{1, 2, 3} do not make it after the switch and that you need IL-4 for survival, that IL-4 acts via a survival gene rather than via the switch machinery?

Vercelli: Yes, I don't know how that could happen, but in principle it could. I was not aware of this problem until I saw these results and now the plan is to find a way to solve it. In this perspective, differential induction of B cell survival might become an issue, even though at this stage we would have no mechanism to explain it.

Aalberse: IgE and IgG₄ are very similar, but there really is a major difference between IgE and IgG₄. This is perhaps best illustrated by looking at the immune response following immunotherapy. If you inject an allergen into people with a pre-existing Th2 response, IgE does not do much, but you do get a tremendous increase in specific IgG₄. So, somehow, in that situation, you do have a switch to IgG₄ and that switch does not go beyond that. So, what you see in early sensitization is that a few B cells leak through this filter of IgG₄-stage B cells and make it to IgE and at a later stage all B cells stop at the IgG₄ stage and you just get IgG₄ produced.

Vercelli: The Davos group, in their study on the production of IL-10 during specific immunotherapy for bee venom allergy, really placed IL-10 in the spotlight for the induction of tolerance. The interesting thing is a very small figure at the bottom of the last-but-one page. What it says is that IL-10 has a small suppressive effect on IgE, but a strong enhancing effect on IgG₄. This would fit very well with the data from Nutman *et al.* on microfilaremic and asymptomatic patients, with no allergy and lots and lots of IgG₄: They have a massive amount of IL-10. So, IL-10 might be a way to get this differential response of IgE and IgG₄. That is, of course, why we need to keep working on that. It is not just IgE and IgG₄, we have to wonder also how IL-4 gets limited in its potential to do basically anything across the board.

Lamers: I have a conceptual problem with sequential switching. IgE will be the end stage and IgG₁ (mouse) or IgG₄ (human) will be the intermediate stage and would not be favored unless you have a mechanism that switches off the recombination. Is there any evidence for that?

Vercelli: No. But it is true that at least *in vitro* the amount of IgG₄ is much more than the amount of IgE. I do not know if this has to do with the fact that the plasma cell can secrete more IgG₄ than IgE, but that is a possibility. There are cytokines that can preferentially activate germline transcription to one or the other isotype.

Lamers: Do you have any evidence for sequential switching through IgG₄?

Vercelli: Our interest in sequential switching arose from the fact that IgG₄ and IgE antibodies have very similar antigen specificities, that in our opinion could not be achieved but through sequential switching. Sequential switching would give you identical VDJ recombined with different C ϵ or C $\gamma 4$. Apart from the clones in which we showed that there was a $\gamma 4$ switch region in the transcript we have no further evidence for sequential switching.

RL: If you isolate those sequential switched cells, are these from a primary or secondary immune response?

Vercelli: These are the questions that a mouse immunologist can ask and a human immunologist cannot. I can tell you how they were isolated. They were isolated from human B cells that were very stringently sorted to be sIgE negative, because we were looking at IgE switching, not at IgG₄ switching. So, we took away all the IgE-switched B cells. Then we amplified with primers in μ and primers in ϵ . The evidence was the presence of leftovers of the switch $\gamma 4$ region in between switch- μ and switch- ϵ regions. This result was later confirmed by Saxon's group with a different approach: sequencing switch circles.

Djukanovic: How easily can you make a B cell to switch; is it something you can do with any donor or with any B cell?

Vercelli: We find sequential switching in cultures with anti-CD40 and IL-4 with all donors that we tested, some more, some less.

Djukanovic: What can you tell about the signal transduction that occurs at the G4 and E stages? The cell sees IL-4, sees CD40L, but somehow responds differently. Can you speculate that there is maybe a transformation in the IL-4 receptor?

Vercelli: I have indirect data to answer that. I find the data difficult to understand, but I will share it with you. We have a B cell line from an X-SCID patient, a patient who was born with a complete absence of the γ chain of the IL-4 receptor. It is a complete absence, the mutation is very early on in the DNA so you don't get even a little protein. I mention this, because I have the suspicion that having a little protein might screw up things downstream in a different way. So, this is a human functional knockout of the γ chain. Of course, this is an EBV B cell line, with all the limitations associated with EBV, and there is

no way of telling how that will affect what you see. We have a lot of EBV controls and they do not behave in the same way, but still, how do you know? Within the limitations of what we have, if you stimulate the EBV B cell line of this patient with IL-4, you get absolutely nothing for ϵ at the level of germline transcription (EBV lines don't switch, so you can only look at germline transcription). However, you get a very enhanced response for $\gamma 4$. I do not know why. If this holds true, it might indicate that the two chains of the IL-4 receptor have a differ-

ent role. This is clearly very preliminary, even if we have done this experiment four or five times, with the same result.

Savelkoul: If B cells are stimulated *in vitro*, they remain IgM positive, but in the meantime they accumulate ϵ germline transcripts. Could these cells through the process of trans-splicing become temporarily positive for IgE and be responsible for the ongoing low level of IgE production?

Vercelli: We have not directly looked for trans-splicing, but I have doubts as to its relevance *in vivo* for normal B cells.