Epitope-specific regulation: the elephant in the bathtub

Leonore A Herzenberg

Modern cellular and molecular studies have made great progress in characterizing the mechanisms that control immune responses. Now it is time to broaden the present views to accommodate evidence from epitope-specific and other immunoregulatory systems, which were well studied some years ago and are still highly relevant to contemporary work.

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Evolution is perforce a veiled process. Although its imprint on the genome can be traced with modern genetic tools, the selective forces responsible for establishing even so narrow a functional entity as the mammalian immune system must be inferred mainly from an understanding of how that system now operates. Similarly, although modern genetic and molecular tools have introduced an amazing array of functional molecules and interactive pathways, understanding of the functions of these molecules and pathways in immune responses depends heavily on present views of how the immune system operates. Nevertheless, perhaps because specialization has its cost, much of what is known about the complex processes that mediate and regulate immune responses seems to be relatively less accessible, or at least relatively less accessed, by investigators now involved in identifying the molecular and evolutionary basis of immune responses.

The apparent loss of contact with what is likely to be crucial information for the interpretation of present data stems in part from the history of immunological studies over the past two decades. In essence, during the 1970s and 1980s, there was a thriving community of investigators focused on charting the cells, molecules and mechanisms that regulate antibody production and other immune functions[1–10]. These issues typically occupied the central agenda at major meetings and the key content space in major journals. However, once the T cell receptor was cloned and the availability of molecular methods opened the way to properly define the structures of the molecules that mediate immune function, focus rapidly shifted to these highly constructive pursuits. A revolutionary generation of molecular immunologists took over the reins of the field and, with the zeal of new converts, collectively agreed to ignore the complex regulatory mechanisms under discussion just a few months before and to start fresh with a more manageable picture of the immune system and its various parts.

Essentially, maintaining detailed knowledge of the confusing array of regulatory cells and poorly defined regulatory molecules was a burden in the new molecular era. It needed to be put aside, at least temporarily. Studies focusing on the suppression of antibody responses were progressively marginalized, even demonized, particularly after attempts to clone or locate the genes for some of the key regulatory proteins proved unsuccessful with the (primitive) methods that were available. Eventually, collective amnesia set in among immunologists, putting the 'S word' (suppression) out of bounds until very recently and treating the whole earlier body of knowledge of regulatory T cells as incorrect, or at least as not relevant to present immunoregulatory thinking. As a result, a stunted view of the immune system developed, one that predominates today and unfortunately conditions expectations in present evaluations of gene function in knockout and transgenic animals.

I must admit that the epitope-specific regulatory mechanisms that my group and I proposed[11] contributed to this cataclysm, mainly because they introduced a level of regulatory complexity that even investigators deeply involved in suppression studies found difficult to accept. The evidence we presented was solid, backed by data from many experiments involving hundreds of mice and thousands of antibody-response assays[3,11–17]. Furthermore, the model we proposed integrated evidence from many of the regulatory systems under study at the time and explained the 'mysteries' published by several highly respected laboratories over the preceding 10 years[11]. However, our contention that antibody responses to individual epitopes on a complex immunogen are individually regulated and, notably, that immunization could induce either persistent positive or persistent negative regulation for responses to individual epitopes clearly conflicted with the ruling paradigm. These ideas were being hotly debated when the molecular era began and study of cell-based immunoregulatory mechanisms was abruptly terminated.

In the intervening years, epitope-specific regulation found a receptive home among vaccine developers, who recognize the potential for inducing long-term suppression for responses to viral or bacterial epitopes when these are presented on carrier molecules to which the subject has been previously immunized. In contrast, among basic immunologists, epitope-specific regulation seems to be mostly unknown (or ignored). Thus, I submit that it constitutes the 'elephant in the bathtub', whose presence must be acknowledged, accounted for and refined if the immune system is to be understood in the real world.

Epitope-specific regulation

The studies to which I refer and which I will summarize here were published in a series of papers[3,12–17] that we ultimately reviewed in detail in a 1983 article in the first edition of *Annual Reviews of Immunology*[11]. We found

Leonore A. Herzenberg is with the Department of Genetics, Stanford University, Stanford, California 94305, USA.
e-mail: leeherz@stanford.edu

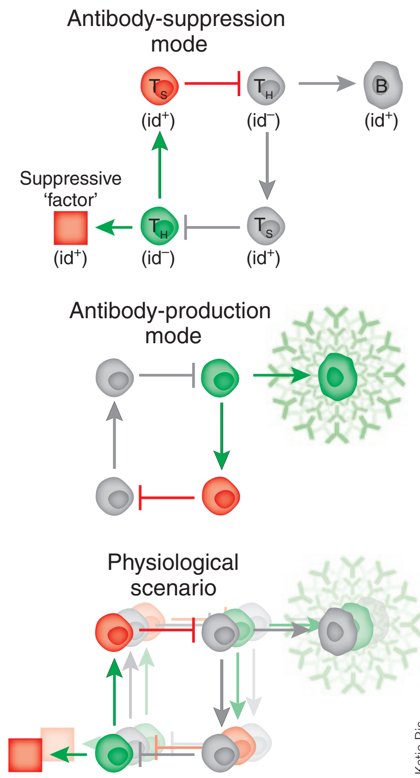

Figure 1 Model T cell circuits that use idiotype recognition to control the epitope specificity of IgG antibody responses. Each circuit is 'keyed' to the combining-site structure (id) of the antibodies produced by a memory B cell clone: helper T cells (T_H cells) have id^- receptors that are complementary to the id^+ epitope-specific IgG receptors on the B cell; suppressor T cells (T_S cells) have id^+ receptors that are complementary to the id^- receptors on T_H cells and hence resemble the id^+ epitope-recognizing antibodies on the B cell. Each circuit acts like an electronic 'flip-flop' circuit with opposing pairs of T_H and T_S cells. Two stable positions, on or off, 'translate' to help or suppress production of antibodies to an epitope. Carrier-specific help induces the positive side of the circuit, which then provides help for antibody production and maintains itself in a positive configuration by providing help for T_S cells that interfere with upregulation of the negative side of the circuit. Carrier-specific T_S cells, in contrast, upregulate T_H cells and T_S cells on the negative side of the circuit, which then turn off help for antibody production and maintain the negative configuration by turning off help for the T_S cells that interfere with suppression induction. In physiological conditions, some circuits are induced into each configuration. Adapted from ref. 18 and reproduced with permission.

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that immunization results in the induction of both positive and negative regulatory mechanisms (**Fig. 1**). Together, these control the primary and subsequent antibody responses to individual structures (epitopes) that are initially encountered on an immunogen (immunizing molecule). The positive regulatory mechanisms induced for some epitopes ensure the rapid production of 'secondary' antibody responses to those epitopes when they are subsequently encountered. The negative (suppressive) regulatory mechanisms induced for other epitopes will, in contrast, ensure that the system will not produce antibodies to those epitopes after a subsequent encounter. Although an initially negative response to an epitope can be shifted to positive and vice versa, the state initially induced is unexpectedly resilient and most often tends to be maintained over many subsequent immunizations, even in the presence of strong immune adjuvants that readily boost the magnitude of the positive responses that are produced.

The stubbornness of the immune system in terms of initiating antibody production to additional native epitopes on immunogens once the initial response pattern is established in a given animal is (was) well known among immunogeneticists and others faced with generating antibodies to poorly immunogenic epitopes. In my experience, which echoes that of many of my colleagues, there is no point in trying to 'convince' an animal to make antibodies to a particular epitope if the animal did not make detectable amounts of antibodies to that epitope after one or two immunizations. The response pattern is fixed by that time and, except in rare circumstances, does not change no matter how many booster immunizations are given. Indeed, if antibodies to a particular epitope are needed, it is far better to immunize more animals than to boost animals that have not responded, even those producing high titers of antibodies to other epitopes on the same immunogen.

These kinds of findings have been explained by the failure to induce memory B cells capable of producing antibodies reactive with the 'poor' epitope, perhaps because precursors of memory B cells with immunoglobulin rearrangements that result in antibodies that can bind the epitope are scarce in some animals. This idea seems logical and is probably true in some circumstances. However, our studies provide an alternate explanation, which is supported by studies demonstrating suppressed responses in animals in which immunization with a 'strong' epitope concomitantly induces the development of mature memory B cells that can be readily demonstrated in adoptive transfer studies[15]. In essence, our findings tie the observed response failure to negative regulatory mechanism(s) that suppress the expression, rather than the induction, of memory B cells producing antibodies reactive with the epitope.

Bringing the system into focus

Our studies of epitope-specific regulation began simply as an attempt to increase high-affinity primary immunoglobulin G (IgG) antibody responses to the dinitrophenyl (DNP) group (also known as hapten) coupled to keyhole limpet hemocyanin (KLH), a large, highly immunogenic molecule. KLH is one of several 'carrier' proteins typically used to induce T cell help for IgG antibody responses, commonly to DNP or other haptens coupled to the carrier. As priming and boosting with DNP-KLH was well known to induce strong high-affinity IgG antibody to DNP (anti-DNP) responses, we reasoned that initially immunizing with only the carrier would increase the amount of T cell help available and hence increase the strength of the antibody response to DNP presented subsequently on the carrier (that is, to DNP-KLH).

In fact, just the opposite turned out to be true[11]: both the magnitude and the affinity of the IgG response to the DNP in the animals immunized with carrier followed by hapten-carrier were well below the primary IgG anti-DNP response that occurred in naive animals immunized with only the hapten-carrier conjugate. Furthermore, subsequent immunizations failed to improve the situation: the IgG anti-DNP responses remained below primary response in animals immunized with KLH followed by DNP-KLH, regardless of whether they were subsequently immunized with priming or booster doses of DNP-KLH or, notably, with DNP on other carrier proteins, such as chicken γ-globulin (DNP-CGG). The same result occurred when animals were immunized with CGG followed by DNP-CGG and subsequently with DNP-KLH. Once animals had experienced DNP in the context of a protein to which they had previously been immunized, they consistently produced small, low-affinity IgG anti-DNP responses.

In contrast, responses to DNP-KLH in naive animals (that is, animals not immunized initially with KLH) increased in magnitude and affinity to reach the rapid, large, high-affinity secondary responses typical in animals primed and boosted with hapten-carrier conjugates. Similarly, naive animals immunized sequentially with DNP-CGG or with DNP-KLH followed by DNP-CGG developed typical high-affinity responses to multiple exposures to the hapten on either, or on other, carrier proteins. Consistent with this finding, responses to native epitopes on the carrier proteins had the usual primary and secondary response patterns regardless of whether the animals were immunized initially with the carrier protein or with the hapten-carrier conjugate. Thus, secondary responses to KLH epitopes are equivalent whether the animal is immunized twice with KLH or once with KLH and once with DNP-KLH.

Overall, the results of these sequential immunization studies demonstrate the induction of persistent support for antibody responses to certain epitopes presented on a carrier protein and

the induction of persistent suppression to other epitopes presented on the same protein, with suppression induction being the rule for epitopes that are presented at a later time[11]. Thus, our findings suggest the existence of a window of opportunity during which positive support for antibody responses to individual epitopes can be established (**Fig. 2**). Once this 'regulatory opening' expires, responses to additional epitopes on the protein will be suppressed and will remain so thereafter, despite reintroduction of the epitope on the same or a different carrier.

Time in, time out

Who holds the stopwatch and what makes it tick? Frankly, no one really knows the answer to that question. Most likely, the regulatory T cells known as carrier-specific suppressor T cells are key in inducing epitope-specific suppression, much as carrier-specific helper T cells are involved in inducing stable antibody responses to the 'successful' epitopes. The helper T cells arise rapidly after immunization. The suppressor T cells, in contrast, arise about a week after immunization with a carrier (or other) protein and persist thereafter. The term 'carrier-specific' is aptly applied to these cells in the sense that they arise in response to immunization with a carrier molecule and induce suppression only for antibody responses to epitopes presented on the immunizing carrier. However, the effector mechanism that mediates the suppression that is induced is epitope specific rather than carrier specific. Once it is induced to suppress an anti-epitope response, it suppresses responses to the epitope essentially regardless of the carrier on which it is subsequently presented.

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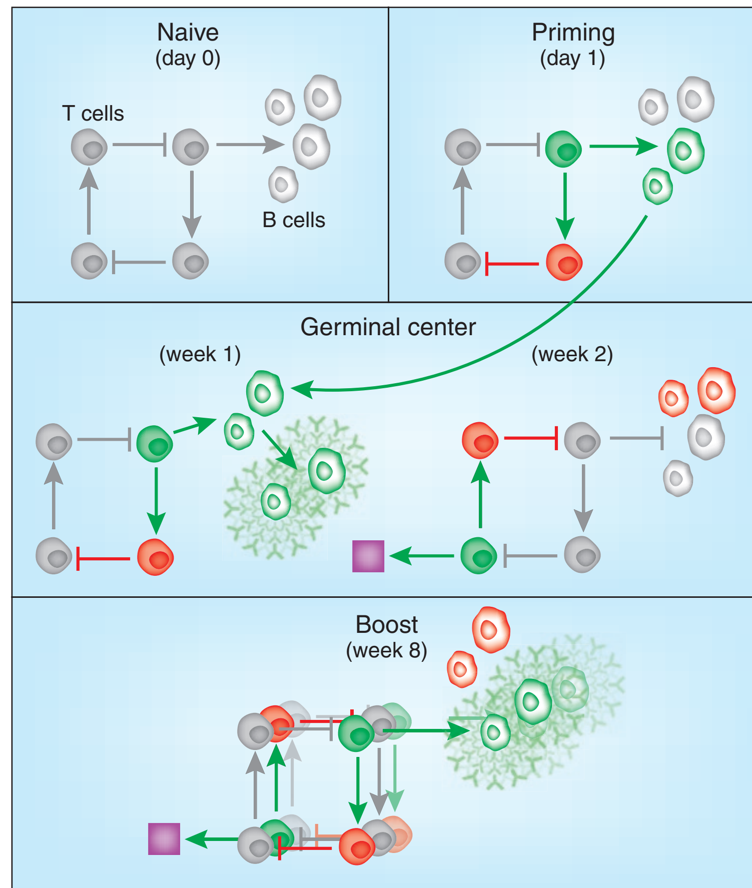

Figure 2 Kinetics of epitope-specific suppression induction. The development of memory B cells is accompanied by the development of stable regulatory circuits that recognize and regulate the production of immunoglobulin combining-site structures (idiotypes) produced by individual memory B cell clones. The initial circuits tend to be stably configured to help antibody production. However, later circuits tend to stabilize mainly in a negative configuration that persistently blocks the necessary help for primary, secondary and subsequent memory B cell responses.

This curious distinction between the specificity of the suppression induction and suppression effector mechanisms was missed in the earlier studies, mainly because the suppression assays were not constructed to detect it. At the time, anti-hapten responses were commonly accepted as representative of antibody responses to all epitopes on a carrier molecule. Thus, in typical assays, a source of memory B cells primed with a hapten on one carrier molecule was combined with sources of helper and/or suppressor T cells primed with a second carrier molecule and challenged with the hapten on the second carrier molecule (or on an unrelated carrier molecule, to test for carrier specificity). These types of assays were used initially to demonstrate that memory B cell responses require carrier-specific T cell help and ultimately to show that such memory responses could be suppressed when sources rich in carrier-primed suppressor T cell activity were introduced in the assay. The latter finding was commonly interpreted as being due to the ability of carrier-specific suppressor T cells to remove carrier-specific help, although in retrospect there was no way of distinguishing this mechanism from the carrier-specific induction of an epitope-specific effector mechanism that, once induced, prevents antibody responses to the hapten presented on any carrier molecule.

Occam's razor, of course, cuts sharply in favor the simpler, carrier-specific mechanism. However, from the present perspective, the available data fit better with the more complex construction, as it can account both for the carrier-specific and the epitope-specific elements of the antibody response regulation demonstrated by sequential immunization with carrier, then hapten-carrier. As we have shown, immunization with KLH, DNP-KLH and finally DNP-CGG results in persistent suppression of anti-DNP responses, even though the last two immunizations (without the initial KLH immunization) result in strong, high-affinity anti-DNP responses. Carrier recognition is obviously required for the induction of this suppression, but the effector mechanism is ultimately epitope specific.

As carrier-specific suppressor T cells arise *in vivo* at about the same time that animals become sensitive to suppression induction for new (or unsuccessful) epitopes on the carrier molecule, it is reasonable to hypothesize that the carrier-specific suppressor cells initiate the induction of epitope-specific suppression. And, conversely, it is reasonable to hypothesize that epitope-specific suppression mediates what has been called carrier-specific suppression. To my knowledge, these hypotheses have never been directly tested with appropriately isolated suppressor cells or molecules. Thus, they remain the best available explanation for evidence gathered in studies that mostly terminated in the mid-1980s.

Viewed broadly, the emergence of carrier-specific suppressor T cell activity shortly after antibody production is initiated can be seen as the ascendance of inductive capability for the negative arm of a comprehensive system that exerts both positive and negative control over antibody responses to individual epitopes on an immunogen. In essence, this system operates both to stabilize the production of rapidly induced

responses and to persistently prevent the initiation of those responses that lag behind long enough to fall prey to emerging suppression-induction mechanisms (**Fig. 2**). Thus, mechanisms that either delay the initiation of antibody responses or speed up the activation of carrier-specific or other suppression-induction mechanisms will result in suppression, whereas mechanisms that enhance the rapid initiation of antibody responses will increase the magnitude and, probably, the breadth of those responses.

Consistent with that formulation, epitope-specific suppression apparently is key in controlling at least one classical antibody response under the well studied control of immune-response genes of the mouse major histocompatibility complex. Antibody responses to a synthetic tyrosine, glutamine, alanine and lysine terpolymer (TGAL), fail in mice with certain major histocompatibility complex haplotypes but succeed in mice with other haplotypes. Antibody responses to DNP-TGAL also fail in the nonresponding mice, even though these mice produce strong anti-DNP responses when immunized with DNP-KLH[14]. Sequential immunization with DNP-TGAL followed by DNP-KLH results in considerable suppression of the strong anti-DNP responses that would normally have been induced by the DNP-KLH immunization but does not affect the responses mounted to the native KLH epitopes on the DNP-KLH. Thus, there is good reason to believe that the epitope-specific system mediates the failure to respond to native and other epitopes presented on TGAL in this well known response system controlled by major histocompatibility complex and most likely in some or all of the other so-called 'genetic nonresponder' systems with similar characteristics. If so, the genetic regulation in these systems could simply resolve to a decrease in the rate at which production of the relevant antibodies can be initiated or to an increase in the rate at which epitope-specific suppression is induced in the nonresponder animals.

The issue of what cells or molecules mediate the actual suppression of antibody responses to the 'lagging' epitopes, as well as how these mediators are induced to specifically do their job, is still wide open. Some years before we fell into working on epitope-specific suppression, we had proposed a 'bi-stable' antibody response–regulation model in which idiotype-specific helper T cells would either promote continued antibody responses by B cells or would be inactivated by suppressor T cells specifically induced to downregulate the helper activity (**Fig. 1**). The thinking we did as we developed the idiotype-based 'bi-stable' circuits introduced in this model[18] provided a guide without which we probably could not have threaded our way through the complex interactions we later identified in the epitope-specific system.

The idea that epitope-specific regulation is mediated by idiotype–anti-idiotype or other immunoglobulin-specific interactions, and hence keys memory B cell immunoglobulin, is attractive. By examining the individual isotype and allotype anti-DNP and anti-KLH responses produced by sequential immunization with KLH, DNP-KLH and related immunizations, we have shown that individual isotype responses are differentially sensitive to suppression induction[11]. IgG1 responses, for example, are much less likely to be suppressed in marginal conditions, and suppression for these responses is easier to reverse by intensive immunization than is suppression for IgG2a responses[11–15]. How this fits with the present ideas of how isotype responses are regulated I do not know (although in analyzing data for very large numbers of such responses after immunization in our conditions, we failed to detect the isotype response shifts noted when conditions are varied in T_H1 or T_H2 studies). In any event, we also found evidence that epitope-specific suppression is key to the chronic IgG2a allotype production that we have studied for years[13].

Although seemingly arcane, the idea of an epitope-specific regulatory mechanism capable of integrating signals from carrier-specific and immunoglobulin-specific systems has much to recommend it from an evolutionary perspective. Regulation of antibody production at the epitope-specific level enables the individuation of responses but minimizes the general dissipation of resources on responses to poorly immunogenic epitopes. Thus, it favors diversity while still maintaining central economy and efficiency. Similarly, the cacophony of carrier-specific, immunoglobulin-specific, idiotype-specific and other immunoregulatory influences that affect antibody responses are all crucial to a broadly functional immune system. However, they cry out for an organizational matrix that can bring order to the void. Thus, although its mechanisms are still shrouded in mystery, there is reason to believe that evolution has fostered the development of an epitope-specific regulatory system capable of mediating among a variety of conflicting regulatory 'claims' and thereby enabling a clear-cut and stable 'decision' as to which responses will prevail.

I hope that this 'express tour' of the epitope-specific regulatory landscape has whetted the reader's appetite for serious critical examination of the evidence summarized in our 1984 review article[11], which provides the basis for the extravagant claims that I have made here. It would be of interest to revive this discussion in a modern context and perhaps to see studies in which transgenic and knockout mice are tested for alterations in the epitope-specific regulation of antibody responses (rather than simply being declared 'whole' because they do not demonstrate any lesions in the development of germinal center and memory B cells, both of which are necessary but not sufficient for high-affinity antibody responses). In any event, it is certainly time to recognize the presence of the 'elephant in the bathtub', to reevaluate the evidence on carrier-specific and epitope-specific suppression with a view to either definitively declaring it incorrect or, as I believe will be the case, incorporating it and other previously identified regulatory mechanisms into modern studies.

Historical note

The bi-stable regulatory circuit model[18] owes much to the idiotype-based regulatory networks introduced by Niels Jerne[19], who was the first to emphasize the role(s) that combining site complementarity could play in shaping and controlling immune responses. Takeshi Tokuhisa (Chiba, Japan) and Kyoko Hayakawa (Fox Chase Cancer Center) also made key contributions. Finally, we owe much to my husband and long-term colleague, Leonard A. Herzenberg, who along with Ray Owen (Caltech) strongly supported my entry into the theoretical arena at a time when women were seldom welcome in such hallowed precincts.

COMPETING INTERESTS STATEMENT
The author declares no competing financial interests.

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