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EPITOPE-SPECIFIC REGULATION

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INTRODUCTION

Long before lymphocytes had been identified with certainty as the precursors of antibody forming cells, immunologists and immunogeneticists were well aware that animals immunized with the complex antigens like bovine serum albumin individually produce antibodies to different subsets of the epitopes (determinants) on the antigen. Over the years, many of the cells and cell interactions that regulate antibody production have been defined in great detail; however, the processes that control the characteristic individuality of antibody responses still remain shrouded in mystery. The epitope-specific regulatory system described here offers a workable explanation of how this variation is generated and maintained. Furthermore, as we shall show, the joint operation of the carrier-specific induction mechanism and the epitope-specific effector mechanism that defines the characteristics of the regulated response provides an explanation for many of the odd observations encountered in studies of immunologic memory and carrier-specific regulation.

Current concepts of how carrier-specific regulation influences antibody responses to the epitopes (immunogenic structures) on carrier molecules date from the early 1970s, when Mitchison (1) and Rajewsky et al (2) first demonstrated that carrier-primed T cells help hapten-primed B cells give rise to adoptive secondary antibody responses. These studies, which showed that ". . . the antigen [hapten-carrier conjugate] is recognized by two receptors, one directed to the hapten and the other to a determinant on the carrier protein" (1), in essence formulated the contemporary definition of a T-dependent antigen, i.e. a macromolecule with at least one "carrier determi-

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nant" recognized and used by T cells to regulate antibody responses to the various epitopes on the antigen.

The hapten-carrier bridge mechanism suggested for carrier-specific helper activity in these early studies is as viable today as it was when first stated (and still remains unproven); however, the simplistic two-cell model (helper T and memory B) introduced initially has undergone considerable expansion. Two functionally distinct carrier-specific regulatory T cells have been added, one which suppresses antibody responses (3–8) and another which contrasuppresses such responses (9). Furthermore, several carrier-specific cells have been identified as part of developmental cascades leading to the emergence of the functional cell types. Finally, on a theoretical level, a series of carrier-specific regulatory circuits have been proposed locating the various functional cells in a self-limiting ("feed back") type system that controls antibody production by controlling the supply of carrier-specific help (10).

This construct is consistent with most of the available data; however, it fails to explain a number of relevant observations (several of which predate its inception). In particular, because it is predicated on the idea that carrier-specific regulatory interactions do not selectively influence antibody production to individual epitopes on an antigen, it tends to trivialize findings that suggest links between carrier-specific and epitope-specific or other regulatory mechanisms. In essence, it has led to consistent disregard of evidence suggesting that immunizing carrier-primed animals with a "new" hapten coupled to the priming carrier results in the induction of specific suppression for antibody responses to the hapten (see below).

Mitchison (1) and Rajewsky et al (2) overtly chose to ignore this peculiar response failure to simplify consideration of the mechanisms involved in carrier-specific help. Thus, although they carefully cite evidence from several studies (including their own) showing that carrier-primed animals often fail to produce detectable levels of antibodies to haptens introduced subsequently on the priming carrier, they put more trust in opposing evidence showing that significant anti-hapten antibody production can be stimulated by this "carrier/hapten-carrier" immunization protocol. Rajewsky et al (2) make this point quite directly by stating: "We suggest that, in general, an animal pretreated with free carrier and receiving a secondary injection of this carrier complexed with a hapten will be found to produce a better anti-hapten response than without the pre-treatment, if the experimental design is aimed at detecting this effect."

This "leap in faith" (although largely incorrect) was probably crucial to the orderly progress of early studies exploring the mechanisms regulating antibody production. The idea that carrier-priming would augment (rather than suppress) subsequent antibody responses to a hapten presented on the

priming carrier cleared away confusion and allowed rational planning of adoptive and in vitro experiments that followed from the newly published carrier-specific help studies. However, although this idea was advanced originally as a prediction and had been shown to be invalid under certain circumstances, it rather rapidly assumed the mantle of truth. Consequently, several years later, we (and most of our colleagues) were quite surprised by the suppressed rather than augmented anti-hapten antibody responses that we obtained following carrier/hapten-carrier immunization (11).

These unexpected findings (e.g. see Figure 1) engendered a rapid series of experiments exploring the mechanism of the suppression and a somewhat more leisurely search through the literature looking for precedents for our observations. Thus, by the time we discovered that previous investigators had ascribed this kind of response failure to impaired anti-hapten memory development (14) or to the presence of anti-carrier antibodies (12, 13), we had already ruled out these possibilities and realized that we were dealing with a previously unrecognized "epitope-specific" regulatory system that selectively controls antibody production to individual epitopes according to the dictates of carrier-specific (and other) regulatory T cells present in the immunologic environment when such epitopes are first introduced (11, 15–22).

In pursuing our studies of this system, we have been concerned primarily with finding out how it works; however, we have also put considerable thought and some experimental effort into determining how it could have escaped notice for so many years. Not surprisingly, the answers to these questions often merge. For example, in attempting to establish adoptive assays for the inducer and effector cells responsible for the suppression, we found that quite strong in situ suppression is difficult to transfer to irradiated recipients, particularly when measured as the ability to suppress a response mounted by a co-transferred cell population (16). This characteristic tendency for help to predominate over suppression in irradiated recipients put several investigators off the track, including Sarvas et al (14) who in 1974 reasoned correctly that carrier-specific suppressor T cells induce suppression for anti-hapten responses in carrier/hapten-carrier immunized

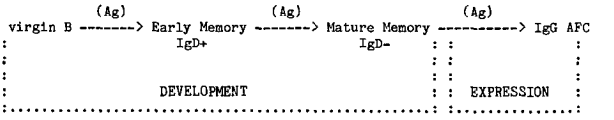

Figure 1 Regulation of IgG (memory) responses. Mechanisms that regulate memory B-cell development regulate the potential for IgG antibody production (22). Mechanisms that regulate memory B-cell expression control which and how many of the memory B cells present in a given animal actually give rise to AFC (17).

animals (see below), but discarded the idea because it "requires postulating that the effect of these suppressor cells is abolished in cell transfer experiments."

The general tendency to measure anti-hapten antibody production exclusively while studying the mechanisms involved in carrier-specific regulation also appears to have played a major role in "hiding" the epitope-specific system. Ishizaka & Okudaira (23), for example, demonstrated that anti-hapten responses were suppressed while anti-carrier antibody responses proceeded to secondary levels in carrier-primed animals stimulated with a hapten on the priming carrier. Discussing this work (in 1973), these investigators referred to work by Tada & Okumura (24–26) demonstrating the existence of suppressor T cells and suggested ". . . that immunization with 1 to 10 μg of ovalbumin may result in the formation of so called [carrier-specific] suppressor T cells that might suppress preferentially the primary anti-DNP response" (23). Immuno-history would probably be quite different had Tada (or others interested in carrier-specific suppressor T cells) taken this suggestion seriously enough to test anti-carrier antibody responses in suppression assays routinely.

All in all, the attractive simplicity of the idea that carrier-specific suppressor T cells regulate antibody production by depleting carrier-specific help appears to be sufficient to explain the willingness of many laboratories (including our own) to ignore subtle inconsistencies and leave a few moss-covered stones unturned. Perhaps this has been mainly for the good, since the mechanisms involved in epitope-specific regulation are considerably more complex and would, in fact, have been difficult to explore without the level of technology and theoretical understanding reached with the last few years. In any event, we now appear to have reached the time when re-evaluation of past evidence is essential to present progress.

The sections that follow describe various aspects of our studies of the epitope-specific regulatory system. We begin with an overview section (immediately below) that broadly outlines the system as a whole, documenting statements with references rather than with evidence. The remaining sections, in contrast, discuss aspects of the system in more detail and include much of the evidence upon which our conclusions are based.

The work we discuss here has mainly been conducted in our laboratory at Stanford; however, studies with carrier-specific suppressor T cells and soluble factors were conducted in Dr. Masaru Taniguchi's laboratory in Chiba, Japan. Almost without exception, the evidence we report derives from experiments that repeat earlier work but include key controls aimed at distinguishing carrier-specific from epitope-specific regulatory effects. Thus, we independently measure both the anti-hapten and anti-carrier antibody responses following various antigenic stimulations, and we confirm the

induction of epitope-specific suppression for a given anti-hapten antibody response by a final immunization with the hapten conjugated to an unrelated carrier molecule. As we shall show, the application of these rather straightforward techniques provides surprising insights into the older data and reveals the outlines of a highly flexible central regulatory system responsible for controlling all aspects of antibody responses.

EPITOPE-SPECIFIC REGULATION: AN OVERVIEW

The epitope-specific system apparently provides a common channel through which carrier-specific, isotype-specific, allotype-specific, and I-region-defined mechanisms exert control over antibody responses (11, 15–21). It plays a key role in regulating IgG antibody responses to haptens and native epitopes on commonly used carrier molecules such as KLH (keyhole limpet hemocyanin) and CGG (chicken gamma globulin) (11, 15–18). Furthermore, it is active in regulating IgG responses to epitopes on the synthetic ter-polymer TGAL, even in genetic "non-responders" to this antigen (20). Carrier-specific interactions induce it to suppress antibody production (to individual epitopes on the carrier); however, once induced to suppress a given anti-epitope response, it will suppress that response even when the epitope is presented in immunogenic form on a different carrier molecule (11, 15–17).

The effector mechanism in this system controls memory B-cell expression (as opposed to development; see Figure 1) and appears to be the ultimate arbitor of which and how many such B cells will be permitted to differentiate to IgG antibody-forming cells (AFC) in response to a given antigenic stimulus. It is epitope specific in that it independently regulates the amount and affinity of the antibody response produced to each of the epitopes on a complex antigen (11, 15–17); and it is Igh restricted in that it selectively controls the IgCh isotype and allotype expression in such responses (17–19). These properties suggest that the overall system is composed of individually specific elements, each charged with the regulation of a subset of B cells committed to produce unique or closely related IgG molecules (17).

The flexibility of this (compound) regulatory system and the nature of its effects on in situ antibody responses are extraordinary. It is capable of suppressing virtually the entire primary and secondary antibody response to a given epitope. Therefore, it can completely conceal the presence of normal anti-epitope memory B-cell populations clearly demonstrable in adoptive transfer assays (11, 15, 16). Alternatively, it can support the expression of a subset of memory B cells and suppress the expression of others, thereby defining the unique spectrum of an antibody response produced by a given animal (17–19).

Carrier-specific and other regulatory mechanisms operative in the immunological environment when an epitope is first introduced determine which components of the antibody response will be suppressed and which will be supported (11, 15–17). Prior immunization history, genetic predisposition toward responsiveness (20) and perinatal immunologic "conditioning" (18) all contribute to this determination. Thus, the epitope-specific system constitutes an ideal candidate for a central integrative mechanism capable of resolving conflicting signals from various peripheral regulatory systems and translating a coherent decision to the antibody-producing apparatus.

In keeping with this role, this system offers a unique regulatory capability that provides both the stability required to maintain a response pattern once induced and the flexibility to modify that response pattern when stimulatory conditions change dramatically. In essence, the individual epitope-specific, Igh-restricted elements in the system appear to behave similarly to bistable electronic binary ("flip-flop") circuits that can be switched initially into an "on" or "off" position by a small electrical force and then require a substantially larger force to switch them to the opposite position. That is, initial immunization conditions that induce epitope-specific elements to suppress a given IgG antibody response will usually fail to do so once the system has been induced to support that response; and, similarly, conditions that induce the system to support a response will be far less effective once the system has been induced to suppress the response. Nevertheless, either suppression or support can be reversed by sufficient stimulation in the opposite direction (17).

The evidence upon which these conclusions (and hypotheses) are based is presented in detail in several publications that have appeared within the last few months (16–19). Therefore, in the discussion which follows (and in Tables 1 and 2), we summarize these findings and concentrate more intensively on several recent studies defining the T cells that mediate epitope-specific suppression and demonstrating some of the more complex aspects that exist within the system.

CARRIER/HAPTEN-CARRIER IMMUNIZATION INDUCES EPITOPE-SPECIFIC SUPPRESSION FOR IgG ANTI-HAPTEN RESPONSES

The epitope-specific regulatory system can be specifically induced to suppress primary and secondary IgG antibody responses to the dinitrophenyl hapten (DNP) without interfering with antibody responses to epitopes on the carrier molecule on which the DNP is presented (see Table 3). Furthermore, once so induced, it specifically suppresses antibody responses to DNP presented on unrelated carrier molecules. The magnitude of a suppressed

primary anti-DNP response is usually about 30% of the normal primary response; however, the affinity of a suppressed response is about 10-fold lower than normal. Suppressed secondary anti-DNP responses are typically less than 10% of normal and have average affinities that are at least 100-fold below normal (11, 16).

For most of our studies, we induce this suppression by immunizing animals sequentially with a carrier molecule such as KLH (keyhole limpet hemocyanin) and then DNP conjugated to the carrier (i.e. with the

Table 1 Induction of epitope-specific suppression occurs under a wide variety of conditions

Variable tested		Result[a]
Epitope	DNP, TNP, NIP	Suppression induced for both epitopes by carrier/hapten–carrier; suppression inducible for KLH epitopes by other protocols
Carrier	KLH, CGG, OVA, TGAL	All prime for suppression induction; 100 μg on alum sufficient; some genetic restrictions (see table 2)
Age	KLH at 8 weeks to >6 months	Suppression equally strong at all ages
Timing	1 to 13 weeks between KLH and DNP–KLH	Suppression equally strong for all intervals between carrier and hapten–carrier
	KLH/DNP–KLH then DNP–KLH or DNP–CGG up to 1 yr later	Suppression equally strong for all intervals between first and second hapten–carrier immunizations
Strains	BALB/C, BAB/14, SJL, SJA, C3H, C3H.SW, A/J, (SJL × BALB/C), C57BL/10, C57BL/6	Suppression inducible in all strains

[a] Summarized from References 1 through 7.

Table 2 Epitope-specific suppression in genetically controlled regulatory conditions

Condition	*Immunization*	*Result*
I-region controlled responses	TGAL/TNP–TGAL in C3H (H-2k) and C3H.SW (H-2b)	Suppression induction occurs in responder and non-responder strains (20)
Impaired response to KLH carrier (non-MHC)	KLH priming in A/J or C57BL/10	Poor suppression induction by KLH/DNP–KLH; normal induction by CGG/DNP–CGG (21)
Allotype suppression for Igh–1b (IgG2a) allotype production in (SJL × BALB/C)F1	DNP–KLH at 8 weeks of age (prior to mid-life remission from allotype suppression)	Igh–1b responses to DNP and KLH specifically suppressed during remission (18)

Table 3 Anti-hapten antibody production is specifically suppressed in carrier/hapten-carrier immunized mice

Immunizations[a]			In situ IgG2a antibody responses[b]		
Carrier	First DNP	Second DNP	Anti-DNP μg/ml (Ka)[c]	Anti-KLH units	Anti-CGG units
K	—	—	<3 (<0.3)	20	—
K	D–K	—	5 (<0.3)	170	—
—	D–K	—	35 (5)	15	—
K	D–C	—	20 (2)	—	21
—	D–C	—	13 (1)	—	11
K	D–K	D–K	9 (0.5)	370	—
—	D–K	D–K	120 (300)	130	—
K	D–K	D–C	6 (<0.3)	—	8
—	D–K	D–C	60 (100)	—	9
—	D–C	D–C	85 (400)	—	100

[a] K = KLH (keyhole limpet hemocyanin); C = CGG (chicken gamma globulin); DNP = 2,4-dinitrophenyl hapten; D–K = DNP–KLH; D–C = DNP–CGG. (BALB/c X SJL)F1 mice injected i.p. with 100 μg of the indicated antigen on alum at approximately six week intervals.

[b] Serum antibody levels measured by RIA two weeks after last indicated immunization. Anti-carrier antibody expressed as percentage of antibody in a "standard" secondary response serum pool.

[c] $K_aM^{-1} \times 10^6$ measured by RIA (22).

KLH/DNP-KLH immunization sequence). Several weeks later we again immunize with DNP, this time either on KLH or on an unrelated carrier molecule such as CGG (chicken gamma globulin). We inject 100 μg of each antigen on alum, usually at 4-week intervals, and compare the IgG anti-hapten and anti-carrier responses obtained 2 weeks after each immunization to the responses raised in (age, sex, and strain matched) control groups that are not primed initially with the carrier protein and thus are immunized only with the appropriate hapten-carrier conjugates.

This carrier/hapten-carrier immunization protocol induces marked suppression for IgG anti-hapten antibody production (as indicated above) but does not interfere with anti-carrier antibody responses or with the development of normal anti-hapten memory B-cell populations. That is, splenic B cells from either KLH/DNP-KLH immunized animals or DNP-KLH primed animals give rise to equivalent anti-hapten memory responses in adoptive recipients supplemented with an appropriate source of carrier-specific help. Furthermore, IgG anti-DNP responses in KLH/DNP-KLH immunized animals are suppressed to below primary level while anti-KLH responses are equivalent to the (secondary) anti-KLH responses obtained from control animals immunized a similar number of times with the carrier protein, i.e. twice with KLH or DNP-KLH (11–16).

Comparison of responses in KLH/DNP-KLH/DNP-CGG immunized animals and DNP-KLH/DNP-CGG immunized controls demonstrates the specificity of the suppression for anti-DNP responses even more dramatically. Control animals produce typical high magnitude, high affinity in situ secondary IgG anti-DNP responses. The experimental group produces IgG anti-DNP responses that are still below primary levels. Nevertheless, all animals (experimental and control) produce equivalent primary IgG antibody responses to the CGG epitopes on the second hapten-carrier conjugate. Thus, carrier-primed animals fail to produce antibodies to a "new" hapten presented subsequently on the priming carrier and animals immunized in this way develop a persistent suppression specific for responses to the hapten, even when presented next on a different carrier molecule (11, 15, 16).

Although the failure of anti-hapten responses in carrier/hapten-carrier immunized animals appears novel from a contemporary perspective, this phenomenon was well known in an earlier era (ca 1970), having been described in the landmark papers demonstrating adoptive carrier-specific help interactions (1, 2). It was later attributed to interference with memory B-cell development (12) and then largely forgotten as attention shifted to using adoptive and in vitro assays for characterizing the carrier-specific (and other) mechanisms regulating antibody responses. We view the loss of this key "immunologic fact" as understandable (17) but nonetheless regrettable, since some serious misconceptions (discussed below) could have been avoided if it had not fallen from sight.

CARRIER-SPECIFIC SUPPRESSOR T CELLS INDUCE EPITOPE-SPECIFIC SUPPRESSION

Carrier-specific suppressor T cells (CTs) that arise shortly after priming with a carrier molecule (4–6) are responsible for inducing the epitope-specific system to suppress IgG anti-hapten responses to "new" epitopes presented on the carrier molecule (15, 16). These well-known regulatory T cells were commonly believed to regulate antibody production by interfering with carrier-specific help; however, by repeating the original CTs transfer experiments (4, 5) with additional controls that define the specificity of the mechanism mediating the suppression in CTs recipients, we have shown that KLH-specific CTs regulate responses by inducing typical epitope-specific suppression for anti-DNP responses when the recipients are immunized with DNP-KLH (15, 16). Thus, whether KLH-primed animals are immunized directly with DNP-KLH (KLH/DNP-KLH immunization sequence) or whether T cells from KLH-primed animals are challenged with

DNP-KLH in (non-irradiated) recipients, anti-DNP responses are persistently suppressed whereas anti-carrier responses proceed normally.

The demonstration of CTs in KLH-primed animals generated some confusion initially because splenic T cells from such animals provide an excellent source of carrier-specific help (CTh) rather than suppression in (irradiated) adoptive transfer recipients. This difference appeared to be due to the use of aqueous KLH for generating CTs and alum-precipitated KLH for generating CTh; however, our results indicate that priming either with aqueous or alum-precipitated antigen induces both CTh and CTs in the immunized animals, that both kinds of regulatory T cells are active in such animals, and that each can be demonstrated independently in the in vitro or adoptive assays developed to reveal its activity.

The aqueous KLH priming protocols usually used to generate CTs did prove to be somewhat more effective in priming for in situ suppression induction than the alum-KLH priming protocols commonly used to generate KLH-specific helper T cells; however, as a rule, we have used alum-KLH priming in the carrier/hapten-carrier sequence and in adoptive studies with KLH-primed T cells, and have generated strong epitope-specific suppression. In fact, as we shall show below, roughly equivalent suppression is induced (by DNP-KLH) in animals primed with KLH on alum, in Freund's adjuvant or in aqueous form.

In essence, these studies collectively demonstrate that the epitope-specific system constitutes the major, if not the only, effector mechanism through which CTs control antibody production. Thus, they define a new role for CTs (as inducers of epitope-specific suppression) and cast these cells as "conditioners" of the immunologic environment that, when present, alter how the epitope-specific system responds to (carrier-borne) epitopes that it has not "seen" before. Coupled with the bistable properties of the epitope-specific system, this construct explains how priming with an antigenic (carrier) molecule can simultaneously prepare the animal to produce typical secondary antibody responses to epitopes encountered initially on the priming antigen and yet to specifically suppress antibody production to "new" epitopes encountered subsequently on the same antigenic molecule. We return to this point in a later section exploring the consequences of bistable regulation.

The revised view of how CTs regulate antibody production discussed above is consistent with recent evidence from studies with T-cell lines and hybrids demonstrating two carrier-specific inductive pathways that terminate in epitope-specific cells (7, 8), one that suppresses antibody production and the other that augments antibody production. Relationships (if any) between these cells and the epitope-specific effector mechanism described here have yet to be established; however, the separate carrier-specific path-

ways they define support the inductive role our evidence assigns to carrier-specific regulatory cells in in situ antibody responses.

EPITOPE-SPECIFIC REGULATION IS IGH-RESTRICTED

The induction and maintenance of suppression (in carrier/hapten-carrier immunized animals) varies in efficiency for individual isotype anti-hapten responses (17–19, 21). IgM responses show no evidence of suppression. IgG2a, IgG2b, and IgG3 responses are easily suppressed whereas IgG1 responses are more refractory to suppression in that they tend to be suppressed in fewer animals under suboptimal suppression-induction conditions and to escape from suppression more frequently than the other IgG isotypes after a given number of restimulations with the hapten. This characteristic isotype hierarchy prevails in animals in which the induction of epitope suppression is either genetically impaired or experimentally minimized by immunizing initially with low doses of the carrier protein. In fact, whenever suppression is weak initially or begins to wane after repeated antigenic stimulation, IgG1 antibody responses are always the first to appear (17, 21).

IgG2a, IgG2b, and IgG3 responses show about the same susceptibility to suppression; however, when suppression is weak or waning, these isotypes often "escape" individually or in random pairs (very occasionally in animals that remain suppressed for IgG responses). This selective expression demonstrates the independent control exerted by the eptitope-specific system with respect to isotype representation in antibody responses. Studies with allotype-suppressed mice similarly show that the epitope-specific system can specifically suppress Igh-1b (IgG2a allotype) responses to individual epitopes in an allotype heterozygote without interfering with production of the (allelically determined) Igh-1a responses to the same epitopes (18). Thus, the individual elements that mediate epitope-specific regulation appear to be restricted to controlling the production of antibodies with the same or closely related combining-site structures and a single heavy chain constant-region structure (allotype/isotype).

Theoretical considerations suggest that this Igh constant-region restriction may be based exclusively on the recognition of allotypic (rather than isotypic) structures. That is, since isotypic structures are shared between allotypically different heavy chains, isotype-restricted regulation cannot explain the selective regulation of Igh-1b allotype antibodies. Allotype-restricted regulation, in contrast, can clearly account for selective isotype regulation since nearly all Igh allotypic structures are unique to (and thus can identify) the heavy chain isotype on which they are found (27). Thus,

it is likely that the selective regulation of both isotype and allotype representation in individual anti-epitope responses derives from a requirement for recognition of polymorphic (allotypic) regions of Igh heavy chain constant regions.

EPITOPE-SPECIFIC REGULATION IS BISTABLE

Bistable systems, by definition, have two alternative steady states with mutually exclusive functions. When confronted initially with a stimulus favoring one state or the other, these systems move rapidly to the favored state. Stabilization mechanisms then maintain the initially induced state, so that a substantially stronger signal is required to move to the other steady state than would have been required to establish that state initially. Thus, bistable systems tend to remain as initially induced but nonetheless remain capable of shifting to the alternate state if stimulatory conditions so dictate (17, 28).

The characteristics of the regulation provided by the epitope-specific system meet these criteria (17). As we have indicated, carrier/hapten-carrier immunization induces suppression for IgG responses to the hapten. Once induced, this suppression tends to be maintained (especially for IgG2a, IgG2b, and IgG3 responses). Repeated stimulation with the hapten (on any carrier), however, eventually induces IgG anti-hapten antibody production (more quickly for IgG2 than for the "more suppressible" isotypes).

Antibody production, once initiated, also tends to be maintained. Carrier/hapten-carrier protocols that induce strong suppression for anti-hapten antibody responses in virgin animals are substantially less effective in animals producing an ongoing primary IgG anti-hapten response (e.g. due to stimulation with the hapten on an unrelated carrier prior to completion of the carrier/hapten-carrier sequence). Under these conditions, detectable suppression is induced in about half the animal and, when induced, mainly affects the more suppressible isotypes. Thus, the initiation of antibody production impairs the subsequent induction of suppression, and the initial induction of suppression tends to prevent subsequent initiation of antibody production (17).

This reciprocal relationship defines a bistable regulatory mechanism that fixes long-term antibody response patterns according to the conditions under which it first "sees" individual epitopes. Thus, it provides a vehicle through which even quite transient conditions in the initial regulatory environment can strongly influence the characteristics of subsequent anti-epitope responses.

CONSEQUENCES OF BISTABLE REGULATION

Although memory B-cell induction and development are required for anamnestic (memory) responses, the epitope-specific system and the mechanisms that induce it to suppress or support memory B-cell expression play a key role in determining which and how many of these B cells will be expressed when an animal reencounters a given epitope. In fact, as a general rule, the characteristics of in situ memory responses provide a much better measure of the status of the epitope-specific system than of the memory B-cell populations that have been generated by a particular priming protocol.

Our evidence on this point suggests that much of the information from studies using in situ secondary responses to evaluate the effects of priming conditions on the development of memory B cells requires re-evaluation to distinguish conditions that truly influence B-cell development from those that influence the in situ expression (epitope-specific regulation) of memory B-cell responses. Carrier/hapten-carrier immunization, for example, induces normal anti-hapten memory populations that can be revealed in adoptive recipients (11, 15, 16); however, these memory cells remain entirely cryptic in situ under the conditions usually used to test for the presence of immunologic memory, e.g. repeated boosting with 1 μg aqueous antigen (unpublished observations). Stimulatory conditions that overcome the initially induced epitope-specific suppression reveal the presence of the cryptic memory populations, but this generally requires several immunizations with priming doses of the antigen (e.g. 100 μg of alum-precipitated hapten-carrier conjugate were used for each immunization in the suppression-reversal experiments discussed above).

Adoptive studies in which transferred spleen cells (co-resident B plus T) are used to evaluate memory development suffer from much the same problem, since suppression is maintained when spleen cells from suppressed animals are transferred to adoptive recipients (16, 21). In essence, we have found that the only reliable way to reveal anti-hapten memory that is not expressed in situ is to transfer T-cell depleted splenic (B cell) populations into recipients supplemented with carrier-primed T cells (preferably from animals primed with alum-precipitated carrier protein plus *B. pertussis*).

These considerations lead us to question the idea that variation in the memory B-cell populations generated in individual animals immunized with a typical protein antigen accounts for the well-known tendency for such animals to produce antibodies to different subsets of the epitopes on the immunizing antigen (17). B-cell "clonal dominance" mechanisms may contribute to this individualization of antibody responses; however, considerable variability will also be introduced by stochastic processes inherent in

the operation of a bistable regulatory system in which the major force inducing the system to suppress antibody production (CTs) matures to full function several days after the initial immunization with a carrier protein and its associated epitopes (17, 19).

That is, since a bistable mechanism tends to maintain itself in its initially induced configuration, anti-epitope responses established prior to the emergence of a functional CTs population will tend to continue despite the presence of these cells; however, anti-epitope responses that could not (or did not) become stabilized rapidly enough will tend to be suppressed once CTs become active. Therefore, the probability that a given epitope on a priming antigen will induce stable antibody production (or stable suppression) in a particular animal will be a function of the rate at which the epitope induces support for antibody production in comparison with the rate at which CTs mature (17).

In practice, this "horserace" can be expected to result in the induction of suppression for responses to essentially random subsets of priming antigen epitopes in individual animals. Some epitopes, however, will tend to be more like the DNP hapten, which induces support so rapidly that stable antibody production is established well before CTs mature in virtually all immunized animals (perhaps because such epitopes bind to a very wide variety of antibody combining sites and consequently can stimulate a large number of B cells). Other epitopes will be notably less successful in establishing antibody production. Thus (in accord with common serologic experience), the frequency of responses to individual epitopes obtained in a group of immunized animals will be relatively reproducible while the combination of epitopes detected by the antibodies produced by individual animals will vary from animal to animal.

TESTING THE HYPOTHESIS

If CTs induce suppression for responses to carrier-borne epitopes that have not as yet induced stable support for antibody production, then antibody responses to the all of the epitopes on a priming antigen (even DNP) should fail if the level of antigen-specific CTs activity is sufficient to initiate the induction of epitope-specific suppression immediately after priming. This condition is rarely (if ever) met naturally since the developmental cascade that results in the appearance of functional CTs only begins after the first (priming) encounter with an antigen; however, it can be approximated experimentally by priming animals with a hapten-carrier conjugate shortly after they have been injected with appropriately specific CTs-secreted factors (CTsF) that mediate the induction of epitope-specific suppression. Under these conditions (if our concept of bistable immunoregulation is

correct), antibody responses to the hapten and to the native epitopes on the carrier should all be suppressed.

Data in Table 4 show that, as predicted, anti-DNP and anti-KLH antibody responses are suppressed in animals that received KLH-specific CTsF shortly before being primed with DNP-KLH. Furthermore, anti-DNP responses predictably remain suppressed in these animals when the hapten is presented subsequently on an unrelated carrier molecule (DNP-CGG), whereas antibody responses to the (CGG) epitopes on the second carrier molecule proceed normally. Thus, this immunization protocol results in the induction of typical epitope-specific suppression for antibody responses to DNP and (by inference) to all other epitopes presented on the priming carrier.

These findings (T. Tokuhisa, M. Tagawa, M. Taniguchi, manuscript in preparation) directly demonstrate that all epitopes on hapten-carrier conjugate are treated equivalently to the hapten in the carrier/hapten-carrier immunization sequence when CTs activity is artificially introduced prior to priming with a hapten-carrier conjugate. Nevertheless, under normal circumstances, the emergence of active CTs shortly after priming does not interfere with antibody production to (at least some of) the epitopes on the priming antigen. This apparent paradox confirms the existence of a bistable mechanism that allows priming antigen epitopes to induce specific protec-

Table 4 Carrier-specific suppressor T-cell factor (CTsF) induces epitope-specific suppression[a]

BALB/c KLH–TsF[b]	Immunizations[c]	Strain	IgG2a antibody responses[d] DNP (μg/ml)	KLH (units)	CGG (units)
—	D–K	BALB/c	73	24	—
+	D–K	BALB/c	18	3	—
—	D–K D–C	BALB/c	102	—	7
+	D–K D–C	BALB/c	19	—	6
—	D–C	BALB/c	22	—	—
+	D–C	BALB/c	30	—	—
—	D–K	C57BL/6	71	—	—
+	D–K	C57BL/6	102	—	—

[a] T. Tokuhisa, M. Tagawa, M. Taniguchi, manuscript in preparation.
[b] KLH-specific suppressor factor (CTsF) prepared from BALB/c thymocytes as previously described (5); yield from 10^8 thymocytes injected per animal 24 hours prior to first immunization. CTsF prepared from BALB/c (H–2d) is not active in C57BL/6 (H–2b) (5).
[c] 100 μg each antigen on alum at two week intervals.
[d] Measured by RIA 2 weeks after last indicated immunization; anti-carrier responses expressed as percentages of "standard" adoptive secondary responses to the indicated antigen.

tion (support) for antibody production before CTs gain sufficient strength to induce specific suppression for unprotected responses.

EXCEPTIONS TO THE RULES

During the course of studies characterizing the epitope-specific system and the conditions that induce it to suppress or support antibody production, we tested the effects of a wide variety of protocol modifications (different antigens, doses, timing, adjuvants, mouse strains, etc). In nearly all cases, the results we obtained (summarized in Tables 1 and 2) were consistent with the properties of the bistable regulatory system defined by our original carrier/hapten-carrier immunization studies; however, we noted two striking exceptions, one concerning adjuvant effects on the carrier-specific suppression induction mechanism and the other (somewhat more surprising) concerning the maintenance of suppression for anti-DNP responses in sequential immunizations with DNP on different kinds of carriers.

The adjuvant studies show that although KLH/DNP-KLH immunization results in the induction of typical epitope-specific suppression when animals are primed with aqueous KLH, KLH on alum or KLH on alum plus complete Freunds adjuvant (CFA), priming with KLH on alum plus *Bordatella pertussis* completely prevents the subsequent induction of suppression for anti-DNP responses (see Table 5). Coupling the *B. pertussis*

Table 5 Carrier immunization with *Bordatella pertussis* prevents subsequent suppression induction

Immunizations with KLH (K) or DNP-KLH (D–K)[a]			Anti-DNP in serum (μg/ml)	
First	Second	Third	IgG2a	IgG1
—	—	D–K alum	64	145
K alum + PV	—	D–K alum	60	125
K alum	—	D–K alum	<13	125
K alum	K alum	D–K alum	<6	15
K CFA	—	D–K alum	<15	63
K aqueous	—	D–K alum	<6	55
K aqueous	K aqueous	D–K alum	<7	<28
—	—	D–K CFA	70	200
K alum	—	D–K CFA	<9	128
—	—	D–K alum + PV	90	225
K alum	—	D–K alum + PV	33	225
K alum	K alum	D–K alum + PV	<13	45

[a] 100 μg each antigen at 4 week intervals; antibody responses measured by radioimmune assay 2 weeks after last immunization.

with the DNP-KLH immunization in the sequence, in contrast, does not appear to interfere substantially with suppression induction since anti-DNP responses are clearly lower than in non-preimmunized controls that received DNP-KLH plus *B. pertussis.*

The interference with suppression induction does not appear to be due to the increased amounts of anti-KLH antibody produced by the KLH/*pertussis* immunized animals since KLH/CFA immunization stimulates roughly the same amount of antibody but does not interfere with suppression induction (see Table 6). These findings suggest that KLH/*pertussis* induces a carrier-specific cell population that prevents epitope-specific suppression induction when animals are subsequently immunized with DNP-KLH. Since this population appears to be functionally similar to the carrier-specific "contra-suppressor" population described by Gershon and colleagues (9), we wonder whether carrier immunization with *B. pertussis* might not be an excellent way to stimulate contra-suppressor cells and whether, in fact, such cells might not be the effector mechanism through which *B. pertussis* acts as an adjuvant to augment antibody responses.

Results from current carrier/hapten-carrier immunization studies with DNP coupled to sheep erythrocytes (D-SRBC) introduce another, more disquieting, exception to the otherwise consistent behavior of the epitope-specific system (19). SRBC/D-SRBC immunization induces what appears to be typical epitope-specific suppression in that the IgG anti-DNP response is substantially smaller and has a lower affinity than control responses (in D-SRBC immunized animals), whereas the IgG anti-SRBC response climbs to secondary levels comparable to those in SRBC/SRBC-immunized controls. Similarly, IgG anti-DNP responses remain suppressed after a second immunization with D-SRBC, and anti-SRBC responses proceed normally. However, when SRBC/D-SRBC-immunized animals are immunized with DNP-KLH, their IgG anti-DNP responses are barely suppressed!

Table 6 Priming with KLH in complete Freund's adjuvant or plus *Bordatella pertussis* yields similar responses

Immunizations[a]		IgG anti-KLH response[b]		
First	Second	Status	IgG2a	IgG1
KLH on alum	DNP–KLH on alum	primary	8	6
		secondary	100	106
KLH plus CFA	DNP–KLH on alum	primary	48	36
		secondary	87	140
KLH on alum plus *B. pertussis*	DNP–KLH on alum	primary	15	18
		secondary	99	236

[a] See legend to Table 5.
[b] Percentage of a "standard" adoptive secondary anti-KLH response.

Several other carriers (including ficoll and certain synthetic amino acid copolymers) yield similar results vis a vis their failure to induce suppression that extends to responses to DNP on KLH or CGG (unpublished observations). Furthermore, although we have not extensively cross-checked these carriers, the suppression they induce does not appear to extend to DNP presented on any of the others. Thus, we are faced with a heterogeneous group of carriers (cells, carbohydrates, artificial proteins) that apparently induce a suppression specific for anti-DNP responses (since anti-carrier antibody responses proceed normally) and yet do not induce suppression for such responses per se (since presentation of DNP on other carriers induces IgG anti-DNP antibody production).

This puzzling set of findings would be explained if the suppression induced in each case affected production of a different subset of the combining sites in the anti-DNP repertoire. This would mean that DNP presented on each of these carriers evokes a substantially different antibody response and a correspondingly unique set of regulatory cells specific for that response. This idea is consistent with theoretical considerations concerning the structure of combining sites likely to bind DNP in different structural environments (29–31; A. Edmondsen, personal communication); however, whether it will prove correct remains to be seen.

EFFECTOR CELLS IN THE EPITOPE-SPECIFIC SYSTEM

Early attempts to characterize the cell(s) responsible for epitope-specific suppression were stymied by difficulties in reliably measuring suppressive activity in adoptive recipients. Recent studies (T. Tokuhisa, M. Tagawa, M. Taniguchi, manuscript in preparation) have had more success using an in vitro assay in which cells from CGG/DNP-CGG immunized animals suppress anti-DNP antibody production by spleen cells from DNP-KLH or DNP-OVA (ovalbumin)-primed mice. These rather elegant studies demonstrate that the epitope-specific suppression is mediated by Thy-1 positive cells that have a specific receptor for DNP and (as Table 7 shows) can be removed on DNP-BSA coated plates.

At present, data are consistent with one or more DNP-specific suppressor T cells being required for suppression in this assay and with the suppressor cell(s) serving as direct mediators of suppression or as inducers of cells that mediate suppression (since the responding spleen cells provide a source of T cells that potentially can be induced to suppress antibody production). However, in either event, these studies clearly define a new category of regulatory cells by demonstrating the existence of epitope-specific suppressor T cells (ETs) that control antibody production independently of the carrier on which the epitope is presented.

Table 7 Epitope-specific suppressor T cells (detected in vitro) bind specifically to DNP-coated plates[a]

DNP–KLH primed spleen[b]	CGG/DNP–CGG immunized spleen		IgG1 anti-DNP (ng/ml)
	Cell population[c]	Cells added[b]	
3	—	None	238
3	T cells (anti-MIg depleted)	1	25
3	T cells (anti-MIg depleted)	2	13
3	T cells bound to DNP–BSA	0.4	50
3	T cells bound to BSA	0.4	288
3	Unseparated spleen	1	88
3		2	100
4	—	None	450
5	—	None	625

[a] T. Tokuhisa, M. Tagawa, M. Taniguchi, manuscript in preparation.
[b] Cells cultured ($\times 10^5$).
[c] Spleen cells that failed to bind to anti-MIg coated plates (anti-MIg depleted fraction) were applied to DNP–BSA or BSA coated plates and incubated 30 minutes at 37°C. Plates were then washed, chilled and the bound cells were eluted by gentle pipetting. About 1 percent of the cells in the original spleen-cell suspension were recovered from the DNP–BSA coated plate.

A MODEL FOR A BISTABLE REGULATORY MECHANISM

Several years ago, we introduced a set of hypothetical immunoregulatory circuits whose operation provides bistable, Igh-restricted, epitope-specific regulation for antibody responses (28). This model proposed a system of central (Core) "circuits," each composed of two helper and two suppressor T cells and each providing the basic "on-off" regulation for production of antibodies carrying a particular antibody-combining site coupled to a particular Ig heavy chain (isotype/allotype). By allowing each suppressor cell in the circuit to attack one of the helper cells and be helped (to differentiate and expand) by the other (see Figure 1), we arrived at a set of cell interactions that approximates the behavior of an electronic binary ("flip-flop") circuit in that the circuit tends to stabilize in a help or suppression mode but nevertheless can shift to the opposite configuration in response to a dramatic shift in stimulatory conditions.

The decision as to whether a given Core circuit in the model permits or suppresses production of the antibody it regulates is partially internal to the circuit but also depends on the relative strengths of positive and negative signals transmitted to the Core circuit from a series of functionally distinct auxiliary regulatory circuits that directly sense the antigenic environment. Thus, this model places Ig-specific regulation to the B cell being regulated and establishes a system whereby a variety of positive and negative auxiliary

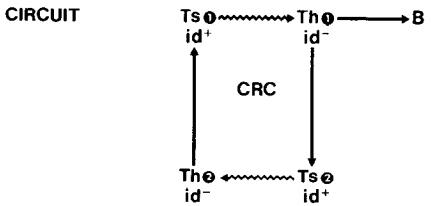

Figure 2 Model for a bistable regulatory circuit. In this theoretical cell-interaction circuit (28), a B cell carrying Id+ VH Ig surface receptors is helped by a T cell, Th1, that has complementary (id–) receptors. Th1 (analogous to an idiotype-specific helper T cell) is depleted by a suppressor T cell, Ts1, (analogous to an idiotype suppressor T cell) which carries id+ receptors similar to the B cell and therefore tends to bind the same haptenic determinant as the B cell. Ts1 is helped by Th2, and Th2 is depleted by Ts2 (which is helped by Th1). Th2 and Ts2 are distinguished from Th1 and Ts1 by non-VH related surface determinants. The configuration of this circuit is such that it will tend to stabilize either with Th1 and Ts2 dominant or Th2 and Ts1 dominant, since either of these pairs will decrease the activity of the other. Thus this circuit will tend to maintain itself either in a help or suppression configuration depending on how it is induced initially by conditions of antigenic stimulation (for further explanation, see 28).

stimulatory signals are integrated (by the Core circuits) to determine whether one or more of the possible antibody populations shall be represented in a response.

In framing the model, we deliberately avoided considering auxiliary circuits containing CTs since we could see no straightforward way of rationalizing the then-current view (that CTs regulate antibody production by depleting carrier-specific help) with the central regulatory system we were proposing. Working now, we would draw a CTs-containing circuit that induces the Core circuits to suppress antibody production (to new epitopes on the carrier). Similarly, concepts of contrasuppression (9) were nascent (or unknown) when this model was drafted but would now be included in auxilary circuits that favor help rather than suppression. Thus, although the model as published "shows its age," the basic principles it embodies are more viable than ever.

We would be pleased to claim that our studies on the epitope-specific system were directly instigated by this "theoretical exercise;" however, serendipity had more to do with the initiation of these studies than rationality. That is, having forgotten the earlier work (1, 2) showing that in situ anti-hapten responses fail following immunization with (what we now call) the "carrier/hapten-carrier" sequence, we set up an allotype suppression experiment in which we hoped to augment anti-hapten antibody production by pre-immunizing with carrier (32). The minimal anti-hapten and normal anti-carrier responses we obtained in control animals intrigued us suffi-

ciently to trigger a further series of experiments. Thus, we inadvertantly reopened an old question and, armed with modern methods for measuring memory B-cell development and expression, wound up some three months later with evidence (11) outlining a previously cryptic (epitope-specific) regulatory system whose properties, we found, were largely predicted by the theoretical regulatory circuits we had proposed earlier.

The coincidence of these properties adds credibility to the proposed model (28) and opens the way for a direct test of some of its more specific predictions (e.g. if the model is correct, epitope-specific regulation as described here should be based on co-ordinated idiotype-specific regulatory interactions that can be dissected by introducing conditions that perturb idiotype representation in anti-epitope antibody responses). However, aside from its value as a guide to future experimentation, this model (correct or incorrect) serves a clear and current purpose. That is, it demonstrates that a workable set of cell interactions can be devised to account for the bistable regulation of antibody responses demonstrated in the epitope-specific system. Thus it de-mystifies this novel regulatory capability and brings it into the realm of possibility for a system that has evolved complex cellular mechanisms to protect the animal against invasion by deleterious agents.

GENERALITY OF EPITOPE-SPECIFIC REGULATION

In essence, the evidence we have presented casts the epitope-specific system as an integrative central mechanism responsible for shaping antibody responses according to the dictates of the regulatory environment when an epitope is first introduced. The status of the carrier-specific regulatory system, as we have shown, plays a key role defining the properties of this environment (11, 15, 16). The status of allotype-specific and I-region defined regulatory mechanisms contribute significantly, apparently by preventing rapid initiation of antibody production and hence allowing CTs activity to predominate (18, 20). Thus, through the agency of this central system, the activities of a variety of independently studied regulatory interactions come together as co-ordinated influences on the antibody responses produced by a given animal (17).

Perusal of the literature suggests that an analogous epitope-specific system centrally regulates cellular immune responses. For example, recent studies demonstrate that the induction of allergic encephalomyelitis (AE) by an encephalitogenic peptide-carrier conjugate can be inhibited (suppressed) by prior immunization with the carrier protein, i.e. by carrier/-hapten-carrier immunization (33). Similarly, the mechanisms regulating delayed-type hypersensitivity (34) show a specificity for epitopes not unlike

the mechanisms described here. Therefore, it is reasonable to suspect that each of the major types of immune responses are controlled by "Core" systems that duplicate the properties of the epitope-specific system controlling antibody responses.

Taking this supposition one step further, the existence of such Core systems would constitute an overall mechanism through which cellular and humoral responses could be co-ordinated, perhaps by direct communication or perhaps by differential responsiveness to common "auxiliary" systems. The complexity inherent in such a mechanism is staggering; however, given the extraordinary versatility of the immune system as it operates even in its earliest evolved form, we (as investigators) will indeed be lucky if immune response regulation proves to be based on such a simplistic view.

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