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VISIONS AND REFLECTIONS

Ten experiments that would make a difference in understanding immune mechanisms

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Abstract Jacques Monod used to say, "Never trust an experiment that is not supported by a good theory." Theory or conceptualization permits us to put order or structure into a vast amount of data in a way that increases understanding. Validly competing theories are most useful when they make testably disprovable predictions. Illustrating the theory–experiment interaction is the goal of this exercise. Stated bleakly, the answers derived from the theory-based experiments described here would impact dramatically on how we understand immune behavior.

Keywords TCR · BCR · Self–nonself discrimination · Autoimmunity · Repertoire · Signaling

Introduction

The immunological community is essentially deriving its experimental program based on a singular set of hypotheses. The purpose of this essay is to highlight the competing set and propose the experiments that would distinguish them.

Hypothesis I Single V-gene segments comprising the $\alpha\beta$ TCR encode recognition of allele-specific determinants expressed on MHC-encoded restricting elements (R). As the TCR docks on the MHC-encoded restricting element (R) in a fixed geometry and as Class II R-elements do not display hybrid or F1 allele-specific determinants [1], each R-element must have the potential to express two allele specific determinants [2]. Therefore, the TCR should be able to signal the cell from either one of two orientations, restrictive recognition via the positively selected V-domain and allorecognition via the entrained V-domain [3–6].

Experiment 1 Isolate as hybridomas, two sets of $CD4^+$ TCRs with identical Vα and Vβ domains from each of two reciprocal allogeneic MLRs, B10.H-2^banti-B10.H-2^s and B10.H-2^santi-B10.H-2^b. As the TCRs from each of the MLRs will have been selected to have identical Vα- and Vβ-domains, one set will be A^b-restricted and A^s-alloreactive, the other set will be A^s-restricted and A^b-alloreactive.

Comment 1 Given fixed docking of the TCR, Vα on RIIβ and Vβ on RIIα, and the absence of hybrid RII alleles, this observation would confirm that single V-gene segments encode recognition of allele-specific determinants and that the TCR can deliver Signal 1 to the cell from either one of two orientations. Sequencing the CDR3 and J-regions would provide the physical basis for the determination of signaling orientation (see Hypothesis IX). A more detailed background can be found in refs [5, 7].

Consider now the competing assumption that VαVβ complementation (analogous to V_LV_H of the BCR) is required to create a unique combining site that recognizes an allele-specific determinant. This implies a unique VαVβ complement for each distinct allele. The alleles of R(MHC) under discussion here are defined by interactions of restrictive and allorecognition with the TCR. The two alleles, A^b and A^s, are distinct when assayed either by restrictive or by allorecognition. Consequently, under this model, which excludes that two distinct alleles be defined by a single VαVβ complement, this assumption would be ruled out. It might be added that the general finding of multiple VαVβ usage by TCRs specific for and restricted to

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a given [P_Y-R^X] ligand supports this conclusion. Multiple VαVβ usage is uniquely consistent with Hypothesis I [6].

Hypothesis II Two models of TCR recognition are described as "Centric" [8] and "Tritope [2]." Under the Centric Model the TCR possesses a single combining site that recognizes a determinant common to R-elements but its alleles are the result of presentation of unique arrays of peptide recognized as such by the TCR. The Tritope Model defines two distinct combining sites on the TCR, one germline-selected and recognitive of the allele-specific determinant, the other somatically derived and recognitive of the peptide (P) presented by R. Interactions at these two sites are integrated by the TCR to signal restrictive reactivity.

Experiment 2 There exists a family of TCR clones isolated from an B6.H-2^b anti-B6.H-2^k MLR that are allorestricted to an allele-specific determinant shared by A^b/E^k [9], discussed in Ref. [2]. These clones should be screened for alloreactivity to a variety of foreign H-2 haplotypes. A most illustrative finding would be an A^b/E^k-allorestricted clone alloreactive to A^k. The existing clones due to the method of selection have a low probability of expressing A^k-alloreactivity. The search for A^b/E^k-allorestricted, A^k-alloreactive clones might best be accomplished by selecting A^k-alloreactive clones and screening them for A^b/E^k-allorestriction using the Felix et al. [9] selection procedure. Those expressing these dual specificities should have their Vα and Vβ gene usage determined.

Another approach to revealing A^b/E^k-allorestricted, A^k-alloreactive clones would be the use of tetramer-binding and FACS sorting. However, in the end, a confirmatory demonstration of functionality would be required.

Comment 2 Under the Centric Model allorestriction to A^b/E^k and alloreactivity to foreign MHC-haplotypes by single clones, is not predicted. Pushing this point to its limit, as this family of TCR clones was derived from B6.H-2^b, both allorestriction to E^k and alloreactivity to A^k by a single TCR is possible [2]. However, such a combination would be deleted in the B6.H-2^k mouse, which is restricted to and tolerant of A^k. Therefore, the VαVβ combination that determines E^k-allorestriction and A^k-alloreactivity used by B6.H-2^b should be absent from B6.H-2^k. Allorestriction and alloreactivity by single clones would rule out the Centric Model as being no longer explicative of allele-specific recognition. Further, such a finding would emphasize that allorestriction should be distinguished from alloreactivity in all analyses of the data.

Hypothesis III Positive selection in thymus extracts from the pool of Vα- and Vβ-domains of TCRs, those that recognize the allele-specific determinants expressed by the host MHC. The family of allele-specific determinants is distinct for Class I(RI)- and Class II(RII)-elements so that during positive selection the effector class is also decided. The TCR has no way of knowing its restriction specificity; only the MHC-encoded restricting element (R) has that information. No signal via the TCR can tell it if it is interacting with an allele-specific determinant on RI or RII. During positive selection, any signal via the TCR to the 4^+8^+ T-cell must be distinct from inactivating Signal 1 in that it is peptide-unspecific. The allele-specific TCR-R interaction can only trigger a default pathway known to be to $CD4^+$ T-helper [10]. The induction of the default pathway includes the expression of the IL-7 receptor. If the TCR-R interaction is with RI then this default $CD4^+$ pathway must be diverted to the $CD8^+$ T-cytotoxic lineage. Therefore, during positive selection, the TCR and the RI must switch roles, in that the TCR acts as a ligand for the RI-element which functions as a receptor [2]. This latter signals the positively selecting medullary thymic epithelial cell (mTEC) to secrete IL-7, the transmitter molecule that diverts the T-cell default pathway from $CD4^+$ helper to a $CD8^+$ cytotoxic phenotype [10].

Experiment 3 Three questions must be answered.

First, is positive selection when initiated by the TCR-RI interaction responsible for a signal transmitted via RI that activates IL-7 transcription in the mTEC?

This is a question answered by revealing a pathway of intracellular signaling from the TCR-RI complex to the initiation of transcription of the IL-7 gene in mTECs. There is a myriad of approaches ranging from the search for associated TCR-induced RI-specific cofactors to the analysis of their post-translational modification by phosphorylation, aceylation, ubiquination, etc. Knockout experiments of defined components in a putative signaling pathway would be confirmatory. Amino acid replacements in the membrane proximal extracellular or cytoplasmic domains of the RI heavy chain that obliterated positive selection of $CD8^+$ T-cells but left the $CD4^+$ default pathway operative and unchanged, would provide formal evidence of an RI-transmitted signal.

Second, is the peptide (P) that is presented by R during positive selection acting as a structural or specificity element?

One general test might be derived from the use of a mouse that expresses a single peptide-Class II MHC ligand, in this case, [P_n-A^b] [11]. If the transgenes for TCR anti-[$P_{H\text{-}Y}$-A^b] are introduced and that TCR is positively selected then it would be safe to conclude that peptide plays a structural, not a specificity role, in positive selection. The outcome should be the same whether one uses males or females, as the self H-Y target would be absent in both cases and any putative H-Y mimotope would be absent in females. If one wishes to argue that the single ligand [P_n-A^b] happened to resemble the [$P_{H\text{-}Y}$-A^b] ligand

for the tgTCR, then the introduction of another tgTCR anti-[P_S-A^b] that undergoes positive selection would make that argument highly unlikely.

Third, how is the target of IL-7 limited to the 4^+8^+ T-cells interacting with RI?

Clearly for this pathway to be viable the IL-7 signal must be limited to the cells being positively selected on RI. Assuming that positive selection occurs on an mTEC that expresses both RI and RII, the TCR-RI interaction (not the TCR-RII interaction) would be expected to form a unique signaling synapse. Within the confines of this synapse, it would be expected that the mTEC would secrete IL-7 of very short half-life and the T-cell would gather its IL-7 receptors. The short half-life implies the presence of an enzyme degrading IL-7. Optical photometry permits an analysis of the surface distribution of the IL-7 receptors. If RI and RII are expressed as functional positively selecting elements on separate and sequestered mTECs, this question would be answered simply by secretion of IL-7 with an appropriate half-life.

Comment 3 Two pathways require two signals. If no signal were delivered via the TCR, then the determination of the two pathways would require an RI- or an RII-specific signal. This appears to have been ruled out [10] leaving a signal (not Signal 1) via the TCR interacting with an allele-specific determinant on RI or RII that initiates a default pathway to $CD4^+$ T-helper. This pathway is diverted by a TCR-RI interaction to the $CD8^+$ T-cytotoxic lineage. On a priori grounds, only specific recognition of the allele-specific determinant is required. In the Tritope framework, the peptide functions as a structural, not as a specificity element in the [PR]-complex involved in positive selection.

Positive selection must depend on allele-specific recognition. There is a general tendency to ignore allele-specific recognition, a term that has all but disappeared from the literature. The above arguments would obtain if TCR recognition were for two determinants, one shared by all RI-elements, the other by all RII-elements. However, this assumption is equivalent to a denial of restrictive recognition of antigen, the phenomenon that we are trying to explain.

Hypothesis IV In order to sort the somatically generated adaptive repertoire by inactivating anti-self (S) cells and activating anti-nonself (NS) cells, two signals are required. Signal 1 resulting from a ligand-TCR/BCR interaction is inactivating. Signal (1 + 2) is activating; Signal 2 is delivered by an effector T-helper that has, itself, undergone a S-NS discrimination so that it is anti-NS. A requirement for Signal 2 activation applies to all naive antigen-responsive cells, including the T-helper (Th) itself. This poses the question, what is the origin of the eTh anti-NS necessary to prime the response?

Two theories have emerged: (1) naive or initial state Th (iTh) are unique in that they undergo a controlled NS-antigen-independent pathway to priming effectors (eTh anti-NS) [12–14], and (2) the B-cell acting as an APC can deliver a priming Signal 2 uniquely to naive iTh (a variation on the Bretscher proposal [15]). The B-cell is acting as a primer eTh uniquely for naive iTh.

Experiment 4 An H-2^b $Rag^{-/-}$ mouse expressing a transgenic A^b-restricted anti-H-Y specific TCR is known to delete that TCR in males (H-Y expressing) and to express it at high level in females (H-Y negative) [16].

Step 1: Immunize a $Rag^{-/-}$ female expressing a transgenic (tg) TCR anti-[$P_{H\text{-}Y}$-A^b] with $Rag^{-/-}$ male non-B-cells. If effector tgT-cells are induced, then B-cells as a unique source of primer Signal 2 would be ruled out and we would be left with the assumption that a priming level of eTh is required to initiate a naive iTh response. If there is no response, immunization with male B-cells would test whether they are a unique Signal 2 source for iTh. No response to male B-cells has several explanations that would require further investigation. In this situation, as H-Y is invisible to B-cells, the BCR is not involved. Consequently, a response would suggest that these male B-cells can function as APC for a T–T interaction (i.e., iTh-B[APC]-eTh) BCR-independently, rather than as the postulated surrogate for primer eTh. Of course functioning as an APC for a T–T interaction would imply an NS-independent pathway of iTh→eTh that primes responsiveness. The same would be implied if H-Y is scavengered from the B-cells and presented by dendritic cells.

Step 2: As cells that leave the thymus are in the naive or i-state, the assay in females for activated and/or effector Th anti-H-Y as a function of age (embryonic to 1 month) would establish or disprove the existence of the NS-antigen-independent pathway.

Comment 4 If primer eTh are required to initiate an adaptive immune response, then the most thorny lacuna in the ARA Model [14, 15] (i.e., the chicken and egg conundrum) would be resolved.

There could well be a difference in presentation of peptide by B-cells between exogenous antigen processed after uptake via the BCR and processed endogenous antigen. The relative presentation on RI versus RII could be dramatically different. It is conceivable that the B-cell receiving Signal 1 by interaction with exogenous antigen would function as an APC for an eTh-iTh interaction before inactivation by apoptosis or activation by Signal 2. The B-cell processing endogenous antigen in the absence of engagement of its BCR might not function as an APC even if that antigen were cross-presented on RI and RII. In the absence of engagement of its BCR, the B-cell is no longer a solution to the problem of the mechanism of ARA.

The answers would come out of the above analysis of the B-cell as an APC.

Hypothesis V There must be a mechanism that links what is to be ridded (the Eliminon [17]) to what is to be induced in response to it. Antigen-receptors, TCR/BCR, recognize epitopes, not Eliminons ("antigens"). The induced biodestructive and ridding effector mechanisms armed by recognitive receptors that see epitopes, operate on Eliminons that are collections of linked epitopes. The sorting of the repertoire (S-NS discrimination) is mediated epitope-by-epitope. The execution of effector activity is mediated Eliminon-by-Eliminon. In order for the response to be specific for the collection of linked epitopes (Eliminon), the family of executive naive i-cells interacting with one set of epitopes on the given Eliminon (Signal 1) must only be activated by regulatory eTh (Signal 2) interacting with a set of epitopes linked on that Eliminon. This referred to as Associative (linked) Recognition of Antigen (ARA). ARA is not only crucial for the sorting of the repertoire (S-NS discrimination) but, to use Bretscher's terminology [18, 19], for a "coherent" and "independent" response to each Eliminon. The question becomes, "How is ARA accomplished?"

The Eliminon is taken up by an antigen-presenting cell (APC), processed to peptides (P_E) that are presented on an MHC-encoded Class II restricting element (RII) as a (P_E- RII)-complex. The first step, the activation of iTh anti-(P_E-RII), requires Signal 2 delivered in ARA between an eTh and an iTh. This requires that the eTh-iTh communicative interaction take place on an APC expressing [P_E-RII]. The popular dendritic cell (DC) acting as an APC is viewed as presenting simultaneously multiple peptides derived from unrelated Eliminons as well as Self components. As it cannot make a S-NS discrimination, "costimulation" is ruled out as being Signal 2. If the third party cell is a DC something must be added to accomplish ARA.

Bretscher who has made major contributions both to the understanding of the S-NS discrimination and to the regulation of effector class has given us a thought-provoking solution [15]. The eTh-iTh interaction occurs only on B-cells that act as APCs. Of course, if the B-cell were the sole APC for an eTh-iTh signaling interaction, then the requirement for ARA would be solved at the level of the helper as the B-cell could be envisaged to present a single antigen, the one taken up as a ligand for the BCR. Unfortunately while B-cells are certainly capable of functioning as APC, it is questionable that they are the sole or even major APC for eTh-iTh interactions. In any case, the behavior of non-B cell APC (dendritic cells) needs detailed analysis with respect to ARA.

Experiment 5 Consider an H-2^b APC presenting peptides from H-Y and from chicken ovalbumin (ova) on Class II R. Two $CD4^+$ T-helper lines are involved: A^b-restricted anti-Pova and A^b-restricted anti-$P_{H\text{-}Y}$.

The TCRs are expressed transgenically in $Rag^{-/-}$ H-2^b female mice.

The naive iTh lines are induced to eTh by immunization and irradiated for experiment.

The various combinations are all analyzed on H-2^b dendritic cells that present both $P_{H\text{-}Y}$ and Pova.

iTh anti-	eTh anti-	Comment (assumes ARA is strict)
1. $P_{H\text{-}Y}$	$P_{H\text{-}Y}$	Response
2. Pova	Pova	Response
3. $P_{H\text{-}Y}$	Pova	No response
4. Pova	$P_{H\text{-}Y}$	No response

If there is a response (lines 3 and 4) then the experiment should be extended to the comparison of the response to two extracellular proteins, for example, tetanus toxoid and ovalbumin. H-Y was chosen because it is a strictly intracellular protein, whereas in this setup ovalbumin would be extracellularly derived, an extreme case to reveal ARA.

Comment 5 If ARA cannot be demonstrated to occur on a professional APC, a whole new look at the question of the mechanism of ARA would be mandated and the role of the B-cell as a sole APC for T–T interactions reexamined (see Experiment 4). The demonstration of T–T interactions in ARA on non-B-cell APC would raise the question of mechanism given that they appear to present concurrently multiple random antigens, self and nonself [13, 17].

Hypothesis VI The transcription factor *Aire* controls the ectopic expression of a family of peripheral self-components in thymus where naive Th anti-self is negatively selected [20]. In *Aire* knockouts a humoral autoimmune attack on some of these peripheral self-components is initiated. Under the ARA model the reason that a subset of peripheral self-antigens is targeted and requires ectopic expression in thymus is due to their delayed expression in the periphery until after the immune system is functional [12, 21].

Experiment 6 Using B-cell hybridomas from $Aire^{-/-}$ autoimmune mice [22] as sources of diagnostic antibody, determine their specific self-targets and when their expression occurs as a function of developmental time. Ideally the study should be extended to the expression of derivative peptides presented on RII that are seen by eTh anti-S because these latter must be induced prior to induction of the B-cell anti-S.

Comment 6 A mature or responsive immune system cannot determine whether a de novo appearing antigen is

self or nonself. Newly appearing antigens are treated by the mature immune system as nonself. Not all peripheral self antigens that are ectopically expressed in thymus need be delayed in expression. To account for *Aire* function only a proportion of (in fact only one) *Aire* regulated self need be delayed. The specificity of *Aire* for its regulatory sequence could be quite promiscuous.

A nomenclature ambiguity might be pointed out. A delayed expression host component referred to as "self" by the immunologist is nonself to the host immune system.

Hypothesis VII The class of humoral response is determined by signals generated by cytopathicity due to host-pathogen interactions. These "trauma" signals are read either by the various classes of Th, which, in turn, instruct the B-cell to switch to a given isotype, or by the activated B-cell itself [23]. If the initiating signals are due to a host-pathogen injury, they should be absent in a response to self.

The term "pathogen" is used generically in this context in that it refers to any noxious element that induces a tissue to call upon an immune system response.

Experiment 7 is divided into two parts.

A. To test whether the B-cell switches to a given isotype consequent to a specific external signal or does it switch randomly to be selected upon subsequently for the optimal isotype? The experiment then is to isolate from adult animals, B-cells of each of the isotypes by FACS sorting or by panning. Using appropriate primers and single cell PCR technology, determine whether the unexpressed chromosome has switched to the same isotype as the expressed chromosome. If both chromosomes switch to the same isotype then a switch signal is implied. If they switch to different isotypes, randomness and subsequent somatic selection is implied. A pioneering experiment carried out for a single isotype showed that both chromosomes switch to the same isotype [24]. However, in the present context, this study establishing feasibility is too limited and a detailed analysis for all isotypes is warranted.

Comment A If it were found that both chromosomes switch to the same isotype in the case of each and every isotype then the conclusion would be, one isotype-one signal. If there were any grouping of isotypes per signal, then the switching of the unexpressed chromosome would be random within the group. Such an analysis would permit a determination of the total number of distinct trauma signals that direct switching. This is what mandates an analysis of the switching pattern to all isotypes of a species.

B. Using the B-cell hybridomas producing antibody specific for given self autoimmune targets, determine the Ig-isotype (see Experiment 6). It is important to distinguish an autoimmune target from an autogenously generated housekeeping target as necrotic waste could well be a source of trauma signals. The anti-self antibodies under analysis here should have cytopathic consequences.

Comment B One very informative result would be that the switch from IgM to Ig-other would be to a fixed or default isotype (e.g., $IgM \rightarrow IgG_1$) independent of the self-target. This would be a prediction of the Trauma Model [23] and would set the stage for experimentally revealing the pathogen-host interaction signals that are postulated to determine the choice of humoral isotype.

If, by contrast, different self-targets induce distinct fixed isotypes, then the Trauma Model would be ruled out and the initiating determinant for choice of isotype would have to be a structural or metabolic property of the self-antigen that is shared with pathogens. This decision must depend on the grouping of targets, (independent of whether they are self or nonself) with isotypes based on recognition of a singular property or determinant shared by each group. As this does not appear to be even remotely likely, let's hope that the Trauma Model obtains before we run out of reasonable hypotheses.

Hypothesis VIII The initiation of an immune response requires uptake of the antigen by an APC and the processing of it to peptide bound to MHC-encoded Class II restricting elements (RII). The [P-RII] complex is the ligand for the induction of eTh. A proportion of the pathogenic universe is recognized for processing by the innate system. However, there is a crucial portion of the pathogenic universe to which the innate system is blind. Among these invisibles are monomeric proteins, exemplified by toxins produced by most bacterial pathogens. In order to respond to them, they must be processed and presented on RII by APC for induction of eTh. Monomeric proteins are, in general, non-immunogenic unless injected with adjuvants or as aggregates. This is probably due to ineffective uptake by APC, as $[Ag\text{-}Ab]_n$ complexes of them are quite immunogenic. If, then, a response to a monomer requires interaction with antibody such that the antibody to it be aggregated, then we have a second primer problem, namely what is the source of the antibody necessary to initiate uptake for processing of monomers.

The hypothesis is that the IgM/IgD B-cell, after it has undergone a S-NS discrimination, secretes antigen-independently primer anti-NS antibody, the level of which is controlled to be sufficiently effective in permitting a response to be initiated.

Experiment 8 In the absence of the chosen monomeric cognate antigens, X and Y, using two transgenic B-cell lines in $Rag^{-/-}$, one IgM anti-X, the other IgD anti-Y, assay as a function of age the serum expression of IgM anti-X or IgD anti-Y.

Comment 8 The priming antibody pool must express a sufficiently large anti-NS repertoire and be at a sufficient concentration to form an aggregate with a bacterial toxin that is lethal at nanogram concentrations. This priming antibody will have no mutations; its only sources of variation would be V_LV_H complementation and CDR3. This raises key questions that will be cited but discussion of them is an aside here. In order for a monomer to aggregate Ig, it must be seen in three or more ways. The virgin repertoire must be large enough after subtraction of anti-S to ensure this (see discussion of polyspecificity, Hypothesis X). Further, the antibody must be at a concentration and affinity high enough to bind effectively. Lastly, the APC must have Fc receptors that see the isotypes of monomeric (as well as pentameric) IgM and/or IgD. This may be a chance to reveal a justifiable role for monomeric IgM and IgD in serum.

One reviewer of this paper, Colin Anderson, made the argument that, while monomeric toxins might not be immunogenic when secreted in the absence of primer antibody, the toxin when associated with the pathogen might be quite immunogenic. In this case the rationale for searching for primer antibody would be considerably weakened. This is certainly true and I encourage him to design an experiment to test it.

Hypothesis IX The D-gene segment of Ig is incorporated into the H-chain in all three reading frames (RF) but appears in antigen-selected cells in a preferred frame. This behavior of D_HRF in Ig is to be contrasted with D_βRF in the TCR where it is expressed in all three reading frames (RF). The question then becomes what is the functional role of a germline-selected framework D-sequence sequestered between somatically-derived N-sequences? One assumption is that it regulates the transmission of Signal 1. In the B-cell the non-signaling D_HRFs would contribute to haplotype exclusion; in the T-cell the D_βRF would determine the signaling orientation of the interaction with ligand, restrictive or alloreactive (see Hypothesis I) [25].

Experiment 9 The sequencing of CDR3β in the family of TCRs isolated in Experiment 1 where the orientation is known, would determine what the relationship is between D_βRF and orientation of signaling. If no relationship is observed, then sequencing CDR3α might reveal the element determining orientation, but the role of D_βRF would be left dangling.

In the case of the murine B-cell, using single cell PCR sequencing compare the distributions of D_HRF expressed in:

1. IgM versus IgG/A B-cells
2. IgM B-cells of athymic or $RII^{-/-}$ mutants versus wildtype

A significant increase in the proportion of cells expressing the preferred RF in IgG/A over IgM or in wildtype over T-helper defective would establish selection by antigen and imply a role in signaling.

Referring back to *Experiment* 7A the distribution of D_HRFs of the expressed chromosomes compared to that of the unexpressed chromosomes should be determined. A finding that there is a preferred D_HRF in the expressed chromosome and a random D_HRF in the unexpressed chromosome would demonstrate that antigenic selection is the determining factor for the expression of a preferred D_HRF.

Comment 9 In spite of the distinctly different properties of D_H and Dβ, two irresistible assumptions drive the thinking leading to the above experiments.

1. The D-gene segment encodes a similar selectable role in the BCR and the TCR.
2. D has been maintained as a separate gene-segment over evolutionary history because waste of 2/3 of cells unable to receive Signal 1 is an essential step in haplotype exclusion at the BCR H-locus [26] and, in the case of the TCR, because determination of the signaling orientation is a key step in sorting out a functionally restricted T-cell population [2].

An effect on antigenic selection by the preferred DRF could be determined either by the control of signal transmission or by physically allowing the CDR3 loop to engage in a recognitive interaction with an epitope. The demonstration that the DRF controls signal transmission consequent to interaction of the TCR/BCR with ligand, would be decisive evidence that the signal is driven by a conformational change. Distinguishing these two explanations would be a next step.

Hypothesis X The antigen-receptor can look at the universe of chemically distinguishable ligands in one of two ways:

1. Antigen-receptors can recognize as signaling a shape common to subsets, the members of which are referred to as "mimotopes." This concept is derived from the lock and key image. The crucial aspect of this view is that each family of mimotopes is either self (S) or nonself (NS) and the antigen-receptor that recognizes a family is either anti-self or anti-nonself. Negative

selection purging the anti-S repertoire has no effect on the specificity of the anti-NS repertoire.

2. Antigen-receptors bind a set of ligands of a distributed size *n* that are random with respect to the property, self or nonself. The antigen-receptors are referred to as "polyspecific" and the members of a set are referred to here as "clantopes." The purging of anti-S receptors by negative selection results in an anti-NS residue of greater specificity (i.e., receptors that are less promiscuous, lower values of *n*).

The mimotope hypothesis [27, 28] has been ruled out for the TCR [14, 29–31] but remains open for the BCR. Induction of a B-cell requires specific uptake and processing of antigen to nonself [PRII], the ligand for eTh. Whether the BCR anti-S sees a mimotope or a clantope is of little import, as the NS-peptide seen by the eTh can be derived from a part of the antigen carried along as an innocent bystander. Given a B-cell anti-S, only antigens that express an S-epitope and can be processed to [P_{NS}-RII] are candidates for breaking B-cell tolerance. Tolerance must be broken at the level of the T-helper in order to establish chronic autoimmunity.

The tools to decide between the two models of polyspecificity are not available for the BCR. However, the two models may lead to different outcomes when considering the origin of autoimmunity. There is a steady state population of T and B cells anti-S on the pathway to inactivation. The time that it takes between receipt of Signal 1 and irreversibility must be carefully controlled. As the delivery of Signal 2 by an eTh involves the slow process of cell–cell interactions, if the half-life of reversibility by Signal 2 of inactivation by Signal 1 were too short, no cell could be activated. If it were too long, the frequency of autoimmunity would be too high. Consequently the half-life of reversibility must be regulated such that Signal 2-driven activation is adequate and the frequency of autoimmunity acceptably low by evolutionary standards. The steady state level of anti-S cells on the pathway to inactivation is referred to as the autoimmune boundary [26].

Experiment 10 There are no direct labtop experiments that, at the moment, can be used to characterize the parameters of the autoimmune boundary. Therefore, for this exercise, *in silico* experimentation or modeling is introduced. A great deal is known about the input to the autoimmune boundary and about the output (frequency of autoimmunity). Further, the breaking of tolerance under a mimotope or a clantope model has predictable differences. Experiment 10, then, is the exploration of the autoimmune boundary of the naive iTh and B-cell over a wide range of parameters. Can reasonable parameters for a stable boundary be revealed without the need to invoke feedback suppression or will the latter prove to be obligatory?

Comment 10 Polyspecificity of the T-helper implies that an anti-S cell on the pathway to inactivation can, in principle, be activated by an ongoing response to an NS-antigen that the polyspecific receptor also recognizes. As the individual is under a steady state immunogenic load and has a steady state population of polyspecific Th anti-S cells on the pathway to inactivation, why is autoimmunity infrequent? The likely answer is that for a polyspecific receptor, the self-ligand can compete with the nonself-ligand to prevent the breaking of tolerance even though the two epitopes are structurally unrelated. This proposal is a special case of allostery. The introduction of T-suppressors (Treg) to regulate the autoimmune boundary must explain how a population of Treg-cells sorted to be anti-NS (like all other i-cells) can turn off polyspecific anti-S cells, yet allow polyspecific anti-NS cells to respond [32, 33]. In the absence of an explanatory model, the assumption of Treg regulation of the autoimmune boundary becomes gratuitous.

The reader will be surprised that a computer modeling experiment has been introduced as a heuristic exercise. It is important to stress that computers are tools to conveniently explore the consequences inherent in a theoretical construct. The power of modeling is largely ignored by the wet experimentalist and the elegant predictive and descriptive modeling studies of the immune system all too often are buried in computer-oriented journals. As this is rapidly changing, describing the need for exploration of the autoimmune boundary by a modeling experiment is appropriate.

Visions and Reflections

Vision

This essay would increase in value if it encouraged the readers to pose competing sets of experiments that would have a more significant input into our understanding of immune responsiveness.

Reflection

Given the present day rush of granting agencies, foundations, universities and research institutions to market themselves by claiming breakthroughs into translational research and development, basic or fundamental investigations designed to understand are receiving ever dwindling support. Translational research requires a knowledge-base from which to translate and this is disciplined curiosity-driven investigation. Illustrating this was an element in writing this essay. The hope was to encourage a return to thinking about basics by putting a greater emphasis on

hypothesis-driven research. In the light of the present day emphasis on salesmanship and impact factors, the experiments proposed here have a higher probability of getting you a Nobel prize than research support to study fundamentals, Alas!

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