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LETTERS AND COMMENTS

Comment on: Ten experiments that would make a difference in understanding immune mechanisms

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Abstract Melvin Cohn provides a list of experiments to test predictions of his associative antigen recognition (AAR) and Tritope models in relation to standard models of immunity. The manuscript highlights important questions and some decisive experiments testing the competing models. A central aspect of good science is the ability to pinpoint the important questions, something Cohn has, and continues here, to do with great clarity. The problems posed are presented succinctly, although knowledge of Cohn's previous theoretical contributions are needed in some areas to fully understand the path he is taking here. The importance of theory, as championed by Cohn, is a message that needs repeating, as it seems increasingly that immunologists favor description over theory. I briefly comment on each of Cohn's ten experiments and then discuss in detail a critical experiment that is missing from his list—an experiment testing the timing of antigen encounter in ontogeny as a central principle of the self non-self discrimination.

Hypothesis/Experiment/Comment I

It is not clear why the ability of the same Vα and Vβ to recognize two different alleles of MHC (b and s) would necessarily lead to the conclusion that "single V-gene segments encode recognition of allele-specific determinants…from either one of two orientations". This observation would not distinguish Cohn's Tritope model and the standard models, both models would predict the existence of such T cells. Perhaps what Cohn means to say here is that his model would predict that the CDR3 sequencing will show that all clones having the same Vα and Vβ will recognize A^b in a single orientation and A^s in the opposite orientation, while the standard model would predict a mixture. The question is not whether individual V segments, Vα and Vβ, or instead the combination of VαVβ, see the allele specific determinant, but instead whether the TCR has to see the allele-specific determinants directly at all. The recognition of two different MHC alleles could occur based on general MHC recognition [1], and therefore the proposed experiment does not allow one to conclude it is allele-specific recognition, rather than generic MHC recognition in the two different orientations. The most important competing hypothesis is not that the TCR acts like the BCR, but that the Vα and Vβ domains individually recognize generic aspects (not allele specific) of MHC. The proposed experiment and sequencing would not distinguish these competing models.

A test that could directly distinguish Tritope from the competing models would examine if a high proportion (approximately 50%) of T cells that recognize their cognate peptide in I-E are able to recognize the peptide independent of the allele of I-E presenting the peptide. Unlike the other MHC chains, I-Eα, and HLA-DRα are essentially monomorphic. Based on the rules of the Tritope model, 50% of T cells positively selected by DR or I-E (i.e., those positively selected by Vβ binding to DRα or I-Eα) would recognize antigen in a fashion that is not restricted to a specific allele of this class-II. Although the class-IIβ is polymorphic, for

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T cells positively selected on the monomorphic class-IIα, any allele should work if Tritope is correct. Thus, Tritope predicts that half of the T cells recognizing antigen in DR and I-E will violate the standard rules of MHC restriction. The competing standard model predicts instead that all T cells, even those recognizing antigen in DR or I-E, will do so in an allele-specific fashion.

Hypothesis/Experiment/Comment 2

The presence of a significant frequency of single TCRs that are both allorestricted and alloreactive to different H-2 haplotypes may indeed be a unique prediction of the Tritope model and the experiments proposed could be very informative.

Hypothesis/Experiment/Comment 3

The experiments proposed to test signaling via MHC class-I in driving CD8 T cell-positive selection, and those to test whether peptide serves a structural role during positive selection are clear and should be informative. Cohn raises the concern that allele-specific recognition, as determined by the thymus, is often ignored. Given Cohn's assertion that positive selection must depend on allele-specific recognition, it would be of interest to see how he explains the success of HLA mismatched thymus transplantation in the restoration of immunocompetence of Di George syndrome patients.

Hypothesis/Experiment/Comment 4

The experiments proposed here are useful in testing the mechanism of induction of 'the first' helper cells, the primer problem. However, it is not clear why the prediction from Cohn's ARA model would not be that all naive Th would eventually become eTh. Such an outcome would already have been observed in various TCR transgenics via upregulation of activation markers etc., over time, such that all T cells in the TCR transgenic appear as eTh or memory cells. Since this has not been observed, one would have to postulate that spontaneous generation of eTh is followed by a return to a state that appears naive phenotypically or that only a small fraction of naive T cells have the capacity to spontaneously become eTh. The rationale for these postulates, needed to explain why massive spontaneous production of eTh hasn't been observed in TCR transgenics, is not yet clear. We (and possibly others) have done experiments similar to those suggested by Cohn, that address a potential need for B cells in turning on a CD4 anti-H-Y T cell response. We grafted naive monoclonal ($Rag^{-/-}$) Marilyn TCR transgenic female mice with $Rag^{-/-}$ male islets. Male islet grafts were specifically rejected, despite the complete absence of B cells in the system [2].

Hypothesis/Experiment/Comment 5

In this section, Cohn suggests the possibility that DCs as APCs may not be sufficiently specific in their presentation of antigen to suffice for ARA, whereas B cells, due to the specificity of BCR uptake of antigen, would be. It is unclear whether Cohn himself thinks DC presentation of antigen is sufficiently specific for ARA. Although B cells, via their BCR, could increase the 'linked' nature of helper and helpee epitopes, they would nevertheless also co-present self antigens, just as DCs do. That the 'professional' at linked antigen presentation, the B cells, are in fact not needed to turn on a CD4 T cell response (DCs, the 'amateurs' of linked presentation seem sufficient), argues against linked recognition as the basis for turning on an immune response. Instead, the linked presentation capacity of B cells makes them ideally situated to control the regulation of class. From this viewpoint, the absence of B cells would not reduce the ability to start up an immune response but instead the ability to generate a coherent immune response in terms of the class generated.

Hypothesis/Experiment/Comment 6

The proposed studies would be a useful test of the ARA model's explanation of AIRE function. The postulate regarding AIRE function also relates to an ARA principle that 'prior and persistent' antigens are treated as self, a principle discussed more fully at the end.

Hypothesis/Experiment/Comment 7

A creative and interesting solution to the initiation of class control is presented, and the experiments suggested would represent an important initial test.

Hypothesis/Experiment/Comment 8

Whether primer antibody is required for generation of responses to monomeric toxins is an important question. The alternative possibility, that such monomers are presented as multimers and hence immunogenic when in the context of the infectious agent, even without primer antibody, is testable. One such test would be to use the

suggested transgenic anti-toxin B cell $Rag^{-/-}$ mice, with the addition that they would be engineered to be incapable of antibody secretion, only surface Ig would be present. B cell activation could be examined, by various means, in the context of infection.

Hypothesis/Experiment/Comment 9

Important unanswered questions regarding the function of the D region of BCR and TCR are discussed along with a logical set of predictions and tests of potential function in signaling. These experiments could contribute to resolving whether BCR/TCR signal via conformational change or only via aggregation.

Hypothesis/Experiment/Comment 10

Cohn postulates here that the 'autoimmune boundary' is governed by the time available to receive T cell help, before inactivation becomes irreversible. This is favored over the hypothesis that Treg control the autoimmune boundary. A potential source of confusion here is in Cohn's assumption that Treg must be anti-non-self. Many in the field would consider they are predominantly anti-Self, and therefore find the argument presented misleading. Nevertheless, even if Treg were selected for self-recognition, the problem Cohn raises (how do anti-non-self escape suppression while anti-self do not?) would still need to be addressed. The competing postulates that the level of danger-induced co-stimulatory signals, or the balance between co-stimulators and co-inhibitors, regulate the autoimmune boundary, are not addressed.

On 'prior and persistent antigen'

It is surprising that Cohn does not propose a test of one of the most obvious predictions of his model, the prediction that an antigen present sufficiently early, before immune system development, should induce tolerance. If such an antigen does not universally induce tolerance, the ARA model is disproved. Many experiments have tested this prediction and would seem to have disproved ARA. Cohn must see faults in the design of all these experiments, and therefore he should provide the experimental design that would satisfy him in terms of the ability to disprove his model. He has previously provided explanations [3–5] for several experiments where prior and persistent antigens generated immunity rather than tolerance (tolerance is the [failed] prediction for these experiments, based on ARA). Cohn's view would be much more appealing if he could provide a single unifying explanation for all these failed tests of ARA. Instead, the previous explanations he refers to require multiple different improbable explanations for each failure, leaving the appearance that these are desperate attempts to save a model that has been disproved.

Referring back to some of Cohn's previously published explanations, the postulate that quail limb buds grafted into a chicken embryo expresses peripheral antigen in a delayed fashion fails to explain why allogeneic limb buds also suffer a rejection response (i.e., immunity is not a consequence of the graft being of xenogeneic origin). That thymus grafting was able to induce tolerance of limb buds, in the experiments of Le Douarin's group [6], simply indicates that central tolerance is a necessity, and cannot be construed as uniquely supporting the ARA model. Similarly, the ability of a male skin graft to be rejected by a female $Rag^{-/-}$ mouse post-immune reconstitution with female hematopoietic stem cells [7], despite the simultaneous successful establishment of self tolerance, cannot be explained by Cohn's postulate that there was insufficient male antigen presented in these experiments. He predicted that if the graft were to be larger, it would induce tolerance. As I pointed out in a previous publication [8], we increased the male antigen-expressing graft by giving both a male skin graft and a male heart graft prior to development of immunocompetence in the recipient, and although the male heart graft alone could induce tolerance, the male skin graft was nevertheless rejected (Anderson and Matzinger, unpublished data). Thus, there was sufficient antigen for tolerance and yet immunity to a prior and persistent antigen was induced.

There is, in contrast, a unifying explanation for almost all of the variety of outcomes of antigen given prior to immunocompetence (e.g., all the studies of transplants given prior to recipient immune system development), where about half of the experiments show tolerance and half show immunity. The defining principle that explains each outcome is central tolerance. Those experiments in which the graft antigens make it to the thymus/bone marrow typically induce tolerance, those where the donor antigens/cells are localized in the periphery instead induce immunity, with few exceptions. This is by far a much simpler explanation for the 'graft prior to immunocompetence' experiments. Parsimony would not favor Cohn's alternative solutions. Certainly if one is to build a model of immunity based on a synthesis of existing data, one would not start with a central principle being developmental time of the organism, a proposal that fails the experimental tests about 50% of the time. Evolution would not favor such an unreliable mechanism. Of all the predictions of Cohn's ARA model, tolerance of prior and persistent antigen is perhaps the simplest to test, and the most central to the validity of his model. It is one of the elegant aspects of Cohn's model that it is in fact so readily testable and

amenable to disproof. Perhaps an alternative a priori argument would be more convincing. It is reasonable to suggest that, a priori, developmental time of the organism cannot be the sole basis for a self-non-self discrimination, precisely because of problems Cohn raises such as antigen dose. If the level of antigen presentation needed to generate immunity is lower than that required to establish tolerance (one of Cohn's proposed explanations for rejection of pre-immunocompetence transplants), then many self antigens present below the cut-off for tolerance would trigger autoimmunity. Clearly, an additional mechanism would be needed to generate tolerance for these antigens if we are to explain why the frequency of autoimmune disease is so low. Cohn often argues there can be no physical or chemical property that distinguishes self from foreign antigens, and yet his antigen dose explanation for the rejection of a pre-immunocompetence graft is precisely an argument using a physical/chemical property to make the distinction between self and the graft antigen.

I do fully agree with Cohn that to understand immunity/tolerance in a broad sense, it is fundamental that we define the validity of the principle that developmental time determines the somatic selection. That at least a few immunologists still debate this concept is important if we are to move beyond description and generate a comprehensive evolution-based understanding of immunity. Lastly, I would support Cohn's reasoning that one should be suspect of the interpretation of the many tests that seem to disprove ARA if there were no other validly competing models (I appreciate the power of a good theory). However, there are validly competing models in this reviewer's opinion, a subject discussed thoroughly in previous debates [9]. Cohn has dismissed other theories based on their utilization of germline mechanisms to control the sorting of the repertoire, a misleading argument. The sorting of the repertoire in ARA is no less governed by germline mechanisms (e.g., a germline determined time for the spontaneous generation of effector helpers) than the competing models (e.g., detection of danger).

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