Cell. Mol. Life Sci. (2012) 69:417–422
DOI 10.1007/s00018-011-0890-4

Cellular and Molecular Life Sciences

LETTERS AND COMMENTS

Reply to Colin Anderson's commentary on "Ten experiments that would make a difference in understanding immune mechanisms"

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Received: 10 November 2011 / Revised: 14 November 2011 / Accepted: 15 November 2011 / Published online: 7 December 2011

Colin Anderson correctly challenges the assumptions that I feel are crucial to understanding immune responsiveness. In order to address his concerns, I will follow the format of his analysis.

Hypothesis/Experiment/Comment I

Two points were made by him:

1. The statement that "single V-gene segments encode recognition of allele-specific determinants……from either of two orientations" is not a necessary conclusion from the experiment.
2. The assumption that the recognition of R (MHC) by the V-domains of the TCR is allele-specific is questionable.

These two challenges can be dealt with together. A formal reply is surprisingly complex because the immunologist defines an allele by one set of criteria (e.g., sequence differences, chromosomal location, association with a distinct MHC-haplotype) whereas the TCR defines an allele by another criterion, namely, the germline-selected recognition by a V-domain of an epitope on R.

Anderson's comment makes me realize that the classical term, allele-specific determinant, used since the earliest studies on alloreactivity and restrictive recognition of peptide, has become ambiguous. Consequently, I will use the term "restriction determinant/epitope".

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Using the murine MHC as example, if two Rs encoded in the same locus, A^b and A^s, are distinguished by the TCR either by restrictive recognition of peptide or by alloreactivity, then by definition the epitope on each R being recognized is an allele-specific determinant. If now the given TCR also restrictively recognizes peptides presented by two Rs encoded at distinct loci, for example, A^b and E^k, the epitope on R being recognized is still the same "allele-specific determinant" but now the terminology is confusing as alleles are not involved. A mimotope shared by A^b and E^k is the restricting epitope. Interestingly enough, as an aside, this defines Tritope. One V plus the anti-P site of the TCR sees P-A^b/E^k (allorestriction); the other V sees A^s (alloreactivity). Allorestriction requires that the anti-P site be engaged; alloreactivity is postulated to be anti-P independent (i.e., P-unspecific). As the MHC and the TCR loci evolved interactively by gene duplication and divergence, it is to be expected that situations will arise in which mimotopes shared by duplicated, diverged Rs will be seen by the TCR resulting in a phenomenon referred to as allorestriction [1] that involves allelic and non-allelic Rs.

Anderson asks us to consider the case in which a subunit of class II R, namely Eα, which is essentially monomorphic, is recognized by a given Vβ-expressing TCR. Being monomorphic, or as a geneticist might say, being monoallelic, means that it has no alleles from the point of view of the TCR. The Eαs encoded in different MHC haplotypes could well be sequence-distinguishable; in fact, this is known, $E\alpha^b$ (defective) versus $E\alpha^k$ (functional). The given Vβ recognizes a restriction epitope on Eα when expressed as R(EαEβ) and the TCR(Vβ) recognizing it will function either via restrictive recognition of P-EαEβ or via alloreactivity to Eα, P-unspecific (see discussion of Table 2 in Ref [1]). Being monoallelic/monomorphic does not challenge the Tritope model; at

best, it questions the semantics of the term "allele-specific determinant".

Could the TCR function by recognizing an invariant epitope shared by all R-elements? The answer is clearly, Yes. So the question is, why doesn't it? Why is R, in general, highly polymorphic when assayed by the TCR? What was the selection pressure that resulted in a highly polymorphic MHC and a highly polygenic TCR. This is not the place to deal with this question (see Ref. [2]) except to stress that the TCR is preoccupied with determinants most often unique to distinguishable Rs, which, in turn, are defined as allelic or nonallelic by independent criteria like sequence or chromosomal location.

Evolutionary selection on the specificity of the TCR-R interaction has resulted in a family of gene loci in the MHC that encode restriction epitopes which vary between the most polymorphic known to monomorphic. The restriction epitope cannot meaningfully be described as being a "generic aspect of MHC". One might ask, why does the individual need roughly 100 V-gene segments to recognize a "generic" determinant on R? Once the MHC and the TCR-loci were established by interactive selection in a primordial animal, it became very difficult for evolution to alter the recognitive relationships during speciation. As a result, we observe the phenomenon of xenoreactivity in which murine TCRs can distinguish human MHC-encoded R-alleles (restriction determinants) (see discussion in Ref. [2]). Ignored in this discussion, is a postulated essentially invariant recognition of R by the TCR (the i-site discussed in Refs. [2–4]; also see below).

In order to address Anderson's Point 1, two facts are background:

1. Vα and Vβ dock on R in a fixed orientation. By way of illustration using class II R, Vα docks on Aβ and Vβ docks on Aα.
2. The two subunits of A sort independently (see Table 1 in Ref. [1]); no F1 hybrid determinants recognized by the TCR exist.

Therefore, each R has the potential to express two restriction epitopes recognized by the germline-selected V-domains of the TCR for signaling function but, of course, a given R may or need not express both. Referring back to the i-site, if Vα docks on the restriction epitope expressed by one domain of R, on what determinant of the opposite domain does Vβ dock? In the Tritope framework, this was postulated to be an essentially invariant determinant (i-site) present on all Rs in addition to the restriction epitope. Given this conjecture, the interpretation of structural studies in terms of epitopes being recognized will require a clear understanding of the functional relationships.

Under the Centric model, P and R interact to form a melded epitope that the TCR, like the BCR, sees as an available piece of R contiguous with P. Therefore all interactions of the TCR (in particular, positive selection and alloreactivity) are P-specific.

Under the Tritope model, P and R are recognized independently by an anti-P site and an anti-R site on the TCR. Positive selection and alloreactivity are anti-P independent or P-unspecific; for these functions, P is only acting as a structural element required for the stability and conformation of R.

Now consider my proposed experiment. The two R-elements, A^b and A^s, are encoded in the same chromosomal locus and are distinguished, under Tritope postulates, by the TCR recognition of two restriction epitopes, in this case two allele-specific epitopes. Under Centric postulates, the TCR sees a mimotope common to P_1-A^b and P_2-A^s. The TCR cannot distinguish them as alleles. To do that requires independent information, like sequence differences in Rs or, as has been proposed, recognition of distinct Ps presented by the two R-alleles [5]. As a definition of an allele, this latter needs rationalization because what the R-element sees when anchoring P and what the TCR sees as a P-epitope are unrelated. The TCR-P interaction cannot determine the alleles of R. Only the P–R interaction can do that.

If a given V can function in two orientations, both allele-specific, one requiring engagement of anti-P, the other anti-P independent, then the Centric model would be in jeopardy. So let me acknowledge Anderson's criticism and add a distinguishing experiment to Hypothesis I. Isolate a family of TCRs that are specific for $P_{H\text{-}Y}$-A^b and screen them for alloreactivity to A^s using females (absence of H-Y or its mimotope as a functional self-ligand). If such lines are found then the Centric model would be ruled out because a peptide-specific interaction is deemed obligatory for alloreactivity. The reciprocal experiment, if doable, would be confirmatory.

Hypothesis/Experiment/Comment 2

No reply needed.

Hypothesis/Experiment/Comment 3

As a challenge to the assumption of allele-specific or restrictive recognition of R, Anderson asks me to explain, why does transplantation of a mismatched thymus restore immunocompetence to Di George patients?

The key here is how the mismatch between the transplanted thymus and the host HLA was assayed and interpreted. If immunocompetence is restored, then clearly some measure of class II MHC sharing of restriction

epitopes must exist between the host and the thymic transplant. I have not examined the data but if they pose a mystery, I would have to argue that the assay of the mismatch missed the shared Class II restriction epitopes on R (i.e., allorestriction).

Hypothesis/Experiment/Comment 4

Given an antigen-independent pathway to primer eTh, Anderson asks, why would not all naive or initial state Th (iTh) be converted to effector Th (eTh)?

The steady-state level of primer eTh is determined by the rates of production versus turnover, which must be tightly regulated. We have published the details of this model with an accompanying computer program to analyze its parameters [6, 7]. If the cognate antigen is not present, the ARA requirement for an eTh–iTh interaction would be absent. Massive spontaneous production of eTh is clearly not indicated under our model in the absence of antigen.

Hypothesis/Experiment/Comment 5

This section deals with the question, "How is ARA accomplished?"

I have tried to make it clear that I doubt that B-cells are the sole or even major APCs for eTh–iTh interactions (see also Ref. [8]). The experiment of Chan et al. cited by Anderson is but one example that rules out B-cells as sole APCs for T–T interactions and requires that we face the dendritic cell as the platform for such interactions. To this end, I have proposed the signaling patch model to preserve ARA [8, 9].

As the B-cell acting as an APC does not solve the problem, I proposed the most elementary of experiments to explore whether or not the professional APC or dendritic cell (DC) has a mechanism that permits an eTh–iTh interaction in ARA. I suspect that they do have such a mechanism and the suggestion of a signaling patch is one possible solution.

Anderson, if I understand his comment correctly, suggests that the DC (nonassociatively by co-stimulation) can initiate a T-cell response (i.e., activate the iT-cell). By contrast, he suggests that, for the B-cell, activation be ARA-dependent because it permits "a coherent response with respect to class generated". This dichotomy is unsatisfactory because, in principle, coherence and independence are also required for the determination of T-cell effector responses.

So let me reformulate the dichotomy. We agree that ARA is required for a coherent and independent response to different antigens. However, Anderson is making an important distinction between activation, which for the T-cell is ARA-independent, and class-determination (for T or B), which is ARA-dependent. For the activation of the B-cell by eTh delivery of Signal 2, ARA is established by the BCR-mediated uptake of NS-antigen for processing. This raises two crucial questions involving the T-cell uniquely:

1. To what degree must Signal 2 be antigen-specific (i.e., dependent on ARA)?
2. Why consider activation, as well as class determination, to be ARA-dependent?

The antigen-receptor, TCR/BCR, has no way of knowing whether it is interacting with a self (S) or nonself (NS) epitope. It must be told. That is the role of Signal 2 delivered by an eTh anti-NS in ARA. Negative selection is mediated epitope-by-epitope, whereas activation is mediated antigen-by-antigen. To argue, as does Anderson, that an antigen-unspecific co-stimulation signal is activating for T-cells receiving Signal 1 is equivalent to assuming that Signal 1 alone is activating. Such an assumption could only obtain if the repertoire were prior sorted to be uniquely anti-NS (i.e., the total absence of anti-S). This would require that Signal 1 be inactivating in a selected space (e.g., thymus or bone marrow) where, or during a developmental time window when, the immune system arises in the presence and persistence of all self. Upon leaving the selected space or after the developmental time window closes, Signal 1 becomes activating. This would be the updating of the Lederberg model. Implied is that peripheral negative selection does not exist, for if it did, Signal 1 would have to be always inactivating.

The proposed experiment assumes the existence of peripheral negative selection (i.e., an inactivating Signal 1) and therefore requires an eTh-iT interaction platform (e.g., the DC) permitting ARA (see further discussion, *Hypothesis 10*).

Hypotheses/Experiments/Comments 6, 7, 8, 9

No reply needed.

Hypothesis/Experiment/Comment 10

Anderson correctly challenges my conclusion that the autoimmune boundary is regulated by kinetic parameters and not by Tregs. He feels that my assumption that the Treg repertoire is sorted to be anti-NS, would be considered "misleading" by most immunologists because they believe that Tregs (i.e., T-suppressors) are "predominantly anti-self". This is equivalent to proposing that the Treg repertoire

is unsorted because, as a matter of fact, there exists many examples of suppressive regulation of nonself antigens. An unsorted suppressive repertoire cannot contribute to a S–NS discrimination. I have dealt with this problem in detail elsewhere [10, 11]. My failure to address "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" is due to my lack of an understanding as to how antigen-unspecific messengers could regulate a discrimination between self and nonself, which *per force* is antigen-specific. A detailed proposal would be welcome as it could be computer modeled to explore its parameters.

The last point of Anderson is outside of the subject of my paper, as he, himself, points out. However, he feels that I should face this gap as it underpins much of the thinking used by me. It deals with what is loosely described as the "developmental time model" for the establishment of "tolerance". I have debated aspects of this question with Jacques Miller [12, 13] but Anderson adds a new perspective. He takes the view that the time model is disproven by the many experiments that fail to establish unresponsiveness consequent to exposure to antigen sufficiently early. Of course, he peripheralizes the equal number of experiments that succeed [14] by asking me to "provide a single unifying explanation for all of these failed experiments". This is an irresistible challenge that has cost me many sleepless nights for which he will never be forgiven. To do this requires a bleak statement of my reasoning.

The S–NS discrimination is a poetic to describe the mechanism by which a somatically generated, random recognitive repertoire is sorted into those specificities (anti-S), which if expressed would debilitate the host, and those specificities (anti-NS), which if not expressed would result in the death of the host by infection. The sorting of the repertoire requires that the anti-S be purged leaving the residue to function as anti-NS. A recognitive receptor interacting with its ligand has no way of knowing if the ligand is S or NS. Therefore, under all models, a S–NS discrimination is an individual-specific (i.e., somatic) process that requires the somatic sorting of the antigenic universe into S and NS. The first question is how is this accomplished? As what is S for one individual of a species is NS for another, it follows that there is no physical or chemical property of antigens that can be used by the immune system of the individual to sort the antigenic universe into S and NS as classes. The only solution that has withstood the ravages of experiment has been the ARA model. As Anderson has not stated it with precision, let me restate the key element that permits me to provide the "single unifying explanation for failure" to establish unresponsiveness following embryonically administered antigen..

It is not "time" but the presence or absence of effector T-helpers (eTh) specific for the antigen that determines whether a naive cell receiving Signal 1 will be inactivated or activated. During ontogeny, the immune system arises in the presence of all self and no nonself, but, above all, in the absence of eTh. All interactions with antigen are deletional. This unresponsive system becomes responsive when eTh anti-NS appear. As T-helpers follow the same rules, the persistence of S maintains the absence of eTh anti-S. "Absence" more precisely means "insufficiency".

This gives me a "single unifying explanation for all failed experiments". The failure means that there was present an unsuspected source of eTh or Signal 2 surrogate that led to a response when unresponsiveness was anticipated. In some cases the antigen used was contaminated with LPS (e.g., bacteriophage); sometimes the antigen, an allogeneic tissue, brought with it as a passenger, an abnormal source of T-help; sometimes the antigen was delayed in its expression as a ligand for T-helpers (i.e., P-RII) compared to its expression as a host component; etc. These are negative experiments and, as such, present no challenge to the developmental time model.

Now let me illustrate this unifying explanation with the best failure to achieve unresponsiveness, the experiment by Anderson himself. To quote him, "….. the ability of a male skin graft to be rejected by a female $Rag^{-/-}$ mouse post immune reconstitution with female hematopoietic stem cells [15], despite the simultaneous successful establishment of self tolerance, can not 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 [16], 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".

In order to explain their experiment, I suggested that the cryptic source of eTh anti-H-Y came from the antigen-independent pathway. In the race between being converted to eTh anti-H-Y and being inactivated by the H-Y of the graft, the former dominated because the injected cells had an insufficient chance to encounter $P_{H\text{-}Y}$-RII in the skin graft before conversion to eTh. This predicted that increasing the skin graft size would result in its acceptance. If accepted, the theory would be confirmed; if rejected, nothing can be concluded. Maybe a finding of increased time for rejection would be a more sensitive test of this explanation.

Anderson points out that the simultaneous grafting of male heart and male skin increased the antigen with the result that the heart was accepted but the skin rejected. From this, he concludes that there was "sufficient antigen for tolerance yet immunity to a prior and persistent antigen was induced".

The failure to reject heart does not require the conclusion that the animal was tolerant. There are many reasons why a graft might not be rejected in a responsive animal, among them inducing a response in an ineffective class or simply being more resistant to rejection relative to skin. After all, the animal, which failed to reject heart, was clearly responsive; it rejected skin. It was not "tolerant". The male heart transplant might not have significantly increased the presentation of the key ligand, $P_{H\text{-}Y}$-RII. My suggested experiment still remains valid as the best test of this explanation.

In contrast to my unifying explanation, Anderson's unifying explanation of success versus failure is as follows:

"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."

Anderson may have produced a valid explanation of the transplantation experiments but, clearly, it cannot be extrapolated to the S–NS discrimination because evolution never faced the artificiality of transplantation as a selective pressure on the immune system. An explanation of transplantation experiments that cannot be extrapolated to normal physiological behavior is an aside for this discussion. So let me try to rework his explanation. It would be an absurdity to propose a mechanism for the S–NS discrimination in which peripheral self-antigens that make it to the thymus/bone marrow typically induce tolerance, whereas those self-antigens that are localized in the periphery induce immunity. Therefore, it must be assumed that all self is expressed centrally as a negatively selecting ligand. Since the discovery of ectopic expression in thymus of peripheral self, the argument has surfaced that peripheral tolerance doesn't exist. We have discussed such a "space model" [17, 18] and concluded that a totally "sequestered self" is unselectable because self-components must be selected to function in the physiology of the individual; they are not selected upon, as a generality, to escape attack by the immune system. It is the immune system that is selected upon not to attack self. This argues that a peripheral tolerance mechanism exists. The revealing of one example of a self component expressed uniquely peripherally as a ligand for T-helpers would settle this still open and pending, albeit crucial question.

To correct a misunderstanding, I never argued that, as a factor in determining the S–NS discrimination "…..the level of antigen presentation needed to generate immunity is lower than that required to establish tolerance……". Signal 1 is the same for both. It is the threshold level of Signal 2 that decides immunity versus tolerance. Below threshold, tolerance dominates; above threshold, immunity dominates.

Lastly, I would like to deal with a lack of understanding of a fundamental general argument. Anderson states, and I quote:

"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)."

It should be clear that all somatic processes are built on germline-selected elements. That is not what is in question. What is in question are which germline-selected elements cannot be used to sort a somatically generated random repertoire? In order to place this in context, a comparison with the S–NS discrimination by the innate system is helpful. The recognitive repertoire of the innate system is germline generated and selected. The consequence is that this system distinguishes the self-of-the-species from nonself. Further, the specificity of its paratopes is selected to recognize epitopes that are common to as many different pathogens as possible. For the recognitive repertoire of the adaptive system, which is somatically generated and selected, the distinction is between the self-of-the-individual and nonself. The specificity of its paratopes is selected to be acceptably able to distinguish host-self from the rest of the universe of antigens. This distinction can be easily visualized as transplants between individuals with only an innate immune system are accepted, whereas transplants between individuals with adaptive systems are rejected.

When discussing the S–NS discrimination by the adaptive system, the somatic process in question is individual-specific. It involves the mechanism by which an individual defines self, an epitope-specific event. No germline-selected recognitive system can be individual-specific; an individual-specific recognitive repertoire can only be arrived at by a somatic selection process. The germline-selected mechanisms of hormonal regulation and glucose

metabolism, the kinetic parameters such as the half-lives of division or death, the recognition of danger, the processes of localization, context, tuning, co-stimulation/co-inhibition, etc., are important elements in immune responsiveness but can not provide a model for the S–NS discrimination by the adaptive system, unless, of course, a germline-selected mechanism can be envisaged to determine individuality. As this has not been forthcoming after over 50 years of debate, I feel justified in rejecting models based on germline-selected recognitive interactions that are claimed to sort a random repertoire such that it becomes individual-specific.

References

1. Cohn M (2011) On the logic of restrictive recognition of peptide by the T-cell antigen receptor. Immunol Res 50:49–68
2. Cohn M (2008) The Tritope model for restrictive recognition of antigen by T-cells: II. Implications for ontogeny, evolution and physiology. Mol Immunol 45:632–652
3. Cohn M (2005) The Tritope model for restrictive recognition of antigen by T-cells: I. What assumptions about structure are needed to explain function? Mol Immunol 42:1419–1443
4. Langman RE, Cohn M (1999) The standard model of T-cell receptor function: a critical reassessment. Scand J Immunol 49:570–577
5. Morris GP, Ni PP, Allen PM (2011) Alloreactivity is limited by the endogenous peptide repertoire. PNAS 108(9):3695–3700
6. Cohn M, Langman RE, Mata J (2002) A computerized model for the self-nonself discrimination at the level of the T-helper (*Th-genesis*). I. The origin of "primer" effector T-helpers. Int'l Immunol 14:1105-1112
7. Langman RE, Mata JJ, Cohn M (2003) A computerized model for the self-nonself discrimination at the level of the T-helper (*Th genesis*) II. The behavior of the system upon encounter with nonself antigens. Int Immun 15 (5):593-609
8. Cohn M (2005) A biological context for the self–nonself discrimination and the regulation of effector class by the immune system. Immunol Res 31(2):133–150
9. Cohn M (2005) The common sense of the self–nonself discrimination. Springer Semin Immunopathol 27(1):3–17
10. Cohn M (2004) Whither T-suppressors: If they didn't exist would we have to invent them? Cell Immunol 227:81–92
11. Cohn M (2008) What roles do regulatory T-cells play in the control of the adaptive immune response? Int Immunol 20(9): 1107–1118
12. Cohn M (2009) On the opposing views of the self–nonself discrimination by the immune system. Immunol Cell Biol 87:113–119
13. Cohn M (2009) The discussion with Jacques Miller: illustrating the limitations of pure empiricism. Immunol Cell Biol 87:435–437
14. Brent L (1997) A history of transplantation immunology. Academic Press, San Diego
15. Anderson CC, Carroll JM, Gallucci S, Ridge JP, Scheever AW, Matzinger P (2001) Testing time-, ignorance-, danger-based models of tolerance. J Immunol 166:3663–3671
16. Anderson CC (2009) Placing regulatory T cells into global theories of immunity: an analysis of Cohn's challenge to integrity (Dembic). Scand J Immunol 70:321–325
17. Langman RE, Cohn M (1996) A short history of time and space in immune discrimination. Scand J Immunol 44:544–548
18. Langman RE, Cohn M (1997) A short history of time and space in immune discrimination: reply to the commentaries. Scand J Immunol 46:113–116