RESPONSE

It is sobering to realize that, even if Gus Nossal had invented us, he could not have made us perfect. This first attempt at a synthesis of humoral responsiveness left us with many loose ends to be reckoned with later, but, not surprisingly, Nossal has fingered all of them. We appreciate how much of an effort that took on his part and are deeply indebted to him. We don’t need to apologize for mistakes and oversimplification; it represents a limitation in our thinking and illustrates the only way we have to arrive, painfully slow as it is, at an acceptable understanding. That is, after all, the function of this dialogue. In commenting on Nossal’s analysis, we will follow his paper but in an attempt to limit length we will deal only with those issues that leave him out-of sorts.

A. THE CONCEPT OF A PROTECTON

Nossal recognizes the importance of having a functional equivalence among B-cell specificities, but he does not go beyond using anatomical measurements, such as between the ear and the toe, in estimating the actual repeat frequency. We used the fluid volume into which antibody diffuses to calculate a minimum frequency of functional repeats assuming a uniform dispersion of B cells. Of course, this willful ignoring of architecture means that we are missing something. Evolution did put a lot of effort into lymphoid organ architecture, and for good reason; we are open to suggestion as to what forced evolution to make such complicated organs. As Nossal correctly points out, if differences in molecular sequence are equated one-to-one with functional specificity differences, then all attempts to attain equivalence via identity at the molecular level quickly reduce to an absurdity; such as having rearranged B cells divide a number of times that is in proportion to the size of the animal. To obtain 100 “exemplars”, or copies of a given specificity, the total number of functionally different specificities must be small; our STAGE I repertoire, which considers D-diversity absurd and makes D-disaster inevitable, offers a solution to the repeat problem. Applying the fudge of inherently more broad cross reactivity of a primary repertoire only makes the repeats forever inestimable. The effects of cooperativity between Protectons we calculated to be rather small, making the larger molecular repertoire of larger animals indeed a luxury, and functionally gratuitous.

We seem to agree that there is some kind of homeostatic mechanism and the buck has to stop somewhere; we assume all B cells to be counted equally, Nossal assumes “independent forces” operating on iB and eB cells. Until these independent forces of homeostasis are identified, it seems worthwhile to stick with the simplest case and see where that leads us (i.e., the Protecton calculation). Nonetheless, if homeostasis is made sufficiently elastic that washout displacement is not a problem, and if D-diversity is used to generate a large repertoire, then engaging a small fraction (less than 0.1%) has to be made sufficient to maintain the repertoire by evolutionary selection; a mechanism is wanting. It also occurs
to us that the germfree animal cannot be as robust at combatting infection without prior antigenic experience; the argument for vaccines depends on the germfree animal being immune-insufficient. Of course the axenic and germfree animal's response might well be "fine" to a novel laboratory antigen. A general comment needs to be made concerning the quantitation of the various parameters. Although we have gone to some lengths to justify our choice of values for the various parameters, these values are not fundamental to Protecton theory, rather they illustrate how parameters are defined and quantitated in an interlinked set of relationships. Our challenge is to find new or different parameters of importance and to cajole experimentalists into making precise measurements of the parameters, and so remove a level of uncertainty presently due to our having to reinterpret data gathered in a quite different framework. The important issue here is to go beyond the "I believe" and "I see," by completing the argument in terms of evolutionary necessity.

B. STAGE I AND STAGE II B CELLS: THE QUESTION OF B-CELL SUBSETS

We have tended to view the experimental definition of B-cell subsets in terms of stages in a linear sequence of differentiation steps. However, Nossal does raise an important point concerning the fate of antigen-selected B cells and how they might contribute to B-cell memory. Protecton theory was developed on the premise that the strongest evolutionary forces would be revealed by analyzing the primary immune response; a secondary immune response and memory presuppose recovery from the primary infection. Thus, we have no real quarrel with Nossal here.

C. THE CELLULAR BASIS OF IMMUNOLOGICAL TOLERANCE

This long discourse is as dear to Nossal as it is to ourselves. However, we will keep our comments to the bare essentials in an attempt to stay focused on the naked truths. At the outset we reemphasize that tolerance, as a mechanism for the self-nonself discrimination, deals only with the anti-self component; the anti-nonself component must be included before we can properly address the complete issue of the self-nonself discrimination.

Nossal refers to our "two egregious omissions": a t-state that precedes the i-state, and a blurring of the S-F (self-foreign) distinction. So long as his t-state is a quantitative condition, meaning that the B cell can be either tolerized or induced above some threshold, we could not, in principle, distinguish it from the i-state. However, the Klinman position does require a separate t-state because he assumes that B cells are tolerizable-only (The Tolerance Workshop, 1986, Vol. I, p. 19) and they oscillate between this and an e-state that is inducible to antibody secretion-only; this is a difference in principle that can be argued. The S-F distinction is exact in the sense that anti-S does not reach an effective (lethal)
concentration whereas anti-F must. We formulated the problem in order to be able to face the fudge of affinity and valence head-on. By establishing a boundary condition that was independent of affinity and valence connotations we dealt with the very problem which Nossal ponders as he tries to shoot Associative Recognition theory down.

To rephrase his conundrum, how can B cells of low affinity anti-S be allowed to escape tolerance by S but, if induced by an F antigen, the pentameric IgM can now react with S and eliminate the individual. Indeed a receptor aggregation model of B-cell induction does generate such lethal effects. This is, in part, why we argue that the resolution of this dilemma can be found in an Ig molecule that has a threshold of binding to trigger a conformational change (a very different assay from aggregation). The threshold for conformation-dependent signaling is set in Ig structure during evolution. Consequently, a single threshold applies to all isotypes (including pentameric IgM). Nossal finds this conundrum cause to convince him of the necessity for a “peripheral” tolerance mechanism in addition to a “central” mechanism (Signal [1]). We recognize that only B cells can be regulated (not secreted antibody), and this raises the question of what “peripheral” tolerance means. The Sinclair model for IgG inhibition of the IgM response is not tenable as a tolerance mechanism because it requires an effective concentration of anti-S (lethal) to bring about the inhibition of IgM anti-S. Thus, Nossal should repent “about ascribing self-tolerance to a variety of mechanisms” and leave the peripheral and failsafe mechanisms to the Jesuit scholars.

It is off the main track of Protection theory but we cannot resist the temptation to comment on Nossal’s treatment of our antigen-independent mechanism for the conversion of iT\(^H\) to eT\(^H\) ("entirely ad hoc") and how we “then state baldly” that anti-S iT\(^H\) are inactivated before they could be converted into anti-S eT\(^H\). If this represents his “deepest worry” about the Associative Antigen Recognition model, we can breath a sigh of relief. We agree wholeheartedly that “the only operational difference between an S antigen and an F antigen is that S is always present ... while F antigen is pulsed unexpectedly.” Our antigen-independent step parallels his antigen-independent conversion of a cell in the t-state (condition) to the i-state. Indeed, since Nossal pointed this out, we feel that our two views are close to coincident.

The distinction between “abortion” and “anergy” is of no physiological interest as far as the self-nonself-discrimination is concerned, if both result from Signal [1] alone and both become irreversible with respect to an interaction with eT\(^H\) in a functional time-frame (e.g., of the order of 10 hours). Given that there is a substantial proportion of steady-state B cells able to bind antigen but unable to secrete antibody we remapped Nossal’s “anergy” experiments onto the existence of non-functional cells. In light of the physiological equivalence of anergy and abortion we have no need to analyze the possible competing interpretations of the anti-\(\mu\) experiments.
Nossal tries to dig in his heels with an “in vivo veritas”; he argues that “the Goodnow model works,” and, therefore, “it will take more than handwaving (i.e., our arguments) to make it go away.” This illustrates how important is the distinction between unresponsiveness and tolerance that we always insist upon. Unresponsiveness describes the experimental finding; tolerance is the conceptual extrapolation of the finding to a theory of the self-nonself discrimination. The argument that the “Goodnow et al. model works” (whatever that means) is irrelevant. We are trying to decide which experimental observations can be extrapolated to a concept of the self-nonself discrimination, and to what extent the “anergic” cell classification fits the nonfunctional mIg+ B cell.

All this having been said, it is not clear to us why our explanation of the Goodnow et al. (1988) experiment was so stupid. They found a measurable level of B cells expressing the transgenic Ig anti-HEL (TG) but these cells were not inducible to eB under their conditions of adoptive transfer. We therefore have two findings to explain. If clonal abortion operates; first, why were any B cells expressing TG detectable and, second, why were they non-inducible? Nossal takes issue with our explanation of the first question, not the second. What was detected by Goodnow et al. (1988) was the level of iB cells in the “lady or tiger” state. Since virtually every iB cell expressed the TG and was receiving Signal [1], which leads to irreversible inactivation, this process cannot be instantaneous as it is a decision step. The eTm-Ag-iB cell–cell interaction initiating activation is slow compared to the Ag-iB interaction initiating inactivation. If the latter were too rapid, activation would be impossible. What is being detected in the Goodnow et al. case are the cells that are on the pathway to inactivation but are reversible. This is detectable only because every cell expresses the TG. Added to this level is the neutralizing effect of doubles, TG+ endogenous Ig; the endogenous Ig engaged by the antigenic load tends to protect some iB cells against clonal abortion. Why is this unreasonable? In any case, the interpretation of these transgenics, while important, is not crucial to the outcome of Protecton calculations.

D. A POPULATION ON NON-INDUCIBLE B CELLS AND “ONE CELL-ONE ANTIBODY” REVISITED

Two conclusions (as shocking to us as they are to Nossal) are brought into question by him, and rightfully so as they drastically change how we view immune behavior, if correct. First, 10% of iB cells are doubles, mostly 1L and 2H. Second, 90% of B cells express an antigen-binding Ig but are non-functional.

We argued that these two conclusions are inexorable consequences of molecular biology, but that statement needs tempering, lest we lose a good friend; rather, we needed to get his attention.

As to the first conclusion, if we are correct that haplotype exclusion is depend-
ent upon only three processes, a “branching ratio” (a probabilistic order of Ig locus expression), an in-frame fusion probability, and an (LH)_{STOP}, then 10% of iB-cells must express two mlg molecules.

To assuage Nossal, we have not gone in a circle. Rather, we have traveled a helical path and can look down with a certain perspective on the earlier studies.

In the early studies of the ’60s to which he refers, the search for iB cells that expressed two functional BArS was carried out by immunizing with two unrelated antigens and assaying at the single-cell level for doubles. Under any model such cells had to be very rare and, in our case, cited by him, we footpad-immunized rabbits for a year using a variety of protocols and carried out our analyses on lymphnodes as big as golf balls. We found specific doubles at the level of 1% of plasmacytes whereas Nossal in another system detected <0.1%. Neither result attacked the clonality theory as a general case; they both supported it.

Today this question is being posed by us at another level. There is no selection pressure, as we point out, that could make clonality perfect as it is driven by the self-nonself discrimination to some defined limit. The molecular biology of the mechanism of haplotype exclusion, fed into the Protecton calculation, leads us to the conclusion that the limit at which that selection pressure operates is around 1% of total B cells expressing two mlgs of all specificities, at least one of which is functional. There is not much room for error in that calculation. So the chips are down; either the molecular biology is flawed or the cellular immunology is wrong. We suggest the latter.

As to the second conclusion, given that the D-segment is expressed in functional Ig in essentially one frame, what conclusion would Nossal prefer, D-diversity or D-disaster?

When you extrapolate a set of data derived from chemistry to cells and come to a surprising conclusion which seems totally solid, the first reaction is to search the literature for an experimental hint that it obtains. Nossal excoriates us for ignoring the evidence that roughly half of splenic B cells can be induced into antibody formation in vitro. Our argument is not based on cellular immunology. We were not trying to use cellular immunology to bolster our conclusion but rather to show that the data are not substantial arguments against the conclusion that only 10% of virgin iB cells are functional. We did, however, show that there are cases where, due to antigenic selection, most iB cells would appear to be functional (Part Four, II.A.3). We chose the most quantitative study we were aware of; those cited by Nossal should be added to it as examples of the same point. They do not establish that virgin iB cells are 50% functional; they are antigen-selected iB cells that probably selectively migrate into splenic germinal centers.

However, we insist that Nossal face our argument. Does he prefer D-diversity or D-disaster? In reality, he walks the fence proposing a large, functional repertoire dependent on D-diversity. They are mutually exclusive concepts (see Re-
If D-disaster obtains, as part of the haplotype exclusion mechanism, then doubles and non-functional iB cells are unavoidable; if D-diversity obtains, the neogermline theory is unavoidable. We claim to have ruled out neogermlineism and it is with those arguments that our dialogue should be pre-occupied.

E. SIGNALING PATHWAYS AND THE ROLE OF LPS

Nossal puts us, once again, in face of the LPS question; it is a major reagent in experimentation. We would not quarrel with LPS being a Signal [1+2] generator; in fact we considered that assumption years ago but decided in favor of its being a Signal [2] generator because of the observed synergy between antigen and LPS both in induction and in the reversal of the establishment of tolerance, both admittedly weak arguments as Moller pointed out. However, to argue that it is a Signal [1+2] generator and then to use that reagent to determine that the proportion of functional B cells is > 50% is a contradiction. The nonfunctional iB cells, which cannot transmit Signal [1] via an interaction with antigen, would be activated by the LPS pathway thus bypassing the normal Signal [1]. This, of course, would not permit the use of LPS to assay whether the iB cell is functional or not with respect to antigen. Further, we analyzed the Sauter & Paige (1987) experiment to show that the LPS-stimulated colonies derived from fetal liver, because of the $\lambda : \lambda$ ratio that they expressed, had to be derived from antigen-stimulated cells (Part Four I.A.2). If LPS were a Signal [1+2] generator then the $\lambda : \lambda$ ratio would have been 1:1, whereas if it is a Signal [2] generator and requires Signal [1] from antigen encounter that ratio would be $\sim 10:1$, that found.

CONCLUSIONS

In the debunker we examine the equation $R = N/YS$ and argue that the repertoire of sequence differences, $R$, is not equal to the number of functionally different specificities, (i.e., the functional repertoire). The STAGE I repertoire is $N^2$ when $N$ equals the number of different $V$ segments; the value of $N^2$ is around $10^6$. The Stage II repertoire contains all the somatic mutants, and, if anyone wishes, can have upward of $10^{40}$ different sequences, even $10^{20}$ different specificities, but coming back to Earth the single amino replacement mutants represent no more than $10^6$ specificities, $5 \times 10^4$ of which are functionally different.
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