Ontogeny of the Clonal Selection Theory of Antibody Formation[a]

Reflections on Darwin and Ehrlich

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This is an idiosyncratically personal account of the origins, about 30 years ago, of the clonal selection theory, a no longer controversial integrating theme of immunological research. As an interested participant, the perspectives I can offer are those within my own ken, inevitably an egocentric one. This will unfortunately understate the independent roles played by a host of others, including several in these proceedings. Other historical accounts[1,2] may give a more objective view. However, some parts of my story have not been told before. It will be of particular interest to students of the philosophy and sociology of science to analyze the processes of resistance and acceptance of clonal selection theory after 1957, until its general acceptance around 1967.[3-5]

My personal mise-en-scène begins in 1955. I had been at the University of Wisconsin since 1947, having gone there directly from my work in Ed Tatum's lab at Yale and Francis Ryan's at Columbia. If I needed any reinforcement about the interest antigens and antibodies would have for general biological theory, I would have received this amply from M. R. Irwin. Ray Owen had left Wisconsin for Caltech just before I arrived, but his intellectual trace was everywhere. However, my own work was strictly confined to the genetics of *Escherichia coli* and of salmonella. The diversity of serotypes in salmonella had been one of the conceptual clues to genetic recombination in bacteria, and I had at least one experimental contact with immunology, namely, serology of flagellar and somatic antigens.[6]

The principal antecedental threads of clonal selection, at least for this microbiologist, were: (1) physicochemical concepts of serological specificity, spanning from Paul Ehrlich to Karl Landsteiner and Linus Pauling; (2) the revalidation of Darwinian models (namely, prior spontaneous mutation and natural selection) in their application to adaptation in microorganisms, such as the development of specific resistance to antibiotics; (3) an emerging understanding of gene expression in protein synthesis, particularly in substrate-induced enzyme synthesis in bacteria; and (4) a developing conception of a genetics of somatic cells by analogy with the genetics of bacteria (Mendelian models).

Karl Landsteiner's "The Specificity of Serological Reactions" focused attention on antigen-antibody reaction as a prototype of biological specificity. Pauling's chapter in the 1945 edition[7] showed how "specificity can arise in the interaction of large molecules as a result of the spatial configuration of the molecules." The seminal value of this stereochemical axiom was unfortunately not matched by well-founded speculations on the mechanism of antibody synthesis. In the early 1950s, there was notably little serious discussion of the mechanism of antibody formation. The most prevalent

[a]Dedicated to the memory of Frank Macfarlane Burnet (1899–1985) and Peter Medawar (1915–1987).

notions were those elaborated by Haurowitz,[8] that the antigen itself acted as a template on which the antibody globulin was molded. Pauling and Campbell[9] had even published experiments in 1942 claiming the synthesis of antibody *in vitro* by the renaturation of globulin in the presence of antigen. One minor variant challenged the need for the continuous presence of the antigen and supposed that an intermediate mold was generated, perhaps in many copies, from the initial antigen conformation. Another gave homage to the central role of RNA and DNA in protein synthesis, but supposed that antigen could be attached to or modify the nucleic acid in directing the course of protein synthesis. These models, which I later classified as "instructive," reflected a miscomprehension of the most basic feature of the genetic coding theory: the linear correspondence of the nucleotide sequence in the DNA/RNA to the amino sequence of a protein.[10]

My own research, starting in 1946, had made extensive use of artificial selection to discover rare recombinant or mutant genotypes in large microbial populations.[11] Francis J. Ryan introduced me to this at Columbia in an investigation on a leucine-dependent mutant of Neurospora. Placed on nutritionally deficient media, this mutant would "adapt" to that constraint on its growth. We established that this adaptation was a genetic reverse-mutation with crossing studies. We presumed that it occurred spontaneously, the deficient medium selecting for the mutants, but we could adduce no compelling evidence. Our thinking was of course influenced by Luria and Delbruck's demonstration in 1943[12] that the statistics of phage resistance in bacteria also agreed with the Darwinian paradigm. Shortly after the Neurospora experiments, a similar method of selection enabled the discovery of genetic recombination in *E. coli* K-12, which achieved a certain reinforcement to "think selection" for a variety of experimental purposes and as a pervasive strategy in natural process.

Many of the aforementioned findings went against contemporary traditions. For example, many bacteriologists still held that drug resistance was evoked by some chemical reaction of the drug with the bacterial protoplasm—a view that continued for many years to be nourished by the authority of Sir Cyril Hinshelwood, President of the Royal Society of London. Several never unraveled the difference between genetic changes in individual cells, changes in the proportion of genotypes in populations, and the reversible regulation of enzyme synthesis by inducing substrates. To others, it was congenial as a last stronghold of Lysenkoism: a direct effect of environment on hereditary traits. Francis Ryan continued to devote much of his energy to studying adaptive mutation in bacteria.[13]

The development of the replica-plating technique in 1952[14] was similarly motivated: it allowed indirect selection of resistant mutants in a fashion that assured their presence among cells that had never been exposed to the drug. As a constructive demonstration it did finally quiet that controversy. It was also a further reinforcement of "think selection."

The study of enzyme induction, and of the genetic control of B-galactosidase, was one of the first tasks I addressed with the use of genetic recombination analysis in *E. coli*. With the help of Karl Paul Link and Martin Seidman, *o*-nitrophenyl galactoside[b] became available as a chromogenic substrate for assay of the enzyme.[15] I was soon struck by the fact that "uninduced" cells, grown in the absence of galactosides, nevertheless showed an unmistakable basal level of the enzyme. Subsequently, I found that neolactose, altrose-β-D-galactoside, was a noninducing substrate that could be

[b]It is curious to recall that W. Goebel and O. T. Avery had synthesized nitrophenyl glycosides in 1929 as intermediates in the synthesis of artificial conjugated haptens.[17]

used to select constitutive mutants that produced full-blown levels of the enzyme without specific induction. These findings supported the view that enzyme specificity was inherent in the bacterial genome; the inducer was a quantitative regulator of gene expression.[16]

Finally, under the stimulus of conversations with G. Klein and H. Koprowski, in 1955 I started to beat the drums for a research strategy of "a genetics of somatic and tumor cells."[18,19] Bacteria had also been thought to be intractable; it seemed certain to me that mammalian cells could be made to fuse, and at least chromosome reassortment could be readily studied.

My first published thoughts about antibodies[20] were a brief statement of possible analogy to induced enzyme formation. The complexity of the animal system seemed to defeat experimental analysis. Then, in November 1955, at a symposium on Enzymes in Detroit, Jacques Monod again posed the question of whether the inducer provided the information needed to mold the enzyme. In my discussion, I responded in the negative, citing the aforementioned evidence. The role of the inducer was to regulate the expression of that genetic information, as we would now all agree. In a spectacularly unprescient fashion, my impromptu discussion went on to contrast the induction of enzymes with the antibody response:

> "The immune response has provoked a similar discussion. Ehrlich had proposed that specific antibodies were normal products, subject to quantitative variation under the influence of the antigen. Pauling and others believe that the antigen plays a direct role in molding the antibody protein. Enzymes are generally less specific than antibodies in their range of complex formation, but more so in their catalytic action. Furthermore, antibodies are constructed from a common gamma globulin, whereas enzymatic specificity can call on a more fundamental variety in structure. We need not assume, therefore, that both syntheses follow the same plan."[21]

Calling on the prevailing common wisdom, that was not my most insightful moment. The only other comment about antibody synthesis at that meeting was Pauling's reiteration of his 1940 model.

When I returned home, I found the November issue of the PNAS and therein Nils Jerne's paper on: "The Natural-Selection Theory of Antibody Formation."[22] I wrote him promptly to apologize for not having cited his paper, and to express my approbation of approaches that avoided an instructional role for the antigen. He responded that I was the only one to date to express any interest in his proposals. Felix Haurowitz had criticized him, on the one hand, for neglecting to mention Ehrlich's precedent in proposing the spontaneous formation of antibodies. On the other, it was just not possible for an animal to be preadapted to form antibodies to artificial haptenes like Landsteiner's azophenyl arsonate. Jerne responded that a million specificities randomly chosen would be far less than the "million million million" globulin molecules in the blood, the supposed targets of selection according to his model. At that point, I was sure that some Darwinian model would handle the problem of antibody formation; I was a bit skeptical of the self-replication of circulating antigen-selected globulin molecules that he was proposing. More plausible targets of selection would have been diversified protein-synthesizing units (in the cell), still bound to their antibody product. It still did not occur to me that the cell itself satisfied that criterion. In fact, not working directly in immunology, it was only at conferences that offered the stimulus of dialectic with people actively working in the field, that I would put much attention into scientific speculation. What perils meetings like this may have for the unwary!

In August 1957, however, I found myself in Macfarlane Burnet's laboratory in

Melbourne, on a trimester's Fulbright fellowship.[c] I had gone there to learn about the influenza virus, and its recombinational processes,[26] and was dismayed to hear that Mac had just closed down his research on flu; he had decided to go full blast into the mechanism of antibody synthesis. We began earnest discussions about the new wrinkle that Mac had placed on Jerne's proposal: it had to be the *cells* that varied and were subject to selection.[25] But, I expostulated, there must be far many more species of antibody than there are cells available! "Mac, how do you know that? How do you know as a matter of experimental fact that there are more than a few thousand species?" I realized instantly how I had taken for granted a spurious "fact" that had misled the entire field. (A complete history would trace the ultimate origin of that ikon, of the infinity of antibodies. Today we would use information-theoretic criteria to measure specificity, and might avoid such pitfalls.)

Our discussion became intense, although somewhat clouded by Burnet's tendency to resist the "simplistic" mechanisms of DNA-based molecular genetics that are today's foundation stone. I would receive his exciting ideas, and then have to translate them into a contemporary idiom to get the full benefit of his marvelous biological intuition.

There was also an opportunity to construct some experiments to test the hypothesis, as difficult as this was in the absence of any reliable procedure to clone antibody-forming cells. Working with Burnet was a young, audacious, postdoctoral fellow: Gus Nossal. He was more than eager to attack the theory. Could we at least study the phenotype of individual cells in animals stimulated with two or more antigens. The Pauling model made no particular exclusion; on a clonal selection model, cells making two kinds of antibodies would be vanishingly rare, barring second order complexities.

I had been doing serological microassays with motile salmonella strains, in this case to study the genetics of the flagellar antigens in single-cell pedigrees of the bacteria.[27] I suggested that we characterize the antibody released by single lymphoid cells by immobilization of the bacteria in microdroplets in paraffin oil. The feasibility of the assay was proven during the brief months I still had in Melbourne, and Nossal continued thereafter until 62 reactive cells had been tested: 33 immobilized *Salmonella adelaide*, 29 *S. typhi*, none both.[28] This was only one step toward proof of clonal selection. Propagable clones would be needed for that. The paper made a few mumbles of alternative possibilities, like an analogy to mutual exclusion of viruses. This was my first and last experimental involvement. I need hardly tell you about Nossal's further career. When I went to Stanford in 1959, I persuaded him to join me for an interval, but his roots in Australia ran very deep and he returned, eventually to succeed Burnet as director of the Hall Institute.

Returning to Wisconsin in November 1957, I had a number of other matters in mind besides antibody synthesis. Sputnik had opened up the exploration of space in ways that were dramatized by an encounter with J. B. S. Haldane in Calcutta, en route[29]; and I saw little evidence that scientific objectives were to be honored in the development of the nation's space programs. It seemed an urgent task to move the National Academy of Sciences to take leadership for this objective and to include biological questions on its agenda. What was later termed "exobiology" was initiated the spring of 1958. I also became engaged in the negotiations that would lead to my going to Stanford. But during 1958, Burnet's ideas came up on a number of occasions

[c]Burnet's memoirs[23,24] have a small factual error—he had me in Melbourne November and December, after he had published his paper on clonal selection theory[25]; in fact, it was August through October 1957. Briefly visiting Melbourne at that time was Carlton Gajdusek, just on his way to New Guinea to study kuru among the Fore—and to discover the slow viruses.

where I felt they would receive greater due after being retranslated into DNA language.[19]

When Bernard Davis invited me to give the Howard J. Mueller memorial lecture at Harvard that November, I decided to use the occasion to frame a critical reformulation of the clonal selection theory. Burnet's uncanny biological intuition was not matched by his resonance with molecular biology or a detailed familiarity with its chemical precepts. At one point he refers to himself as "positively schizophrenic about molecular biology"—his main grievance "the arrogance which defines biology as the chemistry of the nucleic acids." By 1958, I had long since consolidated the philosophical position he had repudiated. Meanwhile, David Talmage, at the University of Chicago, had reached a substantially similar posture. Quite independently of Burnet's revelation of how to read Jerne, he had published a succinct statement of the same theory of clonal selection of cells.[30] In October, I asked him if he would meet in Madison. The upshot was an exchange of manuscripts and an agreement that we would submit papers to *Science*, for publication back to back.[31,32] Meanwhile, I had still other diversions: a surprise invitation to revisit Stockholm once again (I had attended the International Congress of Microbiology in August), this time in December on Alfred Nobel's birthday. I was far too busy to prepare still another paper that would do credit to the occasion; quite literally, I was packing to move my home and my lab to Stanford, targeted for end January. But I did manage to present the Mueller lecture, and was gratified by the interested, if mostly skeptical, discussion it aroused. The talk I finally did present in Stockholm, the next May, was in a similar mood. So much had happened in the 12 years since my initial work on genetics in bacteria that I decided to devote my address[33] not primarily to my own work, but precisely to the extent to which biology had become the chemistry of the nucleic acids, as coding agents for proteins.

Our papers appeared in *Science*, June 1959. Talmage focused on experimental data, including his own important contributions, on the overlapping diversity of antibodies—an essential point in the argument that antibodies are normal globulins. Mine focused on the theoretical framework of the cell selection theory. It is reprinted here (at the end of this article), the more substantial part of this presentation. It generally followed Burnet's reasoning. One deviation was my proposal that clonal diversification was a life-long process; he would have confined that to the perinatal period as part of his model of induced tolerance.

The sharp delineation of "instructive" from "elective models" is now a matter of common understanding. Nevertheless, a reminder is needed to distinguish "elective" from "selective." Purification of a globulin preparation on an affinity column is an elective process. If it permitted replication of the elected units, it would also be selective. Likewise, inducers play an elective role in enzyme synthesis in bacteria, by derepressing the expression of preexisting genes. They are not *ipso facto* selective; substrates may be so when they encourage the differential reproduction of specified genotypes. Thus, the hypothesis analogizing immunogenesis to enzyme induction was an elective one; it did not yet embrace genotypic diversification and selection therefrom. These distinctions are important in efforts to apply these concepts to further domains such as neurobiology.

For some time, many immunologists' reaction was that they could not see what experimental basis there was to support the selection theory. This was entirely legitimate, but the alternatives to be sorted out were not always logically coherent, such as efforts to distinguish our selection theory from one based on "cellular differentiation."[34,35] Even today, to describe a phenomenon as epigenetic rather than genetic[19] is hardly to explain it. The restriction of antibody potentialities that Nossal and I had reported (no more than one antibody species per cell) came under sharp experimental attack, especially by Attardi *et al.*[36] At one point, Nossal and Makela

themselves[37] found a few cells that, depending on the assay method used, seemed to be bipotent. This was not a mortal wound to selection theory: we were, after all, working with diploid cells; but I was acutely uncomfortable with the kinetics of the model needed to accommodate two sequential mutations, one on each chromosome. Of course, other compromises were available—and one has emerged as fact: substantial reduplication of genes for immunoglobulins. Without experimental necessity, I was loathe to multiply entities. But it appears as if immunobiology falls outside the domain of Occam's razor. After 1959, I did not lose interest in immunogenetics, but my medium was an administrative one: the new department of genetics at Stanford. Gus Nossal, Av Mitchison, Walter Bodmer, and Leonard Herzenberg having occupied chairs there, I could confidently direct my own experimental interests elsewhere.

Meanwhile, chemistry was marching ahead. Brenner, Jacob, and Meselson had given us the messenger RNA, and the role of DNA in protein coding began to be shaped in its contemporary form.[10] And in 1962–1964, a number of studies made it clear that the specificity of antibodies was related to their primary structure, an amino acid sequence whose determination could hardly have any other provenience than the DNA. Ollie Makela also stuck to his guns and clarified some of the methodological problems that may have given bipotent cells as artefacts[38]; Benacerraf's group also gave a strong affirmation of unipotency of cells.[39] It appears that Nossal and Lederberg were probably correct in 1958, but in view of the methodological problems, that has to be put down to sheer luck. The experiment had the undeniable virtue of providing a target of skeptical investigation more pointed than the generalities of the theory that was its background.

By the 1967 Cold Spring Harbor Symposium, the clonal selection theory was an undeniable fundament for almost every investigation of the chemistry of antibodies or the biology of immunocytes. It was also clear that further progress would depend on the propagation of antibody-forming cells as clones. We do not have a detailed intellectual biography of the precursors to Kohler and Milstein's famous experiment.[40] Some of the precedent ideas about fusing immunocytes with neoplastic cells to produce such clones have been reviewed by Bodmer.[41] In a *popular* piece I wrote in 1972: "Many products of differentiated cells, such as specific enzymes and antibodies, could become important in medicine if we could produce them in larger, predictable quantities. Cell fusion should enable scientists to increase the rate at which these substances are produced by cells in culture."[42] This remark was inspired by Henry Harris's observation that the dormant nucleus of the chick erythrocyte could be reactivated by fusion with mouse cells. Into the ears of babes . . .?

The immune response stands today as the first epigenetic phenomenon for which a chemical structural interpretation can be given. Nature often returns to the same handbook of tricks; it surely will not be the last to violate the dogma of somatic cell constancy of DNA, the apparent reversibility of cell differentiation notwithstanding.[43,44]

RETROSPECTION: THIRTY YEARS LATER

1. The greatest weakness in reference 32 is its economy of cell types. What sane person would have postulated today's menagerie in 1959?

2. The interpretation of immunological tolerance needs be far more complex, although within the same general conceptual framework as offered there.[45]

3. We would have gotten to a modern theoretical perspective as a direct yield of structural chemical studies of immunoglobulins. Doubtless, these labors get some motivational push and focus from the theoretical context. For example, I would rather

see intensive comparison of DNA sequences of selected sites in samples from differentiated tissues: muscle, neurones, fibroblasts versus gonia, than a mindless traverse of one complete genome. The latter would have told us nothing about immunogenesis.

4. Don't let conflicting and awkward "facts" stand in the way of an esthetically satisfying theory whose fundamentals are consistent with the world model and with one another! And be suspicious of "facts" that seem in the way of any coherent theory. In some measure, the uniformity of the genome among somatic cells may be one of these.

Note added in proof: The last word on the clonal selection mechanism is: TONEGAWA, S. 1988. Somatic generation of immune diversity. Prix Nobel 1987; pp. 203–227. Also appeared in In Vitro Cell. Dev. Biol. **24**(4): 253–265.

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CURRENT PROBLEMS IN RESEARCH

Genes and Antibodies

Do antigens bear instructions for antibody specificity or do they select cell lines that arise by mutation?

Joshua Lederberg

An antibody is a specific globulin which appears in the serum of an animal after the introduction of a foreign substance, an antigen (*1*). Each of the many globulins is specified by its reaction with a particular antigen (*2*). Our present concern is to formulate a plausible mechanism for the role of the antigen in evoking large amounts of a specific complementary globulin. An important element of any theory of antibody formation is its interpretation of self-recognition, the means by which an organism discriminates its own constituents from the foreign substances which are valid stimuli of the immune response.

Recent speculation about antibody formation (*3–8*) has been dominated by instructive theories which suppose that the antigen conveys the instructions for the specificity of the globulin synthesized under its governance. Elective theories date from Ehrlich (*9*) and have been revived principally by Jerne (*10*), Talmage (*2, 11*), and Burnet (*12*). These postulate that the information required to synthesize a given antibody is already inherent in the organism before the antigenic stimulus is received, and the stimulus then functions to stimulate that mechanism electively. Jerne had proposed an elective transport of antibody-forming templates to functioning sites; Talmage and Burnet have explicitly proposed an elective function based on cellular selection. The details which distinguish the various proposals are pointed out in the following discussion.

Immunology does not suffer from a lack of experimental data, but still some of the most elementary questions are undecided, and it is not yet possible to choose between instructive and elective theories. However, the latter have had so little expression in the past few decades that a detailed exposition may serve a useful function, if only as a target for experimental attack. This article is an attempt to formulate an elective theory on the basis of genetic doctrines developed in studies of microbial populations.

Of the nine propositions given here, only number 5 is central to the elective theory. The first four are special postulates chosen as an extreme but self-consistent set; however, they might well be subject to denial or modification without impairing the validity of the elective approach. The last four propositions are stated to account for the general features of antibody formation in cellular terms and may be equally applicable to instructive and elective theories. If this theory can be defended, and I know of no fatal refutation of it, then clearly elective theories of antibody formation perhaps less doctrinaire in detail should have a place in further experimental design, each proposition being evaluated on its own merits. I am particularly indebted to Burnet (*13*) for this formulation, but Burnet should not be held responsible for some elaborations on his original proposal, especially in propositions 1 through 4. A connected statement of the nine propositions is given in Table 1, and each one is discussed in detail in the following sections.

Antibody Globulin

A1. *The stereospecific segment of each antibody globulin is determined by a unique sequence of amino acids.*

This assertion contradicts the more popular notion, and the usual basis of instructive hypotheses, of a uniform sequence subject to differential folding. The chemical evidence is far from decisive. For example, Karush (*14*) rejects this proposition not on analytical evidence but on the cogent argument that miscellaneous antigenic compounds can scarcely convey instructions for sequence. But if instructive-sequence is implausible, this perhaps argues against instruction rather than differential sequence. Karush has also demonstrated the remarkable stability of antibody through cycles of exposure to denaturing concentrations of urea. He attributes the structural continuity to stabilizing disulfide linkages, but determinant amino acid sequences may also be involved.

Elective antibody formation is of course equally compatible with sequence or folding. In such a theory, the mechanism of assembly does not have to be specified, so long as the product (the prospective antibody) recognizes—that is, reacts with—the antigen. Differential sequence is proposed (i) to stress the ambiguity of present evidence and (ii) as being more closely analogous to current conceptions of genically controlled specificity of other proteins (*15*).

The direct analysis of antibody structure by physicochemical methods has been equivocal. The fractionation of globulins by partition chromatography (*16*) might be interpreted by differential exposure of phenolic, amino, and carboxyl groups rather than differences in essential composition. Characterization of amino acid composition has given sharply different results with rabbit globulins, on the one hand, and equine and human globulins, on the other. Rabbit globulins, including various antibodies, apparently have a uniform N-terminal sequence, so far identified for five residues as (*17*):

Alanine-leucine-valine-aspartic-glutamyl

Various antibodies were, furthermore, indistinguishable in over-all composition (*18*). Any chemical differences would then have to attach to a central, differential segment. This possibility is made more tangible by Porter's recent finding (*19*) that rabbit antibody globulin could be split by crystalline papain into three fragments. One of these was crystallizable (and presumably homogeneous), devoid of *antibody* activity, but equivalent as an *antigen* to the intact globulin. The remaining fractions were more heterogeneous and retained the antigen-combining specificity of the intact antibody. As these fractions may well correspond to the differential segments, their

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Table 1. Nine propositions.

A1. The stereospecific segment of each antibody globulin is determined by a unique sequence of amino acids.
A2. The cell making a given antibody has a correspondingly unique sequence of nucleotides in a segment of its chromosomal DNA: its "gene for globulin synthesis."
A3. The genic diversity of the precursors of antibody-forming cells arises from a high rate of spontaneous mutation during their lifelong proliferation.
A4. This hypermutability consists of the random assembly of the DNA of the globulin gene during certain stages of cellular proliferation.
A5. Each cell, as it begins to mature, spontaneously produces small amounts of the antibody corresponding to its own genotype.
A6. The immature antibody-forming cell is hypersensitive to an antigen-antibody combination: it will be suppressed if it encounters the homologous antigen at this time.
A7. The mature antibody-forming cell is reactive to an antigen-antibody combination: it will be stimulated if it first encounters the homologous antigen at this time. The stimulation comprises the acceleration of protein synthesis and the cytological maturation which mark a "plasma cell."
A8. Mature cells proliferate extensively under antigenic stimulation but are genetically stable and therefore generate large clones genotypically preadapted to produce the homologous antibody.
A9. These clones tend to persist after the disappearance of the antigen, retaining their capacity to react promptly to its later reintroduction.

further immunological and chemical analysis will be of extraordinary interest.

In contrast to the uniformity of rabbit globulins, normal and antibody globulins of horse serum proved to be grossly heterogeneous but equally so, a wide variety of N-terminal groups being found in all preparations (*20*). This merely confirms the concept of the plurality of antibodies evoked by a given antigen, which have in common only the general properties of normal gamma globulins and the capacity of reacting with the evoking antigen. The globulins of man, and in particular the characteristic globulins produced by different patients suffering from multiple myeloma, are likewise recognizably different, inter se, in amino acid composition (*21*).

Gene for Globulin Synthesis

A2. *The cell making a given antibody has a correspondingly unique sequence of nucleotides in a segment of its chromosomal DNA: its "gene for globulin synthesis."*

This postulate follows plausibly from proposition A1, and would trace antibody-forming specificity to the same source as is imputed to other specific proteins. As the most deterministic of genetic hypotheses, it should be the most vulnerable to experimental test. For example, a single diploid cell should be capable of *at most* two potentialities for antibody formation, one for each chromosome.

In tests of single antibody-forming cells from rats *simultaneously* immunized against two *Salmonella* serotypes, Nossal and I (*22*) could find only monospecific cells producing one or the other antiflagellin. Coons (*23*) and White (*24*) have reached a similar conclusion in applications of fluorescent labeling technique. However, Cohn and Lennox (*25*) have convincing evidence for some bispecific antibody-forming cells in rabbits *serially* immunized against two bacteriophages. Experiments pertinent to the possibility of a single cell's carrying more than two antibody-forming specificities remain to be done (*26*).

The chromosomal localization of antibody-forming specificity is uncoupled from its elective origin in proposals (*7, 8, 27*) that an antigen induces a mutation in a gene for globulin synthesis, though not necessarily involving a new nucleotide sequence.

Multiple specificity would stand against a simple chromosomal basis for antibody formation (*28*), leaving two alternative possibilities: (i) replicate chromosomal genes or (ii) extrachromosomal particles such as microsomes. These might best be disentangled by some technique of genetic recombination.

The differentiation of microsomes must be implicit in any current statement of a theory of antibody formation that recognizes their central role of protein synthesis. The main issue is whether or not their specificity is dependent on that of the chromosomal DNA. Autonomy of microsomes, in contradiction to proposition A2, is implicit in most instructive theories, the microsome carrying either the original or a copy of the antigenic message. On the other hand, a powerful elective theory is generated by substituting the term *microsomal RNA* for the terms *chromosomal DNA* and *gene* in the various propositions. Since a single cell may have millions of microsomes, this theory would allow for any imaginable multiplicity of antibody-forming information in a single cell. If the potential variety of this information approaches that of the total antibody response, further instructions in an antigenic input would become moot. In addition, the complexities of selection of cellular populations would be compounded by those of microsomal populations within each cell. These degrees of freedom which blur the distinction between microsomal instruction and election favor the utility of the chromosomal hypothesis as a more accessible target for experimental attack.

Genic Diversity of Precursor Cells

A3. *The genic diversity of the precursors of antibody-forming cells arises from a high rate of spontaneous mutation during their lifelong proliferation.*

Three elements of this statement should be emphasized: (i) that antibody-forming cells are specialized, (ii) that their diversity arises from some random process, and (iii) that the diversification of these cells continues, in company with their proliferation, throughout the life of the animal.

Item (i) and its justification by various experiments have already been discussed as an aspect of proposition A2. Talmage (*2*) also stresses the specialization of antibody-forming cells by referring to their progressive *differentiation*. This is entirely consistent with propositions A3 and A4, which then postulate a specific mechanism of cellular differentiation, in this case, gene mutation. If, on Talmage's model, fully differentiated cells are ultimately left with no more than one antibody-forming specificity per chromosome, the general consequences will be the same whether this final state represents the unique activation of one among innumerable chromosomal loci (see *27*) or the evolution of one among innumerable specific alleles at a given locus. Once again, the final resort for decision may have to be a recombinational technique.

If the discrepancy between the experiments of Nossal and Lederberg (*22*) and those of Cohn and Lennox (*25*), as dis-

cussed under proposition A2, is real and depends on the timing of immunization, it may furnish strong support for (ii), the random origin of antibody-forming specificity. If antibody-forming cells can have two (or any small number of) specificities randomly derived, only a negligible proportion will have just the two being tested for. This would correspond to the case of simultaneous immunization with the two test antigens. If, however, a population of cells carrying one specificity is selected for, followed by selection for a second specificity among all available cells, this is the case of serial immunization and is precisely the method one would predict to obtain a clone "heterozygous" for two mutant alleles. Simultaneous versus serial immunization would be analogous to the suppression versus selection of bacterial mutants resistant to two antibiotics (*29*). Further experiments are needed to exclude more trivial reasons for the scarcity of bispecific antiflagellin-forming cells.

Item (iii) diverges from Burnet's proposal that the "randomization" of antibody-forming cells is confined to *perinatal* life, thereby generating a set of then stable clones corresponding to the antibody-forming potentiality of the animal. These clones would then be irreplaceable if lost either by random drift or as a consequence of premature exposure to the corresponding antigen. The arguments against Burnet's proposal are by no means decisive; however, the correspondence between cells and antibodies is made more difficult by having to maintain each clone at a sufficient population size to compensate for loss by random drift. Further, the recurrence of antibody-forming specificity is supported by experiments showing the decay of immune tolerance in the absence of the corresponding antigen (*30*; see comment on proposition A6). Since immune reactivity in these experiments may return during adult life, susceptibility to the induction and maintenance of tolerance by the timely introduction of the antigen may have only a coincidental relationship to the immunological incompetence of the newborn animal.

Hypermutability

A4. *This hypermutability consists of the random assembly of the DNA of the "globulin gene" during certain stages of cellular proliferation.*

This *ad hoc* proposal is doubtless the least defensible of the propositions, and certainly the furthest removed from experimental observation. It is stated to illustrate that accurate replication rather than mutability is the more remarkable phenomenon, whatever the detailed mechanism for the variation. If, as has been suggested, many nucleotide triplets are *non*sensical (*31*), the triplets rather than single nucleotides would have to be posed as the unit of assembly in this case.

To carry this speculation one step further, *heterochromatin* has been proposed to be, on the one hand, a random sequence, and, on the other hand, a dissynchronously assembled segment of the genome (*32*). If both views are correct, proposition A4 might be restated: "the globulin gene is heterochromatic during certain stages of cellular proliferation" (becoming by implication, euchromatic in the mature stages of propositions A8 and A9).

For the theory of microsomal election it might be postulated that globulinogenic microsomes are initially fabricated as faulty replicas of the globulin gene, but are then capable of exact, autonomous replication.

Pending more exact knowledge and agreement of opinion on the morphogenetic relationships of antibody-forming cells, the term *certain stages* cannot be improved upon. On the other hand, as is shown under proposition A8, a model might be constructed even on the basis of a constant but high mutation rate of all antibody-forming cells.

Further insight into the mechanism of cellular diversity in antibody formation may be won by studies on the genetic control of reactivity to various antigens in inbred animals (*33*); two cautions, however, must be stated: (i) for effects on the transport of particles of different size, and (ii) for effects from cross-reactions with gene-controlled constituents evoking autotolerance.

Spontaneous Production of Antibody

A5. *Each cell, as it begins to mature, spontaneously produces small amounts of the antibody corresponding to its own genotype.*

Note the implication that antibody is formed prior to the introduction of the antigen into the antibody-forming cell.

The function of spontaneous antibody is to mark those cells preadapted to react with a given antigen, either to suppress these cells for the induction of immune tolerance (proposition A6) or to excite them to massive antibody formation (proposition A7). Therefore, the antigen need participate in no type of specific reaction with cell constituents other than antibody itself, the one type of reaction available to chemically diverse antigens that requires no further special pleading. There is no agreement whether the reactive globulins found in the serum of untreated animals are produced spontaneously or by casual exposure to cross-reacting antigens (see 2). Accordingly, the spontaneous antibody postulated in proposition A5 may or may not be produced in the quantity and form needed for it to be liberated and detected in the serum. The nonspecific fragment of antibody-globulin described by Porter raises the possibility that the same *determinant* segment may be coupled either to a diffusible or to a cell-bound residue, the latter corresponding to various aspects of cellular immunity, including the suppression or excitation of antibody-forming cells by reactions with the corresponding antigen.

Induction of Immune Tolerance

A6. *The immature antibody-forming cell is hypersensitive to an antigen-antibody combination: it will be suppressed if it encounters the homologous antigen at this time.*

This is the first of four propositions which bear less on the source of antibody-forming specificity than on its subsequent expression in terms of cellular behavior. These propositions are therefore equally applicable to instructive theories.

The duality of reactions of antigens with antibody-forming cells is simply a restatement of the experimental observations of tolerance versus immunity (*34*). It seems plain that every cell of the antibody-forming system must be marked to inhibit its reactivity both to the autologous antigens of the same animal and extraneous antigens introduced and maintained from a suitably early time of development. In the light of current evidence for the persistence of antigenic molecules (*5, 6*) and for the loss of tolerance when a given antigen has dissipated (*30*) there are no more plausible candidates for the self-markers then the antigens themselves. The distinction between the function of an antigen as inhibitor (self-marker) or as inducer of antibody formation is then the time when the antigen is introduced into the potential antibody forming cell. We may profitably define maturity in terms of

the progression of the cell from sensitivity towards reactivity.

The suppression of this process of maturation is a sufficient attribute to account for tolerance, and this need not involve so drastic an event as the destruction of the cell. However, the elective hypothesis proposes that only a limited number of cells will spontaneously react with a given antigen, so that their destruction by premature reaction can safely be invoked as the means of their suppression. It may be hoped that presently documented phenomena of cellular hypersensitivity may furnish a precedent for cellular destruction by such reactions. The cytotoxicity of the antigen to hypersensitive cells is still controversial even in the historical case of tuberculin sensitivity (*35*). However, the destruction of invading *lymphocytes* of the host in the course of rejection of a sensitizing homograft (*36*) supports the speculation of some role of cellular destruction of immature antibody-forming cells in the induction of tolerance.

The nature of immaturity remains open to question. It might reflect the morphogenetic status of the antibody-forming cell—for example, sensitive lymphocyte → reactive plasma cell (*37*), some particular composition of immature sensitizing antibody, or merely a very low level of antibody so that complexes are formed in which antigen is in excess.

Finally, one additional hint of an implication of hypersensitivity in the early stages of the antibody response: the transient skin sensitivity of delayed type (and transferable by cells) appearing in the course of immunization, as observed by several workers (*38*). If these skin reactions reflect the destruction of some antibody-forming cells, it would speak for some overlapping or reversibility of the two stages of maturation.

The implications of proposition A6 in the elective theory may be summarized as follows: If an antigen is introduced prior to the maturation of any antibody-forming cell, the hypersensitivity of such cells, while still immature, to an antigen-antibody reaction will eliminate specific cell types as they arise by mutation, thereby inducing apparent tolerance to that antigen. After the dissipation of the antigen, reactivity should return as soon as one new mutant cell has arisen and matured. As a further hopeful prediction, it should be possible to induce tolerance in clones of antibody-forming cells from adult animals by exposing a sufficiently small number of initials to a given antigen.

Excitation of Massive Antibody Formation

A7. *The mature antibody-forming cell is reactive to an antigen-antibody combination: it will be stimulated if it first encounters the homologous antigen at this time. The stimulation comprises an acceleration of protein synthesis and the cytological maturation which mark a "plasma cell."*

These principles of the cellular response to *secondary* antigenic stimulation are widely accepted and are readily transposed to the *primary* response on the elective hypothesis whereby some cells have spontaneously initiated antibody formation according to proposition A5.

Proliferation of Mature Cells

A8. *Mature cells proliferate extensively under antigenic stimulation but are genetically stable and therefore generate large clones genotypically preadapted to produce the homologous antibody.*

This proposition takes explicit account of the secondary response, the magnitude of which is a measure of the increase in number of reactive cells (*26*). However, the antigen *need* play no direct part in the stabilization of antibody-forming genotype which might accompany the determinate maturation of the cell whether or not it is stimulated. In fact, it may be possible to dispense with the postulate that mature cells are less mutable by adopting a mutation rate which is an effective compromise: to furnish a variety of genotypes for the primary response while selected genotypes may still expand for the secondary response. For example, by mutation of one daughter chromosome per ten cell divisions, on the average, after ten generations about 600 chromosomes of the same type would have been produced, together with 100 new genotypes distributed among the other 400 or so cells. Selection must then compensate for the mutational drift if a given clone is to be maintained.

Persistence of Clones

A9. *These clones tend to persist after the disappearance of the antigen, retaining their capacity to react promptly to its later reintroduction.*

This is a restatement of the possibly controversial phenomenon of lifelong immunity to viruses (*4, 5*). A substantial reservoir of immunological memory should be inherent from one cycle of expansion of a given clone. Its ultimate decay might be mitigated either by continued selection (that is, persistence of the antigen) stabilization of genotypes, or dormancy (to cell division or remutation, or both) on the part of a fraction of the clone.

Discussion

Each element of the theory just presented has some precedent in biological fact, but this is testimony of plausibility, not reality. As has already been pointed out, the most questionable proposition is A4, and it may be needlessly fanciful to forward a too explicit hypothesis of mutability for antibody formation when so little is known of its material basis anywhere.

Theories of antibody formation have, in the past, been deeply influenced by the physiology of inducible enzyme synthesis in bacteria. In particular, instructive theories for the role of the substrate in enzyme induction have encouraged the same speculation about antibody formation. This interpretation of enzyme induction, however, is weakened by the preadaptive occurrence of the enzymes, at a lower level, in uninduced bacteria (*39*).

One of the most attractive features of the elective theory is that it proposes no novel reactions: the only ones invoked here are (i) mutability of DNA; (ii) the role of DNA, presumably through RNA, as a code for amino acid sequence and (iii) the reaction between antibody and antigen, already known to have weighty consequences for cells in its proximity. The conceptual picture of enzyme induction would be equally simplified if the enzyme itself were the substrate-receptor. Clearly, susceptibility to enzymic action is not a necessary condition for a compound to be an inducer —for example, neolactose and thiomethylgalactoside for the β-D-galactosidase of *Escherichia coli* (*39, 40*), but formation of complexes with the enzyme may be. The picture is somewhat complicated by the intervention of specific transport systems for bringing the substrate into the cell (*40*).

Antibody formation is the one form of cellular differentiation which inherently requires the utmost plasticity, a problem for which the hypermutability of a patch of DNA may be a specially evolved solution. Other aspects of differentiation

may be more explicitly canalized under genotypic control. Nucleotide substitution might still play a role here by modifying the level of activity rather than the specificity of neighboring loci, and elective recognition of transient states spontaneously derived then remains as a formal, if farfetched, possibility for other morphogenetic inductions.

References and Notes

1. This definition excludes antibody-like substances such as the hemagglutinins found in normal human sera. These reagents do not, however, pose the problem of the mechanism of specific response which is the burden of this discussion.
2. Talmage, in this issue of *Science*, discusses various aspects of antibody specificity, including the number of antibodies, which may be exaggerated in current immunological thought. For the present discussion, however, this number is left open for experimental determination, for it would embarrass a theory of cellular selection only if it is large compared with the number of potential antibody-forming cells in the organism. To anticipate proposition A1, as few as five determinant amino acids would allow for $20^5 = 3{,}200{,}000$ types of antibody.
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26. An indirect measure of polyspecificity would be the total number of antibodies multiplied by the proportion of competent cells *initially* recruited to yield a particular species. Coons (7) has not attempted to count the antibody-forming cells in primary response, but his statements are compatible with an incidence of 10^{-6} to 10^{-5} of cells forming antialbumin in lymph nodes 4 days after inoculation. Nossal (*Brit. J. Exptl. Pathol.*, in press) found about 2 percent of yielding cells in a primary response after 7 days. These figures are subject to an unknown correction for the extent of proliferation in the interval after inoculation. They perhaps also raise the question whether all the yielding cells are indigenous to the lymph node, or whether circulating cells of appropriate type can be filtered by a node in which locally administered antigen has accumulated.
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