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## THEORIES OF ANTIBODY FORMATION: A REVIEW**

Like many other subdivisions of biology, immunology is in the midst of a period of great productivity and rapid accumulation of knowledge. In an attempt to classify this information in some orderly fashion, several new and controversial theories have been introduced in recent years. A greater understanding of the entire field may result from familiarity with its theoretical foundations. The development of the present theories of antibody formation serves as an outstanding example of the rapid progress of scientific knowledge and its interpretation; accordingly, an historical review may help to clarify current ideas. In order to evaluate current theories, we will refer to some of the recent advances that have been made in the past five years. Most of this information will necessarily be in the form of brief comments or in tabular form with references to the original publications or to more comprehensive reviews.

A unifying hypothesis is central to a system of ordered knowledge, broad theories being especially successful in the physical sciences. Similarly, the cell theory, the germ theory of disease, the theory of evolution, and the like, have advanced understanding in biology. Of course, we must re-emphasize, as did the great physicist, J. J. Thomson, that . . .

> a theory . . . is a policy rather than a creed; its object is to connect or coordinate apparently diverse phenomena and above all to suggest, stimulate, and direct experiment. It ought to furnish a compass which, if followed, will lead the observer further and further into previously unexplored regions. Whether these regions will be barren or fertile experience alone will decide.[1]

### EARLY THEORETICAL FORMULATIONS

Although vaccination had been introduced by Jenner[2] almost a century before, Pasteur must be credited with the concept of preventive inoculation,

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and thus, with having established the scientific foundation of immunology. In 1880, he showed how attenuated microorganisms could be used to prepare vaccines for chicken cholera,[3] and, in the following year, similar developments occurred with anthrax.[4,5] In 1880, he also advanced his "exhaustion theory,"[6] which suggested that immunity is attributable to the disappearance from the body of some nutrient that had been consumed by the first attack of the microorganism. On the other hand, Chauveau's "retention theory," also of 1880, postulated that, in its first invasion, the microbe left behind some substance that prevented a successful subsequent attack.[7] Both of these theories failed to explain the positive response of the organism, the active production of antibody, and soon were discarded. During this period, the Russian biologist, Metchnikoff, working primarily at the Pasteur Institute, was collecting data supporting the theory that specialized cells of the body attack and destroy infecting agents by a process called phagocytosis. The major premises of Metchnikoff's theory have been solidly supported by subsequent data and there is little question of their validity today, although they were bitterly attacked in his lifetime, particularly by those who viewed humoral and cellular defense mechanisms as mutually exclusive. Further reference to Metchnikoff's important work[8] will not be made, since it is of primary importance to focus attention on the production of humoral antibody.

The first description of the bactericidal action of blood was presented by von Fodor of Budapest.[9,10] The material in plasma responsible for this effect was further characterized by Hans Buchner of Munich[11,12] and called alexin (more commonly called complement today). Buchner formulated a doctrine of humoral immunity,[13] based on this work. He suggested that bacteria are destroyed by heat labile, nonspecific alexins in the humors of the body, and that toxins are modified in the body into specific, heat stable antitoxins. Thus, specificity was assured because the antigen became a part of the antibody.[14] This theory had a brief period of popularity but could not be reconciled with the fact that each molecule of toxin or toxoid caused several hundred or even thousands of molecules of antitoxin to be formed. Many years later, when the theory almost had been forgotten, more direct proof of its impossibility became available.[15] Paul Ehrlich elaborated the first hypothesis of antibody formation of more than transient interest; this, together with his many other brilliant contributions, earned for this versatile genius the title of "founder of immunochemistry."

## THE SIDE-CHAIN THEORY OF EHRLICH

Ehrlich first elaborated his "side-chain" theory of antibody formation in 1897 as a digression in describing the assay of the activity of diphtheria antitoxin that he had formulated.[16] Some aspects of the theory may be found in his writings as early as 1885,[17] but the fullest development of the theory appeared in his Croonian Lecture to the Royal Society of London in March 1900.[18]

Ehrlich was a physician with a chemical approach to problems of prevention and treatment of communicable diseases. One of his quantitative studies showed that a toxin maintained its ability to combine with a fixed amount of a standardized antitoxin, but that the toxic properties diminished with time.

> Treated from the chemical standpoint, this circumstance was most simply explained by assuming that the toxin was characterized by the possession of at least two different combining groups: one, which may be designated haptophore, conditions the union with antitoxin, while the other group, which may be designated toxophore, is the cause of the toxic action. From the constancy of the combining capacity, and the diminution in the toxicity, it was to be inferred that the toxophore group was very unstable, but the haptophore group more stable, and also that the deterioration of the toxophore group proceeded of necessity quite independently of any relation to the haptophore group.[18]

Thus, toxoid was a toxin with its toxophore groups destroyed. To Ehrlich, a normal cell had "side-chains" that would unite with certain nutrients that the cell required. In certain organs some of these side-chains had receptors that by pure coincidence were chemically specific for the haptophore, and by analogy, would fit as male and female screw (Louis Pasteur) or as lock and key (Emil Fischer). Such a union prevented the nutritive function of the side-chain and, therefore, this defect was repaired by regeneration. "The antitoxins represent nothing more than the side-chains, reproduced in excess during regeneration and therefore pushed off from the protoplasm—thus to exist in a free state."[18] Certain immune sera, particularly those prepared against erythrocytes or bacteria, could contain immune bodies with two different haptophores, one combining with the specific antigen, and the other combining at a different receptor site exclusively with complement (Bordet) or alexin (Büchner) and leading to lysis of the involved cell. Ehrlich also pointed out that an organism will never make antibodies against itself—his famous doctrine of "horror autotoxicus."

For more than 30 years, Ehrlich's theory served to explain the available facts and to stimulate further inquiry. The studies of Landsteiner[19] showed,

however, that a number of relatively simple chemical groups (haptens) could be covalently linked to animal proteins. When these conjugates were injected into test animals, antibodies specific for the haptenic group were produced. It seemed that the number of chemical groups that could be synthesized and conjugated was limited only by the chemist's industry and imagination. Accordingly, it appeared, and is still presumed by many, that the number of possible antibodies an animal can produce is as great as the possible antigens, *i.e.,* the number is almost infinite. If this be true, it is unlikely that enough side-chains of different specificity could be present in any animal to fulfill this potential demand. Thus, Ehrlich's theory could not meet this objection and its popularity was lost.

### DIRECT TEMPLATE THEORY

In the 30 years after the Croonin Lecture, a considerable body of immunological data accumulated, in addition to those provided by the studies of Landsteiner. Thus, the facts that led to rejection of Ehrlich's side-chain theory also provided the basis for a new hypothesis, born independently in three separate places, that came to be known as the direct template theory. As developed by Breinl and Haurowitz,[20] by Alexander,[21] and by Mudd,[22] it was postulated that the antigenic material is brought to the site of synthesis of globulins after it is injected into the tissues of the animal. In the subsequent synthesis of globulin the peptide chain is composed of those amino acids that would constitute the con-figuration most nearly complementary to the reactive sites of the antigen, allowing a maximum opportunity for interaction between the antigen and the antibody. In this manner, specificity not only is conferred upon the globulin, so that it acquires the properties of antibody, but also specificity is determined by the sequence of amino acids within the protein. Thus, within a given species, antibodies would differ from each other and from normal gammaglobulin by the sequence and nature of the amino acids.[23] This theory was modified in 1940 by Pauling,[24] who assumed that the globulin molecule is a single polypeptide chain, the specificity of which is deter-mined by its three-dimensional configuration. In his own words,

> The effect of an antigen in determining the structure of an antibody molecule might involve the ordering of the amino acid residues in the polypeptide chain in a way different from that in the normal globulin as suggested by Brienl and Haurowitz and Mudd. I assume however, that this is not so, but that all antibody molecules contain the same polypeptide chain as normal globulin, and differ from normal globulin only in the configuration of the chain; that is, in the way that the chain is coiled in the molecule.[24]

Pauling's theory gained widespread acceptance and has been one of the major determinants of subsequent research activity. The concept of antibody-antigen interaction was so brilliantly conceived that the diagrams in Pauling's original article resemble recent electron micrographs of antibody-antigen interactions.[25] When radioisotopes became available, however, it was demonstrated that antibodies against a specific antigen are made *de novo* from recently injected, radioactively labeled amino acids and that no radioactivity can be detected in the preformed globulins,[26] as would be required by the early statements of Pauling.[24, 27] The suggestion that the amino acid sequence (primary structure) of all antibody gammaglobulins is the same, and that specificity is determined by the tertiary structure (folding in space) of the antibody molecule, is no longer tenable. Gitlin and Merler[28] showed that the "fingerprints" of peptides of related antibodies are different, while Koshland and Englberger[29] demonstrated that the amino acid composition of related antibodies is not identical; thus, the primary structure of antibodies is not uniform, as would be required by Pauling's theory. Studies of the completely characterized protein, bovine pancreatic ribonuclease, indicate that its tertiary structure and its biological activity are determined by its primary structure.[30-32] It is likely that these observations soon will be extended to ascertain whether hemoglobin, insulin, and gammaglobulin behave in a similar manner.

## INDIRECT TEMPLATE HYPOTHESES

In a broad-ranging essay and review in 1941, Burnet[33] criticized the direct template theory of Haurowitz and Mudd because it failed to account for the fact that (1) a second or subsequent contact with the same antigen causes increased antibody production (the secondary response), (2) antibody production can continue long after the antigen responsible has disappeared from the body, (3) antibody production is a function not only of the cell originally stimulated, but of its descendants, and (4) the type of antibody produced varies (a) according to the species used, (b) with the age of the animal, and (c) according to the nature and frequency of the antigenic stimulus. Having disposed of this theory, as well as those of Sabin[34] and Jordan[35] (which we will not elaborate upon, because they did not contribute importantly to the development of theories of antibody formation), Burnet suggested a somewhat vague hypothesis in terms of the proteinase theory of protein synthesis. The latter theory, of Bergmann and Niemann,[36, 37] suggested that proteinases are enzymes with both proteolytic and synthetic activities that possibly could even replicate their own

structural pattern. Burnet applied this concept to that of antibody formation by assuming that the serum globulins concerned in immunological reactions are synthesized within the cells of the reticuloendothelial system by proteinase units of the cells. According to this view, proteinases are modified by contact with antigen which destroys the antigen and leads to antibody formation.

In the following eight years, Burnet and Fenner were impressed with (a) the progress in understanding of enzyme induction in bacteria, (b) with the observations of Owen[39] on bovine red cell chimeras, and (c) with the persistence of antibody for many years after exposure in the apparent absence of the antigen. The tentative proteinase theory of 1941 was modified to their indirect template theory. They postulated that in the antibody-forming cell, antigen modifies a globulin-synthesizing enzyme to produce a proteinase that normally is not a constituent of the cell. This enzyme would synthesize the globulin that reacts specifically with the antigen. The proteinase would be reproduced with replication of the antibody-forming cell, so that antibody formation could continue after the antigen had disappeared from the tissues. It was postulated that the same system of cells which eliminated foreign organic material with an antibody response was also concerned with destruction of the body's own aged cells without induction of antibodies. In order to allow this differentiation, expendable body cells would carry "self-marker" components which would allow a recognition of their "self" character. Antigens in general could be substances of similar chemical nature with the addition of the marker conferring a slightly different molecular configuration that results in its specificity. The most important result of this theory was the prediction that any markers to which the fetus was exposed would be recognized as "self-markers." Actually this had already been observed by Owen and colleagues[39-41] in their studies of dizygotic twin cattle with coexisting blood groups of the same system in each circulation as a result of intra-uterine circulatory admixture of stem cells. Owen's observations and the stress placed on them by Burnet and Fenner stimulated Billingham, Brent, and Medawar [42, 43] to expose embryos to foreign donor cells. They showed that after birth these animals would accept skin grafts from the donor whose markers were now recognized as "self."

Although Burnet abandoned his indirect template theory in favor of his clonal selection theory, other workers have continued to expand the indirect template theory. Schweet and Owen[44] suggested that the antigen must modify the DNA of the cell, since it is the DNA that directs protein

synthesis and only in this way can the change be genetically propagated to subsequent cell generations. This propagation either would be directed by an antigen-modified DNA or the antigen could be passed on to daughter cells, with modification of each new generation of DNA. Monod[45] has called attention to the marked similarity between induced enzymes and induced antibody formation. They are both stereospecific. He has suggested that this demonstrates the same fundamental property of the living cell, namely, its ability to learn and to reproduce, in the form of the combining structure of a protein, certain elements of structural information. Both enzyme and antibody production are examples of protein synthesis. In both cases, the proteins are only slight structural modifications of macromolecules ordinarily produced by the cell; thus, the cell would modify a pre-existing and already functioning protein-synthesizing mechanism. It has been established that the new protein is synthesized from amino acids and not from pre-existing proteins.[38] In both systems more than one molecule is synthesized in response to one molecule of either inducer or antigen. All or some part of the inducer or antigen remains fixed in the cell and thereafter is distributed to daughter cells. The differences in the two systems also are considerable. The most telling difference is that the number and variety of antibodies that an organism of a given type can produce is potentially very great. On the other hand, the number, type, and structure of the enzymes that can be induced within one species of bacteria is strictly limited and is determined by the genome. Monod also attempts to draw a parallel between the secondary response to antigens and the presence of permeases in bacterial cells that permit the concentration of inducing molecules, which are then passed on to daughter cells. This could be regarded as analogous to immunological memory, since the permease concentrates its own inducer, which also happens to be an inducer for other proteins in the cell. Under certain conditions it behaves as an auto-catalytic system. By analogy, Monod suggests the possibility of an "antigen-capture" model that behaves like a "pseudogenetic" permease.

## THE NATURAL SELECTION THEORY

In 1955, Jerne[46] proposed the natural selection approach to antibody synthesis. He suggested:

> Among the population of circulating globulin molecules there will, spontaneously, be fractions possessing affinity toward any antigen to which the animal can respond. These are the so called "natural" antibodies. The introduction of an antigen into the blood or into the lymph leads to the selective attachment to the antigen surface of those globulin molecules which happen to have complementary configuration. The

antigen carrying these molecules may then be engulfed by a phagocytic cell. When the globulin molecules thus brought into a cell have been dissociated from the surface of the antigen, the antigen has accomplished its role and can be eliminated.[46]

Presumably the cell then replicates the gammaglobulin that has been selected for it by the antigen.

It will be noted that Jerne's approach is strikingly similar to Ehrlich's theory, although the words are different and the discussion is more modern. After half a century, the pendulum has swung back. One might object to the natural selection theory on the grounds that there is no evidence for the natural antibodies it presumes. In fact, Weiner[47] had reviewed the question in detail with respect to blood group antibodies and demonstrated that all these antibodies either resulted from passive transfer across the placenta or, more commonly, by postnatal stimulation after exposure to the antigen itself or to a cross-reacting antigen. Subsequent experience has served to confirm Weiner's conclusion.[48]

An additional objection to Jerne's theory is that it fails to explain the actual mechanism of antibody synthesis, but implies that an extra-cellular protein (globulin) causes its own replication. Such a concept was acceptable in the 1930's and 1940's, but not in 1955. All recent sudies of protein synthesis have indicated that protein never serves as a template for its own replication. According to current theory,[49-51] the deoxyribonucleic acid (DNA) carries the genetic information that dictates all protein synthesis. Its information is coded as sequences (probably triplets) of nucleotides, which probably are repressed to varying degrees in different cells by histones. The information for directing the synthesis of a specific protein is transferred from the DNA to a ribonucleic acid (RNA) of complementary base composition (with uracil replacing thymine) known as template or messenger RNA.[52] This material attaches to ribosomes and determines the order in which the amino acid-activating enzyme will attach the amino acyl-transfer RNA to the growing peptide chain and thereby will determine the primary structure of the protein. Although this version of protein synthesis is oversimplified, any theory of antibody formation must conform to modern concepts of protein synthesis.

## CELLULAR SELECTION THEORIES

In reviewing the concepts of allergy and immunity in 1957, Talmage[53] re-examined the theories of antibody synthesis and his comments have had a major impact on subsequent ideas in the field. Accordingly they are elaborated in some detail, although this necessarily recapitulates some

of the material previously presented. He classified the theories on the basis of the biochemical mechanism involved in three (oversimplified) categories.

The first type is what he calls the direct template hypothesis which assumes that the antigen is taken into the antibody-producing cell in such a way that it can act as a mold or a template to impress a complementary pattern on the globulin molecules synthesized by the cell. Such a theory has been held or elaborated upon by Breinl and Haurowitz,[20] by Alexander,[21] by Mudd,[22] by Pauling,[24] by Marrack,[54] by Cannon[55] and most recently by Karush.[56, 57]

The second type, the adaptive enzyme theory (also called indirect template hypothesis) assumes that all aspects of protein synthesis are under genetic control and a foreign pattern modifies the globulin-synthesizing apparatus in the cell. The "information" carried by the antigen must be incorporated into the genome, allowing the production of a new ("indirect") template that will persist by replication under genetic control in cells descended from that primarily modified by the antigen. Similar theories had been proposed by Burnet and Fenner[38] prior to Talmage's review and have since been elaborated upon by Schweet and Owen,[44] by Monod,[45] and by Szilard.[58]

A third type of theory based on natural selection suggests that all immunological competence and antibody production are determined wholly at the genetic level. The function of the antigen in these theories is simply to react with those globulin-synthesizing units with which it has the greatest affinity and to induce them to replicate and to produce additional globulin of the same specificity. The theories of Ehrlich and Jerne are in this category. Talmage perferred Ehrlich's theory because of its emphasis on the cell as producer of antibody based upon its own hereditary abilities. Jerne's hypothesis, the direct template theory, and the adaptive enzyme theory, all require the modification of a cell's normal synthetic activity by exogenous substances. Also, the available evidence was in favor of the cell as the basis of the specific anamnestic response.[59] Accordingly, Talmage proposed:

> As a working hypothesis it is tempting to consider that one of the multiplying units in the antibody response is the cell itself. According to this hypothesis, only those cells are selected for multiplication whose synthesized product has affinity for the antigen injected. This would have the disadvantage of requiring a different species of cell for each species of protein produced, but would not increase the total amount of configurational information required of the hereditary process.[58]

In essence, Talmage has returned to a cellular hypothesis similar to Ehrlich's, the important difference being that the antibody-producing cells multiply rather than the antibody itself. This concept was elaborated upon in much greater detail in a series of artcles by Burnet,[60,61] Lederberg,[62] and Talmage,[63] and in lectures[64,65] and popular reviews[66,67] by Burnet. In an expansion of his ideas, Talmage suggested that Haurowitz'[68] conservative estimate of 50,000 different antigenic patterns (therefore, by the antigen-template theory a similar number of antibodies) was excessive. In Talmage's view, immunological specificity is not based on a single molecular species of antibody reacting with a single pure antigen, but rather a series of molecules of slightly differing specificity with varying degrees of complementarity reacting to give the final mixture of antibody molecules that we detect as a specific antiserum. He assumes, based on the large number of cross reactions known,[69,70] that there may be only 5,000 different types of antibody molecules, each of which may react with perhaps one in 100 of all possible antigenic configurations. If this were true, there would be, according to his calculations, $3 \times 10^{120}$ different combinations possible, a number greater than the presumed number of electrons in the universe. This is a difficult concept to understand and may be more readily appreciated with the diagrams in the original paper.[63]

Talmage's speculations were an attempt to meet the objection that selective theories could not account for the almost infinite number of possible antigens. It should be clear, however, that there is little or no evidence as yet to support such speculations. On the next page of the same journal, Lederberg[62] presented his particular approach to a selective theory with some ideas borrowed from studies of bacterial genetics and protein chemistry. He assumed that the stereospecific segment of each antibody globulin was determined by a unique sequence of amino acids. If the active site was as small as five amino acids, it allowed for $20^5$ or 3,200,000 types of antibody molecules. The cell making a given antibody had a correspondingly unique sequence of nucleotides in a segment of its chromosomal DNA, *i.e.,* its "gene" for globulin synthesis. In this hypothesis, the genetic diversity of the precursors of antibody-forming cells arose from a high rate of spontaneous mutation during their lifelong proliferation. The remaining propositions of Lederberg were similar to those of Burnet and will not be considered separately. It should be noted that Lederberg reclassified the types of theories into only two categories, instructive and elective (same as selective). In the instructive group he included both the direct template and adaptive enzyme theories, and in

the elective group he included the selection theories of Ehrlich, Jerne, Talmage, Burnet, and Lederberg. This classification has become popular and is widely quoted. Unfortunately, Burnet has grouped together the theories of Ehrlich and Pauling as examples of instructive theories[64] which seems to be inconsistent with Lederberg's classification and the historical development of concepts of selectivity. Although Burnet's clonal selection theory differs in a great many respects, incorporating new ideas as well as discarding old ones, it is nevertheless, historically and conceptually (whether Burnet likes it or not), a descendant of Ehrlich's side-chain theory.

## THE CLONAL SELECTION THEORY

Burnet has written so extensively in defense of his theories that it is difficult to do them justice and still restate them briefly. One of the best summaries is given in his own words:

> The clonal selection theory is not concerned with how information carried by genetic units is eventually incorporated in the pattern of specifically functional protein. Its contention is that the whole experience of biology indicates that genetic information can be developed by genetic processes only—never by a meaningful impact of the environment on the genome. It seeks therefore to interpret the observed changes in antibody content or in resistance to infection as a result of genetic or epigenetic processes amongst the population of mesenchymal cells in the body.
>
> The main features of the clonal selection theory are: 1. the phenomena of immunity are based on the presence in the body of clones of immunologically competent cells which (a) can react directly with the corresponding antigenic determinant and (b) produce or give rise to cells which can synthesize and liberate antibody. 2. The wide range of clones necessary to cover all possible antigenic determinants is developed in the course of embryonic differentiation by a process based on hypermutability of the relevant region of the genome. This is followed by a progressive reduction in mutability sufficient to stabilize the various patterns produced by random mutation. 3. During embryonic life those clones which can react with antigenic determinants in the body are eliminated. In this way the 'information' needed to differentiate self from not-self is incorporated into the body.[65]

Recent research has caused Burnet to make several major modifications of his theory. The first arose out of the interpretation by Miller[71] that the thymus is responsible for populating the body with immunologically competent cells in some species. Accordingly, Burnet has made this modification in his theory indicating that the immunologically competent cells that may differentiate self from not-self are derived from the thymus shortly after birth,[72] and that the thymus also eliminates any cells that may react with "self" components. In addition, it has been shown by Simonsen[73-75] that approximately one in 20,000 cells of lymphoid character will

produce a reaction on the chorioallantoic membrane of the chick embryo when there are different genetically determined antigens in the embryo and the cells of the donor. This is a graft versus host reaction by the large lymphocytes of the donor. Burnet studied this problem at great length and summarized some of his conclusions, as follows:

> In all discussions of the clonal selection theory, it has been emphasized that the special virtue of the simple form of the theory is that it could be experimentally disproved. While it might be possible to devise *ad hoc* reasons by which the difficulties encountered in fitting the Simonsen reaction into the pattern might be overcome, the overall picture indicates that a straight forward clonal selection theory does not give an acceptable interpretation of the phenomena.
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> To account for the relatively small number of large lymphocytes per specific focus would demand a nonrandom distribution of patterns amongst the cell population. The fact that there is no clear increase in the number of foci on embryos differing by several antigenic factors over the number on embryos differing from the donor by only one factor, requires some rather special pleading for its explanation on the assumption that a subpopulation of competent cells is available for each antigenic determinant. The change in specificity of competent cells in the course of proliferation on passage indicates at least that any immunological pattern initially carried is highly labile.[76]

Some support for the clonal selection theory was provided by the single cell studies of Nossal and Lederberg,[77-80] which showed that single cells from sensitized animals exposed to two antigens produce antibodies to one or the other but never to both. These studies were supported by Coons[81] and by White.[82] In a very careful study, Attardi, Cohn, Horibata, and Lennox[83] found that two per cent of the single cells tested *did* produce two types of antibody against immunologically unrelated phage. This discrepency has not yet been resolved.

Finally, Trentin and Fahlberg[84] have shown that a single cell cloned from the spleen may be propagated in lethally irradiated animals and will repopulate the animal. Thereafter, the animal may react to as many as four different antigens. Assuming that these observations are correct and can be reproduced, Burnet, in the oral discussion following this paper, conceded that "this blows out the *original* clonal selection theory" and that the entire theory will have to be recast.[84] Perhaps the most important contribution of Burnet has been his emphasis on the importance of the differentiation of "self" from "not-self," or immunological tolerance. The explanation of this phenomenon is crucial to any theory of antibody formation. Burnet assumes that during embryonic development, "forbidden" clones that match "self" antigens will be eliminated as they arise. This is a one cell—one antibody theory of tolerance. If the clone or cell is

destroyed, then the organism is tolerant. There is no such thing as a tolerant cell; a tolerant cell is a dead cell.[85] However, in artificially induced tolerance, Smith and Bridges[86] have shown that $10^{10}$ molecules of antigen are required to maintain tolerance, indicating a discrepancy between the theory and the observation. Lederberg explains this by assuming that new mutations occur in the organism throughout its life and that these must be eliminated by antigen. This is still a poor explanation. No explanation thus far satisfactorily explains the hereditary autoimmune disease of NZB mice which develop a spontaneous hemolytic anemia as adults.[87]

Just before the completion of this review, a new hypothesis for the specific response in antibody synthesis appeared. In this theory, L. R. Finch[88] of the University of Melbourne attempts to modify the regulator-operon theory of Jacob and Monod[40] for the regulation of enzyme synthesis through the control of the corresponding messenger RNA in order to explain antibody protein synthesis. Burnet's clonal selection theory is assumed to explain all the other phenomena. The information for specific protein synthesis is present in the DNA of each clone cell but is normally repressed. The antigen is assumed to be an effector capable of combining with the repressor and the resulting de-repression permits messenger RNA synthesis on the operon, which in turn serves as template for polypeptide chain assembly. This is a commendable attempt to establish a firmer biochemical basis for a selective theory and is worthy of serious consideration. It has several deficiencies, most of which are a result of our state of continuing relative ignorance, as new knowledge raises as many questions as it answers.

While the selective theories may meet the problem of immunological tolerance with less than a fully satisfactory explanation, the instructive theories were generally formulated before the question gained prominence and they failed to confront it at all.

In addition to immunological tolerance, there are other specific unresponsive states: (a) radiation-induced tolerance, (b) the Sulzberger-Chase phenomenon, (c) immunological paralysis (*e.g.* pneumococcal polysaccharide), and (d) protein overloading paralysis. The field of tolerance has accumulated an extensive literature in just one decade and several of the recent review articles will provide an introduction to this growing area of knowledge that cannot be attempted here.[89-93] We should note, however, that one of the major objections to instructive theories has been the prolonged persistence of both antibody and immunological memory. Campbell[94,95] has pointed out that antigen can persist in the body cells

for many months or even years. In the case of nonviable antigens, it has been shown that a protein given in proper amount to an animal may first induce tolerance, and then as the amount diminishes to a critical level, antibody may be produced along with immunological memory. Finally, when there is a sufficiently low level of antigen, there will be no detectable effect.[96] The one observation that has not yet been explained away is the persistence of a measurable level of tetanus antitoxin more than ten years after active immunization.[97] In the case of 19S antibody, it has been demonstrated that the persistence of the antigen is necessary for continued antibody production.[98]

In addition to the theories of antibody formation we have reviewed, other theories have been proposed by Speirs,[99, 100] Schultz,[101] Pappenheimer, Scharff, and Uhr,[102] Boyden,[103] Grabar,[104] Weissman and Lustgaard,[105] Karush and Eisen,[106] Najjar,[107] and others. Our failure to comment on them does not imply any lack of merit, but rather indicates that they have not played an important role in stimulating experimentation thus far. Accordingly, their contribution to the development of current theories of antibody formation, for better or worse, has been minor.

### EVALUATION OF MODERN THEORIES

It will be appreciated that, at least in the author's opinion, none of the major theories of antibody formation, even with recent modifications,[108, 109] is fully acceptable because each is incompatible with some of our current experimental data. No alternative theory will be attempted here. Instead, I would like to point out that a series of remarkable advances in our knowledge in the past five years has made it apparent that all previous theories were oversimplifications of an exceedingly complex field. An appreciation of some facets of this new knowledge may indicate how difficult it will be, for the time being at least, to encompass all these data into a new approach to a theory of antibody formation.

### SITE OF ANTIBODY FORMATION

There has been a considerable amount of research done dealing with the cells that are presumed to be involved in antibody formation. When progress in this field was summarized a decade ago,[110] the most impressive evidence for implicating a single cell had been presented by Fagraeus.[111] She showed that antigen stimulated the reticulum cells in the red pulp of the rabbit spleen to undergo proliferative changes that led to the production of antibody by immature plasma cells. With fluorescent anti-

body studies, Coons[113, 113] showed the progressive accumulation of antibody in maturing plasma cells of lymph nodes. Since then, antibody formation has been noted in organs as diverse as the lung, liver, spleen, and bone marrow.[114] The subject is still quite controversial and has been reviewed in detail.[115-117]

Gowans and his colleagues[118-120] have shown that small lymphocytes collected from the thoracic duct of rats are immunologically competent cells that can change into dividing cells with new morphological features. Within 24 hours, a proportion of the donor's small lymphocytes enlarge, develop prominent nucleoli and cytoplasm that stains strongly with pyronin; they then begin to divide. These pyroninophilic cells resemble those that appear in lymphoid tissue during the homograft reaction[121] or with antibody formation.[112] Such "large pyroninophilic cells" do not appear when isologous small lymphocytes are injected into normal hosts.

Recently, Fishman[122-124] showed that if either lymph node cells alone or macrophages alone were exposed to an antigen *in vitro,* they would not make antibody. But if the two together were incubated with antigen, antibody was produced. If macrophages were incubated with antigen alone, washed free of the antigen, and later incubated with lymph node cells that had not been exposed to antigen, antibody was formed. Finally, if macrophages were exposed to antigen and their RNA was extracted, this protein-free RNA could be incubated with nonstimulated lymph node cells and specific antibody formation resulted. These observations seem to demonstrate the transfer of information by RNA. Aronson[125] has demonstrated bridge formation and cytoplasmic flow between phagocytic cells and suggests that this could be a mechanism of information transfer. Continuity of cytoplasm between macrophage and lymphocyte has been seen on electron micrographs, although no actual passage of material from cell to cell[126] was demonstrable. Nossal has shown recently that Salmonella flagellar antigens labeled with carrier-free $I^{131}$ are taken up by macrophages and are rarely detectable in the actual antibody-producing cells.[127] If this is confirmed, it strikes a strong blow at Karush's modification[56] of the template theory, which requires the antigen in the actual antibody-producing cell.

Perkins and Leonard[128] have shown that macrophages demonstrate a degree of selectivity in their phagocytic properties that is only partially modified by the presence of antibody opsonins; this selectivity appears to involve some recognition of the degree of foreignness of cellular antigens. It may be that macrophages ingest the foreign material and then code a

nucleic acid message for transmission to the actual globulin-synthesizing cell.[129] This is in an area of active investigation.

The role of the thymus (and the Bursa of Fabricius in fowl) in the production of immunologically competent cells has been mentioned briefly. There is already extensive literature on this subject.[130-133] More recent work indicates that the thymus also may have a *hormonal* action of importance in the maturation of immunological competence.[134, 135] In the

TABLE 1. SUMMARY OF IMMUNOGLOBULINS

| Synonyms | gamma 2 globulin | gamma 1M globulin | gamma 1A globulin |
|---|---|---|---|
| | 7S gamma globulin | 19S gamma globulin | Beta 2A globulin |
| | | beta 2M globulin | |
| | $\gamma$ss; Igg | IgM | IgA |
| Svedberg sedimentation constant | 7S | 19S | 7-15S |
| Approximate molecular weight | 150,000-160,000 | 1,000,000 | 150,000-500,000 |
| Electrophoretic mobility | gamma globulin | fast gamma globulin | gamma to beta globulin |
| Carbohydrate content | 2.6% | 10.5% | 7.0% to 9.8% |
| Placental transfer | yes | no | no |
| Genetic Gm factors | present | absent | absent |
| Genetic InV factors | present | present | present |
| Paraprotein disease | gamma myeloma | macroglobulinemia | gamma 1A myeloma |
| Average per cent of total | 70-85 | 5-10 | 5-25 |

rabbit, a cellular role similar to that of the thymus has been claimed for the appendix.[136]

## CHEMICAL STRUCTURE OF ANTIBODIES

Each of the theories considered was formulated on the assumption that antibody protein is a single gammaglobulin. At least three types of normal immunoglobulins are produced by immunologically competent cells. Whether the same cell is capable of producing two different kinds of immunoglobulins has not yet been resolved.[137-139] As if the field were not complex enough, more confusion has resulted from simultaneous observations which led to a duplication of terminology. An attempt to simplify the

data is presented in Table 1, which has been modified from a brief review by Mannik and Kunkel[140] and an extensive review by Franklin.[141]

The normal 7S globulin has been studied most extensively. Oudin and colleagues were able to show six hereditary (allotypic) groups in rabbit 7S gammaglobulin.[142-144] Grubb and Laurell, using rheumatoid factor, found that there were hereditary groups in human 7S gammaglobulin; these they called Gm groups.[145,146] Later, other hereditary groups (InV) were found to occur in all the immunoglobulins.[147] A challenging hypothesis for

TABLE 2. PAPAIN DIGESTION FRAGMENTS OF RABBIT GAMMA GLOBULIN

| Synonyms | I & II (Porter)<br>S (Edelman)<br>A and C (Franklin) | III<br>F<br>B |
|---|---|---|
| Molecular weight | 40,000 to 50,000 | 50,000 to 60,000 |
| Crystallizes | No | Yes |
| Biological activity | Antibody-combining sites | Placental transmission sites |
| | Allotypic antigenic sites<br>Some isotypic antigenic sites | Skin attachment sites<br>Most isotypic antigenic sites<br>Complement-fixation sites |
| Association with globulin | Fragment II(C)—<br>   gamma 2 globulin<br>Fragment I (A)—<br>   gamma 1A globulin | Fragment III (B)—different<br>   for each of the globulins |

the genetic control of the synthesis of the gammaglobulins has been proposed by analogy to the hereditary human hemoglobins.[148-150]

It has been found that the Bence-Jones protein excreted in the urine of many patients with multiple myeloma, as well as myeloma proteins and normal immunoglobulins, all can be classified into two (or possibly three) other immunological groups. The significance of this finding is still obscure, but the confused terminology (purposely omitted here) adds to the "air of mystery."[151-154]

If rabbit 7S gammaglobulin is digested with the enzyme papain by the method of Porter,[155] three polypeptide fragments can be separated by starch electrophoresis. Porter has called the slower moving lighter fragments, I and II, and the faster and heavier fragment, III. Edelman, *et al.*,[156] studied guinea pig and human gammaglobulin and suggested that I and II are a mixture of fragments with similar physical properties from different antibody molecules. He called I and II the S (slow) fragment

and III the F (fast) fragment. Franklin[157] isolated the fragments by column chromatography from human gammaglobulin and called them A and C (I and II of Porter) and B (III of Porter). The properties of these fragments are summarized in Table 2, with data taken from Porter[158-160] and Edelman.[161] In the chart, we note that the antibody-combining site and the complement-fixation site are on different parts of the molecule, as suggested more than a half century earlier by Ehrlich.

TABLE 3. PROPERTIES OF POLYPEPTIDE CHAINS OF GAMMA GLOBULIN

| Synonyms | A | B |
|---|---|---|
|  | H | L |
| Molecular weight (approx.) | 50,000-60,000 | 20,000-25,000 |
| Hexose | 4.5 moles/mole | 0.27 moles/mole |
| Hexosamine | 4.0 moles/mole | 0.16 moles/mole |
| Sialic acid | 0.41 moles/mole | 0.001 moles/mole |
| Biological properties | Complement fixation<br>Skin fixation<br>Placental passage<br>Distinct antigenic determinants<br>Genetic Gm factor | Heterogeneity<br>Antigenic cross reactivity<br>  with other gamma<br>  globulins<br>Genetic InV factor |
| Present in "classical" myeloma protein | No | Yes |
| Heavy chain (Franklin's) disease | Yes | No |

Further studies by Porter's group in London and by Edelman's group in New York led to the separation and identification of the polypeptide chains of 7S gammaglobulin by splitting an estimated five interchain-disulfide bonds. Again, each group used a different nomenclature but the essential findings are in close agreement. The London group studied rabbit gammaglobulin and called the heavier polypeptide chain, A, and the lighter, B.[162] The New York group preferred guinea pig and human gammaglobulin for their study and called the polypeptide chains H (heavy) and L (light).[163] The data summarized in Table 3 are taken in part from each group. Porter believes that the A (H) chain has all the antibody-combining activity when tested *in vitro*;[162] Edelman, *et al.*, believe that the antibody-combining activity depends on the presence of parts of both chains,[164] and a group at La Jolla agree.[165] Further study will be necessary to resolve this highly controversial point.

The recognition of the polypeptide structure of normal gammaglobulin prepared the way for studies of myeloma proteins and Bence-Jones proteins.[166] The latter are dimers of B (L) chains and represent the common type of multiple myeloma. It was to be expected that sooner or later a type of heavy-chain myeloma would be recognized; a few cases of this interesting abnormality have been reported during the past year.[167, 168]
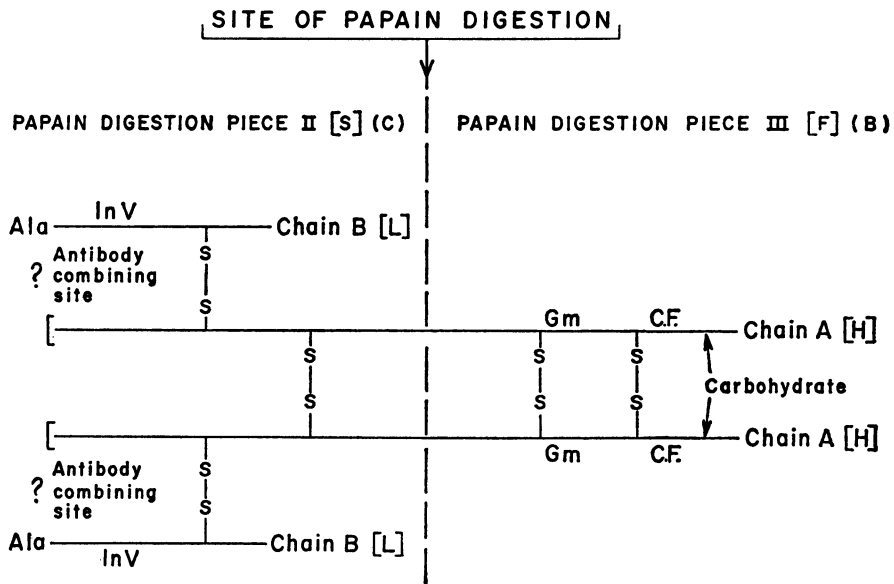


Fig. 1. Diagrammatic structure of rabbit gamma 2 globulin, modified from Porter.[159] The terminology of Porter is used with that of Edelman in brackets and that of Franklin in parenthesis. The half bracket on the A chain indicates an inability to demonstrate a free N-terminal group. C.F. = complement fixation site. Ala = alanine, the predominant N-terminal amino acid. Gm = genetic antigen on gamma 2 globulin H chain only. InV = genetic antigen on B chain of all immunoglobulins. Papain digestion piece I [S] (A) would be similar in gamma 1A globulin. The number of disulfide bonds is an estimate.

On the basis of the information already summarized, Porter[159] made a model of the most likely structure for rabbit gammaglobulin; this is reproduced with some modification in Figure 1. The studies of Palmer, *et al.,*[160] tend to support this structure. Most of the information reviewed thus far has come primarily from studies of rabbit gamma 2 globulin. Recently, there has been great interest in gamma 1 globulin[170] and the macroglobulins[171, 172] (see Table 1 for alternate terminology). Progress in these fields is so rapid that much of what is written may be obsolete or superseded by new concepts between the time of writing and publication.

An interesting comparison of the biological properties of the 7S immunoglobulins in guinea pigs has been presented and the data so far available,[173-176] with a few extrapolations, are summarized in Table 4.

Another area in which there has been rapid progress is the study of synthetic polypeptides. Although very new, this field has been extensively reviewed recently,[177-179] a few important observations should be highlighted It has been demonstrated that polymers of single amino acids, such as

TABLE 4. PROPERTIES OF GAMMA GLOBULINS*

| Synonyms | gamma 2 globulin 7S gamma globulin slow | gamma 1M globulin beta 2M globulin | gamma 1 globulin beta 2A globulin fast |
|---|---|---|---|
| Svedberg sedimentation constant | 7S | 19S | 7S-15S |
| Concentration in serum | high | low | low |
| Passive hemagglutination | + | + | + |
| Precipitation | + | + | + |
| Passive cutaneous anaphylaxis | — | — | + |
| Complement fixation | + | + | — |
| Arthus reaction | + | ? | — |
| Systemic anaphylaxis | — | ? | + |
| Passive hemolysis | + | + | — |
| Placental crossing | + | — | — |

* These properties are not the same in all species and apply in some cases to guinea pig, rabbit, or human proteins. The exact figures are different for the separate species.

poly-*L*-lysine or poly-*L*-glutamic acid, lack antigenicity in guniea pigs, while random copolymers of two or more L-amino acids are antigenic.[177] Some guinea pigs react to dinitrophenyl-poly-*L*-lysine and to DNP-copolymer glutamyl-lysine, while others do not, but an individual animal reacts either to both or to neither. Kantor, *et al.*[180] suggest that the response of the animals is genetically determined and may depend on the inability of their macrophages to split lysyl peptides. Poly-*D*-amino acids are not antigenic in rabbits;[181] it has been suggested that this is due to an inability of their enzymes to break down the *D*-amino polypeptide.[182] Thus, it would seem that the process of antigen degradation plays a definite role in governing antibody synthesis.[183]

An impressive synthesis of data has led to the recent proposal of a genetic hypothesis for gamma globulin variability by Smithies.[184] This theory offers a plausible explanation of the genetic variability, but does not address itself to the question of antibody induction, specificity, and

synthesis. From a review of the available data, it is again obvious that we still do not have enough information for a comprehensive theory of antibody formation.

## CONCLUSION

In this brief review, we have been able to consider, but only very superficially, the highlights of several theories of antibody formation. We have had to omit many important topics such as the delayed hypersensitivity reaction,[185-188] the role of complement,[189] the transfer reaction, [190] reaginic antibodies,[191] immunological deficiency diseases and experiments of nature,[192] transplantation immunity,[193] subcellular changes,[194] the cellular basis of immunological memory,[195] autoimmune disease,[196-198] the heterogeneity of gamma globulins,[199] and the nature of immune mechanisms in germ free animals.[200, 201] We have even failed to comment on reviews of this subject,[202-204] but to consider the matter in any depth would require a book instead of an article.

Burnet, the biologist, has (in a friendly fashion) chastised chemists like Haurowitz, Pauling, and Karush for paying excessive attention to the chemical basis of antibody formation and ignoring the biological data in the formulation of the various direct template theories. Yet the direct template theories have failed mainly because their chemical basis was undermined by the newer chemical information we have reviewed. The selective theories have been purposely vague in this matter and can accommodate the new chemical information. Their failure has been primarily biological, an inability to reconcile their premises with the objections to Ehrlich's side-chain theory as well as with the newer biological data. Thus, a new theory of antibody formation may arise from some synthesis of these opposed and, at present, mutually exclusive theories. Alternatively, a new theory may be formulated as an outgrowth of an indirect template theory, giving due cognizance to the role of allosteric changes in protein conformation as one basis for specificity.[205] We may conclude that Medawar's comments on theories of immunological tolerance apply equally well to theories of antibody formation:

> Within the next few years it should become possible to devise an adequate theory of immunological tolerance—or rather, a theory of the immune process which comprehends the phenomenon of tolerance on the one hand and of immunity on the other. At present we are far too ignorant to do so, and the sole purpose of the present contribution is to attempt to classify our lack of knowledge of the matter in some manageable way.[206]

It is five years since Medawar dreamed of an adequate comprehensive theory, but alas, we have none.

## SUMMARY

The major theories of antibody formation from Pasteur's time to the present are reviewed. The merits of and objections to a direct template theory, a clonal selection theory, and an indirect template (adaptive enzyme) hypothesis are summarized. The sites of antibody formation are considered, including the two cell hypothesis. The relevant highlights of the research of the past five years are outlined, and it is concluded that in the light of this work, none of the theories encompasses both the biological and chemical data presently available.

## ADDENDUM

Since this article was submitted, Edelman and Gally[207] have proposed a model for the 7S antibody molecule that is similar to the model of Porter in some details, but different enough to suggest further productive avenues of experimentation.

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