Mechanism of antigen-induced antibody biosynthesis from antibody precursors, the heavy and light immunoglobulin chains

(antigen persistence/thiol-disulfide transhydrogenation/phenotypic reaction)

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ABSTRACT The immediate precursors of antibody molecules, the heavy (H) and light (L) peptide chains of the immunoglobulins, combine with each other by means of disulfide bonds formed by dehydrogenation of their cysteine residues. In the absence of an antigen this process yields the heterogeneous mixture of normal immunoglobulins. Antigens or their processed derivatives (Ag) interfere with this stochastic process by noncovalent combination with complementarily fitting H chains. The (Ag-H)\textsubscript{n} complexes thus formed, owing to the loss of rotational and translational freedom, combine preferentially with those L chains whose \( \nu \text{L} \) regions have some affinity for the determinants of the antigen molecule. Subsequent release of Ag from the (Ag-H-L)\textsubscript{n} complexes yields free antigen and antibody molecules. Each of the released Ag molecules can be used repeatedly for the same reaction cycle and thus can induce the biosynthesis of a large number of antibody molecules. Any macromolecule, natural or synthetic, that has at least a few polar groups and that can penetrate to the nascent H and L chains can thus act as an antigen. Whereas the structure of the H and L chains is genetically determined and transmitted through the germ line, the process induced by the antigen is a phenotypic phenomenon. The antigen acts in this process as a stereospecific cofactor or regulator of the thiol-disulfide transhydrogenation of the combining H and L chains of immunoglobulins.

Widely held views on the mechanism of antibody formation are based on some or all of the following claims: (i) The ability of producing antibody of a definite specificity is transmitted genetically through many generations of a clone of lymphoid cells; antibody production, once initiated, continues even in the absence of antigen. (ii) The immunocompetent lymphoid cells are unipotent; each of them produces only one type of antibody molecule. (iii) The antigen combines selectively with homologous receptors on the surface of the immunocompetent lymphoid cells; the receptors are or contain cell-bound antibody molecules; the combination of these receptors with the antigen stimulates the cells to divide and multiply at an increased rate, thus accelerating antibody production; the mechanism of this stimulation is not known. Experiments performed in this and in many other laboratories during the last three decades have made it necessary to modify these claims. In the present article an attempt is made to formulate a concept in which the immunogenic action of the antigen and the process of antibody biosynthesis are described in chemical terms.

Dependence of antibody production on continued presence of antigen

Immunity against some of the antigens frequently lasts several years. Long persistence of immunity against pathogenic viruses usually has been attributed to continuous reproduction of the virus in the host in a mitigated form. Yet it was for a long time not clear whether proteins or other unanimated antigens might persist over long periods of time in the host organism. For this reason Burnet (1), in the original form of his clonal selection theory, postulated that each cell of an antibody-producing clone transmits to its daughter cells the ability of antibody production and that this ability is maintained through many generations of cells. Using isotopically labeled protein antigens we were able to show that these antigens or their fragments persist in the spleen of rabbits for long periods of time (2). The slow decrease in the antigen content of the spleen was paralleled by an analogous decrease in the antibody titer of the blood serum (3). The bulk of the radioactive antigen fragments was located in the cytoplasm of the cells; nuclei were free of the radioactive material (4).

Dependence of antibody production on the persistence of the antigen has been convincingly established in cultures of antibody-forming cells and in syngeneic colonies of such cells in lethally irradiated animals (5–10). Antibody production in \textit{vivo} and also in cell cultures strictly depends on the continuous presence of the antigen; short exposure of the cells to the antigen is not sufficient to induce antibody production (9). If antibody formation in \textit{vivo} or in \textit{vitro} stops after elimination of most of the antigen, it is rapidly restored after renewed addition of the antigen. North and Askonas (10) conclude that it is "memory" in the form of precursor cells rather than autonomous antibody production that is transferred through many generations. While the term "memory cell" is usually applied to those cells that had been exposed to prior stimulation by the antigen, the terms antigen-sensitive cell or antigen-reactive cell have been used for cells that had no earlier contact with the antigen and yet bind a definite type of antigen. The common property of all these cells is their content of heavy (H) and light (L) chains whose combination yields antibodies directed against the antigen bound by these cells. Since the property of binding a definite antigen is genetically determined, antibody production is evidently a two-phase process in which the first phase, the production of H and L peptide chains, is directed by the genome. The second phase, the mutual combination of the h and L peptide chains, is a phenotypic process typical of differentiation (11).

H and L chains as precursors of antibodies and other immunoglobulins

Since all immunoglobulins, including antibodies and myeloma proteins, are formed by the combination of H and L peptide chains, these chains are indeed antibody precursors. Both H and L chains contain one NH\textsubscript{2}-terminal variable (V) region of approximately 110 amino acid residues, and a COOH-terminal

Abbreviations: H chains, heavy chains; L chains, light chains; V, variable region; C, constant region; Ag, native or processed form of antigen; Dnp, 2,4-dinitrophenyl.
constant (C) region. The latter consists of three or four domains; each of these is similar in its size and structure to the V_H domain. Fig. 1 shows the structure of an IgG immunoglobulin which consists of only two H chains and two L chains. The domains within each chain are linked to each other covalently by peptide bonds, whereas the four peptide chains are cross-linked by disulfide bridges.

![Fig. 1. Structure of rabbit IgG immunoglobulin. H, heavy chains; L, light chains; V, variable domains; C, constant domains.](image)

It is generally assumed that the combination of the V and C regions within each of the H or L peptide chains occurs at the DNA level, i.e., as combination of V genes with C genes. This process seems to take place during ontogenesis; it involves excision, insertion, or other forms of translocation within certain DNA sequences, but not typical mutations (12). Transcription of the final H and L gene yields the mRNA molecular for the H and L chains; they are translated independently, the H chains on 80S polyribosomes, the L chains on 180S polyribosomes (13). Earlier suggestions that the transcription or translation process might be modified by the antigen (14, 15) are invalidated by these findings. Since the amino acid sequence of the H and L chains reflects the DNA sequence of their H and L genes, the variety of repertoire of H and L chains is genetically determined. This repertoire limits the antigen sensitivity, i.e., the antigen-binding capacity of the lymphoid cells and their responsiveness.

In the normal, nonimmunized organism the nascent H and L chains in the endoplasmic reticulum are rapidly converted into a mixture of normal immunoglobulins (16) which are then secreted into the circulation. The ability to produce antibodies is acquired at a certain state of maturation. In the sheep fetus, whose gestation period is 150 days, production of antibodies against some of the injected antigens begins after day 40; antibodies against other antigens appear at later times of the intrauterine life or after gestation (17). The production of detectable amounts of antibody is preceded by a phase in which added antigen is bound to the surface of the lymphoid cells (18).

Chemical mechanism of immunoglobulin production in absence of antigen

The combination of H and L chains involves merely the formation of interchain disulfide bonds from the two SH groups of the H chains and the single SH of the L chains. The disulfide (-SS-) bond, although usually designated as a covalent bond, is easily cleaved by reducing agents and can be considered as containing the isomeric sulfenium sulfide forms: -S-S- and -S-S-. Each of the domains of the H and L chains contains also intrachain disulfide bonds; these are not involved in the combination of H and L chains.

In the following discussion the IgG molecule shown in Fig. 1 will be represented by the abbreviated symbol L—H—H—L, in which the elongated horizontal lines indicate the disulfide bonds. Immunoglobulins can be formed in vitro by mild oxidation of aqueous solutions of H and L chains. Kinetic analyses reveal that these solutions contain intermediates of the structures H_2, HL, and H_2L (18, 19, 20). In some immunoglobulins the two H chains are linked to each other by two or more interchain disulfide bonds and also by other noncovalent bonds. Other immunoglobulins are polymers of the L—H—H—L structure. In the following discussion only the simple tetrameric L—H—H—L structure will be used as a model. The three interchain disulfide bonds in the H_2L_2 structure are formed by the mild oxidation of thiol groups of the nascent H and L chains. The biological oxidizing agent is either O_2 (reaction I) or a disulfide of the general structure R-S-S-R (reaction II). In reactions I and II the H and L chains are shown by bold print in order to distinguish the chains from the hydrogen atoms of thiol groups.

\[
2H(\text{SH})_2 + 2L(\text{SH}) + 1.5O_2 \rightarrow L-L-L+3H_2O \quad [\text{I}]
\]

\[
2H(\text{SH})_2 + 2L(\text{SH}) + 3R-S-S-R \rightarrow L-L-L+6R-\text{SH} \quad [\text{II}]
\]

While reaction I, in the absence of reducing agents, is irreversible, reaction II can proceed in both directions. The equilibrium of reaction II depends not only on the concentrations of RSSR and RSH, but also on pH, temperature, other physicochemical conditions, and obviously also on the concentrations of any substance that reacts with the nascent H or L chains. Among the three disulfide bonds of the H_2L_2 tetramer the H—H bond is more easily reduced than the H—L bonds; cleavage of the H—H bond yields two identical half-molecules; mild reoxidation restores the H_2L_2 tetramer (21, 22). The stability of this tetramer, like that of the hemoglobin tetramer \(\alpha_2\beta_2\), is attributed to a 2-fold axis of rotatory symmetry (23). The two identical L—H half-molecules are held together not only by their interchain disulfide bonds, but also by noncovalent interaction between the two H chains (24).

In vitro, hydrogen transfers from thiols to disulfides are catalyzed by thiol–disulfide transhydrogenases of the class EC 1.8.4; most of these enzymes are flavoproteins. The best known biological hydrogen acceptor in these reactions is glutathione. It is not yet known whether glutathione and transhydrogenases are involved in immunoglobulin formation in vivo. In vitro the conversion of dissolved H and L chains into immunoglobulins proceeds in the absence of enzymes; it is probably catalyzed by trace metal ions (19).

The normal immunoglobulins of vertebrates are a highly heterogeneous mixture of immunoglobulins which differ from each other chiefly in their variable V_H and V_L domains. Evidently, the nascent H and L chains, in the absence of antigens, combine with each other stochastically. If the genetically determined repertoire of H and L chains consists of \(m\) different types of H chains and \(n\) different types of L chains, and if these combine randomly with each other, the maximum number of H—L variants would be \(m \times n\). Estimates of \(m\) and \(n\) vary from \(10^5\) to \(10^8\), so that \(10^4\)–\(10^5\) different immunoglobulins might be produced. However, electrostatic interaction between positively and negatively charged groups, dipole–dipole induction, formation of hydrogen bonds, and other intermolecular phenomena will render some of the combinations of H and L chains more frequent than others. Indeed, some of the normal immunoglobulins combine specifically with natural or synthetic antigens such as penicillin, insulin, and lysozyme (25) and also with synthetic hapten; thus about 1% of the normal human IgG molecules combines with dinitrophenyl (Dnp) groups, 0.2% combine with 4-hydroxy-3-iodo-5-nitrophenyl-acetyl groups, and 9% combine with p-azobenzeneearsonate residues (26).

Thus small amounts of preformed antibodies are indeed present in the normal organism before immunization.
Interference of antigens with immunoglobulin production

Immunoglobulin production, according to reactions I and II, must be affected by all substances that have a definite affinity for the H chains, for L chains, or for the product H2L2. It has been known for several years that antigens combine specifically not only with the homologous antibody molecules, but also with the free H chains of these antibodies (27). The affinity of the antigen for the L chains is generally much lower than that for the heavy chain. Consequently we must conclude that the antigen interferes with the normal process of immunoglobulin formation by combining first with those nascent H chains that happen to have the highest affinity for one of the determinant groups of the antigen molecule. In reactions III-V the same symbols are used as in reactions I and II. Since it is well known that many antigens need to be "processed" in macrophages before they induce antibody formation, the symbol Ag stands here for the immunogenic form of the antigen; this may be an antigen fragment containing the specific determinant group or a complex of the determinant part of the antigen molecule with molecules that enable the antigen to penetrate into the cytoplasm of the antibody-forming cells, where we find it.

\[
2\text{Ag} + 2\text{H(SH)}_2 + \text{R-SS-R} \rightarrow \text{HS-H(SH)} + 2\text{R-SH} \quad \text{(III)}
\]

The broken lines indicate noncovalent bonds between the H chain dimers and the determinant groups of the two antigen molecules. The reaction product Ag2H2 reacts then with two L chains according to reaction IV.

\[
\text{Ag} \quad \text{Ag}
\]

\[
(\text{HS-H(SH)} + 2\text{L(SH)} + 2\text{R-SS-R})
\]

\[
\rightarrow \text{Ag} \quad \text{Ag}
\]

\[
\text{H-H} + 4\text{R-SH} \quad \text{(IV)}
\]

Dissociation of the Ag2H2L2 complex according to reaction V yields two molecules of the immunogenic Ag derivative and one molecule of antibody, H2L2.

\[
\text{Ag} \quad \text{Ag}
\]

\[
\text{H-H} \quad \text{H-H}
\]

\[
\rightarrow 2\text{Ag} + 2\text{H-H-L} \quad \text{(V)}
\]

The overall reaction of the sequence III-V is:

\[
2\text{H(SH)}_2 + 2\text{L(SH)} + 3\text{R-SS-R} \rightarrow \text{L-H-H-L} + 6\text{R-SH}.
\]

In some animal species a dimer of the H chain is an intermediate in reaction III; in other animals the first intermediate formed in (HS)H-L, which then is converted to (HS)H-H-L and finally to L-H-H-L (16). In each of the equations the hydrogen acceptor R-SS-R can be replaced by \(\frac{1}{2}\) O2; however, this would make the reactions irreversible. The H and L chains in reaction IV must be present in the reduced thiol form so that their oxidation yields the disulfide bridges present in the antibody molecules. Indeed, it has been found that antibody production in cell cultures takes place only in the presence of reducing agents such as mercaptoethanol, CH3(OH)-CH2SH, or \(\alpha\)-thioglycerol, CH3(OH)-CH(OH)-CH2SH (28, 29). In the absence of these or similar thiols, antibody production in cell culture stops. The view that antibody production is directed by the presence of the antigen, or of its processed form, is supported by the observation that the formation of anti-Dnp antibody from its H and L chains is considerably accelerated by the presence of the homologous hapten, Dnp-lysine (30).

Physicochemical bases of antigen interaction

According to reactions III-V, the function of the antigen is that of a specific cofactor or regulator in the formation of disulfide bridges from the thiol groups of selected nascent H and L peptide chains. The course of this dehydrogenation is modified by the affinity of the antigen for those H chains whose VIH domains closely fit the determinant groups of the antigen. Determinations of the affinity of protein antigens for homologous precipitating antibodies indicate affinity constants close to \(10^6\)-\(10^7\) (31). Much lower values, close to \(10^2\), are found when the macromolecular antigens are replaced by haptens of low molecular weight. Similarly, the affinity of the hapten for a homologous antibody decreases by a factor of approximately \(10^2\) when the antibody molecule is replaced by its H chain (32).

The noncovalent bonds shown in reactions III, IV, and V by broken lines are formed by short-range or long-range interaction. Short-range forces are involved in the formation of hydrogen bonds, in dipole induction, and in other forms of mutual attraction between closely fitting, complementary structures in the surfaces of the antigen molecules and the molecules of the free H chains. Electrostatic interaction between positively and negatively charged groups, because of its long range, is not as highly specific as the short-range interactions. The latter decrease by a factor \(r^6\), whereas the long-range forces decrease by a factor of only \(r^4\), where \(r\) is the distance between the determinant group of the antigen and the combining site of the antibody molecule. The low specificity of the long-range electrostatic interaction is clearly demonstrated by hapten-specific antibodies directed against the protein-bound m-azobenzenesulfonyl group; these antibodies precipitate not only azoproteins containing this group, but also considerable amounts of azoproteins containing o- or p-azobenzenesulfonate, m-azobenzene arsonate, or m-azobenzoate groups (33). The higher specificity of the short-range forces is manifested by antibodies directed against the succinyl residue -CO-CH2-CH(OH)-COOH; they combine with the residue -CO-CH=CH-COOH in its trans form, but not in its stereoisomeric cis form (33).

One of the prerequisites of strong immunogenicity is a molecular weight of at least \(\sim 10^4\). The great importance of the high molecular weight of the reactants for the initiation of an immune response is clearly demonstrated by the increase in immunogenicity when polyamino acids, which are not immunogenic, are coupled with gelatin, which also is devoid of immunogenicity; the macromolecular complex formed in this reaction is immunogenic. Similarly, polynucleotides acquire immunogenicity when they are coupled with methylated bovine serum albumin, which, as such, is not immunogenic. The immunogenicity of many of the small protein antigens can be increased considerably by coupling them to larger macromolecules or by adsorbing them to an adjuvant.

Diffusion-controlled reactions such as enzyme-catalyzed processes are enhanced by an increase in the molecular weight of the reactants or by their embedding into a membrane (34). This enhancement is attributed to a decrease in the rotational and translational mobility upon combination of the two reactants. The enhancing action of reactants of high molecular weight in catalysis has been designated by Jencks (35) as "anchor principle" because the strong attractive forces of the
Mechanism of some antigen-antibody interactions

The principal difference between the views presented in the preceding paragraphs and other widely accepted views is our claim that the antigen combines in vivo with H and L chains rather than with cell-bound antibody molecules. If the antigen would combine with cell-bound antibody molecules in receptors or inside the cell, administration of the antigen would cause a rapid immune response. However, antibody production in the rabbit cannot be detected before day 3 after the primary injection. It reaches its peak at day 8. In the secondary response, the antibody titer increases during 3–4 days to its maximum. These immune reactions are quite different from the almost immediate skin reactions or from the rate of anaphylactic shock. The slow increase in the antibody titer indicates that the antigen does not react with preformed antibody molecules, but that it combines with continually formed H and L chains as shown in reactions III and IV. This view is in agreement with the finding (38) that antibody production in an immunized organism is suppressed by the injection of the homologous antibody. This feedback effect indicates clearly that externally administered antibody molecules compete with the H and L chains for the earlier injected antigen molecules and thus prevent combination of antigen with H and L chains.

The view that the administered antigen combines with H and L chains rather than with antibody molecules allows us to reinterpret some phenomena for which hitherto there was no satisfactory explanation. Only some of these can be discussed here. One of them is the lack of responsiveness to an antigen in some, but not all, individuals of a species. It is usually attributed to the inability of an individual to produce the homologous antibody. According to the view presented above, lack of either the specific H chains or suitable L chains would be sufficient to prevent production of the desired antibody molecules. If only the H chains occur in very small amounts, administration of a very small excess of antigen might cause low-dose tolerance, which otherwise is difficult to explain.

Our views concerning the role of the antigen are difficult to reconcile with the original form of the clonal selection theory (1) according to which the ability of producing antibodies does not depend on the presence and persistence of the antigen. However, reactions III–V are compatible with a modified clonal theory which is based on the earlier discussed findings of Nossal (6, 7, 9), Askonas (8, 10) and their coworkers; they definitely proved that antibody formation can take place only in the presence of the antigen. It is clear from these investigations that the genetic characteristic transmitted through many generations of a clone of lymphoid cells is the ability to produce certain genetically determined types of H and L chains which, in the presence of a suitable antigen, but not in its absence, combine to yield predominantly antibodies directed against the administered antigen. Apparently the antigen merely modifies the phenotypic, stochastic combination of H and L chains, but does not affect the genome of the antibody-forming cells.

According to our views concerning the role of the antigen in antibody formation it is clear that almost any macromolecule, natural or synthetic, provided it contains polar groups and provided it can penetrate to the sites of the production of nascent H and L chains, can combine noncovalently with some of these chains and thus can act as an antigen. If an antigen is immunogenic in only some, but not all, individuals of a species, the lack of immunogenicity is usually caused genetically, by lack of the required H or L chains in the nonreactive individuals. This genetically caused lack of an immune response must be distinguished from the earlier discussed lack of an immune response which can be corrected by increasing the molecular weight, the polarity, or any other factor required for the phenotypic phase of antibody production, namely, the noncovalent interaction of the antigen with H and L chains (reactions III–V). Antibodies, according to these views, can be defined as immunoglobulins produced from the genetically determined repertoire of H and L chains in the presence of an antigen that combines preferentially with those H and L chains that have a significant affinity for its determinants. These H and L chains are thus activated for formation of the homologous antibody.

Note Added in Proof. It is not yet clear whether the antibody-producing lymphoid cells are unipotent or pluripotent. The claim of unipotency of the cells is based on the observation that most of the cells of an animal injected with two different antigens produce antibodies directed against one or the other of the two antigens; only very few cells produce both types of antibody (39). However, it has been shown recently that single lymphoid cells combine with two or more different antigens (40) and that two or more antigens compete with each other for the same cells (41, 42). If the cells are indeed pluripotent, the action of the antigen involves intracellular rather than intercellular selection of suitable H and L chains. The action of the antigen in the reaction sequence III–V might be characterized as instructive, a term frequently used in immunology. However, since the purpose of this communication is to describe the biosynthesis of antibodies in terms of chemistry or molecular biology, the action of the antigen is preferably designated as activation of closely fitting H chains for their disulfide exchange with suitable L chains.

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