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ANTIBODY STRUCTURE AND CELLULAR SPECIFICITY IN THE IMMUNE RESPONSE*

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I. Introduction

The idea of immunological specificity was first formulated in molecular terms as a result of the work of Landsteiner (1945). His research on artificial hapten antigens served to ally immunology to chemistry and provided the operational tools necessary for the precise thermodynamic analysis of antigen–antibody reactions. From this analysis, it became clear that immunological specificity results from molecular complementarity between the antigenic determinant group and the antigen-combining site of the antibody molecule. Landsteiner's work was carried out on humoral antibodies against a general background of belief in instructive theories of antibody formation. It has now become apparent, however, that the notion of immunological specificity must be revised and broadened, largely because of two developments: the clonal selection theory of immunity and the detailed analysis of antibody structure.

In this lecture, I shall consider several ideas that have emerged from the structural analysis of antibodies and attempt to relate them to the framework of the theory of clonal selection and to several problems of cellular immunology at the molecular level. The analysis of antibodies can be considered as the first of the projects of molecular immunology, the task of which is to interpret the properties of the immune system in terms of molecular structure. Before this task can be considered complete, however, our knowledge of immunoglobulin molecules must be matched by a comparably detailed knowledge of the cellular and genetic events underlying their synthesis. Although I shall not hesitate

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to put forth working hypotheses on these events, my main purpose is to consider some experimental approaches which, however incomplete, suggest that these events are susceptible to chemical and structural analysis.

According to the theory of clonal selection (Jerne, 1955; Burnet, 1959) molecular recognition of antigens occurs by selection among different lymphoid cells already committed to producing surface antibodies prior to contact with the antigen (Fig. 1). Interaction of the antigen with an appropriately complementary antibody on the cell surface results in clonal expansion, i.e., maturation, mitosis, and increased production of the same kind of antibodies by daughter cells.

For such a system to function effectively, there are two major requirements. First, a sufficient diversity of antibodies with different combining sites must be synthesized by the cell population,

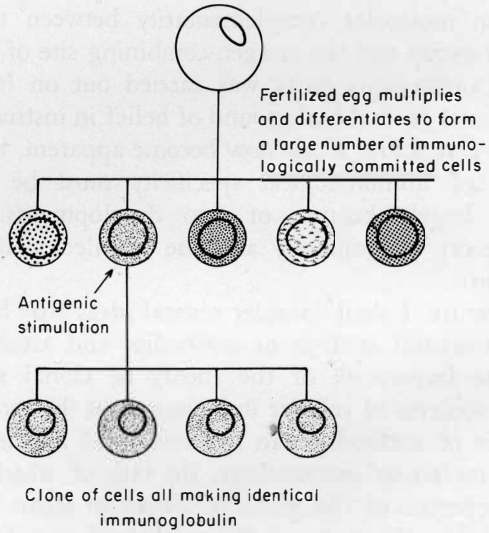


FIG. 1. A diagram illustrating the basic features of the clonal selection theory. The stippling and shading indicate that different cells have antibody receptors of different specificities, although the specificity of all receptors on a given cell is the same. Stimulation by an antigen results in clonal expansion (maturation, mitosis, and antibody production) of those cells having receptors complementary to the antigen.

each cell making surface antibodies of a single specificity that must, in general, be different than that of antibodies on other cells. Second, interaction with an antigen must either specifically trigger or specifically inhibit clonal expansion. Corresponding to these requirements are several fundamental problems of molecular immunology: (1) What is the origin of antibody diversity? (2) What is the mechanism of lymphocyte stimulation or suppression? Although these problems are far from being solved, their consideration in molecular terms, particularly in terms of antibody structure, sheds new light on the nature of immunological specificity.

II. Antibody Structure, Function, and Evolution

Antibodies carry out two main functions in the immune response: antigen binding functions and biologically important effector functions such as interaction with complement. Although antibodies occur in different immunoglobulin classes that mediate different effector functions (Table I), within each class, the immunoglobulins are still enormously heterogeneous and reflect the diversity required for the antigen-binding function. Immunoglobulins in all classes are multichain structures, consisting of either κ or λ light chains and a class-distinctive heavy chain. The most prevalent class in mammals is IgG; detailed structural work indicates that molecules within this class are sufficiently representative to illustrate the basic features of the relationship between antibody structure and function (for reviews, see Cold Spring Harbor Symposium, 1967; Nobel Symposium, 1967; Edelman and Gall, 1969).

Analysis of the complete amino acid sequence of an IgG molecule (Edelman *et al.*, 1969) and comparison with other partial structures (Cunningham *et al.*, 1971) reveals a particularly simple differentiation of the structure to carry out antigen-binding and effector functions (Fig. 2). Both the light and the heavy chains contain variable regions consisting of the first 110–120 amino acid residues, having sequences that differ from molecule to molecule. This observation was first made on light chains from Bence-Jones proteins by Hilschmann and Craig (1965). The remaining portions of the chains are called constant regions because, with

TABLE I

HUMAN IMMUNOGLOBULIN CLASSES

Class	Physiological properties	Heavy chain[a]	Light chains	Molecular formula[b]	Molecular weight ($\times 10^{-3}$) and sedimentation constant	Carbohydrate content (%)
IgG	Complement fixation; placental transfer	γ	κ or λ	$(\gamma_2\kappa_2)$ or $(\gamma_2\lambda_2)$	143–149; 6.7 S	2.5
IgA	Localized protection in external secretions	α	κ or λ	$(\alpha_2\kappa_2)$ or $(\alpha_2\lambda_2)$	158–162; 6.8–11.4 S	5–10
IgM	Complement fixation; early immune response	μ	κ or λ	$(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$	800–950; 19.0 S	5–10
IgD	Unknown	δ	κ or λ	$(\delta_2\kappa_2)$ or $(\delta_2\lambda_2)$	175–180; 6.6 S	10
IgE	Reagin activity; mast cell fixation	ϵ	κ or λ	$(\epsilon_2\kappa_2)$ or $(\epsilon_2\lambda_2)$	185–190; 8.0 S	12

[a] The class distinctive features of these chains are in their constant regions.

[b] IgA can have additional unrelated chains called SC and J; J chains are also found in IgM. For nomenclature, see World Health Organization, 1964.

the exception of certain genetic polymorphisms, they are the same in molecules of a given class and subclass. Experiments on the reconstitution of antibodies from their separate chains (Franěk and Nezlin, 1963; Edelman *et al.*, 1963; Olins and Edelman, 1964) and their completely denatured chains (Haber, 1964) as well as experiments on affinity labeling (Singer and Doolittle, 1966) and on the isolation of antigen-binding fragments consisting of variable regions (Inbar *et al.*, 1972) all indicate that both V_H and V_L regions mediate the antigen-binding function. The effector functions are carried out by constant regions.

Additional hints about the evolutionary origin and functional differentiation of immunoglobulins have emerged from a detailed analysis of their primary structure (Edelman, 1970). A considera-

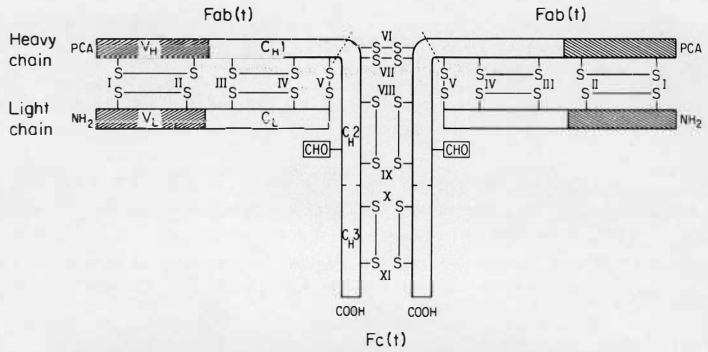


FIG. 2. Overall arrangement of chains and disulfide bonds of the human γG_1 immunoglobulin Eu. Half-cystinyl residues are I–XI; I–V designate corresponding half-cystinyl residues in light and heavy chains. PCA, pyrrolidonecarboxylic acid; CHO, carbohydrate. Fab(t) and Fc(t) refer to fragments produced by trypsin, which cleaves the heavy chain as indicated by dashed lines above half-cystinyl residues VI. Variable regions, V_H and V_L, are homologous. The constant region of the heavy chain (C_H) is divided into three regions, C_H1, C_H2, and C_H3, that are homologous to each other and to the C region of the light chain. The variable regions carry out antigen-binding functions and the constant region the effector functions of the molecule.

tion of the amino acid sequences of immunoglobulin G (Figs. 3 and 4) supports the following conclusions:

1. V_H and V_L regions are homologous to each other but are not obviously homologous to C_H or C_L regions. V regions from the same molecule appear to be no more closely related than V regions from different molecules.

2. The constant region of the heavy chain consists of three homology regions, C_H1, C_H2, and C_H3, each of which is closely homologous to the others and to C_L.

3. Each variable region and each constant homology region contains one disulfide bond, and as a result the intrachain disulfide bonds are linearly and periodically distributed in the structure.

4. The region containing all the interchain disulfide bonds is in the middle of the linear sequence of the heavy chain and has no homologous counterpart in other portions of the heavy or the light chains.

These observations are consistent with the hypothesis of Hill

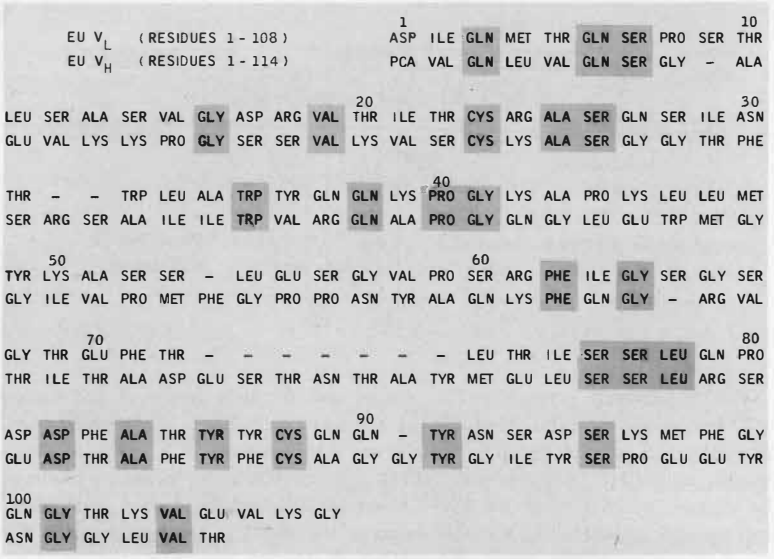


FIG. 3. Comparison of the amino acid sequences of the V_H and V_L regions of protein Eu. Identical residues are shaded. Deletions indicated by dashes are introduced to maximize the homology.

et al. (1966) and Singer and Doolittle (1966) that immunoglobulin chains arose by duplication of a precursor gene about 330 nucleotides in length. Because there is no clear-cut evidence of homology between V and C regions, however, it is somewhat harder to decide whether V and C regions evolved from a single gene. The alternative is that there were two precursor genes for V and C which were originally unrelated but were brought together because of the selective advantages of combining their functions in a single product molecule (Edelman, 1970).

The unusual homology relationships and arrangement of disulfide bonds prompted my colleagues and me to suggest that the molecule is folded in a congeries of compact domains (Edelman, 1970; Cunningham *et al.*, 1971), each formed by separate V homology regions or C homology regions (Fig. 5). In such an arrangement, each domain is stabilized by a single intrachain disulfide bond and is linked to neighboring domains by less tightly folded stretches of the polypeptide chains. A 2-fold pseudosym-

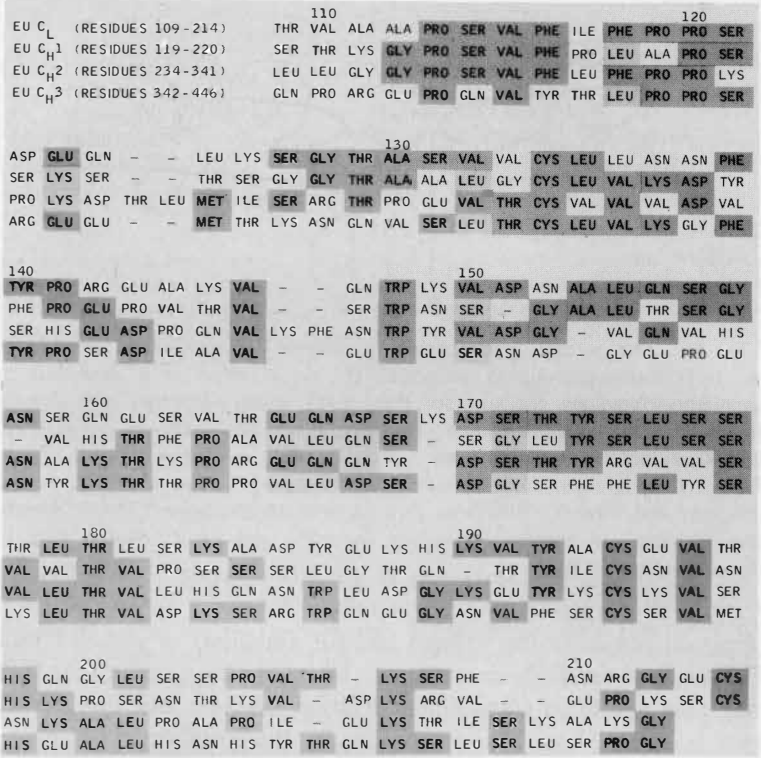


FIG. 4. Comparison of the amino acid sequences of C_H, C_H1, C_H2, and C_H3 regions. Deletions, indicated by dashes, have been introduced to maximize homologies. Identical residues are darkly shaded; both light and dark shadings are used to indicate identities that occur in pairs in the same position.

metry axis relates the V_LC_L to the V_HC_H1 domains, and a true dyad axis through the disulfide bonds connecting the heavy chains relates the C_H2-C_H3 domains. The tertiary structure within each of the homologous domains is assumed to be quite similar to that in the others. Moreover, each domain is assumed to contribute to at least one active site mediating a function of the immunoglobulin molecule.

As I have already mentioned, the V domains have been shown to mediate the antigen-binding function. The proposed similarities in tertiary structure among C domains have not been established,

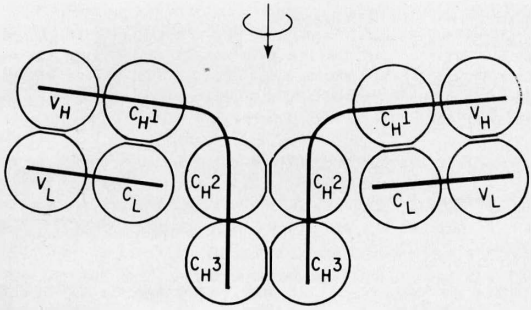


FIG. 5. The domain hypothesis. Diagrammatic arrangement of domains in the free immunoglobulin G molecule. The arrow refers to a dyad axis of symmetry. Homology regions (see Figs. 2–4) which constitute each domain are indicated: V_L, V_H—domains made up of variable homology regions; C_L, C_H1, C_H2, and C_H3—domains made up of constant homology regions. Within each of these groups, domains are assumed to have similar three-dimensional structures and each is assumed to contribute to an active site. The V domain sites contribute to antigen recognition functions, and the C domain sites to effector functions.

however, nor have the various effector functions of the different C domains been fully determined. There is a suggestion that C_H2 may play a role in complement fixation (Kehoe and Fougereau, 1970). A good candidate for binding to the lymphocyte cell membrane is C_H3, the function of which may be concerned with the mechanism of lymphocyte triggering following the binding of antigen by V domains. The C_H3 domain has already been found to bind to macrophage membranes (Yasmeen *et al.*, 1973).

There is now some evidence that lymphocytes can synthesize isolated domains similar to C_H3 as separate molecules. Berggård and Bearn (1968) first isolated a urinary protein called β_2-microglobulin from patients with renal tubular insufficiency. On the basis of the sequence analysis of the first 41 residues of this protein, Smithies and Poulik (1972) suggested that this protein was homologous to immunoglobulins. Work by Peterson *et al* (1972) and Cunningham *et al.* (1973) in my laboratory has defined the complete amino acid sequence of this protein (Fig. 6), strongly confirming the homology. It appears, therefore, that

FIG. 6. Comparison of the amino acid sequence of β_2-microglobulin with the homology regions C_L, C_H1, C_H2, and C_H3 of the γG_1 immunoglobulin Eu. Deletions, indicated by dashes, have been inserted to maximize homologies. Identical residues are enclosed in boxes. Numbers are for β_2-microglobulin.

this protein is a free immunoglobulin domain, and indeed there is now evidence that it is synthesized by lymphocytes (Bernier and Fanger, 1972). Even more exciting is the recent observation (Nakamuro *et al.*, 1973; Peterson *et al.*, 1974) that this protein is the light polypeptide chain of the histocompatibility (HL-A) antigen. This tends to support the hypothesis (Gally and Edelman, 1972) that immunoglobulin genes have arisen in evolution from those specifying the histocompatibility system.

Although many details still are lacking, the gross structural aspects of the domain hypothesis have received direct support from X-ray crystallographic analyses of Fab fragments (Poljak *et al.*, 1972) and whole molecules (Davies *et al.*, 1971), in which separate domains were clearly discerned. Indirect support for the hypothesis has also come from experiments on proteolytic cleavage of regions between domains (Gall and D'Eustachio, 1972; D. Inbar *et al.*, 1972). Whatever the selective advantages of the domain arrangement, it is clear that gene duplication would permit the modular alteration of immunological function by addition or deletion of domains during evolution.

III. The Origin of Diversity and the Arrangement of Immunoglobulin Genes

One of the most satisfying conclusions that emerged from the structural analysis of antibodies is that the diversity of the V regions of antibody chains is sufficient to satisfy the requirements of clonal selection theories.

Comparisons of the primary structures of a great variety of immunoglobulins shows that the diversity of V regions has several distinct features. The fact that V regions fall into subgroups of related sequences was first pointed out for light chains by Milstein (1967) and Hood *et al.* (1967) and is illustrated for heavy chains (Cunningham *et al.*, 1969; Press and Hogg, 1969; Wikler *et al.*, 1969) in Figs. 7 and 8. The sequences of the different V region subgroups are sufficiently different to warrant the conclusion that the subgroups are specified by different genes. Within a subgroup, the amino acid sequence variations may be accounted for by single base substitutions in the genetic code. Moreover,

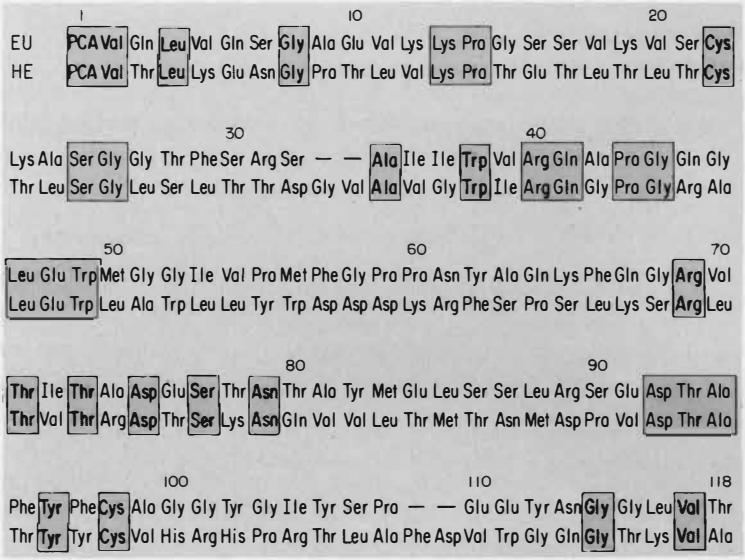


FIG. 7. Comparison of V_H regions of protein Eu (subgroup V_{HI}) and protein He (subgroup V_{HII}). V_H regions within each subgroup are more closely similar in sequence than proteins belonging to different subgroups.

certain positions show many substitutions (the so-called hypervariable regions; see Wu and Kabat, 1970) whereas others, such as the half-cystine residues at positions 23 and 88 of kappa chains, have never been observed to vary at all and may play a role in stabilizing the antigen combining site (Edelman, 1971). This set of observations indicates that both mutation and selection have operated to yield variable region structure.

The ultimate origin of the mutation and selection is one of the two outstanding large problems of molecular immunology. Although this problem is not solved, it is understood that the diversity arises at three levels of structural or genetic organization:

1. Association of various V_H with various V_L regions; i.e., inasmuch as V regions from both heavy and light chains contribute to the antigen-binding site, the number of possible antibodies may be as great as the product of the number of different V_L and V_H regions (Edelman, 1971).

FIG. 8. Comparison of V_{HII} regions showing variations within a single heavy-chain subgroup. Proteins Daw and Cor are described by Press and Hogg (1969) and protein Ou is from μ chains (Wikler *et al.*, 1969). Copyright 1969 by the American Association for the Advancement of Science.

2. The existence of V region subgroups, each specified by at least one separate germ line gene which must have been selected during evolution (Milstein, 1967; Hood *et al.*, 1967).

3. Intrasubgroup variation of unknown origin.

The key problem of the generation of antibody diversity has been converted by the work on immunoglobulin chains and V region subgroups to the problem of the origin of sequence variations within each subgroup. It is still not known whether there is a germ line gene for each V region within a subgroup or whether each subgroup contains only a few genes, and intrasubgroup variation arises by somatic genetic rearrangements within precursors of antibody-forming cells. The various theories supporting one or the other of these alternatives have been discussed elsewhere (Gally and Edelman, 1970). At this time, therefore, we can conclude that only the basis, but not the ultimate origin, of diversity has been adequately explained by the work on structure.

Although the work on the structure of antibodies has not fully resolved the problem of the origin of diversity, it has contributed much to an understanding of the nature of the structural genes for immunoglobulins. There is now considerable evidence to suggest that the arrangement of these genes is unusual, and it may in fact turn out that this arrangement reflects the unusual requirements for the generation of diversity. Briefly, the facts leading to the conclusion that structural genes for immunoglobulins are special are as follows: whereas different V region subgroups are specified by a number of nonallelic genes (Milstein, 1967), the analysis of genetic or allotypic markers suggests that C regions of a given immunoglobulin class are specified by no more than one or two genes. These allotypic markers, first described by Grubb (1956, 1970) and Oudin (1956), provide a means in addition to sequence analysis for understanding the genetic basis of immunoglobulin synthesis. It has been found that V regions specified by a number of different genes can occur in different chains, each of which may have the same C region specified by a single gene. This leads to the remarkable conclusion that each immunoglobulin chain is specified by two genes, a V gene and a C gene (Milstein, 1967; Hood *et al.*, 1967).

Work in a number of laboratories (reviewed by Gally and Edelman, 1972) has shown that genetic markers on the two types of light chains are not linked to those of the heavy chains or to each other. These findings, and the conclusion that there are separate V and C genes, led Gally and me (1970, 1972) to

suggest that immunoglobulins are specified by three unlinked gene clusters (Fig. 9). The clusters have been named translocons to emphasize the fact that a mechanism must be provided to combine genetic information from V region loci with information from C region loci to make complete V-C structural genes. According to this hypothesis, the translocon is the basic unit of immunoglobulin evolution, different groups of immunoglobulin chains having arisen by duplication and various chromosomal rearrangements of a precursor gene cluster. Presumably, gene duplication during evolution also led to the appearance of V region subgroups within each translocon.

In this cursory discussion, I have attempted to show that the work on antibody structure carried out in a number of laboratories has not only satisfied certain basic requirements of the theory of clonal selection, but has also sharpened certain remaining questions. We now understand that the heterogeneity of antibodies is the result of sequence diversity of V regions as well as sequence differences in C_H regions specifying the different immunoglobulin classes. The structure–function problem has been neatly resolved: V domains carry out antigen-binding functions and C domains

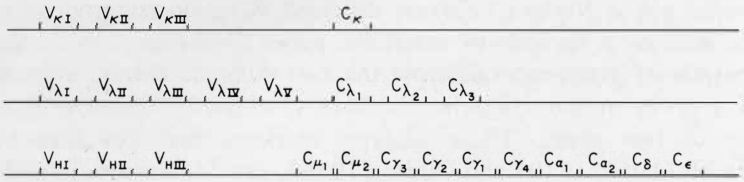


FIG. 9. A diagrammatic representation of the proposed arrangement in mammalian germ cells of antibody genes in three unlinked clusters termed translocons. κ and λ chains are each specified by separate translocons, and heavy chains are specified by a third translocon. The exact number and arrangement of V and C genes within a translocon is not known. Each variable region subgroup (designated by a subscript corresponding to chain group and subgroup) must be coded by at least one separate germ line V gene. The number of V genes within each subgroup is unknown, however, as is the origin of intrasubgroup diversity of V regions. A special event is required to link the information from a particular V gene to that of a given C gene. The properties of the classes and subclasses (see Table I) are conferred on the constant regions by C genes.

mediate effector functions. Furthermore, the evidence suggests that the genes for classes and domains almost certainly arose by gene duplication and chromosomal rearrangements during evolution. As a result of these processes, it appears that separate V and C genes occur in clusters, each cluster specifying a given kind of chain.

Practically all these conclusions and the formulation of the major problem of the origin of diversity have come from work on humoral antibodies. This work is just a beginning, however, for the subject of antibodies on cells and the nature of the specific triggering of these cells comprise a second major problem of molecular immunology. Much less is known in this area of study, but there are some hints that it may be approached at the molecular level.

IV. Cellular Specificity and Cell Surface Antibodies

What is the mechanism by which a particular antigen induces clonal proliferation or immune tolerance in certain populations of lymphoid cells? Although many means are being used by cellular immunologists to study this question, two approaches seem to be particularly suitable for its analysis at the molecular level. The first and most direct approach is to fractionate lymphocytes according to the specificity of their receptor antibodies both for subsequent studies of these antibodies and of the cellular response to antigens of known molecular geometry. The second approach is to analyze the structure and activity of molecules, such as lectins, that can stimulate lymphocytes regardless of their antigen-binding specificity. I shall discuss below some experiments, admittedly preliminary, that my colleagues and I have recently carried out using each of these approaches.

In the attempt to understand cellular specificity, it is particularly important to distinguish between antigen-binding and antigen-reactive cells (Fig. 10). The work of Nossal and Mäkelä (see Mäkelä and Cross, 1970) showed that each antibody secreting cell makes antibodies of a different specificity and the work of Ada and Nossal and others (see Nossal and Ada, 1971) has

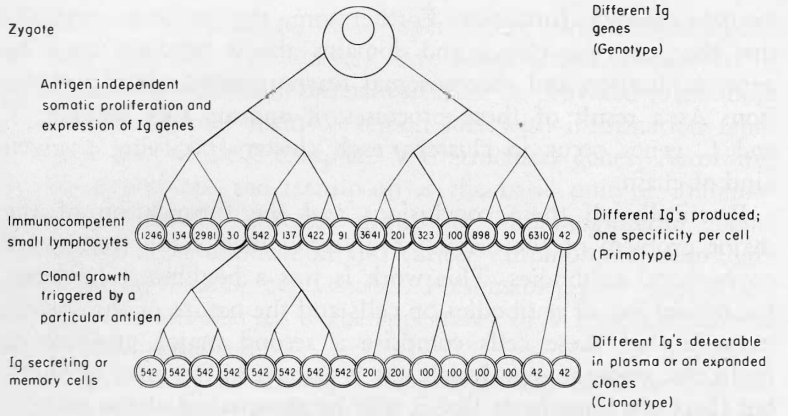


FIG. 10. A model of the somatic differentiation of antibody-producing cells according to the clonal selection theory. The number of immunoglobulin genes may increase during somatic growth so that, in the immunologically mature animal, different lymphoid cells are formed, each committed to the synthesis of a structurally distinct receptor antibody (indicated by an arabic number). A small proportion of these cells proliferate upon antigenic stimulation to form different clones of cells, each clone producing a different antibody. This model represents bone marrow-derived (B) cells, but with minor modifications it is also applicable to thymus-derived (T) cells.

demonstrated the existence of antigen binding cells of different specificities.

Inasmuch as an animal is capable of responding specifically to an enormous number of antigens to which it is usually never exposed, it must contain genetic information for synthesizing a much larger number of different immunoglobulin molecules on cells than actually appear in detectable amounts in the bloodstream. One may therefore distinguish two levels of expression in the synthesis of immunoglobulins that Gally and I (1972) have termed for convenience the *primotype* and the *clonotype* (Fig. 10). The primotype consists of the sum total of structurally different immunoglobulin molecules generated within an organism during its lifetime. The clonotype consists of those immunoglobulin molecules synthesized as a result of antigenic stimulation and clonal expansion. These molecules can be detected and classified according to antigen-binding specificity, class, antigenic de-

terminants, primary structure, allotype, or a variety of other experimentally measurable molecular properties. As a class, the clonotype is smaller than the primotype and is wholly contained within it (Fig. 10).

Although a view of the clonotype is afforded by the analysis of humoral antibodies, we know very little about the primotype. In our laboratory, we have been attempting to approach the problem of the specific fractionation of lymphocytes expressing the primotype by using nylon fibers to which antigens have been covalently coupled (Edelman *et al.,* 1971; Rutishauser *et al.,* 1972). The derivatized fibers are strung tautly in a tissue culture dish (Fig. 11) so that cells shaken in suspension may collide with them. Some of the cells colliding with the fibers are specifically bound to the covalently coupled antigens by means of their surface receptors. Bound cells may be counted microscopically

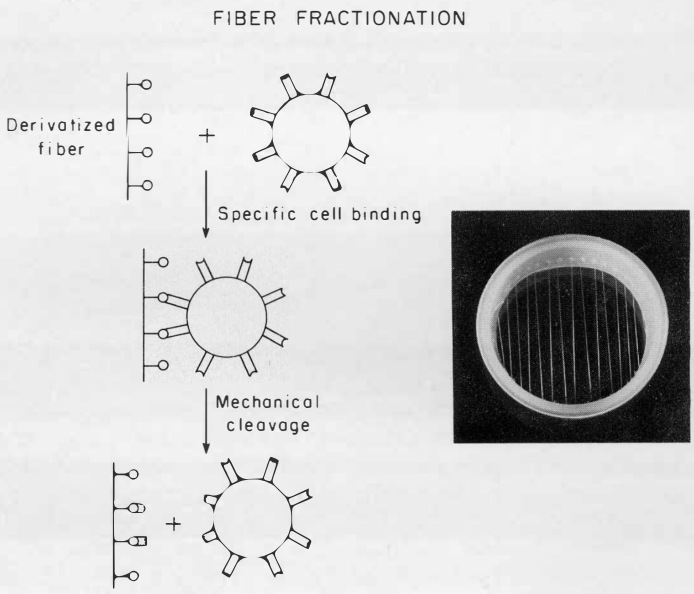


FIG. 11. General scheme for fiber fractionation of cells. Inset shows a tissue culture dish with nylon fibers held under tension in a polyethylene frame.

in situ by focusing on the edge of the fiber (Fig. 12). After washing away unbound cells, the specifically bound cells may be removed by plucking the fibers and shearing the cells quantitatively from their sites of attachment. The removed cells retain their viability provided that the tissue culture medium contains serum.

Derivatized nylon fibers have the ability to bind both thymus-derived lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) (Gowans *et al.*, 1971) according to the specificity

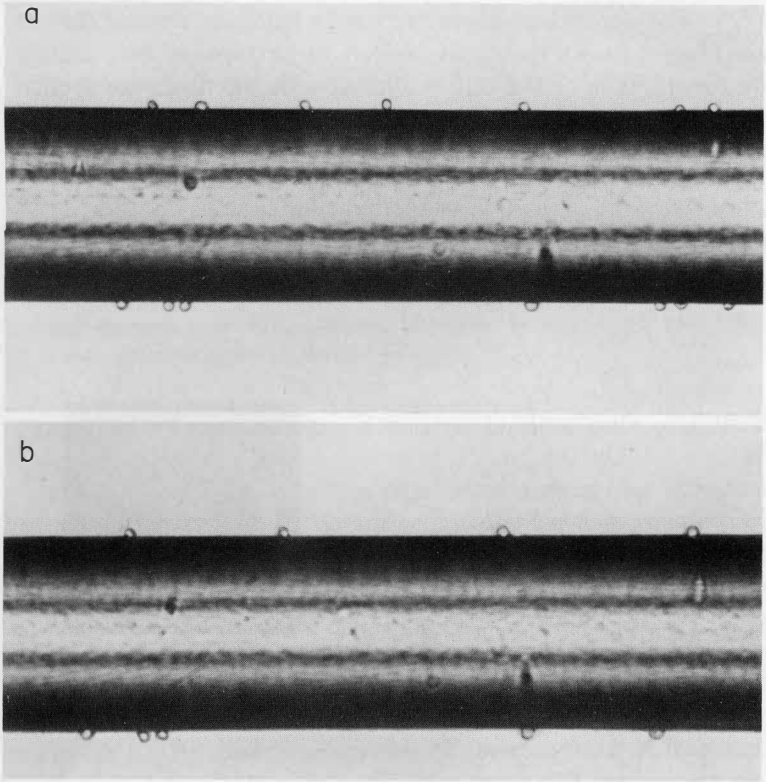
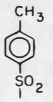


FIG. 12. Lymphoid cells from the mouse spleen bound by their antigen-specific receptors to a nylon fiber to which dinitrophenyl bovine serum albumin has been coupled. Treatment of bound cells in (a) with antiserum to the T cell surface antigen θ and with serum complement destroys the T cells, leaving B cells still viable and attached (b). See Table II. ×130.

of their receptors for a given antigen (Rutishauser and Edelman, 1972) (Fig. 12, Table II). About 60% of spleen cells specifically isolated are B cells, and the remainder are T cells. By the use of appropriate antisera to cell surface receptors, the cells of each

TABLE II

CHARACTERIZATION OF MOUSE LYMPHOID CELLS FRACTIONATED ACCORDING TO THEIR ANTIGEN-BINDING SPECIFICITIES[a]

Antigen on Fiber	Dnp 	Dnp	Tosyl 	Tosyl
Immunization	none	Dnp	none	Tosyl
Cells Bound to Fiber (per cm)	1200	4000	800	2000
% Inhibition of Binding by:				
Dnp	90	95	5	10
Tosyl	1	2	75	87
Anti-Ig	85	93	73	90
High Avidity Cells (per cm)	<100	2800	—	—
Low Avidity Cells (per cm)	1200	1200	—	—
% T Cells	41	39	43	—
% B Cells	59	56	54	—

[a] Nylon fibers were derivatized with hapten conjugates of bovine serum albumin, and mice were immunized with each of the designated haptens coupled to hemocyanin. Inhibition of binding was achieved by addition of hapten–protein conjugates (250 μg/ml) or rabbit antimouse immunoglobulin (Ig) (250 μg/ml) to the cell suspension. High avidity cells are defined as those prevented from binding by concentrations of DNP-bovine serum albumin of less than 4 μg/ml in the cell suspensions. Cells inhibited by higher concentrations are defined as low avidity cells. Virtually complete inhibition occurs at levels of homologous hapten greater than 100 μg/ml.

type can be counted on the fibers and most of the cells of one type or the other may then be destroyed by the subsequent addition of serum complement. In this way, one can obtain populations of either T or B cells that are highly enriched in their capacity to bind a given antigen.

Cells of either kind may be further fractionated according to the relative affinity of their receptors. This can be accomplished by prior addition of a chosen amount of the free antigen, which serves to inhibit specific attachment of subpopulations of cells to the antigen-derivatized fibers by binding to their receptors. As defined by this technique, cells capable of binding specifically to a particular antigen constitute as much as 1% of a mouse spleen cell population. Very few of these original antigen-binding cells appear to increase in number after immunization, however (Rutishauser *et al.*, 1972), and the cells that do respond are those having receptors of higher relative affinities (Fig. 13a). This is in agreement with the conclusions reached by Siskind and Benacerraf (1969) in their analysis of the humoral antibody response.

Using this method together with serological means of distinguishing T and B cells, the cells may be compared for their range of antigen-binding specificities and avidities for antigens. Our studies suggest that T and B cells do not differ in their antigen-binding specificities, at least for several hapten and protein antigens (Rutishauser and Edelman, 1972). A comparison of the avidities of T and B cells for the DNP hapten showed that the avidities of T and B cells were the same for the monovalent ϵ-DNP-lysine (Fig. 13b,c). In contrast, T cells showed a consistently higher apparent avidity for multivalent DNP-bovine serum albumin containing an average of 10 DNP groups per molecule. This suggests the possibility that the receptors of T cells may be arranged or are arrangeable on the cell surface in clusters that differ from those of B cells.

Whether these cell populations correspond to the cells expressing the primotype remains to be determined. It is significant, however, that fiber-binding cells do not include plaque-forming cells (Jerne *et al.*, 1963), and it is therefore possible to fractionate antigen-binding cells from cells that are already actively secreting

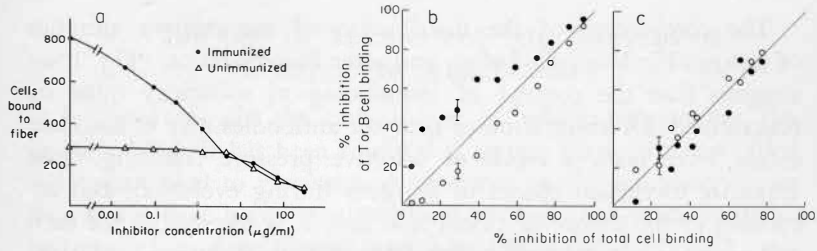


FIG. 13. (a) Inhibition by free DNP-BSA of spleen cell binding to DNP-BSA derivatized fibers. Cell numbers represent fiber edge counts for a 2.5 cm fiber segment. Spleens from immunized mice were removed at the height of a secondary response to DNP-BGG, and cells from several mice were pooled. ●——●, immunized; △——△, unimmunized.

(b and c) Percent inhibition of T cell (●) and B cell (○) binding to DNP-BSA-derivatized fibers as a function of the percent inhibition of total cell binding. Inhibition was obtained with different amounts of (b) DNP-BSA or (c) ε-DNP-lysine. As described (Rutishauser *et al.*, 1972), the percent inhibition of total cell binding increased from 0 to 90% as the concentrations of either inhibitor increased from 0 to 300 μg/ml. T and B cells were identified by cytolytic treatment of the total bound cell population with anti-θ or antimouse immunoglobulin sera. The cells were obtained from the spleens of mice 5 days after secondary immunization with DNP-hemocyanin. Error bars represent the standard deviation from the average values obtained in four experiments. If there were no differences in the binding behavior of T and B cells, the points would fall on the 45° line.

antibodies. Our recent experiments indicate that the antigen-binding cells isolated by this method may be transferred to irradiated animals to reconstitute a response to the antigen used to isolate them. Moreover, they are capable of secondary immune responses in tissue culture. These findings suggest that the antigen-specific population of cells removed from the fibers contains at least some of the precursors of plaque-forming cells. Although these results are encouraging, a number of questions remain to be answered concerning the specificity and function of lymphoid cells isolated by fiber fractionation. In addition, the method must be compared and combined with other means of cell fractionation. This approach should be useful for the isolation of surface antibodies as well as for analysis of populations of lymphoid cells in both developing and adult animals.

The comparison of the distribution of the relative affinities of antigen-binding cells before and after immunization (Fig. 13a) suggests that the concept of immunological specificity must be reexamined. Diversification of receptor antibodies may to a certain extent have been a result of selective pressure resulting from exposure to certain classes of antigens during evolution. But according to the theory of clonal selection, it is impossible for each cell receptor to have been selected for or against during evolution. Instead, a great number of antibody variants have been generated (by whatever process, germ line or somatic) many of which will never be selected during the lifetime of the organism. Under these circumstances how specific can such a system be? This question can be posed in the following terms: Is the probability of cross-reactivity with various antigens the same in the primotype and the clonotype?

The evidence on antigen-binding and antigen-reactive cells suggests that the degree of cross-reactivity may be much greater in the primotype. If this is the case, selection for specificity cannot merely be the result of antigen-binding, but must also depend on a second factor. The most likely candidate is the triggering threshold for stimulation of the cell carrying the antibody receptor. If, for example, a cell population contains cells that can bind two different antigens, specificity could be lost. It would be preserved, however, if a particular cell capable of binding both antigens is more likely to be triggered by only one of them. The trigger threshold might depend on the state of the cell but might also depend particularly on steric factors (reflected in the free energy of binding for each particular antigen) as well as on the surface density of the antigen molecules leading to changes in the avidity of the binding.

Whatever the detailed mechanism of triggering, the implication of this two-factor hypothesis is that variation at the level of the primotype leads to a relatively nonspecific set of immunoglobulin molecules, many of which are capable of binding various antigens with relatively low specificity. According to this idea, the selective forces that yield specificity are a product of both the probability of binding and the probability of lymphocyte stimulation above a certain triggering threshold.

V. The Use of Lectins for the Analysis of Lymphocyte Stimulation

Antigens are not the only means by which lymphocytes can be stimulated. It has been found that certain plant proteins called lectins can bind to glycoprotein receptors on the lymphocyte surface and induce blast transformation, mitosis, and immunoglobulin production (see Sharon and Lis, 1972, for a review). Although their mitogenic properties can be quite similar, different lectins have different specificities for cell surface glycoproteins as well as different molecular structures. In addition, they have a variety of effects on cell metabolism and transport. Such effects are independent of the antigen-binding specificity of the cell, and they may therefore be studied prior to specific cell fractionation.

The fact that antigens and lectins of different specificity and structure may stimulate lymphocytes suggests that the induction of mitosis is a property of membrane-associated structures on the lymphocyte that can respond to a variety of receptors. The process of stimulation appears to be independent of the specificity of these receptors for their various ligands. This implies that the triggering threshold may depend upon the affinity of a receptor, as well as upon its distribution and mode of attachment to the cell membrane in a given type of T or B cell. To understand mitogenesis, it is therefore necessary to solve two problems. The first, to determine in molecular detail how the lectin binds to the cell surface and to compare it to the binding of antigens. The second is to determine how the binding induces metabolic changes necessary for the initiation of cell division. These changes are likely to include the production or release of a messenger which acts as a final common pathway for the stimulation of the cell by any particular lectin or antigen.

In attempting to solve these problems, it would be valuable to know the complete structure of several different mitogenic lectins. This structural information is particularly useful in trying to understand the molecular transformation at the lymphocyte surface required for stimulation. With the knowledge of the three-dimensional structure of a lectin, for example, various amino acid side chains at the surface of the molecule may be modified

by group reagents, which also may be used to change the valence of the molecule. The activities of the modified lectin derivatives may then be observed in various assays of their effects on cell surfaces and cell functions.

My colleagues and I (Edelman *et al.*, 1972) have recently determined both the amino acid sequence and three-dimensional structure (Fig. 14) of the lectin concanavalin A (Con A). This lectin has specificity for glucopyranosides, mannopyranosides, and fructofuranosides, and it binds to glycoproteins and possibly glycolipids on a variety of cell surfaces. Our studies were aimed at determining the exact size and shape of the molecule, its valence, and the structure and distribution of its binding sites.

With this knowledge in hand, we have been attempting to modify the structure and to determine the effects of that modification on various biological activities of the lymphocyte. So far, there are several findings suggesting that such alterations of the structure have distinct effects. Con A in free solution stimulates thymus-derived lymphocytes (T cells) but not bone marrow-derived lymphocytes (B cells) (Andersson *et al.*, 1972a,b). This stimulation leads to increased uptake of thymidine by the cells and to blast transformation. The dose response curve of stimulation of T cells by native Con A shows a rising limb of thymidine uptake and a falling limb (Fig. 15) that probably reflects cellular inhibition and death. The fact that the mitogenic effect and the killing effect are dose dependent suggests an analogy to stimulation and tolerance induction by antigens.

Derivatization of a lectin can alter the dose response curve. When Con A is succinylated, for example, it dissociates from a tetramer to a dimer without alteration of its carbohydrate binding specificity (Gunther *et al.*, 1973). Although succinylated Con A is just as mitogenic as native Con A, the falling limb is not seen until much higher doses are reached (Fig. 15). The dose response curve for this derivative indicates that the mitogenic response approaches saturation at high doses of the lectin.

Succinylation of Con A also alters another effect of this lectin on cell surfaces. Taylor *et al.* (1971) have demonstrated that immunoglobulin receptors are mobile and that they form "patches" and "caps" after addition of divalent antibodies

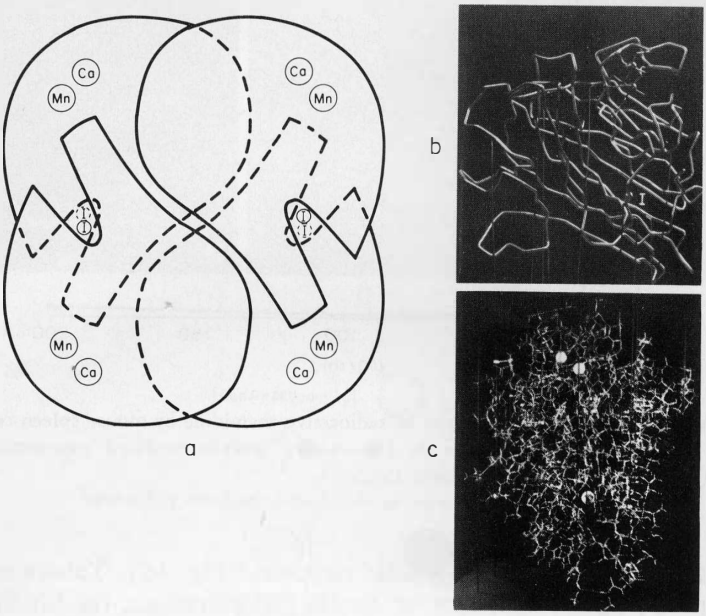


FIG. 1·1. Three-dimensional structure of concanavalin A, a lectin mitogenic for lymphocytes. (a) Schematic representation of the tetrameric structure of Con A viewed down the z axis. The proposed binding site for transition metals, calcium, and the iodine atom of the labeled inhibitor β-(o-iodophenyl)-D glucopyranoside (β-IPG) are indicated by Mn, Ca, and I, respectively. The monomers on top (solid lines) are related by 2-fold axis, as are those below. The two dimers are paired across an axis of D_2 symmetry to form the tetramer. (b) Wire model of the polypeptide backbone of the concanavalin A monomer oriented approximately to correspond to the monomer on the upper right of the diagram in (a). The two balls at the top represent the Ca and Mn atoms and the ball in the center (marked I) is the position of an iodine atom in the sugar derivative, β-IPG, which is bound in the crystal. Four such monomers are joined to form the tetramer as shown in (a). (c) A view of the Kendrew model of the Con A monomer rotated to show the deep pocket formed by the binding site. (The white ball at the bottom of the figure is at the position of the iodine atom of β-iodophenyl glucoside.) The two white balls at the top represent the metal atoms.

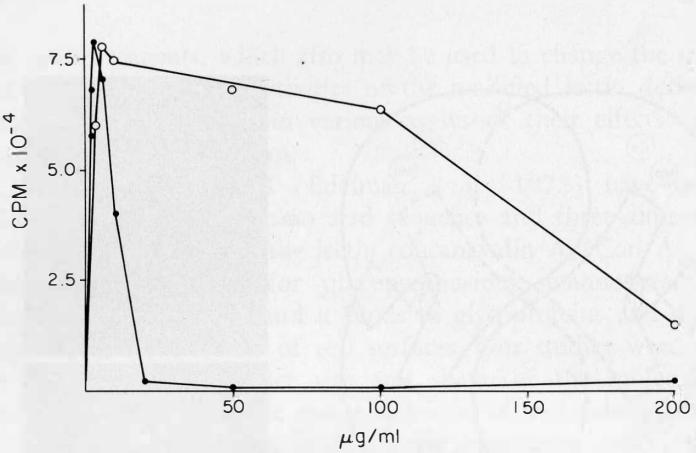
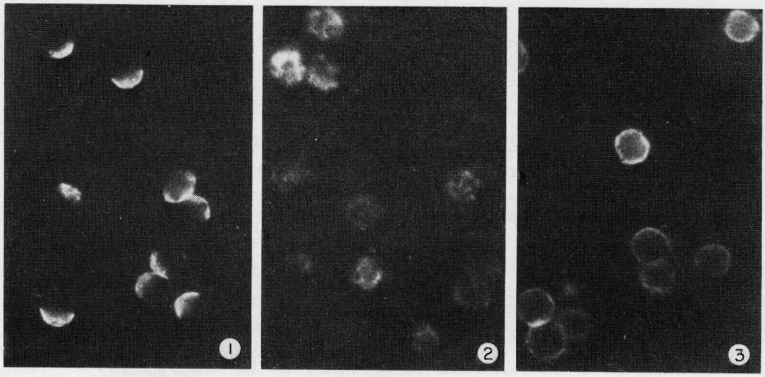


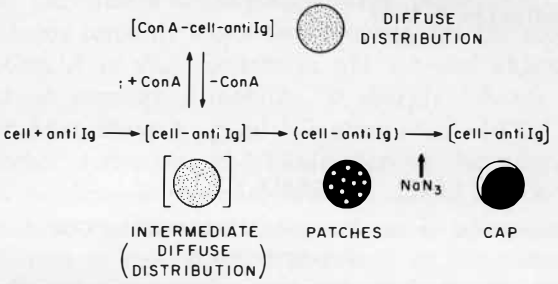
FIG. 15. Stimulation of uptake of radioactive thymidine by mouse spleen cells after addition of concanavalin A (●——●) and succinylated concanavalin A (○——○) in increasing doses (μg/ml).

directed against immunoglobulin receptors (Fig. 16). Yahara and I (1972) have found that, at certain concentrations, the binding of Con A to the cell surface restricts the movement of immunoglobulin receptors, preventing both cap and patch formation (Fig. 16). This suggests that Con A somehow changes the fluidity of the cell membrane, resulting in reduction of the relative mobility of these receptors. In contrast, succinylated Con A has no such effect, although it binds to lymphocytes to the same extent as native Con A molecules and it can also compete with Con A for binding sites.

If Con A is added to cells at 4°C, and the cells with bound Con A are brought to 37° after washing away free Con A, the Con A receptors themselves will form caps. If the Con A is not washed away or if the experiment is carried out at 37°, however, cap formation is inhibited. Succinyl-Con A does not itself form caps under any of these conditions nor does it inhibit cap formation as I have already pointed out. Even more striking is the observation (Gunther *et al.*, 1973) that the addition of divalent antibodies against Con A to cells that have already bound



a



b

FIG. 16. Patch and cap formation in mouse spleen cells and its inhibition by Con A. (a) Patterns of caps (1); patches obtained after addition of fluorescein-labeled antiimmunoglobulin (2); and diffuse distribution of fluorescence when cells are first treated with Con A (3). (b) A model for patch and cap formation and its inhibition by Con A.

succinylated Con A results again in restriction of immunoglobulin receptor mobility.

All these findings (summarized in Table 3 and Fig. 17) suggest that Con A has two antagonistic actions that depend both on the state of the lectin and on the state of the cell. These actions are the induction of cap formation by the Con A receptors themselves and the inhibition of the mobility of Con A receptors as well as other cell receptors. The results appear to be compatible

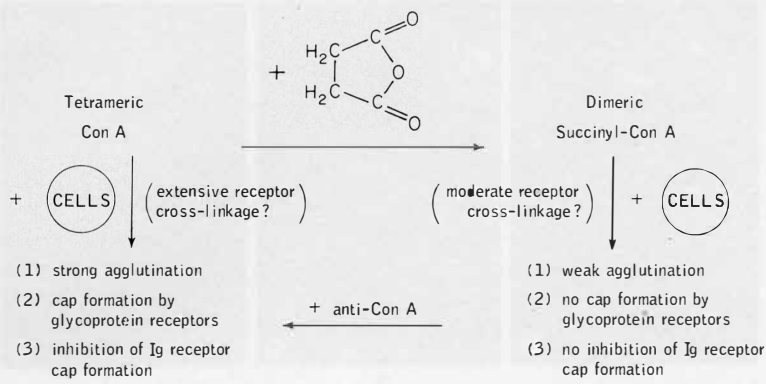


FIG. 17. Schematic comparison of the biological properties of native Con A and succinyl-Con A. The activities listed include the immediate cellular reactions mediated by the lectin.

TABLE III

COMPARISON OF THE BIOLOGICAL ACTIVITIES OF CON A AND SUCCINYL-CON A

Properties	Con A	Succinyl-Con A
No. of binding sites per cell		
Sheep erythrocytes	1.1×10^6	2.8×10^6
Mouse spleen cells	1.4×10^6	4.4×10^6
Percent inhibition of anti-Ig capping		
Lectin (100 μg/ml)	100	0
Lectin (50 μg/ml) + anti-Con A (100 μg/ml)	100	40
Percent of cells forming lectin receptor caps		
Lectin (5 μg/ml, 37°)	0.2–2	0
Lectin (100 μg/ml, 37°)	≤0.2	0
Lectin (170 μg/ml), preincubated in ice bath, washed, then brought to 37°	62	—
Lectin (20 μg/ml) + anti-Con A (100 μg/ml)	18	82
Mitogenesis		
Lectin (5 μg/ml)	Positive	Positive
Lectin (50 μg/ml)	Negative	Positive

with the hypothesis that there are at least two states or types of Con A receptors. According to this hypothesis, attachment of Con A to one type of receptor has no effect on the mobility of other receptors, but attachment to the other type inhibits the mobility of the other receptors. These findings may be related to those of Sachs and his co-workers (M. Inbar *et al.*, 1972) on the agglutination of cells by Con A.

What is the major factor in the alteration of Con A activity by succinylation? The abolition of the inhibition effect in mitogenic assays (Fig. 15) and the failure to inhibit receptor mobility may be the result of a change in valence or a change in the surface charge of the molecule. The restoration of the Con A effects after addition of antibodies against Con A (Fig. 17) suggests that the valence is the major factor. Nonetheless, alteration of side chains certainly has some influence, for the acetyl derivative of Con A is also divalent at pH 7.4 and although it has no effect on membrane mobility, it sharply inhibits thymidine uptake at doses above 5 μg/ml (Gunther *et al.*, 1973). Examination of other derivatives and localization of the substituted side chains in the three-dimensional structure should help to determine the exact causes of these alterations.

In addition to modifications that lead to structures of lower valence, Con A may also be modified by chemically cross-linking several molecules. A very striking effect is seen if the surface density of the Con A molecules presented to the lymphocyte is increased by cross-linking them at solid surfaces (Andersson *et al.*, 1972a). As I have indicated, Con A in free solution stimulates mouse T cells, with resultant increased incorporation of radioactive thymidine, but it has no effect on B cells. When cross-linked at a solid surface, however, it stimulates mainly mouse B cells, although both T and B cells have approximately the same number of Con A receptors. Similar results have been obtained with other lectins (Greaves and Bauminger, 1972). A reasonable interpretation of these phenomena (although not the only one) is that the lectin acts at the cell surface, that the surface density of mitogen binding sites is an important variable in exceeding the threshold for the lymphocyte stimulation, and that this threshold differs in the two kinds of lymphocytes.

VI. A Working Hypothesis to Account for Lectin Activity

Before a detailed model of lymphocyte stimulation can be formulated, much more must be known about the structure and movements of the cell membrane and the chemistry and attachment of the cell surface receptors. Nevertheless, the experiments that I have described and those of other workers do suggest a working hypothesis which may serve to consolidate the disparate findings on lectin activity. This working hypothesis deliberately excludes consideration of the important but complex mass of data on T cell–B cell cooperation after antigenic stimulation as well as the important information on nonspecific factors involved in the immune response (Andersson *et al.*, 1972b). Instead, the hypothesis focuses on the necessary first step of mitogenesis, i.e., the nature and immediate consequences of lectin and antigen binding.

One of the key assumptions made in formulating this hypothesis is that both lectin-induced triggering and antigen-mediated triggering share the same mechanism as well as a final common pathway for surface nuclear interaction. A number of observations suggest that antigens and lectins stimulate lymphoid cells in the same way (Greaves and Janossy, 1972) providing indirect support for the assumption of a shared pathway of stimulation. These observations include the similarity of the time course in culture, the indication that high doses of either lectins or antigens inhibit the response, and the similarity of blast formation and immunoglobulin production. Moreover, as is the case with antigens, the available data suggest that stimulation by lectins is mediated at the cell surface; suppression may be mediated at the cell surface but also by endocytosis of the lectin–receptor complex. Although T and B cells have different thresholds for stimulation and suppression, it is assumed here that the same basic mechanism of stimulation and suppression applies to both cell types.

The assumption that there is a common pathway of stimulation implies that both the mode of attachment of antibody and lectin receptors to the membrane and their perturbation by cross-linkage

at the cell surface may be similar, despite the difference in their specificities and molecular structures. It also implies that triggering or suppression of clonal proliferation is specifically related to the mode of anchorage of the antibody molecule to the cell membrane. The experiments on succinyl-Con A indicate that suppression by this lectin is related to the valence and the state of aggregation of the molecule, but they do not as yet indicate whether these factors are important in stimulation. There are two points to decide: (1) Is intermolecular interaction among receptors in the same cell a necessary and sufficient condition for stimulation or is attachment to single receptors sufficient? (2) Are stimulation and suppression related or are they caused by different mechanisms?

In the present hypothesis, I assume that the necessary and sufficient first step in triggering is the formation of cross-linked aggregates of certain of the cell surface glycoprotein receptors to form a *micropatch.* Such a micropatch would consist of as few as two or as many as 100 receptor molecules; after adequate stimulation, the membrane of a given lymphocyte could therefore have as many as 100–500 micropatches. These micropatches must remain stable (i.e., neither increase nor decrease greatly in size) for sufficient periods of time (up to hours) and diffusion of receptors into and out of a micropatch must therefore be balanced. The formation of a micropatch requires multivalence of the antigen or lectin. Therefore, the formation of a micropatch of proper size and stability depends upon the binding constant, the valence of the lectin or antigen as well as upon its specificity for the proper cell surface receptors. Antigenic determinants or lectin molecules need not, however, be presented in a *regular* array to provide an adequate stimulus; the only requirement is that a sufficient surface density of receptor-lectin or receptor-antigen complexes (Fanger *et al.*, 1970) be maintained.

Formation of larger aggregates would lead to patches, cap formation, and interruption of the stimulation cycle. Failure to form micropatches rapidly enough after binding low doses of antigen or lectin would lead to blockade of stimulation. As I have said, B cells are assumed to have a different threshold of response

to micropatches than T cells, and it is likely that their thresholds for patch and cap formation also differ (Yahara and Edelman, unpublished observations).

In the absence of further experimental tests of these ideas, little can be said in detail about the coupling of micropatch formation to metabolic events initiating cell division. The data indicate that irreversible changes *in vitro* require as long as 12–24 hours of exposure to lectins, and it is likely that a whole series of enzymatic reactions are involved. The assumption that there is a final common pathway for the mitogenic stimulus, however, would suggest a single mediator such as cyclic AMP or cyclic GMP, and there is some evidence that the latter is involved (Hadden *et al.*, 1972). A possible means of coupling micropatch formation to metabolic changes is via interaction with enzymes such as adenyl cyclase or phosphodiesterase. One plausible hypothesis is that the formation of stable micropatches allows interaction of intramembranous particles to which such enzymes might be linked. Such interactions could result in induction of enzymatic action via allosteric or other conformational changes. An alternative mode in which receptor aggregation may be coupled to metabolic events is by means of transport defects, induced after perturbation of the alignment of the phospholipid head groups by the micropatches in the membrane. Movements of ions or small molecules in or out of the cell might then provide the necessary stimulus. Finally, lectin effects may be indirect and require the induction in one cell type of stimulatory or inhibitory factors, which then act on target cells, i.e., two cell types may be required (Andersson *et al.*, 1972b).

The foregoing hypothesis on the necessary initial conditions for stimulation and suppression is compatible with any one of these subsequent metabolic events and it leads to a number of predictions: (1) the valence, state of aggregation, specificity, and binding constant for carbohydrate on the appropriate receptors are overriding in stimulation; the detailed structure of a particular mitogenic lectin plays only a minor role in mitogenic activity. Only certain cell receptors are linked to the mitogenic pathway; some lectins will be nonmitogenic because they bind to receptors that are not so linked. (2) If lectins that are multivalent are

made univalent, mitogenesis will be blocked (an exception to this prediction might conceivably occur if univalent lectins aggregated *after* binding to the cell surface). (3) Lectins that are not mitogenic for B cells but are mitogenic for T cells may be made mitogenic for B cells by cross-linking (this has already received support in two cases, as I have already indicated). (4) Some lectins that are not mitogenic may become mitogenic by cross-linking them to themselves or to other lectins, or after coupling them to Fab fragments of anti-immunoglobulins. (5) In a purified cell population specific for binding to a given antigen, stimulation by an antigen in the proper form should be additive with stimulation by a mitogenic lectin.

Now that fractionated populations of lymphocytes specific for particular antigens are available, it should be possible to determine the connection between lectin-induced and antigen-induced changes by comparing responses to molecular variants of both agents on the same cells and to test various hypotheses on the mechanisms of lymphocyte stimulation. Ultimately, of course, we must return to the cell surface antibody itself and relate its mode of attachment and function at the cell surface to our knowledge of antibody structure.

VII. Conclusion

Our understanding of the origin of immunoglobulin diversity and of the molecular mechanisms of lymphocyte stimulation is in a very early stage. Experiments stemming from the analysis of antibody structure suggest, however, that these two outstanding problems of immunology can be profitably attacked at a molecular level. The results of the experiments on antibody structure provide a basis for studying antibodies on lymphoid cells as well as a molecular framework for the understanding of cellular specificity in the immune response. It appears that immunological specificity results from the interaction of a number of factors including the specificity of the initial binding of the antigen, the affinity and avidity of the antibody receptors and the cellular threshold for stimulation and clonal expansion. In order to understand this interaction in detail, it is necessary to study the structure of the

lymphoid cell membrane and particularly the mode of attachment of its antibody and lectin receptors. Recent developments in the specific fractionation of lymphocytes and in the analysis of the structure and function of membrane probes such as lectins should help to ease this task.

A fuller understanding of the behavior and specificity of lymphocyte receptors and the mechanism of mitogenesis may not only help to solve one of the central problems of immunology, but also be of great importance for a general understanding of growth control and cellular interactions in other areas of cell biology. The pace of recent developments both in this field and in molecular immunology suggests that we can look forward to an exciting decade of research.

ACKNOWLEDGMENT

The work described here has been carried out largely by my colleagues, whose names will be found in association with mine in the references cited. I am grateful for the privilege of that association. Much of the work has received support from the National Institute of Health and the National Science Foundation. Portions of this lecture were presented in a Nobel Lecture, 1972, to be published under copyright by the Nobel Foundation.

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