ADVANCES IN IMMUNOLOGY

Volume 19

F. J. Dixon &
Henry G. Kunkel

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Immunology

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PREFACE

There appears little remaining doubt that we are currently in the midst of what might be termed the "new immunology," the study of the lymphocyte. It is likely that in addition to its interest for immunologists, this ubiquitous cell may well become the prototype for the investigation of all eukaryotic cells. The remarkable stimulatory effect of antigens as well as various lectins through surface interactions makes the lymphocyte uniquely suited for a wide variety of studies. A spectrum of membrane markers have been delineated recently and these too have proven of considerable utility. Three of the four articles of Volume 19 deal specifically with branches of this "new immunology."

The first contribution deals with the broader aspects of membranes and covers the work on other cell types in addition to the lymphocyte. The author, Dr. S. J. Singer, is certainly one of the leaders in this field and he is primarily responsible for the fluid mosaic model of membrane structure. The surface markers of human red blood cells are discussed in considerable detail, since these cells are the primary ones utilized in Dr. Singer's studies. The redistribution of components of cell membranes by a variety of externally added agents is emphasized throughout the section and the importance of this phenomenon in biology is clearly apparent. Special stress is placed on various membrane phenomena of interest to immunologists, such as antigenic modulation, capping, and lectin effects on lymphocytes.

In the second article, Dr. Noel L. Warner deals primarily with the problem of membrane receptors for antigen on B and T lymphocytes. This exhaustive review by one of the leading authorities in the field supplements very well the broader consideration of membranes in the first article. The controversial topic of the character of T cell receptors is covered in special detail and the evidence for the concept of the immunoglobulin nature of these receptors, to which the author adheres, is emphasized. Many other questions concerning lymphocytes and other immunologically important cells are considered in great detail, making this contribution a valuable reference for the cellular immunologist. In addition, its very readable character and illustrations make it a useful review for those less familiar with this branch of immunology.

The third article is by Dr. Victor Nussenzweig and deals with the

field he initiated, the complement receptor sites on lymphocytes and other immunologically important cells. It is clear that the simple technique of rosette formation utilizing red cells coated with complement offers a very useful procedure for enumerating B cells. Considerable evidence is presented indicating that the complement receptors have important biological significance, particularly in facilitating the interaction of immune complexes with B cells and possibly in T and B cell interactions.

The last article is written by Dr. Hans L. Spiegelberg and concerns the many specific biological activities of the different immunoglobulin classes and subclasses. In view of the great interest in the variable portion of the antibody molecule and its relation to antigen binding, the constant part of the molecule involved in these biological activities has not received the attention that it probably deserves. Dr. Spiegelberg, who has contributed very significantly in this area, has brought together the many and diversified properties that are dependent on the constant areas in a very useful review.

The fine cooperation of the publishers in the production of Volume 19 is gratefully acknowledged.

HENRY G. KUNKEL
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Molecular Biology of Cellular Membranes with Applications to Immunology

S. J. SINGER

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I. Introduction

Events occurring at the level of the plasma and intracellular membranes of lymphoid cells have increasingly come to be recognized as critical to the expression of many immune phenomena. The induction of antibody synthesis by antigen, cell-mediated immunity,

histocompatibility and blood group antigenicity, antibody secretion, and complement-induced cytolysis, are only a few of the membrane-associated phenomena of great interest to immunologists. In the last few years, intense research activity has centered on the effects of anti-immunoglobulin antibodies, mitogens, and antigens on the membranes of T and B lymphocytes. While immunologists have been working on these problems, rapid developments have simultaneously taken place in membrane molecular biology. Theoretical and experimental advances have generated new insight into the molecular organization of membranes. This, in turn, has led to novel ideas and speculations about how membranes carry out their manifold functions. The primary object of this review is to discuss the molecular structure of membranes and its bearing on membrane functions as these concepts are presently emerging. In the latter half of this article, the relevance of these concepts to some selected immune phenomena will be discussed.

II. Molecular Organization of Membranes

In two recent articles (Singer, 1971; Singer and Nicolson, 1972), we have presented a detailed analysis of the thermodynamics of membrane systems and of new experimental information which has led us to propose the *fluid mosaic model of membrane structure.* In this review, only a summary of this material will be given; for further details, the reader is referred to the original articles.

A. Thermodynamic Considerations

In many discussions in the past, *ad hoc* assumptions and questionable conclusions derived from electron-microscopic observations have led to arbitrary models of membrane structure. Our own starting point has been thermodynamic. On the assumption that a membrane and its components obey the laws of equilibrium thermodynamics, at least in local domains, we have tried to develop in a systematic fashion a set of thermodynamic criteria, or restrictions, that membrane components must satisfy. For the present purposes, a large body of information about macromolecular interactions in aqueous solutions can be summarized as follows. Three major kinds of interactions must play prominent roles in determining membrane structure: hydrophilic, hydrophobic, and hydrogen-bonding interactions.

1. Hydrophilic Interactions

By hydrophilic interactions we mean a set of interactions that is responsible for the preference of ionic and highly polar groups for an

aqueous rather than a hydrophobic environment (Singer, 1971). It generally costs an unacceptably large amount of free energy to remove an ionic or highly polar group from water into a nonpolar solvent. For example, about 6 kcal./mole is necessary to transfer zwitterionic glycine from water to acetone (which is still a fairly polar solvent). In terms of membrane structure, this means that in the intact membrane the ionic and polar heads of the phospholipids, the ionic amino acid residues of the membrane proteins, and the sugar residues of the glycolipids and glycoproteins, essentially all have to be in atomic contact with water to yield a thermodynamically stable structure.

2. *Hydrophobic Interactions*

The hydrophobic interactions are responsible for the immiscibility of water and nonpolar substances. As a consequence, it costs free energy to remove a nonpolar residue from a nonpolar environment and transfer it to an aqueous one (Kauzmann, 1959). To transfer a single valine side chain from a solvent as polar as ethanol is to water, for example, takes about 2.1 kcal./mole (Cohn and Edsall, 1943). In terms of membrane structure, this means that in the intact membrane, the fatty acid chains of the lipids and the nonpolar amino acid residues of the membrane proteins have to be sequestered to the maximum extent possible into a hydrophobic environment away from contact with water.

3. *Hydrogen Bonding*

For membrane structure, the important point about hydrogen bonding is that in the intact membrane, hydrogen-bond donor and acceptor groups that are *not* in contact with water (for example, any N—H or C=O groups of the protein polypeptide chains that are buried in the nonpolar membrane interior) should be hydrogen bonded to the maximum extent possible to other acceptor and donor groups, respectively (Singer, 1971). To the extent that such internalized hydrogen bonds do *not* form, the membrane is destabilized by about 4 kcal./mole of potential hydrogen-bonding groups (Klotz and Franzen, 1962).

Other factors, such as electrostatic interactions, should also be considered in any detailed theory of membrane structure, but for the level of approximation of the present analysis, they may be neglected.

These few thermodynamic generalizations might seem, at first glance, to be unlikely contributors of any detailed structural insight about membranes. To the contrary, however, they are quite powerful: they place restrictions on membrane models and allow predictions to be made about protein and lipid structures in membranes, as will be

demonstrated after some of the properties of membrane proteins are discussed.

B. Proteins of Membranes

Until relatively recently, most discussions of membrane structure have emphasized the role of membrane lipids. The fact is, however, that of the three major constituents of membranes—protein, lipid, and carbohydrate—proteins have been shown to be the predominant constituent by weight in most well-characterized preparations of functional membranes (Table I). [Among those membrane systems that have been analyzed, myelin is the only exception to this generalization and contains about 4 times as much lipid as protein. But myelin is not a typical membrane; it functions as an electrical insulator rather than as a biochemically active, selective, permeability barrier.] This fact suggests that knowledge of the composition, conformations, and organization of proteins in membranes is of the greatest importance to understanding membrane structure.

TABLE I
Chemical Composition of Cell Membranes[a]

Membrane	Protein (%)	Lipid (%)	Carbohydrate (%)	Ratio of protein to lipid
Myelin	18	79	3	0.23
Plasma membranes				
Blood platelets	33–42	58–51	7.5	0.7
Mouse liver cells	46	54	2–4	0.85
Human erythrocyte	49	43	8	1.1
Ameba	54	42	4	1.3
Rat liver cells	58	42	(5–10)[b]	1.4
L cells	60	40	(5–10)[b]	1.5
HeLa cells	60	40	2.4	1.5
Nuclear membrane of rat liver cells	59	35	2.9	1.6
Retinal rods, bovine	51	49	4	1.0
Mitochondrial outer membrane	52	48	(2–4)[b]	1.1
Sarcoplasmic reticulum	67	33	–	2.0
Chloroplast lamellae, spinach	70	30	(6)[b]	2.3
Mitochondrial inner membrane	76	24	(1–2)[b]	3.2
Gram-positive bacteria	75	25	(10)[b]	3.0
Halobacterium purple membrane	75	25	–	3.0
Mycoplasma	58	37	1.5	1.6

[a] From Guidotti (1972).
[b] Deduced from the analyses.

TABLE II
CRITERIA FOR DISTINGUISHING PERIPHERAL AND INTEGRAL MEMBRANE PROTEINS

Property	Peripheral protein	Integral protein
Requirements for dissociation from membrane	Mild treatments sufficient: high ionic strength, metal ion chelating agents	Hydrophobic bond-breaking agents required: detergents, organic solvents, chaotropic agents
Association with lipids when solubilized	Usually soluble free of lipids	Usually associated with lipids when solubilized
Solubility after dissociation from membrane	Soluble and molecularly dispersed in neutral aqueous buffers	Usually insoluble or aggregated in neutral aqueous buffers
Examples	Cytochrome c of mitochondria; spectrin of erythrocytes	Most membrane-bound enzymes; histocompatibility antigens; drug and hormone receptors

As a first step in an analysis of membrane proteins, we have proposed (Singer, 1971) that at least two major categories of proteins be discriminated—they are termed *peripheral* and *integral*. The criteria suggested for distinguishing them are given in Table II. The main point is that certain membrane-associated proteins (peripheral) appear to be only weakly bound to the membrane, so that very mild treatments release them intact into molecular solution in aqueous buffers; whereas, the majority of membrane proteins (integral) are much more strongly bound and require hydrophobic bond-breaking agents to release them. The division into only two classes of proteins may ultimately prove to be inadequate, and the distinction may be more graduated, but in the absence of much evidence on this point, our purpose is served adequately by considering just the two classes. This classification also helps one to recognize that the structural properties of the more readily isolated and characterized peripheral proteins may not apply to the majority of membrane proteins. For example, the complete three-dimensional structure of cytochrome c of mitochondria has been determined by X-ray diffraction; but because it is released in soluble form from mitochondrial membranes by simply increasing the ionic strength (to 3 *M* KCl), it is a peripheral protein. Its structural features may, therefore, be only remotely related to, and may even be radically different from, those of most membrane-bound integral proteins.

C. Properties of Integral Proteins

By the criteria listed in Table II, generally 70% or more of membrane-associated proteins are integral. This includes most membrane-bound enzymes, antigens, and drug and hormone receptors that have so far been investigated. We assume that it is the integral proteins that are directly involved with the lipids in determining the structure of the matrix of the membrane, and we are therefore especially interested in their properties. The possible functions of peripheral proteins are discussed later. The following are some properties of integral proteins that must be explained by any successful theory of membrane structure.

1. Heterogeneity

It has on occasion been suggested that a particular type of protein functions as an essential "structural" protein in membranes (Richardson *et al.*, 1963; Laico *et al.*, 1970). The use of electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) has revealed, however, that the proteins of any one functional membrane are remarkably heterogeneous with respect to molecular weight (Fig. 1). Furthermore, the distribution of proteins is different for different types of membranes. There is thus no good evidence for specific structural proteins in membranes. Instead, it would appear that

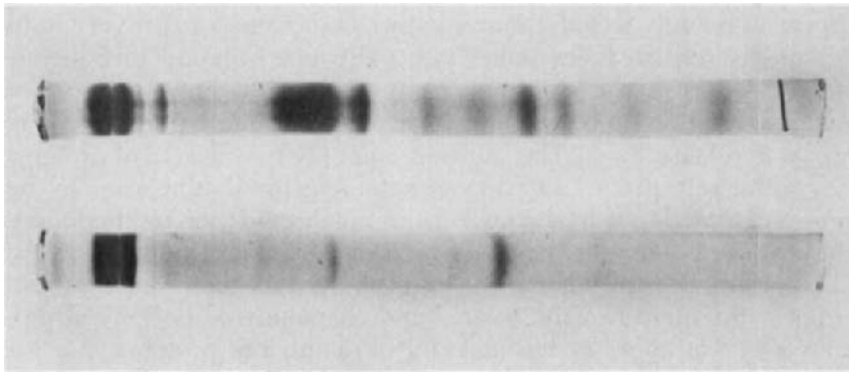

FIG. 1. Polyacrylamide gel electrophoresis patterns in sodium dodecyl sulfate–tris buffer of (top) the total proteins of human erythrocyte membranes and (bottom) the isolated peripheral protein, spectrin, freed of most of its actin-like low molecular weight component. The proteins are distributed according to increasing molecular weight from right to left.

many different proteins (the integral proteins) can be structurally important to membranes.

2. *Amino Acid Composition*

The amino acid composition of total membrane proteins, or of individual ones, is generally not clearly distinguishable from that of cytoplasmic proteins (Engelman and Morowitz, 1968; Rosenberg and Guidotti, 1969), although in a few instances (Capaldi and Vanderkooi, 1972) the amino acid composition is unusually hydrophobic. An important point is that even the unusually hydrophobic proteins do have considerable numbers of ionic residues.

3. *Protein Conformation*

The proteins of intact membranes exhibit on the average a substantial amount of α-helical conformation (Singer, 1971). In the case of the erythrocyte membrane, a careful analysis suggests that on the average about 40% of the protein is α-helical (Glaser and Singer, 1971). Other membrane preparations give similar results. The pronounced helical character suggests that the proteins of membranes are predominantly globular molecules rather than spread largely as monolayers on either surface of the membrane.

D. Structures of Integral Proteins

The properties of integral proteins and the thermodynamic restrictions discussed in the foregoing are all consistently explained if individual integral proteins, or their subunit aggregates, adopt an amphipathic structure in the intact membrane (Lenard and Singer, 1966; Wallach and Zahler, 1966). *Amphipathy* means that different regions of the molecule are distinctly differentiated into hydrophilic and hydrophobic domains, as in the case of a phospholipid molecule with its hydrophilic head group and its hydrophobic fatty acid chains. This notion of amphipathy seems to me to be the crucial key to the problem of membrane structure.

1. *Monodispersed Proteins*

If an integral protein is dispersed in the membrane as an individual polypeptide chain (monodisperse) (Fig. 2a), its three-dimensional structure (or conformation) may exhibit two or three recognizable parts. If it does not span the entire thickness of the membrane, it has a *hydrophilic end,* protruding from the membrane and containing essentially all the ionic and highly polar groups of the protein in contact with water, and a *hydrophobic end,* embedded in the hydrophobic

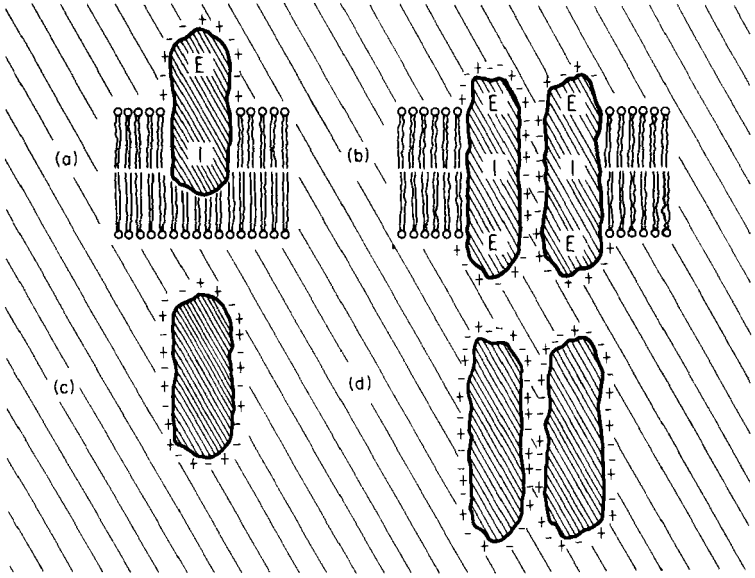

FIG. 2. Schematic representations of the structures of integral membrane proteins that exist in the membrane as (a) single molecules or (b) subunit aggregates compared to cytoplasmic soluble proteins that are (c) single molecules or (d) subunit aggregates. For simplicity, only two subunits are drawn for the aggregate so as to emphasize the central channel through the molecule: E and I refer to exterior (protruding) and interior (embedded) portions of the membrane proteins, respectively. The plus (+) and minus (−) signs represent the ionic charges of the charged amino acid residues of the protein. It is suggested that where the integral protein molecules come into direct contact with the nonpolar fatty acid chains of the membrane lipids, these ionic charges are absent, and this feature distinguishes the integral membrane proteins from otherwise comparable soluble proteins.

interior of the membrane, devoid of ionic groups and predominating in nonpolar amino acid residues. If the polypeptide chain spans the entire thickness of the membrane, it was suggested to possess three recognizable regions: a hydrophilic end, protruding into the aqueous phase from one side of the membrane, followed by a hydrophobic central portion embedded in the membrane, which is, in turn, attached to another hydrophilic end (generally different from the first) protruding into the aqueous phase from the other side of the membrane.

2. *Subunit Aggregates*

If an integral protein forms a specific subunit aggregate in the membrane and each of the subunits spans the thickness of the membrane, the conformation of the subunits may be more complex (Fig.

2b) than just one dimensionally amphipathic. When three or more protein subunits combine to form a specific small aggregate, they may often produce a central channel running through the aggregate. As a rough geometrical analog, the close packing of four cylinders to form a single tetrameric cylindrical aggregate leaves a hole down the center of the aggregate. Even with geometrically asymmetrical subunits, a similar central hole or channel can result. The hemoglobin molecule is a good example. The tetrameric aggregate formed by two α and two β chains generates a channel roughly 10 Å in diameter down a twofold symmetry axis of the molecule (Fig. 3) (Perutz and Ten Eyck, 1971). Other types of soluble subunit aggregates are known, such as lactic

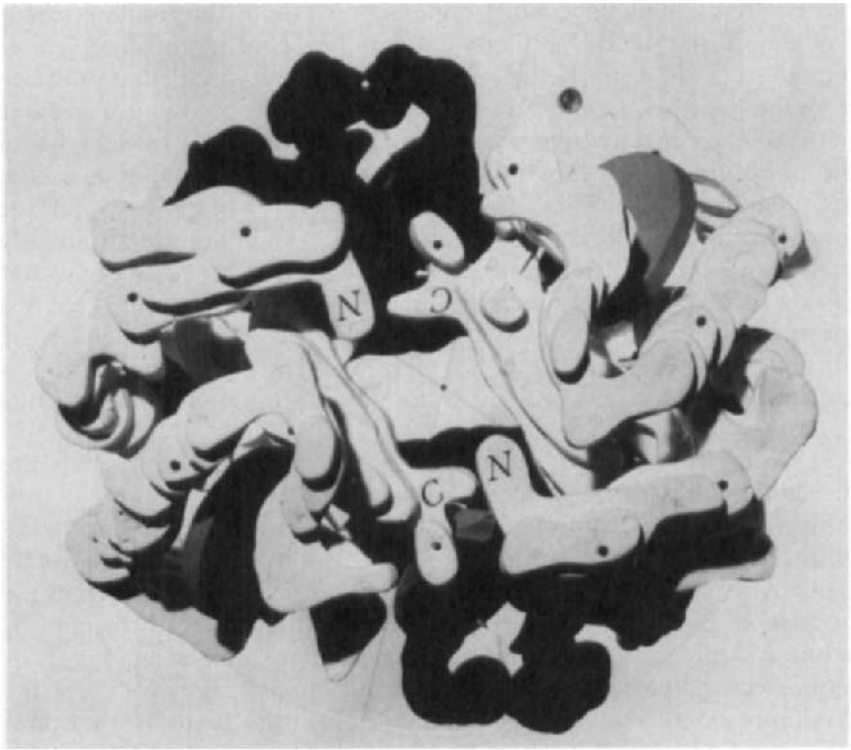

FIG. 3. A view down the molecular dyad axis of the horse hemoglobin molecule, as represented in this model derived from the Fourier synthesis at 5.5 Å resolution. The α chains are in white (N and C representing their amino and carboxyl-terminal residues, respectively) and the β chains are in black. The central cavity or channel runs down the length of the molecule in this orientation. (Courtesy of Dr. Max Perutz.)

dehydrogenase (Adams *et al.*, 1972), that also generate such central channels through them. These channels, if they are large enough, can be continuous through the length of a molecule and be filled with water. Thermodynamic considerations allow highly polar groups to line such pores, since they will be in contact with water.

We have predicted (Singer, 1971) that such subunit aggregates exist in membranes and that their existence is a consequence of their thermodynamic stability; that is, the three-dimensional structures of the individual protein subunits that are involved must favor the formation of such specific aggregates within the membrane. The individual subunits of a specific aggregate may be either identical, or similar, or of several different types, by analogy to the kinds of soluble protein aggregates that have been recognized (Atkinson, 1969). Noncovalent interactions at specific contact regions between the subunits presumably determine the size and geometry of a specific aggregate. Such interactions could occur between the ends of the subunits that protrude from the membrane (and be predominantly hydrophobic interactions with ion-pair and hydrogen-bonding contributions, as in the case of the subunits in the hemoglobin molecule) and also between the nonpolar ends of the subunits that are embedded in the hydrophobic interior of the membrane [the interactions here being predominantly hydrogen bonding (Singer, 1971)]. The aggregates could have water-filled central channels lined with ionic as well as other amino acid residues of the subunits. Such aggregates provide a simple mechanism for generating "pores" through a membrane, which may play important roles in transport and other membrane functions (see Section IV,D,1).

How do the schematic structures shown in Fig. 2a and b satisfy the thermodynamic restrictions and explain the properties of integral proteins described in the foregoing? With respect to maximizing hydrophilic interactions, the ionic residues of the proteins are essentially exclusively situated in contact with water, either protruding from the surfaces of the membranes or lining water-filled channels formed by subunit aggregates. Hydrophobic interactions are maximized by sequestering hydrophobic residues from contact with water to the maximum extent feasible, by situating them either in interior portions of the protruding ends of the protein molecules or in the hydrophobic interior of the membrane.

The *heterogeneity* of integral membrane proteins can be accounted for, because *a priori* any of a large subset of proteins with appropriate amino acid sequences may attain amphipathic conformations such as those shown in Fig. 2a and b.

The average amino acid *compositions* of integral proteins, such as those depicted in Fig. 2a and b, need not be markedly different from those of cytoplasmic proteins. It is rather the amphipathic distribution and, hence, the amino acid *sequences* that have to be distinctive. The overall composition of an integral protein might vary, however, with the depth to which it is embedded in the hydrophobic interior of the membrane relative to the extent to which it protrudes into the aqueous phase. The larger the fraction of the molecule embedded, the larger the ratio of hydrophobic to hydrophilic amino acids that it would be expected to contain, other things being equal.

The extensive α-helical character of membrane proteins can be rationalized by the more-or-less globular molecular structures in Fig. 2a and b; furthermore, there are reasons why their embedded hydrophobic regions may be highly α-helical (Singer, 1971).

An important additional feature of the structures drawn in Fig. 2a and b is that they provide a rational explanation of a question we have not yet explicitly considered, namely, Why are some proteins soluble and present in the cytoplasm, whereas others are integral components of membranes? The proposed distinction is a thermodynamic rather than an *ad hoc* one. Molecules of soluble proteins are known to have a more-or-less uniform distribution of ionic groups on their outer surfaces (Fig. 2c) (as revealed by X-ray crystallographic studies) and to interact with water in a spherically uniform manner (and are therefore soluble), whereas it is proposed that the molecules of integral proteins have markedly nonuniform distributions of ionic groups on their outer surfaces. These molecules interact with water only at the hydrophilic portions of their surfaces and have extensive and continuous areas of hydrophobic surfaces that are embedded in the hydrophobic interior of the membrane.

The same considerations can be readily extended to account for the differences between soluble and membrane-bound subunit aggregates, as depicted schematically in Fig. 2b and d.

E. Properties of Peripheral Proteins

By the criteria given in Table II, peripheral proteins generally constitute less than 30% of membrane-associated proteins. Cytochrome c of mitochondria and the protein complex of mammalian erythrocyte membranes known as spectrin (Marchesi and Steers, 1968) or tektin (Mazia and Ruby, 1968; Clarke, 1971) satisfy the criteria listed in Table II. Spectrin can be cleaved from the erythrocyte membrane simply by adding a chelating agent such as ethylenediaminetetraacetate (EDTA). There are many other examples of what may be

peripheral proteins. An interesting case involves the protein α-lactalbumin that exhibits the structural properties of such a protein (Brew *et al.*, 1968). When in association with the A protein (an integral protein of mammary gland microsomal membranes), it catalyzes the synthesis of lactose:

$$\text{UDP-D-galactose} + \text{D-glucose} \rightarrow \text{lactose} + \text{UDP} \quad (1)^1$$

The A protein by itself, however, catalyzes a different reaction:

$$\text{UDP-galactose} + \textit{N}\text{-acetyl-D-glucosamine} \rightarrow \textit{N}\text{-acetyllactosamine} + \text{UDP} \quad (2)$$

α-Lactalbumin is ordinarily a soluble monodisperse protein in aqueous buffers, but it apparently can bind specifically to the A protein in the membrane, and this association has the important functional result of changing the enzymatic specificity of the system.

The structural properties of peripheral proteins may not be strikingly different from those of soluble cytoplasmic proteins. It seems reasonable to propose that a molecule of a peripheral protein becomes membrane-bound by binding to the exposed surface of some specific integral protein in the membrane. The peripheral protein and the exposed portion of the integral protein to which it binds may both have proscribed surface areas that can combine with one another noncovalently as is the case with soluble subunit proteins such as hemoglobin. It is unlikely that peripheral proteins become membrane-bound by attaching to lipid head groups; if they did, it is not clear how they could mediate specific biochemical functions. The binding of α-lactalbumin to the mammary gland A protein, mentioned in the foregoing, or of an antigen to receptor immunoglobulins on the membranes of lymphoid cells, may be typical of the mechanisms by which peripheral proteins become membrane-bound and functional.

F. Lipids of Membranes

Lipid composition, chemistry, and structure are very important to the properties and functions of membranes. For the purposes of this article, however, only a few aspects of this multifaceted subject are touched upon.

The phospholipids of membranes appear to be arranged largely in a bilayer form (Davson and Danielli, 1952). The evidence for this comes from a variety of experiments, including differential calorimetry (Steim *et al.*, 1969; Melchior *et al.*, 1970), but most definitively by X-ray diffraction (Wilkins *et al.*, 1971). The evidence does not

[1] UDP, uridine 5′-diphosphate.

imply, however, that all of the phospholipid is in bilayer form nor that the bilayer is a continuous uninterrupted one.

Furthermore, under physiological conditions (e.g., 37°C.) the lipids of most functional membranes appear to be largely in a fluid rather than a rigid state. This has been extensively demonstrated by spin-label techniques (Hubbell and McConnell, 1968; Scandella *et al.*, 1972). However, at low temperatures (e.g., 4°C.), the fluidity of many lipid bilayers is markedly reduced. The membranes of cells ordinarily have an extremely heterogeneous population of lipid molecules, which helps to keep the lipid fluid. However, even with certain strains of prokaryotes, which can be made to incorporate large amounts of only one fatty acid into their membrane lipids, the incorporation is apparently regulated so that at least part of the lipid is in a fluid state at the growth temperature (Melchior *et al.*, 1970; Esfahani *et al.*, 1971).[2]

G. Carbohydrate of Membranes

The carbohydrate of most cell membranes is largely in the form of oligosaccharide chains that are covalently attached to membrane proteins (to yield glycoproteins) or to glycolipids. In terms of the thermodynamic arguments considered earlier, these oligosaccharide chains must be in contact with water and, therefore, must be located largely on the membrane surfaces. As is discussed later in Section III,B,1, the oligosaccharides appear to be exclusively localized to only one of the two surfaces of many cell membranes.

III. The Fluid Mosaic Model of Membrane Structure

All of the material so far presented, and additional information discussed later, can be synthesized into a schematic model of membrane structure which we have termed the "fluid mosaic model" (Singer, 1972a; Singer and Nicolson, 1972) (Fig. 4). In this model, globular amphipathic molecules of the integral proteins are partially embedded in a matrix consisting of a fluid lipid bilayer. In effect, the

[2] Note added in proof:

The suggestion has recently been made (Bretscher, 1972) that the major phospholipids of the erythrocyte membrane are asymmetrically distributed across the two halves of the lipid bilayer. While the evidence first presented was not compelling (Schmidt-Ullrich *et al.*, 1973), more recent results lend support to this proposal (Zwaal *et al.*, 1973). By inference, such lipid asymmetry may be generally characteristic of cell membranes. If further evidence confirms this suggestion, the significance for membrane structure and function may be very great.

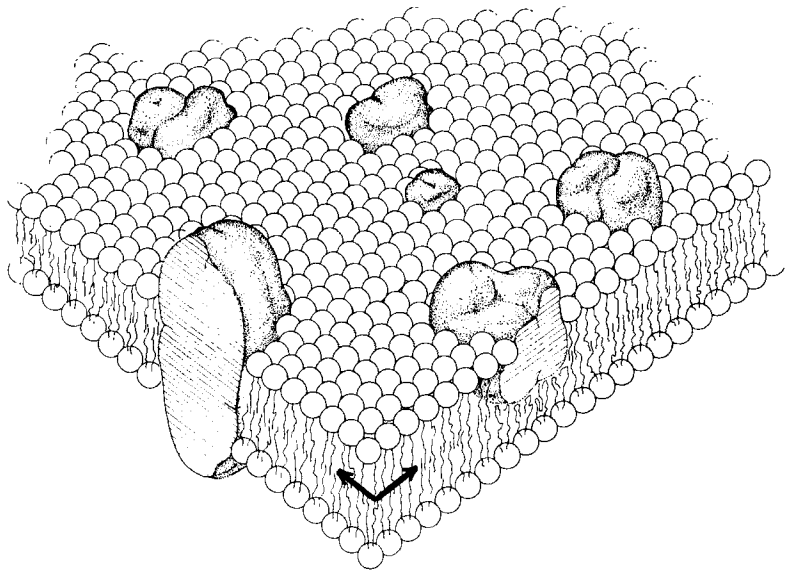

FIG. 4. A schematic representation of the fluid mosaic model of membrane structure. The solid bodies with stippled surfaces represent the globular integral proteins, with their hydrophilic ends protruding from the membrane, and their hydrophobic ends embedded in the membrane (as seen in cross section). At long range, the integral proteins are randomly distributed in the plane of the membrane; at short range, some form subunit aggregates as shown. The spheres represent the ionic and polar head groups of the lipids and the wavy lines their fatty acid chains. The arrows denote the plane of cleavage in freeze fracture experiments. From Singer and Nicolson (1972). Copyright 1972 by the American Association for the Advancement of Science.

membrane is thus pictured as a special kind of two-dimensional fluid solution. Some of the integral proteins may exist in the membrane as single molecules, others as specific subunit aggregates. If the latter have the appropriate size and amino acid sequence, they may create water-filled channels that span the thickness of the membrane. The ionic and oligosaccharide groups of both the integral proteins and the lipids are mostly on the surfaces of the membrane in contact with the aqueous phase. Peripheral protein molecules are attached to some of the integral proteins by specific but weak noncovalent bonds. Additional features and restrictions to be incorporated into the model are discussed later. In the next section, we outline briefly some further experimental information that is consistent with, and strongly supports, the fluid mosaic model.

A. Some Experimental Information Relevant to the Fluid Mosaic Model

1. Amphipathy of Integral Membrane Proteins

As yet only a few proteins that satisfy the criteria of integral membrane proteins have been isolated in a pure state, let alone have had their structures analyzed. In two cases, however, some striking information has been obtained from protein structure studies of purified integral membrane proteins.

One of these is the protein cytochrome b_5 of microsomal membranes. Mild proteolysis of the intact membrane releases a single soluble polypeptide of about 100 amino acids containing the heme group, the amino acid sequence of which has been determined (Ozols and Strittmatter, 1969). However, after this proteolytic treatment, a polypeptide fragment of the original intact cytochrome b_5 molecule is left behind in the membrane. This residual fragment of about 40 to 44 amino acids is highly hydrophobic in composition (Spatz and Strittmatter, 1971). The entire molecule of about 140 amino acid residues can be released from the membrane by detergents (Ito and Sato, 1968; Strittmatter *et al.*, 1972).

The other case involves a major glycoprotein of the erythrocyte membrane which has been named *glycophorin* (Marchesi *et al.*, 1972). This protein contains 60% carbohydrate and 40% polypeptide, the latter consisting of a single chain of about 200 amino acid residues. The amino-terminal half of the chain contains *all* of the carbohydrate and can be released from the intact membrane by limited proteolysis (Morawiecki, 1964; Winzler, 1969). The carboxyl-terminal half of the polypeptide chain, containing no carbohydrate and remaining behind in the membrane on proteolysis, apparently contains two linear stretches—an amino-terminal, highly hydrophobic sequence of about 25 amino acids, followed by another hydrophilic segment (but containing no carbohydrate) at the carboxyl-terminal end of the chain. The glycophorin molecule may then span the entire thickness of the membrane, with its two hydrophilic ends protruding on either side of the membrane and its intervening hydrophobic sequence embedded in the membrane (Segrest *et al.*, 1972).

In both cases, therefore, the results described indicate that the molecules have one or two hydrophilic ends that protrude (and can be proteolytically cleaved) from the membrane and a hydrophobic portion that is embedded in the membrane. Furthermore, this three dimensionally amphipathic structure is apparently the consequence

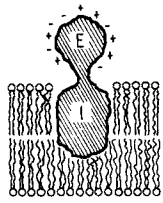

FIG. 5. A schematic representation of a monodisperse integral membrane protein in which the hydrophilic (E) and hydrophobic (I) ends form distinct three-dimensional domains, attached to one another by a region of polypeptide chain that is exposed and perhaps flexible, and is especially susceptible to proteolytic cleavage. The cytochrome b_5 molecule appears to conform to this structure. See text for further details.

of the fact that the molecules are *linearly* amphipathic; that is, their linear amino acid sequences are divisible into two or three distinct parts—one or two hydrophilic and the other hydrophobic. The two or three linear portions very likely each fold up independently into globular domains linked by regions of the polypeptide chain that are especially susceptible to proteolytic cleavage (Fig. 5).

Linear amphipathy may turn out to be a general feature of integral membrane proteins that are monomolecularly dispersed in the intact membrane. The amphipathic conformation that has been predicted for them (Fig. 2a) would be the result of having such independently folding domains. On the other hand, the linear structure of integral membrane proteins that formed subunit aggregates in the intact membrane, for the reasons discussed in Section II,D,2, might be expected to be more complex.

2. *Proteins Embedded in Membranes*

The fracture faces of membranes that are exposed in freeze-fracture experiments (Branton, 1966) have been shown to be *interior* surfaces of the membranes (Pinto da Silva and Branton, 1970; Tillack and Marchesi, 1970). For all types of cell membranes, with the exception of myelin, this interior surface is seen to consist of a large number of more-or-less globular particles randomly intermixed with smooth areas. Examples of these interior surfaces of the plasma membranes of different types of lymphocytes (Mandel, 1972) are shown in Fig. 6. In the case of erythrocyte membranes, these particles have been shown to contain the protein glycophorin (Marchesi *et al.*, 1972) previously discussed. It seems likely that the intramembranous particles in other types of membranes are also protein in nature. All of this experimental information is consistent with the suggestions that (*a*) the interior membrane surface exposed by fracture is that shown by the arrows in

Fig. 4, (*b*) the particles represent deeply embedded integral proteins that cannot be fractured, and (*c*) the smooth areas represent the regions occupied by lipid bilayer, the fracture occurring at the plane where the two lipid layers meet. Clearly, these results provide strong evidence for the validity of the fluid mosaic model.

It is clear that the uniform-sized intramembranous particles in the erythrocyte membrane are too large to consist simply of single molecules of glycophorin, with a molecular weight of only about 55,000.

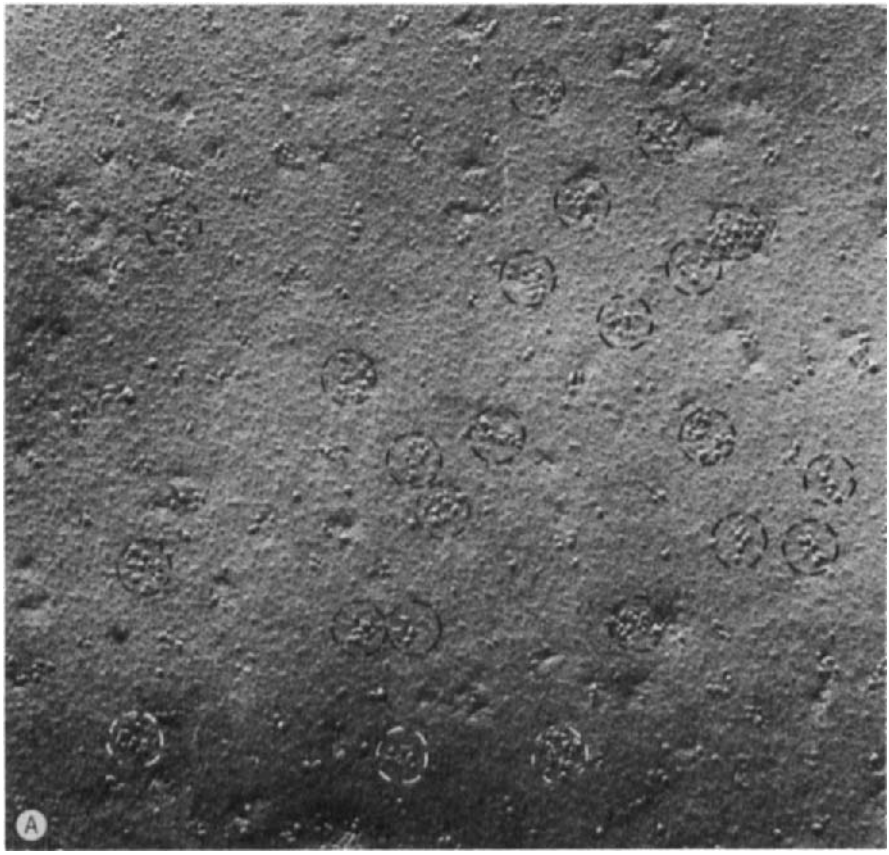

FIG. 6A. Freeze-fracture electron micrograph of the inner faces of plasma membranes of various mouse lymphocytes: "immature" (cortisone-sensitive) thymus cell. The intramembranous particles have distinctly different distributions in the three types of cells, often appearing as small clusters (circled), as much larger patches (circled in Fig. 6B), and almost always individually dispersed (Fig. 6C) (arrows). (Courtesy of Dr. Thomas E. Mandel; Mandel, 1972).

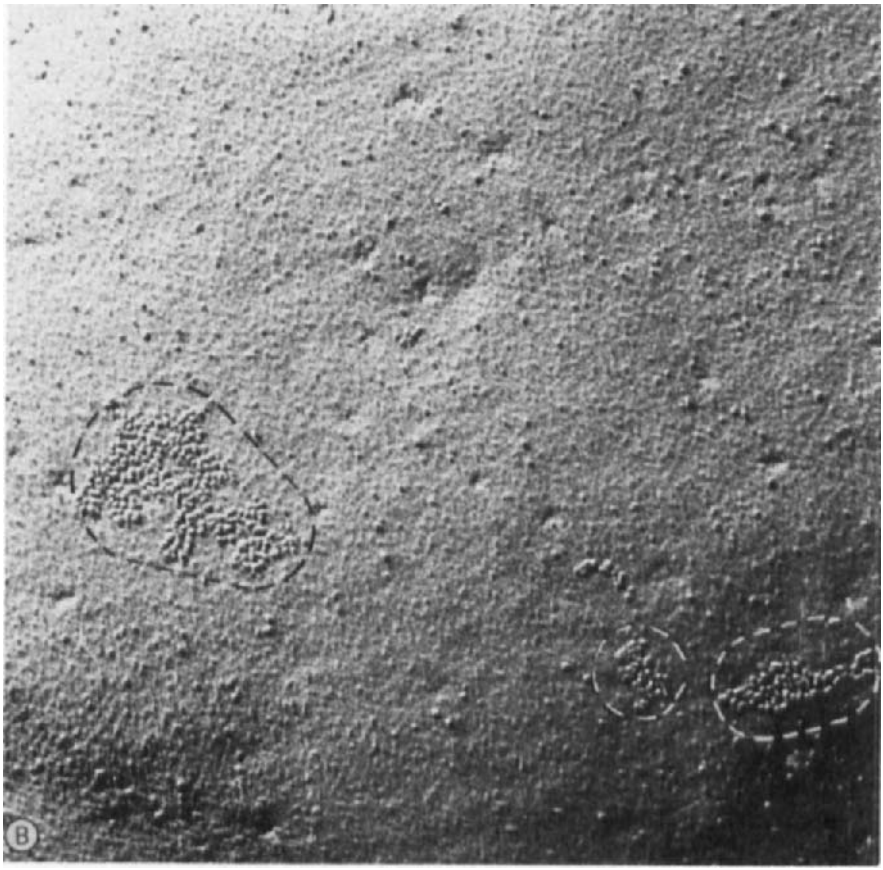

FIG. 6B. Same as Fig. 6A for "mature" (cortisone-resistant) thymus cell.

Some kind of specific protein subunit aggregates, containing glycophorin and perhaps other integral proteins, must be postulated to account for the particle size (see Section II,D,2).

There is also chemical evidence (Steck *et al.*, 1971; Bretscher, 1971) that certain proteins of the erythrocyte membrane completely span the thickness of the membrane and have regions exposed to the aqueous medium on both membrane surfaces.

3. *Two-Dimensional Distribution of Proteins on Membrane Surfaces*

There have as yet been very few direct studies to determine how a particular membrane protein is distributed over the surface area of a

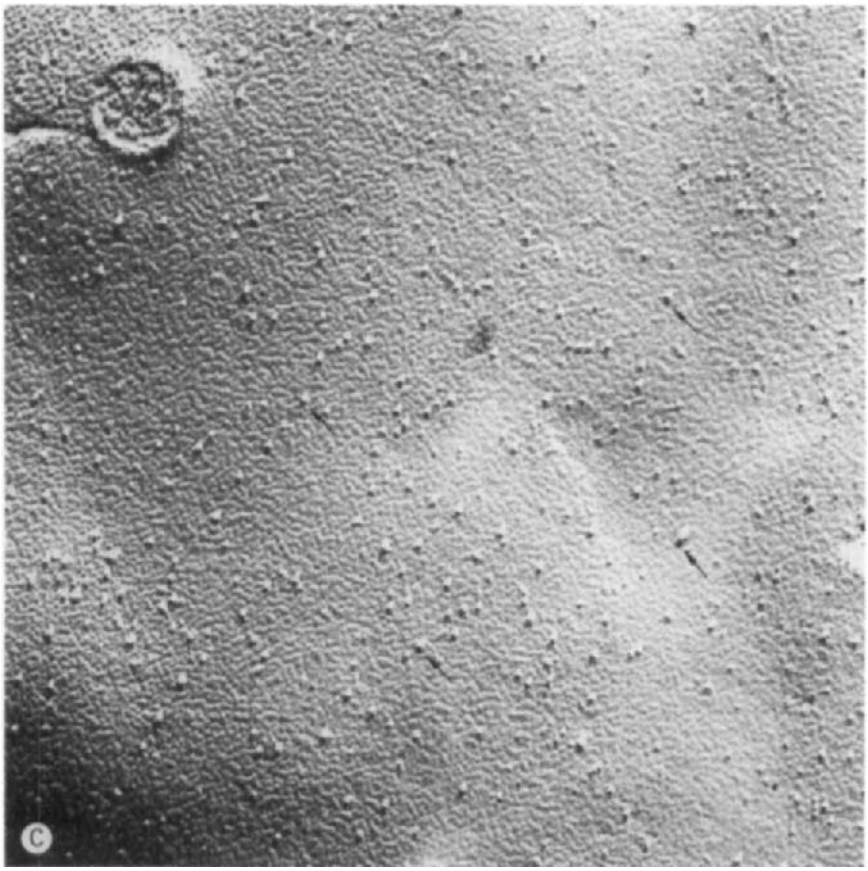

FIG. 6C. Same as Fig. 6A for a splenic lymphocyte.

membrane. With the use of ferritin–antibody conjugates and special membrane preparative procedures, the two-dimensional distributions of the Rh_o(D) antigen on human erythrocyte membranes (Nicolson *et al.*, 1971a) and of the *H-2* histocompatibility locus antigen on mouse erythrocyte membranes (Nicolson *et al.*, 1971b) have been determined. The Rh_o(D) antigen was found to be dispersed as single molecules in the membrane, in agreement with earlier observations (Lee and Feldman, 1964). The *H-2* antigen, however, appeared to exist in small irregular clusters in the membrane surface. However, both the individual Rh_o(D) molecules and the individual clusters of *H-2* were found *at long range* to be randomly and uniformly distributed on the membrane surfaces.

By contrast, Aoki *et al.* (1969), using a hybrid ferritin–antibody technique found that the apparent distribution of the *H-2*, TL, and *θ* antigens on thymocyte and lymphocyte membrane surfaces was highly nonuniform. These antigens appeared to be present in very large patches on isolated portions of the membrane. It seems likely, however, that the labeling procedures of Aoki *et al.* induced a large part, if not all, of the observed nonuniform patching (Davis, 1972), in a manner related to the capping phenomenon of lymphocytes (see Section III,A,5). The results of Singer and his colleagues were obtained with membranes of *intact erythrocytes,* in which membrane components appear to be unusually immobile (Section VI,E), a fortuitous circumstance for these experiments.

In studies of the two-dimensional distribution of antigenic components on membrane surfaces using labeled antibodies, it is evident that Fab fragments of the antibodies and low temperatures are required to minimize any artifactual redistributions of the antigens.

Since the Rh_o(D) (Green, 1967, 1968) and the *H-2* histocompatibility (Cullen *et al.*, 1972) antigens are most likely integral protein components of membranes, their random and relatively uniform distribution over large distances in the erythrocyte membrane is consistent with expectations from the fluid mosaic model, because in that model there are no constraints operating to fix the spatial distribution of membrane proteins at long range.

4. Molecular Fluidity in the Membrane

Although the fluidity of membranes has long been discussed by cell physiologists, it is apparent that in using that term they were concerned with the plasticity, deformability, and flow properties of the membrane as a whole. Our usage of "fluidity" in this article refers to the rotational freedom and rapid long-range translational mobility of *individual molecules* within membranes, phenomena that only recently have been considered to be widespread. It is possible for a body to be plastic even if its molecules are not individually mobile (e.g., a latex rubber ball).

The fluidity of membrane lipids has already been discussed in Section II,F. However, one can conceive of membrane models in which the lipid is fluid, while the proteins remain in place in a rigid framework. (In fact, this appears to be the case with the intact adult erythrocyte; Section VI,E.) Therefore, a significant and separate question is, Do the integral proteins of membranes have rotational and

translational freedom as is expected in the fluid mosaic model? The answer is clearly yes in several independent cases. In the case of the protein rhodopsin, which is the predominant protein in retinal rod disc membranes and which satisfies the criteria for an integral membrane protein (Blasie, 1972), X-ray diffraction methods (Blasie and Worthington, 1969) and fluorescent measurements (Cone, 1972; Brown, 1972) have shown that the molecule is in a fluid matrix, free to rotate in the membrane. Similar conclusions have been derived for the cytochrome oxidase of mitochondrial inner membranes (Junge, 1972). In particularly striking experiments with cell fusion heterokaryons between two mammalian cells, Frye and Edidin (1970) have shown that membrane antigens can apparently diffuse rapidly over long distances within the membrane at 37°C., but only very much more slowly at 4°C. Among these antigens is the mouse *H-2* histocompatibility antigen. The diffusibility of components in membranes under physiological conditions has also been demonstrated with intact muscle cells (Edidin and Fambrough, 1973).

5. *Capping Phenomena*

More recently, experiments on the capping of immunoglobulin and other receptors on lymphocyte membranes and of components of other mammalian cells have provided impressive evidence for the mobility of integral proteins in membranes. A detailed discussion of capping effects with lymphocytes is deferred until Section VI,F, but it is relevant to describe the outlines of the phenomenon here. Outer membrane surfaces of unprimed B lymphocytes contain an IgM-like receptor, presumably with a specific antigen-binding capacity for each cell. These receptor immunoglobulins can be visualized by binding to them antimouse immunoglobulin (MIg) antibodies labeled with either highly radioactive (Santer *et al.*, 1972), fluorescent (Taylor *et al.*, 1971; Loor *et al.*, 1972; Unanue *et al.*, 1972), or electron-dense (Karnovsky *et al.*, 1972; de Petris and Raff, 1973) markers. If the labeled anti-MIg is reacted with the cells at 4°C., the label is found uniformly distributed over the cell surface. When such labeled cells are warmed to 37°C., however, within a few minutes the label migrates to a pole of the cell membrane, forming a cap, which after about 30 minutes is often endocytized. If labeled Fab fragments of the anti-MIg are used, the labeling of the cell surface remains uniform at 4°C. or at 37°C. If endocytosis follows cap formation, the receptor immunoglobulin is swept from the surface, but no apparent changes in

the amounts of other membrane antigens or receptors are observed. The capping and endocytosis appear to be energy-dependent processes, as discussed in Section IV,C.

In the context of this section, the critical features of these experiments are that (*a*) the redistribution and capping require bivalent antibody, the univalent Fab fragments being incapable of inducing these effects; (*b*) the redistributions are inhibited by low temperatures; and (*c*) capping of one component by the binding of its specific multivalent ligand to the membrane generally does not grossly affect the distribution of other components in the membrane. These results strongly suggest that the bivalent anti-MIg antibody initiates a specific antigen–antibody aggregation reaction in the membrane, and this aggregation requires the lipid to be in a fluid state. Such direct capping effects occur with other ligand–receptor combinations of lymphocytes. For example, the mitogen concanavalin A (Con A) produces capping of its receptors on both T and B lymphocytes (Unanue *et al.*, 1972; Greaves and Janossy, 1972). On the other hand, anti-*H-2* antibodies do not directly cap the *H-2* antigen receptors on T lymphocytes; if, however, an anti-antibody is subsequently added, then capping occurs (Unanue *et al.*, 1972). Capping effects are not confined to lymphocyte membranes but have also been observed with antibodies to the receptor IgE on basophiles (Sullivan *et al.*, 1971) and to the A antigen and the *H-2* antigen on monkey kidney (Sundqvist, 1972) and mouse L cells (Edidin and Weiss, 1972), respectively. Furthermore, capping, endocytosis, and related effects have most likely been observed many times in the past, but, because the critical experiments with univalent ligands (Fab fragments) or to test the effect of temperature on capping were not performed, it is not possible to identify the observed effects unambiguously as capping. Examples of such studies include the effects of antibodies directed against amoeba plasma membranes on the stimulation of pinocytosis by the amoeba (Wolpert and O'Neill, 1962), of anti-cell surface antibodies on pinocytosis of Krebs ascites tumor cells (Easton *et al.*, 1962), and of antibodies to blood group A substance on the mature A+ erythrocytes of newborn humans (but not of adults) (Blanton *et al.*, 1968). Further discussion of related phenomena is found in Section V,B and Sections VI,D,E,F, and G.

All of the evidence quoted in this section therefore lends strong and detailed support to the fluid mosaic model as a working model of membrane structure. In some respects, however, the model may be extended and refined in the future as more information becomes available. These suggested refinements are discussed in the next section.

B. Some Restrictions and Extensions of the Fluid Mosaic Model

1. Asymmetry of Membranes

A variety of experimental studies has suggested that cell membranes have a different composition and structure on their two exterior surfaces. For example, following earlier related studies (Rambourg and Leblond, 1967), we have found that the oligosaccharide components of several mammalian cell membranes are exclusively localized to only one of the two membrane surfaces (Nicolson and Singer, 1971, 1974). These oligosaccharides are predominantly attached to glycoproteins, which appear to be integral proteins of the membranes (Winzler, 1969; Marchesi *et al.*, 1972). This asymmetry of glycoprotein distribution has important implications for membrane biogenesis, which is discussed later in Section V,A. At this juncture, we point out that the asymmetry suggests that the integral glycoproteins are not free to rotate from one surface of the membrane to the other at any appreciable rate; otherwise, their oligosaccharide moieties would be found on both surfaces. On the other hand, it appears that under certain conditions at least some of these glycoproteins are free to diffuse *in the plane* of the membrane (Marchesi *et al.*, 1972).

This rotational restriction has its counterpart for the phospholipids of membranes. It has been shown by elegant spin-labeling experiments (Kornberg and McConnell, 1971) that individual phospholipid molecules rotate at only very slow rates from one surface to the other in synthetic phospholipid vesicles.

The restrictions on transmembrane rotations may be quite general for all integral proteins of membranes. If, as postulated in the fluid mosaic model, the integral proteins are *all* amphipathic molecules, one would expect there to be a very large free energy of activation for the rotation (or diffusion) of the hydrophilic end(s) of the protein through the hydrophobic interior of the membrane to reach the other membrane surface. If this generalization is correct, then the fluid mosaic structure is an *oriented solution*, its components being mobile in the plane of the membrane, but (except for predominantly hydrophobic substances) unable to rotate through the membrane from one surface to the other.

2. Restrictions on Translational Diffusion of Membrane Components

One restriction on the translational mobility of membrane components is already implied in the fluid mosaic model but might best be

explicitly stated. If a protein or lipid molecule is part of a protein subunit aggregate within the membrane, then the aggregate will be the mobile unit within the fluid lipid matrix. Most such aggregates are likely to contain small numbers of subunits, but it is possible that subunit aggregates may occasionally be very large two-dimensional structures within a membrane. Such large aggregates may form from identical or very similar subunits if they have the proper geometrical orientation of their binding sites with respect to one another and may include some lipid molecules or micelles intercalated within them. Such specialized, large, two-dimensional aggregates, relatively homogeneous in their protein content and containing substantial amounts of lipid, appear to be present in the membranes of purple bacteria (Blaurock and Stoeckenius, 1971; Osterhelt and Stoeckenius, 1971) and in gap junctions between the membranes of mouse liver cells (Goodenough and Stoeckenius, 1972). These aggregates, however, occupy only a fraction of the entire membrane surface of which they are a part; presumably the remainder of the membrane is a fluid mosaic structure containing the many other integral proteins necessary for the range of membrane functions.

It is also possible that other kinds of obstructions to the free diffusion of components in a continuous membrane exist in specific instances. For example, the intact adult human erythrocyte, in contrast to the lymphocyte, does not exhibit capping effects (see Section III,A,4) when reacted with antibodies or mitogens directed to erythrocyte surface antigens or receptors, respectively (Loor *et al.*, 1972). On the other hand, with erythrocyte membranes prepared from the cells lysed in hypotonic media, the intramembranous particles can be redistributed by trypsinization (Speth *et al.*, 1972) and by changes in pH (Pinto da Silva, 1972), and Con A receptors can be redistributed by proteolysis (Nicolson, 1972). This relative immobility of components in the intact adult erythrocyte membrane may be due to the attachment of a uniquely large amount of the peripheral protein complex spectrin (Marchesi and Steers, 1968) to the inner cytoplasmic surface of the erythrocyte membrane (Nicolson *et al.*, 1971c). Aggregates of the spectrin molecules, attached noncovalently to arrays of otherwise independent integral proteins, may act to obstruct the free diffusion of components in the plane of the membrane. This underlying spectrin structure in the intact cell may be partially disrupted when the membranes are isolated from the lysed erythrocyte, thereby permitting increased mobility of the components in the isolated membranes. The possible role of spectrin-like components in capping and pinocytosis is discussed further in Sections IV,C and VI,F.

It is also possible that other intracellular or extracellular structures attached to membrane sites can produce local immobilization of certain integral components of the membrane (Yahara and Edelman, 1972).

We have tried to demonstrate in this section that specific restrictions on the fluidity of certain membranes and the presence in some membranes of highly organized large structures, such as gap junctions and synapses, and smaller aggregates, such as electron transport complexes, can be reasonably accommodated into the fluid mosaic model and are not in conflict with it. Our point of view is that where such diffusional restrictions or differentiated structures are found in membranes, specific molecular mechanisms must be responsible for them and should be investigated and elucidated.

IV. Functional Consequences of a Fluid Mosaic Membrane

The consistency of the fluid mosaic model with recent experimental information about the properties of cell membranes warrants a more detailed examination of possible structural and functional consequences of the model. Some of these ideas are now discussed.

A. Thermodynamic Accounting and Membrane Components

The basis for the fluid mosaic model was originally and still is the thermodynamics of macromolecular systems in an aqueous environment. Our point of view is that the proteins of membranes are not there by accident, nor are their properties altered in some random fashion. Their presence and properties in membranes are taken to be expressions of their lowest attainable free-energy states under particular conditions.

1. Embedment of an Integral Protein in the Membrane

The degree to which an integral protein molecule is embedded in the membrane, and the degree to which it protrudes from it, for example, are postulated to be under thermodynamic control. We would expect that in order to embed the molecule further into the membrane, without changing its covalent structure or its interactions with ligands, would require an expenditure of free energy in order to transfer some of the ionic groups on the protruding hydrophilic end from a water environment to a nonpolar membrane interior (See Fig. 2a). Similarly, to pull the protein molecule further out of the membrane would cost the free energy required to expose some of the hydrophobic residues to an aqueous environment. In related fashion,

the degree of embedding, the surface orientation, the interactions with lipids, and all such structural details are therefore presumed to be thermodynamically controlled and characteristic properties of the covalent structure and noncovalent interactions of the integral membrane protein in question.

These considerations are particularly relevant to the problem of "cryptic sites" in membranes, as discussed in Section IV,B,3.

2. *Proteins in Membrane-Bound and Soluble States*

An important corollary of these thermodynamic considerations has to do with proteins that in some situations appear to behave as integral molecules and are *firmly* bound to membranes (and are therefore not likely to be peripheral) and that in other situations are quite soluble and molecularly dispersed in free solution. If an integral membrane protein is an amphipathic molecule, however, it would generally be in a much lower free-energy state when bound to the membrane than when molecularly dispersed in free solution because of the strong hydrophobic interactions of the nonpolar end of the molecule and the nonpolar interior of the membrane. One would, therefore, not expect the *identical* molecule to be present in significant concentrations in both membrane-bound and soluble states.

An example of interest to immunologists is IgM. A monomeric IgM-like receptor is apparently bound to the plasma membranes of unprimed B lymphocytes where it is presumed to function as an antigen receptor. IgM molecules are also secreted by the progeny of precursor B cells once they differentiate to become antibody-producing plasmacytes. It is possible that the IgM molecules are essentially identical in the two situations and that it is the membranes of precursor B lymphocytes and plasmacytes which are significantly different, the former being capable of binding IgM molecules, the latter not, perhaps because of the presence in the former membrane of some other IgM-binding integral protein. This is not likely, however, since secreted IgM molecules added to precursor B lymphocytes do not generally become bound. It seems more likely that the membrane-bound and secreted IgM molecules are not entirely identical. Although they might be identical in much of their covalent structure, they must somehow be structurally different enough to account for the large free-energy difference that must be involved between the membrane-bound and soluble states. There are a variety of ways a given molecule could be made to differ sufficiently to exist predominantly in one or the other of these two states. The covalent modification of a protein, such as by adenylation, phosphorylation, acylation, glycosyla-

tion, or by other means; the presence of an additional length of polypeptide chain in one state which is proteolytically cleaved to convert the protein to the other state; or attachment to another polypeptide chain are a few of such possibilities. We consider this problem with IgM molecules in greater detail in Section VI,B.

With peripheral proteins, the differences in free energies between the membrane-bound and soluble states are likely to be smaller than for integral proteins (i.e., peripheral proteins are only weakly bound to the membrane). It is possible that even the noncovalent binding of a ligand to the peripheral protein, for example, could provide the free-energy difference between the membrane-bound and soluble states. In some instances, a peripheral membrane protein may exist in free solution because it is present in excess over the number of membrane receptor sites to which it is noncovalently bound.

B. Redistributions of Membrane Components

The characterization of a membrane as a two-dimensional solution of integral protein molecules partially embedded in a fluid lipid bilayer suggests many functional consequences that would not otherwise be expected. This was stated in a very general way (Singer and Nicolson, 1971, 1972), "The physical or chemical perturbation of a membrane may affect or alter a particular membrane component or set of components; a redistribution of membrane components can then occur by translational diffusion through the viscous two-dimensional solution, thereby allowing new thermodynamic interactions [involving] the altered components to take effect." The observation of capping phenomena produced by reacting anti-immunoglobulin antibodies with lymphocytes (Section III,A,4) provides, at least in the initial stages, remarkable confirmation of these suggestions, but there are in addition other known and potential expressions of such mechanisms that are worth considering.

That these redistribution phenomena can and do occur in cell membranes, there is little doubt. What is not certain is whether they have functional significance, and if so, by what mechanisms they produce their effects. Considerable attention is given in this article to redistribution effects in membranes, because the intuition we share with many others is that such membrane changes do have profound consequences for the metabolism of the cell. In discussing these possible consequences and the mechanisms by which they might be produced, however, we must be aware that there are yet few concrete facts to guide such speculations.

1. Redistributions Induced by Binding of Multivalent Ligands

In principle, the addition to a cell suspension of any external ligand, with multiple binding sites per molecule, specific for a mobile integral protein in the cell plasma membrane could, under physiological conditions, induce a two-dimensional aggregation reaction between the ligand and the protein in the membrane surface. Among such external ligands could be (*a*) a multivalent antibody directed to a cell surface antigen, (*b*) a multivalent antigen to a cell surface antibody receptor, (*c*) a multivalent lectin specific for a saccharide determinant on a membrane glycoprotein, (*d*) a multivalent hormone for a specific cell membrane receptor, or (*e*) a polymeric ion (such as polylysine or a polynucleotide) capable of binding to ionic groups of opposite charge on membrane proteins. These aggregation reactions can be viewed roughly as two-dimensional analogs of similar reactions occurring through diffusion-mediated interactions in ordinary aqueous solutions, but with important differences as outlined in the following.

The factors involved in such two-dimensional aggregation reactions can be illustrated for the reaction of an externally added, multivalent antigen and a membrane-bound receptor immunoglobulin.[3]

a. If the receptor immunoglobulin had only one binding site for the antigen, binding could produce only small aggregates including one antigen molecule and several immunoglobulin molecules, but no large-scale aggregation reactions could occur in the membrane unless they were of a secondary nature (see following).

b. With a multivalent antigen and a bivalent receptor immunoglobulin antibody, there should be antibody excess and antigen excess zones as in three-dimensional precipitin reactions. With very small amounts of added antigen, small aggregates should predominate containing one or two antigen molecules and several antibodies. With very large amounts of added antigen, small aggregates should again predominate containing mainly two antigen molecules attached to each antibody. In between these zones, large antigen–antibody aggregates should form. The interesting consequence is that with either very small or very large antigen concentrations, small aggregates

[3] Our terminology attempts to distinguish between *receptor immunoglobulins*, immunoglobulins that function as receptors (for antigens, for example), and *immunoglobulin receptors*, receptor molecules that are capable of binding immunoglobulin molecules.

would form which might be expected to remain mobile and dispersed, whereas at intermediate antigen concentrations, larger aggregates of the antigen-bound antibody receptors would form.

c. Any redistribution or aggregation reactions occurring after the antigen is bound would require the membrane to be fluid and, hence, would depend on temperature. At temperatures around 4°C, the effective viscosity of the lipids of most mammalian membranes appears to become very great and to inhibit molecular mobility in the membrane (Frye and Edidin, 1970).

Furthermore, if the receptor molecules are integral proteins of the membrane, they would have a defined orientation with respect to the plane of the membrane, contrasted with the random orientation that a protein molecule can take on in three-dimensional aqueous solutions. This can impose additional restraints on redistribution or aggregation reactions. For example, consider the system involving a bivalent *antibody* as the added external ligand directed to an antigen in the membrane. For a single antibody molecule to bridge two antigen molecules would require that the maximum distance between the two antibody active sites [of the order of 100 to 120 Å (Green, 1969)] be equal to or larger than the distance of closest approach of the identical epitopes of two adjacent antigen molecules. If in some circumstances the antigen was part of a subunit aggregate in the unperturbed membrane, this distance of closest approach of two antigen molecules could be too large for an antibody molecule to span and thus could prevent aggregation reactions that are intrinsically capable of occurring. However, if an *anti-antibody* is now added (indirect antibody or sandwich technique), cross-linking of the first antibody might become sterically possible and capping of the antigen would result.

These considerations can clearly be generalized to other types of external ligands and membrane receptors.

An additional possible complication that might be involved in certain cases is an aggregation of the external ligand itself *on the membrane surface*. Many lectins, particularly the plant seed globulins, have a strong tendency to aggregate in ordinary aqueous solutions. (Concanavalin A, for example, is highly aggregated in aqueous buffers at pH greater than 6.5.) When combined with a membrane receptor, the thermodynamic environment (e.g., the surface pH) may produce aggregation *of the lectin itself* on the membrane surface, thereby generating a lectin aggregate of higher effective valence (binding sites per "molecule") than would be anticipated from its properties in the supernatant solution. With hormones such as insulin, which also have a strong tendency to aggregate, this possible complication should also

be considered in connection with their reactions with specific membrane receptors (Cuatrecasas, 1971).

2. *Redistributions Produced by Nucleation*

The binding of an external *monovalent* ligand, such as a small protein or small molecule hormone, to a membrane receptor protein might change the conformation of the receptor protein or otherwise alter it so as to increase markedly the thermodynamic tendency of the receptor to aggregate with itself or with other specific components in the membrane. If the receptor molecules and other components are mobile, they would then serve as nucleation centers and, after diffusing together, form specific aggregates within the membrane. (The tendency to aggregate, conversely, might markedly *decrease*, and *dissociation* of existing aggregates might result upon binding the ligand.) The effects of certain colicins on bacteria (Cramer and Phillips, 1970) and of growth hormone on erythrocyte membranes (Sonenberg, 1971) have been suggested to involve such a nucleation mechanism (Singer and Nicolson, 1972).

In a related manner, molecules of a new component inserted into a membrane (as a result of viral infection or other metabolic changes in a cell) might serve as nucleation centers for the aggregation of integral proteins in the membrane.

Each nucleating agent, whether acting on some integral component or itself inserting into the membrane, may produce a specific and limited type of aggregation or redistribution, involving different specific integral components with different agents.

3. *Redistributions Produced by Mild Proteolysis*

Great interest has attended observations (Burger, 1969; Inbar and Sachs, 1969) that normal cells that have been subjected to mild proteolysis (e.g., with trypsin) acquire some of the properties of cells malignantly transformed by infection with an appropriate virus. The trypsinized normal cells show an abrogation of growth control in dense monolayers, apparently until they metabolically repair their surface membranes; and they show, as do transformed cells, a marked increase in agglutinability by certain plant lectins that can combine with specific cell surface oligosaccharides. One explanation that has been given for such an increase in agglutinability is the presence of additional "cryptic sites" for lectin binding in normal membranes that are exposed by proteolysis (Fig. 7A) (Burger, 1969). These tryptic digestions can be very mild; the enzyme is dilute and the treatment is brief, and under such circumstances there is no detectable release of

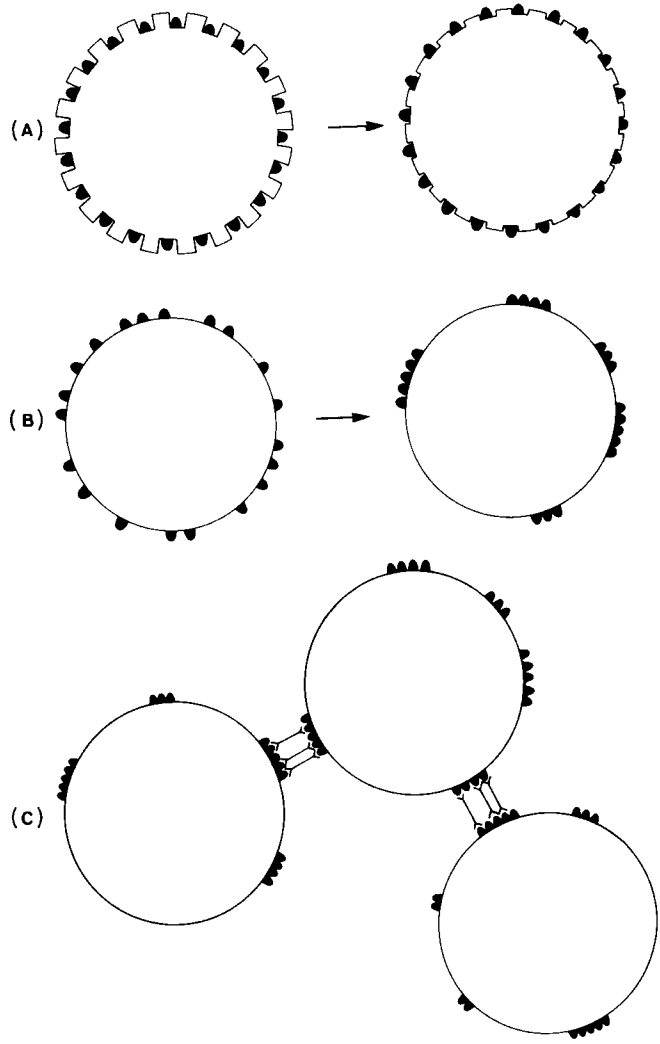

FIG. 7. A schematic representation of two different proposed mechanisms for the effect of mild trypsinization on the agglutinability of normal mammalian cell membranes by lectins: (A) Exposure of "cryptic sites" for the binding of lectin molecules; (B) redistribution and clustering of already exposed lectin-binding sites in a fluid mosaic membrane. In part C, the enhanced agglutinability of cells altered, as in part B, is ascribed to the formation of multiple lectin bridges between clusters on two opposed cells. (After Singer and Nicolson, 1972.)

peptide or glycopeptide fragments from the cell surface. In the light of the thermodynamic considerations discussed in Section IV,A, it is therefore difficult to understand how a molecule of a lectin receptor could be unavailable or "cryptic" in the surface of the normal cell and become "exposed" upon mild proteolysis. A large free-energy input would be required to convert the receptor from a cryptic to an exposed state. In fact, in some instances, there is no apparent increase of lectin-binding sites upon mild proteolysis of normal cells (Ozanne and Sambrook, 1971; Sela *et al.*, 1971).

In considering the implications of the fluid mosaic model of membrane structure, I therefore suggested as an alternative explanation for such results that mild proteolysis of intact normal cells, by altering one or more integral protein components of the cell surface, might increase the tendency of that component to aggregate with itself or with other membrane proteins. This suggestion was incorporated into the publication by Singer and Nicolson (1972). If the lectin receptors were originally uniformly dispersed in the normal cell membrane but became clustered upon mild proteolysis (Fig. 7B), the increased agglutinability could be accounted for by an increased probability of agglutinin bridges forming between clusters of receptors on two opposed cells (Fig. 7C). Following this suggestion, Nicolson (1972) has obtained direct evidence, using ferritin-labeled lectins (Nicolson and Singer, 1971) that there is a marked clustering of Con A receptors on mildly trypsinized mouse 3T3 cells compared to untreated cells and that the regions of cell–cell contact upon agglutination of the trypsinized cells are, indeed, regions where the receptors are clustered.[4]

In a related fashion, it has been shown that proteolysis produces clustering of the intramembranous particles of erythrocyte ghost membranes seen in freeze-fracture experiments (Marchesi *et al.*, 1972). These particles have been shown to include an integral glycoprotein.

The metabolic effects of mild proteolysis, including abrogation of growth control, may therefore mimic at least some of the effects of hor-

[4] Note added in proof:

More recently, several investigations (Nicolson, 1973; Rosenblith *et al.*, 1973; Noonan and Burger, 1973) have indicated that while a difference in clustering of lectin-bound sites does exist between malignantly transformed and trypsinized normal cells, on the one hand, and normal cells, on the other, the clustering on the surfaces of the former cells is *induced* upon binding of the lectin. Apparently, the difference is that the *mobility* of the lectin binding sites on transformed and trypsinized normal cells is much greater than on normal cells.

mones, mitogens, or other agents acting upon cells, if all of these effects involve initially a similar redistribution of components in the plane of the membrane. This introduces a cautionary note in connection with current experiments on the apparent effects of hormones and of mitogens on lymphocytes, which are discussed further in Section VI,G.

C. Mechanochemical Properties of Plasma Membranes

Cell membranes exhibit various kinds of mechanical behavior that are under metabolic control. Cell ruffling and locomotion, elongation and retraction of cell processes, and cell division are only a few examples. Much current interest focuses on the possibility that some kind of actomyosin-like system is associated with the plasma membranes of eukaryotic cells and imparts mechanical and contractile activity to the membranes. Some such system may be involved in capping and endocytotic phenomena in lymphocytes.

It has been suggested (Guidotti, 1972; Singer, 1972b) that spectrin (Section II,E), a protein complex isolated from erythrocyte membranes, functions as such an actomyosin-like system in the intact erythrocyte. Spectrin can be removed from the erythrocyte membrane by mild treatments such as the addition of 10^{-3} *M* EDTA (see Fig. 1). It consists of two large myosin-like components with molecular weights of about 250,000 and an actin-like component with a molecular weight of about 40,000. The complex is attached to the inner cytoplasmic surface of the membrane (Nicolson *et al.*, 1971c), apparently by way of specific integral proteins which may form part of the intramembranous particles in the membrane (Nicolson and Painter, 1973). When γ ^{32}P-labeled adenosine 5′-triphosphate (ATP) is added to erythrocyte ghosts, ^{32}P becomes covalently attached to one of the two myosin-like components (Williams, 1972). This phosphorylation also occurs in the intact erythrocyte when the cells are incubated with phosphate-^{32}P (M. Sheetz and S. J. Singer, unpublished observations). These biochemical events, which involve ATP and Ca^{2+}, appear to be correlated with marked mechanical and shape changes in the erythrocyte membrane (Weed *et al.*, 1969; Hochmuth and Mohandas, 1972).

It is proposed that proteins similar to spectrin are associated with the plasma membranes of other eukaryotic cells and make up at least part of the latticelike, ~50-Å diameter microfilaments that appear on electron micrographs to be in contact with a variety of plasma membranes (Wessells *et al.*, 1971). An especially clear and important instance of such microfilament activity is in the contractile ring formed

in cell division (Schroeder, 1970). This ring of microfilaments is attached to cytoplasmic surfaces of plasma membranes just at the cleavage furrow where the cell is dividing and appears to be exerting a "purse-string" contraction of the membrane across the equatorial plane of the cell. It is remarkable that this ring appears only at the furrow, and only at the time of cell division, suggesting that it is part of a reversibly aggregating and disaggregating protein system roughly analogous to (but clearly different from) microtubules.

The fungal antibiotic cytochalasin B (Carter, 1967) inhibits a great many mechanical activities of cells and tissues in a reversible manner (Wessells *et al.*, 1971). According to several investigators, the effects of the drug are correlated ultrastructurally with a disappearance of the latticelike microfilaments just mentioned (Schroeder, 1970; Wessells *et al.*, 1971), but the precise mechanism of action of the drug is still not known (Carter, 1972). It is therefore of some interest that cytochalasin B has been reported to inhibit lymphocyte-mediated cytotoxicity reversibly (Cerottini and Bruner, 1972), which may suggest a role for mechanochemical activity in that process (see comment on microprojections later).

The mechanochemical components of membranes and their mechanisms of function are being intensively investigated at the present time.

Another aspect of the mechanochemical properties of cell membranes is the formation and retraction of microprojections and other morphological differentiations. Lymphocytes in particular seem to have an exceptional capacity for such differentiation (McFarland, 1969). Projections from the cell surface can become very ramified and appear to be directly involved in interactions of lymphocytes and their target cells under physiological conditions (Ax *et al.*, 1968; Able *et al.*, 1970). It is, therefore, important to think about membrane surfaces not as simple, more-or-less planar and uniform structures but to be aware of the possible special functional roles of microprojections, microvilli, etc., as well as to learn more about the molecular bases for their formation. The exocytosis of such microprojections may also be involved in the surprisingly rapid "shedding" or turnover of membrane-bound immunoglobulins (Cone *et al.*, 1971; Wilson *et al.*, 1972; Vitetta and Uhr, 1972).

D. Some Possible Biochemical Consequences of Redistributions

One focus of interest in the redistribution of integral proteins and other components in cell membranes is the possibility that the redis-

tribution could have profound direct consequences on certain membrane-associated functions of the cell. There are a number of ways this could conceivably occur, but all of these are largely speculative at present.

1. Transport Activity Changes

One possibility is that the specific transport of ions and small hydrophilic molecules and other metabolites may be affected by redistributions of integral protein and other components in the membrane. To appreciate this possibility, we must first digress briefly and consider some suggested mechanisms for the transport of small hydrophilic molecules through membranes.

Two general classes of molecular mechanisms have been proposed to account for protein-mediated active transport across membranes. One is the so-called carrier mechanism (cf. Wilbrandt and Rosenberg, 1961) in which a protein of the membrane specifically binds the ligand on one side of the membrane and then by rotation in the membrane carries the ligand to the other side where it is released. For the reasons discussed in Section III,B,1, in general we consider it very unlikely that transmembrane rotational motions of amphipathic integral proteins occur at any appreciable rate, since such motions require very large free energies of activation. We therefore do not favor this class of transport mechanisms.[5]

The other class of mechanisms invokes the presence of protein "pores" through the membrane. Previously, in Section II,D,2, we suggested that specific subunit aggregates of suitable integral proteins can provide a feasible way to generate such protein-lined pores (Singer, 1971). These transmembrane aggregates could also provide a reasonable mechanism for facilitated diffusion or active transport (Singer, 1973). The specific binding of a ligand (X in Fig. 8) on one side of the membrane to an active site within the pore, followed in the case of active transport by some enzymatic energy-yielding step, might result in a significant *quaternary rearrangement* of the subunits of the aggregate. This rearrangement might then "translocate" (Mitchell, 1957) the active site to face the other side of the membrane, releasing X.

[5] Note added in proof:

Recent experiments in our laboratory (Kyte, 1974) show that the enzymatic activity of the Na^+, K^+-dependent ATPase of kidney cell membranes is unaffected by the binding of specific antibodies to the enzyme. Since the ATP hydrolysis by this enzyme and the coupled transport of Na^+ and K^+ are considered identical events, this evidence argues against a transmembrane rotation mechanism for the transport process.

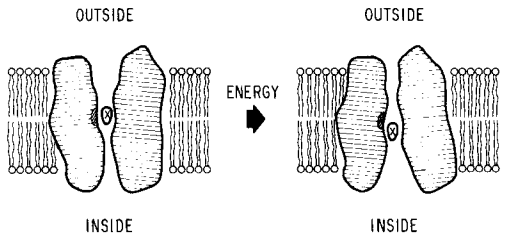

FIG. 8. A proposed mechanism of active transport in which a subunit aggregate of integral membrane proteins forms a water-filled pore (Figs. 2b and 3), with a specific binding site for the transportable ligand X. Some energy-yielding process (an enzymatic phosphorylation, for example) might produce a quaternary rearrangement of the subunits and result in a translocation of X across the membrane. (From Singer, 1973.)

The significant feature of this mechanism is that in appropriate cases protein subunit aggregates are ideally suited to convert *a small free-energy input into a large structural alteration*, by producing considerable rearrangement of the subunits relative to one another. Again, hemoglobin provides an example of these effects. Oxygenation and deoxygenation of hemoglobin do not greatly alter the conformations of individual subunits but are accompanied by large rotations of the subunits relative to one another (Perutz and Ten Eyck, 1971). For example, an α subunit of one $\alpha\beta$ protomer unit turns 13° relative to the β subunit of the other protomer.

If this general mechanism for facilitated diffusion or active transport phenomena is correct in its broad outlines, then it is conceivable that redistribution or aggregation of integral proteins of the membrane could directly or indirectly affect the properties of such specific transport aggregates. For example, the latter may become intercalated into larger aggregates involving other integral proteins when redistribution occurs. Or, the effects of redistribution may be less direct. They may result in a changed local membrane environment for the transport aggregate, perhaps through a redistribution of surface charge density or a redistribution of lipids. Or, the redistribution of some components in localized regions of the membrane may produce rapid changes in the surface area per molecule of lipid in the remaining regions. Such changes could influence the ligand binding or the quaternary rearrangements of the transport aggregate depicted in Fig. 8.

Although these suggestions are highly speculative, several reports are consistent with them. The experiments of Sefton and Rubin (1971) showed that trypsinization of normal chick embryo fibroblasts caused a *rapid* large increase in the rate of influx of 2-deoxyglucose and

glucose but had only small effects on the rates of thymidine, uridine, and α-aminoisobutyric acid transport.[6]

Experiments have also been performed on the effect of mitogens on transport activities of lymphocytes. For example, van den Berg and Betel (1973) have shown that very rapid stimulation (twofold in 5 minutes) of the active influx of 2-aminoisobutyric acid (a nonmetabolizable amino acid) into rat lymphocytes is produced by the addition of phytohemagglutinin (PHA) and Con A, but not by pokeweed mitogen (PWM). On the other hand, the transport of another such compound, aminocyclopentanecarboxylic acid, is not affected by any of these mitogens.

There is an apparently bizarre but very interesting system involving Na^+-K^+ transport in sheep erythrocytes that is relevant in the context of this discussion. The allelic blood group antigens, M and L, are associated with high K^+ (HK) and low K^+ (LK) levels, respectively, in the erythrocytes. Isoimmunization of a sheep that is homozygous at one allele with the blood of a sheep homozygous at the other produces isoimmune antisera (anti-M and anti-L). The binding of anti-M antibodies to HK cells is not affected by ouabain, an inhibitor of active Na^+-K^+ transport, nor does it change active or passive cation transport in HK cells. By contrast, anti-L antibodies stimulate active cation transport in LK cells four- to sixfold. The further remarkable finding, however, is that this stimulation requires bivalent antibody or the $F(ab')_2$ fragment; univalent Fab fragments or Fc do not stimulate (Snyder *et al.*, 1971). These results show that the effect of the antibodies on stimulating transport cannot be simply the result of binding to the Na^+-K^+ transport component, for the Fab fragment would then have been as effective as intact antibody. Furthermore, the stimulating effect of the anti-L antibodies shows antigen and antibody excess zones; that is, only in a narrow concentration range are the antibodies maximally effective. All of these results are consistent with the suggestion that whether the anti-L antibodies bind to the transport components themselves or to some other antigen receptor in the erythrocyte membrane, a redistribution of the receptors occurs which is the direct cause of the stimulation of Na^+-K^+ active transport. One

[6] In these experiments, the presence of cycloheximide at the time of trypsinization (Sefton and Rubin, 1971), or the addition of mitogen (van den Berg and Betel, 1973) prevented the rapid stimulation of the transport activities observed. It seems unlikely, however, that such rapid changes require new protein synthesis, and the possibility should be investigated that cycloheximide in these cases has a direct structural effect on the plasma membranes.

would predict from this proposal that low temperatures would markedly inhibit the rate at which these transport changes appear . It is also noteworthy that while we are proposing that at least some antigens are translationally mobile in the membrane of the intact sheep erythrocyte, this does not appear to be the case with the human adult erythrocyte (Section VI,E). This can also be tested experimentally.

We have examined three apparently unrelated experimental systems in which some perturbation of a cell has produced very rapid changes in certain selective transport activities. What these systems may share in common is that proteolysis in the first case, lectin binding in the second, and antibody binding in the third are all capable of producing redistributions of integral components in the membrane of the cell, as discussed in Section IV,B. The differential effects of these agents on certain transport systems in each case is particularly interesting. These results therefore lend some support to the hypothesis that redistributions of membrane components may have as a direct consequence differential effects on transport activities. Similarly, differential distributions or redistributions of membrane integral proteins may be directly responsible for the differences in specific transport activities between normal and malignantly transformed cells (cf. Foster and Pardee, 1969; Martin *et al.*, 1971) as well as for the differential effects of lectins on these transport activities (Inbar *et al.*, 1971)[4].

2. *Enzymatic Activity Changes*

In addition to possible effects on transport activities, redistributions of some integral proteins in a membrane might also affect one or more critical enzyme activities in that membrane, by mechanisms similar to those just discussed for transport components. Membrane-bound ATPases and adenyl cyclase are two examples of ubiquitous integral proteins with active sites that must be oriented to the cytoplasmic surfaces of their plasma membranes so that the expressions of their enzymatic activities are directed to cytoplasmic constituents. If these molecules are embedded sufficiently deeply in the membrane from the cytoplasmic side, their properties could be affected by a redistribution of components from the exterior surface of the membrane embedded deeply enough to interact with them. In this connection, it may be relevant that Burger and his colleagues (1972) have reported that mild trypsin treatment, which stimulates growth of normal 3T3 cells, is correlated with a *decrease* in intracellular cyclic adenosine 5′-monophosphate (AMP); furthermore, the addition of dibutyryl cyclic AMP to the trypsin-treated cells inhibits this growth

stimulation. Possibly also related are the results of Smith *et al.* (1971) who found that, within 2 minutes after the addition of PHA to human peripheral blood lymphocytes, there was a significant *increase* in intracellular cyclic AMP levels.

It is also possible that changes in transport rates occur as a result of, rather than independently of, changes in enzyme activities. For example, the activation of adenyl cyclase may lead to phosphorylation of a membrane transport component and affect its properties (Gardner *et al.*, 1974).

V. Some Aspects of Membrane Biogenesis and Remodeling

Having discussed some aspects of the molecular organization of membranes and its bearing on the molecular mechanisms of membrane functions, we turn now to consider some features of membrane biosynthesis and alteration.

A. Plasma Membrane Biogenesis

Membranes of eukaryotic cells are of several different kinds. There are the limiting or plasma membranes of cells; the intracellular cytoplasmic membranes making up the rough and smooth endoplasmic reticulum; and the membranes of nuclei and several intracellular organelles and granules. For the present purposes, attention will be confined to the first two kinds of membranes.

There is substantial reason to believe that the plasma membranes of eukaryotic cells are not synthesized *de novo* but rather are formed from intracellular cytoplasmic membranes by a kind of assembly-line process, as was first suggested by Palade (1959). By analogy with secretory mechanisms which have been extensively studied in the pancreatic acinar cell, it is proposed (Fig. 9) that membrane elements are first assembled in (perhaps a specialized part of) the rough endoplasmic reticulum; they are then transmitted and converted into smooth membrane and Golgi elements; following this, Golgi elements are pinched off into specific vesicles; and, finally, new plasma membrane is generated by the fusion of these specialized vesicles with already existing plasma membrane.

As expected from the analogy of an assembly-line process, on passage of the membrane through the cell, components in the membrane may be altered by enzymatic modification or they may be inserted into or deleted from it. This could account for differences found in the protein distribution and enzymatic activities of the different intracellular and plasma membranes (Meldolesi and Cova, 1972). There might also

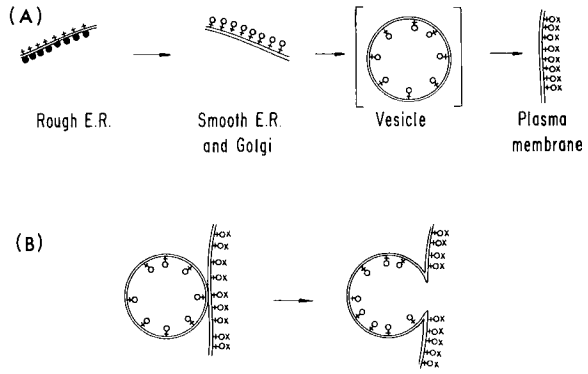

FIG. 9. The assembly-line mechanism for the biogenesis of plasma membranes of eukaryotic cells. (A) Membrane synthesis is initiated in the rough endoplasmic reticulum (E.R.), and such membrane is conveyed on an assembly-line leading penultimately to the formation of precursor vesicles. (B) The fusion of such vesicles with already existing plasma membrane leads to the formation of new plasma membrane. At different stages along the assembly line, saccharide units (+, O, X) are added successively to growing oligosaccharide chains on integral glycoproteins and glycolipids. (After Hirano *et al.*, 1972.)

be several different assembly lines each specialized for plasma membranes, secretory granules, and other elements.

This assembly-line mechanism has not yet been definitively established, but consistent with it are the findings (Ray *et al.*, 1968) that, with rat liver cells, cycloheximide treatment stops within minutes the further incorporation of a previously applied pulse of leucine-^{3}H label into the *cytoplasmic* proteins but does not markedly affect for several hours the continued incorporation of label into the plasma membranes.

Further evidence favoring this mechanism has been obtained by a study of the distribution of oligosaccharides on plasma and intracellular membranes (Hirano *et al.*, 1972). It has been shown by electron microscopy that the oligosaccharide moieties of the plasma membranes of a variety of eukaryotic cells are exclusively localized to the exterior surfaces of the membranes (Nicolson and Singer, 1971; 1974). On the other hand, it is known from other investigations (for reviews, see Spiro, 1970; Kraemer, 1971) that the covalent attachment of saccharide residues to membrane glycoproteins proceeds in a stepwise manner, with specific residues becoming attached in different intracellular membrane compartments. The exclusive localization of oligosaccharides to the *exterior* surface of the plasma membrane can be explained if it is postulated that new plasma membrane

is generated by the fusion of a precursor vesicle with already existing plasma membrane (Fig. 9), and the oligosaccharides of the vesicle are exclusively localized to the *interior* surface of the vesicle membrane. In turn, the assembly-line model for plasma membrane biogenesis would then require that the first sugar residues attached to membranes are exclusively localized to the cisternal side of the rough endoplasmic reticulum. The latter prediction has been verified by Hirano *et al.* (1972) with homogenates of a myeloma cell in culture.

In terms of the thermodynamic considerations discussed earlier, it makes sense that an integral protein of a plasma membrane, which must be synthesized on ribosomes in the rough endoplasmic reticulum, is directly incorporated into the reticulum membrane and transported to and incorporated into the plasma membrane, as indicated in Fig. 9. An alternative mechanism, involving the release of the integral protein from the ribosome and its subsequent diffusion through the cytoplasm and incorporation into the plasma membrane, is fraught with problems, including the very low aqueous solubility of the nascent integral protein molecule.

These mechanisms bear directly on many problems in cellular immunology, including the resynthesis of membrane-bound receptor immunoglobulins, the switchover from IgM to IgG production in B cells, and the secretion of immunoglobulin molecules.

B. Antigenic Modulation

One means of introducing radical changes in membrane structure in certain cases is referred to as antigenic modulation. In these instances, specific antibody directed to a cell surface antigen eliminates that antigen from the cell surface. This was encountered in the serotype transformation of paramecia exposed to type-specific antisera (Beale, 1957), and later, in the modulation of the TL antigen on thymocytes and leukemic cells (Old *et al.*, 1968). It seems likely that antigenic modulation occurs by mechanisms very similar to those involved in the capping and endocytosis by anti-immunoglobulin antibodies of receptor immunoglobulins on B lymphocytes (Section III,A,5). In the case of the TL antigen system, for example, antigenic modulation occurs at 37°C, but is inhibited at 0°C., suggesting the need for membrane fluidity, and it is sensitive to actinomycin D and iodoacetamide, suggesting a dependence on certain metabolic activities. The modulation of the TL antigen from TL+ to TL− is accompanied by an increase in the amount of *H*-2(*D*) on the leukemic cell, but, whereas modulation was complete in about 1 hour, *H*-2(*D*) continued to increase for about $4\frac{1}{2}$ hours, an effect which may be ex-

plained by the assembly-line mechanism of plasma membrane biogenesis (Section V,A). The only observation about antigenic modulation of TL that is difficult to reconcile with the suggested mechanism is that the Fab fragments of the anti-TL antibodies induce specific modulation as effectively as the intact antibody (Lamm *et al.*, 1968). This is in marked contrast with the behavior of Fab fragments in the receptor immunoglobulin capping experiments (cf. Taylor *et al.*, 1971; Loor *et al.*, 1972), although de Petris and Raff (1973) report a slow and partial endocytosis, but no capping, of ferritin-labeled Fab fragments of anti-MIg antibodies bound to lymphocytes.[7] The results of Lamm *et al.* may reflect a relatively unique ability of the Fab fragments to aggregate when bound to the surface of the leukemic cell (Section IV,B,1).

VI. Some Applications to Immunology

In the preceding sections, we have discussed in detail some facts and speculations about membrane structure, function, and biosynthesis. There are many respects in which this discussion is relevant to phenomena of immunology, but it would require a much longer article than this one to give all of these phenomena due consideration. Our object here, therefore, is not to make an exhaustive application of current concepts of membrane molecular biology to immunology but rather to illustrate their applicability and usefulness. It is the approach, as well as the content, that we wish to emphasize in the following sections.

A. Membrane Proteins of Interest in Immunology

It seems likely that most membrane proteins of direct interest in immunology, such as various protein-based antigens (histocompatibility proteins, blood group glycoproteins), receptor immunoglobulins, and glycoprotein, lectin, and mitogen receptors, are *integral* proteins (Table II) by the criterion that mild treatments do not *rapidly release them from the membrane in a molecularly intact and molecularly dispersed form.* For other antigens expressed on different lymphoid cells [θ (Thy-1), Ly, TL, MBLA, etc.], there is as yet insufficient information to characterize them as protein-based or as perhaps lipid-based.

[7] In order for a ferritin conjugate of Fab fragments to be effective as a monovalent control for experiments with ferritin-labeled antibodies, it must be demonstrated that in the conjugation reaction no more than one Fab fragment is attached to each ferritin molecule.

In this connection, the general problem of solubilization of membrane-bound antigens and other proteins might usefully be mentioned. Solubilization is a term that has often been used very loosely in the past. For example, sonication breaks a membrane into small pieces that no longer sediment in moderate centrifugal fields; this has sometimes been referred to as solubilization but is better viewed simply as fragmentation.

Antigenic activities have sometimes been released in soluble form from membranes by proteolysis, but if the protein antigen is amphipathic (Fig. 5) then only its protruding portion may be proteolytically cleaved from, and its embedded portion retained in, the membrane (see Section III,A,1). The released antigen, which may be quite useful immunologically, is then, nevertheless, not in its molecularly intact state. A case in point is the *H-2* histocompatibility antigen of the mouse. Its antigenic activity can be released from mouse spleen cells in significant amounts by papain digestion, but the solubilized polypeptide is a (large) fragment of the apparently intact protein that can be released from the membrane by detergent treatment (Cullen *et al.*, 1972; Schwartz *et al.*, 1973).

Since membranes themselves often contain proteases as integral components (Bernacki and Bosmann, 1972) or even as contaminants, prolonged incubation of cells without any added enzyme may release antigens as a result of proteolysis.

Nonionic detergents, such as Triton X-100 and Nonidet P-40, have been successful in a growing number of instances in releasing integral proteins from membranes in an apparently molecularly dispersed form (although bound to the detergent) with retention of specific antigenic or enzymatic activities. The histocompatibility antigen is an example (Cullen *et al.*, 1972). With ionic detergents, however, most such activities are lost. This may be a reflection of the amphipathic character of the integral protein. The nonionic detergent may interact with the hydrophobic end of the protein and solubilize it, but may not interact at all with the hydrophilic end, leaving unaffected its expression of antigenic or enzymatic activity. An ionic detergent may, however, interact with and alter the conformation of the hydrophilic as well as the hydrophobic ends of the integral protein. This suggested differential action of detergents on membrane proteins would be entirely consistent with the facts that nonionic detergents generally to not interact or bind to simple soluble proteins (Helenius and Simons, 1972), whereas ionic detergents usually bind to and denature them.

Another aspect of membrane proteins, about which there is as yet little direct information, is the short-range distribution of specific pro-

teins in the plane of a membrane. The methodology for obtaining such *two-dimensional* information by electron microscopy is at hand, however, and such studies have been initiated, with ferritin-labeled antibodies and related specific staining techniques (Nicolson *et al.*, 1971a,b). As mentioned in Section III,A,3, this methodology must be refined (through the use of Fab fragments of antibodies, low temperatures for the staining reactions, etc.) to ensure that the added antibody reagents do not of themselves induce redistribution of the antigens they label. The two-dimensional distribution of the Rh_0(D) and *H*-2 antigens on erythrocyte membranes has been shown to be interestingly different *at short range:* the former is molecularly dispersed in the membrane (Nicolson *et al.*, 1971a), whereas the latter appears to be present in small patches (Nicolson *et al.*, 1971b).

An indirect method by which some idea of the short-range interactions of different membrane antigens can be derived is to determine the effect of the binding of antibodies of one specificity on the subsequent binding of antibodies of a different specificity (Boyse and Old, 1969). This blocking method has been used to "map" some of the alloantigens on the surface of mouse thymocytes. Another indirect method that could be used is to determine whether the capping of one particular membrane antigen by a specific antibody causes the coincident capping of an antigenically non-cross-reactive component (Taylor *et al.*, 1971).

The topological distribution of components on membranes is an area that will certainly be intensively investigated in the coming few years.

B. Receptor Immunoglobulins

Of central interest in cellular immunology are the receptor immunoglobulins on B and presumably on T lymphocytes: their structure, their mechanisms of attachment to the plasma membranes, the effects of their interaction with specific antigen, their involvement in T–B cell and T–T cell interactions, and other critical problems.

The presence of receptor immunoglobulins on B cells has been firmly established by direct labeling methods, and their numbers have been estimated to be about 10^5 per cell (see reviews by Katz and Benacerraf, 1972; Miller, 1972). There is little question that T cells also have some kind of antigen receptors, but these are apparently present in very much smaller numbers than on B cells and/or are unusual and difficult to detect by direct labeling methods. The nature of these T-cell receptors has not yet been universally agreed upon, but is likely to be some kind of immunoglobulin. As a result the few direct experimental studies that have so far been successful have been with

the receptor immunoglobulins on B cells. Here it is agreed that on unprimed cells the receptor is basically a monomeric form of the IgM species (Vitetta *et al.*, 1971; Marchalonis *et al.*, 1972).

Let us consider the possible structure and mode of attachment of IgM-like receptors to membranes, in the light of the thermodynamic discussion in Section IV,A,2. From the means required to release it from the membrane, the IgM-like receptor has the properties of an integral, rather than a peripheral, protein. As was pointed out earlier, there must therefore be some substantial difference in structure between the IgM-like receptor and the soluble IgM monomers that exist in cytoplasmic compartments of secretory cells, in order to account for the likely large difference in free energy between the membrane-bound and free (soluble) states of the molecule. What might this difference in structure be? And how might the receptor form be attached to the membrane?

Since the antigen-binding sites, located on the Fab portions of the immunoglobulin receptor, must be exposed and accessible on the outer surface of the membrane for the receptor to function, it has been assumed that the receptor is attached by its Fc region. In *secreted* IgM molecules, however, the Fc region of μ chains is rich in carbohydrate. There are four oligosaccharide chains of two distinctly different chemical types attached at specific amino acid residues which are distributed along the whole length of the Fcμ amino acid sequence (Shimizu *et al.*, 1971a). In Section II,A,1, however, it was emphasized that it would be too costly in free energy to bury saccharide groups away from contact with water. Therefore, if the Fcμ region of an IgM-like receptor has essentially the same carbohydrate content and distribution as in secreted IgM, it is not likely that a substantial portion of Fcμ is the site of binding to the membrane. Although the carbohydrate content of the IgM-like receptor remains to be determined directly, the μ chains of receptor and secreted IgM on SDS–polyacrylamide gel electrophoresis are not distinguishable (Vitetta *et al.*, 1971). Furthermore, the Fcμ region (Shimizu *et al.*, 1971b) shows no especially hydrophobic linear segment of amino acid sequence. Another possibility is that the IgM-like receptor has a separate polypeptide chain or fragment covalently attached to it. There is no definitive evidence as yet for or against this possibility. The J-chain which is covalently attached to α and μ chains of secreted IgA and IgM polymers, respectively, and which is not present in the monomeric IgA and IgM species, could be an analog for such an attached chain, which might be unusually hydrophobic.

Another intriguing possibility is suggested by the work of Milstein *et al.* (1972), who provide evidence for a possible precursor of L

chains apparently containing an additional 15 or so amino acids at the amino terminus of the chain. The authors speculate that this extra region may provide an anchor point for the L chain in the membrane and predict that a like structure is present on H chains. If the IgM-like receptor were membrane-bound by highly hydrophobic amino-terminal regions of the L and μ chains, the active sites on the Fab regions could be accessible to specific antigen binding at the membrane surface. The Fc regions would, of course, also be exposed to the aqueous medium. The regulated action of a specific protease in the membrane might cleave the chains at the appropriate peptide bonds and release the IgM from the membrane during secretion.

It is of interest in this connection that in the experiments of Vitetta *et al.* (1971), with the lactoperoxidase-iodination technique of Marchalonis (1969) and Phillips and Morrison (1970) used on intact lymphoid cells, the IgM-like receptors bound to the membranes show a ratio of ^{125}I label in their μ and L chains that is similar to the ratio found on the chains of soluble secreted IgM labeled in the same manner. This appears to suggest that most of both the μ and L chains of the *membrane-bound* IgM-like receptor on B cells is exposed to the aqueous medium—a conclusion that is at least consistent with the findings and speculations of Milstein *et al.* (1972).

The antigen receptors on T cells have been less well studied than those on B cells. As concluded from labeling methods, there must be fewer than 10^3 Ig-like receptors per T cell (Vitetta *et al.*, 1972; Santer *et al.*, 1972). Although anti-L-chain antisera interfere with T-cell functions (Lesley *et al.*, 1971), conflicting results, but generally negative ones, have been obtained with antisera directed to the known types of H chains (for review, see Katz and Benacerraf, 1972). It is possible that the receptor on T cells is Ig-like but contains a unique class of H chain, or that, if it is an IgM-like molecule, it is attached to the T-cell membrane by a mechanism very different from that of the B cell. If the antigen receptor on T cells is indeed similar to that on B cells, but is somehow attached differently to the membranes of the two cells, this may be in some manner connected with the *Ir* gene product. The location of the *Ir* gene within the major *H-2* histocompatibility gene complex (Benacerraf and McDevitt, 1972) may signify an association of the *Ir* and *H-2* gene products in the membrane of the mouse T cell.

In helper functions mediated by T cells, the initial event is thought by some to involve *simultaneous* binding of the antigen to receptors on a specific B and a specific T cell. It is remarkable, however, that this binding together of B and T cells by antigen would occur if there are so few antigen receptors on T cells. Even more paradoxical, how-

ever, is the evidence that at least one of the roles of the T cell in such helper functions is to *concentrate* the antigen onto the B cell[8] (Mitchison, 1971). The specific binding of erythrocytes to rosette-forming T cells (Greaves and Möller, 1970; Elson and Bradley, 1971) is also remarkable for the same reason. If the receptors on T-cell membranes are dispersed as individual molecules, the probability of forming stable antigen bridges between the T and B cells, or between rosette-forming T cells and erythrocytes, should be unfavorable (see following section). One possibility is that all of the receptors are clustered in a few small patches on the membranes of *resting* T cells, that is, in the absence of the antigen. This could explain the "concentrator" role of the T cell and may relate to the observations of Santer *et al.* (1972), who found only one or a few sites of ^{125}I-labeled anti-L-chain antibodies on thymus lymphocytes. Such proposed patches may be mobile in the membrane (Ashman and Raff, 1973) and may or may not be related to the patches of intramembranous particles seen in mature T-cell but not B-cell membranes (Fig. 6) and to the occurrence of microvilli on T cells. This proposal could be tested with a ferritin-conjugated Fab fragment of an anti-κ-chain antibody to localize the receptor immunoglobulins on the T-cell surface.

C. Agglutination, Cell–Cell Interaction, and Complement-Mediated Cytolysis

No doubt many factors are involved in the capacity of antibodies directed to cell surface antigens to agglutinate the antigen-bearing cells, including surface charge properties of the cells, degree of exposure of the antigen epitopes, and valence and affinity of the antibodies. One factor that has long been recognized is the antigen density on the cell surface. The hemagglutinating capacity of IgG antibodies directed to specific erythrocyte antigens correlates reasonably well with the antigen density on the red cell; e.g., IgG anti-Rh antibodies do not agglutinate directly (so-called incomplete antibodies), whereas IgG antibodies to the ABO blood group antigens do. The role of epitope density in agglutination has been investigated by coupling different amounts of a hapten to intact erythrocytes and examining the specific hemagglutination of these modified cells by antihapten antibodies (Leikola and Pasanen, 1970). Whereas IgM an-

[8] There is evidence to suggest that the T cell–B cell interaction is indirect, occurring through the mediation of the macrophage (cf. Unanue, 1972). The macrophage may either take up receptor–antigen complexes shed from activated T cells (Feldmann, 1972c) or may directly bind and concentrate the antigen for presentation to, and activation of, the T cell (Katz and Unanue, 1973). In either case, it would still be remarkable for the proposed process to occur with so few antigen receptors on T cells.

tibodies were effective in agglutinating cells with both low and high hapten densities, the IgG antihapten antibodies agglutinated only the ones with high hapten densities.

For effective agglutination, it may be required that *several* antibody bridges in a relatively close-packed array be formed between two cells (Fig. 7C); a single antibody bridge may not be adequate to make a stable bond between the cells. This could explain the greater effectiveness of multivalent IgM than bivalent IgG molecules in hemagglutination, since even a single IgM molecule can make a multiply bonded bridge between isolated antigens situated on two cells. If indeed *clustering* of antibody bridges is important in agglutination, then two additional factors not previously recognized may have to be taken into account in understanding the phenomenon. One is the distinction between an antigen, such as $Rh_0(D)$ on human erythrocytes (see Section III,A,3), which is dispersed as single molecules in the membrane, and others which may be present in clusters as subunit aggregates in the membrane. In such circumstances, the *overall* density of epitopes on the cell surface might not be as important as their *local* density. For a clustered antigen, the overall density might be very low, but the local density at specific regions on the membrane might be high, and IgG antibodies might be able to form multiple local bridges between such antigen clusters on two adjacent cells.

A second factor is the mobility of the antigen in the membrane. Even if an antigen is molecularly and uniformly dispersed in the membrane of an unperturbed cell, the addition of specific IgG antibodies can cause clustering or aggregation of the antigen in the membrane, with a number of different potential effects on agglutination. If capping occurs and is followed rapidly enough by endocytosis of the antigen, the antigen density on the surface of the cell may be so diminished that agglutination would be inhibited (see Section V,B on antigen modulation). On the other hand, if the antigen is clustered into patches by the antibodies but is not endocytized, the patched antigen may enhance multiple antibody bridge formation and agglutination. In this connection, the relative *immobility* of surface antigens of the intact adult human erythrocyte (Section VI,E) may make hemagglutination a special case of agglutination phenomena.

The clustering of lectin-receptors in the surface of cells undergoing agglutination by lectins, a subject of great current interest in connection with malignant transformation, has been discussed earlier (Section IV, B,3). The clustering of membrane receptors may also be a critical event in cell–cell interactions. For example, it has been found (Wekerle *et al.*, 1972) that the adherence of rat T-lymphocytes to monolayers of intact mouse fibroblasts, involving the recognition by the rat cell of specific mouse histocompatibility antigens, oc-

curred at 37°C. but not at 4°, and furthermore did not occur at 37°C, in the presence of dinitrophenol or sodium azide. These authors therefore suggest that this apparent "energy dependence of specific adherence might be related to surface migration and concentration of membrane receptors," related to cap formation on B-lymphocytes induced by anti-immunoglobulins.

In connection with another important immunological phenomenon, complement-mediated cytolysis, the static or dynamic clustering of antigens in membranes may play an important role. The binding and activation of complement components requires that a suitable aggregate of immunoglobulin molecules is formed when IgG antibodies bind to specific cell surface antigens. The formation of such an aggregate of antibody molecules clearly requires the close apposition of at least several molecules of the membrane antigen in question. For a membrane antigen that is only sparsely present in the membrane, however, this would clearly require antigen clustering to occur either in the resting cell membrane, or to be induced by the bivalent antibodies.

D. Phagocytosis and Pinocytosis

It seems very likely that the translational mobility of components in cell membranes plays a critical role in phagocytosis by macrophages and pinocytosis by other types of cells. In fact, phagocytosis and pinocytosis may be only specialized versions of more general endocytotic events such as follow the addition of antibodies or mitogens to lymphocytes and other cells at 37°C. (Sections III,A,5 and VI,F). Macrophages are specialized to contain in their membranes specific receptors for the Fc regions of certain classes of immunoglobulin molecules (cytophilic antibodies) and other receptors for certain complement components. With human macrophages, these receptors bind IgG1 and IgG3 (Huber and Fudenberg, 1968) and C3 (Huber *et al.*, 1968), respectively. By virtue of these receptors in their membranes, macrophages are capable of attaching antigen–antibody complexes and opsonized cells, either with or without complement present. The act of binding itself, however, does not lead to phagocytosis. If opsonized erythrocytes (in the absence of complement) are added to macrophages at 0° or 22°C, they become firmly attached without interiorization, but, if the temperature is raised to 37°C, rapid interiorization results (Berken and Benacerraf, 1966). This is consistent with the suggestion that the fluidity of the membrane at the higher temperature allows aggregation of the phagocyte immunoglobulin receptors bound to the immunoglobulin that is attached to the opsonized cell. This aggregation would then initiate a mechanochemical process in the phagocyte membrane that leads to clustering (and

perhaps, but not necessarily, capping) followed by endocytosis of the clustered regions. The stimulation of phagocytosis by small amounts of cationic polymers (de Vries *et al.*, 1955; Ryser and Hancock, 1965) may also be rationalized if the binding of the polymer to the membrane results in a clustering of negatively charged components (sialoglycoproteins?) in the plane of the membrane with subsequent endocytosis. Analogous mechanisms may be involved in the release of lysosomal granule enzymes from neutrophils stimulated by antigen-antibody complexes (Janoff and Zeligs, 1968) and by immunoglobulin aggregates of specific classes (Henson *et al.*, 1972).

Although multiple-site binding has been invoked (Phillips-Quagliata *et al.*, 1969, 1971) to explain the markedly enhanced binding of antigen–antibody aggregates as compared to that of individual immunoglobulin molecules to macrophages, the possible role of receptor mobility in phagocytosis has not been recognized heretofore.

Stimulation of pinocytosis in *Amoeba proteus* by the addition of antibodies directed to surface antigens of the amoeba (Wolpert and O'Neill, 1962) has been referred to earlier as an example of capping and endocytotic phenomena.

The picture that emerges is that given the proper stimuli and the proper membrane receptors, pinocytosis is a very general phenomenon of eukaryotic cells. It is tempting to speculate that similar phenomena are involved in at least some situations where proteins appear to be specifically transported across membranes that are normally impermeable to large molecules. For example, the entry of specific yolk proteins into mosquito oocytes (Roth *et al.*, 1972) is correlated with a specialized, energy-requiring micropinocytosis of the oocyte membrane. Other proteins, such as ferritin, do not enter the oocyte unless exogenous yolk proteins are present; presumably the ferritin then becomes engulfed in the micropinocytotic vesicles. It is suggested that the mosquito oocyte membrane contains specific receptors for one or more yolk proteins, which when bound to the receptors, induce aggregation of the mobile receptors and consequent micropinocytosis of the membrane.

The possibility that the transfer of specific IgG molecules across the placenta involves such specific pinocytotic activity of the appropriate cell membranes might be investigated.[9]

[9] Note added in proof:

Since this was written, two reports have appeared showing that indeed IgG uptake by the fetal rabbit yolk sac (Sonoda *et al.*, 1973) and the neonatal rat intestine (Rodewald, 1973) occurs by a mechanism involving micropinocytotic vesiculation of the surface membranes of the cells involved.

E. Erythrocyte Immunology

The membranes of *intact* adult human erythrocytes do not appear to exhibit redistribution or capping effects when treated with antibodies to surface antigens or mitogens directed to saccharides on the surfaces, or when subjected to various perturbations such as a change in pH (Loor *et al.*, 1972). This makes the erythrocyte relatively unique among eukaryotic cells so far examined. On the other hand, the components of the membrane of the *lysed* erythrocyte appear to be mobile (Pinto da Silva, 1972). It is proposed that this unique immobility in the intact cell membrane is attributable to the peripheral protein complex, spectrin, and that spectrin is bound to integral protein components on the cytoplasmic surface of the membrane (Nicolson and Painter, 1973), thereby obstructing the translational diffusion of components in the membrane of the intact cell (Fig. 10). Upon lysis, the spectrin structure may become somewhat disorganized and allow the integral components to move extensively in the plane of the membrane. The difference in membrane properties of the intact and lysed cells cannot be attributed to gross changes in the physical state of the lipids, since the lipids of the membrane of the intact and lysed cells appear to be equally fluid (Landsberger *et al.*, 1972).

This relative immobility of membrane antigens of intact adult

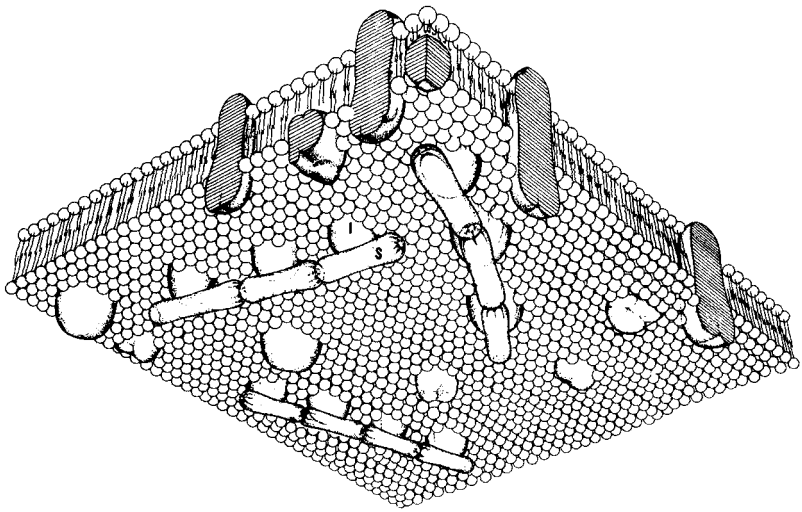

FIG. 10. A schematic representation of the cytoplasmic surface of the erythrocyte membrane, the surface opposite to that depicted in Fig. 4. It is proposed that aggregates of the protein complex spectrin (S) are noncovalently attached to integral protein molecules (I) protruding from the cytoplasmic surface. This effectively ties together several of the otherwise independent I molecules into individual clusters.

erythrocytes may have important consequences for the properties of these cells in a variety of immunological phenomena, such as agglutination, complement fixation and lysis, and erythrophagocytosis (Section VI,C,D). For example, consider the $Rh_0(D)$ antigen on Rh+ human adult erythrocytes. On erythrocytes with about 10,000 monomolecularly dispersed $Rh_0(D)$ molecules per cell, the average distance of separation of the molecules is about 1200 Å, significantly larger than the largest distance of separation of the two sites of a single IgG anti-$Rh_0(D)$ antibody molecule (~ 100–120 Å). Closed-packed clusters of several anti-$Rh_0(D)$ molecules could not be bound to the cell under such circumstances if the $Rh_0(D)$ antigen was immobile in the membrane. Indeed, Hugh-Jones (1970) found that the same molar quantities of intact bivalent anti-$Rh_0(D)$ antibodies, or of their Fab fragments, were bound to Rh+ cells, and with about the same affinity, indicating that bridging of two $Rh_0(D)$ sites by a bivalent antibody molecule did not occur to any significant extent. In view of the discussion in Section VI,C, it is therefore understandable that IgG anti-$Rh_0(D)$ antibodies do not agglutinate or promote complement-induced hemolysis of human adult Rh+ erythrocytes.

Anti-$Rh_0(D)$-coated Rh+ erythrocytes, in the absence of complement, are bound to monocytes forming rosettes without much erythrophagocytosis even at 37°C. (Douglas and Huber, 1972). If phagocytosis by monocytes requires clustering or capping of the immunoglobulin receptor sites on the monocyte membrane (see preceding section), the *fixed separations* of antibody-bound $Rh_0(D)$ sites on the Rh+ erythrocyte could prevent clustering or capping of the immunoglobulin receptor sites that were bound to the antibody and thereby prevent erythrophagocytosis. These fixed separations of the bound anti-$Rh_0(D)$ antibodies on coated Rh+ erythrocytes bound to monocytes were indeed observed in the electron-microscopic experiments of Douglas and Huber (1972). On the other hand, erythrocytes coated with antibodies to the Forsmann antigen (of which there are about 600,000 molecules per cell) are readily phagocytized at 37°C. The close proximity of antigens at such high density may lead to a dense, closely packed coat of anti-Forsmann antibodies on the erythrocyte membrane; in turn, this may be sufficient to cause clustering of the immunoglobulin receptors on the monocyte membrane that become bound to the antibodies. Furthermore, because the Forsmann antigen is a lipid, it may be translationally mobile in the erythrocyte membrane even if protein antigens are not; if so, this would help induce clustering of immunoglobulin receptors.

It is especially interesting, therefore, that at least some of the membrane components of the intact mature erythrocytes of *newborn* humans may be much more mobile than in membranes of

erythrocytes in *adult* humans. This is suggested by the studies of Blanton *et al.* (1968), who showed that, whereas ferritin-labeled anti-A antibodies attached uniformly to the surfaces of intact A+ adult human erythrocytes with no sign of vesiculation, the same labeled antibodies showed a patchy distribution on the A+ erythrocytes of newborns, and a high incidence of intracellular vesicles internally lined with ferritin particles was observed. Apparently, micropinocytosis (but not capping) of the newborns' cells was induced by the specific binding of the antibodies to the A antigen. To correlate these results with redistribution, capping, and endocytotic effects of antibodies reacting with lymphocytes, further experiments with the newborns' erythrocytes, including those with Fab fragments of the antibodies, should be carried out. But the inference is certainly clear that the mobility of at least the A antigen [an integral glycoprotein (Pinto da Silva *et al.*, 1971)] in newborns' erythrocyte membranes is greater than in adults'.

This mobility, if general for other components in the newborns' erythrocyte membrane, could have important consequences in immune hemolytic anemias of the newborn, such as in erythroblastosis fetalis. The mobility of antigens in the membrane could allow clustering of antibodies bound to the antigens and thereby promote complement binding and hemolysis or erythrophagocytosis which might not occur with comparable adult erythrocytes. It is also clearly important to determine the molecular basis for the observed difference in properties of newborn and adult erythrocyte membranes: possibly a different content or state of aggregation of the spectrin exists on the cytoplasmic surfaces of the two types of membranes.

F. Redistributions of Membrane Components of Lymphocytes

We have discussed (Section III,A,5) the outlines of the capping phenomenon induced by the specific binding of bivalent antibodies to receptor immunoglobulins on B lymphocytes. A detailed analysis of the events at the cell membrane accompanying the binding of fluorescent-labeled anti-MIg antibodies to receptor immunoglobulins on mouse B lymphocytes has been made by Loor *et al.* (1972). They recognize three events following the binding of the antibodies which, although at least partly sequentially connected, can usefully be distinguished (Loor *et al.*, 1972):

"1. the formation of multiple spots or patches of immunoglobulins throughout the lymphocyte membrane.

2. the formation of polar caps.

3. the disappearance of the immunoglobulins from the membrane."

The first event, which is clearly distinct from capping in that the receptors are still *dispersed throughout* the membrane, appears to be a localized antigen–antibody microprecipitin reaction mediated by the diffusion of the receptor in the plane of the membrane. It does not occur with univalent Fab. With bivalent antibodies, it is inhibited at 0°C. but occurs rapidly at 22° or 37°C. It is not affected by NaN_3 at 37°C., implying that it is not an energy-requiring step. It is inhibited by an excess of anti-MIg (analogous to an antibody excess zone in precipitin reactions).

The second event is, however, inhibited by NaN_3 at 37°C. The polar cap is observed to form over the uropod of the cell, and it has been suggested (Taylor *et al.*, 1971) that in the course of cell movement the mechanochemical flow of the membrane toward one pole of the cell leaves behind the antibody-immobilized receptors to cluster into the cap. Capping, in this view, is a kind of indirect process; its apparent energy requirement is the energy required for normal cell movements of the unaffected regions of the cell membrane. If there is an actomyosin-like (spectrin-like?) mechanochemical system operating in normal cell movements (Section IV,C), it would be important to determine whether such a system is preferentially attached to, or preferentially dissociated from, the regions of the membrane that have been capped. If capping were in this sense an indirect process, one might expect that the spectrin-like components would be bound to the unaffected regions of the membrane but not extensively under the capped regions. On the other hand, if such spectrin-like components were found specifically associated with the capped regions, a direct process for capping would have to be considered.

The third event involves endocytosis (or, on occasion, perhaps exocytosis) of the aggregated receptor. Endocytosis is not observed with NaN_3-treated cells. It is not an obligatory consequence of capping; the treatment of lymphocytes with rabbit anti-lymphocyte IgG followed by sheep anti-rabbit immunoglobulin produces capping but little or no endocytosis (Unanue *et al.*, 1972). Nor does endocytosis, when it occurs, require that a polar cap be formed first. In different systems, spots or patches, in the absence of cap formation, can apparently be endocytosed (Blanton *et al.*, 1968; Santer *et al.*, 1972) while the remainder of the patches are still on the membrane surface. Therefore, the processes of capping and endocytosis, although related, are clearly separable.

If the process of endocytosis is carried far enough, the receptor immunoglobulins which are aggregated by anti-immunoglobulin antibodies can be completely swept from the membrane. If such cells

are cultured in the absence of antibodies, the receptor immunoglobulins reappear in about 6 to 8 hours, a time that would be consistent with new immunoglobulin protein synthesis and attachment in the rough endoplasmic reticulum, followed by an assembly-line transfer to the plasma membrane (see Section V,A). It is interesting that the receptor immunoglobulin density after this reappearance is significantly greater than that on the original cells and that the receptors can again be capped and swept from the membrane by anti-immunoglobulin antibodies. This effect is to be compared with that of antigenic modulation (Section V,B). In the latter, there is a permanent loss of the surface antigen in question; subsequent culture of the modulated cells does not restore it. In the case of receptor immunoglobulins on lymphocytes, however, the modulation is only transient. This important phenomenon is considered further in Section VI,H.

In some cases, direct antibody reactions at 37°C. with antigens or receptors in cell membranes [anti-lymphocyte antibodies on lymphocytes (Unanue *et al.*, 1972); anti-θ antibodies on T cells (Taylor *et al.*, 1971)] appear to produce spots or patches on the membrane, but not capping. If however, a further indirect antibody is then added, capping occurs. It may be that a critical size or surface area coverage of microprecipitin spots is required to initiate capping, and if a membrane antigen or receptor is too deeply embedded in the membrane or is ordinarily present within a subunit aggregate, the size attained by the spots is limited.

In all of these studies the properties of the receptor immunoglobulins on the entire B-cell population have been studied using anti-MIg reagents. A separate and functionally critical question is whether an antigen can, upon combining with its specific receptor immunoglobulin on the membrane of a clonally selected lymphocyte, produce similar redistribution and capping effects on that cell. Direct observations of cap formation by radioactivity-labeled antigens have been made with polymerized flagellin (Diener and Paetkau, 1972) and with keyhole limpet hemocyanin and a glutamic acid–alanine–tyrosine polymer (Dunham *et al.*, 1972). In these cases, only 1–10 in 10^4 lymph node cells showed significant antigen-binding. If immunogenic doses of antigen were used at 4°C., the photographic grains marking the location of the antigen were dispersed over the entire membrane surface of the specific cell but, on raising the temperature to 37°C., the grains were found in a cap. The specific receptors were swept from the surface if the antigen-bound cells remained at 37°C. and reappeared several hours later on the cell

membrane at a higher density than previously present (Diener and Paetkau, 1972). At flagellin concentrations known to produce high zone tolerance, caps did not form at 37°C. Therefore, in these three different cases, it is evident that antigens, upon binding to their receptor immunoglobulins, produce the same kind of redistributions of the receptors on the surfaces of their selected specific B cells as do the anti-immunoglobulin antibodies.

That the nature of the redistribution of the receptor immunoglobulins is important in determining the type of interaction of an antigen with its specific immunocompetent B cell is suggested by the studies of Feldmann (1972a,b) using dinitrophenylated flagellin of different degrees of hapten substitution. With a low degree of dinitrophenylation (about 1 group per flagellin subunit) an *in vitro* primary response to DNP (in the absence of T cells) was obtained, but no tolerance even at high concentrations. With a high degree of dinitrophenylation (about 4 groups per subunit), the molecule was a tolerogen at all concentrations, and no primary response to DNP was obtained.

G. Lymphocyte Activation

Activations of lymphocytes by antigens, anti-immunoglobulins, and mitogens are complex multifaceted phenomena presumably occurring in a stepwise and continually ramifying fashion. The focus of interest in this section is on the earliest events in these processes. It is clear that the first event is the specific binding of the externally added agent to receptors in the cell membrane. What events then follow to trigger the cell response? And how is the response affected by the particular ligand that sets it off?

In order to explore these questions, information is needed about the activating effects of different agents on the different types of lymphocytes. Two very useful recent reviews of the present state of the art of lymphocyte activation, particularly by mitogens, have appeared (Greaves and Janossy, 1972; Andersson *et al.*, 1972), and we shall therefore only summarize some of their salient observations and conclusions. The experimental situation may be complicated by the heterogeneity of both T- and B-cell populations (Stobo, 1972); nevertheless, the following points seem to be established:

1. The patterns of response of T and B cells are very different; in other words, the nature of the response is in part determined by the cell. This is true even with mitogens for which the T and B cells appear to have roughly the same number and quality of receptor sites and which produce similar capping effects on the two classes of cells.

2. For a given cell type, some mitogens in a soluble form produce activation, others do not. However, if the mitogens are insolubilized by covalent attachment to a solid matrix, these activation effects sometimes appear to be *reversed.* Thus, B cells cannot be activated by soluble Con A but (presumably the same cells) are activated by Con A attached to plastic petri dishes or Sepharose bead particles.

3. When a given type of cell is activated, the pattern of response appears to be very similar whether a specific or nonspecific agent is used. For example, bacterial lipopolysaccharide (LPS) stimulates the synthesis of an IgM anti-2,4,6-trinitriphenyl (TNP) antibody response in B cells (Andersson *et al.*, 1972), although LPS and TNP do not cross-react. If correct, this is very important. It would mean that while the stimulation of IgM antibody synthesis normally involves the binding of an antigen to its specific receptor immunoglobulin on a B-cell membrane, it is not a *necessary* event. (Presumably, this also would mean that internalization of the antigen is not necessary for stimulation of antibody synthesis.) However, it is not clear whether binding to the receptor immunoglobulin is a necessary event or whether binding to an *entirely unrelated* receptor in the same membrane is sufficient to initiate the same process. It is not known to which receptors pokeweed mitogen (PWM) and LPS bind; these might be oligosaccharide or other moieties on the receptor immunoglobulin or to unrelated receptors.

4. There is a strong correlation between the multivalence of an agent and its capacity to activate B cells. Thus, bivalent or higher-valent antibodies, but not Fab fragments, activate rabbit lymphocytes. Certain multivalent hapten carriers can function as T-cell-independent antigens, but not poorly substituted carriers (Feldmann, 1972a,b). The inference is that cross-linking of receptors by the added agent is important in triggering the response.

5. On the other hand, capping per se and endocytosis are not correlated with activation. If cross-linking of receptors by the added agent is involved, neither the formation of a cap nor the internalization of the agent or the receptor appears to be sufficient to produce activation.

Let us consider the activation of B lymphocytes first. In order to rationalize the findings just summarized, the following (and still rather vague) suggestions may be made about the early events in that process. The added agent, if it is to activate the cell, must first bind to a specific receptor in the membrane and then cause it to redistribute in the plane of the fluid membrane. [I have elsewhere called this a cis-type cooperative effect (Singer and Nicolson, 1972), as opposed to a

trans effect acting locally across a membrane.] The requirement for multivalence of the added agent strongly suggests that redistribution involves clustering or patching of the initially uniformly dispersed receptors. It is proposed that such *energy-independent* clustering into small patches is necessary (but may not be sufficient) to perturb some important transport components and/or enzymes in the membrane and change some transport rates and/or enzyme activities (see Section IV,D) and that these changes, in turn, lead to a whole cascade of subsequent biochemical events that ultimately results in activation.

It is not yet clear whether the clustering of receptor immunoglobulin molecules is required for B-cell activation. Such clustering could be produced directly by multivalent ligands (antigens or mitogens) binding to the receptor immunoglobulins or indirectly as a result of perturbations of other membrane components. Alternatively, the possibility exists that the redistribution need not involve the receptor immunoglobulins at all and that clustering of appropriate integral proteins entirely unrelated to the receptor immunoglobulins produces the same end result.

If *patching* of the appropriate receptors in the membrane is indeed the crucial early event in triggering the response of the B lymphocyte, then it can be appreciated why under certain circumstances *capping* of the same receptors might actually be inhibitory. If all of the receptors are moved into a cap, the remainder of the membrane may be in a physical and chemical state not very different from that in the resting membrane, compared to the perturbed state when the receptors are clustered into small patches all over the cell surface. Thus, the transport components and enzymes left behind in the uncapped portion of the capped membrane may exhibit the same properties as in the resting membrane, but be altered in the patched membrane. This could be the reason why soluble Con A caps but does not activate B cells, whereas insolubilized Con A does activate. With its Con A molecules rigidly spaced, insoluble Con A may prevent or at least significantly alter the capping of the Con A receptors produced by soluble Con A.

In this scheme one can also explain why different mitogens, each of which might produce patching of its specific receptors, may or may not activate the cell. It could depend on the molecular properties of the particular receptor and whether formation of its patches in the membrane significantly affects the transport components and/or enzymes that are critically involved.

One can also reason that the B-cell-activating effect of a given added agent might be potentiated by another agent if the latter

enhanced the effects of patching by the former. For example, a specific humoral antibody might potentiate the activating effect of a low concentration of antigen by bridging the antigen molecules and promoting the antigen-induced patching of the receptor immunoglobulins in the membrane. On the other hand, the activating effect of a particular agent might be inhibited by another, if the latter heavily cross-links and immobilizes membrane components so as to inhibit patching (Yahara and Edelman, 1972). The possible role of proteases in potentiation should also be appreciated, since mild proteolysis can, in certain circumstances, itself produce patching of at least some membrane components (Section IV,B,3).

Although these mechanistic considerations can be extended to the activation of T cells, there is not enough information available about the comparative properties of T- and B-cell plasma membranes to make meaningful speculations feasible. Clearly, the patterns of activation of T and B cells by the same mitogens, for example, are different (Greaves and Janossy, 1972).

There is some evidence (Mandel, 1972) (Fig. 6) that T- and B-cell plasma membranes are structurally distinguishable. It could well be that the amount and distribution of different receptors in the resting cell membrane and the nature and effects of their redistribution upon the addition of an external agent are different for T and B cell membranes. We have suggested (Section VI,B) that the small numbers of antigen receptors on the membranes of resting T cells may already be clustered into patches, which might be connected with the activation of T cells by doses of antigen roughly 1000-fold smaller than for B cells (Mitchison, 1971).

These speculations about the early events in lymphocyte activation may turn out to be incorrect in many details, but if in a broad sense they are on the right track, then the important conclusion is that further progress in understanding these processes will require much more detailed insight into the fundamentals of membrane structure and of membrane functions (such as transport) than we now possess.

H. Immunological Enhancement

It has been known for a long time that humoral antibodies directed to specific surface antigens of a tumor cell can inhibit or block a cell-mediated cytotoxic immune response to the tumor and thus enhance its growth. This phenomenon has its counterpart in other cell-mediated immune responses, such as normal tissue graft rejection and delayed hypersensitivity. This general subject, of critical importance in cancer immunology, deserves an extended analysis, but this will

not be attempted here (for a recent review, see Feldman, 1972). In this section, we want only to point out the relevance to immunological enhancement of some of the material discussed in previous sections.

It has often been considered that the effect of humoral antibodies (with or without the contributions of other factors such as soluble antigen or complement) in the enhancement phenomenon is to modulate the tumor-specific antigen on the tumor cell surface (cf. Möller, 1964; Kaliss and Suter, 1968), although the molecular mechanisms of modulation were not then understood. However, in general after serial inoculation of tumors into several generations of recipients, the expression of the tumor-specific antigen remains unaltered, so *permanent antigenic modulation* cannot be occurring. On the other hand, recent studies of the modulation of receptor immunoglobulins on B lymphocytes by antibodies (Loor *et al.*, 1972) or by antigen (Diener and Paetkau, 1972) have shown that *transient modulation* of membrane antigens can occur (Section VI,F). As long as the anti-MIg antibodies, for example, are present in suitable concentration, the receptor immunoglobulin is continually swept from the surface of the lymphocyte. But, if the modulated cells are then cultured in the absence of the antibodies, the receptors reappear on the surfaces of the cells after several hours. We interpret this to mean that on removal of the perturbing influence of the anti-MIg antibodies, or the antigen, intracellular synthesis of the receptor immunoglobulin is reinitiated in the rough endoplasmic reticulum, and, by the assembly-line mechanism of plasma membrane biogenesis (Section V,A), the receptor appears several hours later on the outer surface of the cell. In this view, the difference between permanent and transient modulation is only whether synthesis of the modulated membrane component is permanently repressed or is derepressed, respectively. It is possible that the latter is a much more frequent occurrence, but, since the phenotypic expression of the cell is regained, it may often go undetected.

We suggest that similar effects may be involved in immunological enhancement. The ability of very small amounts of humoral antibodies (much less than are required to saturate the tumor-specific antigen on the cell surface) to enhance tumor growth (Haughton and Nash, 1969) shows that enhancement is not owing simply to blocking the tumor antigen. It is consistent with the idea that these antibodies (with or without other potentiating factors) produce redistribution of the antigen and its removal or marked depletion from the tumor cell surface by endocytosis. As long as humoral antibodies are present, the antigen is not quantitatively reexpressed on the cell surface, and the tumor is resistant to specific cell-mediated immune attack. But on inoculation of the tumor into an unprimed recipient, in the absence of

the humoral antibodies, the tumor-specific antigen is reexpressed on the cell surface as the cells multiply. These suggestions are amenable to experimental study with techniques that have been employed for lymphocyte receptor immunoglobulins.

VII. Concluding Remarks

I have attempted in this article to present some current ideas about how membranes are organized at the molecular level and, in particular, to discuss the fluid mosaic model of membrane structure. The thermodynamic basis for this model and some of the experimental evidence supporting it have been considered. From this base, which I feel (despite its recent development) is relatively secure, I have tried to project some of the functional consequences of the fluid mosaic model. Here we enter an area which is certainly highly speculative at present, but for which one can muster bits and pieces of information from the literature that are relevant and intriguing. What emerges from this analysis is the clear indication that redistributions of components in the fluid membrane that are produced by a variety of externally added agents play a critical role in cell biology in general. At present, perhaps, the best-studied system in which these redistributions occur is the lymphocyte system, providing one more example of the fruitful cross-fertilization of immunology and cell biology.

In the final portion of the article, I have applied the fluid mosaic model and the facts and speculations about membrane redistribution effects to some problems of interest in immunology. Although this has been done in some detail, the primary object was not so much to provide explanations for particular problems, as to show that the approach to membrane structure and function advocated in this article may be useful in understanding a large variety of immunological phenomena and in suggesting new experimental approaches to problems in immunology. In the process, many other important immunological phenomena have not been considered in this article, but might be thought about in similar terms.

Finally, it should also have become evident that there are many fundamental aspects of membrane molecular biology about which entirely too little is known at present and that further developments in this very active area should be of great and continued interest to immunologists.

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Membrane Immunoglobulins and Antigen Receptors on B and T Lymphocytes

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I. Lymphocyte Heterogeneity and Antigen Recognition

A. INTRODUCTION

The initiation of all types of immune responses is dependent in part on the direct interaction of antigen with an immunocompetent cell. The subsequent differentiation of the effector cells in the various forms of immunity then involves a further complex series of events including the interaction or collaboration among different cell types. In particular, immune responses are characterized by their exquisite

specificity for the immunogen, and it is now generally considered that this is directly related to the nature of the receptor for antigen on the surface of the original immunocompetent cell.

It was postulated over 70 years ago that these receptors for antigen on the immunocompetent cell are preformed membrane-attached antibodies (Ehrlich, 1900), and the model developed at that time bears a striking resemblance to current demonstrations of lymphoid cell surfaces (Fig. 1) and the interaction of labelled antigens with lymphoid cells. This concept of specific preformed receptors for antigen was not followed up in the ensuing decades; instead, instructional theories of antibody formation were in vogue (Breinl and Hurowitz, 1930; Alexander, 1932; Mudd, 1932; Pauling, 1940). It was thought that the antigen itself in some manner directed the formation of complementary structures by the antibody (protein)-synthesizing system of the cell. However, these general views are not consistent with the current understanding that the information for specific protein synthesis is encoded in the genome of the cell.

In the 1950s, selection theories of immunity (Jerne, 1955; Talmage, 1957; Burnet, 1959), returning to the concept of precommitment of immunocompetent cells for antigen, were proposed. It was considered that precursor cells exist bearing the receptors for antigen on their surface. Each precursor cell carries antibody receptors of only one specificity, which is identical to the antibody whose synthesis is induced when the cell is triggered to proliferation and clonal expan-

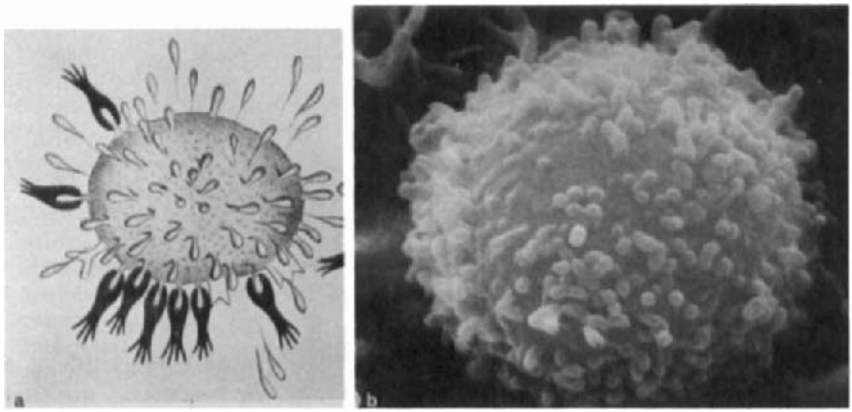


FIG. 1. A comparison of the (a) model of cell surface, antibody receptors for antigen proposed by Ehrlich (1900) with (b) a scanning electron micrograph of a typical normal B lymphocyte. (Polliack *et al.*, 1973).

sion by interaction with the specific antigen. More recently, this aspect has been clearly discussed by Mitchison (1969) as the "receptor hypothesis" which states that antigen interacts with cell membrane-associated, receptor antibody molecules and that this reaction induces transformation and multiplication of the cells leading to their clonal expansion including development of plasma cells and cell-bound antibody. In considering induction, it must be noted that interaction of antigen with immunocompetent cells can also lead to the alternative pathway of paralysis (Bretscher and Cohn, 1968; Bretscher, 1972; Feldmann and Nossal, 1972), and, for the purpose of this discussion, the receptor hypothesis is considered essential for the induction of immunity, but it is not in itself of necessity the complete description of immune induction.

One of the major goals of current immunological research is to elucidate the detailed cellular and molecular events involved in the initiation of immunity. This area could be arbitrarily divided into several aspects, concerning the elucidation of the nature of the receptor for antigen on the cell surface, the initial events following receptor–antigen union at the cell surface, the problem of induction versus paralysis discrimination, and the regulatory control of other events including cell interactions in the complete differentiation of antibody-producing cells. The general scope of the present review concerns the first of these aspects, namely, a consideration of the presence, derivation, and significance of membrane-bound immunoglobulin (M-Ig) on lymphoid cells and its relation to the receptor for antigen on all recognized types of immunocompetent cells. Various other relevant reviews have been presented on antigen recognition (Ada, 1970; Bach, 1973; Davie and Paul, 1973; Greaves and Hogg, 1971a, Modabber, 1973; Möller and Sjöberg, 1972; Nossal and Ada, 1971; Paul, 1970; Roelants, 1972; Wigzell, 1970; Wigzell and Andersson, 1971), M-Ig and Ig expression by lymphoid cells (Greaves, 1970; Lerner, 1972, Mäkelä, 1970; Mäkelä and Cross, 1970; Marchalonis and Cone, 1973; Sell, 1970a; Sell and Asofsky, 1968; Warner, 1972a; Warner and Harris, 1973), immunity–tolerance considerations (Bretscher, 1972; Feldmann and Nossal, 1972), and cellular interactions among lymphoid cells (Basten and Howard, 1973; Claman and Chaperon, 1969; Davies, 1969; Katz and Benacerraf, 1972; Miller, 1972, Miller and Mitchell, 1969; J. F. A. P. Miller *et al.*, 1971; Mitchell, 1974). In considering the nature of M-Ig and expression of Ig's by lymphoid cells, considerable attention will be given to aspects of phenotypic restrictions of the various Ig classes and allotypes. The nature of Ig class heterogeneity and genetic polymorphisms of Ig's have been extensively

reviewed elsewhere (Edelman and Gall, 1969; Fudenberg and Warner, 1970; Herzenberg *et al.*, 1968; Hood and Prahl, 1971; Natvig and Kunkel, 1973).

B. The Two-Cell System of Immunity

It has been recognized for many years that immune responses are of two general types, broadly termed *cellular immunity*—delayed-type hypersensitivity responses, transplantation immunity, and immunity to some viral, bacterial, and parasitic infections—and *humoral immunity*, the production of circulating antibody molecules. In all types of immune responses, lymphocytes have been shown to play a central role in both the initiation and development of the response (Gowans and McGregor, 1965). Lymphocytes, however, can no longer be considered to be one homogeneous cell population, and a large body of current research is attempting to analyze these cells into discrete functional subpopulations ranging from the immunocompetent or antigen-sensitive cell that initially reacts with antigen to the effector cells in cell-mediated immunity.

At a more peripheral level than the direct specific interaction of antigen with immunocompetent cells are various other cell types that play accessory roles either in the early phases of antigen handling or at effector levels. Thus, cells of the reticuloendothelial system, macrophages, monocytes, and dendritic reticulum cells in lymphoid follicles play various roles in the development of some (Unanue, 1972), but not all (Shortman *et al.*, 1970), humoral antibody responses; whereas mast cells, for example, play an accessory effector role in certain hypersensitivity responses (Austen and Becker, 1971; Bloch, 1967).

In considering the heterogeneous nature of specific immune responses, studies in birds first indicated the existence of two distinct, lymphoid cell differentiation pathways that were respectively concerned with the two broad types of immune responses. The pioneering studies of Glick *et al.* (1956) showed that neonatal removal of the avian bursa of Fabricius considerably depressed the subsequent ability of the bird to produce specific antibodies on immunization. These studies were extended by Mueller *et al.* (1960) using hormonal methods to prevent bursal development. Around this time, Miller (1961) made the basic observation that in mice, neonatal removal of the thymus markedly depressed aspects of cellular immunity, namely, the rejection of skin homografts. A similar effect of neonatal thymectomy was also found in the chicken (Warner and Szenberg, 1962), and the concept of a qualitative dissociation of immunity in chickens then

emerged (Warner *et al.*, 1962), in that the bursa of Fabricius controlled the development of cells of the antibody-forming series, whereas the thymus controlled the differentiation of lymphoid cells involved in cellular immunity (Aspinall *et al.*, 1963; Cooper *et al.*, 1966; Warner, 1967). These studies did not, however, indicate any role for thymus-derived cells in the development of antibody production, and, although subsequent studies have demonstrated that this does occur in chickens (Ivanyi and Salerno, 1971; Rouse and Warner, 1972a; Weinbaum *et al.*, 1973), the basic concepts and analysis of lymphoid cell interactions have arisen from studies in mammals, principally mice.

In view of the absence of a well-characterized bursal equivalent in mammals (cf. Cooper and Lawton, 1972a), the analysis of mammalian lymphoid cells into two functionally distinct series has followed a more tortuous path. Various studies had shown that neonatal thymectomy could influence both cellular immunity and some humoral antibody responses (Miller and Osoba, 1967). Davies, however, showed that although thymus-derived cells (T cells) proliferate in response to stimulation by various antigens (Davies *et al.*, 1966), they do not themselves differentiate into antibody-forming cells (Davies *et al.*, 1967; Davies, 1969). The immunological relevance of this T-cell proliferation to antigen was then indicated by the studies of Claman *et al.* (1966), who showed a synergistic effect for antibody production when T cells, bone marrow cells, and antigen were injected into irradiated recipients. The specific roles of the bone marrow-derived cells (B cells, bursal or bursal equivalent-derived cells) and T cells in the production of antibody were then clearly defined in a series of experiments by Miller, Mitchell, and colleagues (Miller and Mitchell, 1969; Miller, 1972). Cell transfer experiments involving several types of marked cells clearly showed that the antibody-producing cells differentiated from B cells under the influence of activated T cells.

Another method of approach has also been used to demonstrate T–B cell collaboration in humoral antibody responses. For a decade, it has been recognized that the degree of an antihapten antibody response to hapten–protein conjugates in primed animals is dependent on the carrier used for the hapten–carrier boost (Ovary and Benacerraf, 1963). *In vivo* studies indicated that two cell populations were involved, in that immunization of hapten–carrier 1 primed animals with carrier 2 and then hapten–carrier 2 gave marked antihapten antibody responses (Katz *et al.*, 1970; J. F. A. P. Miller *et al.*, 1971; Mitchison *et al.*, 1970; Rajewsky *et al.*, 1969). Various cell transfer and other studies clearly defined that two distinct classes of

lymphocytes cooperate in the induction of antihapten antibody responses. Antihapten antibody-producing cells derive from hapten-sensitive B cells under the facilitating influence of carrier-sensitive T cells (J. F. A. P. Miller *et al.*, 1971; Miller, 1972; Mitchison, 1971a). Various studies have also demonstrated that specific immunological memory and tolerance can be induced in both T and B lymphocytes.

C. Immunocyte Differentiation

The available evidence at present indicates that antigen-sensitive T and B cells represent independent lines of differentiation that are relatively fixed—cells of one line do not differentiate into the alternative pathway. However, both lines ultimately trace back to a common precursor cell termed the *hematopoietic stem cell,* and it is a problem of some current interest to determine the early stages of differentiation from this stem cell to the point where the two pathways irrevocably diverge. A general outline of immunocompetent cell differentiation has previously been reviewed (Warner, 1972b) and briefly may be summarized as follows. Hematopoietic stem cells originally derive in the yolk sac from precursor hemangioblasts, and during fetal to postnatal life this pool progressively moves from yolk sac to fetal liver to the adult bone marrow. Hematopoietic stem cells are capable of self-renewal, and under specific inductive influences, possibly derived from local microenvironments, these cells progressively differentiate into the mature elements of the hematopoietic system (Metcalf and Moore, 1971). Marker studies have formally shown that hematopoietic stem cells will give rise to all hematopoietic elements, including the lymphoid, erythroid, and myeloid series (Wu *et al.*, 1968).

Under the influence of the bursa of Fabricius in birds (Warner, 1967, Cooper *et al.*, 1971a) or some equivalent in mammals (Cooper and Lawton, 1972a), differentiation of the stem cell to the B lymphocyte is induced (see Fig. 2). This stage of differentiation is antigen-independent and occurs initially during embryonic life. Further differentiation to the activated B-cell stage (recognized as immunoblasts or plasmablasts) is initiated by antigen with resulting antibody secretion. Continued differentiation with cell division ultimately leads to the formation of plasma cells that are generally considered to be end cells incapable of further division or differentiation. The role of T-cell influences on this plasma cell differentiation will be considered later in this review. An alternative pathway of differentiation for the hematopoietic stem cell involves induction by the thymic environment and leads to the formation of T cells. As indicated in the foregoing, antigenic stimulation of this pathway leads to the develop-

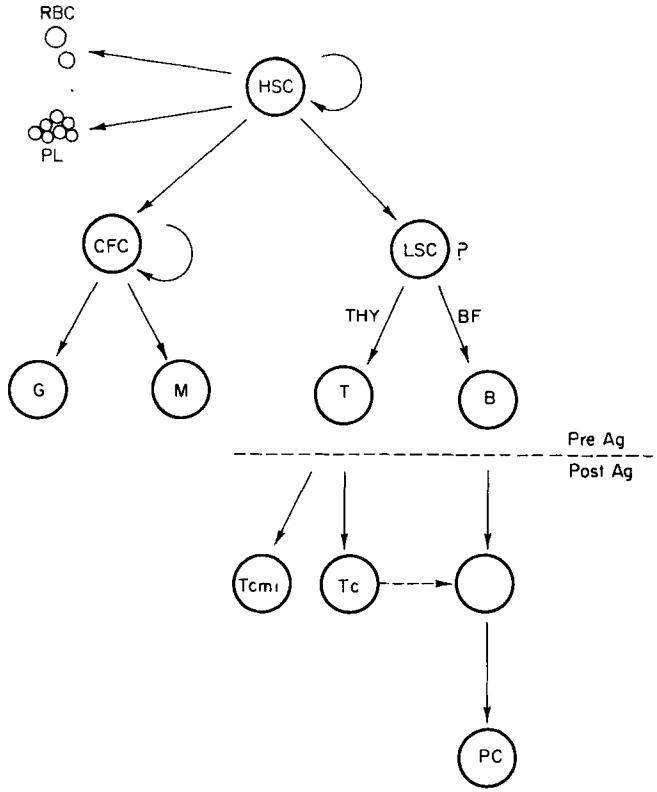


FIG. 2. Proposed scheme of immunocyte differentiation. Hematopoietic stem cells (HSC) capable of self-renewal can differentiate into either erythroid elements (RBC), megakaryocytic elements (PL), the myeloid series or into lymphoid cells. Myeloid differentiation proceeds through an intermediary cell detected *in vitro* as a colony-forming cell (CFC) which under suitable direction differentiates to granulocytes (G) or monocytes/macrophages (M). Lymphoid differentiation may also proceed through an intermediary, lymphoid, stem cell (LSC) which under either thymic (THY) or bursal (equivalent) (BF) direction proceeds in the absence of antigen (Pre Ag) to T lymphocytes or B lymphocytes. Following antigenic stimulation (Post Ag), B cells pass through an activated stage into maturing plasma cells (PC). The T cells can differentiate either into activated T cells detected in cell-mediated immunity (T_{CMI} or into helper cells (Tc) that direct B-cell maturation. (T_{CMI} and Tc could be different expressions of the same cell.)

ment of two functionally recognized types of T cell—one directly involved in cell-mediated immunity and the other collaborating with B cells in antibody production. Whether these two functional activities are expressions of the same or different cells is not fully resolved.

Several observations have indicated that immunocompetent cells (as determined by expression of Ig antigen receptors) appear in cen-

tral lymphoid organs within 1 or 2 days after hematopoietic stem cell immigration into these primary sites of lymphoid differentiation (Dwyer and Warner, 1971, Cooper *et al.*, 1972a). It has not yet been determined whether the hematopoietic stem cell directly transforms into the committed immunocompetent B or T cell in the respective inducing environment, or whether there are several distinct differentiation stages between these two types, such as the existence of a true lymphoid stem cell that can differentiate into either a B or T cell (given the right inducer) but not into the erythroid or myeloid series. In the myeloid system, various studies (see Metcalf and Moore, 1971) have clearly defined the existence of a stem cell with a more restricted differentiation potential to either granulocyte or macrophage pathways (Fig. 2). Several recent studies have indicated that some form of lymphoid precursor cell may exist. El-Arini and Osoba (1973) studied the differentiation of T cells from bone marrow cell suspensions that had first been depleted of T cells. It was shown that T-cell progenitors were present that lacked the θ antigen and could not respond to alloantigens (both properties of antigen-sensitive T cells). The density profile of these progenitor T cells was different from that of the pluripotential hematopoietic stem cell. Furthermore, Lafleur *et al.* (1972) identified a cell type with the same density profile as the precursor T cell of El-Arini and Osoba that was also present in bone marrow and could give rise to cells capable of collaboration with T cells and antigen (i.e., B cells). Whether these T precursors and B precursors are the same or separate populations still needs to be resolved. In chickens, Toivanen and Toivanen (1973) have identified a bursal stem cell capable of restoring antibody-forming capacity to cyclophosphamide-treated chickens. This cell type requires contact with a bursal environment for its further differentiation, although it might also be possible for this cell to differentiate to a T cell given the right environment. Further studies on this general problem of T- and B-cell differentiation pathways from stem cells are required.

Cellular heterogeneity also exists within the T and B compartments, in that the B-cell series ranges from antigen-sensitive B cells to plasma cells (see Ellis *et al.*, 1969; Cooper *et al.*, 1971a) and the T-cell series clearly shows functional heterogeneity (see Raff and Cantor, 1971; Asofsky, 1974; Gershon, 1974). Although immunocompetent T and B cells appear relatively similar, fine structural differences have been revealed (Mandel, 1972), and further studies on the possible correlation of structural components with surface markers on these cells would be warranted.

For most practical purposes, T and B cells are best distinguished

by phenotypically expressed surface markers (see Raff, 1971). Cells of the B-cell series in mice carry the heteroantigen MBLA (Raff *et al.*, 1971; Niederhuber and Möller, 1972) and on differentiation to plasma cells express the heteroantigen MSPCA (Takahashi *et al.*, 1971a; Watanabe *et al.*, 1971) and the alloantigen PC-1 (Takahashi *et al.*, 1970). The T cells are characterized by the alloantigens θ (Reif and Allen, 1964; Raff, 1971) and the Ly-A, B, and C systems (Boyse *et al.*, 1968), whereas thymocytes also express antigens of the TL system (Boyse and Old, 1969). It appears evident now that phenotypic expression of these respective allo- and heteroantigens changes quantitatively with cell differentiation, and the phenotypic expression of these antigens thus helps to characterize the cellular subpopulation.

II. Receptors for Immunoglobulins on Cell Surfaces

Before considering in detail the possible demonstration and significance of M-Ig on lymphoid cells, it is essential to realize that the mere presence of M-Ig does not in itself necessarily prove that the M-Ig was derived from the cell. An alternative possibility is that the cell surface bears a receptor structure that will bind with varying degrees of avidity to portions of the immunoglobulin (Ig) molecule. As will become evident in later sections, this aspect is particularly crucial when considering a cell type, such as T cells, that may synthesize only very small amounts of M-Ig. The concept that cell surfaces have receptors for Ig was first expounded for macrophages. The presence of cytophilic antibodies for macrophage surfaces was clearly documented by Boyden and Sorkin (1960) and subsequent studies by various groups have confirmed and extended these observations to a wide range of species including man (see reviews, Nelson, 1969; Unanue, 1972; Tizard, 1972b). It has subsequently become clear that macrophages are not unique in possessing receptors for Ig and that varying proportions of B cells, T cells, mast cells, granulocytes, and basophiles also possess receptors of various types for Ig. Before considering the more relevant aspects of receptors on B and T cells, it may be useful, for comparison, briefly to review macrophage receptors for Ig.

A. Macrophage Receptors

The interaction of macrophages with Ig has been studied in two different ways, and several of the controversial aspects of cell surface interaction with Ig may relate directly to these methodological differences. At the end stage of both general methods, a complex of macrophage–antibody–antigen is usually observed. However the dif-

ference relates to the order of interaction of these three components. The strict definition of cytophilic antibodies, as proposed by Boyden (1963), states that the binding of antigen to cytophilic antibody takes place subsequent to antibody–macrophage interaction; that is, cytophilic antibodies can bind directly to macrophage surfaces without combination with antigen. The alternative technique, usually termed *opsonic adherence*, is to prepare antigen–antibody complexes (antigen either radiolabeled or large particulate such as red blood cells) and then to interact these with the cells. The basic difference between the two tests is that the cell surface receptor is binding to Fc of Ig (see in following) either as free Ig or as an antigen–antibody complex. Berken and Benacerraf (1968) considered that these are essentially the same tests, as Benacerraf (1968) showed that, in guinea pigs, cytophilic antibody eluted from macrophages can opsonize sheep erythrocytes and cause their adherence to macrophages. However, several studies have indicated that this is not the complete picture and that, whereas cytophilic antibodies will always be opsonic, opsonic antibodies may not necessarily be cytophilic. Thus, in mice, Parish (1965) indicated that there were electrophoretic differences in the migration of these antibodies. Tizard (1969) showed that mouse cytophilic antibodies formed during early primary immunization were macroglobulins and during secondary responses were of IgG size, and of fast γ mobility, possibly indicating IgG1 (Tizard, 1971a). Opsonic adherence antibodies were only IgG. Similarly, Lay and Nussenzweig (1969) have reported that, in mice, both IgG and IgM are cytophilic. These studies contrast with opsonic adherence tests, in which it was shown that, in guinea pigs, 7 S γ_2 antibodies are responsible (Berken and Benacerraf, 1966) and, in mice, IgG (Berken and Benacerraf, 1968) and possibly only IgG2a (Cline *et al.*, 1972a) antibodies are active, whereas IgM is definitely inactive.

Studies with bacterial opsonization have, however, shown that IgM can act most efficiently in opsonization (Del Guercio *et al.*, 1969; Robbins *et al.*, 1965; Rowley and Turner, 1966). The IgG subclass specificity is also in question since Shevach *et al.*, (1972a) showed inhibition of binding erythrocyte–antibody complexes by IgG1 and IgG2b but not by IgG2a proteins. Similar results on IgG binding have been found in man using either macrophages (LoBuglio *et al.*, 1967) or monocytes (Jandl and Tomlinson, 1958; Archer, 1965). In most studies, the opsonic adherence test was used rather than the strict cytophilia approach. It was clearly shown that IgG and not IgM antibodies were capable of opsonic adherence (see Huber and Fudenberg, 1970; LoBuglio *et al.*, 1967, Abramson *et al.*, 1970; Inchley *et*

al., 1970). Furthermore, subclass analysis using inhibition studies with myeloma proteins indicated that only IgG1 and IgG3 proteins were active (Huber and Fudenberg, 1968; Abramson *et al.*, 1970). These studies indicate that the Fc portion of the molecule is responsible for binding to the cell surface receptor as has been amply shown in inhibition studies with fragments and the inability of Fab antibodies to bind (Berken and Benacerraf, 1966; LoBuglio *et al.*, 1967).

Recent studies (Yasmeen *et al.*, 1973) have shown that the location of the site of cytophilic activity of human IgG1 for guinea pig macrophages is in the C_{H3} homology region of the γ chain. The specificity of human subclass inhibition studies was confirmed by Hay *et al.*, (1972) using a direct binding assay. Radiolabeled myeloma proteins were directly interacted with monocytes, and only IgG1 and IgG3 proteins were found to bind. This binding could be inhibited by soluble immune complexes. Thus, for IgG, the uptake of immune complexes and the binding of cytophilic Ig is to the identical receptor sites on macrophages, and conformational changes in the antibody (due to antigen interaction) are not essential to create binding sites. Philips-Quagliata *et al.* (1971) showed that markedly enhanced binding of antibody to macrophages occurred when the antibody was bound to divalent or polyvalent haptens at equivalence, but not when bound in antigen excess or to monovalent haptens at any concentration. As normal Ig could inhibit the binding, these studies suggest that enhanced binding of complexes is due to an increased energy of binding resulting from summation of individual binding sites and not from conformational changes in Ig structure.

Various sources of reticuloendothelial cells have been shown to possess these receptors, although it is essential to be aware that other cell types may also be involved. For some time, it was considered that lymphocytes could transform into macrophages (Maximow, 1902; Bloom, 1938), and one study (Coulson *et al.*, 1967) shows that *in vitro*-transformed guinea pig cells in a mixed lymphocyte culture have receptors for cytophilic antibody. It was rather tentatively suggested that this supported the concept of lymphocyte-to-macrophage conversion, because of the contention that only macrophages possess this receptor. Unanue (1968) then showed, however, in mice, that similarly transformed cells with cytophilic antibody receptors were not killed by a specific antimouse macrophage serum, and subsequent studies (see in following) indeed confirmed that lymphocytes can have this receptor.

Certain cells in the lymphoid follicles of rats have been implicated in antigen localization involving an antibody-mediated opsonization

(Ada *et al.*, 1967). These cells are termed dendritic cells of lymphoid follicles, and direct uptake of radiolabeled Ig by surface receptors has been demonstrated (Herd and Ada, 1969). Using rabbit or rat antibodies in rats, it was shown that antigen–antibody complex binding was due to Fc interaction with the cell surface and that both IgM and IgG could bind. Binding of Fab, but not of L chains, was possibly due to the presence of natural homoreactants against Fab determinants. It was shown that the C3 cyanogen bromide fragment of rabbit H chain localized very well in the follicles, possibly suggesting a role for the carbohydrate moiety on the chain (present on the C3 fragment). Whether these cells are analogous to the recently described dendritic cells present in adherent cell populations derived from mouse spleen and lymph nodes (Steinman and Cohn, 1973) is not known, and it will be relevant to examine these latter cells also for receptors for Ig.

The precise nature of the cell membrane receptor for Ig is not resolved, but several enzymatic treatments of cell membranes have been reported. The receptors are not susceptible to treatment with proteolytic enzymes but are susceptible to phospholipase A and reagents that react with free sulfhydryl groups (Howard and Benacerraf, 1966; Davey and Asherson, 1967; Kossard and Nelson, 1968). In most of these studies it was, in fact, found that treatment with proteolytic enzymes, such as trypsin, increased the uptake of cytophilic antibody (Arend and Mannik, 1972). Further studies have shown that alveolar macrophages have about 2×10^6 receptor sites for IgG per cell (Philips-Quagliata *et al.*, 1971; Arend and Mannik, 1973) and that increased adherence of soluble complexes to trypsin-treated cells from male rabbits was due to an increase in the average association constant of the receptor sites for IgG, whereas in females, the increase was due to an increased number of receptor sites (Arend and Mannik, 1973).

Trypsin treatment also distinguishes between cell surface reactivities to cytophilic IgM or IgG in mice: whereas increased uptake of IgG occurs after trypsin treatment of the cell, the receptor for IgM is destroyed (Nelson and Boyden, 1967; Lay and Nussenzweig, 1969; Tizard, 1969). Further studies are clearly needed to characterize the relationship of the receptor sites for IgM and IgG.

If antigen presentation to lymphocytes by macrophages is a phenomenon of biological significance, then it might be expected that these two cell types should show close anatomical associations. Aspects of this were recently discussed by Unanue (1972), and in particular Schmidtke and Unanue (1971) have shown that B cells will adhere to macrophages by virtue of the surface Ig on B cells (see in following) binding to the receptor on macrophages. Thus, it was

shown that only lymphocytes with detectable Ig on their membranes would bind to macrophages, provided that the Ig was IgM, IgG1, or IgG2 but not IgA. The binding was inhibited by free Ig and was specific for macrophages and not fibroblasts. If binding is for the same receptor as indicated with human antibody (Yasmeen *et al.*, 1973), it implies that virtually the entire Ig molecule (to the C terminus) must be exposed on these B cells. Further studies on the possible biological significance of this cell interaction are clearly required.

In this discussion of macrophage receptors for Ig, we have specifically considered serum (B-cell derived) Ig. Studies by Marchalonis and associates (Marchalonis and Cone, 1973; Cone *et al.*, 1974) have suggested that macrophages bear a receptor that is specific for T-cell-synthesized Ig but not for B-cell Ig. As their studies suggest that both of these cells bear membrane-bound IgM, it is difficult to reconcile the failure of the B-cell IgM to bind in view of the previously cited reports for mouse cytophilic IgM. The discrepancy may relate to the 8 S monomeric form of the cell membrane-associated IgM. Further aspects of the macrophage binding of T-cell M-Ig are discussed in Section III,D.

B. B-Cell Receptors

Although several early studies on the binding of antigen (Ag)- or erythrocyte (E)–antibody (Ab) complexes to macrophages also noted binding to some lymphocytes, this latter aspect was not systematically studied until relatively recently. In 1965, Uhr showed that lymphocytes could bind AgAb complexes and that this differed from macrophage AgAb binding in several ways: the lymphocyte AgAb complex appeared less stable in that it was reduced on washing; the receptor was trypsin-sensitive; mercaptoethanol treatment of the antibody destroyed binding; and complement augmented binding.

Subsequent studies have particularly concentrated on three aspects of this phenomenon: (*a*) the nature of the lymphocyte type involved, (*b*) the specificity of the receptor, and (*c*) its possible functional role. The indication that complement augments binding has particularly led to some controversy over the specificity of the receptor and, although not fully resolved, the current information (see in following) suggests that B lymphocytes can bear two receptors—one for Ig and one for complement.

1. Methods of Receptor Detection

In studying the interaction of lymphocytes with Ig's, three general approaches have been used, involving soluble AgAb complexes, aggregated Ig, and antibody-coated erythrocytes.

In terms of the discrimination discussed previously between cytophilic antibody and opsonic adherence, virtually all approaches used with lymphocytes have been of the opsonic adherence type. Thus, Basten *et al.* (1972a) found that when lymphocytes were incubated with antibody and then exposed to ^{125}I-labeled antigen, complex binding to the cells, as detected by radioautography, was demonstrable only if the cells were not washed free of antibody before addition of antigen. In the studies of Eden *et al.* (1973a) preformed soluble AgAb complexes were used, and the efficiency of binding was related to defined AgAb ratios. These studies seem to indicate that, as shown previously (Uhr, 1965), lymphocytes bind complexes but not free Ig. This may not, however, be a true qualitative picture, but rather that the avidity of binding of the receptor to Ig is much greater when the Ig is in polymeric form (see in following).

Paraskevas *et al.* (1972a) have used inhibition of the reverse immunocytoadherence (RICA) (Paraskevas *et al.*, 1971a) to detect the receptor for Fc. In this system, the presence of M-Ig (see Section III) is revealed by use of a hybrid antibody, one part directed to mouse Ig and the other to an antigen. The antigen is linked to erythrocytes, and rosettes are formed between lymphocytes bearing M-Ig and erythrocytes by the hybrid antibody. Reverse immunocytoadherence can be inhibited by treatment of the lymphocytes with soluble AgAb complexes, implying that the Fc receptor is relatively close to M-Ig on the cell surface or is itself M-Ig. This latter possibility has been virtually disproven by other studies (Basten *et al.*, 1972b; Dickler and Kunkel, 1972).

If aggregated Ig appears to the receptor as Ig in AgAb complexes, then direct visualization of the receptor interaction might be made by incubation of lymphocytes with labeled aggregated Ig. This has been observed by Brown *et al.* (1970) using aggregated human Ig in mice *in vivo*, and has been developed as a B-cell marker in the homologous human system by Dickler and Kunkel (1972) using fluorescent-labeled aggregated Ig.

Lastly, a method has been introduced that employs erythrocyte–antibody complexes (EA) and is based on the fact that the receptor binds firmly to preformed soluble AgAb complexes (Basten *et al.*, 1972a; Eden *et al.*, 1973a,b) and accordingly rosette formation between lymphocytes and EA should occur. This approach is particularly complicated by the question of whether complement is also bound to EA (EAC). The literature is somewhat controversial on this point as some studies (LoBuglio *et al.*, 1967; Cline *et al.*, 1972b; Yoshida and Andersson, 1972; Brain and Marston, 1973) have clearly shown that

EA can bind to lymphocytes, whereas others have not detected EA binding (Nussenzweig *et al.*, 1971; Shevach *et al.*, 1972a; Eden *et al.*, 1973a). When EA binding was found, it depended on the presence of the Fc in the Ab (LoBuglio *et al.*, 1967; Cline *et al.*, 1972b) and, since binding could be inhibited by competition with soluble mouse myeloma proteins (Cline *et al.*, 1972b), it is not likely that undetected complement on the EA was involved. On the other hand, the presence of a receptor for complement on some lymphocytes, *complement receptor lymphocytes* (CRL) has been clearly shown using rosette formation with EAC (Bianco *et al.*, 1970; Nussenzweig *et al.*, 1971) or soluble AgAbC complexes (Eden *et al.*, 1973a). Current understanding on this interaction might be briefly summarized as follows: (*1*) all CRL are B lymphocytes and not T cells (Bianco *et al.*, 1970; Dukor *et al.*, 1971; Nussenzweig *et al.*, 1971); (2) not all B lymphocytes are CRL (Dukor *et al.*, 1971; Ross *et al.*, 1973); (3) the CRL receptor recognizes C3 component (Bianco *et al.*, 1970) and possibly C3 split products (Eden *et al.*, (1973c); and (*4*) the CRL receptor is independent of the receptor for Fc and is distinguished from it in that the receptor for EAC is trypsin-sensitive (Uhr, 1965; Eden *et al.*, 1973a), that binding of AgAbC but not of AgAb inhibits EAC interaction with CRL (Eden *et al.*, 1973a), and that EAC prepared with cobra venom factor serum does not bind to CRL (Bianco *et al.*, 1970) whereas cobra venom factor does not affect AgAb binding to lymphocytes (Basten *et al.*, 1972b).

Although some aspects of EA binding to lymphocytes are not fully resolved, the current data clearly indicate that some lymphocytes can possess at least two receptors—one for Ig and one for C3. Accordingly, in studying AgAb interactions of any type with lymphocytes, it is essential to ascertain whether or not complement is involved in the particular reaction.

2. *Lymphocyte Type*

The identity of lymphocytes binding antibody was established by examining cell populations either enriched for T or B cells or containing known numbers of the two cell types (Basten *et al.*, 1972a). When T-cell content of various preparations, as assessed by θ antigen, was compared with the number of AgAb binding cells, an inverse relationship was clearly established with the two values totalling around 100%. Furthermore, almost all thoracic duct cells from athymic mice (T-depleted) labeled with the complex, whereas complex-binding cells were severely depleted in bursectomized chickens (B-depleted). Studies in mice with aggregated human Ig have also shown normal binding of the Ig to lymphocytes from thymus-depleted mice (de

Jesus *et al.*, 1972). These results suggest that AgAb complex binding cells are only B lymphocytes, and that *all* B lymphocytes have this receptor. However, the question of B-cell heterogeneity in terms of Fc receptor and CRL is not resolved. The data of Dukor *et al.* (1971) and Ross *et al.* (1973) imply that not all B lymphocytes are CRL. However, Eden *et al.* (1973a) show that depletion of CRL from the population virtually eliminates cells binding AgAb, whereas more cells than the number of CRL in the population bound AgAbC. These data suggest that some lymphocytes have complement receptors but cannot bind AgAb. This is not consistent with the data of Basten *et al.*, (1972a), unless the subpopulation of CRL lacking AgAb receptors does not circulate through lymph. Further studies on this aspect are needed.

The method used by Paraskevas *et al.* (1972a) by definition implies that the cells bearing the Fc receptor carry readily detected M-Ig and are thus (see Section III) B cells. The studies of Cline *et al.* (1972b) with EA binding also used purified B lymphocyte preparations derived from thymectomized or nude mice, and purified T lymphocytes from thymus or educated thoracic duct cells (T-TDL). The results again imply that most B cells have Fc receptors and T cells do not (but see data by Yoshida and Andersson in Section II,C).

Studies with human lymphocytes have also indicated that receptors for Ig are present only on B cells. Using either aggregated Ig binding (Dickler and Kunkel, 1972; Dickler *et al.*, 1973) or EA binding (Brain and Marston, 1973) with human peripheral blood lymphocytes, it was found that most Ig-binding cells possessed readily detectable M-Ig, although a small proportion of overlap in either direction can occur. Using sheep erythrocyte binding to human lymphocytes as a marker of T cells (Lay *et al.*, 1971; Wybran and Fudenberg, 1971; Froland, 1972; Jondal *et al.*, 1972; Wittingham and Mackay, 1973) it was found that T cells did not bind, including aggregate binding cells that lacked detectable M-Ig (Dickler *et al.*, 1973). In general it appears that aggregate binding to human lymphocytes may be the most sensitive and comprehensive marker for human B cells.

3. *Plasma Cells*

Uhr (1965) noted that AgAb binding also occurred on some plasma cells. Whether this binding was due to AgAb or really AgAbC was not, however, clarified. Basten *et al.* (1972b) examined eight plasma cell tumors with ^{125}I-labeled AgAb and found all but one to be quite negative. Similarly, Cline *et al.* (1972b) using EA binding found strong binding to only one of six plasma cell tumors (the same tumor HPC-6 as studied by Basten *et al.*). This particular tumor has been maintained in tissue culture for some time, and at the time of study was secret-

ing only L chains with minimal IgA detectable on the membrane (Warner and Harris, 1973); morphologically this cell appeared lymphocytic rather than markedly plasmacytoid. These observations seemed to indicate that plasma cells have usually lost the receptor for Fc of Ig on differentiation from B cells, and, as the marker is also absent from hematopoietic stem cells (Basten *et al.*, 1972b), it was suggested the marker pinpoints B cells at the differentiation stage of immunocompetent but not fully mature cells. However, recent tests for the receptor on cultured myeloma cells have shown (Harris, 1974) a rough inverse correlation between Ig-secreting activity and the cell's ability to form EA rosettes. The availability of the receptors also seems to be related to the cell generation cycle, since the proportion of cells capable of forming such rosettes was considerably increased by Colcemid-induced metaphase arrest. These results suggest that the plasma cell may still possess the receptor for Ig, but that it is relatively inaccessible when the cell is actively synthesizing and secreting Ig.

4. Specificity of Receptor

Although the receptor for Ig has not been purified or chemically characterized, some indications of its specificity of binding have been made. The antibody specificity of the target Ig appears to be of no relevance providing the specific antigen is used in the AbAg complex (Basten *et al.*, 1972a). Various species combinations have been successfully used, such as mouse lymphocytes with mouse, rabbit, or chicken antibodies, all showing similar degrees of binding (Basten *et al.*, 1972a). The observation that chicken antibody can be used is consistent with the thesis that complement is not involved, as mammalian complement is not fixed to avian Ig. The lymphocyte receptor does not appear to bind to free Ig but only to aggregated or complexed Ig. Optimal visualization of the aggregate binding involves aggregates of over 300 S (Dickler and Kunkel, 1972; Dickler *et al.*, 1973). In some studies (Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972a), free 7 S Ig did not even inhibit aggregate or complex binding, although Paraskevas *et al.* (1972a) found that Fc fragment isolated from rabbit antibody did inhibit. It was suggested that the Fc was aggregated, and Fc crystals were found to adhere directly to lymphocytes. Purified mouse myeloma proteins were found to inhibit EA rosettes by Cline *et al.* (1972b) and, in the studies of Yoshida and Andersson (1972), mouse IgG also caused inhibition. Purified mouse myeloma proteins were also found to inhibit AgAb complex binding (Basten *et al.*, 1972b), but again it is quite feasible that the preparations contained aggregated protein.

Although the dynamics of receptor Ig interaction are not fully re-

solved, it does appear that as "valency" or aggregation of the Ig preparations increases, the more avid is the binding to the receptor. It is likely that, just as for the macrophage (Philips-Quagliata *et al.*, 1971) and the mast cell (Warner and Ovary, 1971) binding of AgAb, similar requirements for multipoint binding will be involved rather than true conformational changes in the Ig molecule. These considerations are particularly relevant to the question of the possible extrinsic origin of M-Ig found *in vivo* on lymphoid cells. If only aggregated or complexed Ig were capable of binding, then this might not constitute a major source of M-Ig. However, it must be recognized that species differences may exist in the avidity of binding of complexed versus free Ig to the lymphocyte receptor. As will be discussed in more detail in Section III, controversy has existed over the origin of M-Ig on rabbit peripheral blood lymphocytes (PBL), particularly concerning the presence of parental allotypic Ig in heterozygotes.

Although it was concluded in some studies (Wolf *et al.*, 1971; Davie *et al.*, 1971) that serum Ig does not bind to rabbit lymphocytes, recent studies of Jones *et al.* (1973a) have shown that this may occur. Peripheral blood lymphocytes from homozygous b^9b^9 rabbits were incubated in 50% b^4b^5 serum and then stained with fluorescinated anti-b^4 or -b^5 antibodies. The appearance and number of stained cells was not significantly different from that of cells derived from b^4b^5 rabbits. Separate staining with rhodamine anti-b^9 reagents revealed that at least as many cells as had their own M-Ig were capable of binding exogenous Ig. Incubation of cells in the presence of AgAb complexes did not show any increased intensity of binding, although possible differences in binding with progressively lower concentrations of complexed versus free Ig have not been determined. The results at present indicate that, in rabbits, binding of Ig to B lymphocyte receptors may involve free 7 S Ig and not predominantly aggregated or complexed Ig as in man and mouse.

The Ig class specificity of receptor binding also appears to be controversial at present. This has been investigated primarily by inhibition studies using myeloma proteins rather than testing direct binding of AgAb complexes involving purified Ig class antibodies. Thus these studies have not strictly asked whether distinct receptors exist for each given class of Ig but rather whether receptors exist that can bind competitively to different Ig classes. For example, although IgA has never been found to inhibit AgAb binding, the present data do not eliminate the possibility that a distinct and separate site exists for the binding of only IgA. Furthermore, if binding to mouse and human lymphocytes is strictly a property only of aggregated Ig, differences in

degrees of aggregation of the myeloma protein preparations could influence the results. Within these limitations, the following observations have been made. Although EA complexes of IgM antibodies were not found to give B-cell rosettes (Cline *et al.*, 1972b), some binding of AgAb complexes of purified IgM antibodies were found by Basten *et al.* (1972b). Grain count studies showed weaker binding of IgM complexes than of IgG, and it may be that the avidity of IgM binding to receptor is less than that of IgG, falling below the threshold required for forming stable rosettes. Basten *et al.* (1972b) also found that an IgM myeloma protein gave partial inhibition of AgAb binding. Immunoglobulin G antibody complexes with antigen give good binding to B cells provided that the Fc fragment of the antibody is not removed by papain or pepsin treatment (Basten *et al.*, 1972b; Cline *et al.*, 1972b; Paraskevas *et al.*, 1972a; Thunold *et al.*, 1973). Free light chains or IgA proteins do not inhibit complex binding (Basten *et al.*, 1972b; Cline *et al.*, 1972b). The reactivity of different IgG classes is not at all resolved at present. In the studies of Basten *et al.* (1972b), purified IgG2 antibody–Ag complexes gave some binding, but less than with complexes containing also IgG1. Inhibition studies indicated that binding was principally due to IgG1 and to a lesser extent with IgG2b, although Cline *et al.* (1972b) found similar inhibition by IgG1, IgG2a, and IgG2b. Paraskevas *et al.* (1972a) also noted that IgG2a antibody complexes could inhibit binding. There may be quantitative rather than qualitative differences in IgG class binding to the B-cell receptor, as Basten *et al.* (1972b) found that IgG1 was about 10 times more efficient at inhibiting than IgG2b proteins.

A practical question is whether discrimination of receptor binding to IgG classes can be used as a functional marker for distinguishing cells, particularly malignant cells, of the B lymphocyte and macrophage series. At present some controversy still exists on this point. Basten *et al.* (1972b) claim the B-cell receptor primarily binds IgG1 with weaker binding to IgG2 and IgM; Cline *et al.* (1972b) showed inhibition of B-cell binding with IgG1, IgG2a, and IgG2b and of macrophage binding with only IgG2a (Cline *et al.*, 1972a); whereas Shevach *et al.* (1972a) claim macrophages bind IgG1 and IgG2b but not IgG2a. Resolution of this problem is clearly required. Studies with human Ig aggregate binding with indicated (Dickler and Kunkel, 1972) that at least IgG1 and IgG2 bind to B cells, whereas IgG1 and IgG3 proteins inhibit monocyte EA complex binding (Huber and Fudenberg, 1970). By using cell surface radiolabeling of M-Ig (see Section III), Cone *et al.* (1973a) have shown that B cells bind neither B-cell- nor T-cell-derived M-Ig, although these studies have exam-

ined the 7 S monomeric IgM and do not eliminate the possibility that polymeric IgM of either source might bind to B cells.

Relatively little information is available on the nature or membrane location of the B-cell receptor for Ig. Basten *et al.* (1972b) visualized the location of complex binding with electron-microscopic radioautography. Patterns of a patchy distribution were found that were similar to antigen binding to B cells (see Section V), with only a small proportion of the cell surface involved. Eden *et al.* (1973b) observed that the complexes capped to one pole of the cell and remained on the membrane for several hours at 37°C. in culture. The receptors, therefore, appear capable of similar membrane movement following interaction with multivalent reagents as found for M-Ig (see Section V). It was suggested (Eden *et al.*, 1973b) that the complexes could be removed by incubation of the cells with either excess antigen or monovalent (Fab) antibody to Ig, but not with excess antibody (of the type in the complex) or with divalent antibody to Ig. This latter treatment in fact stabilizes the complex on the membrane and renders it resistant to removal by either antigen or monovalent anti-Ig antibody. This phenomenon is analogous to modulation of the M-Ig by anti-Ig (Takahashi *et al.*, 1971b; see Section V) and may represent interiorization of the complexes after stable linking to membrane components as a result of M-Ig changes. The binding of free Ig to rabbit B cells is also quite stable persisting for many hours (Jones *et al.*, 1973a). These authors also found that the binding sites are susceptible to pronase digestion but regenerate in culture.

Several studies have attempted to inhibit the binding site with antisera directed to other membrane components, with variable results. Paraskevas *et al.* (1972a) found that anti-Ig would inhibit, but because their method of detection involved an indirect approach of binding of hybrid antibodies to the M-Ig, this is to be expected. Basten *et al.* (1972b) and Dickler and Kunkel (1972) showed that anti-Ig did not inhibit complex or aggregate binding, indicating that M-Ig and B-cell receptor for Ig are independent sites. Paraskevas *et al.* (1972b) observed that antilymphocyte serum (ALS) at low concentrations *in vivo* or *in vitro* inhibited the binding of Fc (as detected by Fc inhibition of RICA). However, Brain and Marston (1973) did not find any effect of ALS on EA uptake by human B cells. Thunold *et al.* (1973) observed inhibition of EA uptake to lymphoid tissue sections by treatment with ALS (absorbed with thymocytes) but not with ALS absorbed by spleen cells or with anti-θ serum. Inhibition of AgAb binding by antisera to membrane components may not necessarily indicate identity or proximity of that component to the Fc receptor. A. Basten (personal

communication) has shown that inhibition with anti-H-2 sera can occur through shedding of the anti-H-2:H-2 antigen complex that then directly binds to the Fc receptor.

5. *Functional Role of the Ig Receptor*

The functional significance of the receptor for Ig is at present rather speculative. A series of possibilities have emerged.

a. Lymphocyte Activation. Clonal selection concepts hold that interaction of antigen with specific preformed antibody receptors on the cell surface trigger clonal proliferation. It would not be expected according to this view that triggering would occur as a result of antigen union with M-Ig that had been derived exogenously by the cell and bound to it through the Fc receptor. Basten *et al.* (1972a) tested this aspect in terms of immunological memory and showed that, whereas primed lymphocytes (TDL) and antigen gave secondary responses on transfer to irradiated recipients, unprimed TDL coated with AgAb complexes did not do so. Thus, immunological memory and perhaps by inference primary induction are not related to cytophilic binding of antibody. Eden *et al.* (1973b) suggested that, although the actual triggering stage would occur only in the specific precommitted lymphocytes, the initial recognition of antigen may be by circulating antibody (cf. Jerne, 1955), and the complexes are then nonspecifically brought into contact with many B lymphocytes, which act to concentrate the antigen. It has also been observed that antigen–antibody complexes can stimulate DNA synthesis in normal lymphocyte cultures (see Section III; Bloch-Shtacher *et al.*, 1968; Möller, 1969) although the significance of this *in vivo* is not clear.

b. Antigen Presentation. Since B lymphocytes are recirculating cells (Sprent and Miller, 1972; Howard, 1972) it is possible that they may effectively transport antigen to appropriate sites, particularly to lymphoid follicles. Basten *et al.* (1972a) found that normal lymphocytes coated with AgAb complexes and injected into recipients localized in marginal zones of the spleen, and, within 4 hours, the antigen was eluted into the surrounding white pulp. Brown *et al.* (1970) labeled mouse lymphocytes with aggregated human Ig and concluded that these cells could transport the Ig into the white pulp of the spleen where it becomes concentrated in germinal center areas. Further data showed that this activity was B-cell-associated as lymphocytes from thymus-depleted mice were also able to transport aggregated Ig (de Jesus *et al.*, 1972).

c. B-Cell Suppression. Various studies (reviewed by Diener and Feldmann, 1972) have shown that in distinction to antibody-mediated

peripheral suppression (Uhr and Möller, 1968), a central suppression of the immune competence of B cells may occur when cells are exposed to a mixture of antigen and antibody at low concentrations. *In vivo* antibody-mediated suppression has also been shown to depend on the Fc fragment (Chan and Sinclair, 1971) and as suggested by Basten *et al.* (1972a) it is possible that a sufficiently high epitope density may be achieved on the B cell by virtue of the receptor for Ig.

d. Antibody-Dependent Lymphocyte-Mediated Cytotoxicity. Lymphocyte-mediated cytotoxicity of target cells has been frequently studied as being a possible mechanism involved in allograft and tumor immunity systems (Perlmann *et al.*, 1972; Maclennan, 1973). It has become quite apparent that there is not just one mechanism for lymphocyte-mediated cytotoxicity, but at least two or three mechanisms have been recognized. In general, it appears that cytotoxic reactions to allogeneic target cells are mediated by T cells (Cerottini *et al.*, 1970; Golstein *et al.*, 1972a) and at least in some syngeneic tumor immunity situations, *in vitro* cytotoxic destruction of target cells and *in vivo* rejection of tumors can be mediated solely by T cells (Rouse *et al.*, 1973b; Rollinghoff and Wagner, 1973; Berke and Amos, 1973). It was also recognized that antibody-dependent target cell killing by nonsensitized lymphoid cells can occur (Perlmann and Perlmann, 1970; Maclennan *et al.*, 1970), and studies suggested that T cells were not involved in these reactions (Harding *et al.*, 1971). Van Boxel *et al.* (1972a) confirmed that lymphoid cells from populations depleted of T cells were active in these latter systems and showed that these effector cells probably carried surface Ig. Similarly, Lamon *et al.* (1972) showed that these effector lymphoid cells from nonsensitized animals could be removed by passage over an anti-Ig column (see Section III) again suggesting that they carried M-Ig. Thus, both of these studies suggest that the effector lymphoid cell is not a T cell but probably is a B cell. The presence of the Fc receptor on these cells therefore provides a reasonable explanation for the ability of these cells to bind to antibody-coated target cells and then mediate lysis. However, it must be noted that, although these data are consistent with the concept of B-cell involvement, it is also quite possible that another non-T, non-B lymphoid-type cell carrying receptors for Ig and some M-Ig is involved (A. C. Allison, cited in Perlmann *et al.*, 1972). Some evidence for this was given by Wisloff and Froland (1973) who showed that normal human lymphocytes depleted of B cells or lymphocytes from hypogammaglobulinemic patients lacking B cells were capable of giving antibody-dependent cell-mediated cytotoxicity. In view of studies on receptors for Ig on T cells (see Section II,C), it is not

excluded that T cells were active in this system, and clearly the main conclusion is that various cell types must be examined (with an appropriate series of markers to define the cell types) to determine their ability to induce antibody-dependent target cell lysis.

C. T-Cell Receptors

One of the most controversial questions in cellular immunology at present concerns the nature of the antigen recognition site on T lymphocytes, and this particularly concerns the question of the presence and significance of M-Ig on T cells. These aspects are discussed in more detail in Sections III and V; the present section deals specifically only with attempts to determine the uptake of exogenous Ig by T cells. As for the B-cell receptor for Ig, several methods have been used in these studies, basically involving antigen-complexed or aggregated Ig or free Ig.

1. Complexed Ig

The distribution of mouse lymphoid cells forming rosettes (RFC) with EA using rabbit antibodies was studied by Yoshida and Andersson (1972). Whereas 2–5% of normal thymus cells showed RFC, about 70% of activated T cells from spleen or lymph nodes gave rosettes. This rosette formation could not be significantly inhibited by free mouse Ig but could be inhibited by mouse AbAg complexes. It was thus concluded that antigen-activated mouse T cells had receptors for antigen-complexed Ig. However, similar studies by Cline *et al.* (1972b) also using activated mouse T cells but derived from the thoracic duct totally failed to detect rosette formation using EA complexes with mouse antibodies. This discrepancy might imply that the subpopulation of T cells with receptors for Ig does not recirculate; this possibility needs to be evaluated. However, it was also observed in the studies of Basten *et al.* (1972a), using the sensitive technique of radioautography, that ^{125}I-labeled AgAb complexes did not bind to T cells from several sources, including activated thoracic duct T cells. The level of 2 to 4% of cells with receptors in C57 Bl mouse thymus could well be due to immigrant B cells (Yoshida and Andersson, 1972). By using ^{125}I-labeled, aggregated, mouse IgG2b myeloma proteins, H. M. Grey (personal communication) has, however, found that about 90% of activated T cells derived from spleens of irradiated F_1 mice given parental thymus cells bound the Ig, as compared to binding of Ig by 20% of the normal thymus cells.

A recent observation by Bentwich *et al.* (1973) may also bear on the question of T-cell receptors for Ig. It was found that, whereas the up-

take of sheep erythrocytes is a marker of human T cells (Wybran and Fudenberg, 1971; Lay *et al.*, 1971; Froland, 1972; Jondal *et al.*, 1972; Wittingham and Mackay, 1973), an additional population of human PBL acquired the receptor for erythrocytes after neuraminadase treatment of the lymphocytes. These lymphocytes also possessed surface Ig and the ability to bind aggregated Ig. These cells may represent a B-cell subpopulation that has a hidden receptor for erythrocytes or a T-cell subpopulation that has receptors for human Ig.

In the studies of Paraskevas *et al.* (1972a), it was observed that Fc crystals formed around mouse lymphocytes including thymocytes. The specificity of this observation is not certain, although crystal formation did not occur around polymorphs. During primary immunization of mice to a range of antigens, it was observed by Paraskevas *et al.* (1972c) that 6 hours after stimulation there was a significant increase in cells with M-Ig, as detected by the RICA technique (see Section III). It was also concluded (Lee and Paraskevas, 1972) that the increase represented an acquisition of M-Ig by cells previously not bearing M-Ig. That these cells are T cells was indicated by the reduction of θ-bearing cells in the spleen by the same proportion as the increase of M-Ig cells. It was also found that normal spleen cells treated *in vitro* with serum taken 6 hours after immunization showed the same changes (Lee and Paraskevas, 1972; Orr and Paraskevas, 1973). If the spleen cells were first treated with anti-θ serum, they did not show this increase. About 5% of thymus cells were also found capable of binding the cytophilic Ig. Fractionation studies of the 6-hour serum indicated that the active Ig is 7 S IgG, possibly IgG2a. In view of the somewhat indirect methods used in these studies (which have also given data on surface Ig at variance with other reports; see Section III) the significance of the observations in terms of T-cell receptors for Ig is not certain at present.

2. *Free Ig*

If the hypothetical receptor for Ig on T cells bears any functional resemblance to B-cell receptors, it might be expected that the uptake of free Ig would be at best rather minimal, and this has been the general indication. Studies on RFC have been performed by Webb and Cooper (1973) with chicken cells that were first incubated with 7-day primary chicken antisheep erythrocyte serum and then mixed with erythrocytes. Because four washings of the cells before application of erythrocytes did not affect the results, it appears that this system studies a true cytophilic Ig. Using cell preparations presumed to be totally devoid of B cells (from anti-μ-treated bursectomized chickens),

some RFC were observed although these represented only 1% of PBL preparations or 0.5% of thymocytes. It was indicated that these cells were not phagocytic nor could they be removed on adherence columns, although, in view of the small proportion of the T-cell population involved, it can only be provisionally concluded that these RFC are T cells. Inhibition studies indicated that the cytophilic Ig involved is IgM. In view of the potential of this model of B-cell-deficient populations, it will be of particular interest to determine whether or not a significantly increased proportion of T cells might show the receptor, either if presented with an AgAb complex, or with activated T-cell preparations. In previous studies, Ivanyi (1970) had observed uptake by chicken lymphoid cells of soluble AgAb complexes formed early in immunization. These involved 7 S antibody, but the nature of the lymphoid cells was not characterized.

Cytophilic binding of anti-β-galactosidase antibody to thymocytes was also shown by Modabber and Coons (1972), using a cell population fluorometric technique. It was shown that mouse thymus cell suspensions bound about the same amount of antibody units per cell as mouse spleen and that the binding to thymus was inhibited by pretreatment of the cells with normal mouse serum. However, again a more direct visualization and use of specific anti-T markers will be required to prove conclusively that this binding is to T cells.

Using cell surface iodinated proteins, Cone *et al.* (1973a) found that as for B cells, T cells do not bind 7 S M-Ig derived from B cells. Goldschneider and Cogen (1973), using *in vitro* cultures of rat lymphocytes, did not observe significant binding of rat Ig to pokeweed mitogen-stimulated cultures that potentially also contained a large number of antibody-secreting B cells. The cultures, however, also contained human serum, and human Ig was found on the surface of nonstimulated, antigen-stimulated, and mitogen-stimulated rat T cells. Many activated mouse T cells (T-TDL) derived from the thoracic duct of hybrid mice injected about 5 days previously with parental thymus cells bear M-Ig (Bankhurst *et al.*, 1971; Pernis *et al.*, 1973; see Section III). Treatment of these cells with trypsin removed the Ig coat which did not reappear in 18-hour cultures (Pernis *et al.*, 1973). Similarly, when immunoglobulin determinants were removed from T (T-TDL) and B (nude mouse TDL) cells by capping with anti-Ig sera, subsequent overnight incubation of the cells resulted in reappearance of M-Ig on the B cells but not on the T cells (Hudson *et al.*, 1973, cited in Miller 1973b). It was also shown in this study that pretreatment of the thymus cell population through an anti-Ig column led to a considerable reduction in the number of M-Ig bearing T-TDL. It

was suggested (Miller 1973b) that these experiments indicated that the activated T cells bound anti-H-2 antibody produced by B cells in the original inoculum. This might possibly occur either through T cell Fc receptor binding of complexes, or by anti-H-2 antibody binding to host H-2 antigen that was bound to the T cell antigen receptor.

Although not directly demonstrating receptors on T cells for Ig, recent studies of Vuagnat *et al.* (1973) have suggested that a regulatory function of the Fc portion of IgG1 anticarrier antibody complexes with antigen may be expressed at the T-cell level. Whereas guinea pigs given passive IgG1 antihapten antibodies show delayed enhanced responses to haptens (on hapten–protein conjugates), IgG1 anticarrier antibodies resulted in sustained suppression of the active IgG1 antihapten response. This may represent direct effects of the IgG1–Ag complex on the T cell, mediated through a receptor for Ig on the T cell. Further direct studies will be required to verify this aspect. It might also be relevant to note in this context that T cells can also be rendered tolerant by AgAb mixtures (see Feldmann and Nossal, 1972).

3. *Receptors for Ig on T-Cell Tumors*

It is evident that one of the main problems in ascertaining specific properties of subpopulations of lymphoid cells is in obtaining homogeneous pure populations of these cells. Lymphoid tumor lines may represent such homogeneous populations although it must be recognized that malignant cell lines may not necessarily show the phenotypic expression of their normal counterparts. Three θ-positive mouse lymphomas have been examined for presence of M-Ig by Grey *et al.* (1972b) (see Section III) using quantitative measurements of the amount of M-Ig. Whereas all lines derived from *in vivo* passages showed M-Ig, one tissue culture line examined did not contain any detectable M-Ig. This suggested the possibility that all the positive M-Ig was cytophilically derived. Incubation of the tissue culture line cells with either normal mouse serum or aggregated IgG2 and subsequent washing showed the presence of as much M-Ig as was present on the *in vivo*-derived cells, indicating the presence of receptors on the cells for Ig. Harris *et al.* (1973) have examined tissue-cultured θ-positive murine lymphomas, using lines derived from thymomas and determining uptake of Ig was by rosette formation with EA. One cloned cultured tumor line was found to bear and synthesize M-Ig, and this was clearly not of cytophilic derivation. However, this line (WEHI-22) also gave RFC with EA using mouse IgG antibody but not with IgM antibody. Rosette formation was inhibited by free mouse myeloma proteins with IgG1 and IgG2b being consistently the most

efficient. Of several other T-cell lines examined in culture, one has shown no receptor activity and two (S49 and WEHI-112) have shown the presence of the receptor. From these two studies it is likely that further investigations of cultured T-cell lymphomas will be of value in determining and characterizing membrane receptors on subpopulations of T cells.

The existence of T-cell receptors for Ig is, thus, at present far from being proven. The few positive reports are countered by negative results using similar cell sources. Where receptors appear to have been found, there is no clear uniform picture of specificity for a particular Ig class or for free versus complexed Ig. In view of the clear evidence for the existence of receptors for Ig on virtually all B cells and macrophages, it is going to be essential to ensure that any demonstration of T-cell receptors prove the T cell itself (and not contaminating cells) is implicated. It is likely at present that, if receptors for Ig exist on T cells, only a subpopulation of T cells is involved, probably representing antigenically stimulated T cells. Whether the expression of such a receptor on activation need be of physiological significance is also debatable, as binding sites for various structures may appear when cell membranes are altered as a result of transformation. Insulin-binding sites, for example, have been shown to appear on the lymphocyte surface during mitogen-induced transformation (Krug *et al.*, 1972), although these, in turn, may be involved in controlling cell growth and division.

III. Membrane Immunoglobulins on Lymphoid Cells

If the receptors for antigen on lymphoid cell surfaces are Ig molecules of some type, then it would be expected that the presence of Ig on plasma membranes could be detected using specific anti-Ig reagents. Such has been the case in a wide range of studies using many different methods and animal species. The interpretation of these observations is, however, fraught with complexities. Do all lymphoid cell types carry M-Ig? Is the M-Ig, derived from the cell itself or from other sources, bound either through the Fc receptor or as a result of antibody activity to another cell membrane component? Even if M-Ig is detected, is it necessarily the antigen receptor? Although these are all rather interwoven questions, some separation of the problems will be made in the following discussion. This section will specifically consider studies aimed at demonstrating the presence of M-Ig of various Ig classes and in different species, separately considering normal, abnormal, and malignant, B, T, and plasma cells.

A. Methods for Detecting Membrane-Bound Immunoglobulin

As a wide range of methods have been used to demonstrate M-Ig, these will be briefly enumerated, without attempting in this section to consider all studies using each method nor the nature of the lymphoid cell types involved. At the essential core of all methods is the use of specific anti-Ig sera. Although Ig's in general have been characterized by functional, antigenic, physicochemical, and detailed sequence and structure studies, for all practical purposes, the definitive demonstration of M-Ig in rather limited amounts must make use of the specific antigenic properties of the Ig polypeptide chains. Accordingly, the validity of M-Ig detection is very much dependent on the specificity of the anti-Ig sera used. Several general points, which are all fairly obvious, nevertheless bear repeating in this context.

1. As sera from many species contains natural antibodies cross-reactive to cell membrane components of other species, it is essential to demonstrate that binding of an anti-Ig serum to a cell membrane is due to the anti-Ig antibody. This is best controlled by the parallel use of another sample of the same antiserum of which the specific anti-Ig in question has been removed on a solid phase immunoabsorbent of the purified Ig type under study. Competitive inhibition studies in the test system may not be valid, as soluble Ig–anti-Ig complexes could bind to the Fc receptors.

2. In view of the binding of aggregated Ig to the Fc receptors, as previously described, it is important in studies on the direct binding of anti-Ig antibodies to ensure that any binding detected is due to antibody site binding and not to the uptake of some aggregates in the antiserum. This is particularly relevant for fluorescent or radiolabeled preparations, and use of $F(ab')_2$ preparations of the Ig antibodies to the target Ig can eliminate this problem.

3. When attempts are made to study class or allotype specificity of M-Ig, it is essential to verify the specificity of the anti-Ig serum, by a method of equal or greater sensitivity than the test M-Ig method itself. Ideally, the specificity of the uptake of anti-Ig should be demonstrated in the test system.

4. Immunoglobulin molecules do not necessarily possess only one antigenic determinant that defines the class or allotype of the molecule, and before concluding that a particular cell type lacks detectable M-Ig, several different anti-Ig sera should be used, preferably detecting different regions of the Ig molecule. This is particularly relevant to the possibility that not all of the M-Ig molecule may be exposed on the cell surface.

TABLE I
METHODS OF DETECTING MEMBRANE-BOUND IMMUNOGLOBULIN ON LYMPHOID CELLS

A.	Direct cell-anti-Ig visualization:
	Fluorescent conjugation of anti-Ig (light)
	Radio-iodination of anti-Ig (light, E/M)
	Hybridization of anti-Ig, antivirus (E/M)
	Ferritin conjugation of anti-Ig (E/M)
	Enzyme conjugation of anti-Ig (light, E/M)
B.	Intermediary layers:
	Mixed anti-Ig (single or double)
	Reverse immunocytoadherence (direct or mixed)
C.	Activation of biological process:
	Lymphocyte transformation
	Cytotoxicity
	Opsonic adherence
D.	Physical methods:
	Electrophoresis
	Anti-Ig columns
E.	Cell population Ig quantitation and/or characterization:
	Hemagglutination inhibition
	Radioimmunoassays
	Cell surface iodination

1. Lymphocyte Stimulation

Addition of anti-Ig sera to cultures of lymphoid cells *in vitro* stimulates metabolic changes and cell proliferation, and this was interpreted to imply that the antisera are reacting to Ig determinants on or in the lymphocyte (Sell and Gell, 1965). Lymphocyte stimulation was usually determined by the transformation of cells to a blastlike morphology or, in a more quantitative manner, by the incorporation of tritiated thymidine. The original studies were performed with rabbit PBL and showed that either anti-L-chain allotype sera (Sell and Gell, 1965) or anti-class specific heterosera (Sell, 1967) could induce stimulation. Anti-H-chain allotype sera (*a* locus) also stimulate although are usually weaker than anti-*b* locus sera (Gell and Sell, 1965). Stimulation of human lymphocyte cultures by anti-Ig sera also occurs with various heteroantisera (Adinolfi *et al.*, 1967; Oppenheim *et al.*, 1969). In both human and rabbit cultures, stimulation appears to require multipoint binding or at least cross-linking on the membrane as Fab or Fab' fragments of the anti-Ig antibodies do not stimulate (Greaves *et al.*, 1969; Fanger *et al.*, 1970) although the bivalent $F(ab')_2$ fragments do stimulate (Fanger *et al.*, 1970). Stimulation in the latter system can occur with initial Fab' binding if a second het-

eroantibody to the Fab' fragment is then applied (Fanger *et al.*, 1970). Similarly, augmentation of antiallotype transformation has been described (Sell *et al.*, 1970b) by the use of a second antiallotype serum directed against allotypic determinants of the first anti-Ig. However, lymphocyte stimulation as a general method for detection of M-Ig suffers from the disadvantage that a response is measured at the cell population level rather than visualizing direct interactions at single cell levels. Accordingly, other components introduced into the reaction mixture may positively induce lymphocyte stimulation, or may interfere at some level with cell proliferation. In particular AgAb complexes have been reported (Bloch-Shtacher *et al.*, 1968; Möller, 1969) to stimulate lymphocyte proliferation, and it may be that aggregated Ig at suitable concentrations could also stimulate. Unsuspected antibody activities to other non-Ig antigens in the sera could potentially cause false reactions in terms of M-Ig interpretation, as for example, antibody to rabbit α-macroglobulin has also been described (Sell, 1970b) to stimulate rabbit PBL.

2. *Immunofluorescence*

The use of fluorescent (Fl)-labeled antibodies to detect surface antigens on viable lymphoid cells was first introduced by Möller in 1961 in studying the presence of isoantigens of the *H*-2 system at the cell membrane. Cell suspensions were treated with anti-*H*-2 sera and, after washing away free serum proteins, were reacted with Fl-labeled rabbit antimouse Ig. It was observed that lymph node and bone marrow cells were unique among tissues studied in showing a low frequency of "nonspecific" reactions when the anti-*H*-2 serum was omitted. It was interpreted by Möller (1961) to signify that mouse serum proteins, possibly γ-globulin, were present at the cell membrane. These observations have been amply confirmed and extended to many other species and this technique has been one of the most frequently used methods for studying M-Ig, particularly on B cells (see in the following). Raff *et al.* (1970) described the reaction of Fl-labeled rabbit antimouse Ig on mouse lymphoid cell suspensions and observed a plateau effect with increasing concentrations of reagent in terms of the proportion of cells labeled in a given suspension. This method is thus ideally suited for quantitating proportions of M-Ig-bearing cells provided that an optimal concentration of reagent is used. Möller (1961) observed that some of the cells showed a crescentlike staining reaction, and Raff (1970) clearly showed that direct staining of lymphoid cells with Fl-labeled anti-Ig resulted in a characteristic caplike staining on one pole of the cell. This aspect, found to

be temperature-dependent, has since been studied in far more detail as it relates to membrane mobility of the M-Ig, and is discussed in detail in Section V. Immunofluorescence can be used with different Fl-staining reagents to determine whether two different M-Ig are present on the same cell (e.g., Pernis *et al.*, 1970). Fluorescein and rhodamine conjugates of IgG fractions of antisera are usually prepared by the method of Cebra and Goldstein (1965) and can be used together with appropriate combination of filters to evaluate staining of the same cells with the two reagents. Recently, a fluorescence-activiated cell sorter has been used to separate cells bearing M-Ig of a particular allotype (Jones *et al.*, 1973b), and it is clearly shown that viable cells can be stained with Fl-labeled anti-Ig reagents, separated, and then used in *in vitro* or *in vivo* functional studies.

Two general limitations of immunofluorescence might be noted. Particularly when cell populations containing low proportions of M-Ig-bearing cells are studied, it is important to verify that staining is due to Fl-labeled anti-Ig–M-Ig union rather than to the uptake of fluorescent aggregates of Ig to the Fc receptor. Moreover, negative reactions with Fl-labeled anti-Ig cannot be construed to imply absence of M-Ig, but rather that, if M-Ig is present, it is so in relatively low amounts. As will be discussed later, the use of other methods has shown the presence of M-Ig on cell types that did not stain with Fl-labeled anti-Ig.

3. *Radioimmunolabeling of M-Ig*

Purified IgG fractions of antisera to Ig have been iodinated with ^{125}I and the uptake of these preparations to viable cell suspensions determined by either bulk counting of the cells or radioautography of cell smears, following incubation of cells and reagents and then extensive cell washing. Raff *et al.* (1970) compared the uptake of ^{125}I-labeled anti-Ig to mouse spleen cells with uptake of Fl-labeled-anti-Ig. In both cases, increasing the concentration of reactants gave increased labeling of cells until a plateau level of percent cells labeled was reached. Marked differences in sensitivity were observed—500 μg./ml. of Fl-labeled Ig was required to reach plateau level uptake compared to 0.05 μg./ml. of ^{125}I-labeled antibody. Jones *et al.* (1970) also compared uptake of Fl–anti-Ig with ^{125}I-labeled anti-Ig using rabbit antiallotype antibodies, and they also observed that radioautography was more sensitive in detecting M-Ig.

In general, radioautography is far more sensitive than immunofluorescence and this, in turn, poses an even greater need for control of specificity of the reaction. Bulk counting of cell suspensions labeled with ^{125}I anti-Ig reagents are often difficult to interpret as in-

tensive binding of Ig to damaged cell membranes frequently occurs and, accordingly, radioautographic counting of percent labeled cells is preferable. Although most studies use only a single concentration of labeled anti-Ig reagents, it must be verified that these reach plateau labeling (Jones *et al.*, 1971; Bankhurst and Warner, 1971), even though saturating conditions are not necessarily used. With some cell sources, such as bone marrow, it has been found that the kinetics of uptake do not always reach plateau conditions (Osmond and Nossal, 1973).

The radioautographic method is particularly suited to situations of low M-Ig amounts, and in an attempt to increase sensitivity further, a sandwich method of detection was studied (Nossal *et al.*, 1972). Rabbit antimouse Ig sera were bound to the surface of lymphocytes and then exposed to a heterologous ^{125}I-labeled antirabbit Ig reagent. It was found that little difference existed between the direct and sandwich methods when using radioautography, but for bulk scintillation counting the sandwich method was more specific.

Combined fluorescence and radioautography have also been used to detect the possible presence of different M-Ig specificities on the same cell (Davie *et al.*, 1971).

4. Lymphocyte Cytotoxicity

In studying the presence of various isoantigens on lymphoid cell surfaces, cytotoxic killing of cells by treatment with antibody and complement has been frequently used. Several studies have also shown that this can be applied to the detection of M-Ig on at least some lymphoid cells. Klein *et al.* (1967) showed that anti-μ or anti-κ antibodies and complement would kill a line of Burkitt lymphoma cells derived from either biopsy or a tissue cultured line. The lysis of some human PBL by rabbit antihuman IgG Fc was also demonstrated (Welsh *et al.*, 1971), although a necessary condition for lysis was incubation of the cells at 37°C. prior to addition of the antiserum. Lysis of some lymphoid cells from mouse tissues can also occur with anti-Ig sera (Kaplan and Batchelor, 1971; Takahashi *et al.*, 1971b), but it was noted (J. F. A. P. Miller *et al.*, 1972) that preincubation of the cells at 37°C. before incubation with sera greatly enhances lysis. Thus, a limiting condition of cytotoxic killing might be the amount of Ig present on the cell, in that insufficient Ig prevents suitable complement fixation (see below, J. F. A. P. Miller *et al.*, 1972) and too much Ig secretion, as in plasma cells may also hinder suitable binding and fixation (see IIIc). It is obvious that another limitation is the class of the

anti-Ig antibody—it must be of the type that can fix complement. A further essential control concerns the source of complement. Many animal sera contain natural lytic antibodies to other heteroantigens (e.g., rabbit and guinea pig sera for mouse), which must be completely removed from the sera used as complement source (J. F. A. P. Miller *et al.*, 1972).

5. *Mixed Antiglobulin*

The previous four methods all dealt with direct interactions of free anti-Ig molecules with lymphoid cell surfaces. Several other methods have been developed that involve a coupling of the anti-Ig to large, macroscopically visible particles such as red cells or bacteria. Immunocytoadhesion between sheep erythrocytes and lymphocytes was first developed for the demonstration of antibodies to the erythrocytes by cells from immunized animals (Nota *et al.*, 1964). This direct technique has been modified by Coombs *et al.* (1969, 1970) using the mixed antiglobulin technique (Coombs and Gell, 1968) to detect M-Ig on lymphocytes of several species. Accordingly lymphocytes are washed free of serum proteins and then treated with anti-Ig reagents of defined specificities. After further washing the cells are incubated with red cells that are coated with a subagglutinating dose of an antierythrocyte antibody containing the determinant recognized by the anti-Ig serum used to treat the lymphocytes. If the lymphocytes bind the anti-Ig, free anti-Ig groups should be exposed on the lymphocyte and could then bind to the Ig on the sensitized erythrocytes. This system has been used to detect H- and L-chain classes and allotypic determinants. There is a marked dose dependence between the concentration of the antiglobulin reagents used to treat the lymphocytes and the number of rosetting lymphocytes (Coombs *et al.*, 1970). This may well be a limitation of the method, as it depends on sufficient Ig sites being exposed after union with M-Ig. Furthermore, as will be discussed, this method has given results that conflict with results of other methods in terms of proportions of cells stained and allotype or class restrictions (e.g., Wolf *et al.*, 1971; Heller *et al.*, 1971). With some allotypic determinants on rabbit cells, it has been shown (An and Sell, 1973) that an increased detection of M-Ig-bearing cells occurs with the use of a double, indirect rosette formation (DIRF) system. In this method, two antiallotype molecules are interposed between the lymphocyte and red cell. Thus, b^4 lymphocytes are coated with b^5 anti-b^4 Ig and then reacted with a second antiallotype antibody directed to the first, i.e., b^6 anti-b^5. Rosettes are then formed by addition of sheep red cells sensitized with b^5 antibody.

6. *Reverse Immune Cytoadherence (RICA)*

The RICA test also depends upon the interaction of M-Ig with an anti-Ig molecule that then bridges to an erythrocyte, forming rosettes. However, in this method, rather than sensitizing erythrocytes with Ig (as in mixed antiglobulin), the erythrocytes are coated with an antigen, and the anti-Ig antibody used is hybridized with an antibody to the antigen coating the erythrocyte. Thus in the single RICA test (Paraskevas *et al.*, 1971a,b), a hybrid 5 S antibody is prepared, with one site to Ig and the other to the antigen used to sensitize the erythrocyte, and this antibody bridges the cells and forms rosettes. A modification of this test was then devised (mixed RICA) that permitted the detection of two different M-Ig types on the same cell. In this system (Lee *et al.*, 1971), one hybrid antibody had anti-Ig specific to mouse γ_{2a} chain and antibody to ferritin, and a second hybrid antibody to anti-γ_1 chain and to egg albumin. Two different indicator particles were used, e.g., sheep and chicken erythrocytes, one coated with ferritin and one with egg albumin. Lymphocytes were treated, in turn, with one hybrid antibody and its indicator and then with the other antibody and its indicator (sequential mixed RICA) or with both antibodies simultaneously (simultaneous mixed RICA). Somewhat different results were obtained between these two methods, and mixed RICA also produces results at variance with results of other methods in terms of Ig class expression (see later sections).

7. *Opsonic Adherence*

The reaction of antibodies with cell surface antigens can be detected using the ability of macrophages to bind the Fc of IgG when complexed to antigen (see Section II). This has been applied by Greaves (1970) to demonstrate an opsonizing effect of anti-Ig sera on lymphocytes. Mouse lymph node cells treated with anti-Fab serum adhere to the surface of macrophages and the percentage of adherence cells can be determined by comparing the mean number of nonopsonized cells from macrophage monolayers with the number of control lymphocytes (no macrophages). This test is particularly sensitive, although again it is essential to ensure that activity in the anti-Ig serum is due to the specific anti-Ig antibodies. Greaves (1970) showed that absorption with insoluble Ig removed opsonizing activity from the anti-Fab serum. The presence of complement in the reaction mixture does not affect the proportion of cells opsonized but does increase the opsonizing titer.

8. *Anti-Ig Columns*

In studies to be discussed later in this article, it was shown by Wigzell (1970) that antigens bound to Degalan bead columns can be used to deplete antigen-sensitive immunocompetent cells specifically. In a reciprocal approach, the presence of M-Ig on cells can be demonstrated by their ability to bind to columns coated with anti-Ig antibody. In studies with anti-Ig antisera directly bound to polyacrylamide beads, some selective removal of M-Ig-bearing lymphocytes was achieved (Sell and An, 1971) although the separation was not absolute. In studies with mouse spleen cells on anti-Ig plastic bead columns, a 90% depletion of M-Ig-bearing cells was obtained on single passage, and 99.8% depletion after serial passaging (Campbell and Grey, 1972). In column treatment of cells, a considerable degree of attachment may be nonspecific (Shortman *et al.*, 1971), and thus removal of certain adherent non-M-Ig-bearing cells can occur. A more efficient removal of M-Ig-bearing cells was developed by Wigzell *et al.* (1972) using a double-layer column. These authors found that coating of beads with just the antiserum or even the IgG fraction of the antiserum did not lead to a very efficient removal of M-Ig cells. However, if columns were coated with M-Ig and then an excess of antibody to the M-Ig, selective binding of the anti-Ig occurred, and this column efficiently removed cells bearing M-Ig of the type coated on the column. This method is rather similar to the use of AgAb complex columns described by Basten *et al.* (1972c) who claimed removal of B cells by virtue of binding of complexes to the Fc receptor. Some controversy on this point exists, as Wigzell *et al.* (1972) could not confirm this using bovine serum albumin (BSA)–anti-BSA columns, and they suggested that removal of B cells by Basten *et al.* may have been due to antihuman Ig antibodies (used in the complex) cross-reacting with mouse Ig.

9. *Enzyme-Labeled Antibodies*

Quantitative determinations of the amount of M-Ig on lymphoid cells have been made by the binding of horseradish peroxidase-labeled antibody (Avrameus and Guilbert, 1971). Washed lymphocytes are incubated with varying amounts of peroxidase-labeled antibody and incubated for various periods of time. Samples of the washed cells are then quantitated for amount of peroxidase using standard chemical procedures. When the degree of peroxidase conjugation to antibody is known, the amount of antibody bound to the cell

can then be calculated. With increasing concentrations of antibody, a plateau is reached where no further antibody can be fixed.

The binding of enzyme-labeled antibodies can also be determined visually by interacting lymphocytes with labeled antibodies, revealing peroxidase by standard cytochemical techniques, and then preparing suitable light or electron-microscopic sections (Gonatas *et al.*, 1972). In this manner the proportion of M-Ig-bearing cells can be determined.

10. Ultrastructural Examination of M-Ig

The binding of Fl-, isotope-, and enzyme-labeled anti-Ig antibodies to lymphocytes has been detected by suitable processing of the treated cells for light microscopy. Similarly, antibodies can be tagged with various markers that can be visualized in the electron microscope (E/M) provided that suitable fixation of the cells can be achieved without loss of the label. Jones *et al.* (1970) showed the detection of M-Ig on rabbit cells with ^{125}I-labeled antiallotype antibody followed by processing for radioautographic electron microscopy. Santer *et al.* (1972) described more quantitative studies on the uptake of isotope-labeled rabbit antimouse class-specific antibodies to various sources of mouse lymphoid cells. Just as for light-microscopic radioautography, studies with the E/M can use either direct labeled antibodies or indirect sandwich labeling (Santer *et al.*, 1972).

Indirect ferritin-labeled antibody methods have also been used for demonstrating M-Ig on either human (Biberfeld *et al.*, 1971) or rabbit lymphocytes (An *et al.*, 1972). In the rabbit system, b^4 lymphocytes are labeled with excess of an anti-b^4 serum, followed by the addition of b^4 antiferritin–ferritin-soluble complexes and processing the washed cells for E/M. As mentioned before, the use of soluble complexes, however, always introduces the possibility of AgAb binding to Fc receptors rather than to true M-Ig. In studies with human lymphocytes, Biberfeld *et al.* (1971) used an indirect method of cell treatment with rabbit antihuman Ig followed by ferritin-conjugated sheep antirabbit Ig. In considering several E/M methods, Gonatas *et al.* (1972) suggest that the use of peroxidase-conjugated antibodies followed by E/M has several advantages over other methods, in that radioautographic electron microscopy has a limited resolution, ferritin methods are relatively less sensitive, and the ferritin conjugate is too large to penetrate the cell surface coat.

Another approach to E/M studies of surface antigen is the use of hybrid antibodies of which one specificity is to Ig and the other to an E/M detectable marker particle such as virus. Hammerling and Ra-

jewsky (1971) used hybrid antibodies to mouse Ig and southern bean mosaic virus. Cell suspensions are incubated in the hybrid antibody, washed, and resuspended in viral preparations and then washed and prepared for E/M examination. In this system $F(ab')_2$ fragments are used for hybrid antibody preparation, thus minimizing nonspecific uptake of hybrid antibody–virus complexes. Specificity controls also include specific absorptions of the hybrid antibody with Ig fragments.

Electron-microscopic studies can therefore be used both to quantitate proportions of cells bearing M-Ig (although this is a more tedious method than, e.g., Fl–antibody binding) and particularly for detailed ultrastructure studies of the location and movement of M-Ig.

11. Electrophoretic Mobility of Cells

Cells placed in an electric field will move according to the net surface charge. It was shown by Bert *et al.* (1969) that pretreatment of human PBL with a polyvalent antiserum caused a reduction in the net negative surface charge of the cells. Antisera to individual H-chain classes did not, however, show this effect. Although the results are consistent with the view that at least some of the PBL have M-Ig, the method does not seem to be applicable to determining proportions of cells with particular types of M-Ig.

12. Hemagglutination Inhibition

In hemagglutination inhibition systems, specific antisera are quantitatively absorbed with varying amounts of test and standard inhibitors, and the residual capacity of the sera to agglutinate antigen-coated red cells is determined. This was applied by Klein *et al.* (1970) to quantitate amounts of IgM on several human lymphoid cell types. The hemagglutinating system involves IgM(K)-coated erythrocytes and specific anti-μ or -κ sera; absorption is then performed with varying number of cells and is compared to inhibition by reference purified IgM. The residual anti-κ (or -μ) activity is then determined—with the usual limitations of detecting hemagglutination end points.

By comparing the degrees of inhibition, the amount of IgM per number of lymphoid cells can be determined. Two main limitations affect this assay. First, the calculated amount of Ig per cell is a mean value and cannot detect heterogeneity within the population (although this is less of a problem for tumor lines as studied by Klein); and, second, the calculation of amounts of Ig on cells assumes the molecule is as effective an inhibitor in the free state as when it is cell-membrane bound. Because the latter is probably not true, as discussed by Klein *et al.* (1970), therefore several assumptions must be made in

the calculations. The method is, however, quite sensitive and is relatively free of nonspecific uptake of the anti-Ig, as this is presented to the cell as free Ig, not as a complex. If bivalent $F(ab')_2$ antibodies were used, the possibility of cytophilic removal of any anti-Ig would be completely removed.

Cooper *et al.* (1973) further developed this approach by automated (Technicon autoanalyzer) hemagglutination assays. The method is basically as outlined in the foregoing in that viable cell suspensions are incubated with a dilution of specific anti-Ig sera, and the residual activity in the serum is then determined by hemagglutination with appropriately Ig-coated red cells. The method can detect amounts of Ig in the nanogram range. Again, the main limitation is that an *average* value of nanograms Ig per given number of cells is obtained and does not reflect possible homogeneity.

With this limitation in mind, the method is likely to be of considerable value in quantitating M-Ig expression on cells, particularly if, as a check for possible gross heterogeneity, it is combined with one of the direct methods for visualizing the M-Ig in the cell population.

13. Radioimmunoassays

In any particular system requiring quantitation of a biological substance, radioimmunoassays, if they can be developed for the given substance, are among the most sensitive and accurate methods. In the context of detecting M-Ig, these methods are essentially similar to hemagglutination inhibition, except that isotope-labeled Ig is precipitated by the specific anti-Ig rather than using red cell agglutination. In all variants of this method, as for hemagglutination inhibition, the cell population as a whole is being studied and mean values for the population only can be obtained. One of the problems in this method is to ensure that only cell membrane Ig is detected and not also intracytoplasmic Ig. Smith *et al.* (1970) used a modified Farr assay (ammonium sulfate precipitation) to detect cell-associated Ig on human PBL. Lymphocyte preparations were tested for their ability to block the binding of a standard anti-$F(ab')_2$ serum to ^{125}I-labeled Fab; the complex was precipitated by ammonium sulfate (Cerottini, 1968). Comparisons were made with the ability of purified Ig to inhibit the reaction. A similar system was described by Lerner *et al.* (1971) where intact cells were used to inhibit the capacity of rabbit anti-IgG to bind to labeled L chains or Fc fragments. Quantitation of as little as 10^{-8} gm. of Ig chains is possible with this method. By using cloned lines of human lymphocytes, Lerner *et al.* (1971) showed that the M-Ig detected was probably not due to adsorption of secreted Ig to plasma

membranes, as two cell lines with different M-Ig phenotypes (e.g., K+Fc+, K+Fc−) when suspended in medium harvested from the other line, maintained their own phenotypes. (These types of studies with Ig classes do not, however, eliminate the possibility that the phenotype depends in part on the ability of a given cell to bind exogenous Ig in a manner that does or does not permit the exposure of all or part of the Ig molecule.) Allotypic studies will be required to eliminate completely or confirm the passive adsorption question when such sensitive techniques are used. In these studies L/H chain ratios of M-Ig can be determined, and the significance of these studies will be considered in later sections.

Inhibition of labeled mouse IgG2 precipitation by specific rabbit anti γ_2, using mouse lymphoid cells, has been described by Rabellino *et al.* (1971). In this system, ammonium sulfate precipitation cannot be used, and the complexes are precipitated by heteroantibodies to rabbit IgG. Viable cell suspensions are similarly tested for their ability to remove anti-Ig antibody by using standard amounts of antisera and comparing to inhibition by purified Ig. The same limitations as for hemagglutination assays apply, and it is to be stressed that these assays measure the *average* amount of cell-bound Ig that is *available* for anti-Ig binding.

14. Radiolabeling of Cell Surface Proteins

If M-Ig exist on lymphoid cells, it should be feasible to purify cell plasma membranes and in some chemical manner release and then detect the Ig. This has only been followed to a limited extent; Merler and Janeway (1968) showed that Ig-like fragments were released from human tonsillar small lymphocytes in suspension by mercaptoethanol treatment. An alternative approach, which has recently received much attention, is to label cell surface proteins directly on living cells with ^{125}I, then to disrupt cells, and to characterize immunologically or physicochemically the labeled protein. This method is based on the ability of lactoperoxidase to catalyze the covalent binding of radioactive iodide to accessible tyrosines on the surface of living lymphocytes (Marchalonis *et al.*, 1971; Baur *et al.*, 1971), and E/M studies demonstrate that radioactive iodine is incorporated solely into cell surface components (Marchalonis *et al.*, 1971). Many of the problems that have been raised with this method concern either the ability to release the labeled Ig from the cell membrane, usually using metabolic release (Cone *et al.*, 1971), urea–acetic acid dissociation (Marchalonis *et al.*, 1971), or lysis with nonionic detergents (Baur *et al.*, 1971), or the quantitation of the labeled Ig, usually specific im-

munological coprecipitation (Marchalonis *et al.*, 1972a) or "sandwich" techniques of precipitation (Baur *et al.*, 1971). General details of these methods have been recently reviewed (Marchalonis and Cone, 1973; Uhr and Vitetta, 1973), and specific aspects of the controversial results are discussed in Section III,D.

B. B Lymphocytes

The expression of M-Ig has been studied in a variety of animal species and in man, with particular emphasis on the possible selective expression of different classes and allotypes, using many of the methods described in the foregoing and employing lymphoid cells derived from animals at varying stages of immunization. One may wonder whether or not a consistent pattern of Ig expression emerges across species regardless of methods used. In the main, this is the case, with the general conclusion that IgM is the predominant class expressed on the cell membrane of immunocompetent cells and that lymphocytes express predominantly only one class and one allotype. In this section these aspects will be considered in some detail for several of the species most intensively studied, giving consideration to the evidence that B cells are the principal cell type that expresses high-density M-Ig. Aspects of M-Ig expression following antigen activation is dealt with in Section IV.

1. Human

a. Do Human B Cells Have M-Ig? Well over fifty independent studies have demonstrated that some, but not all, human lymphocytes bear readily detected M-Ig. The characterization of these positive cells as being analogous to the recognized B-cell series in animals is dependent on the development of non-Ig markers for human B- and T-cell series. Several of these markers are now available.

However, the use of these markers to identify M-Ig bearing cells as B cells is a somewhat circular argument, as the definition of some of the markers partly assumes that M-Ig cells are B cells. A more strict definition of cell type depends on the use of cell populations of defined thymic or bursal equivalent derivation. The closest to this situation in man is achieved in certain immunodeficiency disease states, and these are discussed in detail in Section III,F. It has been shown that the *in vitro* proliferative response to anti-Ig sera appears to be a property of a cell population independent to that stimulated by phytohemagglutinin (PHA) (Daguillard *et al.*, 1969) and that, in many studies (see Section III,F), Bruton-type agammaglobulinemic patients completely lack M-Ig-bearing B cells. In reciprocal fashion, patients

with Di George syndrome, deficient in T-cell function, have been shown to possess greatly elevated numbers of M-Ig-bearing cells in peripheral blood (Gatti *et al.*, 1971), but, after thymic transplantation, the proportion of non-M-Ig-bearing cells in blood increases (Gajl-Peczalska *et al.*, 1973a). Although the nature of a bursal equivalent in man is not resolved, it has been shown that bone marrow in man (Klein *et al.*, 1970; Abdou and Abdou, 1973) contains a population of cells particularly bearing IgM-type M-Ig, and the authors equated this finding with similar studies on the origin of B cells in animals. These studies, when compared to animal experiments in which more formal proof of B- and T-cell origin can be obtained, are consistent with the thesis that cells bearing readily detectable M-Ig in man are of the nonthymic-derived B-cell series.

Studies with available independent markers have further confirmed these views. In man, T-cells have been identified by their ability to form rosettes with nonsensitized sheep erythrocytes (Wybran and Fudenberg, 1971; Lay *et al.* 1971; Jondal *et al.*, 1972; Froland, 1972; Wittingham and Mackay, 1973; Bentwich *et al.*, 1973) and by their reaction with specific antisera prepared by immunization of rabbits with either fetal human thymus (Williams *et al.*, 1973; Aisenberg *et al.*, 1973) or PBL from Bruton agammaglobulinemia (Aiuti and Wigzell, 1973a) followed by absorption with cells from chronic lymphocytic leukemia patients; B cells have been identified by receptors for Ig and for complement (see Section II). The use of these markers has clearly shown that cells bearing readily demonstrable M-Ig (usually by immunofluorescence) do not carry T-cell markers but frequently carry B-cell markers. Thus, in combined sheep rosette tests with M-Ig detection, the two populations were totally independent (Papamichael *et al.*, 1972; Ross *et al.*, 1973). The relative distribution in 35 normal subjects of M-Ig-bearing cells and cells reacting with specific anti-T serum in PBL was described by Williams *et al.* (1973) and again indicated that the two populations were separate, the sum of the two ranging from 70 to 119% (average 98.6%). Removal of cells binding to anti-Ig-coated columns led to a specific enrichment of cells reacting with specific anti-T serum (Aiuti and Wigzell, 1973b). Studies of the association of M-Ig and binding to complement (CRL) indicated an overlapping but not identical population (Ross *et al.*, 1973); whereas 24% of PBL cells had M-Ig, only half of these were CRL. In spleen and thoracic duct, most M-Ig cells were also CRL, but in spleen 10–20% of CRL did not have detectable M-Ig. These marker studies are, therefore, completely consistent with the view that readily detectable or high-density M-Ig is a property of B cells,

but that some heterogeneity within the B-cell population may exist. This aspect will be referred to in discussion of M-Ig on plasma cells (i.e., activated B cells).

b. Lymphoid Tissue Distribution of M-Ig-Bearing Cells. Many groups have determined the proportion of M-Ig-bearing cells in normal adult peripheral blood, and a wide range of values have been found. These are summarized in Table II, and show an overall average of about 20%, which compares favorably to the reciprocal value of around 70 to 80% of human T cells. Some of the discrepancies in the values between reports may be due to methodological differences. In particular, the mixed antiglobulin technique clearly

TABLE II
PERCENTAGE OF MEMBRANE-BOUND IMMUNOGLOBULIN-BEARING CELLS IN HUMAN PERIPHERAL BLOOD

% M-Ig-positive cells (mean)	Method[a]	Reference
5	Mixed antiglobulin	Coombs *et al.* (1969)
5	Mixed antiglobulin	Heller *et al.* (1971)
8	Mixed antiglobulin	Litwin (1972)
11	Fl-anti-F(ab')$_2$	Froland and Natvig (1972a)
14	Fl-polyvalent	Aisenberg and Bloch (1972)
15	Fl-polyvalent	Pernis *et al.* (1971)
16	Fl-sum $\kappa + \lambda$	Preud'homme and Seligmann (1972a)
16	Fl-anti-Fab	Van Boxel *et al.* (1972b)
17	Fl-sum $\kappa + \lambda$	Piessens *et al.* (1973)
22	Fl-polyvalent	Siegal *et al.* (1971)
23	Fl-sum heavy chain	Williams *et al.* (1973)
24	Fl-polyvalent	Ross *et al.* (1973)
28	Fl-sum $\kappa + \lambda$	Grey *et al.* (1971)
29	Fl-polyvalent	Papamichael *et al.* (1971)
30	Fl-sum heavy chain	Gajl-Peczalska *et al.* (1973b)
30	Cytotoxicity	Wernet *et al.* (1972)
32	Fl-sum heavy chain	Cooper *et al.* (1971b)
34	^{125}I-AR polyvalent	Wilson and Nossal (1971)
	20 = Average of all tests	
% T cells		
53	Cytotoxicity	Aiuti and Wigzell (1973a)
75	Cytotoxicity	Williams *et al.* (1973)
87	Cytotoxicity	Aisenberg *et al.* (1973)
80	Sheep erythrocyte rosettes	Wybran *et al.* (1972)

[a] Fl, fluorescent label; AR, radioautography.

TABLE III
PROPORTION OF B CELLS IN HUMAN LYMPHOID TISSUE

Tissue	% B cells	Method[a]	Reference
Spleen	27	Fl-anti-Ig	Ross *et al.* (1973)
Spleen	37	CRL	Ross *et al.* (1973)
Lymph node	23	Fl-anti-Ig	Verma *et al.* (1971)
Lymph node	31	CRL	Pincus *et al.* (1972)
Thoracic duct	18	Fl-anti-Ig	Ross *et al.* (1973)
Thoracic duct	24	CRL	Ross *et al.* (1973)
Bone marrow	6	Fl-anti-Ig	Klein *et al.* (1970)
Bone marrow		Fl-anti-Ig	Hijmans *et al.* (1971)
Tonsil	60	CRL	Pincus *et al.* (1972)
Thymus	1–2	CRL or Fl-anti-Ig	Ross *et al.* (1973)

[a] Fl, fluorescent label; CRL, complement receptor lymphocyte.

seems to underestimate the total number, possibly due to fixation of both anti-Ig specificities of the bivalent molecule to the lymphoid cell and not leaving sufficient binding activity available for coated red cells. It is possible that excessively high values could be due to the detection of some non-B cells that bear sufficient amounts of M-Ig to permit their detection, such as monocytes or T cells (see later section), particularly when sensitive techniques such as radioautography are used. Variations due to population or environmental differences may also exist. However, in general, the average range of 15 to 30% would encompass most observations. Only limited data are available on the proportions of B cells in normal human lymphoid tissues, and these are listed in Table III. It is of interest to note that the proportion of CRL in tissues is as high as or higher than M-Ig-bearing cells, although it is lower in blood. Whether this is due to detection of nonlymphoid cells, such as monocytes, needs to be resolved. The proportion of B cells in all tissues is roughly comparable to murine studies although somewhat different from rabbit (see below).

c. Anti-Ig Stimulation of Lymphocytes. In 1967, Adinolfi *et al.* showed that about 6% blast cells developed in human PBL cultures stimulated by anti-γ chain but not by anti-μ chain. Stimulation of human lymphocytes has also been achieved with anti-L-chain antibodies (Greaves *et al.*, 1969) and anti-F(ab')$_2$ antibodies (Froland and Natvig, 1970). In studies with a range of class-specific sera produced in monkeys, Oppenheim *et al.* (1969) also found around 2 to 8% blast-transformed cells using either anti-γ- or anti-μ-chain sera, but considerably less with anti α,κ or λ. It was observed that leukocytes washed free of serum Ig responded to a greater degree to the

anti-Ig stimulation, and this may be analogous to the need of preincubation of lymphocytes before anti-Ig-induced cytotoxicity tests (Welsh *et al.*, 1971). It may be that effective stimulation or cytotoxicity is dependent on firm binding of the anti-Ig to the cell membrane and not to loosely adhered (? cytophilically adsorbed) M-Ig. In most of these stimulation studies, the degree of tritiated thymidine incorporation is significantly less than that achieved by T-cell mitogens, consistent with the lower proportion of B cells in blood.

Lymphocyte stimulation has also been induced by the addition of 19 S anti-γ-globulin rheumatoid factors (RF) (King *et al.*, 1969). The stimulation is variable and less than that induced by rabbit anti-γ-globulin. Rheumatoid factors can also cause stimulation of human lymphocytes (Mellbye and Williams, 1972) that have been coated with substimulating doses of antilymphocyte antibody, although in this latter system the RF may only be acting as a cross-linking agent. As RF often have very sharply defined specificities to Ig antigens, stimulation by these agents may be useful in defining the conformational arrangements of Ig surface membranes.

d. Lymphocyte Cytotoxicity. Anti-Ig-induced cytotoxicity of lymphocytes has been particularly effective in mice, although it has not been exploited to a very great extent in man. Klein *et al.* (1967, 1968) clearly showed that Burkitt lymphoma cells bearing IgM could be killed by anti-μ or -κ and complement. Kaplan and Batchelor (1971), however, could not detect any lysis of human PBL with anti-IgG sera. Welsh *et al.* (1971) then showed that prior incubation of the cells at 37°C was necessary before addition of antiserum and C′. Both rabbit anti-human IgG Fc and IgG serum gave lysis, and absorption studies indicated anti-IgG activity was due to anti-Fc. Anti-Fab had minimal activity, and it was suggested that the orientation of IgG determinants on the cell surface is such that Fab is relatively inaccessible. This does not agree with most studies using fluorescent or radioautographic observation, and in mice anti-L-chain antibodies effectively lyse cells (J. F. A. P. Miller *et al.*, 1972). Wernet *et al.* (1972) also showed that an antiserum with anti-μ specificity would kill about 10% of cells and that an antiserum against idiotypic antigens (presumably on Fab) was efficient in lysing a high proportion of the Ig-bearing cells of patients producing the monoclonal IgM protein of the idiotype.

e. Ontogenic Development. In fetuses, IgM and IgG synthesis has been detected as early as the twentieth week of gestation (Van Furth *et al.*, 1965). The appearance of M-Ig bearing B cells, however, appears to occur well before this time. At 4 months of gestation, Klein *et al.* (1970) found a few M-Ig cells in thymus (up to 3%) fetal liver (approximately 3%) and in bone marrow (~ 2%). Lawton *et al.* (1972a)

have observed that M-Ig cells can be found by the ninth week of embryonic life, and that by 14 weeks human embryos have adult proportions of lymphocytes bearing membrane-bound IgM, IgG, and IgA in their spleen and blood; they concluded that this is consistent with the concept of Ig class differentiation prior to antigen-induced differentiation (see Section IV). Froland and Natvig (1972b) also observed a similar relative distribution of Ig classes in lymphocytes of the newborn, although the mean percentage value of M-Ig-bearing cells was claimed to be somewhat higher than of adults.

An interesting situation has arisen in regard to the ontogenic expression of IgD. In normal adult serum, IgD is present only at low levels and is rarely even detectable in cord plasma (Rowe *et al.*, 1968). However, in cord blood lymphocytes, 14.5% of cells have IgD-type M-Ig, whereas only 8.5% have IgM, 2.9% has IgG, and 0.8% has IgA. Thus the IgD cells constitute at least 50% of the M-Ig-bearing cells (Rowe *et al.*, 1973). This contrasts to the adult situation where about 3% of PBL carry IgD-type M-Ig (Van Boxel *et al.*, 1972b; Aisenberg and Bloch, 1972; Piessens *et al.*, 1973; Rowe *et al.*, 1973), which still, however represents a disproportionately high number of cells as compared to adult serum levels of IgD. It has not been established whether the newborn lymphocytes that carry IgD are a separate population or carry other Ig classes. However, it is relevant to note that Froland and Natvig (1972b) found only 14.3% (average) of cord blood lymphocytes to react with anti-$F(ab')_2$ which is the same value as found by Rowe *et al.* (1973) for IgD. This may suggest that all lymphocytes from newborns bear IgD and another Ig class.

f. Immunoglobulin Class Distribution of M-Ig. Using specific anti-H-chain sera, various groups have studied the proportions of IgM-, IgA-, and IgG-bearing cells in peripheral blood. The results are summarized in Table IV showing the percent of cells in a given class as a percentage of the sum of the individual H-chain values. Only limited information is available on the percent of IgD- and IgE-bearing cells, but, as noted in the preceding section, the values for IgD do not reflect serum levels and this is also the case for IgE even when considering the lower of the three values. This might be interpreted to imply that the intracellular activation of the different H-chain genes in the immunocompetent cells is of a more equal nature than would be anticipated from serum studies and that the low serum values of IgD and IgE reflect a lesser activation or selection by antigen of the cells expressing these H-chain genes. This approach is consistent with the view of Cooper *et al.* (1971a, 1972a) that different H-chain expression does not depend on antigen stimulation.

The results for μ, γ, and α chains vary among the different groups,

TABLE IV
PROPORTION OF MEMBRANE-BOUND IMMUNOGLOBULIN CELLS WITH DIFFERENT IMMUNOGLOBULIN CLASSES

% M-Ig cells with indicated H chain[a]					
μ	γ	α	δ	ϵ	References
25	54	21	–	–	Papamichael *et al.* (1971)
26	53	21	–	–	Grey *et al.* (1971)
27	27	46	–	–	Hijmans and Schuit (1972)
28	52	19	–	–	Lawton *et al.* (1972b)
30	30	23	16	3	Piessens *et al.* (1973)
30	49	17	–	4	Gajl-Peczalska *et al.* (1973b)
33	53	14	–	–	Lindstrom *et al.* (1973)
45	18	9	14	14	Aisenberg and Bloch (1972)
45	14	3	–	–	Siegal *et al.* (1971)
50	42	8	–	–	Froland and Natvig (1972a)
68	22	10	–	–	Preud'homme and Seligmann (1972a)
74	25	11	–	–	Pernis *et al.* (1971)

[a] All values have been calculated to the nearest whole number and are proportions of the total number of M-Ig-positive cells based on the published values for total M-Ig-bearing cells using polyvalent reagents, or where these are not available, using the sum of the individual H-chain values.

particularly in terms of the relative numbers of IgM- and IgG-bearing cells (with only one exception the IgA-bearing cells are around 10 to 20% of the M-Ig cells). The results for μ and γ are expressed as a ratio in Fig. 3. With this mode of presentation, it appears that the results fall into two distinct groups: there are either fewer M than G cells (μ/γ ratio averages 0.75 for group I) or considerably more M than G cells (μ/γ ratio averages 2.8 for group II). These results do not simply reflect a normal range of distribution, and 35 individual μ/γ ratios from the data of Williams *et al.* (1973) are shown for comparison in Fig. 3. Furthermore, the average ratio of κ/λ from several studies is 1.9 (see Fig. 3) and, with the limited data available, does not show any clear trend into separate groups. [A value of 11 for the κ/λ ratio has, however, been found by Pernis *et al.*, (1971) but was not included in the calculation as this does not agree with any other published values.]

The existence of two separate μ/γ ratio groups implies that either the results reflect unknown influences that can drastically change IgM/IgG relative expressions in different groups of normal humans or that methodological procedures vary and affect the result. The latter situation would occur if either too few or too many M or G cells were being estimated. In considering the four possibilities, ex-

cessive G or M values could be due to cytophilic binding of Ig, and, in view of previous considerations (see Section II), this would be expected for IgG rather than for IgM. However, if this were the case, then many cells would bear γ chain as well as other classes (assuming only B cells bound Ig *in vivo*). This would be reflected in a low μ/γ ratio, and also in a ratio considerably lower than unity when the total

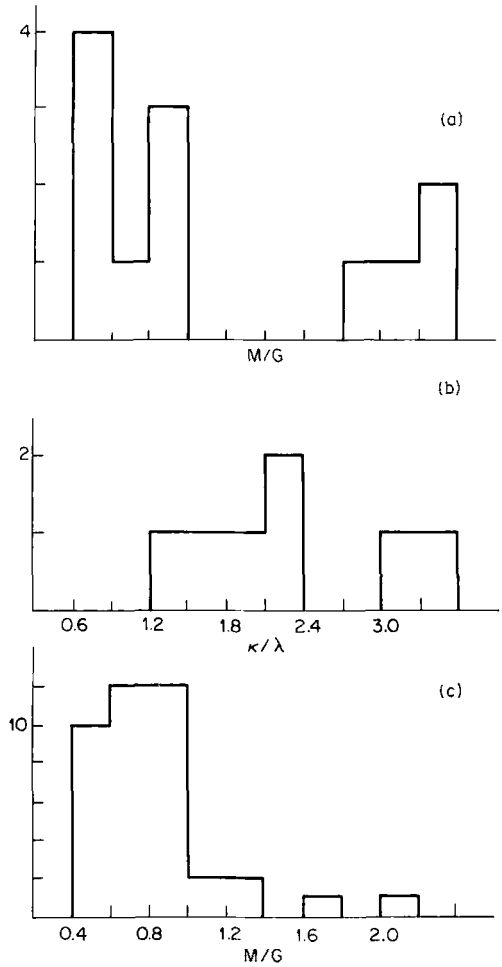


FIG. 3. Expression of IgM and IgG in human peripheral blood lymphocytes shown as ratio of M/G-bearing cells. The values are shown as a histogram for all available published studies, showing (a) M/G, (b) κ/λ, and (c) M/G ratios for 35 individual cases in one study. (Williams *et al.*, 1973.)

number of cells bearing M-Ig, as determined with polyvalent antisera, was compared to the sum of the cells bearing individual H chains. This does not seem to be the case; for example, Grey *et al.* (1971) found about twice as many G as M cells but did not find any evidence for cells bearing multiple Ig classes. The available data thus do not suggest that overestimation of γ or μ is made by studies of either group I or II (Fig. 3). Underestimation is, however, more likely, particularly when the exposure of the H chain on the cell surface is incomplete. As different anti-H-chain sera can react to determinants on different parts of the H chain the following thesis might be considered. If, for example, IgG on some B cells permitted exposure of the entire H chain, but on other cells the C_{H3} region was buried, the latter cells would only be detected if the anti γ-chain serum used had specificities for either the C_{H1} or C_{H2} region, whereas the former cell type could be detected with a serum specific for determinants only on the C_{H3} region. Froland and Natvig (1972c) have indeed shown that antisera reacting to determinants corresponding to the C_{H3} region (GmA, "non-A," and pFc') react with only very few or usually none of the B cells known to bear IgG-type M-Ig. Similar arguments could be made for IgM, particularly considering "hinge region" determinants (cf. Hogg and Greaves, 1972). In view of the available data on M-Ig classes in animals and the work of Hogg and Greaves (1972) concerning murine IgM-type M-Ig, it might be proposed that the discrepancy between group I and II in Fig. 3, is due to underestimation of μ chain by some groups possibly due, in turn, to the specificities of the antisera used. This implies that group II values may represent the true normal situation. However in view of the observations of Froland and Natvig (1972c), underestimation of G is equally possible and it is quite apparent that further studies are needed in this area, particularly to investigate the other major alternative, that unsuspected factors (environmental, subclinical infections, etc.) can alter M/G relative expression.

The possible existence of multiple H chains on B cells is discussed in Section III,B. It may be noted here, however, that, in humans, in the majority of studies comparing total number of M-Ig-bearing cells to the sum of individual H-chain values, evidence for a major proportion of cells bearing multiple classes has not been found, although it is evident that *some* cells may express more than one class.

The expression of IgG subclasses on B cells has been studied by Froland *et al.* (1971) and Froland and Natvig (1972c) using hetero subclass-specific reagents. Of the two to four normal PBL preparations studied with the four reagents, the following distribution of the

subclass determinants expressed as average percentages of the IgG-bearing cells was found: $\gamma_1 - 18\%$; $\gamma_2 - 54\%$; $\gamma_3 - 14\%$; and $\gamma_4 - 14\%$. The expression of IgG2 on the cell surface thus appears to be quite disproportionate to its relative expression in serum Ig, and the significance of this aspect will require further investigation. It was also observed in these studies that when double staining was performed with two different fluorochrome-labeled reagents, no double stained cells were observed for the combinations tested (γ_1/γ_2, γ_2/γ_3). Similar distributions of subclass determinants on cord blood lymphocytes were found (Froland and Natvig, 1972b).

The presence of different Ig classes on human B cells has also been studied with other methods. Using the mixed antiglobulin technique, Litwin (1972) observed that only 4% of the IgM- or IgG-bearing cells and only 2% of the cells with IgA or IgG have both classes. Abdou (1971) showed that binding of PBL to anti-γ-chain-coated bead columns specifically depleted cells capable of blastogenic responses *in vitro* to anti-IgG but not to anti-IgM, indicating that IgM and IgG were carried on different cells. In comparing PBL and bone marrow cells, Abdou and Abdou (1973) observed a greater uptake of ^{125}I-labeled anti-IgG to human PBL than of anti-IgM, but the reverse situation applied to bone marrow. It was suggested that this is consistent with other studies in animals concerning the predominant nature of the IgM class on antigen receptor cells (see Section V).

Using quantitative hemagglutination studies Cooper *et al.* (1973) found an average of 11 ng. of γ chain per 10^7 PBL but did not detect μ chains (<7 ng./10^7), whereas on tonsil both μ, γ, and κ chains were detected. The significance of these observations is uncertain, as sensitivity differences presumably exist between the groups in that whereas γ chain was detected on PBL, κ chain was not.

g. Allotypic Studies on M-Ig. Two reports of allelic exclusion of M-Ig on human PBL have appeared. Both involve the Gm markers of the human IgG1 subclass. Litwin (1972) used a modified mixed anti-Ig technique and found that of 162 lymphocytes bearing Gm(a) or Gm(f) only 3 had both allotypes. Similarly, Froland and Natvig (1972c) using immunofluorescence observed no lymphocytes bearing both Gm(f) and Gm(z) in heterozygotes.

2. *Mouse*

In 1961 Möller observed that a proportion of viable mouse lymphoid cells reacted directly with Fl-labeled anti-Ig reagents and showed membrane staining. Subsequently many studies have amply confirmed the existence of readily detectable Ig on cell membranes of

some mouse lymphocytes. The question regarding the nature of these cells in mice is at face value more complex than in other species, because, as discussed in Section III,D, there is some evidence to suggest that mouse T cells carry M-Ig. For the purpose of this section, we will be considering only the proportion of cells that show readily detectable, presumably high surface density M-Ig.

a. Are High-Density M-Ig Cells in Mice, B Cells? The answer is unequivocally yes. In mice there are both many markers available for distinguishing B and T cells and also clearly defined experimental techniques or animal strains available that offer relatively purified populations of B and T cells.

Raff (1970) showed that the percentage of M-Ig-bearing cells in lymph nodes and spleen was considerably increased in ALS-treated and thymectomized mice. In different lymphoid tissues, the proportion of θ-bearing or M-Ig-bearing cells were observed to be inversely related. These results have been amply confirmed and extended using thymectomized, ALS-treated, or congenitally athymic nude mice (Bankhurst and Warner, 1971, 1972; Jones *et al.*, 1971; Unanue *et al.*, 1971; Nossal *et al.*, 1972; Lamelin *et al.*, 1972; Osmond and Nossal, 1973). Removal of M-Ig-bearing cells on anti-Ig bead columns has also been shown to increase considerably the number of cells bearing specific T-cell determinants (Wigzell *et al.*, 1972). The results of these studies leave no doubt that high-density M-Ig-bearing cells are nonthymic-derived B cells.

b. Lymphoid Tissue Distribution of M-Ig Cells. Varying proportions of cells from different lymphoid tissues show high-density M-Ig. The results of several such studies are summarized in Table V. With only a few exceptions, there is general agreement between most of the studies on the proportion of B cells present. Spleen cells have been most frequently studied and show an average range of about 25 to 55% B cells. When the results of all studies are averaged for each tissue, they show a considerable similarity to the content of B cells as determined by the receptor for Fc of Ig (Basten *et al.*, 1972a). Thus, spleen has the highest proportion of B cells, around 40%, whereas thoracic duct lymph, lymph nodes, and peripheral blood all contain around 15 to 25%. Thymus clearly contains very few high-density cells but, in several studies, was shown to contain a few percent B cells (see Section III,D). The situation with bone marrow may be a little more complex. Several studies showed around 10 to 25% of M-Ig-bearing cells. Osmond and Nossal (1973) have examined bone marrow in some detail using quantitative studies with ^{125}I-labeled anti-Ig. Where, with spleen or lymph node cells, well-defined plateau levels of percent

labeled cells were found with increasing concentration of reagent, with bone marrow, a linear increment in labeled cells occurred throughout the entire dose range of reagent used. At high concentration, approximately 50% cells were labeled. These results indicate that the bone marrow cells vary considerably in either their densities of M-Ig or in the presentation or accessibility of the Ig.

Mouse colonies are notoriously variable in their exposure to subclinical infections, and it may be expected that proportions of B cells

TABLE V
PROPORTION OF MEMBRANE-BOUND IMMUNOGLOBULIN-BEARING CELLS IN MOUSE LYMPHOID TISSUES[a]

% M-Ig-positive cells							
Spleen	Lymph node	PBL	TDL	BM	Thymus	Method	Reference
57	36	44	–	27	>10	Cyto-toxicity	Takahashi *et al.* (1971b)
49	7	14	–	9	>1	Fl	Rabellino *et al.* (1971)
48	–	–	–	–	–	Fl	Wigzell *et al.* (1972)
47	21	30	20	–	–	AR	Osmond and Nossal (1973)
46	–	–	15	–	–	AR	Nossal *et al.* (1972)
40	20	–	15	15	–	Fl/AR	Raff *et al.* (1970)
35	27	–	–	–	1	Fl	Lamelin *et al.* (1972)
34	15	–	–	–	0	Cyto-toxicity	Huber *et al.* (1971)
34	–	–	–	–	–	RICA	Lee *et al.* (1971)
33	–	–	30	–	4	AR	Bankhurst and Warner (1971)
29	18	34	–	–	4	AR	Jones *et al.* (1971)
24	23	11	–	23	0	Fl	Yamana *et al.* (1973)
–	–	–	18	–	–	Cyto-toxicity	Miller *et al.* (1972)
–	20	19	–	17	1	Mixed anti-Ig	Coombs *et al.* (1970)
–	–	24–45	–	–	–	Mixed anti-Ig	Yakulis *et al.* (1972)
40	21	27	19	18	1	(Average for all studies)	
42	21	19	17	15	1	AgAb receptor	Basten *et al.* (1972a)

[a] PBL, peripheral blood lymphocytes; TDL, thoracic duct lymphocytes; BM, bone marrow; Fl, fluorescent label; AR, ^{125}I label by radioautography; RICA, reverse immunocytoadherence.

in different organs will be affected by natural or deliberate immunization. Osmond and Nossal (1973) observed a slight increase in the B-cell proportion of germfree mice, suggesting that immunization with natural antigens can either selectively increase T cells or induce differentiation to more mature B cells lacking detectable M-Ig (see Section III,C).

c. Ontogeny of Mouse B Cells. The site of the presumed bursal equivalent function in mice has not been conclusively determined. Using the criterion of the first site of detectable Ig synthesis, N. L. Warner and M. A. Moore (unpublished observations) found that spleen and mesenteric lymph node of 3 to 5 day old mice showed IgM synthesis by radioimmunoelectrophoresis of ^{14}C-labeled short-term cultures, whereas IgA synthesis in spleen and Peyer's patches developed at around 2 weeks of age. These studies do not indicate a bursal-like function—at least *in situ*—of gut-associated lymphoid tissue. Spleen cells from newborn mice contain around 10 to 20% of M-Ig-bearing cells (Nossal *et al.*, 1972; Osmond and Nossal, 1973), but they are absent from spleen, liver, or thymus of 15-day-old fetal mice. Therefore B cells, as determined by M-Ig, first originate between these times. Studies by Nossal and Pike (1972, 1973) have shown an increase in the proportion of M-Ig-bearing cells in the blood, spleen, and liver of fetal mice between 16.5 days of embryonic life and several weeks after birth; B cells were first detected in low numbers at 16.5 days in the spleen and blood. The bone marrow and fetal liver contained detectable M-Ig cells somewhat later than blood and spleen. These studies are consistent with the short-term culture synthesis studies of Warner and Moore and together suggest that murine bone marrow, Peyer's patch area gut tissue, and fetal liver are not *in situ* sites of a mammalian equivalent of the avian bursa of Fabricius.

d. Immunoglobulin Class Expression in Mouse M-Ig. Several groups of investigators have studied the class distribution of M-Ig, principally using splenic B cells. The results are summarized in Table VI, as the percentage of total M-Ig-bearing cells (determined by anti-polyvalent Ig or anti-κ) that carry a given class. The values for a given study do not therefore necessarily add to 100 and would do so only if cells expressed a single H-chain type. Before considering this point, it should be noted that, in mice, the proportional expression of cells bearing a given H-chain type may be dependent on the natural antigenic encounters of the animal (see Section IV), and this may particularly affect the ratio of μ chain to the other chains. Furthermore, as discussed for human M-Ig class expression, the results could depend on the specificity of the antisera used for determinants that may or

TABLE VI
PROPORTION OF H-CHAIN TYPES OF MURINE B CELL MEMBRANE-BOUND IMMUNOGLOBULIN

% M-Ig-positive cells with indicated H-chain type[a]						
μ	γ_1	γ_2	α	γ_3	Method of detection[b]	Reference
88	5	61	76	–	Indirect AR	Nossal *et al.* (1972)
76	–	44	40	–	AR	Bankhurst and Warner (1972)
75	<20	<20	<20	–	Cytotoxicity	Takahashi *et al.* (1971b)
62	<3	35	14	–	AR	Jones *et al.* (1971)
57	4		6	–	Fl	Lamelin *et al.* (1972)
48	21	21	10	–	Fl	Grey *et al.* (1972a)
–	–	–	–	2	Fl	Rabellino *et al.* (1971)

[a] The IgG value of Lamelin *et al.* includes both γ_1 and γ_2; values of individual classes of Rabellino *et al.* are omitted because these have been updated by Grey *et al.* (1972a).

[b] AR, ^{125}I label by radioautography; Fl, fluorescent label.

may not be partially hidden on the cell surface. The results show some range of IgM expression; most studies indicate that the majority of cells bear IgM (average of all values ~65%). The proportion of IgG cells varies considerably between the G subclasses. Only one study has reported on IgG_3, and as for the serum levels, this is only a minor component of the M-Ig. The great majority of membrane-bound IgG is of the IgG2 class, with only one of five studies indicating a significant amount of membrane-bound IgG1. In three reports on class distribution of M-Ig on PBL cells (Bhoopalam *et al.*, 1971; Yakulis *et al.*, 1972; Jones *et al.*, 1971), it was shown that 20–45% of PBL cells carried IgG-type (IgG2a by Jones *et al.*, 1971)M-Ig, whereas a lower proportion (4–25%) carried IgM type. The expression of IgA is quite variable among groups, and whether this is due to methodological differences or to various intercurrent infections affecting the proportions needs to be resolved.

Labeling of surface proteins with ^{125}I, followed by disruption and analysis in either acrylamide gels or antigenically with anti-Ig, has consistently shown that the predominant Ig label on B cell populations from normal spleen or nude mouse spleen cells is IgM (Baur *et al.*, 1971; Vitetta *et al.*, 1971; Marchalonis *et al.*, 1972a; Marchalonis and Cone, 1973). In the studies of Baur *et al.* (1971) and Vitetta *et al.*, no significant γ chain was detected, although in the studies of Marchalonis *et al.* a significant but small amount of γ chain was found. These

studies are therefore consistent with direct anti-Ig-labeling studies in showing that IgM is the predominant class carried on the B-cell membrane of mouse spleen cells. Results from several groups (Vitetta *et al.*, 1971; Esekland and Klein, 1971; Marchalonis *et al.*, 1972a,b) have shown that the membrane-bound IgM is in the form of a 7 S monomer.

Although most studies agree that in splenic B cells, IgM is the predominant class of M-Ig, there is some question as to the pluripotentiality of the cells in terms of expression of other classes. The studies of Jones *et al.* (1971), Rabellino *et al.* (1971), Grey *et al.* (1972a), Lamelin *et al.* (1972) do not suggest that a large proportion of cells carry more than one Ig class. However, the reports by Bankhurst and Warner (1972) and Nossal *et al.* (1972) indicate that a sizable proportion of cells, perhaps 40–50%, may carry IgM and another (or several other) classes. These studies have been discussed elsewhere (Warner, 1972a, Warner and Harris, 1973) and will be considered in Section III,B. Lee *et al.* (1971) using the RICA method have suggested that most of the IgG-bearing cells in spleen (~20%) carry both IgG1 and IgG2 and that about 10% carry only IgG1. The significance of these observations is not clear at present, particularly as this group found a far higher overall IgG1 proportion than most other studies.

3. *Rabbit*

Studies with rabbit lymphocytes have the particular advantage of the availability of antiallotype reagents to determinants on L chains (*b* locus κ chain), all H chains (*a* locus common to all H chains), and specific H chains (*D* locus γ Fc, *Ms* locus μ chain) (Kelus and Gell, 1967). A disadvantage is that relatively few markers are available for distinguishing B- and T-cell series.

a. Are Rabbit M-Ig-Bearing Cells, B Cells? As in other species the answer is yes, although direct evidence is based on only a few studies. By using the functional marker for T cells of PHA stimulation *in vitro*, Daguillard and Richter (1969) concluded that different populations of cells responded to PHA or to anti-Ig-induced stimulation. Treatment of splenic lymphocytes with anti-Ig and complement totally prevented subsequent stimulation by anti-Ig but did not affect PHA stimulation. By using an antiserum prepared in goats against rabbit thymocytes, Fanger *et al.* (1972) showed that this serum completely suppressed PHA or concanavalin A (Con A) stimulation but did not affect stimulation by anti-Ig. These two studies strongly indicate that cells bearing Ig receptors are distinct from the PHA/Con A-sensitive thymus-derived population. Furthermore, by means of radioau-

tographic examination of the cells stimulated by anti-Ig sera, Elfenbein *et al.* (1973) showed directly that these same cells had the receptor for complement—a B-cell-specific marker. It is, therefore, reasonably clear that M-Ig-bearing cells in rabbits are B cells.

b. Tissue Distribution of Rabbit B Cells. The majority of studies with rabbit lymphocytes have used peripheral blood, and only limited information is available on tissue distribution. Stimulation of rabbit lymphocytes by anti-Ig sera of various specificities clearly indicate that a sizable proportion of PBL bore M-Ig receptor (Sell and Gell, 1965; Sell, 1967). Studies with lymphocyte stimulation do not, however, provide reliable information on proportions of the original population bearing M-Ig [in fact, recent studies show that a continuous action of anti-Ig is required for maximal stimulation (Sell *et al.*, 1973)]. However, the method does give an estimate as to whether a cell population contains some M-Ig-bearing cells, and, by using lymphocyte stimulation, Kaplan and Thorbecke (1970) showed that the ability to respond developed in spleen only after the third week. This was also true for rabbit appendix, indicating that this organ may not be the site of early B-cell development (bursal equivalent). Various groups have used immunofluorescence to determine proportions of M-Ig-bearing cells, particularly using antiallotype sera to the κ L-chain (*b*) locus. Although the majority of rabbit Ig molecules express H-chain-*a*-locus determinants, antiallotype sera to the *a* locus have been considerably less effective in direct studies (Wolf *et al.*, 1970; Sell *et al.*, 1970a; An and Sell, 1973), perhaps indicating that the Fd region is relatively hidden in the M-Ig. A series of results are summarized in Table VII showing for peripheral blood a range of 24 to 85% M-Ig-bearing cells, but with an average and clear cluster around the 50% value. In limited tests, a similar value is found for rabbit spleen and Peyer's patch. It thus appears likely that the proportions of B cells in rabbit spleen, lymph nodes, bone marrow, and Peyer's patch may all be similar to those tissues of mice, but in PBL, about twice as many cells are B cells in rabbits than in mice or man.

c. Class and Allotype Distribution. Relatively little information is available on rabbit H-chain classes on lymphocytes. Pernis *et al.* (1970, 1971) have shown that about 90% of the M-Ig-bearing cells have IgM, although two other studies using mixed agglutination suggest lower values. However, it might be noted that this latter method seemed to underestimate IgM cells in mice and man. Some controversy has appeared over the question of multiple Ig types (classes or allotypes) on rabbit cells. The studies of Sell *et al.* (1970b) were interpreted to indicate that a sizable proportion of cells pos-

TABLE VII
PROPORTION OF MEMBRANE-BOUND IMMUNOGLOBULIN-BEARING CELLS IN RABBIT LYMPHOID TISSUES

% M-Ig-positive cells					
L chain (b)	IgM	IgG	Tissue	Method[a]	Reference
85	–	–	PBL	AR	Jones *et al.* (1970)
76	–	–	PBL	Transformation	Marcusson and Roitt (1969)
65	–	–	PBL	Fl	Jones *et al.* (1973a)
53	–	–	PBL	AR	Davie *et al.* (1971)
50	–	–	PBL	Fl	Jones *et al.* (1970)
50	40	4	PBL	Fl	Pernis *et al.* (1970)
40–80	–	–	PBL	Transformation	Sell and Gell (1965), Sell *et al.* (1970a,b)
42	23	30	PBL	Mixed anti-Ig	Coombs *et al.* (1970)
27	–	–	PBL	Mixed anti-Ig	Wolf *et al.* (1970)
24	–	–	PBL	Mixed anti-Ig	An and Sell (1973)
40	35	5	Spleen	Fl	Pernis *et al.* (1970)
13	–	–	Bone marrow	Fl	Pernis *et al.* (1970)
–	3	18	Lymph node	Mixed anti-Ig	Coombs *et al.* (1970)
48	–	–	Peyer's patch	Fl	Jones *et al.* (1973a)
52	33		PBL/Spleen (average)		

[a] AR, ^{125}I label by radioautography; Fl, fluorescent label.

sessed multiple H-chain classes, and, in heterozygotes, both parental allotypes. Direct Fl–anti-Ig studies, however, showed that IgM and IgG were in separate cells (Pernis *et al.*, 1970, 1971). Similar results were found by Bona *et al.* (1972) using combined ^{125}I-labeled anti-IgM and peroxidase-labeled anti-IgG. In heterozygous rabbits, Wolf *et al.* (1971) suggested that around 3 to 69% of cells could bear both allotypes as shown by mixed anti-Ig tests, and, by using combined rhodamine and fluorescein-labeled reagents, Jones *et al.* (1973a) observed a range of 7 to 63% of cells with both labels. These results are in contrast to Pernis *et al.* (1970, 1971) and Davie *et al.* (1971) who found only a minimal number (~3%) if any of double-stained cells in heterozygotes. These results have been recently discussed by Jones *et al.* (1973b) who also showed (see Section II) that rabbit cells could cytophilically bind Ig. Furthermore they demonstrated that when the cells bearing both parental allotype M-Ig were separated by an automated fluorescence-activated cell sorter and placed in culture with pronase, the majority of cells lost the M-Ig but then regenerated only one parental allotype per cell.

It therefore appears that in rabbits as in mice and man, the majority of cells—at least 90–95%—produce and bear only one H-chain and one L-chain gene product (i.e., one class and allotype) unless the cell binds additional Ig from serum or extracellular sources.

4. *Other Species*

Limited information is available on distribution of M-Ig bearing cells in other animal species. In general, it is likely that (*1*) M-Ig-bearing cells in all species will be B cells, and where examined this is the case, (2) proportions of B cells in tissues may vary somewhat, perhaps due to antigenic exposure, but will be of generally similar proportions in man, rabbits, and mice, and this also appears to be so, and (3) M/G ratio of M-Ig will be similar in most species unless a given species tends to bind cytophilically a particular class.

a. Chickens. In this species, formal proof of the B-cell origin in high-density M-Ig-bearing cells has been obtained. Lymphocyte stimulation studies of Ivanyi *et al.* (1969), Skamene and Ivanyi (1969), and Alm and Peterson (1969) clearly showed that, whereas PBL from normal chickens could be readily stimulated by anti-Ig sera (particularly with anti-μ), virtually no stimulation occurred with PBL from bursectomized chickens that lacked serum Ig. Direct examination of spleen or PBL cells with Fl- or ^{125}I-labeled anti-Ig sera has confirmed that M-Ig-bearing cells are virtually absent from agammaglobulmemic chickens (for references, see Table VIII). Avian spleen contains a similar to slightly lower proportions of B cells than mice, man, and rabbit, and the PBL value is closer to rabbit values. As expected from Ig synthesis studies (Thorbecke *et al.*, 1968), the bursa contains a very high proportion of M-Ig-bearing cells, and the cecal tonsil resembles studies on Peyer's patches in rabbits and on tonsils in man. The ontogenic development of M-Ig-bearing cells in the embryonic bursa has been described by Hudson and Roitt (1973), and is fairly similar to studies on the Ig content of bursal cells shown by Kincade and Cooper (1971). The latter group observed IgM-containing cells on the fourteenth day of incubation—the time when recognizable lymphoid cells first appear—whereas Hudson and Roitt first detected M-Ig on day 16. A progressive increase in percentage of M-Ig-bearing cells occurred till hatching.

b. Rat, Guinea Pig, and Sheep. Lymphocyte stimulation by anti-L-chain antibody in rats (Koch and Neilson, 1973), and by anti-IgG2 antibody in guinea pigs (Foerster *et al.*, 1969; Elfenbein *et al.*, 1973) has been reported. In the latter report it was also shown that the responding cells had the B-cell CRL marker. Proportions of cells reacting

with various labeled antisera are shown in Table VIII. The results in sheep suggest that multiple chains are present on most lymphoid cells, and the possibility of cytophilic attachment as in rabbits (Jones *et al.*, 1973b) will need to be examined in this species.

In general, the values for these four species are comparable to those of mice and man.

5. *Are B Cells Restricted for M-Ig Class and Allotype?*

Mature antibody-forming plasma cells are clearly restricted in their expression of Ig structural genes, with most of the cells producing only one type of L-chain and H-chain gene product at both the class and allotypic levels. Immunofluorescent studies of Ig-containing plasma cells from heterozygous rabbits revealed only one H-chain and one L-chain type per cell (Pernis *et al.*, 1965; Cebra *et al.*, 1966). By developing Jerne plaques to sheep erythrocytes with plasma cells from heterozygous or homozygous rabbits, Chou *et al.* (1967) showed that only one L-chain allele was expressed per cell in the heterozygote and also gave data indicating this to be true in homozygous animals. Restriction of Ig, using electrophoretic analysis of proteins from single antibody-forming cells, has shown homogeneity comparable to that obtained with single myeloma cells (Marchalonis and Nossal, 1968). Analysis of the class of antibody made by single cells has revealed that over 95% of the cells produce specific antibody of only one H-chain class (see section IV,B) (Cosenza and Nordin, 1970; Merchant and Brahmi, 1970; Nordin *et al.*, 1970; Nossal *et al.*, 1971b; Nussenzweig *et al.*, 1968). The question that arises is whether this restriction develops during the maturation of the antibody-forming plasma cells or whether it is present at the initial stage of the immunocompetent cell before antigen is encountered. Studies in rabbits using lymphocyte stimulation suggested that the B lymphocyte might be pluripotential for Ig expression and that restriction therefore developed as a result of antigenic stimulation. Some studies on antibody-forming lymphocytes also suggested the expression of cell-bound antibody of multiple gene products (Biozzi *et al.*, 1969).

Examination of Ig classes and allotypes on the cell surface of B lymphocytes added further to the controversy in that in some studies it was evident that the sum of individual H-chain types far exceeded the total number of M-Ig-bearing cells as shown by polyvalent anti-Ig reagents. On the other hand, this was not always found, and in studies that used double-labeling techniques, double-stained cells were rarely observed except sometimes in man (Heller *et al.*, 1971), rabbits (Wolf *et al.*, 1971; Jones *et al.*, 1973a), and mice (Lee *et al.*, 1971) (see

TABLE VIII

MEMBRANE-BOUND IMMUNOGLOBULIN-BEARING CELLS IN LYMPHOID TISSUES OF SEVERAL ANIMAL SPECIES[a]

% M-Ig-positive cells								
Spleen	PBL	Thymus	B of F	BM	Cecal tonsil	Donor (chicken) treatment	Method	Reference
40	40	4	78	2	–	–	Fl	Hudson and Roitt (1973)
40	–	< 10	80	–	–	–	Cytotoxicity	McArthur *et al.* (1971)
35	18	0.4	93	–	52	–	AR	Rouse *et al.* (1973a)
26	42	0.4	68	–	–	–	Fl	Rabellino and Grey (1971)
–	18/15	–	–	–	–	–	Fl	Kincade *et al.* (1971)
1	2	0	–	–	–	SBX + 1RR	Fl	Rabellino and Grey (1971)
4	3	–	–	–	–	HBX	AR	Rouse *et al.* (1973a)
–	0/0[b]	–	–	–	–	Anti-μ + SBX	Fl	Kincade *et al.* (1971)
–	–	–	–	–	–	HBX	AR	Bankhurst *et al.* (1972)

% M-Ig-positive cells					
Fab/L	μ	γ	Species/tissue	Method	Reference
12	7	6	Pigs/PBL	Mixed anti-Ig	Binns *et al.* (1972)
20	23	18	Sheep/lymph	AR	Ey (1973)
–	21–25	24–46	Rat/lymph node	Peroxidase Ab	Gonatas *et al.* (1972)
30	–	–	Guinea pig/lymph node	Fl	Shevach *et al.* (1972d)

[a] PBL, peripheral blood lymphocytes; B of F, bursa of Fabricius; BM, bone marrow; Fl, fluorescent label; AR, ^{125}I label by radioautography.

[b] Values of Kincade *et al.* are for IgM/IgG-positive cells.

previous sections). It must be concluded from the number of double-labeling techniques that fail to show double cells using either allotype (man and rabbit) or H-chain markers (man, rabbits, and mice) that allelic and class restriction applies to the majority of B lymphocytes. Where double-labeling cells are found or inferred from population studies, rigorous examinations will have to be made to determine whether or not this is due to the additional acquisition of Ig from the serum or extracellular fluid, as has clearly been shown to be the case for rabbit allotypes (Jones *et al.*, 1973a). There is, however, a real possibility that a small proportion of B cells may bear M-Ig of multiple classes, and whether or not these represent a distinct subpopulation of B cells, possibly of a precise stage of maturation, will need to be further evaluated (see Section IV).

The possibility of a small proportion of B cells expressing multiple allelic products has been inferred from some studies on inhibition of antigen binding (e.g., Greaves, 1971b), and these studies are considered in Section V.

6. *Quantitative Estimates of M-Ig on B Cells*

Determinations of the amount of M-Ig on B cells have been made by various groups using several different methods. In all cases, several assumptions have been made and a generally constant picture has emerged. In studies using intact cells, the values could represent underestimates if some of the surface M-Ig determinants are inaccessible. Studies on uptake of labeled antibodies must reach saturating conditions, and approaches of direct surface labeling must make assumptions regarding specific activities of the labeled Ig. Within these limitations, the results summarized and referenced in Table IX, show a range of only about tenfold, from 20,000 to 200,000 molecules per cell, including estimates of κ, μ, or γ with an average value of 10^5 molecules per cell.

C. Plasma Cells

Direct interaction of specific antigens with the surface of normal (Mäkelä and Nossal, 1961; Wigzell and Andersson, 1969; McConnell, 1971) plasma cells has been shown by several techniques such as bacterial adherence, antigen-coated columns, rosetting, and radiolabeled antigen uptake. These studies suggest that M-Ig of intrinsic origin should be demonstrable on the surface of the cells, because Mäkelä and Nossal (1961) concluded that the antibody on the cell surface was not cytophilically derived.

TABLE IX

ESTIMATES OF AMOUNTS OF MEMBRANE-BOUND IMMUNOGLOBULIN ON B CELLS

Species	Cell source[a]	Method[a]	M-Ig molecules $\times 10^{-4}$ per B cell[b]	Reference
Man	Burkitt (Daudi)	HI	16.0 (μ)	Klein *et al.* (1970)
	CLL	Cell disruption	8.0 (μ)	Esekland *et al.* (1971)
	PBL	HI	6.0 (γ)	Cooper *et al.* (1973)
	Cultured lines	RIA	1.8–22.0 (κ)	Lerner *et al.* (1972)
	Burkitt (Daudi)	Surface labeling	11.6 (μ)	Sherr *et al.* (1972)
Mouse	Splenic lymphocytes	Surface labeling	5.1–14.3 (γ)	Rabellino *et al.* (1971)
	Splenic lymphocytes	Surface labeling	5.0–10.0 (μ)	Marchalonis and Cone (1973)
Rabbit	PBL	Mixed antiglobulin	< 100 (κ)	An and Sell (1973)
	PBL	^{125}I-Ab uptake	0.7–4.0 (κ)	Jones *et al.* (1970)
Rat	Lymph node	Peroxide label Ab	22.4 (γ)	Avrameus and Guilbert (1971)
Sheep	Efferent lymph	^{125}I-Ab uptake	7.4 (μ)	Ey (1973)
	Efferent lymph	^{125}I-Ab uptake	1.9 (γ)	Ey (1973)

[a] CLL, chronic lymphocytic leukemia; PBL, peripheral blood lymphocytes; HI, hemagglutination inhibition; RIA, radioimmunoassay.

[b] Chain type assayed is indicated in parentheses.

1. *Human Plasma Cells*

By using cytotoxicity testing of human myeloma or Burkitt cells, Klein *et al.* (1968) showed that, whereas Burkitt cells had M-Ig but little intracytoplasmic Ig, myeloma cells had a lot of intracellular Ig but were not killed by anti-Ig sera and failed to show uptake of Fl-labeled anti-Ig on living cells. Pernis *et al.* (1971) also failed to detect M-Ig on cells from two myeloma cases, but did detect M-Ig on tumor cells from one case of Waldenstrom's macroglobulinemia. This latter aspect has been further studied by Preud'homme and Seligmann (1972a) who performed immunofluorescence analysis of viable cells from marrow or blood of 25 cases of Waldenstrom's macroglobulinemia. It was observed that, whereas intracytoplasmic IgM was restricted to plasma cells, the vast majority of the proliferating lymphoid cells and the plasma cells bore M-Ig. A high proportion of blood lymphocytes bore the monoclonal M-Ig. Similarly, Lindstrom *et al.* (1973) and Heller *et al.* (1972) have reported that in some myeloma cases around 20 to 25% of the peripheral lymphocytes stained with anti-Ig specific for the idiotype of the myeloma; Wernet *et al.* (1972) reported that, in 2 monoclonal IgM cases, cytotoxicity studies showed a moderate proportion of blood lymphocytes to be typed by anti-idiotype sera. The significance of these observations in relation to origin of the M-Ig is not clear. Similar observations have been made in mice (Yakulis *et al.*, 1972) and it has been claimed that this effect is not due to cytophilic binding of the myeloma protein but may be associated with some activity of plasmacytoma RNA (Bhoopalam *et al.*, 1972). Further studies on this aspect are clearly needed.

2. *Animal Studies*

Although several reports of failure to detect M-Ig on significant numbers of normal or malignant mouse plasma cells have been made (Paraskevas *et al.*, 1970; Matter *et al.*, 1972; Hammerling and Rajewsky, 1971; Lamelin *et al.*, 1972), many positive reports have appeared, and the controversy regarding presence or absence of M-Ig most likely rests in quantitative consideration of M-Ig exposure and threshold requirement for the assay methods.

Although cytotoxic killing of myeloma cells has not been found in man, Takahashi *et al.* (1971b) have demonstrated lysis of normal and malignant mouse plasma cells. Plaque-forming cells and RFC from immunized mice were both shown to bear the plasma cell-differentiation antigen MSPCA (Takahashi *et al.*, 1971a) and could be eliminated by treatment with anti-Ig sera. Whereas most (>90%) RFC reacted

with anti-κ and 50% with anti-μ, only the PFC producing IgM antibodies were eliminated with anti-κ (or anti-μ). Using either E/M detection of radiolabeled antibodies (Bosman and Feldman, 1970; Perkins *et al.*, 1972) or peroxidase-labeled antibodies (Gonatas *et al.*, 1972), plasma cells from rat lymph nodes or mouse spleen were observed to bear M-Ig, but considerably less than that carried on (B) lymphocytes (Perkins *et al.*, 1972). In rabbit spleen, Pernis *et al.* (1971) observed that of plasma cells containing intracellular IgG or IgM, all those with IgM had membrane-bound IgM and 15% of those with intracellular IgG had membrane IgM. No plasma cells were detected with membrane IgG. Similarly, Jones *et al.* (1973b) showed that a small proportion of rabbit Peyer's patch cells with intracytoplasmic IgA, also had M-Ig of IgM type. In immunized mice, they observed that most cells with intracellular IgM had IgM on the membrane, and about half of those with intracellular IgG had membrane-bound IgM. The implications of these observations in regard to sequential Ig expression in B-cell differentiation is discussed in Section IV. In terms of the present consideration, these results show that Ig-containing cells can have M-Ig.

A recent quantitative study on the uptake of ^{125}I-labeled anti-Ig by 258 single plaque-forming cells (Nossal and Lewis, 1972) has shown a highly variable degree of binding between cells and with cells taken at different stages of the immune response. Thus at 3 to 4 days after primary or secondary antigen injection, 60–90% of plaque-forming cells had detectable M-Ig, whereas around 50% of cells were unlabeled at 9 to 10 days after primary or 6 to 8 days after secondary stimulation. No differences were observed between IgM and IgG plaque-forming cells compared at the same time after immunization. It was suggested that the results are consistent with the thesis that cells still able to be driven to further proliferation retain antigen receptors, whereas on maturation the cells lose both receptors and ability to be influenced by antigen.

Studies of murine plasma cell tumors at first seemed to show variable results that were related to the Ig type produced by the cell. Thus, Takahashi *et al.* (1971b) lysed only IgG1-producing tumors; Shevach *et al.* (1972c) reported that of 4/8 tumors showing M-Ig by immunofluorescence, 2 were IgG1 type; and Reif (1970) showed uptake of anti-Ig by viable IgG1 cells using the paired label antibody technique. Recent studies, however, indicate that all plasma cell tumors bear some M-Ig. Princler and McIntire (1973) have shown that of 7 IgG1-, 9 IgG2-, 2 IgA-, and 1 IgM-producing plasma cell tumors of mice, all were lysed by anti-κ antibodies and complement, but only

the IgG1 and IgM tumors were lysed by specific anti-H-chain sera (to the type produced by the cell). Hannestad *et al.* (1972) have also shown by a rosette-inhibition method that all plasma cell tumors tested showed M-Ig, regardless of the Ig class produced. Cell surface radio-iodination studies of three plasma cell tumors showed that surface-bound Ig was present on all and appeared to be relatively exposed, as the Fc fragment was found to carry radio-iodine labeling (Baur *et al.*, 1972). *In vivo* studies of immunity to plasma cell tumors have shown that anti-idiotype antibodies can inhibit tumor cell growth (Lynch *et al.*, 1972), indicating the accessibility of the M-Ig on the cells, although it should be noted that M-Ig probably does not represent the major tumor-specific transplantation antigen of murine plasma cell tumors (Rouse *et al.*, 1973b).

The general impression that might be gained from these studies in both man and animals is that antibody-producing plasma cells do bear some Ig on the plasma membrane, but in decreasing concentrations as maturation to the end plasma cell stage of B-cell differentiation is approached. In view of the murine tumor studies, it may be that IgG1 is retained on the cell membrane to a greater degree than other classes. Alternatively, A. W. Harris (unpublished observations) (see Section II) has shown that plasma cells may still possess the receptor for the Fc of Ig, and if, as suggested by Basten *et al.* (1972b), this primarily binds to IgG1 proteins, Harris has proposed that in IgG1 producing plasma cell tumors some of the secreted IgG1 may then become rebound to the cell membrane through the Fc receptor, thereby providing a higher surface density of M-Ig than those tumors that carry only cell synthesized M-Ig.

D. T Cells

From the previous considerations, it appears quite evident that B cells, but not T cells, bear high-density M-Ig. A major point of controversy has, however, arisen over the question of whether T cells bear *any* M-Ig. In considering this problem, three particular complexities must be borne in mind: (*1*) most (if not all) T-cell populations studied may contain a small but definite number of B cells; (2) T cells are a heterogeneous population as shown by other markers, and the degree of activation of the T cells may be a very relevant matter; and (3) a practical consideration for studies with anti-Ig sera is that many animal sera contain "natural" antibodies that react with cell membranes from other species (Takahashi *et al.*, 1971b) and, accordingly, specificity controls become very important.

With these considerations in mind, the problem is therefore to question whether T cells—some or all—carry any M-Ig, perhaps only a few hundred molecules per cell, and if so, whether this is really due to T-cell expression of Ig genes, and not to contaminating B cells or to binding of extracellular Ig through an Fc receptor (see Section II, C).

1. Membrane-Bound Ig in Thymus Cell Populations

Incubation of thymus cell suspensions with various labeled anti-Ig reagents has in most studies either failed to detect any labeled cells or, more frequently, some cells with M-Ig were found, usually less than 2% of the population (Pernis *et al.*, 1970; Coombs *et al.*, 1970; Raff, 1970; Rabellino *et al.*, 1971; Takahashi *et al.*, 1971b; Huber *et al.*, 1971; Hammerling and Rajewsky, 1971; Lamelin *et al.*, 1972, Matter *et al.*, 1972; Gonatas *et al.*, 1972; Yamana *et al.*, 1973). From these studies, two questions might be asked, respectively, concerning the few percent of positive cells and the large bulk of apparently negative cells: (*1*) Are the thymus cells with readily detectable M-Ig T cells or B cells? and (2) Do the remaining cells have some but much less M-Ig?

1. By using non-Ig markers of B cells (Fc or C3 receptors), Basten *et al.* (1972a) and Ross *et al.* (1973) showed that thymus cell suspensions in mouse and man contain about 0.3 to 2.0% of B cells. Strain differences in mice were noted by Basten *et al.* with CBA mice having around 0.3% and C57BL having 2% B cells. These two studies clearly imply that up to 2% of M-Ig-bearing cells might be expected to be observed in thymus cell suspensions, and that they would be B cells. It might, therefore, appear that the situation is quite simple and that any M-Ig-bearing cells that are present are immigrant B cells. However, there are several indications that not all of these high-density M-Ig cells may be typical B cells. Unanue *et al.* (1973b) have shown that these cells in thymus bearing M-Ig are not killed by anti-θ-serum and complement, and they suggested that, as an alternative to the B-cell nature of these cells, they could be mature T cells with only low θ concentration. Perkins *et al.* (1972) observed with E/M radioautography that cells with the morphology of the labeled thymus cells were not observed in spleen or lymph nodes. Osmond and Nossal (1973) found that the proportion of labeled M-Ig cells was not increased in thymuses from cortisone-treated mice, but rather was lower than controls. It may be of value to compare the relative steroid sensitivities of these cells with M-Ig cells of proven B origin.

Particularly, in view of the studies of Unanue *et al.* (1973b), of Basten *et al.* (1972a), and of Ross *et al.* (1973), there seems little doubt

that at least some of the high-density M-Ig-bearing cells in thymus are immigrant B cells, although, in the absence of double-staining studies, it cannot yet be concluded that B cells account for all of the high-density M-Ig thymic cells.

2. Several studies have indicated that when methods of increasing sensitivity are applied, a higher proportion of thymus cells show M-Ig. Thus, in the radioautographic studies of Bankhurst and Warner (1971) and Jones *et al.* (1971), up to 6% of labeled thymus cells were found and when the methods were pressed to further sensitivity, the number increased to around 15% of thymus cells (Bankhurst *et al.*, 1971); with an indirect sandwich method, around 50% showed specific labeling with anti-Ig and possibly even all cells (Nossal *et al.*, 1972) gave some degree of binding of anti-Ig. In both of the latter studies, a low level of binding was observed with the anti-Ig reagents that had been preabsorbed with insoluble Ig, possibly indicating a low level of binding of natural antibody to membrane components. The values of cells showing M-Ig were calculated from grain counts in excess of the level shown with these blocked reagents and clearly indicated that a major proportion of thymus cells carry some M-Ig. Quantitative studies, using uptake of ^{125}I-labeled antibodies (Nossal *et al.*, 1972), indicated that B-cell populations had about 140 to 440 times more anti-Ig binding sites than thymus cell populations. Based on the average number of molecules of M-Ig on B cells, this would imply that T cells have around 500 molecules of M-Ig per cell.

Quantitative inhibition studies were performed by Grey *et al.* (1972) who compared the amount of M-Ig with percent cells bearing readily detectable M-Ig determined by fluorescence, using thymus or spleen cells that had or had not been passed over anti-Ig bead columns. All of the data indicated that more Ig was present in the cell populations than could be accounted for by the small percent of fl-positive cells. Assuming that both fl-positive and -negative populations were homogeneous in terms of M-Ig, the positive cells were calculated to have 5–14 ng. N/10^6 cells and the negative cells in thymus 0.01 ng. N/10^6 cells, i.e., an average of about 260 molecules per cell of M-Ig for the latter cell type. To explore the possibility that thymic T cells had much larger amounts of M-Ig that were hidden or inaccessible on the cell membrane, Grey *et al.* (1972b) repeated the quantitative inhibition studies on thymus cells that were lysed by detergent, urea–acetic acid, or freeze-thawing. A two- to fourfold increase in measurable Ig was found, which is the same order of increase as found with treated B-cell sources. It was, therefore, concluded that T cells do not have large amounts of Ig bound on the cell membrane.

The nature of the chain class of thymus cell M-Ig has not been fully resolved. Most studies indicate that it would be IgM. In the observation of Bankhurst *et al.* (1971), only κ-chain reactivity was detected on thymus cells, and none of the anti-H-chain sera reacted, whereas, with the indirect radioautographic studies of Nossal *et al.* (1972), reactivity of T-cell M-Ig with anti-κ and anti-μ was observed. Quantitative inhibition studies have produced some controversial results. Grey *et al.* (1972a) found as much γ_2- as μ-chain activity, and, in man, Cooper *et al.* (1973) detected γ chain in thymus cell preparations but no μ chain. If it is accepted that some B cells are also present in thymus-cell preparations, perhaps derived as immigrant cells from blood, then, considering the relative amounts of IgG in B-cell M-Ig; particularly from blood (see Section III,B), much of the IgG detected in thymus cell suspension may be due to the B-cell content. By this argument, however, some of the IgM reactivity would also be due to B cells, and the general conclusion might tentatively be made that the H-chain type present on the bulk of the T cells is IgM, but the μ-chain determinants are relatively inaccessible. This view was proposed by Greaves and Hogg (1971b) in considering anti-Ig inhibition of antigen-binding T cells (see Section V) and by Marchalonis and Cone (1973), using radiolabeling of cell surface protein.

The technique of enzymatic activation of radiolabeling of cell surface protein has led to some controversial results on the nature of T-cell Ig. These aspects have recently been reviewed in depth by Marchalonis and Cone (1973) and will be only briefly summarized here. Studies of Vitetta *et al.* (1972) and Grey *et al.* (1972b) have totally failed to detect labeled Ig from various T-cell preparations. By adding known numbers of B cells to thymocyte suspensions, Vitetta *et al.* (1972) calculated that, if thymocytes bear any M-Ig, it would be less than 250 molecules per cell. This value is close to the estimates of Nossal *et al.* (1972) and Grey *et al.* (1972a) for T-cell M-Ig, and, accordingly, it might be concluded that the surface labeling and membrane elution technique, as performed by Vitetta *et al.* (1972) and Grey *et al.* (1972b), just falls short of threshold detectability for T-cell M-Ig. Marchalonis *et al.* (1972a,b,c; Marchalonis and Cone, 1973) have, however, readily demonstrated M-Ig from human and mouse thymus cell preparations using a similar method of cell surface labeling. From considerations of the maximum number of B cells that may have been present in the thymus cell preparations and compared to the labeling (or lack of labeling) of this number when B-cell preparations are studied, it is quite clear that the M-Ig detected by Marchalonis *et al.* from thymus cells cannot have been solely due to B-cell contamination, if indeed any of it was derived from this source.

Recent studies of R. E. Cone and J. J. Marchalonis (unpublished observations) have shown that B-cell Ig can be readily extracted from the cell surface with either nonionic detergents, such as nonidet, or by acid urea, whereas T-cell Ig cannot be isolated by the detergent treatment but can be obtained by carefully controlled conditions of urea–acetic acid extraction. At present, therefore, the possibility arises that the apparent discrepancies in the literature concerning isolation of T-cell M-Ig may be methodological in nature.

However, such considerations cannot explain the interpretation of Marchalonis *et al.* (1972a,b) that T cells and B cells have *equal* amounts of M-Ig, when all other approaches have clearly shown considerably less M-Ig in T-cell than B-cell preparations. It was suggested by Marchalonis and Cone (1973) that these discrepancies may relate to the presentation of M-Ig on the cell membrane. There is evidence to suggest that the cell coat on thymus cells is about twice as thick as that of B cells (Santer *et al.*, 1972; Wioland *et al.*, 1972), and it was considered that in thymus cells only part of the M-Ig may have been exposed (see also Greaves and Hogg, 1971b). This possibility is certainly consistent with the data of Bankhurst *et al.* (1971) in showing reactivity of thymus cells with anti-κ but not with anti-μ, and the Fc region of the H chain may, therefore, be inaccessible to the anti-Ig molecule. However, many of the studies with thymus cells have used anti-κ-chain serum, and if the postulate is correct that M-Ig on T cells is (all or part of) the antigen-recognition unit, then it becomes a little difficult to conceive of T cells being able to recognize and react with Ig molecules as antigens but not for Ig molecules (as antibodies) to recognize and bind to T cells. In their recent review, Marchalonis and Cone (1973) emphasize that the method of analysis used (surface iodination, M-Ig elution, and detection in coprecipitation assays) does not allow them to state that T and B lymphocytes possess the same number of M-Ig molecules, although they suggest that if a difference does exist, it is not of the order of magnitude suggested by the anti-Ig-binding studies.

A particular problem with quantitation of M-Ig in the cell surface iodination method is that there is no information on the specific activity of the labeled M-Ig detected in the coprecipitation assays. It may be that equal radioactivity content of the Ig detected from T and B cells represents very different numbers of Ig molecules but with differing specific activities (i.e., higher specific activity on the T-cell M-Ig).

In view of the controversial nature of some of these aspects, the existence, nature, or amount of T-cell M-Ig cannot be said to be fully

resolved. However, most of the available data are consistent with the thesis that in mammals, thymus cell preparations usually contain a small proportion of B cells that contribute to the total M-Ig detected in the population, and that many if not all, of the bulk of θ-positive T cells in the thymus also bear some M-Ig, probably differing in amounts, but with an average of around 200 to 500 molecules per cell.

Recent studies have suggested that the expression of M-Ig on thymus cells may quantitatively differ for mammalian and for amphibian species. DuPasquier *et al.* (1972) using immunofluorescence have shown that M-Ig are present on about 60 to 80% of thymus lymphocytes of 20 to 50 day postfertilization amphibian larvae. Ontogenic studies showed a sharp increase from less than 1% at 8 days postfertilization to around 70% by day 20. It was also noted that the degree of fluorescense was considerably less than that found in reactive spleen cells (? B cells). It will be of interest further to study thymus cells from more primitive animals to determine whether a relative change in degree of T-cell M-Ig expression has occurred through evolution or whether the results in amphibian larvae are associated with the nature of fetal animals that lack maternal–fetal interactions.

2. *Membrane-Bound Ig on Peripheral or Activated T Cells*

As the majority of studies demonstrating B-cell M-Ig have in essence shown that the non-B lymphoid population differs in this regard, it is evident that with most methods used, T cells from any source studied including peripheral activated population, lack high-density M-Ig. However, two questions might then be asked: (*1*) Do peripheral T cells as a population have less, the same, or more M-Ig than thymus cells? and (2) Does activation of T cells lead to increased M-Ig expression?

Comparisons of grain count distribution following radioautography of ^{125}I anti-κ-treated T cells from thoracic duct lymph of F1 animals given parental thymus (T-TDL) indicated that, in 20–50% of the cells, M-Ig appeared after long exposure times (Bankhurst *et al.*, 1971; Nossal *et al.*, 1972), and, by immunofluorescence with labeled anti-κ sera, 20–70% of activated T-TDL showed labeling as faint discrete spots distributed in clusters on the cell surface (Pernis *et al.*, 1973). In this latter study no cells with heavy diffuse staining (as for B cells) were detected. In the radioautographic studies of Bankhurst *et al.* (1971), whereas most of the labeled cells from thymus were large cells, large and small lymphocytes labeled similarly in T-TDL, although this latter population as a whole, is considerably rich in large

blastlike cells (Pernis *et al.*, 1973). In comparing normal TDL with the activated T-TDL population, Bankhurst *et al.* (1971) did not observe any significant labeling of the non-B-cell compartment in TDL, suggesting the importance of activation of T cells for expression of detectable M-Ig. Using hybrid antibodies in E/M studies, Hammerling and Rajewsky (1971) observed that, whereas only 2% of thymus cells bore M-Ig, about 95% of murine lymph node lymphocytes had surface-bound κ and μ determinants. Although it was noted that their method does not reflect quantitative differences of M-Ig per cell, it can be concluded from their studies that all peripheral T cells carry some IgM-type M-Ig that is greater in amount than that present on thymus cells (if the latter possess any M-Ig), but cannot be contrasted with amounts of B-cell M-Ig. However, in the studies of Nossal *et al.* (1972), no significant differences between labeling of thymus and T-TDL cells were observed, and Grey *et al.* (1972a) did not find significantly greater amounts of M-Ig on spleen or lymph node cells depleted of B cells by anti-Ig columns, as compared to thymus cells.

Several short-term *in vitro* culture studies have indicated the presence of M-Ig on PHA-activated human lymphocytes (Biberfeld *et al.*, 1971; Hellström *et al.*, 1971). Although normal peripheral blood contains around 20% of B cells, PHA-activated cultures after several days of incubation are presumed to contain T cells predominantly (see Greaves and Janossy, 1972). Hellström *et al.* (1971) found 35–50% of cells from 2-day cultures stained with anti-κ and 15–20% with anti-λ, whereas control cultures had only 3–5% M-Ig cells. A similar trend was observed by Biberfeld *et al.* (1971) although higher values were recorded, particularly in the control cultures. Titration of antisera indicated that the amount of accessible light chain increased on stimulation (Hellström *et al.*, 1971). Similar studies with mice, rats, or rabbits (Vischer, 1972; Jones and Roitt, 1972; Goldschneider and Cogen, 1973) did not confirm these observations, in that, whereas a marked increase in θ- or T-antigen-bearing cells was observed following PHA stimulation, the increase in M-Ig-bearing cells was concluded to represent some B-cell stimulation. With insolubilized PHA, it has been clearly shown that B cells can be stimulated by PHA (Greaves *et al.*, 1972). However, since these M-Ig studies used immunofluorescence, it cannot be excluded that the stimulated T cells carry small amounts of M-Ig. In cultures of rat lymphocytes stimulated by antigens, Goldschneider and Cogen (1973) have observed, with indirect immunofluorescence, that around 90% of the large blastlike lymphocytes had specific T-cell antigenic markers and also that 70–90% of these cells have detectable M-Ig. These studies clearly

showed that antigen-activated but not mitogen-activated T cells express detectable M-Ig.

As with thymus cells, the use of cell surface radioiodination has given conflicting results; for example, Vitetta *et al.* (1972) failed to detect any M-Ig on several sources of peripheral T cells, whereas Marchalonis and Cone (review, 1973) detected similar if not greater amounts of M-Ig on peripheral and on antigen-activated T-cell preparations. The considerations of the discrepancy and problem of quantitation of amounts is similar in this situation as for thymus (see preceding section). From these foregoing considerations, it might be tentatively concluded that peripheral T cells in general bear at least as much M-Ig as thymus cells, and probably a little more, particularly during antigen-induced blast transformation. However, at no time does the amount of M-Ig approach that of B cells.

3. *Origin of T-Cell M-Ig*

If it is assumed that the increased amount of M-Ig expression on peripheral T cells is no more than tenfold that of thymus cells, then T cells in general carry only of the order of 200 to 5000 molecules of M-Ig. In view of this small amount and of observations suggesting than even erythrocytes may carry some M-Ig (Nossal *et al.*, 1972), the origin and significance of the T-cell M-Ig are critical questions. The alternatives include derivation by synthesis from the cell bearing the M-Ig, binding of Ig from extracellular sources perhaps through Fc receptors, or a combination of both.

The presence of M-Ig in small amounts on several different cell types, such as red cells and thymus cells, does not necessarily imply that binding is nonspecific, as different mechanisms may be involved. Some data suggests that the Ig on red cells is predominantly IgG (Frommel *et al.*, 1967) in nature and this would be consistent with the concept of a different origin from T-cell M-Ig. The possibility of cytophilic binding of Ig to T-cell receptors was discussed in Section II,C, in which evidence clearly favors the existence of such receptors. However, in the studies on M-Ig of Vischer (1972) and Goldschneider and Cogen (1973), mitogen-stimulated T cells did not appear to bind any Ig from cultures that contained (pokeweed mitogen-) activated B cells. In discussing the experiments of Pernis *et al.* (1973), Miller (1973b) suggested that the M-Ig present on T-TDL was cytophilically derived, although experiments failing to demonstrate redevelopment of M-Ig on trypsinized T cells do not necessarily imply cytophilic binding of the original M-Ig. It may be that after antigen stimulation of T cells (via the M-Ig receptor), differentiation and activation may in-

volve activation of other systems, e.g., lymphokinesis, and further functional activity of T cells does not require M-Ig expression. Pretreatment of thymus cells on anti-Ig columns inhibited the development of M-Ig in T-TDL, although further studies will be necessary to validate the implication that specific cell removal was responsible.

Definitive proof of intrinsic origin of T-cell M-Ig will require synthesis studies on purified T-cell populations, and some data on this with malignant T cells have been obtained (data of Harris *et al.*, 1973, Section III,F,2), although other evidence also suggests that T-cell tumors can have M-Ig of cytophilic origin (Grey *et al.*, 1972b). Studies of Marchalonis *et al.* (Cone and Marchalonis, 1973) have indicated that the M-Ig eluted from antigen-activated T cells has specific binding properties for the immunizing antigen (Cone *et al.*, 1972; Feldmann *et al.*, 1973), but this evidence alone does not discriminate between synthesis of the M-Ig by the T cell or by a small proportion of B cells that produce an antibody that bind to the T-cell surface. As mentioned previously (Section II), Cone *et al.* (1974) have shown that M-Ig from B cells is not, however, cytophilic for T cells, at least in the 7 S monomeric form as derived from cell surface labeling of B cells. Furthermore, as the T-cell M-Ig (IgM type) was found to be in the 7 S monomeric form, this does not suggest cytophilic binding of polymeric serum (B-cell derived) IgM. Further evidence for the distinction of T-cell M-Ig from B-cell M-Ig is the property of binding of T-cell M-Ig to macrophages (see Section II). However, as discussed in Section II, in view of other studies, it is not clear why B-cell IgM did not bind; further investigation of possible requirements of other factors (Lay and Nussenzweig, 1969) and comparison between monomeric and polymeric IgM would be of interest.

At the present time, the indications are that, although some of the M-Ig on T cells may be derived from extrinsic sources, much of the detectable M-Ig is of intrinsic T-cell origin.

E. Lymphoid Cells In Culture

Many studies have shown that lines of lymphoid cells can be established in culture that continue to synthesize and secrete Ig. Often multiple classes of Ig are synthesized, even from cloned lines (Tanigaki *et al.*, 1966; Wakefield *et al.*, 1967; Finegold *et al.*, 1968; Bloom *et al.*, 1971). Much of the evidence suggests that regardless of the origin of the cells, i.e., from normal or malignant patients, most of the human lymphoid cell lines are derived from nonmalignant cells, although several clearly malignant tumor lines are in culture (Fahey *et al.*, 1971). Information on tumor lines will be considered in the fol-

lowing section. The human cultured lines are generally lymphoblastic in appearance and probably represent early stages in lymphoid cell maturation. Whether the multiple nature of Ig synthesis in some lines indicates that the lines arise from cells in the state of switch from expression of one class to another, whether the cells represent early stages before restriction has occurred, or whether a specific mechanism of derepression of Ig loci *in vitro* occurs, is not resolved.

Whereas total protein synthesis is usually fairly constant throughout the cell cycle, several studies have shown that Ig is synthesized only during the late G1 and early S portions of the cell cycle (Buell and Fahey, 1969; Lerner and Hodge, 1971). Several studies have been made to determine whether cultured cells bear M-Ig and how this might relate to synthesis and secretion of Ig by the cells (see review by Lerner, 1972). By using quantitative inhibition radioimmunoassays, Lerner *et al.* (1971) have studied M-Ig on several cultured diploid lines that appear to be arrested in differentiation somewhere between the G_0 lymphocyte and the plasma cell. Considerable variation was found among different cell lines and among different clones of a given cell line. As every cloned line studied had some M-Ig, it was suggested that every cell was capable of synthesizing M-Ig and that cells found negative by immunofluorescence were simply in a phase of the cell cycle where Ig was not synthesized. The amount of M-Ig κ chain was frequently in excess of γ Fc determinants suggesting either incomplete membrane presentation of the Ig molecule, presence of other H-chain classes, or free L chains. In other studies, Hutteroth *et al.* (1972), using the mixed antiglobulin technique, demonstrated M-Ig on ten lines, all having κ chain, and eight with μ chain; they claimed that the proportion of cells with κ and μ remained relatively constant throughout the cell cycle.

Cultured cell lines may be of particular advantage for studies on the role of the M-Ig receptor in relation to Ig activation of the cell. Several studies have suggested that the maintenance of M-Ig and secretion of Ig by the cell may be under independent genetic control. Lerner *et al.* (1972) showed that, with various inhibitors of protein synthesis or of cell cycle, cytoplasmic and secretory Ig can be a considerably depleted without affecting M-Ig. It was suggested that during logarithmic growth of the cells at least 90% of the cellular Ig is to be secreted and is not necessary for saturation of M-Ig sites. A lack of relationship between M-Ig and secreted Ig was also noted by Litwin and Cleve (1973) in that several lines with κ or $\kappa\mu$ M-Ig secreted large amounts of γ chains. These results are reminiscent of the studies of Pernis *et al.* (1971) and Jones *et al.* (1973b) in showing

IgM-type M-Ig on IgG-secreting plasma cells and further raise the possibility that these cell lines may represent useful models of activated normal lymphoid cells possibly at the stage of Ig H-chain switch.

F. Neoplastic Lymphoid Cells

Studies of M-Ig on malignant lymphoid cells have been actively pursued with two general aims in view. (*1*) If malignant cells represent a clonal proliferation of normal lymphoid cells arrested at a specific point in differentiation of either T or B pathways, studies of a range of these tumors may provide pure populations of cells representing these various stages of differentiation and, accordingly, permit many critical studies of the nature of the M-Ig expressed at this stage. This premise considers that gene expression in these malignant cells is representative of that in the normal lymphoid counterpart. (2) From the viewpoint of the malignant cells, these studies may help to characterize the nature of the malignant cell type and in so doing, perhaps, indicate subtypes of the disease, which may, in turn, assist in discriminating between some of the more heterogeneous aspects of these various lymphoproliferative disorders.

1. Human Lymphoproliferative Disorders

The majority of published reports on M-Ig in lymphoid cell neoplasms of man have concerned Burkitt's lymphoma (BL), chronic lymphocytic leukemia (CLL) and lymphosarcoma (LS), although a few other conditions have been examined. In both BL and CLL, considerable variations in the incidence of cases with M-Ig-positive cells and in the class of Ig involved have been found. It appears at present, however, that many of the variations are due to secondary effects and that, in general, both BL and CLL express M-Ig of one L- and one H-chain class, predominantly IgM.

a. Burkitt's Lymphoma. Studies of M-Ig on fresh biopsy samples of many BL patients frequently showed the presence of several Ig's, usually IgM and IgG on the cell surfaces (Klein *et al.*, 1966). However, when tissue culture lines of BL biopsy samples were developed, it was found (Klein *et al.*, 1968) that the membrane-associated IgG was not maintained, and it became evident that this was predominantly antibody to antigens (viral-associated) of the BL cell (Klein *et al.*, 1969). It was observed, however, that the IgM component of the M-Ig persisted for many months in culture (Klein *et al.*, 1968), and if the cells were treated with trypsin, the M-Ig soon reappeared on culture (Osunkoya *et al.*, 1969), clearly indicating its intrinsic origin from the

cell. Similar observations on loss of initial IgG coating but retention of IgM-type M-Ig have been made with BL lines established in serial passage in immunodeficient athymic nude mice (Poulsen *et al.*, 1973). The amount of IgM on the cell surface was calculated from various cell disruption or direct hemagglutination inhibition studies to be about 10^5 molecules per cell, with the IgM being in monomeric form on the cell membrane (Klein *et al.*, 1970; Esekland and Klein, 1971). A similar value was determined by Sherr *et al.* (1972) using radioiodination of cell surface proteins, and, in these studies, it was further inferred from iodination ratios of μ/κ chains that the membrane-associated IgM was well exposed on the cell surface. Comparisons of intracellular Ig with M-Ig suggested that only a small proportion of the total Ig is associated with the cell surface, an observation consistent with the thesis of Lerner *et al.* who, using normal cultured cells, suggested that M-Ig may be independently regulated from total cellular Ig. Studies on Ig synthesis by BL biopsies or cultures have indicated (Van Furth *et al.*, 1972) that some lines can synthesize and secrete IgG, but bear membrane-associated IgM—another example of the situation shown by Pernis *et al.* (1971), Jones *et al.* (1973b), and Litwin and Cleve (1973).

Although there is still some controversy over the question of monoclonality of the origin of BL cells in a given patient (see Fialkow *et al.*, 1971; Van Furth *et al.*, 1972), it is likely that many of these lines bear some IgM-type M-Ig, possibly at very low levels in some cases, although the cells can synthesize other Ig classes. These studies have also clearly indicated a mechanism for extrinsic acquisition of M-Ig by the cell, namely, as antibodies directed to other viral-associated cell surface antigen, and this possibility will need to be carefully considered in any situation where M-Ig is detected on a malignant cell.

b. Chronic Lymphocytic Leukemia. In following their studies of BL, Klein *et al.* (1970) observed that many of the PBL cells from occasional CLL patients had IgM-type M-Ig, which was again monomeric in type and at a cell surface concentration of around 10^5 molecules per cell (Esekland *et al.*, 1971). Although their original studies showed only 1 of 11 CLL patients to have M-Ig on most of the PBL cells (Johannson and Klein, 1970), it was noted that most of the patients had some but very weak membrane staining of their cells with fluorescent anti-Ig. Further studies by various groups have clearly indicated that most patients with CLL have M-Ig associated with the majority of the CLL cells in blood but that the amount of M-Ig on the CLL cells is usually less than that on the average normal B cell. These results are summarized in Table X, and it can be seen that a total of

171/192 patients were recorded as having M-Ig associated with the CLL cells, and in the majority of these studies it was concluded that almost all cases of CLL are malignancies of the B-cell series. This interpretation might be questioned on several grounds. Theoretically, if activated T cells can express some M-Ig, then CLL may be very stimulated T cells. It has also been suggested (Rubin and Schultz, 1972) that, since some data indicate functional deficiencies in T-cell activities (PHA stimulation in CLL patients; Rubin, 1970), the cells could be incompletely functioning or incompletely differentiated T cells. The observation on deficient PHA reactivity has now been shown to be due to a low percentage of T cells in the blood, and, if enriched populations of T cells are used (Wybran and Fudenberg, 1973), their reactivity is seen to be normal. However, to be certain of the nature of the CLL cells, non-Ig T or B marker studies are necessary. Results for these have now been obtained and conclusively indicate that virtually all cases of CLL studied are of B-cell origin. Thus, it was shown that CLL cells do not carry specific T-cell antigens detected with heterologous antisera (Williams *et al.*, 1973; Aisenberg *et al.*, 1973; Touraine *et al.*, 1973; Rabellino *et al.*, 1973; Ross *et al.*, 1973) nor do they generally react with sheep erythrocytes to form T-cell rosettes (Dickler *et al.*, 1973; Ross *et al.*, 1973), although one such case was found (Dickler *et al.*, 1973). Using B-cell markers of complement receptors (CRL) or binding of aggregated Ig, it was observed in four studies (Pincus *et al.*, 1972; Shevach *et al.*, 1972b; Ross *et al.*, 1973; Dickler *et al.*, 1973) that 37 of a total of 42 cases of CLL were clearly of the B-cell type. In all of the CRL studies, it was noted that the use of mouse complement was necessary to provide sufficient sensitivity. In the studies of Ross *et al.* (1973), where 8 of 11 cases gave CRL, it was noted that with sensitive methods, all showed M-Ig. It is thus likely that in all of the cases studied except the one case of Dickler *et al.*, the cells were B cell in type.

These studies on lack of T markers and presence of B markers in virtually all cases of CLL clearly confirms the interpretation that the presence of M-Ig on most of these cells is indicative of B-cell origin. As noted in the studies of Wilson and Nossal (1971), Aisenberg and Block (1972), Cooper *et al.* (1973), Dickler *et al.* (1973), and Ross *et al.* (1973) the amounts of M-Ig on the CLL cells appears to be somewhat less than that of the bulk of normal B cells, and a plausible thesis might be that these cells are of the B-cell series but arrested at the stage of differentiation just prior to plasma cell development, but after activation. It is also relevant to note that, whereas CRL comprise only about half of the normal B cells, most of the CLL cells have this

TABLE X

INCIDENCE OF ELEVATED PROPORTION OF MEMBRANE-BOUND IMMUNOGLOBULIN-POSITIVE CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Incidence of patients with elevated M-Ig[a]				
Any Ig	Predominantly IgM	IgM and other Ig	Predominantly other Ig	Reference
1/11	–	–	–	Johansson and Klein (1970)
3/3	–	–	–	Wilson and Nossal (1971)
10/11	–	–	–	Dickler *et al.* (1973)
13/16	7/7	–	–	Pernis *et al.* (1971)
20/20	15/20	–	2/20 (IgD)[b]	Grey *et al.* (1971)
25/25	22/25	3/25 (IgD)	–	Aisenberg and Bloch (1972)
19/19	15/19	3/19 (IgG)	1/19 (IgG2)	Froland *et al.* (1972)
70/73	28/73	13/73 (IgG)	25/73 (IgG)	Preud'homme and Seligmann (1972b)
10/14	6/14	4/14 (IgG + IgD)	–	Piessens *et al.* (1973)
87%	57%	18%	25%	(% of all cases studied)

[a] The values show the number of patients with significantly elevated proportions of M-Ig-positive cells over the total number of patients studied for each of the Ig classes examined.

[b] The values of IgD are from H. M. Grey (personal communication).

property—again indicating that CLL do not represent the range of B-cell heterogeneity.

Studies on the class distribution of M-Ig on CLL cells have demonstrated some interesting complexities in relation to Ig expression by these cells. The results are summarized in Table X and show that a total of 116 of 158 cases examined with different μ-chain reagents were of IgM-type M-Ig, with 93 of the 116 being only of IgM as the predominant cell type represented in the PBL. In studies of κ/λ distributions, it is usually observed that where only one class is present on the cell, only one L-chain type is also present—of a total of 120 cases examined in the groups studied in Table X, 67 were of κ type and 53 of λ type.

The existence of a single H- and L-chain type in the M-Ig is clearly consistent with a monoclonal origin of the cell and with restriction of Ig expression by the cell. In the studies of Froland *et al.* (1972) the one case with IgG was shown to be only IgG2, and probably also showed allelic exclusion. Some discrepancies exist between the studies in terms of relative expression of chain types, although recent studies have suggested that these apparent differences do not necessarily indicate major subtypes in the CLL series. Grey *et al.* (1971) found that, of 20 cases with M-Ig, 5 showed only L-chain expression, although, as noted in Table X, recent data using quantitative immunoprecipitations on radio-iodinated cell surface protein preparations (H. M. Grey, personal communication) have indicated that at least 1 and probably 2 of these cases have predominantly IgD as the M-Ig. In this context, it was also observed by Aisenberg and Bloch (1972) that 3 of 25 cases had grossly elevated proportions of cells bearing IgD and that several others, and some in the studies of Piessens *et al.* (1973), had moderately elevated proportions of IgD. The association of membrane-bound IgD on some CLL cells and normal cells of newborn PBL (Rowe *et al.*, 1973) is reminiscent of the demonstration of other fetal lymphocyte antigens on CLL cells, and the significance of IgD expression is at present unknown but fully warrants further investigation.

The studies of Preud'homme and Seligmann (1972b) indicate that about one-third of the cases had only IgG-type M-Ig, and that 20% of the cases had multiple Ig classes present. It is possible, however, that, in many of these cases, the M-Ig synthesized by the cell may only be IgM. In 7 cases of monoclonal IgG-type M-Ig, trypsinization of the cells followed by culture showed synthesis of γ chains in 5 cases but in the other 2 cases membrane-bound IgM appeared. When similar studies were performed on cells from cases where both IgM and IgG were de-

tected on the surface, it was found that the cell synthesized only IgM and that in some cases the IgM had antibody activity against normal IgG (Preud'homme and Seligmann, 1972c). In another case the IgG was probably bound to the cell surface as an AgAb complex, and studies on the distribution of IgG on the 2 cases of IgG-type M-Ig with synthesis of IgM type indicated that the original IgG may have been bound to the membrane as an antibody to a cell surface antigen.

Although it is not excluded that there may be true cellular heterogeneity in the cases of CLL, the bulk of the studies could be interpreted as indicating that the great majority—perhaps over 90%—of CLL synthesize IgM as their membrane Ig. In some cases, the additional presence of IgG, may mean that (*a*) the cell synthesizes and secretes IgG, (*b*) the IgM has antibody activity to normal IgG, (*c*) the cell has bound AgAb complexes, or (*d*) the IgG is an antibody to a cell surface component (?tumor antigen). The existence of a few lines synthesizing only IgG or possibly IgD also appears to occur, and, in a few cases, a true biclonal population may be present (Preud'homme and Seligmann, 1972b). This interpretation therefore favors the view that most CLL are B cells, possibly being comparable to the stage of activated B cells that have become arrested or blocked from full differentiation into antibody-secreting cells. In this context, it would be of particular interest to determine whether the property of CRL, as shared by most CLL, is that of early or late differentiated B cells in the normal population. It should also be noted that occasional cases of CLL appear to lack any B-cell markers, although careful examination for low levels of M-Ig should be made. One probable case of a T-cell CLL has been reported (Dickler *et al.*, 1973).

c. Other Tumors. Several groups have commented briefly on M-Ig in acute lymphocytic leukemia (ALL). Wilson and Nossal (1971) and Gutterman *et al.* (1973) did not detect any M-Ig on ALL cells, and Cooper *et al.* (1973) mention the presence of only very small amounts, although these authors claim there is some evidence for these cells to be considered as B cells. Preud'homme and Seligmann (1972b) reported that 3 cases of acute "lymphoblastic" leukemia all had M-Ig. It is again evident that absence or presence of M-Ig will not be sufficient to characterize the B- or T-cell nature of the disease, and multiple marker studies will need to be performed.

In three reports of lymphosarcoma, it was again demonstrated that some cases had detectable M-Ig (totaling 20/34) whereas others did not (Aisenberg and Bloch, 1972; Piessens *et al.*, 1973; Cooper *et al.*, 1973). Many cases described by Piessens *et al.* seemed to have multiple Ig classes, and Cooper *et al.* (1973) showed that the amount on

the surface was considerably more than that on the surface of CLL cells, possibly suggesting these cells were more analogous to activated B cells as found, for example, in tonsil.

Finally, as observed at the start of this discussion, (Klein *et al.*, 1968) tumor cells can carry M-Ig derived as an antibody response to tumor antigens. This has now been inferred in several other situations, such as acute myelogenous leukemia (Gutterman *et al.*, 1973), and again it should be noted that several explanations other than cell synthesis can explain presence of M-Ig.

2. *Animal Lymphoid Tumors*

Extensive studies on the induction and characterization of surface antigens of mouse lymphoid tumors have been made, although only relatively recently have these tumors been examined for M-Ig. The presence of other markers can be used to distinguish T-cell tumors (TL,θ) although distinction between B-cell tumors and monocytic tumors is a more controversial matter. The presence of receptors for Ig on both of these latter types has made their distinction difficult (see Section II). Shevach *et al.* (1972a) concluded that tumors binding EA were of monocytic derivation, although studies of Cline *et al.* (1972b) suggest they could be B cells. In comparing 24 mouse lymphomas and leukemias, Shevach *et al.* (1972c) observed only 1 of these to have M-Ig and this line did not have the θ antigen. Of 12 tumors with θ antigen, none had detectable M-Ig. However, studies by Harris *et al.* (1973) have shown a θ-positive thymoma that also synthesizes and bears M-Ig of IgM type. This tumor also binds EA rosettes, and an interesting possibility, in the light of studies by Preud'homme and Seligmann (1972c), is that this tumor, like some CLL, has an IgM of anti-γ-globulin specificity (Warner and Harris, 1973). Many of the θ-bearing murine thymomas studied by Harris *et al.* (1973) did not, however, synthesize Ig or bear detectable M-Ig when conventional methods were used, but several of these tumors appear to have some M-Ig as detected by surface radiolabeling (A. W. Harris and J. J. Marchalonis, unpublished observations). Further studies are clearly necessary, but it is suggestive that, in mice, malignant lymphoid cells, just as normal lymphoid cells, may have either high-density M-Ig if B cells or low-to-moderate density if T cells, possibly representing activated T cells.

Studies in other species are limited. One report on bovine lymphosarcoma cells suggests these have at least L-chain surface determinants (Tsuyuguchi *et al.*, 1973), and a report on a guinea pig lymphatic leukemia indicates clearly that it is a B-cell leukemia in bearing

receptors for complement and having IgG2-type M-Ig (Shevach *et al.*, 1972d).

G. Immunodeficiency and Other Disease States

Studies on M-Ig-bearing cells in experimental animals have clearly shown that T- and B-cell deficiency states are reflected, respectively, in an increase or decrease in the proportion of high-density M-Ig cells in blood and lymphoid tissues. Thus, for example, complete failure of thymus development in athymic nude mice leads to a proportion of about 95% M-Ig-bearing cells in thoracic duct lymph (Bankhurst and Warner, 1972), whereas total prevention of bursal development in chickens results in a virtual abolition of M-Ig cells in blood (Rabellino and Grey, 1971; Kincade *et al.*, 1971; Bankhurst *et al.*, 1972).

These studies suggest that similar results would be found in man if T- or B-cell function had totally failed to develop. However, as the development of antibody-forming plasma cells requires further differentiation after the appearance of B cells, selective failures at these later steps may occur. Much of the interest in studying M-Ig on cells in human disease states is to determine (*a*) the cellular level of abnormal changes in immunological disease states and (*b*) from this to determine whether all similar clinical expressions of an immunodeficiency necessarily indicate a common abnormality at the cellular level.

1. Human Immunodeficiency States

In man as in mice, it appears that total or near total failure of thymic development is associated with a marked increase in M-Ig-bearing cells in peripheral blood. Cooper and Lawton (1972b), studying a di George patient (Gatti *et al.*, 1971), found 91% M-Ig-positive cells, and Gajl-Peczalska *et al.*, (1972) with a similar patient found a total of 84% positive cells. Two patients with severe combined immunodeficiency also showed averages of 84% (South *et al.*, 1972) and 94% (Preud'homme *et al.*, 1973) M-Ig-positive cells. It is, therefore, evident from these few cases, that absence or marked reduction of T cells in man does not prevent expression of B-cell M-Ig.

In studying patients with various humoral immunodeficiency disease states (see Fudenberg *et al.*, 1971, for WHO classification), a wide range of possible M-Ig patterns has been observed. The results of 139 patients are summarized in Table XI, and several patterns are evident. In X-linked agammaglobulinemia (Bruton), functional

studies show in general the obverse situation to T-cell deficiencies, and, as anticipated from the avian studies, around 90% of these patients show either a total lack or considerable reduction in M-Ig-bearing cells in blood. Thus the immunodeficiency in most of these patients can be ascribed to a failure to develop B lymphocytes. Three patients reported by Siegal *et al.* (1971) and at least 1 (probably 3) patients described by Geha *et al.* (1973) present an unexpected situation. The clinical picture of these cases was not felt to differ from that of the others in this general group. However, it is clear that, in these 6 patients, (?B) cells bearing M-Ig develop but apparently do not function. This indicates that an X-linked gene may in some manner control an intracellular event concerned in assembly, transport, or secretion of the Ig molecule. It is of interest to note in this regard that examples of X-linked gene control of Ig production or expression have been described (Amsbaugh *et al.*, 1972; Grundbacher, 1972).

In the variable immunodeficiency disease category, several different patterns are evident. In about one-third of the cases, M-Ig-bearing cells fail to develop, but, in the remaining two-thirds, B-cell expression of M-Ig is normal or somewhat reduced. Studies on Ig synthesis, with this latter group (Choi *et al.*, 1972) show that Ig synthesis occurs but secretion of the Ig fails to develop. It is relevant to consider the thesis of Lerner *et al.* (1972) that regulation of M-Ig and secretion of Ig may be under independent regulation (see Section III,E). It would be of interest to determine whether this group is homogeneous in possessing B cells as shown by other markers, and, thus, whether the variability in M-Ig expression reflects quantitative differences in Ig activation. Alternatively, these different patterns may reflect completely different abnormalities in B-cell differentiation that lead, however, to a similar clinical presentation.

Studies with selective Ig deficiencies have either revealed an occasional depression in M-Ig cells, e.g., in Wiskoff Aldrich syndrome (Preud'homme *et al.*, 1973), or more usually normal if not elevated proportions. The selective failure in serum IgA levels, would seem to be related to a failure of IgA-bearing B cells to differentiate into IgA-secreting plasma cells. Whether this is a defect in the B-cell lineage itself or in another cell system that is involved in controlling activation of IgA-bearing cells, such as T cells (Crewther and Warner, 1972), is unresolved at present.

2. Other Diseases

In many disease processes where immunological activity occurs, it may often be of value to determine whether the activity or lack thereof

TABLE XI
MEMBRANE-BOUND IMMUNOGLOBULIN-BEARING CELLS IN HUMAN IMMUNODEFICIENCY DISEASE STATES

Disease	No. patients	No. with M-Ig-bearing PBL[a]			Reference
		Absent	Low	Normal	
X-Linked agammaglobulinemia	4	4	0	0	Grey *et al.* (1971)
	6	3	0	3	Siegal *et al.* (1971)
	2	2	0	0	Aiuti *et al.* (1972)
	7	7	0	0	Cooper and Lawton (1972b)
	6	6	0	0	Froland and Natvig (1972a)
	11	4	7	0	Gajl-Peczalska *et al.* (1973a)
	10	7	0	3	Geha *et al.* (1973)
	9	9	0	0	Preud'homme *et al.* (1973)
Total	55	42 (76%)	7 (13%)	6 (11%)	
Variable immunodeficiency	2	0	0	2	Cooper *et al.* (1971b)
	6	0	4	2	Grey *et al.* (1971)
	6	0	4	2	Siegal *et al.* (1971)
	4	2	1	1	Aiuti *et al.* (1973)
	2	2	0	0	Froland and Natvig (1972a)
	9	1	7	1	Gajl-Peczalska *et al.* (1973a)
	18	7	3	8	Preud'homme *et al.* (1973)
Total	47	12 (29%)	19 (38%)	16 (33%)	
Wiskott-Aldrich syndrome	6	0	2	4	
Selective Ig deficiency: IgA	23	0	1	22	
Ataxiatelangiectasia	8	0	0	8	

[a] The values show the number of patients reported with virtually no M-Ig-bearing cells detected (absent, i.e., 1%); with significantly reduced levels (low, around 1–10%), or in the normal range (above 11%). PBL, peripheral blood lymphocytes. Values for latter three diseases are summaries from several reports.

involves either or both of the T- and B-cell series. Several studies have reported on proportions of M-Ig-bearing cells in certain diseases. In rheumatoid arthritis, although the proportion of B cells in peripheral blood is approximately normal (Mellbye *et al.*, 1972; Williams *et al.*, 1973), many patients show the presence in blood of a considerable proportion of cells lacking either M-Ig or the T-cell-specific antigen (Williams *et al.*, 1973). The nature of these "null cells" is not resolved, and a similar picture of a high proportion of lymphocytes lacking both θ and M-Ig in autoimmune NZB mice has been described by Stobo *et al.* (1972).

Proportions of M-Ig-bearing lymphocytes in systemic lupus erythematosus also appear to be normal, and in these patients only a few examples of the existence of null cells was found (Williams *et al.*, 1973).

Four studies of mycobacterial infections have been reported. In the one report of active tuberculosis (Williams *et al.*, 1973), no significant changes in B-cell proportions were observed, whereas in all three studies of lepromatous leprosy (LL), a significant increase in the proportion of M-Ig-bearing cells was observed: (*1*) 31–61% of LL positive, controls 18–31% (Verma *et al.*, 1971); (2) 28–80% LL positive, controls 20–33% (Dwyer *et al.*, 1973); and (3) 32–86% LL positive, controls 29 ± 7% (Gajl-Peczalska *et al.*, 1973b). These studies suggest either a marked stimulation of the B-cell series or an overcompensation in the face of a possible T-cell deficiency.

Studies on sarcoidosis (Papamichael *et al.*, 1972) and early infectious mononucleosis (Piessens *et al.*, 1973) both showed also elevations in proportions of M-Ig-bearing cells.

It is likely that further exploitation of studies of *both* T- and B-cell proportions in various disease states may provide useful information in understanding the processes involved in the particular condition and, possibly, may also be of value in monitoring therapeutic approaches for a return to normality.

H. β_2-Microglobulin

Several recent studies have indicated that lymphoid cell lines may not only be expressing conventional Ig on the cell membrane, but may also bear products of other genes that were possibly evolutionarily related to Ig H-chain genes. Such is the case for β_2-microglobulin.

In various patients with renal tubular disorders, moderate amounts of a β_2-globulin have been found in the urine, and this protein has been purified and characterized as a single polypeptide chain of mol. wt. 11,600, termed β_2-microglobulin (Berggard and Bearn, 1968). This protein has since been sequenced (Smithies and Poulik, 1972a; Pe-

terson *et al.*, 1972) and clearly shown to be homologous to the constant portions of Ig chains, particularly to the C_{H3} region of the γ chains. Peterson *et al.* (1972) suggested β_2-microglobulin is analogous to a free Ig domain, and it is of particular interest that the molecule does not exist as a covalent bonded dimer, as found in the Fc fragments of H chains. Although this molecule shows no antigenic cross-reaction with any Ig proteins (using either anti-Ig sera or specific anti-β_2-microglobulin sera), computer analysis of the sequence clearly shows little doubt that the β_2-microglobulin gene is evolutionarily related to Ig genes. A related protein of similar sequence has also been isolated from the dog (Smithies and Poulik, 1972b).

The relation of this protein to lymphoid cells has been shown using cultured lines of human lymphoblastoid cells or stimulated normal lymphocytes. It was shown by Bernier and Fanger (1972) that normal human lymphocytes cultured *in vitro* synthesized and secreted β_2-microglobulin that was antigenically and physicochemically identical to the urinary protein. Stimulation of lymphocytes with PHA markedly increased elaboration of this protein. Cultured human lymphoid cell lines were also shown to secrete β_2-microglobulin, and, using specific anti-β_2-microglobulin antisera, the presence of the protein on the cell membrane of viable cultured lymphoid cells was shown (Poulik and Bloom, 1973). Similarly, lymphoid cells from normal individual fetal thymocytes and from CLL patients carried membrane β_2-microglobulin (Poulik *et al.*, 1973). These studies imply that β_2-microglobulin is produced by and carried on the membrane of both T and B lymphocytes. The membrane-bound β_2-microglobulin does not, however, appear to be under the regulatory controls that affect M-Ig. Hutteroth *et al.* (1973) showed that a series of lymphoid cell lines secreted relatively uniform amounts of β_2-microglobulin but differed markedly in Ig expression. Modulation with anti-Ig sera also failed to affect membrane-bound β_2-microglobulin.

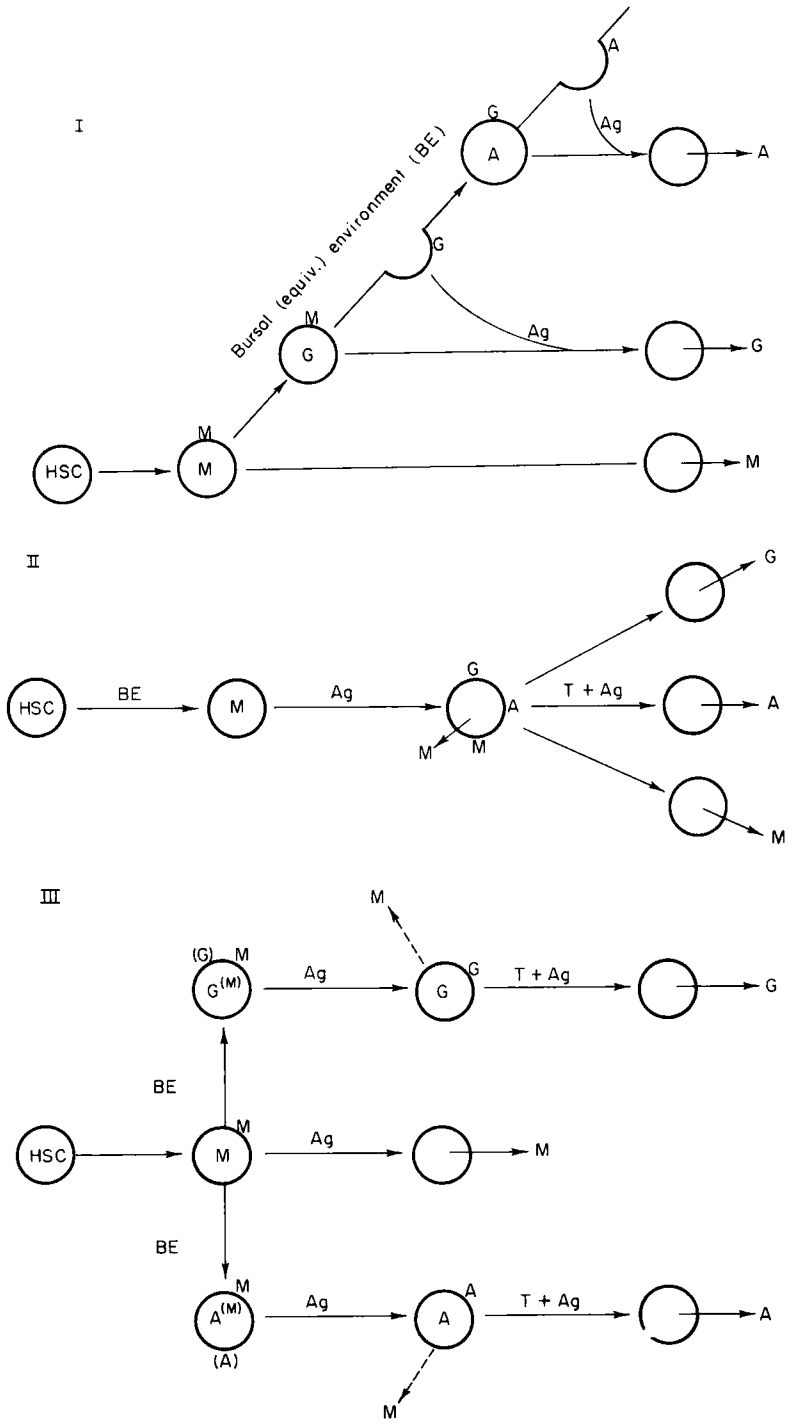
The function of this protein in lymphoid cells is at present quite unknown. However in view of its expression on the membrane of T and B lymphoid cells and possibly in greater amounts after activation, it will be of particular interest to determine whether or not it has a role in any of the membrane activities or properties of these cells, such as regulation of release of M-Ig or binding of various entities to the cell surface, e.g., complement, the Fc of aggregated or complexed Ig, or even antigens.

IV. B Lymphocyte Maturation

The data in the preceding sections on M-Ig-bearing cells indicate that at the immunocompetent B-cell level, most cells are expressing

either IgM, IgG, or IgA as M-Ig. When antigen-induced differentiation occurs, the formation and secretion of specific antibody is usually initially of IgM type followed by other classes of Ig (see Nossal *et al.*, 1971a; Sell, 1970a). At the cellular level, the bulk of antibody-forming cells are producing antibody of only one class and allotype (see Section III). In considering the relationship between these two stages of B-cell differentiation, two alternative views have been considered which essentially differ on the role of antigen in inducing differential Ig class expression in lymphoid cells. The first view is an extension of the clonal selection concept that receptor equals antibody. This receptor hypothesis (see Makelä and Cross, 1970) essentially considers that (*a*) at the time of immunocompetent cell differentiation, one L- and one H-chain gene are expressed by the cell and this same pair of genes continues to be expressed as long as the clone is functional, and (*b*) that these Ig gene products are held on the cell surface as antigen receptor and, on combination with antigen, the cell is triggered to transformation leading to clonal proliferation and secretion of an antibody that is identical in specificity (as shown by Dutton and Eady, 1964; Brownstone *et al.*, 1966; Mitchison, 1967), class, and allotype to the receptor antibody (Mäkelä, 1970). This thesis thus assumes that, in the immunocompetent animal prior to antigen presentation, separate specific IgM, IgG, and IgA precursor cells exist. This thesis must therefore consider the origin of these cells, and Cooper *et al.* (1971a, 1972b) have proposed a model of plasma cell differentia-

FIG. 4. Models of B cell-to-plasma cell differentiation. (I) Proposed model of Cooper *et al.* (1971a, 1972b). Hematopoietic stem cells (HSC) differentiate within the bursal environment by clonal development involving first IgM (M) activation with some IgM on the cell surface followed by expression of IgG (G) and *then* IgA (A). The earlier stage of IgG-producing cells may still bear membrane IgM and, similarly, the early IgA cells, membrane-bound IgG. Peripheralization of all of these elements is occurring during embryonic–fetal life, and subsequent antigen activation of each precursor will lead to plasma cell differentiation. (II) Differentiation of plasma cells via multipotential activated B cells. In this scheme (Warner, 1972a), IgM-bearing immunocompetent cells activate IgG and IgA expression only *after* antigenic stimulation, and the model applies to clonal proliferation in the adult animal for each specific antigen response. The B cells are activated by antigen to express IgG and IgA, and at this stage the cell bears M-Ig of several classes, then, under further antigen stimulation and T-cell control, separate IgA and IgG differentiation to plasma cells occurs. (III) This scheme envisages an activation of IgG and IgA genes during embryonic B-cell differentiation. However, the differentiation is random and not sequential G to A as in Scheme I. Furthermore, the membrane-bound receptor remains predominantly IgM on all cells. Activation with antigen then induces all cells to secrete IgM and, in cells containing IgG or IgA, these Ig classes are then carried as the membrane receptor, and, under the influence of antigen *and* T-cell factors, will further differentiate into IgA- or IgG-secreting plasma cells.


I
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tion that incorporates a sequential activation of μ-, γ-, and α-chain genes with cell differentiation principally in the fetal and neonatal period. This is depicted as Scheme I in Fig. 4. A second view of plasma cell differentiation differs from the receptor equals product concept only in regard to Ig class, in that a cell ultimately producing IgG antibody is not necessarily derived from a precursor cell bearing surface IgG but rather from a cell bearing IgM (see Warner, 1972a; Pierce *et al.*, 1973). This is depicted as Scheme II in Fig. 4 and, therefore, differs from Scheme I in proposing that antigen is one of the essential components in inducing expression of other H-chain classes.

Before considering these models in more detail, several experimental approaches to this problem will be described.

A. Effects of Anti-immunoglobulins on B-Cell Functions

Studies of the possible inhibitory activity of anti-Ig of varying specificities on the differentiation and on functions of B cells have involved three general approaches which will be briefly considered. Studies on the possible *in vivo* or *in vitro* effects of anti-Ig on T-cell functions will not be included in this section (see Section V,D).

1. Allotype Suppression

The phenomenon of allotype suppression results from the exposure of the fetal or neonatal animal to antiallotype antibody directed against one or both of the parental allotypes in the genome of the animal. This phenomenon was first described in rabbits (Dray, 1962; Mage and Dray, 1965) and involved the mating of females immunized with Ig allotypic antigens (*a* or *b* loci) to males bearing that particular allotype. The progeny were found to be extremely deficient in serum levels of the paternal allotype for many months or years. Suppressed rabbits usually compensate for the reduction of Ig by the production of greater amounts of the maternal allelic product (Mage, 1967) or, in the case of suppressed homozygotes, by more of the other Ig classes (Dubiski, 1967; David and Todd, 1969). In relation to our consideration of M-Ig, it might be questioned whether suppression of serum Ig results from a complete absence of the particular B-cell lineage producing that Ig or, as in some of the variable hypogammaglobulinemic patients, whether failure of B-cell differentiation to plasma cells is involved. At the mature plasma cell level, it was shown that a decrease in number of plasma cells producing the particular suppressed allotype occurs (Lummus *et al.*, 1967). From *in vitro*

lymphocyte stimulation studies with antiallotype sera, it was inferred that B lymphocytes bearing M-Ig of the suppressed allotype were also considerably reduced in number (Sell, 1968; Marcusson and Roitt, 1969). This has recently been directly demonstrated by Harrison *et al.* (1973), who showed that b^5-bearing PBL were virtually absent from completely b^5-suppressed rabbits and that, during spontaneous escape from suppression, the appearance of b^5 M-Ig-bearing cells rapidly recovered to normal levels, whereas the circulating serum level remained chronically and disproportionately depressed. These four studies imply that antiallotype suppression in rabbits may be caused by the interaction of anti-Ig with immature M-Ig-bearing cells and, in some manner, eliminate all M-Ig-bearing cells of that specificity and thereby prevent appearance and development of that particular lineage. Whether other mechanisms are involved at later stages is unknown, but the presence of nearly normal numbers of M-Ig-bearing cells during recovery from suppression (but with low serum levels) suggests that another block is involved. Particularly, in this latter instance, it may be relevant to note that many selective IgA deficiency patients who have normal numbers of M-Ig IgA-bearing cells, also produce anti-α allotype antibodies (Vyas *et al.*, 1969; Kunkel *et al.*, 1969).

Studies on allotype suppression in mice using identical approaches have shown that two different types of suppression occur (reviewed by Herzenberg and Herzenberg, 1974). In most strain combinations studied, only short-term suppression can be achieved, and after a few weeks or months, serum levels of the suppressed allotype return to normal (Herzenberg *et al.*, 1967). This type of suppression has been induced with antiallotype antibodies to either IgG1 or IgG2a determinants, and in each case the suppression is specific for the particular class. It might be noted that all studies in mice have involved antibodies to Fc determinants, whereas in the rabbit studies antibodies to Fab (L chain or Fd antigens) are used. Whether this difference may affect ability of the antisera to eliminate effectively the target M-Ig-bearing cell is unknown, but if the expression of Fab versus Fc determinants were different, it might affect the ability of the anti-Ig serum to induce the removal or diversion of the precursor cells. Whatever the final mechanism involved, it is suggestive at present that short-term suppression in mice may be due to interaction of anti-Ig with M-Ig-bearing cells, although the presence or absence of M-Ig cells in suppressed animals is not known.

In (BALB/c × SJL)F_1 hybrid mice, chronic suppression of the SJL parental allotype can be regularly achieved and persists for most of

the life of the animal (Jacobson and Herzenberg, 1972). As for short-term suppression, specificity for the class of Ig that bears the allotypic antigen can be demonstrated and, in fact, only occurs for IgG2A. Cell transfer studies (reviewed by Herzenberg and Herzenberg, 1974) have shown that suppression is an active phenomenon mediated by T cells, and whether or not this situation is unique for the SJL combination—a mouse strain showing many immunological abnormalities—is not clear at present. It is again of interest to determine the possible nature of the membrane-bound antigen on B cells that might be the target for this suppressor T cell. Although suppression is specific for a particular Ig class, the suppressive activity of activated T cells cannot be inhibited by the free serum Ig, indicating that the target Ig may be a particular conformational arrangement of the M-Ig. How this problem relates to the nature of the Ig expression of the target cell is not yet clear, although it may well be a fruitful line of inquiry, particularly to determine whether the stage of B-cell activation is in some manner different in the IgG2a M-Ig-bearing cell.

2. *Immunoglobulin Suppression with Heterologous Anti-Ig*

Just as injections of neonates with antiallotype sera can cause allotype suppression, so it has been found that injection of suitable neonates with heterologous anti-Ig-class-specific sera can cause suppression of the development of that Ig class and, in some instances, of other Ig classes as well. This field has recently been extensively reviewed by Lawton and Cooper (1974) and the approach used will only be briefly outlined here.

In the first series of studies, embryonic chickens were injected with purified goat anti-μ-chain antibodies, and a profound depression in the synthesis of both IgM *and* IgG was found (Kincade *et al.*, 1970). That these effects are related to bursal activity, is indicated by the observations that, whereas early embryonic bursectomy, either hormonal or surgical, can lead to complete abolition of IgM and IgG synthesis (Warner *et al.*, 1969; Cooper *et al.*, 1969), surgical bursectomy at slightly later stages, preferentially suppresses IgG but not IgM (Cooper *et al.*, 1969). Moreover, it has frequently been observed that chickens with supranormal levels of IgM but little or no IgG can be found following bursectomy (Warner *et al.*, 1969; Van Meter *et al.*, 1969). Also studies of Ig synthesis in chicken embryos (Thorbecke *et al.*, 1968) show that IgM and then IgG synthesis occur first in the bursa. Recent studies on chicken IgA have shown that either early bursectomy or anti-μ treatment and neonatal bursectomy will prevent IgA production later in life (Kincade and Cooper, 1973). In contrast to

these general results, bursectomy at hatch *followed* by treatment with anti-μ brought about significant depression of serum IgM concentrations but not of IgG synthesis (Kincade *et al.*, 1971). It was concluded from these studies (Cooper *et al.*, 1971a, 1972a) that a progressive development of IgM- to IgG- to IgA-producing cells occurred in the bursa during late embryonic life and that throughout this time cells bearing these M-Ig classes are respectively seeded out to peripheral tissues. Similar studies were then performed in mice using anti-H-chain treatment of neonatal germfree mice (Lawton *et al.*, 1972c), conventional mice, or athymic nude mice (Manning and Jutila, 1972). In both studies treatment with anti-μ chain resulted in marked depression of IgM levels and considerable depression of other H-chain class levels in serum, at the antibody-producing cell level and at the level of M-Ig-bearing cells. The degree of depression is dependent on the time and duration of administration of the antiserum, and it was concluded that effective suppression depends on contact between the antibody and the M-Ig-bearing lymphocyte at an early and perhaps critical stage of B-cell differentiation. Treatment with anti-γ_1 chain antibodies in both of the preceding studies resulted in depression of IgG1 production but variable and inconsistent effects on other classes. Anti-γ_2 has little effect, and anti-α-chain serum has in some studies been shown to have marked but transient suppressive effects on IgA synthesis (Manning, 1972; Murgita *et al.*, 1973).

The relationship between IgG and IgA sequential development was studied by Lawton and Cooper (1974) using animals first treated with anti-μ and, then, at a later time when IgG levels were low given anti-$\gamma_1\gamma_2$ serum. Many of these animals showed complete absence of IgA, and this seemed to be correlated with depressions of IgG. Frequency of IgA plasma cells in gut was also considerably lower in animals given anti-$\gamma_1\gamma_2$ than in animals given only a short course of anti-μ. It was tentatively concluded that a switch from γ to α synthesis occurs during primary B lymphocyte differentiation.

Suppression of Ig formation by mouse spleen cells using heterologous anti-Ig sera has also been studied in an adoptive cell transfer system using allotype congenic mice (Herrod and Warner, 1972). Treatment of normal spleen cells with anti-μ-chain antisera resulted in a tenfold or greater suppression of donor type IgG1, IgG2a, and IgG2b production after transfer of the cells to sublethally irradiated, allotype congenic recipients. In these studies additional antigenic stimuli were not given, although from studies on the radiosensitivity of this allotype transfer (Anderson and Warner, 1973) it is evident that B-cell proliferation is involved and that this system does

not simply reflect secretion of Ig from already differentiated plasma cells. With the reservation that possible anti-Ig effects on T cells could be involved, the most likely explanation of this observation is that the IgG-secreting cells of all classes derive from cells carrying IgM as part if not all of their M-Ig.

3. *Suppression of Antibody Production by Anti-Ig*

In considering the concept that cells carrying receptor antibody can react with antigen, it was proposed that they should also be able to react with anti-Ig that would suppress their function, and this was shown by Mitchison (1967) in that anti-Fab or anti-IgG serum inhibited antigen stimulation of hapten carrier-primed cells. Similarly, pretreatment of unprimed cells with anti-κ or anti-μ serum prior to their transfer to irradiated recipients markedly suppressed the subsequent antibody response (Warner *et al.*, 1970).

As these latter studies included thymic-independent antigens (Brucella, Pol, see Crewther and Warner, 1972), it was concluded that the anti-Ig sera were inhibiting through binding on B-cell M-Ig. Similar conclusions were reached by Takahashi *et al.* (1971c) using anti-Ig suppression of antibody production to *Brucella,* and in these studies anti-θ serum was shown to be ineffective. It was further shown (Mond and Thorbecke, 1973) that in this system, bone marrow B cells were far less sensitive than splenic B cells to anti-Ig inhibition, and further studies on this aspect may be useful in defining the stage at which M-Ig becomes accessible and functional as an antigen receptor (see Section IV,B).

Inhibition with anti-Ig sera of primary or secondary *in vitro* responses to sheep erythrocytes has been reported by various groups (Fuji and Jerne, 1969; Greaves, 1970; Lesley and Dutton, 1970; Hartmann *et al.*, 1970; Warner and Dwyer, 1971; Mond *et al.*, 1972) although in most of these studies only IgM responses were analyzed. In view of the T-cell participation in this response, the results are difficult to interpret, although Mond *et al.* (1972) showed restoration with anti-θ-treated spleen cells implying the anti-Ig effect is on M-Ig-bearing B cells. These studies will be considered in the next section, and here the studies of Pierce *et al.* (1972a,b, 1973) are of more relevance in discussing the effect of anti-Ig treatment on the development of all classes of antibody-forming cells in primary and secondary *in vitro* systems. The results showed that with unprimed cells, anti-μ chain antibodies suppressed responses of all Ig classes, whereas anti-γ_1 and γ_2 suppressed only IgG1 and IgG2 responses, and anti-α suppressed only IgA responses. The effect of anti-μ was shown to be

on the antibody-forming precursor cells. In studies with primed cells *in vitro*, anti-μ chains had progressively less effect with time after priming, and with 10d-primed spleen, anti-μ suppressed IgM and IgA responses but not IgG1 or IgG2 responses, which were in turn, however, susceptible to suppression by anti-γ_1 or anti-γ_2.

In considering their results, Pierce *et al.* (1972a,b) suggest that some cells bearing IgM receptors are precommitted to differentiate into cells secreting one of the other classes of Ig. The alternative possibility that activation of an IgM-bearing cell is required to activate the IgG precursor cell that need not bear IgM is rendered unlikely by the observation with primed cells that anti-μ treatment suppressed IgM but did not suppress IgG production.

Somewhat conflicting results with rabbits were found by Kishimoto and Ishizaka (1971) in that anti-γ suppressed secondary IgM as well as IgG responses whereas anti-μ suppressed IgM. Studies with anti-Ig columns, however, showed that passage over either anti-γ or anti-μ reduced both IgM and IgG responses (Kishimoto and Ishizaka, 1972). These studies suggest that many of the IgM-producing memory cells have both IgM- and IgG-type M-Ig, whereas of the cells committed to IgG synthesis, most have only IgG on the surface, although some have both IgM and IgG. In view of the studies described in Section III of Jones *et al.* (1973a), it must, however, be cautioned that rabbit cells seem more capable than some species of cytophilic binding of serum Ig. In comparing precursors of IgG1- and IgG2a-producing cells from primed mice, Walters and Wigzell (1970), using anti-Ig inhibition of binding to antigen columns, concluded that the receptor class on the cell was the same as that to be secreted.

B. Models of B-Cell Differentiation

As discussed in the foregoing, the basic distinction between the two models of Ig class expression in B-cell differentiation (Fig. 4) concerns the role played by antigen. In Scheme I (Fig. 4) (Cooper *et al.*, 1971a, 1972b), sequential expression of IgM to IgG to IgA occurs only in embryogenesis and the respective precursor cells seed out during this period. Antigen will stimulate cells that will then produce antibody identical in class (and allotype) to the receptor on the precursor cells. The alternative view, Scheme II (see Nossal *et al.*, 1964, 1971b; Wang *et al.*, 1970a; Warner, 1972a; Pierce *et al.* 1972a,b, 1973; Warner and Harris, 1973), is that, in the adult animal, virgin precursor immunocompetent cells bear IgM-type receptors, and, on antigenic stimulation, differentiation is initiated that results in expression of the other classes, and this is deliberately depicted as al-

ternative pathways, i.e., μ to γ, μ to α, rather than μ to γ to α in the antibody-forming clone (see in the following).

In considering these alternatives several other relevant observations must be noted.

1. Although most antibody-forming cells produce only one class of antibody, approximately 1–2% of cells secrete both IgM and IgG antibodies (Nossal *et al.*, 1964, 1971b; Nordin *et al.*, 1970; Cosenza and Nordin, 1970; Merchant and Brahmi, 1970; Ivanyi and Dresser, 1970), suggesting that these cells are in a state of "switch" of constant region H-chain genes and at the particular time of study may have still possessed RNA coding for both H chains.

2. Several patients with biclonal myeloma protein of different classes have been reported to show identical *V*-region sequences and idiotypes of the two proteins (Wang *et al.*, 1969, 1970a,b; Penn *et al.*, 1970; Yagi and Pressman, 1973; Seon *et al.*, 1973). Immunofluorescent studies have shown that different cells produce the two proteins (Wang *et al.*, 1970a; Silverman *et al.*, 1973) in each patient. The Ig classes of the pairs of proteins studied include IgM with IgG2 (Wang *et al.*, 1970a), IgM with IgG3 (Penn *et al.*, 1970), and IgM with IgA (Yagi and Pressman, 1973). In each case, it is suggested that the two lines of cells in the patient derived from a common precursor cell that was undergoing a state of differentiation or "switch" in regard to C_H genes but not to V_H, V_L, or C_L, and that at some time, possibly early after the malignant change, differentiation into two lines occurred.

3. In considering M-Ig expression in relation to intracellular Ig, as previously noted in Section III, various studies have shown that cells can bear IgM-type M-Ig derived by synthesis from the cell but also contain and secrete Ig of another class (Pernis *et al.*, 1971; Litwin and Cleve, 1973; Jones *et al.*, 1973a,b; Preud'homme and Seligmann, 1972b).

In considering the receptor equals product hypothesis, recognition must be made of changes in affinity of antibodies, particularly IgG (Siskind and Benacerraf, 1969) during the course of an immune response. In terms of this thesis, comparable changes in avidity should occur at the level of the cell population, and several studies have shown that this does occur. By inhibiting hapten at the level of plaque-forming cells, an average increase of avidity of the population occurs with time after immunization (Andersson, 1970, 1972; Davie and Paul, 1972a, Doria *et al.*, 1972). Furthermore, alterations in the average affinity of antibodies, induced by the effect of the paralysis or passive administration of antibodies, were also reflected at the antibody-forming cell level (Walker and Siskind, 1968; Andersson and

Wigzell, 1971). Increase in avidity of IgM-producing cells was not observed in some studies (Huchet and Feldmann, 1973; Möller *et al.*, 1973), although some maturation of IgM plaque-forming cells was found by Claflin *et al.* (1973). These latter authors also concluded that for IgG as for IgM, commitment to the synthesis of antibody production having a given specificity and affinity occurs before antigenic presentation (Claflin and Merchant, 1973). In general, these studies are consistent with the thesis that maturation involves preferential selection of high-affinity cells. However, in terms of our present consideration, these data primarily concern the antibody-secreting cell, not the immunocompetent cell. Studies of avidity of cell receptors at the level of antigen-binding lymphocytes have been made by Davie and Paul (1972a,b; Davie *et al.*, 1971) and showed parallel increases in avidity for antigen-binding lymphocytes, plaque-forming cells, and serum antibody.

Are these studies cited above consistent with either or both of the alternatives I or II? The latter considerations of cellular avidity and secreted antibody are consistent with alternative I, but are not necessarily inconsistent with alternative II, if the switch event occurs relatively soon after stimulation; most studies do not show a very marked avidity change with IgM. Studies concerning avidity at the lymphocyte level in guinea pigs may be complicated by observations (Coates and Lennon, 1973) that, in this species, marked cytophilic binding of antibodies, stable to cell washing, is observed in immunized animals particularly when given adjuvants. It is, accordingly, proposed that this general line of study does not lead to clear discrimination between the alternatives.

In considering the thesis that precursor cells of all classes exist in the virgin animal, Lawton and Cooper (1974) have considered the problems posed by the existence of cells bearing IgM-type M-Ig but containing IgG (observation 3 in the preceding) and studies showing anti-μ inhibition of subsequent IgG production in the *adult* animal. They have proposed that in the course of the embryonic differentiation, cells bearing IgM differentiate into IgG producers and that for a time these cells will continue to bear some IgM receptors. Thus, in the adult animal, it is proposed that IgG committed cells are present in two populations; one bearing IgM-type M-Ig and the other, IgG-type receptors. It must, however, be noted that the *stable* persistence of a μ-bearing γ-secreting cell requires simultaneous expression of two H-chain genes or existence of a very long-lived messenger RNA, since M-Ig turnover is quite rapid (see Section V). If it is assumed that the precursor cell population contains all the elements depicted in

Scheme I (Fig. 4) prior to antigen presentation, then it is necessary to explain on this thesis why IgM is the major antibody class that is first secreted. Why are not all precursors equally stimulated? It was suggested that the IgM receptor may have considerably greater antigen-binding capacity by virtue of its polymeric structure, and, depending on the nature of the antigen, would accordingly be preferentially selected (see Mäkelä, 1970). However, in view of the monomeric nature of the IgM on the cell surface (see Section III), this argument is not tenable. At this point it is essential to consider a major secondary factor that is of considerable relevance in controlling the expression of IgA and IgG antibody responses, namely T-cell helper function. It is beyond the scope of this review to consider in detail all the studies on this aspect, and these have been discussed in several recent reviews (Katz and Benacerraf, 1972; Warner, 1972a; Mitchell, 1973). For the present considerations, suffice it to say that the expression of antibody or Ig production of the different classes shows a marked differential sensitivity to the requirement for T-cell helper activity, with, in order of decreasing need of T-cell help (in the mouse), IgA and IgG1, IgG2, and IgM. Lawton and Cooper (1974) have suggested that the differential triggering of precursor cells is related to the requirements for T-cell help and that in most situations where this may be somewhat limited, e.g., *in vitro,* as in the studies of Pierce *et al.* (1972a) or *in vivo* where antigenic stimulation is not additionally provided (Herrod and Warner, 1972), preferential triggering of IgM-bearing IgG cells over IgG receptor cells will occur. When primed situations develop, T-cell activity will not be so limiting and IgG-bearing precursors can be readily activated. This interpretation permits consistency of alternative I with the observations of anti-μ inhibition of IgG. However, it must also be noted that T-cell requirements for class activation are not incompatible with alternative II and imply that direction of further differentiation of the activated B cell is now determined in some manner by the amount or nature of a combination of several factors, including local environment, T-cell factors, and antigen.

In similar fashion, the rather moderate antiallotype suppression induced in most mouse strain combinations with anti-IgG, Fc, allotype sera would be expected if many of the IgG precursor cells carried IgM receptors rather than IgG. Again, however, this assumption does not necessarily discriminate between alternatives I and II.

Several recent studies, cited in the preceding observations (2 and 3) pose a severe problem for the concept of sequential M → G → A differentiation as in Scheme I (Fig 4). As indicated in Scheme I, cells

containing IgA should only bear IgG or IgA receptors. It has been reported, however, that biclonal IgA and IgM myeloma can occur (Yagi and Pressman, 1973) and that, in rabbits, a considerable proportion of the Peyer's patch cells containing IgA synthesize IgM surface receptors (Jones *et al.*, 1973b). Pierce *et al.* (1972b) also noted that in primed animals treatment with anti-μ suppressed IgA development, whereas anti-γ did not. These observations all imply that IgA-secreting cells can derive from IgM-bearing precursors in the adult animal, and that direction of commitment to IgA production may depend on other local environmental factors (see Craig and Cebra, 1971). These results are therefore not consistent with alternative I.

Observations made in chickens that partial or early surgical bursectomy can lead to animals producing large amounts of IgM that never show switch to IgG have been interpreted to argue against antigen-driven switch (Lawton and Cooper, 1974). However, some observations (Warner *et al.*, 1969) have indicated that the IgM produced by these birds is not representative of all potential IgM precursor cells, because several birds with very high IgM levels could not even mount 1% of a control, IgM antibody response. This result suggests that the precursor IgM-bearing cells are very restricted and that antigen-driven switch to IgG may not occur because the suitable *V* region was not represented in the precursor pool. Further studies on this aspect are clearly needed.

Observations on the Ig class distribution of M-Ig bearing cells in human PBL indicate that a relatively high proportion of cells bear M-Ig of classes that are only relatively very minor proportions of serum Ig, i.e., IgD, IgE, and IgG2. Although the situation with IgD may well be more complex in view of the data on newborn cells (i.e., does IgD expression precede even IgM?), the results with the other classes could indicate that precursor cells bearing each of the Ig types exist in relatively equal proportions (consistent with alternative I) but that selection to proliferation (thereby proportionately depleting the population of precursor cells) may be antigen controlled. On the other hand, the concept of sequential gene activation may become somewhat difficult when considering all of the subclasses, and, indeed, L. A. Herzenberg (personal communication) has noted both IgG1- and IgG2-containing cells can carry membrane-bound IgM.

It is evident that at the present time a firm decision between the two alternative theses cannot be made, and the main virtue of proposing such models, and a further variant, Scheme III (Fig. 4), is perhaps to enable the planning of suitable experiments to elucidate this problem.

In Fig. 4, a modified version of Ig class differentiation in relation to M-Ig expression is proposed. In the bursal environment, IgM expression first occurs, and some antigen-independent activation of other H-chain genes occurs in random cells, so that the cell population, as peripheralized, contains predominantly cells bearing IgM receptors, some of which have cytoplasmic IgG or IgA, and probably in these cells, some membrane-bound IgG or IgA. Thus, alternative paths for other H-chain class expression are considered rather than sequential activation. Antigen then triggers cell proliferation and antibody secretion primarily in the IgM secretors and release of the remaining IgM in the lines committed to other H-chain class expression. Depending on other factors, such as T-cell factors, possibly local environmental factors, and antigen presentation, activation of these cells now bearing and secreting IgG or IgA may occur.

V. Antigen Receptors on Lymphoid Cells

If it is accepted that both T and B cells bear some amount of M-Ig of intrinsic origin from the cell, it must then be questioned whether this M-Ig is the sole functional receptor for antigen on the cell, whether the cell carries both M-Ig and an unrelated receptor for antigen, or whether the M-Ig and another cell surface component together play a role in the process of antigen recognition leading to activation of the cell. Each of these possibilities must be considered both for T and B cells, particularly the question whether or not antigen recognition by T and B cells shows the same specificity (see *Transplantation Reviews*, Vol. 10). Studies of this general problem are chiefly based on the original Ehrlich–Burnet concepts that specific lymphoid cells bear surface-associated receptors for antigen, and in recent years, direct evidence for this contact between antigen and a small subpopulation of lymphoid cells has been amassed from various laboratories. In determining whether or not M-Ig is involved in the cell surface binding of antigen, the basic procedure has been to attempt to inhibit this binding with defined anti-Ig sera. As might be anticipated from the relative difficulty in demonstrating anti-Ig binding to cells, antigen binding to T cells is also a rather controversial issue, and several other approaches to determine the nature of antigen recognition sites on T cells have been used, particularly inhibition of T-cell functions *in vivo* or *in vitro*. The subject of antigen-binding cells has been dealt with in depth in recent reviews (Wigzell, 1970; Greaves, 1970; Ada, 1970; Paul, 1970; Roelants, 1972b; Möller and Sjöberg, 1972; Bach, 1973; Davie and Paul, 1973) and, accordingly, the following discussion will consider most of the approaches used to the problem without reviewing in depth all individual studies.

A. Antigen-Binding Cells

To determine the nature of antigen recognition sites on lymphoid cells, suitable methods for the detection of antigen–lymphocyte interaction had to be developed that permit consideration of the following points: (*a*) Do both T and B lymphocytes bind antigen in the particular system and, if so, can methods be developed of detecting or identifying separately T and B cell union with antigen? (*b*) Does preimmunization of the animal (or tolerance induction) affect the number of antigen-binding cells (ABC) and, if so, are any of the changes due to passive adsorption of antibody? and (*c*) Are the cells detected in ABC assays at all related to the precursor cells that are triggered by antigen in *in vivo* immunization. Some of the following studies have attempted to assess these and other questions, and it appears at present that the direct union of antigen with B cells can be readily detected and includes the antibody precursor cells that recognize antigen *in vivo* through an M-Ig surface receptor. There is compelling evidence that T cells can also bind antigen and that, although the number of binding sites may be less than on B cells, they involve at least some M-Ig components.

1. Detection of Antigen-Binding Cells

The direct adherence of large particulate antigens to the surface of lymphoid and plasma cells from immunized animals has for many years been recognized as a means of enumerating the numbers of antibody-forming cells in the animal. These studies have primarily used bacteria (Hayes *et al.*, 1951; Mäkelä and Nossal, 1961; Russell and Diener, 1970) or red cells (Nota *et al.*, 1964; Zaalberg, 1964), the latter technique is referred to as immunocytoadherence or rosette formation. Whereas studies were primarily concerned with antibody-forming cells from immunized animals, it has also been observed that RFC are present in lymphoid tissues of unimmunized animals, and it was thus assumed that these are immunocompetent lymphocytes (see Section V,A,4, review of Bach, 1973).

Various other methods of visualizing the interaction of antigens with lymphocytes have since been developed and these are listed in Table XII. Rosette formation can be used either with heterologous erythrocytes (see Bach, 1973) or with various soluble antigens that are coated onto the surface of the erythrocytes. These include protein antigens (Bankhurst and Wilson, 1971), polysaccharides (Sjöberg and Möller, 1970; Howard *et al.*, 1969), and haptenic determinants (Möller and Sjöberg, 1972).

Studies with soluble antigens are made possible by the use of iso-

TABLE XII
METHODS FOR DETECTING ANTIGEN-BINDING CELLS

1. Rosette formation:	heterologous erythrocytes
2. Rosette formation:	antigen-coated erythrocytes
3. Radioautography:	^{125}I-labeled antigens
4. Radioautography:	^{3}H-labeled antigens
5. Fluorescence:	direct or indirect
6. Enzymatic:	fluorogenic/chromogenic substrate
7. Enzymatic:	bacterial colony formation
8. Solid phase:	antigen-coated beads or fibers
9. Solid phase:	affinity columns

tope or fluorescent labels in similar fashion to the use of anti-Ig antibodies for detecting M-Ig. The isotopic approach was first applied to the specific problem of enumerating antigen-reactive cells in normal animals by Naor and Sulitzeanu (1967) and was then extensively studied in several other laboratories (Byrt and Ada, 1969; Humphrey and Keller, 1971; Dwyer and Mackay, 1970; Davie and Paul, 1971; see reviews, Ada, 1970; Roelants, 1972a; Davie and Paul, 1973). Combined studies of RFC and ^{125}I-ABC indicate the anticipated greater sensitivity of the radioautographic method (Bankhurst and Wilson, 1971), and, as with the use of radioautography in studying M-Ig, many technical problems arise in relation to the extreme sensitivity of the technique. Thus the actual number of positive ABC counted will depend on the contact time of cells and antigen, the labeled antigen concentration and its specific activity, and the exposure time for radioautography. Quantitative aspects of this problem have been considered by Byrt and Ada (1969) and by Dwyer and Mackay (1972) who clearly showed that over a wide dose range of antigen, an increasing proportion of labeled cells occurs, although at any one concentration, differences between tissues and sources of cells (normal, immune, tolerant) can be determined. This problem of sensitivity of the assay is particularly relevant to the question of possible detection of receptors on T cells that may bind only of the order of 10 to 50 molecules of antigen (Roelants, 1972b) (see Section V,A,3). To avoid discrepancies among studies due to these technical matters, Roelants (1972a) has suggested routinely expressing data as the number of cells labeled in specific categories of numbers of molecules of antigen bound per cell (using calculations of Ada *et al.*, 1966). The ABC values for given cell suspensions can thus be presented either as percent labeled cells, preferably using several concentrations of reagents to ensure that plateau levels of labeling are reached, or as histograms of grain counts

per labeled cells using standard conditions. Specificity of this technique has been shown by the ability of large concentrations of the specific unlabeled antigen to block the uptake of label (usually requiring 10^3–10^4 more cold than hot antigen) (Sulitzeanu and Naor, 1969; Byrt and Ada, 1969). Binding of a given antigen appears to be a property of a distinct subpopulation of cells because the use of mixtures of labeled antigen shows values approximately equal to the sum of the results with each individual antigen (Sulitzeanu and Naor, 1969; Dwyer and Mackay, 1972). A practical problem of uptake of antigen by nonlymphoid cells can be considerably lessened by performing the reaction at 0°C. in the presence of sodium azide (Byrt and Ada, 1969). Visualization of ABC in the E/M can also be used and shows the majority of binding cells are typical small and medium lymphocytes (Mandel *et al.*, 1969; Mandel and Byrt, 1971). In attempting to follow fine details of antigen localization on the cell surface, the use of ^{125}I is somewhat restrictive owing to the long tracks produced in the photographic emulsion. The use of biosynthetically labeled tritiated antigens has recently been described (Diener and Paetkau, 1972) and provides considerably better resolution.

The use of fl-labeled antigens for detection of ABC has been rather limited and the combined use of an automated fluorescence-activated cell sorter (Julius *et al.*, 1972) has made this a practical marker for screening large numbers of cells and for obtaining the cells for further functional studies.

Several alternative methods have been developed for the detection of binding of the enzyme β-galactosidase, the differences in methodology depending on the substrate used: the fluorogenic substrate, fluorescein-di-β-galactoside (Modabber *et al.*, 1970; Sercarz *et al.*, 1971); an indigogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (A. Miller *et al.*, 1971); or riboflavin galactoside with bacterial colony growth of an auxotrophic mutant requiring riboflavin (Rotman and Cox, 1971). The method can also be adapted to other haptenic antigens that are conjugated to the enzyme, and the general concept of binding of antigens that are enzymes followed by detection with substrate can probably be further developed, e.g., horseradish peroxidase (A. Miller *et al.*, 1971).

The attachment of antigens to solid particles such as glass beads was used by Wigzell and Andersson (1969) to deplete selectively antigen-reactive cells to specific albumins by passage of the cell suspension through an antigen-coated bead column. Although this method does not permit enumeration of numbers of antigen-binding cells, inhibition studies with haptens or anti-Ig clearly indicate that the

method detects specific antigen-reactive cells (reviewed by Wigzell, 1970). As recent studies have also shown that under certain conditions untreated glass beads (Rosenthal *et al.*, 1972) or nylon wool columns (Julius *et al.*, 1973) can deplete selectively populations of high-density M-Ig-bearing cells (B cells), the use of similar antigen-coated columns must always be carefully controlled for evidence of antigen-specific depletion of cells. By using small ligands attached covalently to large polyacrylamide beads, Wofsy *et al.* (1971; Truffa-Bachi and Wofsy, 1970; Henry *et al.*, 1972) have developed affinity columns whereby specific antigen-reactive cells cannot only be bound but can then be removed by hapten elution, providing considerably enriched cell populations useful for functional and characterization studies on the specific, antigen-reactive, precursor population. A further modification of the solid phase method has used, as supporting medium for antigen coating, taut monofilament nylon fibers (Edelman *et al.*, 1971). Cells are adsorbed to fibers covalently coupled to antigens or lectins and can then be removed by mechanical plucking of the taut fibers.

Do all these methods detect the same type of antigen-reactive cells and with equal sensitivity? It is clearly evident from many studies that this is not the case and that the type and proportions of cells detected with each assay depend on the conditions used. For some time, it seemed that RFC assays could detect T and B cells, whereas other methods detected only B cells (see in the following). However, as for similar studies with M-Ig detection, it appears that sensitivity thresholds for binding to cells bearing different numbers of receptors may be involved and that quantitative considerations of assay condition are most essential. The evidence for T- or B-cell binding will be considered next, with the RFC studies being separately discussed.

As previously noted for radioautographic analysis, the estimation of the number of antigen-binding cells detected in a given population is dependent on achieving plateau conditions, and, even then, a wide range of binding will be detected in terms of grain counts (i.e., molecules of antigen bound per cell). The recording of a given percent ABC is, therefore, subject to the conditions used, and when these are set at maximal sensitivity, i.e., detection of cells that bind only a few molecules of antigen, it cannot necessarily be assumed that the avidity of binding to the particular antigen is equal for all cells, and, in turn, it cannot be assumed that all cells showing antigen binding are triggered to activation by antigen *in vivo* (see Section V,C).

A series of ABC values for a range of antigens is summarized in Table XIII. With only a few exceptions (possibly including evidence

TABLE XIII
PROPORTION OF ANTIGEN-BINDING CELLS (ABC) IN LYMPHOID TISSUES

Species/tissue	Method	Antigen[a]	%ABC	Reference
Mouse spleen	RFC	SRBC	0.120	Bach (1973), Roelants (1972b)
Mouse spleen	RFC	NNP	0.060	Möller and Sjöberg (1972)
Mouse spleen	RFC	HGG	0.020	Stout and Johnson (1972)
Mouse spleen	RFC	LPS	0.010	Sjöberg and Möller (1970)
Mouse spleen	RFC	CGG	0.007	Bankhurst and Wilson (1971)
Mouse spleen	RFC	SIII	0.004	Howard *et al.* (1969)
Mouse spleen	^{125}I-ABC	DNP–Hb	1.00	Rolley and Marchalonis (1972)
Mouse spleen	^{125}I-ABC	DNP–HGG	0.99	Lawrence *et al.* (1973)
Human PBL	^{125}I-ABC	FLA	0.500	Dwyer and Mackay (1970)
Mouse spleen	^{125}I-ABC	TIGAL	0.280	Humphrey and Keller (1971)
Mouse spleen	^{125}I-ABC	HCY	0.140	Humphrey and Keller (1971)
Mouse spleen	^{125}I-ABC	DNP–Lys–Tyr	0.090	Lawrence *et al.* (1973)
Guinea pig spleen	^{125}I-ABC	BPM	0.070	Coates and Lennon (1973)
Mouse spleen	^{125}I-ABC	HSA	0.070	Sulitzeanu and Naor (1969)
Mouse Spleen	^{125}I-ABC	BSA	0.050	Naor and Sulitzeanu (1969)
Guinea pig spleen	^{125}I-ABC	DNP (GPA)	0.030	Davie and Paul (1971)
Human PBL	^{125}I-ABC	HCY	0.030	Dwyer and Mackay (1972)
Mouse spleen	^{125}I-ABC	FLA	0.020	Ada *et al.* (1970)
Human PBL	^{125}I-ABC	Tg	0.020	Bankhurst *et al.* (1973)
Mouse spleen	^{125}I-ABC	MurGH	0.009	Unanue (1971a)
Mouse spleen	^{3}H-ABC	FLA	0.002	Diener and Paetkau (1972)
Mouse spleen	Affinity column	N_2Phlac	0.003	Henry *et al.* (1972)
Mouse spleen	Enzyme	β-gal	0.002	Rotman and Cox (1971)
Mouse spleen	Enzyme (unfixed)	β-gal	0.002	Modabber *et al.* (1970)

[a] SRBC, sheep erythrocytes; NNP, 4-hydroxy-3,5-dinitrophenyl acetic acid; HGG, human γ-globulin; LPS, lipopolysaccharide; CGG, chicken γ-globulin; SIII, purified pneumococcal polysaccharide Type III, DNP, dinitrophenyl; Hb, hemoglobin; FLA, flagellin; TIGAL, iodinated copolymer of L-tyrosine, L-glutamic acid, L-alanine, and L-lysine; HCY, hemocyanin; BPM, basic protein of myelin; HSA, human serum albumin; BSA, bovine serum albumin; GPA, guinea pig albumin; Tg, thyroglobulin; MurGH, murine growth hormone; N_2Phlac, azophenyl β-lactoside; β-gal, β-D-galactosidase.

of natural exposure to the antigen concerned; Dwyer and Mackay, 1970, 1972), the values generally show around 0.002 to 0.07% of cells binding antigen, this being equivalent to an average of 25,000 ABC per spleen in the mouse. When it is further considered that not all of these cells may be activated by antigen *in vivo*, the values are clearly consistent with clonal selection concepts that a very small population of cells are responsive to antigen *in vivo*.

It must be noted that several situations have been described where the number of ABC is excessively high. Approximately 2% of mouse spleen cells bind either β-galactosidase or horseradish peroxidase (A. Miller *et al.*, 1971). The significance of these values in relation to the values of around 0.002% using different methods (Rotman and Cox, 1971) or using non-formalin-treated cells (A. Miller *et al.*, 1971) is not clear. It was indicated by Melchers and Kohler (cited in Roelants, 1972a) that many nonlymphocytic cells of mouse spleen bind β-galactosidase, and critical examination of the lymphoidal nature of binding cells with this antigen might be necessary. Values of around 0.5% ABC for human PBL with flagellin may well be accounted for by natural exposure to some common antigenic determinants because natural antibodies to the flagellin are frequently found (Dwyer and Mackay, 1972). Two studies have recorded values of around 1% ABC of mouse spleen cells binding dinitrophenyl (DNP)–carrier complexes, using either mouse hemoglobin (Rolley and Marchalonis, 1972) or human γ-globulin (HGG) (Lawrence *et al.*, 1973). As these ABC could be inhibited by free hapten, they would seem to be specific, and further studies are warranted to determine whether specific conformational groupings of the hapten on these carriers permit wider recognition than of hapten alone.

2. *Antigen-Binding B Cells*

In this and the following section on T cells, evidence concerning the ability of T or B cells to bind antigen will be considered for all methods except for RFC, which, in view of several controversial aspects, will be discussed separately.

There is no doubt that B cells can bind antigen directly and be detected as ABC with all available methods.

Using the ABC assay methods under relatively standard conditions (i.e., without pressing for maximal limits of sensitivity), most of these studies show that the major proportion of ABC are B cells, and the more appropriate question is whether these methods can detect any T-cell antigen binding (see Section V,A,3). Some of the data consistent with B-cell antigen binding are summarized in Table XIV,

TABLE XIV
EVIDENCE FOR ANTIGEN BINDING BY B LYMPHOCYTES[a]

1. Absence of ABC in X-linked agammaglobulinemia
   (Naor *et al.*, 1969; Dwyer and Hosking, 1972)
2. Presence of ABC in congenitally athymic nude mice
   (Dwyer *et al.*, 1971)
3. Persistence of (most) ABC after anti-θ serum treatment
   (Roelants, 1972b)
4. Ontogenic origin of ABC in avian bursa of Fabricius
   (Dwyer and Warner, 1971)
5. Presence of ABC in human bone marrow
   (Abdou and Abdou, 1973)
6. Selective retention of antibody-forming precursors over "helper" cells on antigen columns
   (Wigzell, 1970)
7. Purified ABC (Fluorescent-labeled cell sorter) are antibody-forming cell precursors (require T-cell help)
   (Julius *et al.*, 1972)
8. Hapten–carrier binding ABC are inhibited by free hapten
   (Davie and Paul, 1971)
9. Fluorescent-anti-Ig labels all ABC
   (Davie and Paul, 1971, Lamelin *et al.*, 1972)
10. Antigen-binding cells are MBLA-positive, MSLA-negative
    (Lamelin *et al.*, 1972)
11. Sedimentation velocity distribution profile of ABC is identical to AFC profile
    (Diener *et al.*, 1973)

[a] ABC, antigen-binding cells; MBLA, mouse B lymphocyte antigen; MSLA, mouse specific lymphocyte antigen.

and it is quite evident that hapten-specific, M-Ig-bearing, antibody-forming, precursor B cells are ABC. [It has previously been noted (Section II,C) that plasma cells bearing M-Ig can also bind antigen, e.g., Mäkelä and Nossal, 1961; Bystryn *et al.*, 1973; Hannestad *et al.*, 1972]. As noted in Table XIII, several autoantigens (thyroglobulin, basic protein of myelin and murine growth hormone) also demonstrate ABC in normal animals under the conditions that primarily detect B cells. These observations are relevant to considerations on the nature of self–not self discrimination and are consistent with hypotheses (see Fudenberg, 1971; Allison, 1974) inferring that control of autoreactivity may be at the T cell level and that there are no restrictions on the *emergence* of B cells with autoreactive, variable gene conformations.

The expression of antigen-binding capacity exactly parallels ontogenic expression of constant-region genes (see Section III). In embryonic chicken bursa, ABC appear in the bursa around the time of first recognizable lymphoid cells (14-day embryo; Dwyer and Warner,

1971), and this is also the time of detection of intracellular Ig. The appearance of ABC in lethally irradiated animals restored with single, spleen, hematopoietic colonies has also been shown to occur (Yung *et al.*, 1973) at a time interval after transfusion (18–20 days) comparable to the expression of M-Ig in fetal mice (Nossal and Pike, 1973). These studies, particularly considered in the light of antiglobulin inhibition of ABC (see Section V,B), strongly imply that variable Ig gene expression occurs in the development of B cells at the same time as constant-region gene expression, leading to the assembly and membrane location of intact Ig which acts as the receptor for antigen (see Warner, 1972a; Warner and Harris, 1973).

3. *Antigen-Binding T Cells*

The recognition of antigen by T cells as well as by B cells is now quite firmly established (see Miller, 1972). Much of the controversy over T-cell antigen recognition concerns (*1*) the number of recognition receptor sites on T cells relative to B cells, (2) the nature of the recognition site, in particular the role of M-Ig on T cells, and (3) the gene library of recognition sites expressed in T cells—Does it differ from *V*-gene expression in B cells? (see *Transplantation Reviews*, Vol. 10).

The demonstration of antigen binding by T cells has been a relatively difficult matter, at least for soluble protein antigens. Many studies using cellular antigens have claimed to show specific T-cell binding, and, although there have been some denials of this (see Section V,A,4), it is now clearly proven that T cells can bind cellular antigens such as heterologous erythrocytes (Ashman and Raff, 1973). Although binding of T cells to antigen columns has not usually been detected (Wigzell, 1970; but there is a possible exception, see Davie and Paul, 1970), the specific binding, and in some cases elution, of sensitized lymphoid (T) cells to allogenic antigens on cell membranes has recently been described by several groups (Berke and Levey, 1972; Golstein *et al.*, 1972b; Wekerle *et al.*, 1972; Altman *et al.*, 1973; Stulting and Berke, 1973). For reasons that are not yet fully clear, binding of soluble antigens to T cells has been far more difficult to demonstrate. Several recent studies give fairly strong indications that the weaker binding of antigen by T cells is associated with a lower number of receptors, which possibly, in addition to specificity differences, results in a weaker avidity of antigen binding by the T cells (Haskill *et al.*, 1972; Roelants, 1972b; Hammerling and McDevitt, 1974; Hammerling *et al.*, 1973; Lawrence *et al.*, 1973). These and other data relating to direct demonstrations of T-cell antigen binding

are listed in Table XV (for rosette formation and radioactive antigen suicide, see Sections V,A,4 and V,C).

Although several studies on ABC in either B-cell-depleted or -enriched populations (Table XIV) seem to indicate that only B cells are binding antigens, the possibility that low levels of antigen binding by T cells occurs cannot be excluded (Bankhurst and Wilson, 1971). In a careful analysis of ABC in mouse spleen cell suspensions treated with anti-θ serum, Roelants (1972b) clearly showed that about one-quarter of the splenic ABC were T cells and that the number of molecules of antigen bound by the T cells was frequently (but not exclusively) of the order of 10 to 50 molecules per cell. These data are extremely consistent with the observations of M-Ig on T cells as compared to B cells and strongly suggest that the M-Ig and antigen receptor on T cells are the same. In studies with (T,G)-A--L, Hammerling and McDevitt (1974) also showed that T cells bind antigen, but considerably less than is bound by B cells, and the amount of binding was found to be dependent on the temperature of the reaction, with increased T-cell binding (but not B cell) being observed at 37°C. If only few antigen-binding sites are exposed on T cells, could they be due to cytophilic binding of B-cell antibody? This has been denied by Hammerling and McDevitt (1974) and by Marchalonis and Cone (1973) who failed to demonstrate directly binding of serum IgM anti-(T,G)-A--L or B-cell surface IgM protein, respectively, to T cells. Inhibition studies with related antigens have also clearly indicated

TABLE XV

ANTIGEN RECOGNITION BY T CELLS[a]

1. Some RFC are θ-positive
   (Ashman and Raff, 1973)
2. Radioactive antigen suicide of normal T cells or antigen-activated helper T cells
   (Basten *et al.*, 1971; Roelants and Askonas, 1971; Cooper and Ada, 1972)
3. Some splenic ABC are θ-positive
   (Roelants, 1972b)
4. Number of ABC in thymus, particularly fetal, is well in excess of possible B-cell contaminant contribution
   (Modabber *et al.*, 1970; A. Miller *et al.*, 1971; Dwyer *et al.*, 1972; Lawrence *et al.*, 1973; Unanue *et al.*, 1973b)
5. Purified T-cell preparations contain ABC showing different antigen specificity to B-cell ABC
   (Hammerling and McDevitt, 1974)
6. Radioactive-labeled cell surface proteins from activated T cells show specific antigen binding
   (Cone *et al.*, 1972; Feldmann *et al.*, 1973)

[a] ABC, antigen-binding cells; RFC, rosette-forming cells.

differences in specificity or avidity of T-cell versus B-cell antigen binding. Thus, Hammerling and McDevitt (1974) showed T-ABC with (T,G)-A--L could be inhibited only by the identical cold antigen, whereas B-cell binding was also inhibited by (H,G)-A--L and (Phe,G)-A--L. In studying DNP-HGG binding to thymic T cells, Lawrence *et al.* (1973) observed that although DNP-BSA could inhibit both T- and B-cell binding, free hapten was far more efficient at inhibiting B-cell ABC than T-cell ABC, suggesting a lower avidity of T-cell binding to antigen. Similar evidence of weaker avidity of T-cell than B-cell binding to heterologous erythrocytes has been shown by Haskill *et al.* (1972).

Several studies (Table XV) have shown a relatively high level of binding of antigens to thymus cells, and in one of these studies, some direct evidence with anti-θ serum strongly indicates that these ABC were not immigrant B cells (Lawrence *et al.*, 1973). In all but one of these studies (Modabber *et al.*, 1970), the number of binding cells in thymus is less than that observed in spleen. The results with β-galactosidase are rather contrary to other studies and this problem needs to be resolved. It is of interest that in the studies of Lawrence *et al.* (1973) the T-ABC were equally proportioned between the subpopulations of cortisone-sensitive and -resistant cells, indicating that in distinction to functional studies on T-cell activity (e.g., in graft-versus-host reactions; Warner, 1964a) where the cells are predominantly found in the medulla, the appearance of antigen receptors on T cells may occur in the thymic cortex. Furthermore, although direct comparisons have not been made, it is suggestive from the various reports that, like M-Ig, the expression of antigen receptors on thymus versus peripheral T cells is not grossly, if at all, different.

The antigen-binding specificity of cell surface-labeled M-Ig from activated T cells has been shown by Marchalonis and co-workers for a range of antigens including allogenic cells (Cone *et al.*, 1972), tumor cells (Röllinghoff *et al.*, 1973), and protein antigens (Feldmann *et al.*, 1973) (see Section V,B,2), and in all cases it was concluded that the activity could not be accounted for by B-cell contamination. Moreover, as mentioned in Section II, labeled antigen-specific receptor from T-cell preparations can also be removed by (cytophilic) binding on macrophages, but normal B-cell M-Ig cannot be eliminated this way.

4. Rosette-Forming Cells

Although rosette formation has principally been used to study antigen-binding cells from animals primed with the erythrocytes, lym-

phoid tissues from unimmunized animals do contain some RFC (see Table XII). Comparisons of RFC and plaque-forming cells (PFC) from immune animals indicated that they were predominantly two distinct populations (Wilson, 1971), and in most studies the RFC are specific and are not due to cytophilic attachment of antibodies (Biozzi *et al.*, 1966; Greaves, 1971a).

When functional studies were performed on the immunocompetence of RFC, it was generally found that addition of other cells (T cells) was required to generate the usual level of responses, thus indicating that the RFC were predominantly, if not exclusively, antibody-forming cell precursors (B cells) (Brody, 1970; Osoba, 1970; Gorczynski *et al.*, 1971).

In attempting to characterize the T- and/or B-cell nature of RFC, anti-θ serum was used extensively, but the results were found to be inconsistent among laboratories, particularly concerning RFC from normal animals. These and other marker studies of RFC have recently been reviewed (Bach, 1973) and will be only briefly summarized here. Theta-positive RFC from immunized animals were found by some groups (Schlesinger, 1970, Greaves and Möller, 1970; Bankhurst and Wilson, 1971; Wilson and Miller, 1971; Bach and Dardenne, 1972) but not by others (Takahashi *et al.*, 1971c, McConnell, 1971; Russel *et al.*, 1972; Hunter *et al.*, 1972). In unimmunized animals, Wilson and Miller (1971) and Schlesinger (1970) failed to find T-RFC, but they did find T-RFC in immune animals. Absence of T-RFC was also observed when other markers or species were used in that bursectomized birds lacked RFC (Good *et al.*, 1971; Hemmingsson and Alm, 1972) and hapten-carrier-primed guinea pigs that gave delayed hypersensitivity reactions had hapten-specific RFC but not carrier-specific RFC (Roberts *et al.*, 1971). Using H-2 or chromosome markers in reconstituted animals, Greaves and Möller (1970) and Charreire *et al.*, (1973) both gave evidence for thymic-derived RFC in immunized animals.

Variability in the results with anti-θ serum may be due in part to the specificity of the sera (Greaves and Raff, 1971; Baird *et al.*, 1971), although recent studies of Elliott and Haskill (1973) and Haskill *et al.* (1972) indicate that the main problem may be the relative instability of the T-cell rosette, particularly with cells from unprimed mice. Using direct uptake of Fl-anti-θ serum, Ashman and Raff (1973) showed that many of the RFC from immune animals and (in one experiment) from normal animals, are θ positive. As the specificity of their antisera was verified using the θ-congenic A mouse strains, it is thus fairly conclusive that T cells can bind erythrocytes to give spe-

cific rosettes. However, in view of the variability among groups, any study attempting to use T-RFC for evaluation of the nature of the receptor should only be interpreted if reliable direct data on the T-cell nature of the rosette are available.

5. *Antigen-Binding Cells in Immunity and Tolerance*

Immunization of animals and man results in a marked increase in the proportion of specific ABC, whereas the induction of immunological tolerance has been varyingly reported to either have no effect or lead to a reduction in number (see reviews by Ada, 1970; Möller and Sjöberg, 1972; Davie and Paul, 1974). Widely varying degrees of increase have been noted ranging from about twofold to over a hundred-fold. The nature of the ABC from immune animals tends to include higher proportions of blastlike cells or even plasma cells (Naor and Sulitzeanu, 1969; Humphrey and Keller, 1971; Dwyer and Mackay, 1970; Ada *et al.*, 1970) and, by using density gradient analysis of lymph node cells, J. J. Miller *et al.* (1972) observed similar distributions of ABC with some of the long-lived nonmigrating lymphocytes. Increased numbers of ABC in immune animals may be due to either clonal expansion with retention of the receptor on the cell surface or the binding of secreted antibody onto many other cells. Evidence for both mechanisms has been obtained, and some species differences have been noted in the relative role of cytophilic binding. Whether the development of increased ABC in mice is due to binding of early AbAg complexes (Ivanyi *et al.*, 1970) or of free antibody is not known. Immunization of guinea pigs with DNP–guinea pig albumin (GPA) was shown (Davie *et al.*, 1971b) to produce a tenfold rise in number of ABC, and a marked increase in the avidity of the cell receptor, as shown by concentrations of free hapten required for blocking ABC, was noted (Davie and Paul, 1972a,b). The interpretation of these findings is complicated by observations of cytophilic binding as a cause of ABC in immunized guinea pigs (Coates and Lennon, 1973), Freund's adjuvant treatment alone causing both an increase in the number of ABC and rendering cells more liable to bind immune serum. It has also been noted that normal guinea pigs have around 3% of PBL cells that can bind ^{125}I-purified protein derivative (PPD) (Donald *et al.*, 1973). Studies with rats (Coates and Lennon, 1973), mice (Ada, 1970), and man (Dwyer and Mackay, 1972) have, however, usually failed to show cytophilic antibody involvement in ABC. Although this problem is not fully resolved, it is evident from studies of the Fc receptor on cells that cytophilic binding might well lead to increased numbers of ABC, particularly under conditions where small amounts of circulating antigen are still available, thus

permitting more stable binding of complexes formed in antibody excess. Consequently, variations among studies might depend on quantitative aspects of proportions of available complexes still bearing free antibody-combining sites.

The presence of ABC in tolerant animals has also been a matter of some contention. It is essential to consider these results in the light of evidence that the cellular defects in tolerance can reside in either T- and/or B-cell populations, depending on dose of antigen and time of examination (Chiller and Weigle, 1972). Whereas Ada *et al.* (1970), Sjöberg and Möller (1970), Möller *et al.*, 1971, and M. G. Cooper *et al.* (1972) failed to find any significant reduction in ABC in tolerant animals, Humphrey and Keller (1971) observed a selective reduction in heavily labeled ABC; Naor and Sulitzeanu (1969) found fewer ABC in BSA tolerance; Katz *et al.* (1971) and Davie and Paul (1974) reported an absence or marked reduction of specific antigen-binding cells in a model system of B-cell tolerance to DNP hapten; Möller and Sjöberg (1972) studied B- and T-cell RFC in mice tolerant to dinitrophenyl acetyl BSA and found few T-RFC but an increase in B-RFC; and Louis *et al.* (1973) observed a marked decrease in the numbers of ABC in animals tolerant to HGG using a scheme known to induce B- and T-cell tolerance. It thus seems that, consistent with clonal selection concepts (Burnet, 1959), when tolerance has been induced in either B- or T-cell populations, a marked reduction of immunocompetent cells in the respective population exists.

B. Anti-immunoglobulin Inhibition of Antigen-Binding Cells

If T and B cells carry on the cell surface both Ig and receptors for antigen, it must next be questioned whether these are one and the same molecules. Three general alternatives might be considered for both of the cell types: (*1*) M-Ig is the antigen receptor; (2) M-Ig and another cell surface component together act as the complete antigen receptor; or (3) M-Ig plays no role in the receptor for antigen. In the following section, these three alternatives will be considered by several approaches, leading to the thesis that alternative *1* is the case for B cells and alternative 2 for T cells.

1. Inhibition of B-Cell ABC

If the antigen receptor on B cells is M-Ig, then pretreatment of the cell with anti-Ig should inhibit uptake of antigen by the cell. Binding of antilymphocyte serum (Ada, 1970; Walters and Wigzell, 1970; Roelants *et al.*, 1973) or anti-H-2 serum (Hammerling and McDevitt, 1974) does not inhibit antigen uptake, indicating that the presence of additional bound Ig does not sterically hinder antigen–receptor bind-

ing. However, in most of the studies, the possibility that Ig is positioned extremely close to a non-Ig antigen receptor cannot be ignored, although studies of Raff *et al.* (1973) have virtually eliminated this possibility.

Pretreatment of ABC from several species with anti-Ig of varying specificities (Table XVI) has generally shown that virtually all ABC are inhibited with polyvalent anti-Ig serum (Byrt and Ada, 1969) and that IgM is the predominant antigen receptor on B cells from unimmunized animals, although in guinea pigs most of the cells, whether from adult or fetal animals, are inhibited by anti-γ_2 reagents. Apart from this exception, the predominance of IgM as antigen receptor is consistent with the predominance (at least in mice and rabbits) of this class as M-Ig on B cells, and, in most of the studies with immunized cells, there is little evidence for the presence of multiple classes on the cell membrane.

Following injection of antigen, there is, however, a clear shift to IgG-type receptors, although in a few of the studies, summation of the individual H-chain values indicates that many cells may have multiple H-chain classes (e.g., Greaves, 1971a, Bona *et al.*, 1972). In both of these studies, the presence of multiple Ig class receptors occurs only at a particular stage of immunization, around days 1–7. Control studies showed no evidence of passive uptake of specific antibody with serum taken at this time, and following immunization with two unrelated red cells, double rosettes were rarely observed. This demonstration of simultaneous expression of multiple Ig class determinants on the surface of recently activated B cells is in essence the scheme depicted for B-cell maturation (Scheme II, Fig. 4), and the time during which the cell expresses multiple classes may well vary in different immunization schemes and, accordingly, not be detectable in studies where only a few time points are examined. This situation is clearly not resolved at the present time, and further critical studies employing double-label techniques will need to be applied to the problem. The multiple nature of Ig class expression at this stage of immunization also involves the expression of allelic genes, as it was observed (Greaves, 1971b) that, with cells from heterozygous donors, each antiallotype antiserum gave the same degree of inhibition as the appropriate class-specific antiserum. An extensive series of controls were run in this study including radiation chimeras developed from injection of mixed inocula of *H*-2-compatible but allotype distinct donors. These animals did not show multiple allotypes on B cells by RFC inhibition studies, and it was concluded that activation of the B cell does not involve allelic exclusion and that restriction of

TABLE XVI
ANTI-IMMUNOGLOBULIN INHIBITION OF B-CELL ANTIGEN-BINDING CELLS

Tissue source	Antigen[a]/method	% ABC inhibition				Reference
		PVT (or Fab)	κ	μ	γ (sum)	
Mouse spleen (normal)	FLA-ABC	>90	70	70	5	Warner *et al.* (1970)
Mouse spleen (normal)	KLH-ABC	–	78	28	76	Unanue (1971a)
Rat spleen (tolerant)	FLA-ABC	91–98	–	–	–	M. G. Cooper *et al.* (1972)
Mouse spleen (normal)	DNP HGG-ABC	84	71	55	81	Lawrence *et al.* (1973)
Mouse spleen (normal)	DNP% Ig of ABC	–	–	91	11	Davie and Paul (1973)
Mouse spleen (normal)	TGAL	90	–	67	0	Hammerling *et al.* (1973)
Guinea pig lymph node (normal)	DNP% Ig of ABC	–	–	25	81	Davie and Paul (1973)
Rabbit spleen (normal)	RFC	91	95	46	40	Bona *et al.* (1972)
Rabbit lymph node (normal)	RFC	95	80	90	–	Ferrarini *et al.* (1973)
Mouse spleen (5d 1°)	RFC	95	85	50	>100	Greaves (1971a)
Mouse spleen (10d 2°)	ABC TGAL	–	–	0	70	Hammerling *et al.* (1973)
Mouse spleen (primed)	ABC TGAL	–	91	50	–	Roelants *et al.* (1973)
Mouse spleen (primed)	AG Columns	–	–	–	++	Walters and Wigzell (1970)
Rabbit spleen (1d 1°)	RFC	94	92	92	64	Bona *et al.* (1972)
Rabbit spleen (7d 1°)	RFC	84	86	26	60	Bona *et al.* (1972)
Rabbit lymph node (7d 1°)	RFC	98	95	60	–	Ferrarini *et al.* (1973)

[a] ABC, antigen-binding cells; FLA, flagellin; KLH, keyhole limpet hemocyanin; DNP, dinitrophenyl; HGG, human γ-globulin; TGAL, copolymer of L-tyrosine, L-glutamic acid, L-alanine, and L-lysine; RFC, rosette-forming cells; PVT, polyvalent anti-Ig sera.

class and allotype level develops on maturation. These results seem quite incompatible with most other M-Ig studies, but in view of the controls used, warrant further resolution.

Employing an identical approach with rabbits and L-chain allotype sera, no evidence for a major proportion of B cells expressing both allotypes as anti-sheep red blood cells (SRBC) antibodies was obtained (Ferrarini *et al.*, 1973).

Studies involving inhibition of ABC provide reasonably striking evidence that the receptor for antigen is M-Ig. However, it is also relevant to question whether all of the M-Ig acts as the antigen receptor, i.e., whether it is completely homogenous in this regard. Making use of the phenomenon of capping or movement of antigen receptors following union with multivalent antigens at 37°C. (see Section V,F), Raff *et al.* (1973) have shown that when polymerized flagellin (POL) is used to cap receptors for POL on B cells, double labeling with a different fluorescinated anti-Ig serum shows that, on about 90% of the POL-capped cells, all of the detectable M-Ig has moved into the cap. When it is also taken into consideration that most other pairs of cell surface proteins studied move independently on the cell membrane, e.g., M-Ig and *H-2* (Taylor *et al.*, 1971), M-Ig and HLA (Preud'homme *et al.*, 1972), and *H-2D* and *H-2K* (Neauport-Sautes *et al.*, 1973), it is most likely that all of the M-Ig on the B-cell membrane is specific antigen receptor.

2. *Inhibition of T-Cell ABC*

Inhibition of RFC formation by anti-Ig was first shown by Biozzi *et al.* (1967), Zaalberg *et al.* (1968), and McConnell *et al.* (1969), and usually showed complete inhibition. If some of these RFC were due to T cells, then the antigen receptor on T and B cells may be quite similar. By using semipurified (cotton wool-filtered) preparation of T cells, Greaves (1970) clearly showed that virtually all T-RFC could be inhibited by anti-Fab serum but not by anti-H-chain serum. This was further examined by Hogg and Greaves (1972), who found that, whereas some anti-μ-chain sera could cause up to 85% inhibition, other anti-μ sera gave only around 20% inhibition and that no other anti-H-chain sera produced significant inhibition. By absorption studies with Fab or $F(ab')_2$ fragments from IgM, it was shown that the anti-μ-chain sera capable of inhibiting T-RFC had antibody to "hinge region" determinants of the μ chain. These data implied that the IgM antigen receptor molecule on the T cell is only partially exposed on the cell surface, with the C-terminal end of the H chain "buried" or inaccessible (Greaves and Hogg, 1971). A similar interpretation of T-cell presentation of membrane-bound 7 S IgM was given by Marchalonis and Cone (1973) in considering radiolabeled cell surface Ig.

With the clear description of antigen-binding T cells in other studies, inhibition by anti-Ig has been further examined (see Table XVII), and the results all clearly show that inhibition of T-RFC or T-ABC can be produced by anti-Fab, -$F(ab')_2$, -κ chain, or -μ chain, but not by any other anti-H-chain serum, even when T cells from primed animals are used. Thus in distinction to B cells after priming, a phenotypic change in the class of the Ig receptor on T cells is never observed (Hammerling and McDevitt, 1974). Inhibition of T-cell binding of antigen by anti-Ig was also more readily obtained (higher serum dilutions) than B-ABC inhibition (Dwyer *et al.*, 1972; Roelants *et al.*, 1973).

One report of a peculiar change in L-chain expression on T-RFC (Hogg and Greaves, 1972) indicated that between days 2 to 6 after injection of SRBC, 40–80% of the T-RFC were inhibited by anti-λ serum, with the value then falling to around 20% for the following month. This is the only report on λ-chain expression at the T-cell level and clearly needs further investigation. In man, inhibition of flagellin-binding thymic cells by anti-κ and anti-λ sera showed the expected (from serum ratios) 60:40 ratio (Dwyer *et al.*, 1972).

Inhibition of ABC by anti-Ig sera is, thus, extremely similar for T cells and B cells, differing only in minor quantitative aspects and failure of T cells to convert to IgG expression. Because it is generally accepted that M-Ig is the B-cell antigen receptor, these data provide *prima facie* evidence that the T-cell antigen receptor is also M-Ig (and IgM).

More direct evidence has also been provided in studies on the association of M-Ig with antigen-binding specificity either at the cell surface level (Roelants *et al.*, 1973) or with radioiodinated cell surface protein (Cone *et al.*, 1972; Feldmann *et al.*, 1973).

When M-Ig on B cells was capped with noninhibitory concentrations of anti-Ig, the antigen-binding receptors were also found to be in the capped region. In similar studies on T cells, antigen binding was again detected in cap regions, even though, with the fluorescent anti-Ig used, M-Ig could not be visualized on the T cell (Roelants *et al.*, 1973). These data strongly suggest that, on B cells and on T cells, the antigen receptor is partly if not entirely composed of M-Ig. Similar conclusions were reached in the cell surface iodination studies of Marchalonis and collaborators. The antigen-binding receptors produced by activated T cells are completely removed by coprecipitation with specific anti-Ig reagents (Cone *et al.*, 1972; Feldman *et al.*, 1973), and in the latter study it was concluded that the Ig class of T-cell receptor is IgM. On the basis of these and other studies (Feldmann and Basten, 1972a; Feldmann, 1972), a scheme of T-cell collaboration was developed that proposes antigen-induced release of T-cell

TABLE XVII
ANTI-IMMUNOGLOBULIN INHIBITION OF T-CELL ANTIGEN-BINDING CELLS

Tissue source	Antigen/method (immunization)	% Inhibition					Reference
		PVT (Fab)	κ	λ	μ	γ	
Mouse spleen	RFC (14d 1°; 85% θ+)	98	83	26	85	0	Hogg and Greaves (1972)
Mouse spleen	RFC (3d 1°)	–	35	79	–	–	Hogg and Greaves (1972)
Mouse spleen	ABC TGAL (primed)	–	>95	–	>95	–	Roelants *et al.* (1973)
Mouse lymph node	ABC TGAL (normal)	79	–	–	60	4	Hammerling and McDevitt (1974)
Mouse lymph node	ABC TGAL (primed)	56	–	–	70	0	Hammerling and McDevitt (1974)
Mouse spleen	RFC (primed; 37% θ+)	99	–	–	–	–	Ashman and Raff (1973)
Mouse spleen	RFC (primed; 50% θ+)	–	95	–	–	–	Marchalonis *et al.* (1973)
Mouse fetal thymus	ABC – FLA	–	100	–	85	0	Dwyer *et al.* (1972)
Human fetal thymus	ABC – FLA	–	60	40	95	3	Dwyer *et al.* (1972)
Mouse thymus	ABC DNP HGG	63	50	–	74	0	Lawrence *et al.* (1973)
Mouse thymus	ABC TGAL	75	–	–	78	3	Hammerling and McDevitt (1974)

[a] RFC, rosette-forming cells; ABC, antigen-binding cells; TGAL, copolymer of L-tyrosine, L-glutamic acid, L-alanine, and L-lysine; FLA, flagellin; DNP, dinitrophenyl; HGG, human γ-globulin. PVT, polyvalent anti-Ig sera.

IgM, complexing with antigen, binding of the complexes to the surface of macrophages (through the macrophage receptor for T-cell IgM), and presenting to B cells a suitable immunogenic lattice of appropriately spaced antigenic determinants on the surface of the macrophage. This scheme therefore includes an essential role for antigen-specific T-cell IgM as the initial receptor for antigen. It is released from the T cell and is distinct from other T-cell-derived factors that have been reported to be involved in B-cell activation in certain situations but which are usually not specific for the antigen (Dutton *et al.*, 1971; Katz *et al.*, 1971a; Kreth and Williamson, 1971; Schimpl and Wecker, 1972; Britton, 1972; Gorczynski *et al.*, 1972; Feldmann and Basten, 1972b; Sjöberg *et al.*, 1972; reviewed by Katz and Benacerraf, 1972). Two observations might be made regarding the T-cell IgM receptor in this system. If the property of macrophage binding can be firmly established to be due to a specific region of the H chain that is not represented on B-cell IgM, then the possibility arises that there is a distinct H-chain subclass of IgM controlled by a separate μ-chain gene that is activated only in T cells. This may in part explain the distinction between sequential expression of μ and γ in B cells but not in T cells. Moreover, it might be noted that although the T-cell receptor may be IgM, the concept of a *secretion* of this material, attachment to macrophages, and *then* interaction with B cells is not compatible with evidence favoring a physiological cooperation only between histocompatible T and B cells (Katz *et al.*, 1973a).

Several studies outside the realm of this review have considered an alternative action of T-cell populations, namely suppression of B-cell responsiveness (Gershon and Kondo, 1971; Okumura and Tada, 1971; Gershon *et al.*, 1972; Rich and Pierce, 1973; Katz *et al.*, 1973b; Herzenberg and Herzenberg, 1974). Although it is by no means clear whether this represents a distinct subpopulation of T cells, they do appear to act in an antigen-specific fashion, and analysis of the nature of the receptor in this situation needs to be resolved. In one recent study (Tada *et al.*, 1973), it was shown that the suppressive activity could be extracted from T-cell populations and was *antigen-specific*, but did *not* appear to be Ig in nature.

C. Inactivation by Radioactive Antigens

Although the direct demonstration of uptake of radioactive antigens by lymphoid cells indicates the presence of cells bearing receptors for the antigen, it does not necessarily imply that all of these cells would be activated by antigen. In order to determine whether any or all of radioactive antigen binding has any significance for *in*

vivo immunocompetent cell triggering by antigen, Ada and Byrt (1969) developed the radiolabeled antigen suicide technique. This requires that immunocompetent cells bind sufficient ^{125}I-labeled antigen so that electrons from the disintegrating iodide can damage the processes of DNA synthesis and prevent cell replication. Cells are treated with high specific activity-labeled antigen, transfused into inactivated syngeneic mice, then challenged with the same or unrelated antigens, and serum antibody followed. Pretreatment with the specific labeled flagellar antigen either abolished or significantly reduced the response to that antigen but not to a serologically unrelated antigen. As the primary response studied is relatively T-cell independent, antigen suicide was most likely at the B-cell level. Similar observations were made by Humphrey and Keller (1971) using the synthetic antigen (T,G)-A--L and by Humphrey *et al.* (1971) using spleens from primed mice. Grain count studies of radioautographic analysis of labeled cells reveal a wide range of antigen uptake per cell, and it is not yet known where the threshold lies for the amount of radioactive antigen necessary to cause lethal inactivation of the cell, although it is generally believed that only heavily labeled cells are involved. Similar antigen-specific inactivation of bone marrow cells from nonimmune mice (Unanue, 1971b) indicates that some degree of differentiation to immunocompetent cells has occurred in bone marrow, although in other studies (Basten *et al.*, 1971) suicide of peripheral B cells but not bone marrow cells by ^{125}I fowl γ-globulin (FγG) was demonstrated.

Radioactive antigen suicide of T cells has also been shown. Using cell transfer systems with separate sources of T cells (thymus) or B cells (spleen from thymectomized, bone marrow-restored mice), it was shown that treatment of the thymus cell suspension with ^{125}I-FγG specifically inactivated the T-cell collaborative effect for the FγG response but not for an anti-human red blood cells (HRBC) response. Supplementing the cell inoculum with normal T cells showed reversal of the inactivation, indicating the requirement for attachment of the labeled antigen to the target T-cell surface. That the receptor on the target T cell contains Ig components was shown by the ability of F(ab')$_2$ fractions of rabbit IgG antimouse κ chain to inhibit specific antigen suicide. Suppression of T-cell activity with cells from primed animals has also been shown (Roelants and Askonas, 1971) using the carrier helper system of Mitchison *et al.* (1970) with hapten–carrier antigens. Pretreatment of hemocyanin (Hcy)-primed cells with ^{125}I-Hcy prevented their ability to augment the response of DNP–ovalbumin-primed cells to DNP–Hcy, provided that suitable numbers of cells were used for the transfers. Excessive numbers of helper cells will override the suppression indicating that somewhat less than

100% of helper activity has been abolished. Radioactive antigen suicide of T cells mediating delayed type hypersensitivity was demonstrated by Cooper and Ada (1972) who used ^{125}I-POL treatment of activated T cells from POL-treated mice. Similar inactivation of thymus precursors of the anti-POL-responsive cells was shown, and anti-L-chain Ig inhibited the suicide of activated T cells.

From the preceding studies, it can be concluded that both T and B cells, whether of central or of peripheral lymphoid organ derivation, and whether primed or unprimed, bear specific Ig receptors for antigen that, on union with antigen, lead to cell replication.

D. Anti-immunoglobulin Inhibition of T-Cell Functions

In view of the relative difficulty of directly demonstrating antigen binding by T cells, several approaches to inhibition of T-cell functions by anti-Ig sera were developed. The T-cell activity can be assessed *in vitro* by helper function in hapten–carrier antibody production, by stimulation of DNA synthesis with either antigens or mitogens, and by cytotoxic activity of activated T cells. *In vivo* functions include induction of delayed hypersensitivity and of allogeneic reactions. All of these systems have been studied for susceptibility to pretreatment with anti-Ig sera, but there is a considerable lack of uniformity in the answers obtained by different groups. Restrictions on the significance of positive effects have been noted elsewhere (Crone *et al.*, 1972), and it must be stressed that, in view of the presence of natural antibodies to membrane components in heterologous sera, which usually are particularly reactive with T cells, it is essential to control the specificity of the reaction by showing that *purified* Ig can absorb the suppressive action from the serum. On the other hand, inability to demonstrate an effect of anti-Ig serum does not necessarily imply that M-Ig is not the receptor site involved but rather that the particular Ig determinant recognized by the serum, *if* present on the cell, is not exposed or accessible to the serum.

In the initial studies with this approach, it was shown that both *in vitro* (Greaves *et al.*, 1969) and *in vivo* (Mason and Warner, 1970) activities of T cells could be inhibited by anti-L-chain sera but not by anti-H-chain sera. In virtually all of the subsequent reports using this approach, only polyvalent or anti-L-chain sera have been found effective.

Anti-L-chain sera have been shown to suppress the stimulation of human lymphocytes by PPD and by allogeneic cells (Greaves *et al.*, 1969). Because anti-Ig can itself be stimulatory (see Section III), Fab monomers of anti-Ig were used and also shown to have marked suppressive effects on the mixed lymphocyte reaction, provided that the responder cells were treated at the time of or prior to contact

with the allogeneic cells (Greaves *et al.*, 1971). In these studies, no suppressive effect of anti-L-chain on PHA stimulation was noted, although anti-Ig inactivation of target cell lysis by PHA-activated lymphocytes has been observed (Holm *et al.*, 1969). In other studies (Mond *et al.*, 1973), anti-Ig failed to inhibit proliferative responses to antigen. However, in view of the marked efficiency of anti-Ig in stimulating rabbit cells and the requirement in the studies of Greaves for substimulatory or Fab fragments of anti-Ig, positive effects in the rabbit system may be difficult to obtain. Pretreatment of normal mouse spleen cells with anti-L-chain serum and complement inhibits *in vitro* responses to trinitrophenyl (TNP) and erythrocyte antigens (Lesley *et al.*, 1971). By enhancing hapten responses *in vitro* using thymus-derived carrier-primed cells, it was shown that the inhibitory effects of anti-Ig is on the T cell, although, in other studies (see Section IV,A), effects of anti-Ig on B-cell responses *in vitro* have been clearly observed. Inhibition of both hapten-sensitive (B) and carrier-primed (T) cells with anti-Ig have been observed in one study (Cheers *et al.*, 1971).

These results contrast with the total failure to observe inhibition of either the generation of cytotoxic T cells *in vitro* (M. Rollinghoff and H. Wagner, personal communication) or the effector stage of T-cell cytotoxicity to allogeneic antigens (Chapuis and Brunner, 1971).

In vivo studies of suppression of graft-versus-host reactivity (GVHR) with anti-Ig sera have produced conflicting results. In three studies in mice, a selective effect of anti-Ig in suppressing GVHR but not hematopoietic, stem cell colony activity has been reported (Warner, 1971; Tyan, 1971; Cole and Maki, 1971). Suppression with Fab fragments of anti-Ig has been observed (Riethmuller *et al.*, 1971), although failure to suppress GVHR in mice has also been noted (Sternberg, 1970). Similar variability in the effectiveness of anti-Ig in suppressing GVHR in chickens has been observed, with failures to suppress (Crone *et al.*, 1972; Ivanyi *et al.*, 1970) as well as marked suppression by anti-L chain (Rouse and Warner, 1972b) being observed.

The studies in both mice and chickens are, however, uniform in that all the sera observed to cause suppression have anti-L-chain activity and that this suppressive effect of the sera is due to the anti-L-chain component (Warner, 1971; Rouse and Warner, 1972b). It was also noted in both of these studies that not all anti-L-chain sera caused suppression and that the ability of an antiserum to suppress was not related simply to its antibody titer. Although further studies on this aspect are still required, it seems that suppression depends on recognition of a particular L-chain antigen, and whether this is a

unique, T-cell-expressed, L-chain, variable-region determinant or another determinant is not known.

Studies with delayed-type hypersensitivity (DTH) have also yielded variable results. Mason and Warner (1970) using histological quantitation of the transfer of DTH in mice reported a suppressive effect of anti-L chain sera, although this was not observed by Cooper and Ada (1972). Anti-IgA suppression of cell-mediated demyelination in tissue culture using lymph node cells from rats immunized with nerve tissue and adjuvants has been noted (Winkler and Arnason, 1966).

An excellent model system for studies of suppression of T-cell function in the absence of B-cell influences is the totally agammaglobulinemic bursectomized chicken (Theis and Thorbecke, 1973).

Once maternal-derived Ig has disappeared, these animals can be injected *in vivo* with anti-Ig sera without the antisera being immediately eliminated as a complex with serum Ig. Injection of bursectomized chickens in this manner considerably depressed the ability of these animals to elicit DTH responses to ferritin, and it was suggested in these studies that anti-L-chain antibody was responsible for this effect (Theis and Thorbecke, 1973). Prior blocking of the antiserum with IgG removed its inhibitory ability. Previous studies in fetal lambs using this approach did not show suppression of homograft responses (Silverstein *et al.*, 1963), although this could have been due to more rapid elimination of the antibody. Further studies with the agammaglobulinemic chickens could provide useful information on the nature of the T-cell receptors.

In vivo or *in vitro* inhibition of T-cell responsiveness by anti-Ig sera has thus been demonstrated in a variety of systems, but neither reproducibly in terms of different antisera nor consistently with a given method. However, in several cases the inhibitory effect has been clearly shown to be due to the anti-Ig antibody and strongly indicates that the T-cell carrier M-Ig is the receptor site. In the *in vivo* studies, coating of the T cell with anti-Ig may well lead to opsonization of the cell and, thus, remove it from suitable antigen contact, without requiring that the M-Ig actually be the antigen receptor. In *in vitro* studies this cannot be duplicated, and, particularly in view of the timing requirement for anti-Ig treatment in relation to antigen (Greaves *et al.*, 1971), it is most probable that the receptor for antigen is the M-Ig. Furthermore, in the light of studies on the fluidity of M-Ig (e.g., de Petris and Raff, 1973; see Section V,F), it is rather unlikely that steric inhibition by M-Ig–anti-Ig can be invoked as a cause of blocking a hypothetical non-Ig antigen receptor. On the other hand, biological effects of anti-Ig *in vivo* are hardly the most direct system

and, thus, will never in themselves resolve the problem of the nature of the T-cell receptor.

E. T- and B-Cell Receptor Specificities

From the preceding considerations there can be little doubt that both T cells and B cells have cell surface receptors for antigen that are either in whole or part composed of the M-Ig carried by the cell. It is also evident that apart from the considerable controversy on the Ig class nature of receptor versus secreted product, the specificity of the receptor is identical or closely similar to that of the cell product in terms of binding with the antigen. Does this necessarily imply the specificity of T cell and B cell receptors is identical? There is no *a priori* reason why this should be so, although minimal theories of immune recognition would appear simpler in that one immune recognition system need have evolved and be expressed in the lymphoid cells of the body. A variety of experimental studies have attested to the fact that phenotypically, the specificity of T-cell-associated immune reactions appear to be different from that of B cells, and this problem has been discussed in depth in several other reviews with more ample documentation than is given in the following (Paul, 1970; Brondtz, 1972; Schlossman, 1972; McDevitt and Landy, 1973). Although several studies have emphasized the similarity of specificity of T- and B-cell responses to certain antigens (Taylor and Iverson, 1971; Rajewsky and Pohlit, 1971), the more important question is whether there are differences, because, if there are two independent systems, they may well occasionally show similar receptor specificities for some antigens. As many of the recent data in this field are derived from genetic studies on the immune response to defined antigens, these aspects will be separately considered.

1. Antigen Specificity Differences in T- and B-Cell Receptors

Information on the relative specificities of T- and B-cell responses might be most usefully considered in the framework of the following five concepts of the nature of T-cell receptors. These basically revolve around one question: Do B cells and T cells express the same *V*-region sets of genes?

1. The same sets of Ig *V*-region genes are expressed in T cells and B cells, differences in apparent specificity being related only to the density or avidity of receptors at the cell surface. Different triggering thresholds of antigen for the cells may thus be involved.

2. Just as for concept 1, identical *V*-region genes are expressed in T and B cells, but in one of these cell series, an additional cell surface component plays a role in the binding and cell triggering by antigen.

The expression of this second component may differ either quantitatively or qualitatively on the cell membrane between T and B cells.

3. The T-cell receptor is composed of the same *V*-gene products expressed in B cells but is not assembled in the same way on the cell surface, e.g., free L chains or possibly only *V*-region gene products.

4. The antibody-combing site of the T-cell receptor is encoded by variable genes that are not expressed in B cells. Thus in addition to the three recognized sets of *V*-region genes, V_κ, V_λ, and V_H, a fourth set (V_T) is expressed only in T cells.

5. The receptor for antigen on T cells is not an Ig by present concepts of Ig, i.e., it does not consist of L- and H-chain genes of recent common phylogenetic derivation to the recognized L + H chains.

The general concept of differences in specificity of T cells and B cells was principally founded in considering differences in the specificity of humoral antibody versus cell-mediated immunity (delayed hypersensitivity) to hapten–protein conjugates. Many of these data have been reviewed by Paul (1970) and Schlossman (1972) and will not be reiterated here. It seems clear from these studies that delayed sensitivity responses, helper affects of T cells, and T-cell-mediated cytotoxicity (Henney and Nordin, 1971) all show identical specificities that are predominantly carrier-specific and distinct from the predominantly hapten-specific humoral antibody responses. However, this is not always the case (Taylor and Iverson, 1971; Rajewsky and Pohlit, 1971), and hapten-specific helper effect of T cells to anticarrier antibody responses have been observed (Rubin and Wigzell, 1973). Are these apparent carrier specificity differences primarily due to quantitative aspects of receptor density or of receptor avidity?

Clear differences in the sensitivity of T cells and B cells to antigenic stimulation have been noted in either immunity (Parish, 1971; Falkoff and Kettman, 1972) or tolerance (Weigle *et al.*, 1971; Mitchison, 1971b) situations. There can be little doubt that the preceding concept 1 can be operative and, under certain dosage situations, only one of the two immune systems may be triggered into activation and the other immune pathway may either not be activated or may even be rendered tolerant to the antigen (Parish and Liew, 1972). However, considerations of this nature cannot explain all situations of T- and B-cell differences in antigen recognition. With more defined and preferably limiting conditions for T-cell activity, several studies have clearly shown that real differences in recognition of antigen occur. These include responses to erythrocytes (Playfair, 1973; Haritou and Argyris, 1972; Hoffmann and Kappler, 1972, 1973), protein antigens (Senyk *et al.*, 1971; Thompson *et al.*, 1972; Cooper, 1972; Cooper and Ada, 1972), and well-defined polypeptide antigens (see Section V,E,2). For example, the classification of *Salmonella*

flagella antigens is based on serological studies with well-defined antisera and yet when serologically different flagellins are studied, either in delayed hypersensitivity transfer systems (Cooper, 1972) or by radioactive antigen suicide (Cooper and Ada, 1972), complete cross-reaction is observed. The latter approach strongly implies that the difference in specificity between humoral antibody and T-cell immunity is at the T-cell *recognition* level. Another possible gross distinction between T-cell recognition and humoral immunity is the extremely high proportion of cells that are reactive to histocompatibility-associated antigens (Szenberg and Warner, 1962; Warner, 1964b; Nisbet *et al.*, 1969; Wilson and Nowell, 1970), as determined by GVHR. However, whereas the nature of the antigen in these immune responses is not yet clearly defined (see Klein and Park, 1973), this system of specificity recognition may have separate and distinctive features. Direct antigen-binding data for proportion of B cells binding these antigens have not been obtained, if, indeed, B cells do bind the same determinants at all. Furthermore, recent studies with tuberculin antigens have shown (Donald *et al.*, 1973) that this may not be unique to allogeneic antigens, as approximately 3% of PBL in nonimmune guinea pigs bind labeled PPD, and further studies on the cell type involved in this system would be well warranted. In considering the receptor specificity of cellular immunity to allogeneic antigens as compared to humoral immunity (Ramseier and Lindenmann, 1972), antisera have been prepared against humoral alloantibodies raised by skin grafting allogeneic animals. These antisera have been termed *antialiotypes*, and, using ^{125}I-labeled preparations of these sera, it was shown (Binz and Lindenmann, 1972) that alloantibodies and cellular receptors were antigenically similar, the recognition structures being termed *aliotypes*. If these structures can be shown on T cells, then the high proportion of T cells reactive to allogeneic antigens does not necessarily imply existence of a unique T-cell recognition unit. Some studies have, however, suggested that the antialiotypic antibodies do not inhibit T-cell allogene reactions (Lindahl, 1972), and the general nature of this system is still unresolved.

In considering the evidence for T- and B-cell specificity differences at the level of antigen recognition, Greaves and Janossy (1972) have drawn an analogy to mitogen activation of B and T cells. Whereas soluble PHA and Con A stimulate only T cells, locally concentrated PHA (Greaves and Bauminger, 1972) or locally concentrated Con A (Andersson *et al.*, 1972) also stimulate B cells, even provoking marked IgM synthesis (Andersson and Melchers, 1973). Analysis of T and B cells for receptors for soluble PHA or Con A has shown that both cell types have approximately the same numbers of membrane receptor sites (Greaves *et al.*, 1972; Möller *et al.*, 1973).

Thus both B + T cells can similarly bind mitogens, but lymphocyte activation is dependent on the physical form of the mitogen. By comparison, it was suggested (Greaves and Janossy, 1972) that the same may be true for antigen receptors on B and T cells and that apparent differences in stimulation need not be due to basic differences in the receptor sites. This line of argument is quite consistent with concept 2 described in the foregoing. Of the remaining concepts listed, 3 and 5 can be dismissed at present. Although some of the studies on T-cell M-Ig or on inhibition of T-cell function seem to indicate that only L chains are expressed on the cells, the positive studies that show μ-chain presence should be emphasized, and, considered together, are fully consistent with the view that the T-cell receptor is an IgM molecule with most of the μ-chain determinants being relatively inaccessible. On the other hand, the presence of M-Ig on T cells and the data of association of T-cell antigen binding with M-Ig clearly eliminate concept 5. It is therefore proposed that the most likely alternatives are concepts 2 and 4.

A decision between these two alternatives could be made either by positive evidence for the existence of variable-region determinants present on T cell receptors but not on serum Ig (concept 4) or by demonstrating that another membrane component is involved in T-cell antigen binding (concept 2).

2. *T-Cell Antigen Recognition and Ir Genes*

The most likely approach to the resolution of these alternatives lies in studies on the genetic control of the immune response. This field has been extensively reviewed elsewhere (McDevitt and Benacerraf, 1969; McDevitt *et al.*, 1971; Benacerraf and McDevitt, 1972; Grumet and McDevitt, 1973; Lieberman and Paul, 1973; McDevitt and Landy, 1973). Briefly, the ability of an animal to respond to certain antigens, such as well-defined synthetic polypeptides, is under genetic control of loci termed the immune response (*Ir*) genes. Responsiveness is controlled by dominant autosomal genes that are situated between the *H-2D* and *H-2K* loci, usually between the *SS-Slp* locus and *H-2K* (McDevitt *et al.*, 1972; Lieberman and Paul, 1973). Although there is still some controversy (Shearer *et al.*, 1972), most studies indicate (see the reviews just cited) that the *Ir* genes are expressed only in T cells. At the level of antigen-binding cells, it has clearly been shown (Davie *et al.*, 1972a; Dunham *et al.*, 1972; Warner, 1972a; Hammerling *et al.*, 1973) that both unimmunized responder and nonresponder strains bear IgM-type M-Ig receptors on B cells, in equal proportions, and that both strain types produce similar amounts of IgM antibody on primary immunization (see Grumet and McDevitt, 1973; N. L. Warner, S. Mason, and A. W. Harris, unpublished observations).

Inhibition studies of B-cell ABC with serologically related polypeptides show inhibition of binding of labeled (T,G)-A--L, whereas T-cell ABC could not be so inhibited (Hammerling and McDevitt, 1974). These observations clearly imply a specificity difference in T-cell versus B-cell antigen recognition, which is, in this instance, of narrower specificity for the T cell than the B cell (cf. narrower B- than T-cell specificity for flagellins, Cooper and Ada, 1972). In this respect, the argument of whether the T cell "sees" more or less of the antigen than the B cell is probably not consistent for different antigens—the main point is that there is a *difference* in recognition.

In considering the two main alternatives of describing the T-cell receptor (see concepts 2 and 4), these genetic studies are most relevant. The *Ir* genes are extremely specific in their control of antigen-induced lymphocyte stimulation, and it is most likely that this level of control is exerted at the stage of antigen recognition. If this is solely a T-cell-expressed genetic control, it would appear most likely that a different recognition system was involved, and, in view of the equisite specificity and Ig nature of the receptor, this would strongly favor concept 4 (Warner, 1972a). It might also be argued on this thesis, that, if this unique T-cell-expressed region is associated with linked H chains (as described for allotype–idiotype linkage of B-cell-expressed Ig; see McDevitt and Landy, 1973) the lack of *H-2* : Ig H-chain locus linkage (Herzenberg *et al.*, 1968) would be consistent with the implication (see Section V,B,2) that T-cell IgM is composed of a μ chain coded by a different gene than for serum (B-cell) IgM. Thus, at some stage in the evolution of the Ig genes, a translocation or separation occurred between the H-chain constant-region genes and this unique T-cell expressed gene. At this time, these are perhaps rather far-fetched speculations and at best indicate the need for a more complete chemical characterization of T-cell μ chains in comparison to serum-derived μ chains.

If concept 4 is considered improbable at present, is there any supportive evidence for the existence of ancilliary structures in the T-cell recognition site? That is, if *Ir* does not code for any *V* gene, does it code for a receptor molecule that acts in concert with T-cell IgM but not with B-cell IgM in the recognition of antigen? Genetic studies have clearly shown that the *Ir* gene is not *H-2D* or *H-2K* (Bach *et al.*, 1972; Klein and Park, 1973; Lieberman and Paul, 1973). Studies on the inhibition of T-cell antigen binding have shown (Hammerling and McDevitt, 1974) that antibody to either the *H-2D* or *H-2K* products will inhibit antigen binding. Similar studies using antigen-induced T-cell proliferation have been performed in guinea pigs by Shevach *et al.* 1972f) and both lead to the conclusion that a histocompatibility antigen may be associated with the specific antigen receptor. Ham-

merling and McDevitt (1974) also showed that, when the cells were treated with anti-*H-2* sera under conditions that should have induced capping of *H-2*, inhibition of antigen binding still occurred. These results are in contrast to B cells where anti-*H-2* had no effect and to anti-θ serum on T cells where inhibition occurred only in the absence of capping conditions (indicating steric hindrance but independence of θ and the antigen receptor). Similar studies have also been performed with radioactive antigen suicide of T cells (A. Basten, personal communication), wherein it was shown that anti-*H-2* serum inhibited T-cell suicide but not B-cell suicide. Basten also found that this inhibitory activity of the anti-*H-2* serum could be adsorbed by mouse fibroblasts, thus suggesting that the antiserum is not reacting to unique lymphoid cell-expressed receptor structures (cf. Ramseier and Lindenmann, 1972) but to some type of histocompatibility structure. Recent data of Hauptfeld *et al.* (1973) have shown the production of alloantisera between mouse strains that differ only in the middle portion of the *H-2* complex. These sera may recognize antigens controlled by *Ir* loci that seem to be present on only a subpopulation of lymph node or spleen cells.

In attempting to consider these various data on T-cell antigen recognition, it is evident that a definitive picture of the T-cell receptor has not yet been drawn. In this author's view, the most likely thesis (concept 2) is as depicted schematically in Fig. 5. The model essentially claims that, whereas B cells bind antigen only by M-Ig receptors, T cells bind antigen either by M-Ig or M-Ig and ancilliary components that are coded for by the polymorphic *Ir* genes. Whereas B-cell activation can be triggered by antigen binding alone, the degree of T-cell triggering is dependent on whether antigen binds only to the M-Ig (present at low density on the surface and probably with only single-site binding) or to the M-Ig and another cell surface component. The specificity of *binding* is thus controlled only by the M-Ig, but the degree of activation depends on additional interaction of the antigen with a suitable complementary *Ir* gene product, possibly by some type of net charge interaction, as reported by Karniely *et al.* (1973). This scheme also implies that the anti-*H-2* sera have antibodies to this *Ir* product, which may well show variable expression on different cell types. The scheme depicted in Fig. 5 implies that B cells could also express this ancilliary structure, but that it is not necessary for binding nor for activation. Alternatively, the *Ir* product may only be expressed in T cells (although the data of Basten with absorption by fibroblasts would not be consistent with this). If there is any reality to this scheme, the compromise situation of quantitative differences in expression of *Ir* product is more likely and raises the question of whether any of the other known T-cell membrane components

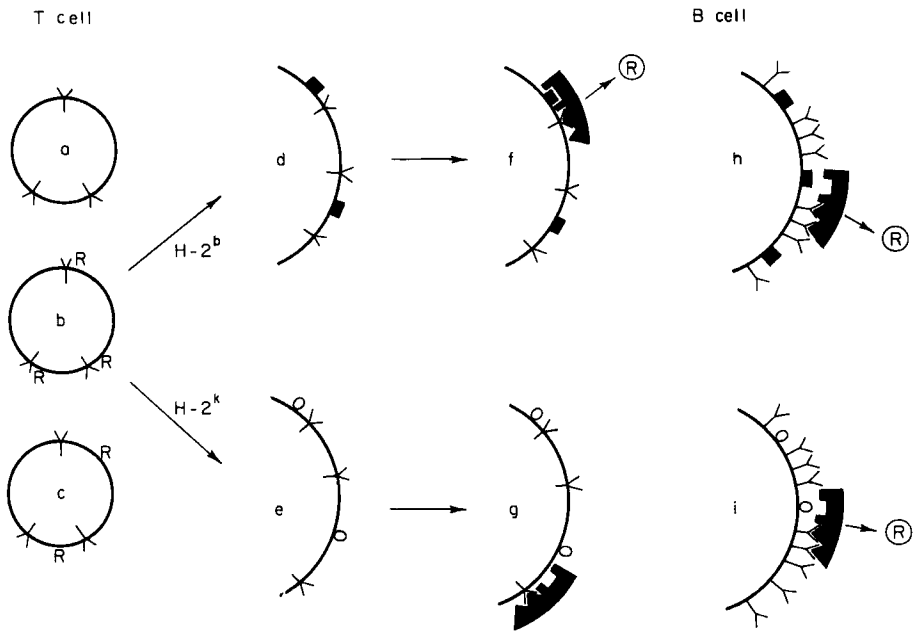

FIG. 5. Antigen binding and membrane-bound Ig (M-Ig) expression in T cells. Three possibilities of T-cell antigen receptors are indicated: (a) cell bears M-Ig as the receptor; (b) M-Ig and another cell surface component bind antigen; and (c) the cell bears M-Ig and another component (R) that is the sole receptor. In considering possibility b, a polymorphic *H-2*-associated product is depicted in two variants, d and e. This product and Ig are randomly present on the cell membrane. When an antigen is used that stimulates response (R) only in, for example, $H\text{-}2^b$-type animals, the antigen binds both to the M-Ig *V*-gene receptor and to the *H-2*-associated structure leading to cell activation (f). In the presence of the other *H-2*-associated structure, antigen is still bound to the M-Ig but the cell is not activated (g). In B cells (h and i), the density of the M-Ig receptor is considerably increased, and regardless of the nature of the *H-2*-associated cell surface component, multivalent binding of the antigen occurs in both cases leading to cell activation.

might be the *Ir* product, such as β_2-microglobulin, the Fc receptor, or any of the other protein or hormone receptors recognized.

F. MOBILITY OF MEMBRANE ANTIGEN RECEPTORS

One of the basic unanswered questions of cellular immunology concerns the process of activation of the lymphocyte following antigenic stimulation. The initial phase clearly involves interaction of the antigen with the M-Ig receptor of the cell. As the nature of this receptor is becoming reasonably well defined, considerable attention is now being focused on the actual changes that may occur at the cell membrane level following interaction of the antigen with its receptor.

In studying this problem it has become clear that M-Ig is neither

rigidly held in fixed positions nor is it just loosely bound in a state of secretion. Rather there is considerable fluidity and movement of the receptors in the plane of the membrane, and particular interest concerns changes in this movement after receptor–antigen union. In normal lymphocyte populations, synthesis and secretion of Ig is proceeding, with a portion of the secreted Ig having a transient phase on the cell surface (Vitetta and Uhr, 1972). As noted previously, there is some suggestion that the synthesis and secretion of Ig is distinct from the synthesis and turnover of M-Ig (Lerner *et al.*, 1972).

Current models of membrane structure envisage a fluid mosaic model of the membrane, composed of membrane proteins partly embedded in a double layer of phospholipids and free to move within the two-dimensional plane of the double layer (Frye and Edidin, 1970; Singer and Nicolson, 1972; Siekevitz, 1972). Studies on the metabolic turnover of M-Ig using a variety of methods have shown that both T cells and B cells turnover M-Ig at a relatively rapid rate (half-life 6–8 hours); mostly the Ig is released into the medium (Cone *et al.*, 1971; Lerner *et al.*, 1972; Wilson *et al.*, 1972; Loor *et al.*, 1972; Milton and Mowbray, 1972; Vitetta and Uhr, 1972). In the studies of Marchalonis and Cone (1973), in which T-cell M-Ig was also investigated, it was observed that B cells show a relatively faster rate of release of M-Ig than do activated T cells. Selectivity in turnover rates of various cell surface proteins is clearly evident with only minimal release of *H*-2 components (Vitetta and Uhr, 1972,) but marked release of mitogen receptors (Jones, 1973). Consistent with the conclusion of Lerner *et al.* (1972), Vitetta and Uhr (1972) observed that IgG secreted from lymphocytes did not appear to show any surface phase of binding in contrast to IgM. It was also shown in these studies that the released cell surface IgM is noncovalently bound to a fragment of cell membrane, and it was suggested that attachment of protein to Golgi vesicles, followed by reverse pinocytosis, is the major pathway for transport of proteins to the cell surface. It thus appears that in the normal cell membrane, IgM receptor is being continually turned over and released into the medium by a process that may be independent of active secretion by the stimulated cell.

How is the M-Ig located or positioned in the cell membrane? Observation with fluorescent anti-Ig sera indicated that the M-Ig is located in a crescent arrangement on one pole of the membrane on many of the cells (Möller, 1961), whereas when anti-*H*-2 sera were interspersed between the cell membrane and the anti-Ig, uniform ringlike staining of the cell occurred. Further detailed analysis of the cell M-Ig mobility has used labeled anti-Ig reagents followed appropriately by fluorescence, radioautography or electron microscopy. A consistent picture of M-Ig cell surface changes following M-Ig–

anti-Ig union has emerged (Taylor *et al.*, 1971; Loor *et al.*, 1972; de Petris and Raff, 1972; Karnovsky *et al.*, 1972) and can be considered in three stages: (*1*) the formation of multiple spots or patches of Ig over the cell membrane, (2) the formation of caps of M-Ig over one pole of the cells, and (3) the elimination of M-Ig from the capped area, and, in the absence of any further anti-Ig, of renewal of the M-Ig in an original diffuse arrangement.

Patch formation of M-Ig was first observed by Pernis *et al.* (1970) using immunofluorescence of rabbit lymphocytes, and similarities to local microaggregates of other cell surface proteins, such as *H*-2 (Stackpole *et al.*, 1971), were evident. The concept that the M-Ig (or *H*-2, or θ, etc.) might normally be located in the membrane in localized discrete sites has now been shown to be incorrect and results from linking of M-Ig by divalent anti-Ig molecules. Thus, when monomeric Fab fragments of anti-Ig are used (Taylor *et al.*, 1971, Loor *et al.*, 1972; de Petris and Raff, 1973), a diffuse and random distribution of M-Ig is observed. These observations strongly suggest that most membrane proteins including M-Ig move randomly in the plane of the membrane with noncovalent bonds attaching the Ig to other membrane components "floating in the lipid bilayer." Patch formation is induced by divalent antibodies, can occur in the cold, and in the presence of metabolic inhibitors such as sodium azide (Loor *et al.*, 1972; de Petris and Raff, 1973). Ingestion by the cell of M-Ig can probably occur at this stage without any requirement for cap formation (Santer *et al.*, 1972; Linthicum *et al.*, 1973), and active degradation by the cell of most of the anti-Ig–Ig complex appears to occur (Engers and Unanue, 1973). Patch formation can be inhibited by Con A (Yahara and Edelman, 1972, Loor *et al.*, 1972), and it was suggested that Con A binding leads to a change in the cell surface that results in an alteration of either the anchorage or the free path of the Ig receptors, possibly by binding of Ig receptors to particle-associated structures. Structural changes have been observed in PHA-stimulated cells as an increased density of globular particles in the cell membrane (Scott and Marchesi, 1972).

The next stage of M-Ig–anti-Ig movement involves an active metabolic process. Cap formation is temperature dependent, and when cells treated with anti-Ig in the cold are warmed, rapid flow of M-Ig to one pole of the cell occurs. Cap formation can be inhibited by a variety of agents including sodium azide, DNP, drugs that inhibit glycolysis and oxidative phosphorylation, Con A, partially by cytocholasin B, and not at all by protein synthesis inhibitors (Loor *et al.*, 1972; Taylor *et al.*, 1971; Unanue *et al.*, 1973b; Yahara and Edelman, 1972).

Quantitative aspects of amount of anti-Ig used are also relevant (Taylor *et al.*, 1971). It therefore appears that cap formation may partially involve microfilament activity, and it was suggested (de Petris and Raff, 1972) that the mechanism of flow of Ig to the uropod area involving the Golgi complex may be the same as that for membrane flow during cell movement.

Are these studies with anti-Ig-induced changes at all relevant to immunological activation of the cell by antigen? Several studies clearly indicate that multivalent antigens induce exactly the same changes in M-Ig movement in that they are temperature dependent and require active metabolism but not protein synthesis (Taylor *et al.*, 1971; Loor *et al.*, 1972; Ashman, 1973). As noted also by Roelants *et al.* (1973), when cells are studied for antigen binding after treating with anti-Ig in capping conditions, antigen is bound predominantly in capped locations.

Cap formation is rapidly followed by pinocytosis of the Ig determinants (Taylor *et al.*, 1971) and catabolism of the anti-Ig (Unanue *et al.*, 1973a; Engers and Unanue, 1973). This disappearance of surface determinants after cap formation induced by antibody probably explains the phenomenon of antigenic modulation of cell surface proteins, i.e., antibody-induced disappearance of the receptor from the cell surface (Old *et al.*, 1968).

Following anti-Ig-induced capping and pinocytosis of M-Ig, a rapid reappearance of new M-Ig occurs. Although one study showed resynthesis of M-Ig to about the same level (Elson *et al.*, 1973), other studies have shown an increased concentration of M-Ig following capping and regeneration (Diener and Paetkau, 1972; Loor *et al.*, 1972). The phenomenon of capping, per se, is not thought to be directly responsible for cell stimulation (Greaves and Janossy, 1972). The *resulting* resynthesis and recapping following initial antigen-induced capping has, however, not been observed to occur in cells given tolerogenic doses of antigen (Diener and Paetkau, 1972), thus clearly indicating that high dose levels of antigen can lead to a degree of cross-linking that restricts further capping.

Katz and Unanue (1972) also observed that anti-Ig-induced capping of primed cells did not in itself stimulate the cells in the absence of antigen, but after reaction with anti-Ig, the efficiency of the response to antigen was significantly increased, possibly due to a more efficient presentation of concentration of the newly formed receptors.

Further investigations into the area of lymphocyte stimulation (see *Transplantation Reviews*, Vol. 11) must consider this dynamic state of

the surface receptors in attempting to resolve the nature of the biological events immediately subsequent to the antigen–receptor interaction on the cell membrane.

VI. Conclusions

Although there are still several unresolved aspects concerning the origin and characterization of M-Ig, a fairly constant picture of M-Ig as the essential component of the antigen receptor site on T and B cells has emerged. There are several qualitative and quantitative distinctions between T-cell and B-cell M-Ig, which may explain many of the apparent differences in reactivity of these cells without requiring the existence of a different set of *V* genes or non-Ig receptors in T cells.

Both T and B cells derive from hematopoietic, precursor, stem cells by antigen-independent differentiation. This involves activation of L-chain and μ-type H-chain genes in both cell types.

The T-cell and B-cell M-Ig differ primarily in density per cell and possibly in relative exposure on the cell surface with much of the H-chain Fc region inaccessible on T cells. The B cells average 10^5 molecules of M-Ig per cell, and T cells of the order of 10^2 to 10^3 molecules per cell.

The presence of M-Ig need not indicate intrinsic origin, as all B cells possess a receptor for binding the Fc region of certain Ig classes. The avidity of binding to Ig is greatly augmented in some species when the Ig is aggregated or complexed with antigen. It is not resolved whether T cells also have receptors for binding Ig, although it is reasonably likely that at least activated T cells may be able to bind some Ig.

The presence of receptor for binding Ig complicates the interpretation of the origin of M-Ig on B and T cells. In all but a few instances, it appears that a given B cell carries only one class and one allotype of L- and H-chain genes. In unstimulated cells, the predominant M-Ig is of IgM class, although a proportion of IgM-bearing cells can secrete IgA or IgG. With antigen-induced differentiation of B cells, both quantitative and qualitative changes in M-Ig occur. The density of M-Ig progressively declines with increasing cell maturation, although plasma cells still bear some M-Ig. A shift in the proportion of cells bearing IgM- or IgG-type M-Ig occurs, with IgG becoming predominant. During clonal expansion, some cells appear to bear multiple classes of M-Ig, although the origin of this Ig is not completely determined.

Whether a true sequential activation of H-chain genes occurs during clonal proliferation or whether IgG-secreting cells are less readily activated has not been resolved.

On antigen stimulation, T cells do not show any change in Ig class

expression, although a moderate increase in receptor density may occur.

Membrane Ig's are in a dynamic state on the cell membrane and the relationship of surface changes on interference with M-Ig movement to lymphocyte activation has yet to be fully clarified.

The antigen-recognition receptor of both T and B cells involves their M-Ig. On B cells the receptor is solely the M-Ig, whereas on T cells it is suggested that, whereas the specificity of recognition is controlled primarily by the M-Ig and binding of antigen to the cell can be solely by the M-Ig receptor, activation of the T cell requires an additional interaction of the antigen with another cell surface structure that is coded for by a polymorphic *H*-2 linked gene. Although the nature of this second component is not clear, it must be stressed that there can be little doubt that M-Ig of T cells is a *part* of the T-cell receptor for antigen.

Although much is known about the expression of M-Ig on normal, malignant, and abnormal lymphoid cells, and information of this type is particularly useful in characterizing the differentiated stage of the cell, several aspects of antigen recognition and M-Ig require further analysis.

1. Are there independent IgM and IgG precursor cells in the adult nonstimulated animal or does IgM act as the primary membrane-bound antigen receptor site on virgin cells, even if the cell is destined to secrete IgG or IgA?

2. Is T-cell IgM composed of a μ-type H chain that is not encoded by the same μ-chain locus as the B-cell IgM?

3. Is there a second structure on T-cell membranes that is involved in the recognition or binding of antigen in a manner that leads to lymphocyte activation?

4. Are any of the changes observed at the cell membrane level by antigen–receptor interaction directly responsible for lymphocyte activation?

Considering the ever increasing interest and activity in this field of immunological research, it would not be too unlikely to expect that these and other related questions of the nature and function of M-Ig on T and B cells might be resolved by the time this article is published.

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Receptors for Immune Complexes on Lymphocytes[1]

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I. Introduction

The functions of the cells of the lymphoid system are being gradually elucidated (for reviews, see Miller and Mitchell, 1969; Claman, 1972; Katz and Benacerraf, 1972). The discovery that lymphocytes can be grouped into subpopulations (B and T lymphocytes) with specific properties and functions has been firmly established and has opened the way to new investigations. Some of the key findings in this area have been the observations that, in the mouse, certain lymphocytes possess on their surface relatively large amounts of immunoglobulin (Ig) molecules (Raff *et al.*, 1970), which serve as specific recognition units for antigen (Ada, 1970; Greaves, 1970; Mäkelä, 1970; Paul, 1970; Wigzell, 1970; Raff *et al.*, 1973). These same cells (B lymphocytes) are

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the precursors of cells that secrete antibody after antigenic stimulation, whereas T lymphocytes, which in the mouse bear the θ antigen on their membranes (Reif and Allen, 1964; Raff, 1969; 1971; Schlesinger and Yron, 1969), induce cell-mediated immunity (Warner *et al.*, 1962; Cooper *et al.*, 1966; Miller and Osoba, 1967; Good *et al.*, 1971).

Another membrane marker, identified in B lymphocytes, and operationally defined as a complement receptor, has been described and studied in this laboratory (Lay and Nussenzweig, 1968; Bianco *et al.*, 1970). Membrane receptors for complement have been detected through the binding of sheep erythrocytes sensitized with antibody and complement, leading to the formation of rosettes [a cell surrounded by sensitized erythrocytes – erythrocyte – antibody – complement (EAC) complexes] which can be seen and counted under the microscope. Immune complexes prepared with bacterial antigens can also bind *in vitro* to some lymphocytes. For example, it has been observed that flagella–antiflagella complexes bind to the membrane of guinea pig lymphocytes. In this case, complement participation in the binding was not formally demonstrated, but it was reported that the binding diminished when complement fixation was inhibited (Uhr, 1965; Uhr and Phillips, 1966).

Lymphocytes can also interact with immune complexes by means of receptors for antigen–antibody complexes or for aggregated immunoglobulin (LoBuglio *et al.*, 1967; Brown *et al.*, 1970b; Basten *et al.*, 1972a; Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972). In this review I will discuss some aspects of the interaction between lymphocytes and immune complexes, the use of membrane receptors for complexes in the detection, identification, and isolation of B lymphocytes, and the possible participation of the cell receptors in the induction of the immune response *in vivo*. Only studies dealing with the surface membrane of the lymphocyte are reviewed here, although other bone marrow-derived cells, such as granulocytes, monocytes, macrophages, and platelets, display membrane receptors for immunoglobulin and C3 with similar general properties (reviewed in Rabinovitch, 1970; Henson, 1972; Unanue, 1972).

II. Interaction between Lymphocytes and Particulate Immune Complexes

A. Distribution and Origin of Complement Receptor Lymphocytes

Complement receptor lymphocytes (CRL's) have been found in man and other mammals (Bianco *et al.*, 1970; Michlmayr and Huber, 1970). They are rare (1–2%) in the thymus and present in different proportions in various lymphoid organs. In outbred CF1 mice, 8–12

weeks old, CRL's comprise 30–40% of the cells of the spleen, 10–25% of the lymph node, 10–20% of the thoracic duct, and about 5% of the mononuclear cells of the bone marrow. Lower proportions of CRL's are found in the lymphoid organs of certain inbred strains of mice. No significant changes occurred in the frequency of CRL's in the spleen, lymph nodes, and bone marrow of CBA strain mice from 200 to 500 days of age (Stutman, 1972).

Complement receptor lymphocytes are small, having an average diameter of 7 μm. Large lymphocytes, greater than 9 μm., account for about 1% of CRL's in the spleen and about 4% of CRL's in the lymph nodes. Morphologically, it is difficult to distinguish CRL's from other lymphocytes either with the light or with the electron microscope (Chen *et al.*, 1972). However, some quantitative differences have been reported between CRL's and non-CRL's in their nuclear structure and in the number of coated vesicles in their cytoplasm (Suter *et al.*, 1972).

The points of contact between the membranes of CRL's and EAC are made through numerous fingerlike projections (microvilli) or through nonvillous areas of the plasma membrane of the lymphocyte. This is shown by section electron microscopy (Fig. 1) or by scanning electron microscopy (Fig. 2). The multiple points of attachment probably contribute to the stability of EAC–CRL rosettes which resist vigorous agitation in a Vortex mixer. The strength of attachment is sufficiently great to deform the membrane of the red cells and when rosettes are taken apart (see Section II,F), fragments of erythrocytes may be found on the lymphocyte membrane. The finding that the primary points of attachment are the tips of the microvilli suggests that these areas may have specialized recognition functions. However, it is not known whether the microvilli, which are more prominent and numerous on B than on T lymphocytes (Lin *et al.*, 1973; Polliack *et al.*, 1973), have some degree of stability or whether they are transient structures continuously emerging from the cell surface.

Histologically, CRL's can be detected in frozen tissue sections by overlayering them with EAC. The erythrocytes firmly adhere to areas rich in CRL's (Dukor *et al.*, 1970) in certain well-delineated anatomical sites: in the follicular areas of the white pulp and the marginal zone of the spleen, in the cortex of lymph nodes, and in the follicles of Peyer patches. These are the so-called thymus-independent areas of these organs (Parrot *et al.*, 1966). Although these areas may also contain some macrophages that also bind EAC, electron-micrographic studies have shown numerous contacts between EAC and small lymphocytes. The same method has been recently used to determine the nature of cells in frozen sections of tissues from patients (Shevach *et al.*, 1973). Under the conditions employed, erythrocyte–antibody

complexes (EA), prepared with relatively low concentrations of 7 S rabbit antibodies to erythrocytes, do not bind to human or mouse lymphocytes but readily adhere to the macrophages in the sections of lymph nodes and spleen. On the other hand, EAC's prepared with 19 S rabbit antibodies to erythrocytes bind to lymphocytes as well as to

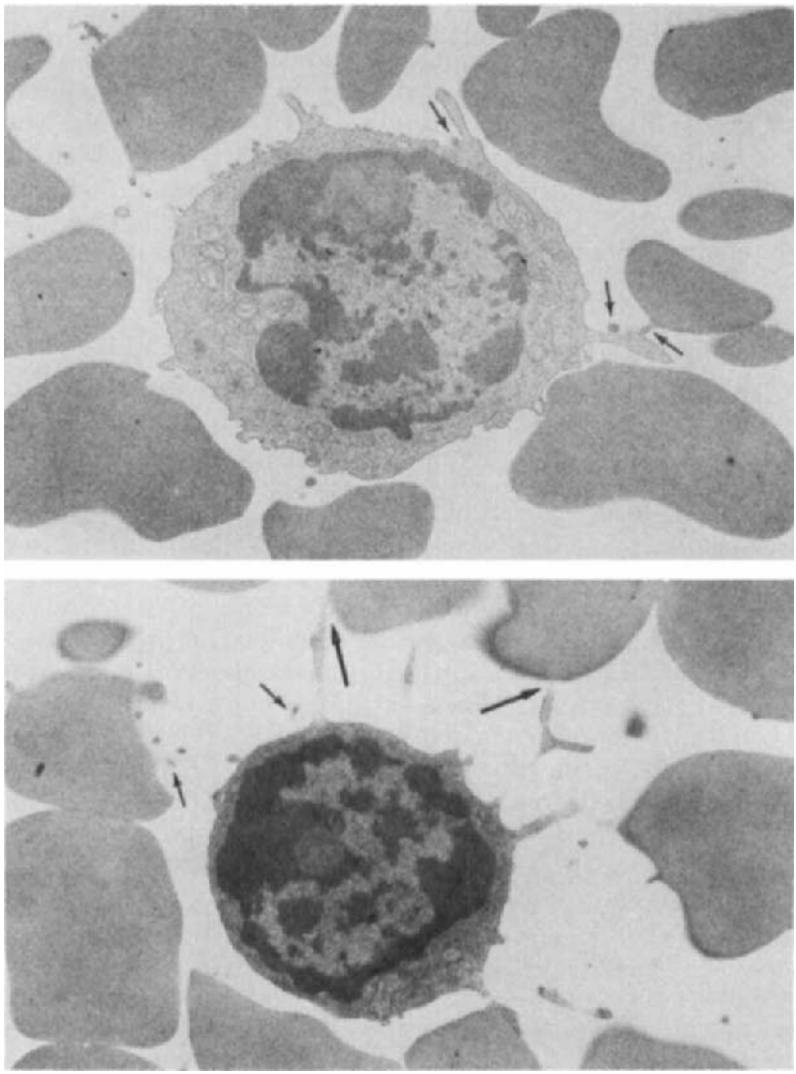

FIG. 1. Rosettes between erythrocyte-antibody-complement complex (EAC) and mouse complement receptor lymphocytes (CRL's). Several EAC projections (thin arrows) adhere to the membrane of CRL's. Long cytoplasmic projections of CRL (microvilli, thick arrows) bind to the sensitized cells. Magnification: ×8,500. (From Chen *et al.*, 1972.)

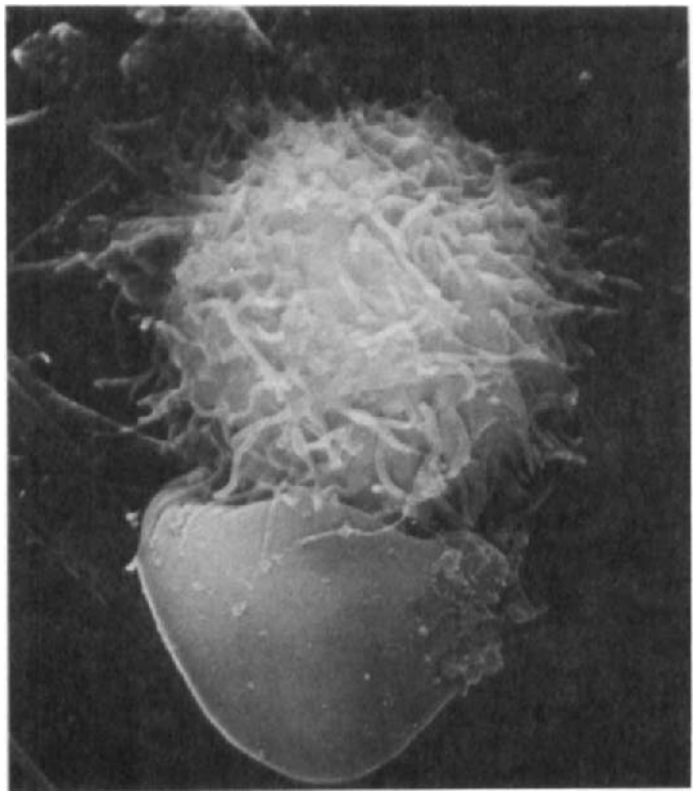

FIG. 2a. Scanning electron microscopy of erythrocyte–antibody–complement complex (EAC)–complement receptor lymphocyte rosettes with human lymphocytes. Lymphocyte from the peripheral blood, with numerous long microvilli (approximately 2 μm.). Magnifications $\times$ 10,000.

histiocytes of the lymph node. Curiously, it appears that spleen macrophages lack the C3 receptor. Thus, in order to study the nature of the mononuclear cells in an infiltrate, serial sections of a biopsy can be exposed to EA (7 S), EA (19 S), and EA(19 S)C. In this way, CRL's were identified as the major cell type in cutaneous infiltrates in a patient with a B-cell leukemia (Edelson *et al.*, 1973) and in infiltrates of the minor salivary glands of the lip of patients with Sjögren's syndrome (Chused *et al.*, 1974). Similarly, evidence has also been obtained that a nodular lymphoma originated from follicular CRL's (Jaffe *et al.*, 1974) (Figs. 3 and 4).

The finding that EAC's do not bind either to T areas of lymphoid organs or to sections of the thymus itself suggests their extrathymic origin. The question was further investigated through the use of other

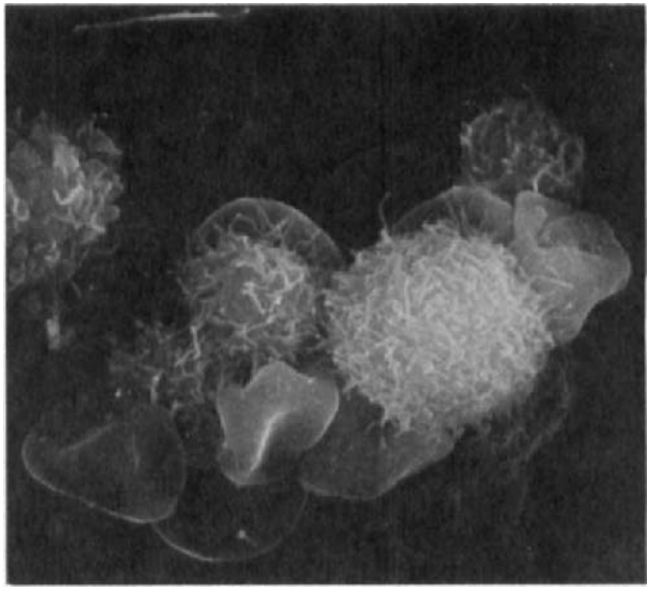

FIG. 2b. Scanning electron microscopy of erythrocyte-antibody-complement complex (EAC)-complement receptor lymphocyte rosettes with human lymphocytes. Lymphocytes from tonsils, with long microvilli, clustering around EAC. Magnification: ×4250.

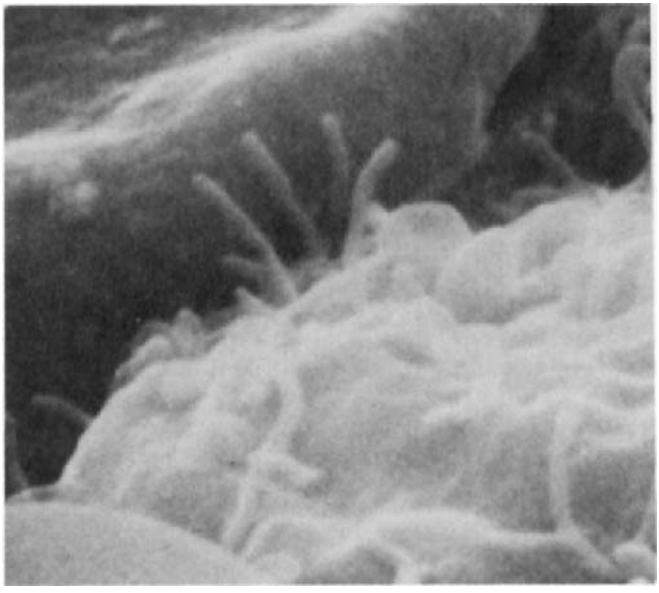

FIG. 2c. Scanning electron microscopy of erythrocyte-antibody-complement complex (EAC)-complement receptor lymphocyte rosettes with human lymphocytes. Lymphocyte from the peripheral blood binding EAC via microvilli. Magnification: ×17,850. (From Lin *et al.*, 1973; Wortis *et al.*, 1973.)

FIG. 3a. Normal human spleen and lymph node, dark-field microscopy. Spleen after treatment with erythrocyte–antibody (19 S)–complement [EA(19 S)C]. *Cells in follicle of white pulp bind the red cells.*

known membrane characteristics of mouse B and T lymphocytes. In some experiments, advantage was taken of the fact that rosettes can be selectively removed from other cell populations by differential flotation in gradients of bovine serum albumin or Hypaque-Ficoll. Because of their higher density, they did sediment under conditions where free lymphocytes did not. In this way it was found that most mouse CRL's, like B cells, have Ig on their membranes, whereas non-CRL's do not (Bianco *et al.*, 1970). Also, passage of mouse spleen cell suspensions through anti-Ig-coated columns, which are reported to bind B lymphocytes selectively, deplete the lymphocytes of CRL's (Wigzell *et al.*, 1972). In addition, mouse CRL's do not bear the θ antigen, a marker for T cells, on their membranes (Bianco and Nus-

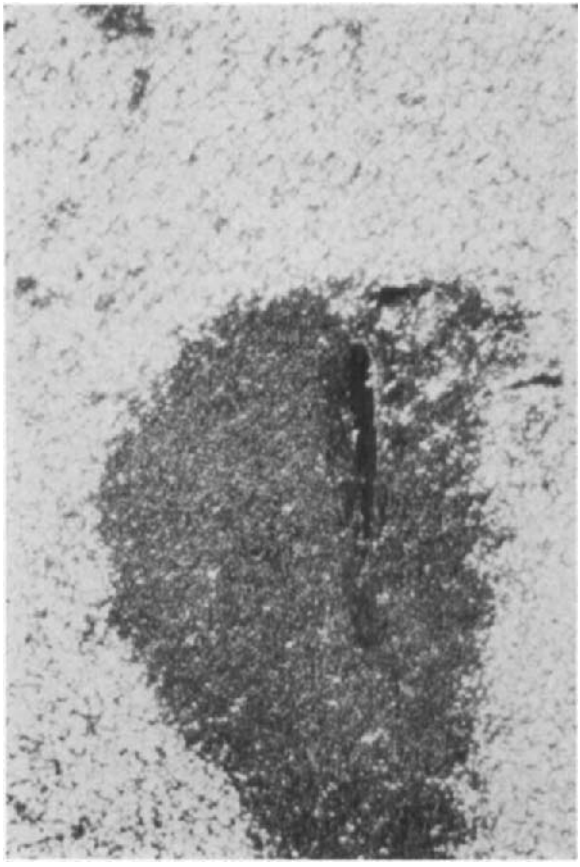

FIG. 3b. Normal human spleen and lymph node, dark-field microscopy. Adjoining section of spleen covered with EA(7 S). Macrophages in red pulp bind the red cells.

senzweig, 1971), and cell transfer experiments indicate that they are derived from the bone marrow and do not require the thymus for their development (Dukor *et al.*, 1971). Complement receptor lymphocytes constitute a large proportion of cells found in the spleens of nude athymic mice. All these observations strongly suggest that the complement receptor is a marker of a B-cell population.

The life-span of CRL's was investigated by injecting mice with ^{3}H-thymidine according to two different schedules that labeled either long-lived or short-lived lymphocytes. The grain distribution in radioautographs of CRL's was not different from that of total lymph node populations. Thus CRL's comprise both long- and short-lived cells (Bianco *et al.*, 1970).

Complement receptor lymphocytes can also recirculate from blood

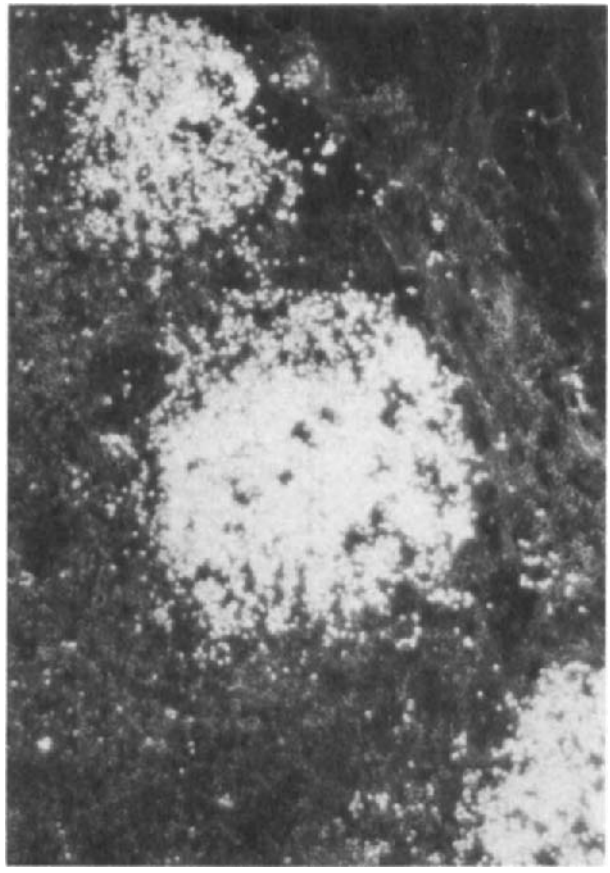

FIG. 3c. Normal human spleen and lymph node, dark-field microscopy. Lymphoid follicles of lymph node covered with EA(19 S)C. In these pictures the adherent red cells appear as bright refractile bodies against a dark background. (From Jaffe *et al.*, 1974; Edelson *et al.*, 1973.)

to lymph. This was established by injecting ^{51}Cr-labeled rat thoracic duct lymphocytes intravenously into syngeneic rats. These rats were subsequently canulated, and ^{51}Cr-labeled CRL's were recovered from their lymph. As shown previously for rat B lymphocytes (Howard, 1972), rat CRL's took a longer time than the other lymphocytes to reappear in the lymph (F. Quagliata, G. W. Miller, and V. Nussenzweig, unpublished observation).

B. Nature of Lymphocyte Receptor and of Complement Component Involved

Receptors for complement have been demonstrated in (*a*) normal lymphocytes from many animal species, (*b*) human and guinea pig

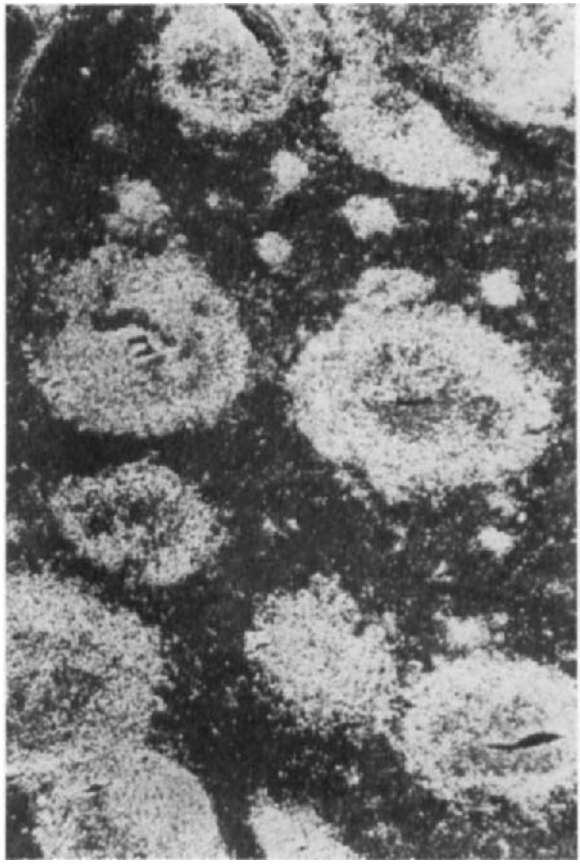

FIG. 4a. Identification of mononuclear cells in sections of pathological specimens from human patients. Nodular lymphoma in spleen. Section was covered with erythrocyte–antibody(19 S)–complement [EA(19 S)C]; abnormal follicles covered with erythrocytes, indicating that CRL's are present underneath. Dark-field microscopy.

leukemic cells (Pincus *et al.*, 1972; Shevach *et al.*, 1972a,b; Ross *et al.*, 1973c), and (*c*) human cell lines (Shevach *et al.*, 1972b). Interestingly, the C3 receptor was not found in any mouse lymphomas or leukemias induced either by carcinogens, virus, or oil injection (Shevach *et al.*, 1972c).

The complement receptor is probably a protein because it is destroyed by trypsin treatment (Bianco *et al.*, 1970). It does not disappear after prolonged cultivation *in vitro* as shown by its presence in numerous human cell lines. The complement receptor probably is not the membrane-bound Ig found on CRL's because the EAC–CRL in-

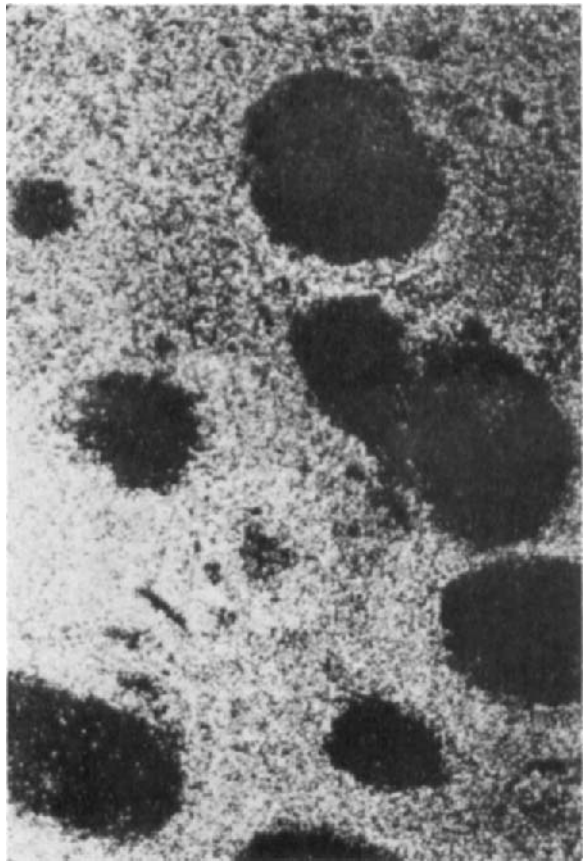

FIG. 4b. Identification of mononuclear cells in sections of pathological specimens from human patients. Adjoining section of this nodular lymphoma covered with EA (7 S), which bind to macrophages in red pulp; dark-field microscopy.

teraction is not affected by the presence in the medium of antibodies to Ig (Bianco and Nussenzweig, 1971). Also, no correlation could be established in several human cell lines between the presence of Ig and of the C3 receptor on the cell membrane. For example, lymphocytes from one cell line (Raji), shown to lack any cell-associated Ig by a sensitive Farr technique (Lerner *et al.*, 1971), display the C3 receptor (Shevach *et al.*, 1972b).

The complement component involved in the interaction between EAC and CRL is probably C3 for the following reasons: (*1*) EAC43, but not EAC4, prepared either with human or with guinea pig puri-

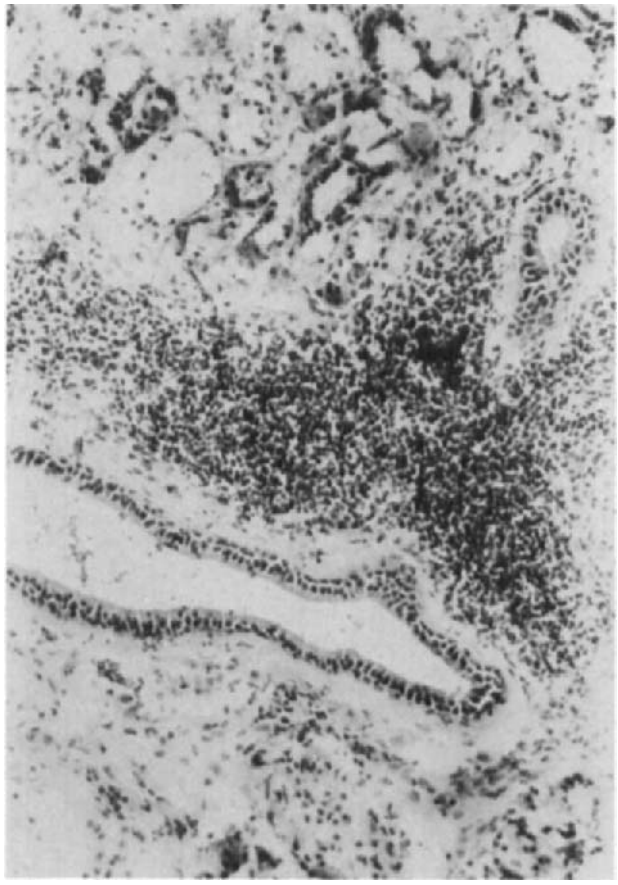

FIG. 4c. Identification of mononuclear cells in sections of pathological specimens from human patients. Lip biopsy from Sjögren's syndrome covered with EA(19 S)C; bright-field illumination.

fied C components, binds to lymphocytes (Bianco *et al.*, 1970; Eden *et al.*, 1973c); (2) the number of clusters formed depends on the concentration of C3 used to prepare EAC43 (Eden *et al.*, 1973c; Ross *et al.*, 1973c); (3) antisera to mouse C3 inhibit and reverse EAC–CRL interaction (Eden *et al.*, 1971) (Fig. 5); (4) C3i, obtained in the fluid phase by interaction of EAC142 with purified C3 (Eden *et al.*, 1973c), as well as C3b (Eden *et al.*, 1973d), obtained by limited hydrolysis of C3 with trypsin (Bokisch *et al.*, 1969), inhibit rosette formation between EAC43 and CRL (Fig. 6); (5) cells from human lymphoblas-

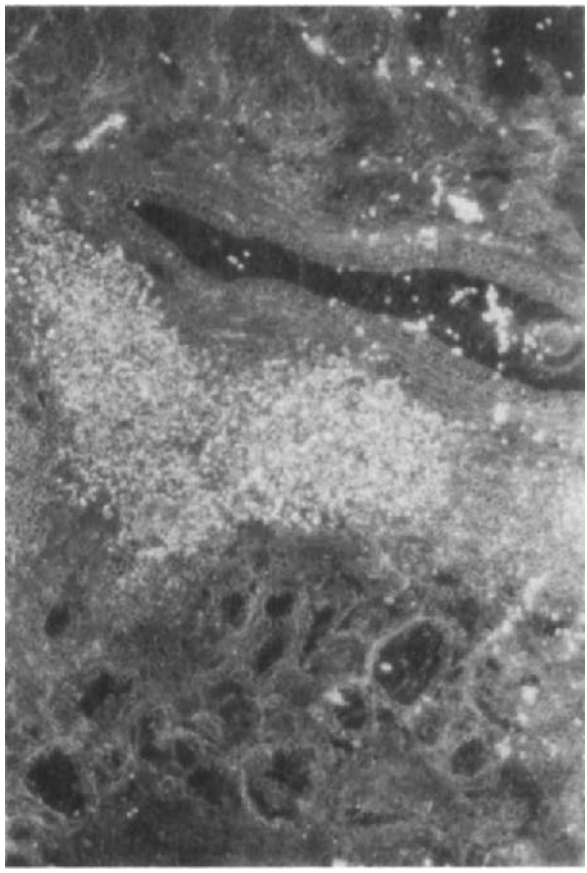

FIG. 4d. Identification of mononuclear cells in sections of pathological specimens from human patients. Same section, dark-field illumination. The lymphocytic infiltrate is covered by red cells, showing that it contains complement receptor lymphocytes. (From Jaffe *et al.*, 1974; Chused *et al.*, 1974.)

toid cell lines bind radioactively labeled C3 and C3b (Bokisch and Theofilopoulos, 1973).

It has recently been shown that human CRL's bind EAC43b(human) as well as EAC43d(human) (Eden *et al.*, 1973d; Ross *et al.*, 1973b). The EAC43d is a cell intermediate that results from the interaction of EAC43b with a serum enzyme, C3 inactivator, or KAF (Tamura and Nelson, 1967; Lachmann and Müller-Eberhard, 1968; Ruddy and Austen, 1969). Since human C3b, obtained by treatment of purified human C3 with trypsin inhibits EAC43b–CRL as well as

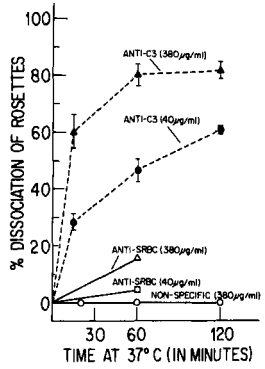

FIG. 5. Dissociation of erythrocyte–antibody–complement complex and complement receptor lymphocytes rosettes by papain fragments of Ig from a rabbit antiserum to mouse C3. The Ig fragments were used at a final concentration of 380 μg./ml. or 40 μg./ml. The controls were prepared either with papain fragments of normal rabbit γG or with papain fragments of the γG fraction of a rabbit antiserum to sheep red blood cells (SRBC). (From Eden *et al.*, 1971a.)

EAC43d–CRL interaction (Eden *et al.*, 1973d), CRL's might have either (*a*) only one membrane receptor for a structure present both on C3d and on C3b molecules or (*b*) two separate receptors on the lymphocyte membrane for two different sites on the C3b molecule, one of them destroyed by C3 inactivator. Several observations give support to the second hypothesis: lymphocytes from patients with chronic lymphatic leukemia preferentially bind EAC43d; normal

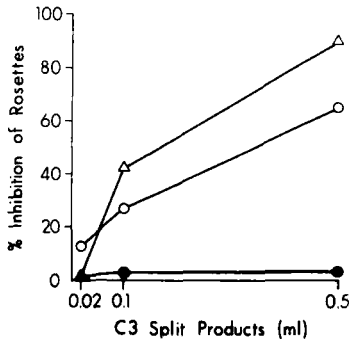

FIG. 6. Inhibition of rosette formation between erythrocyte–antibody–complement (EAC$\overline{1423}$) (prepared with purified guinea pig complement components) and mouse spleen lymphocytes by the addition of various amounts of guinea pig C3-split products. Split products were generated by interaction of EAC$\overline{142}$ with 1500 units (△) or 750 units (○) of C3. Controls were incubated with buffer (●). Similar results were obtained when guinea pig spleen lymphocytes were used. (From Eden *et al.*, 1973c.)

TABLE I
EFFECT ON ROSETTE FORMATION AND ON IMMUNE ADHERENCE OF TREATING ERYTHROCYTES–ANTIBODY–COMPLEMENT [EAC43] COMPLEX WITH C3b INACTIVATOR[a]

Type of leukocyte	Indicator cells[b]	Dilution of EDTA serum (or purified C3b inactivator) used to treat indicator cells	Rosettes (%)[d]	Immune adherence between human type O erythrocytes and indicator cells
Granulocytes	EA	No treatment[c]	1.4	–
	EAC4	No treatment	9.8	–
	EAC43	No treatment	44.0 (27.0)	+
	EAC43	1/64	19.3	±
	EAC43	1/32	8.3	–
	EAC43	1/16	8.0	–
	EAC43	1/8	7.4 (4.0)	–
Blood lymphocytes	EAC4	No treatment[c]	2.3	–
	EAC43	No treatment[c]	11.6 (13.1)	+
	EAC43	1/4	11.9 (15.8)	–
Tonsil lymphocytes	EAC4	No treatment	1.2	–
	EAC43	No treatment	37.4 (32.0)	+
	EAC43	1/4	38.8 (27.8)	–

[a] From Eden *et al.* (1973d).

[b] Erythrocytes–antibody–complement (EAC43) complexes were prepared with purified human complement components.

[c] The cell intermediates were incubated in ethylenediaminetetraacetate (EDTA)–GVB.

[d] The numbers in parentheses refer to results obtained with $EAC43_{(human)}$ treated with a purified preparation of C3b inactivator (initial concentration: 500 units/ml.).

lymphocytes bind both EAC43b and EAC43d prepared with human C3 (Ross *et al.*, 1973b); and human granulocytes preferentially bind EAC43b (Eden *et al.*, 1973d) (Table I). Also, it has been reported that antisera can be obtained that specifically inhibit the interaction of human lymphocytes with either EAC(mouse), which presumably bears the C3d site (see Section II,C), or with EAC43b(human) (Ross *et al.*, 1973a). Antisera obtained by immunizing Rhesus monkeys with normal human spleen lymphocytes inhibit the reaction of normal lymphocytes with mouse or human complement. These antisera, after absorption with human erythrocytes (which have the classic immunoadherence receptors for human C3b), inhibit rosette formation with the erythrocytes sensitized with mouse complement but not with EAC43b(human). Other antisera, prepared against lymphocytes from patients with chronic lymphatic leukemia, strongly inhibit rosette formation with EAC(mouse) but not at all or only weakly with EAC43b(human).

In short, it appears that after activation, the C3 molecules bear several distinct regions that can interact with cell membranes:

A "nonspecific" binding site with a short half-life (Müller-Eberhard *et al.*, 1966) which is generated after the interaction of C3 and antibody-sensitized cells that bear on their membranes the enzyme C3 convertase. This enzyme cleaves C3 into two fragments, one of them (C3b) able to bind to membranes and leading to the formation on the cell surface of a new enzyme, $C\overline{4,2,3}$. All cells that can be lysed by antibody and complement (including thymocytes) must bear receptors for the nonspecific C3 binding site. Fluid phase C3i, as well as C3b obtained by trypsin treatment of C3, have lost this nonspecific site and are hemolytically inactive.

Two other regions of C3, present on C3i or C3b, can interact specifically with leukocytes. The B lymphocytes have receptors for both, whereas granulocytes and human red cells recognize mainly one, probably the classic immunoadherence site (Nelson, 1953).

C. Detection of Complement Receptor Lymphocytes

Some contradictory results about the proportion of CRL's in peripheral lymphoid organs and in the blood of normal individuals and of patients with leukemia may be due to the use in the rosette test of different, and sometimes inadequate, preparations of EAC.[2] Some of the following factors should be considered when preparing this reagent:

1. Erythrocytes from sheep are usually employed. However, if CRL's from man are to be identified, it is important to be aware that sheep erythrocytes, at temperatures below 37°C., can bind and form clusters with human T lymphocytes (Brain *et al.*, 1970; Coombs *et al.*, 1970; Lay *et al.*, 1971). To avoid this interaction, the sheep erythrocytes may be treated with trypsin before incubation with antibody and complement, since this treatment removes from the red cell surface the presumed site for human T lymphocytes (Weiner *et al.*, 1973). Alternatively, the experimental tubes can be kept at 37°C. before counting the rosettes. Erythrocytes from other species have been used to prepare EAC's (Jondal *et al.*, 1972). However, the use of human red cells should be avoided because they have the classic immune-

[2] For example, the proportion of CRL's in the blood of patients with chronic lymphatic leukemia has been reported to be much above normal (Pincus *et al.*, 1972; Shevach *et al.*, 1972b; Ross *et al.*, 1973c) or much below normal (Michlmayr and Huber, 1970; Nishioka, 1971). The proportion of CRL's found in normal human peripheral blood varied from 7 to 30% (Jondal *et al.*, 1972; Pincus *et al.*, 1972).

adherence site for immune complexes (Nelson, 1953). After sensitization with complement components, human red cells may form clusters with each other or with the red cells contaminating the lymphocyte preparations and thus make enumeration of EAC-CRL rosettes very difficult.

2. Antibodies cytophilic for phagocytic cells, such as monocytes and macrophages, should be avoided in the preparation of EAC. For example, to detect mouse or human CRL's, EA prepared with 19 S rabbit anti-Forssman antibodies have been employed because this reagent binds to leukocytes in suspension only *after* interaction with complement. On the other hand, EA prepared with rabbit or mouse 7 S antibody form clusters with mouse monocytes, macrophages, and granulocytes without participation of complement (Lay and Nussenzweig, 1968). It has been shown that as few as 10^3 molecules of rabbit 7 S antibodies are sufficient to sensitize sheep erythrocytes to interact with mouse macrophages (Mantovani *et al.*, 1972). However, EA prepared with 20 times as many molecules of either mouse or rabbit 7 S antibodies do not bind to mouse lymphocytes when the cells are incubated together in suspension at 37°C. for 30 minutes (C. Bianco and V. Nussenzweig, unpublished observations). However, when rat or mouse lymphocytes are incubated with EA prepared with hyperimmune rabbit antibodies to erythrocytes and subsequently *centrifuged*, a large number of rosettes are formed. Thus, a close packing of the lymphoid cells and the erythrocytes is crucial for the formation of EA rosettes but not of EAC rosettes (Kedar *et al.*, 1974). Interestingly, when the centrifugation method for the detection of EA-binding cells was used, equal numbers of EA and EAC rosettes were formed. However, it has not been determined whether the same cell population bound both reagents. Based on these findings, a relatively simple procedure has been proposed to separate B and T lymphocytes. Lymphocytes are centrifuged onto EA monolayers prepared on polystyrene Petri dishes previously treated with poly-L-lysine. More than 90% of lymphoid cells from different organs and bearing the receptor for EA were shown to adhere to the monolayers, and they could be recovered following lysis of the erythrocytes (Kedar *et al.*, 1974).

It is not clear why monocytes and macrophages recognize EA more efficiently than lymphocytes do, since both cells have membrane receptors for aggregated Ig (see Section III). The simplest explanation may be that relatively few functional receptors for complexed IgG exist on the lymphocyte surface and that clustering or aggregation of Ig may be necessary to increase their binding to the lymphocyte

membrane, either by allosteric mechanisms or more likely by cooperative effects (Phillips-Quagliata *et al.*, 1971). Whatever the reason, only EA's prepared with agglutinating doses of 7 S antibody bind and form rosettes in suspension with mouse lymphocytes (Cline *et al.*, 1972). In this case, the EA-binding lymphocytes were identified as B cells because they were the predominant population of cells (40–60%) obtained from the thoracic duct of mice that had been neonatally thymectomized or from congenitally athymic nude mice. In addition, EA's did not bind to thymocytes or to activated T cells obtained from the thoracic duct of (CBA×C57Bl) F_1 mice heavily irradiated and injected with CBA thymocytes. In contrast, others have reported that EA's bind to some thymocytes and to most (90%) activated T cells, obtained by the procedure described in the foregoing, that is, by allowing thymus cells to proliferate in allogeneic hosts (Yoshida and Andersson, 1972). The reason for this discrepancy is not clear.

3. Complement does not have to originate from the same mammalian species as the lymphocyte donor. It appears that the structure of the postulated receptors for C3 products has been well-preserved in evolution, since, for example, EAC's prepared with mouse complement react with B lymphocytes from mouse, rat, guinea pig, rabbit, and man. However, instances of species specificity have been found. Thus, EAC(human) bind to guinea pig, rabbit, and human lymphocytes but do not form rosettes with mouse lymphocytes (Bianco *et al.*, 1970).

The EAC can be prepared with either whole serum as a source of complement or with purified complement components. To prepare EAC(mouse), relatively high concentrations of serum are generally used as a source of complement. In this way, it is presumed that many C3b sites are generated on the erythrocyte membrane (Müller-Eberhard, 1968). The binding to the erythrocytes of late mouse complement components can probably be avoided by using serum from C5-deficient mice (Herzemberg *et al.*, 1963; Cinader *et al.*, 1964) as a source of complement. However, it should be stressed that if the complement source is whole serum, it is possible that the C3b molecules bound to the erythrocyte membrane are further modified through the activity of the serum enzyme, C3 inactivator. This enzyme splits membrane-bound C3b into two fragments: C3c, which is released into the medium, and C3d, which remains associated with the cell surface (Ruddy and Austen, 1971). For this reason, EAC(mouse) or EAC(human) prepared with whole serum as a source of complement probably display both C3b- and C3d-combining regions. When human or guinea pig purified complement components are used,

EAC43b cells are generated exclusively or predominantly. As mentioned earlier, it has recently been found that normal human lymphocytes bind both EAC43d and EAC43b prepared with human C3, whereas human granulocytes and erythrocytes preferentially bind red cells bearing C3b (Eden *et al.*, 1973d; Ross *et al.*, 1973b). In other words, the classic immunoadherence receptor (for C3b) (reviewed by Nelson, 1963; Nishioka, 1971) is found on human granulocytes, erythrocytes, and lymphocytes, but lymphocytes have in addition receptors for C3d. These recent findings may be the explanation for the observations that (*a*) EAC(mouse) is a better reagent than EAC prepared with purified human complement components for the detection of CRL in the blood of patients with chronic lymphocytic leukemia (Ross *et al.*, 1973c); (*b*) EAC43b(human), but not EAC(mouse), binds to human granulocytes (Pincus *et al.*, 1972); and (*c*) some cultured lymphoid cell lines from Burkitt lymphoma (such as Daudi cells) bind EAC(mouse) but not EAC43(guinea pig), that is, these lymphoma cells presumably have on their membranes the receptors for C3d but not for C3b (Okada and Nishioka, 1973). Since most normal lymphocytes appear to have two C3 receptors (see Section II,B), it is possible that the cells from these lymphoma lines and from leukemia patients have lost one of the receptors or, alternatively, they are clonally derived from precursors that had only one receptor.

In summary, many factors have to be considered when preparing EAC. For example, in preparing a reagent to detect human B lymphocytes (*a*), if sheep erythrocytes are used, they should be trypsin-treated; (*b*) EA should be prepared with subagglutinating doses of rabbit antibody (preferentially 19 S) to sheep erythrocytes; and (*c*) C3d sites should be generated on the red cell membrane either by using mouse serum as a source of complement or, if purified human complement components are employed, the EAC43b intermediate should be treated with C3 inactivator to generate EAC43d.

D. Immunoglobulin-Bearing Lymphocytes and Complement Receptor Lymphocytes

These cell populations overlap extensively. I mentioned before that most or all CRL's have relatively high concentrations of Ig on their membranes. Among guinea pig lymph node cells (Shevach *et al.*, 1972a) and human tonsilar lymphocytes (Broome *et al.*, 1973), those cells with surface Ig as detected by immunofluorescence or autoradiography, also form rosettes with EAC. In addition, there is close agreement in the percentage of B cells among mouse spleen antigen-binding cells as determined by two independent assays; rosette for-

mation with EAC and presence of surface Ig (Rutishauser and Edelman, 1972). However, it is a common observation that, in the normal mouse, somewhat fewer CRL than Ig-bearing cells are detected in the peripheral lymphoid organs, suggesting that CRL's may be a subpopulation of B lymphocytes. Before the existence of such a subpopulation is accepted, other possibilities have to be excluded. For example, rosette formation may not detect all cells that have complement receptors. The binding of EAC by lymphocytes probably depends on the distribution and accessibility of the C3 receptor on the lymphocyte membrane, as well as on other cell properties, such as their electrical charge. In support of this hypothesis is the observation that more cells bearing complement receptors are detected by interaction with soluble antigen–antibody–complement complexes than with EAC (Eden *et al.*, 1973a). On the other hand, it should be pointed out that the interesting studies of Gelfand *et al.* (1974a) demonstrated that lymphocytes bearing membrane Ig and complement receptors do not appear simultaneously in spleens of young BALB/c mice. For example, at 3 days of age, 23.8% of the cells displayed membrane Ig and in very few the complement receptor was detected. At 6-12 weeks of age, 37.3% had membrane Ig and 25.2% had complement receptors (Table II). It would be of interest to determine whether these changes reflect the gradual appearance of the C3-receptor on Ig-bearing cells or the differentiation or migration into the spleen of a distinct subpopulation of cells bearing both receptors.

Among lymphocytes from human peripheral blood, the discrep-

TABLE II
RELATIVE RATE OF APPEARANCE OF IMMUNOGLOBULIN-BEARING AND COMPLEMENT RECEPTOR LYMPHOCYTES IN SPLEENS OF YOUNG BALB/c M10 MICE[a]

Age	Ig-bearing lymphocytes (%)	CRL[b] (%)
3 days	23.8	< 1
1 week	35.8	< 1
2 weeks	46.5	2.6
4 weeks	38.8	13.5
6–12 weeks	37.3	25.2

[a] From Gelfand *et al.* (1974a).
[b] CRL, complement receptor lymphocytes.

ancy between the proportion of CRL's and that of Ig-bearing lymphocytes, as detected by antisera to Ig, is even greater (see, for example Ross *et al.*, 1973c). However, in this case, other complicating factors arise, such as (*1*) the purity of the lymphocyte preparations, (2), in pathological conditions, the binding of autoantibodies to the membrane of T lymphocytes (Thomas, 1972; Gutterman *et al.*, 1973), and (3) the binding of immune complexes or complement-split products to lymphocytes in the blood. This may be the explanation for the observation that exposure of lymphocytes from peripheral lymphoid organs to normal serum (C. Bianco and V. Nussenzweig, unpublished observations) or to synovial fluids from patients with rheumatoid arthritis (Mellbie *et al.*, 1972) markedly reduces the number of CRL's detectable by rosette formation with EAC. If the same phenomenon also occurs *in vivo*, it may be one of the causes of the difficulty in detecting complement receptors on some peripheral blood lymphocytes that bear membrane-bound Ig.

E. Frequency of Complement Receptor Lymphocytes in the Spleen of Various Inbred Strains of Mice

At 2 weeks of age, AKR mice have a high frequency of CRL's (27.9%), DBA/2 mice have a low frequency of CRL's (4.8%), and F_1 hybrids (AKD2 mice) an intermediate frequency (14.8%). Analysis of offspring of F_1 and DBA/2 mice reveal that 25% of the mice are of the low CRL type, suggesting that AKR and DBA/2 mice differ at two loci important in CRL differentiation. By *H-2* typing of backcross progeny, it has been revealed that low CRL mice are homozygous for the *H-2* type of the low CRL parent, whereas intermediate and high CRL mice may be either heterozygous or homozygous. This indicates that one of the two CRL genes is *H-2* linked. This gene was termed *CRL-1* (Gelfand *et al.*, 1974b).

Investigation of frequency of CRL's in 2-week-old *H-2* congenic mice confirms the *H-2* linkage of *CRL-1*. Thus, C57BL/10 ($H\text{-}2^b$) mice have low frequency of CRL's at 2 weeks of age (7.6%), whereas the congenic B10.A ($H\text{-}2^a$) mice have an intermediate frequency (14.5%). Similarly, A/WySn mice ($H\text{-}2^a$) have an intermediate CRL frequency (12.0%), whereas the congenic A.By ($H\text{-}2^b$) mice have a low CRL frequency (4.6%).

Complement receptor lymphocyte frequencies in 2-week-old congenic mice with recombinant *H-2* chromosomes suggest that *CRL-1* is located to the "right" of (telomeric to) the genes for *Ss-Slp* and, indeed, may be outside of the *H-2* complex itself. Thus, B10.A (2R) mice, which derive the "left" side of their *H-2* region (i.e., the *K*,

Ir-1, *Ir-IgG*, and *Ss-Slp* genes) from the $H\text{-}2^a$ parent, and the *H-2D* gene from their $H\text{-}2^b$ parent resemble the $H\text{-}2^b$ parent in the frequency of CRL's at 2 weeks of age.

These studies are particularly interesting as it is already known that *Tla*, a gene controlling surface markers of differentiation T lymphocytes, is to the right of *H-2D*. Thus it is possible that this region may control a variety of differentiation steps of lymphocytes.

F. Specific Isolation of Complement Receptor Lymphocytes

The possibility of dissociating EAC from CRL with antibodies to C3 (Fig. 5) is the basis of a method for isolating CRL's from a mixed population of mouse cells (Eden *et al.*, 1971). In this procedure (*a*) rosettes are allowed to form and are separated from free lymphoid cells and free EAC by sedimentation ($1 \times g$) in a linear bovine serum albumin (BSA) gradient, (*b*) the isolated rosettes are treated with papain fragments of antibodies to C3 to dissociate them, and (*c*) erythrocytes are removed from the free CRL population by differential flotation in a continuous BSA gradient. The recoveries of CRL vary between 15 and 30%, and the purity is quite high. More than 95% of the purified cells have Ig on their membrane and none have the θ antigen as detected by cytotoxic reactions with specific antisera and guinea pig complement. When reincubated with fresh EAC, purified CRL's reform rosettes. Therefore, the C3 receptors have not been destroyed or irreversibly modified after being in contact with EAC at low temperatures for up to 20 hours. The contaminating cells are macrophages, granulocytes, which also have the complement receptor, and a few plasma cells. The large majority of purified CRL's are not grossly damaged, as shown by electron-microscopic studies (Chen *et al.*, 1972), but there is no information about their functional properties.

III. Interaction between Lymphocytes and Soluble Antigen-Antibody-Complement Complexes

A. Receptors for Antibody and for Complement

Lymphocytes from several animal species, including man, have receptors for antigen–antibody (AgAb) complexes prepared in the absence of complement (Brown *et al.*, 1970b; Basten *et al.*, 1972a,c; Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972). The binding involves the Fc region of the Ig molecules. This interaction can be demonstrated either by incubation of cells with complexes or with

heat-aggregated γ-globulin or by reverse cytoimmunoadherence. In normal mice, lymphocytes that bind the complexes through the Ig receptor are B lymphocytes. They belong to the CRL population (Eden *et al.*,1973a), do not bear the θ antigen, constitute the predominant population of cells in the thoracic duct of nude athymic mice (Basten *et al.*, 1972a), and bear Ig on their membranes (Paraskevas *et al.*, 1972). In addition, it has been shown that all human peripheral blood lymphocytes that bind aggregated Ig also have membrane Ig. However, in certain pathological conditions in man, significant numbers of peripheral lymphocytes can bind aggregates but not stain for membrane Ig (Dickler and Kunkel, 1972). The morphology of cells that bind immune complexes has been studied by electron microscopy autoradiography in a sample of cells from the thoracic duct of mice which had been previously thymectomized, irradiated, and reconstituted with bone marrow. Examination of stained specimens revealed that 65–70% of cells were lymphocytes and most were labeled, regardless of size. In contrast, plasmablasts and plasmacells failed to bind the labeled immune complexes (Basten *et al.*, 1972c).

The receptor for Ig on the lymphocyte surface is unaffected by treatment with relatively high concentrations of trypsin. Since B cells bear Ig on their membrane, the question arises of whether the receptor for the Fc portion of antibody is the membrane-bound Ig itself. This does not seem likely as the receptor is not destroyed by trypsin, a treatment known to digest the Ig from the membrane of B lymphocytes (Pernis *et al.*, 1971). It may be argued that fragments with affinity for aggregated Ig may still remain associated with the cell membrane after trypsin treatment. However, other findings suggest that the AgAb receptor is not membrane-bound Ig. For example, pretreatment of the cells with anti-Ig does not modify the binding of AgAb (Basten *et al.*, 1972a). Also, it has been shown with human leukemic cells that the cell surface Ig determinants could be induced to accumulate on one pole of the cells and form caps. If these cells are subsequently incubated with aggregated Ig, it was found all over the cell surface and not concentrated on the cap. The conclusion was again that the receptor for aggregated Ig is not the membrane-bound Ig (Preud'homme and Seligmann, 1972).

Recently, liposomes were used as targets to study the interaction between Ig and cell membranes. After mild aggregation, Ig of certain classes and subclasses (IgG1, IgG3, IgG4, but not IgG2 and IgM) induced increased release of markers trapped within the liposomes, and this effect is mediated by the Fc region of the molecules (Weissman *et*

al., 1974). These results suggest that the phospholipids of cell membranes may be the receptors for aggregated Ig, and this hypothesis is supported by the trypsin insensitivity of the receptor as well as by the observation that the receptors for cytophilic antibodies on macrophages are destroyed by phospholipase A (Davey and Asherson, 1967).

It is still unresolved whether the property of binding to the lymphocyte surface is an exclusive property of certain subclasses of 7 S Ig. In the mouse, lymphocytes bind IgG1 (Basten *et al.*, 1972c), as shown by experiments in which purified IgG1 myeloma proteins effectively inhibited the binding to lymphocytes of immune complexes prepared with antibodies to fowl Ig (FγG) and the antigen. Other classes of myeloma did not inhibit the binding as effectively as IgG1 myelomas. However, since the class of mouse Ig forming part of the anti-FγG–FγG complex was not determined, absence of inhibition cannot be unequivocally interpreted. If, for example, mouse antibodies to FγG belong to the IgG1 class, other classes of myelomas may be ineffective in inhibiting the binding of this particular type of immune complexes.

A practical way of depleting mouse B lymphocytes, using their property of binding AgAb complexes, has been proposed (Basten *et al.*, 1972b). Plastic beads were coated with human γ-globulin (HGG), washed and poured into a column. Lymphocytes together with rabbit antibodies to HGG were added to the column. After an appropriate incubation period, the cells were eluted, and it was found that the cells that had been retained were B lymphocytes. The assumption was made that these cells were bound to the beads by means of their receptors for HGG–anti-HGG complexes formed on the beads. However, it was pointed out that mouse and human Ig may cross-react immunologically, and the depletion of B lymphocytes might result from the presence of antibodies to mouse Ig in the preparation of rabbit antihuman Ig. This explanation was given for the failure of attempts to deplete B cells on similar columns but using BSA–anti-BSA complexes (Wigzell *et al.*, 1972).

Soluble immune complexes prepared in the *presence* of complement (^{125}I-labeled BSA–mouse anti-BSA–mouse complement) bind to lymphocytes by means of membrane receptors (Fig. 7) which can be distinguished operationally from the receptors for AgAb (Eden *et al.*, 1973a). Thus, (*a*) trypsin treatment of lymphocytes destroys the receptor for AgAbC (and for EAC) but not for AgAb; (*b*) lymphocytes bearing AgAbC complexes do not interact with EAC to form rosettes (in contrast, AgAb bound to lymphocytes does not interfere with

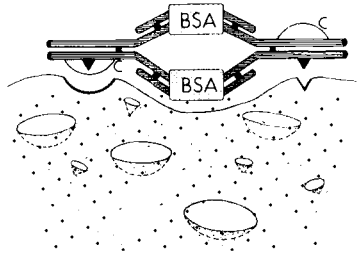

FIG. 7. Schematic representation of antigen–antibody–complement complex interacting with the membrane of lymphocytes. The bovine serum albumin (BSA)–anti-BSA–C complex can interact with the membrane through a site in the Fc region of the antibody molecule, or through complement, presumably a C3-split product. Complement molecules may sterically hinder the interaction between Fc and the site on the membrane of the lymphocyte. (From Eden *et al.*, 1973a.)

rosette formation); and (*c*) the presence of heat-aggregated Ig does not interfere with the binding of AgAbC (and of EAC) to lymphocytes, but prevents the interreaction of AgAb with the cells. The nature of the complement component(s) involved in the binding of the soluble complexes has not been formally established, but the participation of C3 is considered to be likely because of the similarities, previously pointed out, between the binding characteristics of AgAbC and of EAC to lymphocytes.

Other investigators (Dukor *et al.*, 1973b) have reported similar findings: mouse spleen lymphocytes bind ^{125}I-labeled aggregated HGG which has been previously incubated with fresh mouse serum, and the binding is abolished by the addition of antibodies to mouse C3. The cells bearing the aggregated γ-globulin–complement complexes do not form rosettes with EAC. In the absence of fresh mouse serum or in the presence of heat-inactivated mouse serum, the binding is very much reduced.

Most lymphocytes that bind AgAbC belong to the class of B lymphocytes, since they overlap extensively with CRL's. This has been shown by depletion experiments in which the specific elimination of CRL's is followed by a simultaneous decrease in the proportion of cells that bind AgAbC (Eden *et al.*, 1973a).

B. Fate of Membrane-Bound Immune Complexes

Complexes consisting of ^{125}I-BSA–anti-BSA–C remain on the lymphocyte membrane after incubation in tissue culture medium at 37°C. for several hours. The ^{125}I-containing complexes can be quantitatively removed from the cell surface by treatment with excess cold BSA (Eden *et al.*, 1973b).

The release from the cells of most of the labeled BSA in the presence of excess BSA is probably due to the dissociation and rearrangement of the multivalent immune complexes and formation of products with lower affinity for the membrane. The possibility that in these experiments an exchange takes place on the cell surface between hot and cold antigen was ruled out experimentally. The membrane-bound complexes can also be removed by treating the complexes with papain fragments of antibody to mouse Ig. The reason for this is perhaps that the interaction between the immune complexes and the membrane is mediated either directly by the antibody molecules, which are part of the complexes, or by complement products, which are also probably bound to the antibody molecules. Thus, binding of the papain fragments to these antibodies may interfere with the uptake of complexes by the lymphocytes.

Bound immune complexes accumulate at one pole of the cell and form caps. Cap formation appears to be the result of the redistribution and accumulation at one pole of the cells of membrane constituents and is brought about by their cross-linking with divalent and polyvalent reagents (Taylor *et al.*, 1971). It was shown that cap formation may be followed by rapid interiorization of the cross-linked complexes (Unanue *et al.*, 1972). Such rapid interiorization does not occur in the case of the AgAbC bound to CRL, showing that cap formation is not necessarily followed by endocytosis. These results resemble other experiments (Mantovani *et al.*, 1972) in which the role in phagocytosis of the C3 and IgG receptors of the membrane of macrophages was studied. Monolayers of mouse macrophages were overlaid with erythrocytes, EA, and EAC prepared with 7 S mouse or rabbit antibodies against erythrocytes and fresh mouse serum as a source of complement. Attachment and ingestion of erythrocytes by the macrophages were measured separately and the results clearly showed that C3 is primarily involved in particle attachment, whereas only IgG was able to promote markedly the ingestion of the attached particles. Thus, addition of complement to EA substantially increased the binding to the macrophages, whereas ingestion was increased to a much smaller extent (Fig. 8). Moreover, although both binding and ingestion of EAC (prepared with mouse antibodies to erythrocytes and mouse complement) were inhibited by antibodies to C3 by preventing the initial contact between EAC and the macrophages, antibodies to mouse 7 S IgG reduced the ingestion of the particles but not their attachment to the macrophages. The implication of these findings is, of course, that specific signals are necessary to trigger the interiorization process.

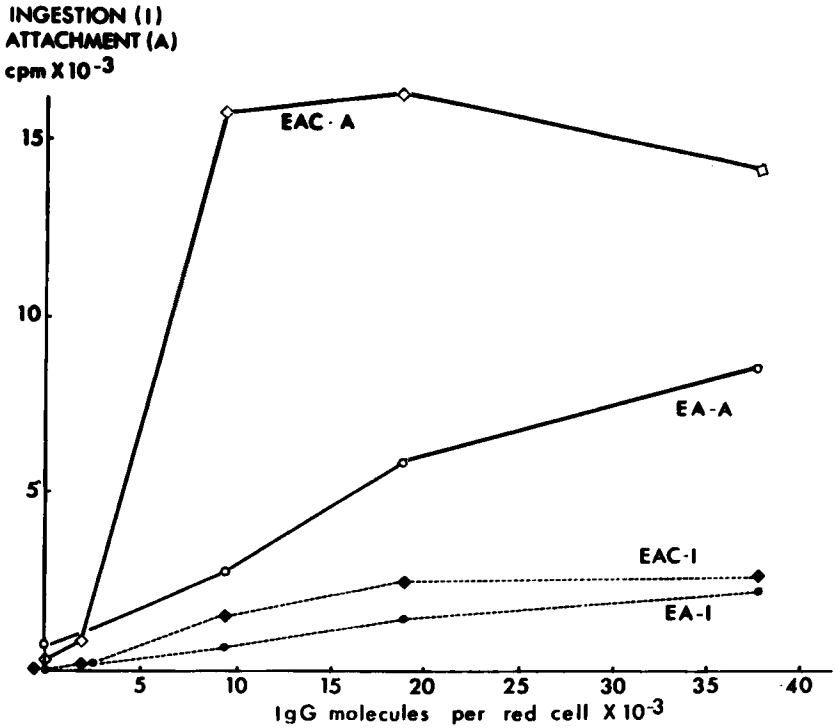

FIG. 8. Attachment and ingestion by mouse peritoneal macrophages of sensitized erythrocytes as a function of the number of IgG molecules bound to the red cell membranes. Separate suspensions of ^{51}Cr-labeled sheep erythrocytes were incubated with increasing concentrations of ^{125}I-labeled IgG antibody, washed 3 times by centrifugation and overlayered on monolayers of mouse peritoneal macrophages. Separate aliquots of the sensitized erythrocytes were incubated with fresh mouse serum as a source of complement, washed 3 times, and incubated with the macrophages. The graph represents the attached and ingested erythrocytes. Complement greatly enhanced attachment but not ingestion of the red cells. EAC, erythrocyte–antibody–complement. (From Mantovani *et al.*, 1972.)

C. Complement as a Regulator of the Interaction between Soluble Complexes and Cell Membranes

Membrane-bound soluble immune complexes can also be released from the cell surface by means of a complement-dependent mechanism, involving the alternate or properdin pathway of complement fixation (Pillemer *et al.*, 1954; Gewurz *et al.*, 1968; Sandberg *et al.*, 1970; Götze and Müller-Eberhard, 1971; Marcus *et al.*, 1971). In these studies (Miller *et al.*, 1973b), mouse lymphocytes bearing ^{125}I-BSA–anti-BSA-C complexes are incubated at 37°C. with fresh serum at various dilutions. At different intervals of time, the cell suspensions

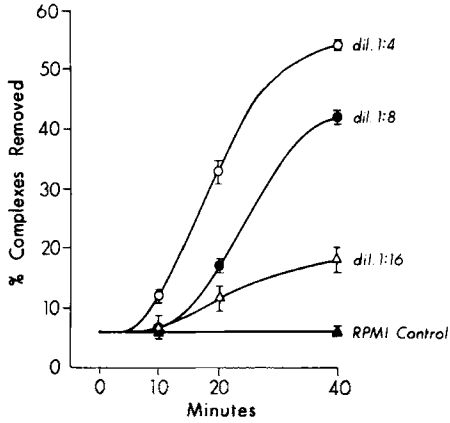

FIG. 9. Kinetics of release of soluble immune complexes [^{125}I–bovine serum albumin (BSA)–anti-BSA–C] bound to the surface of mouse lymphocytes, by incubating the cells at 37°C. in different dilutions of guinea pig serum. After incubation, the tubes were centrifuged and the pellets and supernatants, containing the released labeled complexes, were counted in a gamma counter. The guinea pig serum had been previously absorbed with mouse spleen cells at 0°C. (From Miller *et al.*, 1973b.)

are centrifuged and the labeled BSA released in the supernant is determined. The release activity is found in serum from all mammalian species tested including man (Fig. 9). One component of human serum required for the release is C3 because a unique genetically C3-deficient human serum released the complexes only after addition of purified C3. Purified C3 fragments, obtained by trypsin hydrolysis of C3 do not activate the C3-deficient serum. Also, neither C3, nor C3b, nor a mixture of C3 and C3b in the absence of serum releases the complexes. The involvement of the alternate pathway in the release activity is indicated by the demonstration that (*a*) C4-deficient guinea pig serum releases the complexes; (*b*) the release activity is dependent on the presence of Mg^{2+} but not of Ca^{2+}; and (*c*) pretreatment of the serum at 50°C. for 30 minutes abolishes its activity. Serum activity can be restored by addition of purified factor B (or C3PA), a thermolabile protein required for the activation of complement in the alternate pathway. Although the mechanism of the release activity is not understood at the molecular level, these findings indicate that complement regulates the interaction between immune complexes and the lymphocyte surface. It appears possible that, *in vivo*, when antigen combines with antibody and complement, a series of products is formed which at first have increasing then decreasing affinities for the cell membrane. In support of this hypothesis, it was shown that when sol-

uble complexes are injected intravenously into mice, the complexes are rapidly picked up by circulating cells and then released in the plasma (Fig. 10). About 50% of the complexes are taken up by platelets and the rest presumably by other cells that have C3 receptors on their membranes, such as B lymphocytes, monocytes, and granulocytes. However, complexes that have been previously released *in vitro* from the membrane of lymphocytes when injected *in vivo*, do not bind to circulating cells (Miller and Nussenzweig, 1974)(Fig. 10).

Thus, there are at least two distinct control mechanisms for inhibiting the complement-mediated binding of immune complexes to the surface of leukocytes (immune adherence).

1. The first involves the activity of C3b inactivator. As mentioned earlier, this serum enzyme hydrolyzes C3b molecules associated with cell membranes, such as those found on EAC43b, into two peptides, C3c and C3d. There is good experimental evidence that the clearance and destruction of antibody-coated red cells *in vivo* may involve complement activation and binding of C3b to the erythrocytes followed by

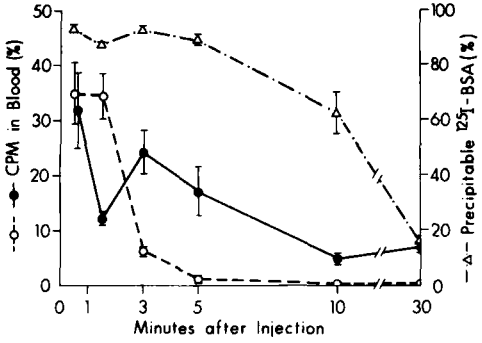

FIG. 10. Short-term fate of immune complexes in the mouse circulation. CBA mice were injected intravenously with ^{125}I-labeled bovine serum albumin (BSA)–anti-BSA–C complexes. At short intervals thereafter, 50-μl. blood samples were taken from the retro-orbital plexus and immediately mixed into 1.5 ml. of 0.15 *M* NaCl. The diluted samples were centrifuged and the supernatants and cell pellets measured for radioactivity. The supernatants were mixed with equal volumes of saturated ammonium sulfate, refrigerated overnight, and centrifuged. The pellets, which contain the BSA–anti-BSA complexes (△), and the supernatants were measured for radioactivity. Cell-bound (○) and cell-free (●) labeled BSA in total blood are expressed as percent of injected counts. Each point represents the mean ± s.e.m. for 3 mice. At thirty seconds, about 70% of the injected counts were found in the circulation and half were free in the plasma. At one minute, most of the counts were cell-bound. However, at three minutes a very large proportion of the injected ^{125}I-BSA is found free in the plasma still in the form of immune complexes, since the counts can be totally precipitated by 50% ammonium sulfate.

their recognition and capture by phagocytic cells that have receptors for C3b, such as liver macrophages. In addition, similarly to what was shown to occur with injected soluble complexes (Fig. 10), it has been observed that the C3b-sensitized red cells which are not phagocytized gradually return to the circulation, probably as a consequence of the transformation of C3b into C3d by the action of C3b inactivator (Brown *et al.*, 1970; Schreiber and Frank, 1972a, Tedesco *et al.*, 1972).

2. As mentioned earlier, a second mechanism, which does not involve C3b inactivator, can interfere with the immune adherence of *soluble* complexes to leukocytes. Further complement utilization, by means of the shunt pathway, is necessary for the deactivation of the immune complexes. Thus, when soluble complexes are involved, complement components participate both in their binding and in their release from leukocytes. Participation of C3b inactivator in the release can be formally excluded because (*a*) this enzyme is not generated through complement activation but it is found preformed in serum; (*b*) C3b inactivator does not depend on the presence of divalent ions for its activity; (*c*) C3b inactivator is not destroyed by treating the serum at 50°C. for 30 minutes; and (*d*) a C3-deficient serum that had normal levels of C3b inactivator was devoid of this activity (Miller *et al.*, 1973b). These observations imply that soluble immune complexes might accumulate on the membranes of cells if the mechanism of complement-mediated inhibition of immune adherence (release activity) is not functioning properly. In support of this hypothesis, it has been recently found that in NZB/NZW mice, as well as in lupus patients, the serum release activity is profoundly altered. In the case of the NZB/NZW mice, the changes in the release activity precede most manifestations of the autoimmune disorders affecting these animals. Preformed soluble complexes injected intravenously into old or young NZB/NZW mice are handled in quite different ways. Although in both young and old animals the complexes bind to circulating cells, only in the young are the complexes rapidly released into the plasma (Nussenzweig *et al.*, 1974). It is probable that the complexes remaining on the cell surfaces will profoundly alter the fate and accelerate the destruction of these cells.

IV. Function of the Receptors for Immune Complexes

Few studies have examined the role of CRL's in the immune response. It is known that part of the antigen-binding cells, which are postulated to include precursors of antibody-forming cells, have C3 receptors. From 60 to 70% of the antigen-binding cells isolated by specific attachment to antigen-derivatized nylon fibers were shown to

consist of lymphocytes that had both Ig on their membranes and formed rosettes with EAC (Rutishauser and Edelman, 1972).

When cells from guinea pigs immunized with soluble proteins or hapten–protein conjugates are cultivated in the presence of antigen, the migration-inhibition factor (MIF) is produced. This factor can be assayed by its capacity to alter the behavior of macrophages of normal animals in culture. The question of whether B or T cells are involved in MIF production was recently examined by Yoshida *et al.* (1973). They found that after antigenic stimulation, MIF production appears to be a function of non-CRL's which include a large proportion of T lymphocytes. On the other hand, purified CRL's from normal as well as from immune animals produce MIF when stimulated by lipopolysaccharide (LPS) or tuberculin. The factors produced by CRL's and non-CRL's could not be differentiated on the basis of a few physicochemical criteria. Thus, lymphokine production is not an exclusive property of T cells as also shown by the isolation of MIF or MIF-like substances from replicating cultures of nonlymphoid cells (Papageorgiou *et al.*, 1972; Tubergen *et al.*, 1972). These observations support the idea that lymphokines play a more general biological role apart from their functions in cell-mediated immunity and inflammation.

In addition, indirect evidence suggests that CRL's are functionally important in antibody production but they do not mediate graft-versus-host reactions (Levy *et al.*, 1972). The experiments consisted of incubating a population of rat spleen in rat serum and cobra venom factor (Co-F), which is known to activate the terminal complement components after interaction with a heat-labile serum component. The lymphocytes treated with Co-F plus serum lost their capacity to bind EAC *in vitro*, presumably because the membrane C3 sites were occupied by C3-split products generated through the activation of complement by Co-F. These functionally altered lymphoid cells were then injected into syngeneic, lethally irradiated rats together with an antigen, sheep red blood cells. The capacity of the depleted cells to differentiate in the recipient animal into antibody-forming cells was found to be much diminished as compared to controls. However, the Co-F-incubated cells retained their ability to induce a graft-versus-host reaction. Another functional test which is thought to be T cell-dependent, the macrophage disappearance reaction, is mediated by cells other than CRL's (Sonozaki and Cohen, 1972).

From the observations on the interaction between B lymphocytes and immune complexes several other points emerge that may be of relevance to the ideas currently held about the initiation of humoral

immune responses. It is reasonable to suppose that the specific signals for activation of a B cell, leading to proliferation, increased antibody synthesis, and secretion (or to its inhibition), are the same in a primary and in a secondary antibody response. On the other hand, during a secondary response, it is almost certain that the antigen will meet circulating antibody before it comes in contact with the relatively few lymphocytes that bear specific membrane receptors. Recent evidence suggests that resting B lymphocytes may continually synthesize and release antibody molecules (Vitetta and Uhr, 1972), which are probably identical to the antibody molecules to be produced by the cell after antigenic stimulation. The leakage of Ig from B lymphocytes may represent the source of "natural" antibody present in serum, and the circulating antibody and cell-bound antibody must compete for available antigen. These simple considerations strongly suggest that an antigen-binding cell encounters the antigen as an immune complex, either free in circulation or bound to the surface of other cells that have membrane receptors for immune complexes (other B lymphocytes, macrophages, platelets, granulocytes). Thus, antibody production may be modulated by membrane disturbances due to the recognition not only of the antigen but also of C3 and the aggregated antibody molecules that are part of the complexes.

Pertinent to this hypothesis (Nussenzweig *et al.*, 1973a) are the following findings:

1. Complement-mediated binding of particulate or soluble immune complexes to B lymphocytes may not be followed by rapid interiorization; in other words, *in vitro* observations suggest the possibility that immune complexes (and antigen) may remain for some time bound to the outer surface of certain cells.

2. Complement regulates the interaction between immune complexes and cell surface receptors.

3. Membrane receptors for immune complexes (both for aggregated Ig and for C3) probably play a role in the triggering of other effector mechanisms, such as the release of vasoactive amines from platelets and of lysosomal enzymes from granulocytes. For example, certain aggregated human myeloma proteins (IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2) and normal human IgG can react with human neutrophiles in serum-free medium and induce the release in the medium of some lysosomal enzymes. Incubation of neutrophiles with these aggregates results in their adherence to the cell surface (Henson *et al.*, 1972). Heat-aggregated, but not monomeric Ig's elicit the release of serotonin from platelets (Pfueller and Lüscher, 1972). Adherence of rabbit platelets to particulate antigens, such as zymosan or erythro-

cytes, which have fixed complement through the third component of complement is followed by histamine release (Henson and Cochrane, 1969). In addition, it has been shown that immune complexes enhance DNA synthesis of lymphocytes *in vitro* (Bloch-Shtacher *et al.*, 1968; Möller, 1969).

4. The extent of the *in vitro* proliferative response of normal rabbit peripheral blood lymphocytes induced by antibodies to membrane-bound Ig is greatly increased by adding a second antiserum that reacts with the first antibodies (piggyback effect) (Sell *et al.*, 1970). These observations imply that a second interaction, the cross-linking of antibody molecules that have already reacted with the Ig present on the cell membrane, further stimulates cell division. This finding is of particular interest in view of the suggestion that the intrinsic, mitogenic, nonspecific properties of immunogens and the multiplicity of attachment points to the B lymphocyte can decisively influence triggering of B lymphocytes (see in the following).

Because in the presence of antibody, immune complexes must form before an antigen meets the rare cell that is precommitted to it, I believe that, contrary to what has been generally assumed, the first contact between antigens and B lymphocytes may not be determined on a clonal basis but through receptors for immune complexes. Enhanced antibody synthesis will follow recognition of the antigen present in the complexes by a few precommitted lymphocytes. In other words, a signal for differentiation will depend on the relative binding affinity between the antigen in the complexes and the specific membrane-bound Ig of the B lymphocyte. Other findings also suggest that at least two signals are necessary for antibody production by B cells. In the case of thymus-dependent antigens, one of the signals can be provided by activated T cells. However, certain antigens are immunogenic in the absence of T cells. It has been recently shown that all known thymus-independent antigens are B-cell mitogens, that is, they are capable of stimulating the proliferation of B cells *in vitro*, whereas thymus-dependent antigens are inactive (Coutinho and Möller, 1973). In addition, all thymus-independent antigens were able to induce polyclonal antibody synthesis (against a variety of different antigens) in nonprimed B-cell populations, suggesting that these antigens are somehow capable of binding to *all* B cells. Since these antigens differ widely in structure, a puzzling question concerns the nature of their interaction with the membrane of B lymphocytes. One explanation may be that small amounts of natural antibody, produced during the cell culture, formed immune complexes with the antigens and bound to B lymphocytes triggering

cell division and nonspecific production of antibody. The observations that *in vitro* responses to polymeric and monomeric flagellin can be profoundly influenced by the addition of antibody to the cultures support this hypothesis (Diener and Feldmann, 1970).

The possibility that C3 and the C3 receptors may participate in the cooperation between T and B lymphocytes has been raised by the finding that *in vivo* depletion of C3 by injection of Co-F inhibits the response of mice to some thymus-dependent, but not to thymus-independent, antigens (Pepys, 1972). It has been suggested that the interaction of the antigen with a T-cell-specific receptor (IgT?) might lead to the activation of the complement system, fixation of C3 to the complexes, and secondary interaction of the complexes with B cells or with macrophages. According to this hypothesis, the IgT–antigen–C3 complexes trigger the activation of B lymphocytes. Implicit assumptions are that B lymphocytes recognize the unique configuration that is given to these complexes by the presence of the postulated IgT and that other antigen–antibody (of B-cell origin)–complement complexes are not as effective in triggering antibody synthesis.

Some recent additional findings indicate that B-cell activation may be complement-dependent. Purified Co-F in its soluble form or coupled to Sepharose beads, as well as LPS, are strongly mitogenic for B cells in the presence of C3-containing but not C3-depleted fetal calf serum (Dukor *et al.*, 1974). Also, some plant and bacterial polysaccharides and LPS's, which can induce an IgM response without T cells (T-independent antigens; reviewed in Katz and Benacerraf, 1972), are known to be able to activate the alternate or properdin pathway of complement utilization.[3] For these reasons it has been suggested that the activation and binding of C3 is necessary for the initiation of antibody production by B cells and also that the release of proteases from activated T cells might generate C3-split products that would subsequently bind to B lymphocytes and render them susceptible to activation by T-independent antigens (Dukor *et al.*, 1974). In

[3] Lipopolysaccharides are also mitogenic for B lymphocytes in *in vitro* cultures (Andersson *et al.*, 1972). Within the structure of the LPS, consisting of lipid A, a core polysaccharide, and the θ-specific chains of repeating units of oligosaccharides, the lipid A was found to be the mitogenic part (Andersson *et al.*, 1973). This is the nonpolar hydrophobic portion of the molecule, which can probably interact with the lipid bilayer of the plasma membrane and which is also probably responsible for some of the endotoxic properties of the whole molecule. In the context of the idea that B-cell activation is complement-dependent, it is of interest that lipid A is the portion of the molecule that can activate the complement sequence (Galanos *et al.*, 1971).

the case of T-independent antigens, such as LPS, the C3-split products would be directly generated by the interaction between the antigen and the complement system.

However, recent findings that a patient with genetically determined absence of C3 in the serum, had normal levels of Ig and was capable of responding to antigenic stimulation (Alper *et al.*, 1972) strongly suggest that the presence of C3 in serum is not an *absolute* requirement for antibody production. Furthermore, it has been shown that all known T-independent antigens are mitogenic *in vitro* for normal lymphocytes or lymphocytes from congenitally athymic nude mice in the *absence* of any serum supplementation (Coutinho and Möller, 1973). When different preparations of LPS were compared for their mitogenicity for B lymphocytes and for their capacity to initiate the alternate pathway of activation of the complement system, it was found that these two properties were not associated, as would be predicted from Dukor's hypothesis. For example, one preparation of LPS from *Salmonella* mR345 was incapable of activating human complement through the shunt pathway, but it stimulated division in as high a proportion of B lymphocytes as in other preparations of LPS that did interact with the complement system. In addition, the mitogenic activities of the various LPS preparations were as great for mouse spleen cells cultured in 10% zymozan-treated or heated serum as in unheated serum, as well as in the presence or absence of excess antimouse C3 in cultures (Janossy *et al.*, 1973).

In summary, the idea that immune complexes and complement may participate in the initial events that take place on the lymphocyte surface during antigenic stimulation and lead to antibody production is currently under investigation. In addition to their role in the initiation of the immune response, other functions have been suggested for the receptors for immune complexes on B lymphocytes. For example, both Fc and C3 receptors may contribute to the follicular localization of antigen (Brown *et al.*, 1970b; Bianco *et al.*, 1971), an antibody-dependent mechanism that brings antigenic substances to certain areas in the lymphoid organs, presumably for the induction of the immune response (Mitchell and Abbot, 1965; Hanna and Szakal, 1968; Nossal *et al.*, 1968; White *et al.*, 1970; reviewed in Unanue, 1972). It has been suggested that the AgAb complexes, found to be accumulated in these areas, are associated with the plasma membrane of cells that have been termed *dendritic cells* because of their numerous, long, cytoplasmic infoldings. However, the same areas contain densely packed B lymphocytes, and sometimes it is impossible to decide the precise location of the antigen (Nossal *et al.*, 1968). Re-

cently, a new cell type with the morphological characteristics of the dendritic cells has been isolated from lymphoid organs (Steinman and Cohn, 1973) and these cells do not bind AgAb complexes (EA or EAC) *in vitro* (Steinman and Cohn, 1974). These observations raise the question of whether B lymphocytes, which have the ability to bind immune complexes, play an important role in antigen localization (Bianco *et al.*, 1971). The observation that depletion of lymphocytes by thoracic duct drainage reduces the uptake of antigenic material by the follicles and that antibody injections only partially restore localization support this idea (Williams, 1966). Also, heat-aggregated human Ig, injected into mice, is concentrated within hours in germinal center areas of the spleen in a dendritic or reticular pattern (Brown *et al.*, 1970a). The cells showing surface staining at 6 hours after injection are round cells more characteristic of lymphoid cells than macrophages (Brown *et al.*, 1973).

The extent of participation of the complement and Fc receptors in the process of localization cannot be evaluated from these experiments because complement will probably be added *in vivo* to the aggregated Ig shortly after injection. Also it is not clear whether the aggregated Ig is taken up by circulating lymphocytes, which afterward localize in the germinal centers, or directly by the lymphoid cells in the nodes. The observations of Williams (1966), mentioned previously, suggest that circulating lymphocytes play a role in localization. However, Brown *et al.* (1973) believe that localization is a function exclusively of lymphoid cells already present in the spleen at the time of injection, based on the observation that spleens shielded during whole-body X-irradiation were capable of localizing aggregated Ig but that local irradiation of the spleen with protection of the rest of the body prevented localization.

We mentioned before (Section III,C) that complement activity influences not only the binding but also the release of soluble complexes from cell membranes. Thus, BSA–anti-BSA–C complexes adhere firmly to the surface membrane of B lymphocytes (and other cells), where they remain for long periods of time without being interiorized if the cells are incubated in tissue culture medium at 37°C. However, in the presence of relatively large concentrations of fresh serum, most, but not all, complexes are rapidly released from the membrane by a mechanism involving the activation of complement through the shunt pathway. These findings obviously raise an additional complexity in understanding the nature of the bonds that keep complexes associated for days or weeks with cell membranes within follicles. If complement participates in this binding why are not the

complexes subsequently released? The answer is clearly not known, but it should be pointed out that not all of lymphocyte-bound complexes are released *in vitro* through complement activity. Some cells retain complexes, probably by means of additional and possibly different (not complement-dependent) bonds, and the same might happen in follicles. Furthermore, there is no information about complement activation in lymphoid organs during antigenic stimulation, when immune complexes are probably being constantly formed within a confined environment. Under these circumstances, it is conceivable that shunt-pathway-mediated functions, such as the release activity, may be inhibited by previous activation by fluid phase complexes. Alternatively, the complexes in the follicles might be in such a form that they cannot activate the shunt pathway.

An additional functional property of lymphoid cells may be related to their membrane receptors for immune complexes. It has been suggested that these receptors play a primary role in triggering the cytotoxic activity of nonimmune lymphocytes for target cells that have been coated with antibody (Möller, 1965; Perlmann and Perlmann, 1970; MacLennan, 1972; Möller and Suehag, 1972; Van Boxel *et al.*, 1972). The cytotoxic effect is complement-independent and not mediated by T lymphocytes, since elimination of these cells does not affect the cytotoxic activity of the remaining lymphoid cells for target cells coated with antibody. The aggessor cells can be inhibited in their function by the presence in the incubation medium of unrelated, soluble, immune complexes or aggregated Ig, but not by antigen or antibody alone. Thus, it is probable that the initial step in the sequence of events leading to cytolysis of the target cell is the recognition by the lymphoid cells of the antibody-coated target cell via the Fc receptor. However, there is some controversy about the nature of the effector cells. It has been recently shown that mouse lymphoid cells depleted of Ig-bearing cells (B lymphocytes), after passage through a dextran column coated with anti-Fab, are more cytotoxic for antibody-coated target cells than the original population. In addition, B cells recovered from the column by dextranase digestion are inactive (Greenberg *et al.*, 1973). These studies as well as others (Evans *et al.*, 1972; Perlmann *et al.*, 1973) suggest that monocytes or a separate class of lymphoid cells that share some membrane properties with monocytes (Fc and C3 receptors) might be the effector cells.

There is suggestive evidence that similar mechanisms may be operative in cell-mediated immunity to certain tumors in man. Lymphoid cells from patients with some carcinomas of the bladder are cytotoxic *in vitro* toward autologous or allogeneic tumor-derived target

cells but not toward cultured cells from normal bladder epithelium. The specificity of the cytotoxic reaction is further indicated by the lack of activity of lymphoid cells from normal individuals for malignant cultured bladder cells (O'Toole *et al.*, 1973). In this case the effector mechanism depends on the presence of B cells because it is inhibited after the removal of the B cells by passage in anti-Ig columns. These findings indicate that mononuclear cells other than T lymphocytes may be functionally important in the response of humans to certain forms of cancer. As for the mechanism of target cell killing by peripheral blood cells from patients with tumors, it has been suggested (Perlmann *et al.*, 1972) that the effector cells may bear membrane-bound immune complexes that have been captured *in vivo*. Antibody molecules that are part of the complexes and that dissociate from the antigen *in vitro*, subsequently could bind to the membrane of target cells of homologous antigenicity and trigger a cytolytic reaction. In other words, it is possible that mononuclear cells transport on their membranes, as part of nonspecifically bound immune complexes, specific antibodies to tumor antigens, and these could be locally reutilized on encounter with a relevant target cell.

In conclusion, the study of the mechanism of interaction of immune complexes with lymphocytes may be of relevance to several important areas of immunological research as well as to the understanding of mechanisms of disease. Further insights may derive from experiments that test directly the hypothesis that immune complexes of certain structural composition play a central role in the triggering of diverse cell functions.

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Biological Activities of Immunoglobulins of Different Classes and Subclasses[1]

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I. Introduction

In the past 15 years, much has been learned about the structure and function of immunoglobulins. It has been shown that all immunoglobulins are composed of two types of polypeptide chains, heavy and light (Edelman and Poulik, 1961), which are linked by disulfide bonds (Fig. 1). Both chains have variable amino acid sequences in the amino terminal region and constant amino acid sequences in the carboxy terminal region. Digestion of im-

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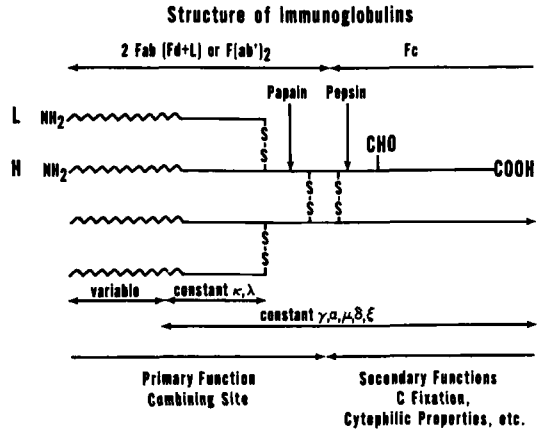

FIG. 1. Schematic diagram of immunoglobulin molecules and localization of structures responsible for primary and secondary functions of antibodies. H = heavy chain; L = light chain; CHO = carbohydrate.

munoglobulins with papain (Porter, 1959) results in formation of two Fab fragments and one Fc fragment; the Fab fragment is composed of one light chain and the amino terminal half of the heavy chain, called the Fd fragment. The Fc fragment consists of the carboxy terminal halves of the heavy chains, which are linked by inter-heavy-chain disulfide bonds and noncovalent bonds. Pepsin splits immunoglobulins on the carboxy terminal side of the inter-heavy-chain disulfide bonds (Nisonoff *et al.*, 1960), resulting in formation of a large fragment called $F(ab')_2$ and degradation of the Fc fragment.

The biological activities of immunoglobulins may be divided into two categories: (1) the specific reaction with antigen and (2) the consequences of antigen–antibody reactions. The reaction with antigen has been called the *primary function* of immunoglobulins and is a property of the Fab fragment. The purpose of this review, however, is to summarize the biological activities that follow antigen–antibody reactions and which have been called *secondary functions* of immunoglobulins. Structures of immunoglobulin molecules that govern these secondary functions have been shown to be localized in the constant portion of the heavy chain, particularly in the Fc fragment. Even before such detailed structural information was available, it was recognized that immunoglobulins could be divided into different classes according to antigenic properties, and it is now well documented that these antigenic determinants are also present in the Fc fragment and reflect different specific amino acid sequences of the

constant portion of the heavy chains. Five classes of immunoglobulins have been delineated on the basis of non-cross-reacting antigenic determinants on the Fc fragment and have further been divided into so-called subclasses according to minor antigenic differences in the Fc fragment. These minor antigenic differences, however, also reflect a distinct amino acid sequence in the constant region of the heavy chain, and classification into classes and subclasses according to antigenic properties is therefore somewhat arbitrary. It would be more logical if immunoglobulins having a specific constant region of the heavy chain were each designated as a class of immunoglobulins. Further support for eliminating the term "subclass" comes from studies of different species: whereas in man an extensive cross-reaction indeed exists between subclasses, in other mammalian species this cross-reaction is less pronounced. In horses, a type of immunoglobulin, the T component (Tiselius and Kabat, 1939), has been found which in structure is clearly a subclass of IgG (Weir *et al.*, 1966) but does not cross-react antigenically with other horse IgG. However, since class and subclass are presently recommended by the World Health Organization committees on nomenclature, and the analogy between subclasses of different species is as yet not fully established, these terms will be used in this chapter.

All antibodies described to date are immunoglobulins; however, it is not certain that all immunoglobulin molecules function as antibodies. Immunoglobulins having no demonstrable antibody activity are often analyzed for secondary biological activities, since it is assumed, probably correctly, that immunoglobulins in general as well as antibodies of the same subclass do not differ in their secondary activities which are mediated by structures not participating in the antibody-combining site.

II. Myeloma Proteins

Myeloma proteins have played an important part both in the discovery of the rare classes IgD and IgE and subclasses of IgG and IgA and in the characterization of the structure and function of different immunoglobulins. These monoclonal, chemically homogeneous representatives of one or another class or subclass of immunoglobulins are a tumor product found in sera of patients with multiple myeloma. With relative ease, myeloma proteins can be isolated in a purer form than normal immunoglobulins and, at present, are the only source of large quantities of IgD, IgE, and the IgG and IgA subclasses. There is no evidence that myeloma proteins are different from their normal counterparts in the constant region of the heavy and light chains, and

the variable regions may be comparable to a single antibody molecule.

Myeloma proteins have been widely used to produce antisera to immunoglobulin classes and, in particular, to subclasses both as antigens as well as reagents to absorb antisera and render them specific. Only rarely do myeloma proteins show antibody activity, e.g., anti-DNP activity (Eisen *et al.*, 1968) or rheumatoid factor activity (Grey *et al.*, 1968b); therefore they must be aggregated artificially in order to test their secondary biological activities. Although heat aggregation at 63°C. for 30 minutes produces biologically active myeloma proteins, unfortunately many myeloma proteins do not aggregate upon heating (Morse, 1965). Aggregation with bisdiazotized benzidine (BDB) (Ishizaka *et al.*, 1967) has therefore been often substituted, but amounts of BDB that form suitable aggregates vary from protein to protein and must be predetermined (Hensen *et al.*, 1972). In our laboratory, aggregated proteins that show a slight turbidity, indicating formation of some insoluble aggregates, have been proved to be most active. As will be shown later in this chapter, the activities of myeloma proteins vary to some extent within a given subclass, and it is therefore necessary to analyze a significant number of proteins in order to establish a definitive activity.

III. Nomenclature of Subclasses in Different Species[2]

The nomenclature of classes and subclasses in different species is summarized in Table I. Immunoglobulins have been studied and defined best in man, probably because of the availability of large numbers of myeloma proteins, and the nomenclature has been standardized by the World Health Organization (1964). The five classes of IgG, IgA, IgM, IgD, and IgE, the four subclasses of IgG, numbered 1 to 4 (Grey and Kunkel, 1964; Terry and Fahey, 1964), and the two subclasses of IgA (IgA1 and IgA2) (Feinstein and Franklin, 1966; Kunkel and Prendergast, 1966; Vaerman and Heremans, 1966) are well established. Additional subclasses have been reported but have as yet not been universally accepted, awaiting confirmation in other laboratories. Antigenic data suggesting a third IgA subclass (Grey, 1969) and a subclass division of IgD (Rivat *et al.*, 1971) have been reported. Two types of IgM proteins differing in the ability to fix complement have been described (Linscott and Hansen, 1969; Mac-

[2] Where possible, the nomenclature used in this article is that suggested by subcommittees of the World Health Organization. Alternative nomenclatures for mouse immunoglobulins are $\gamma 2aG = \gamma G$, $\gamma 2bG = \gamma H$, and $\gamma 1G = \gamma F$.

TABLE I
NOMENCLATURE OF IMMUNOGLOBULIN CLASSES AND SUBCLASSES

Species	Immunoglobulin class and subclass								
Man	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgM	IgD	IgE
Mouse	γ2aG	γ1G	γ2bG	J606	IgA	–	IgM	–	IgE
Rat	γ2aG	γ1G	γ2bG	–	IgA	–	IgM	–	IgE
Rabbit	γ2G	γ1G	–	–	IgA	–	IgM	–	IgE
Guinea pig	γ2G	–	γ1G	–	IgA	–	IgM	–	IgE
Dog	γ2aG	γ2bG	γ2cG	γ1G	IgA	–	IgM	–	–
Horse	γ2aG	γ2bG	γ2cG	T-γG	IgA	–	IgM	–	–
Cow, sheep, etc.	γ2G	–	γ1G	–	–		–		
Birds	IgG	–	–	–	IgA	–	IgM	–	–
Reptiles	IgG	–	–	–	IgA	–	IgM	–	–
Fish	IgG	–	–	–	–	–	IgM	–	–

kenzie *et al.*, 1969), but structural differences in the constant portion of the μ chain, which would support the existence of these subclasses, have not as yet been found.

The immunoglobulins of the mouse have also been studied extensively, again because of the availability of myeloma proteins. Although four classes analogous to those in man, IgG, IgA, IgM, and IgE, have been documented in mice (Fahey *et al.*, 1964a, b; Potter *et al.*, 1965); IgD has not yet been found in mice or any species other than man. The four subclasses of murine IgG listed in order of concentration analogous to those in man are γ2aG, γ1G, γ2bG, and J606. The fourth subclass was named after a myeloma protein, J606, which led to its discovery (Grey *et al.*, 1971). No IgA subclass is known in mice. Rat immunoglobulins are similar to those of mice and are named in the same way (Bloch *et al.*, 1968; Bristany and Tomasi, 1970). In other species, where no or few myeloma proteins are available, the IgG subclasses can only be distinguished by electrophoretic mobility but usually cannot be separated further. Immunoglobulin G of fast anodal electrophoretic mobility is usually called γ1G, and the cathodal IgG is called γ2G (Benacerraf *et al.*, 1963; Coe, 1968). Extensive studies have been made on horse (Rockey *et al.*, 1964; Montgomery *et al.*, 1969) and dog immunoglobulins (Johnson and Vaughan, 1967; Johnson *et al.*, 1967), and several subclasses of these species have been recognized antigenically (Table I). The rabbit shows predominantly one IgG class which is probably analogous to γ2G of mice.

γ1G is a minor component in this species (Zvaifler and Becker, 1966; Rodkey and Freeman, 1969). Relatively little is known of subclasses in lower vertebrates, and since few studies have been performed on the secondary functions of these immunoglobulins, they will be mentioned only if such activities have been shown in the homologous species.

Analogies between immunoglobulins of human and other species are usually made according to physicochemical similarities of the antibodies or by antigenic cross-reactions. For example, IgG has the slowest electrophoretic mobility and a relatively low molecular weight, IgA forms polymers and binds to secretory component (Tomasi and Zigelbaum, 1963), and IgM is a macroglobulin of about 900,000 daltons. Immunoglobulin E elicits an anaphylactic reaction in homologous species and has a slightly higher molecular weight than IgG. No relationship between human and other mammalian IgG subclasses has been established, even though IgG1 and IgG3 are probably analogous to γ2aG and γ2bG, respectively. Immunoglobulins G2 and G4 resemble γ1G in electrophoretic mobility but differ in biological activities from γ1G in rodents.

IV. Concentration of Immunoglobulins in Different Body Fluids

A. Serum

The concentration and distribution of immunoglobulins in body fluids are regulated in part by the constant region of the heavy chain and can therefore also be considered secondary activities or properties of immunoglobulins. The concentration of an immunoglobulin of a particular class in the serum depends on (*1*) the number of plasma cells forming a particular class, (2) the rate of synthesis of that class per plasma cell, (3) the rate of catabolism of that class, and (*4*) the rate of its exchange between intra- and extravascular spaces. The rate of synthesis of immunoglobulin per plasma cell appears to be similar for all classes (Nathans *et al.*, 1958; Osserman *et al.*, 1964), and therefore, the number of plasma cells and the rate of catabolism determine the concentration of immunoglobulins in the serum. For some unknown reason, though, the number of plasma cells forming immunoglobulins of each class is different. The rate of catabolism has been determined for all classes and subclasses and is described in Section VI.

When rate of exchange between intra- and extravascular spaces was studied for some immunoglobulins as well as other serum proteins (Nakamura *et al.*, 1968) the distribution between plasma and lymph space was found to depend primarily on the diffusion coeffi-

cient of the protein, whereas the primary structure of the protein did not appear to be important for this parameter. There is an inverse relationship between the diffusion coefficient of a particular immunoglobulin and the ratio of its concentration in serum versus extravascular fluid, e.g., IgG has a high diffusion coefficient and is predominantly located extravascularly, whereas IgM has a low diffusion coefficient and is found predominantly in the serum. Relatively large concentrations of IgD have been reported in the intravascular space as compared to the extravascular compartment (Rogentine *et al.*, 1966), suggesting that IgD has a low diffusion coefficient compared to its molecular weight. This might result from asymmetry of the molecule

TABLE II
BIOLOGICAL PROPERTIES AND ACTIVITIES OF HUMAN IMMUNOGLOBULINS[a]

Characteristics	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgM	IgD	IgE
Serum (mg./ml.)	5–12	2–6	0.5–1	0.2–1	0.5–2	0–0.2	0.5–1.5	0–0.4	0–0.002
Secretion	−	−	−	−	+	+	(+)	−	?
Cerebrospinal fluid (μg./ml.)		2.5–7.5 IgG					not detectable		
Half-life in days	23	23	16	23	6	6	5	3	2
Fractional turnover (%)	7	7	17	7	25		18	37	89
Synthesis (mg./kg./day)	25		3.4		24		7	0.4	0.02
Placental transfer	+	+	+	+	−	−	−	−	−
Classic C	++	+	++	−	−	−	+	−	−
Alternate C	−	−	−	−	+	+	−	±	±
Prausnitz-Küstner	−	−	−	−	−	−	−	−	+
Reverse passive cutaneous anaphylaxis	+	−	+	+	−	−	−	−	−
Macrophages	+	±	+	±	−	−	−	−	−
Neutrophiles	+	+	+	+	+	+	−	−	−
Platelets	+	+	+	+	−	−	−	−	−
Lymphocytes	+	±	+	±	−	−	−	−	−
Staphylococcal A	+	+	−	+	−	−	−	−	−
Cystic fibrosis factor	+	+	−	−	−	−	−	−	
Rheumatoid factor (antigen)	++	+	−	+	(+)		(+)		(+)
Rheumatoid factor (antibody)		IgG+			IgA+		+	−	−

[a] Symbols used: −, negative; (+), occasional positive reaction; ±, weakly positive; +, positive; ++, strongly positive; blank space, not tested.

caused by the three carbohydrate side chains (Spiegelberg *et al.*, 1970). Immunoglobulin A is relatively more concentrated in lymph than in serum which is probably explained by local synthesis of IgA in the intestines and drainage into the thoracic duct (Vaerman and Heremans, 1970). The concentrations of human immunoglobulins in the serum are shown in Table II. Immunoglobulin G1 is found in the highest concentration followed by IgG2, IgA1, IgM, IgG3, IgA2, IgD, and IgE. The concentrations vary considerably from individual to individual, especially among the rare classes and subclasses such as IgG4 (Kunkel *et al.*, 1970; Morell *et al.*, 1970; Schur *et al.*, 1970) or IgD (Rowe and Fahey, 1965); e.g., IgD cannot be detected in about 10% of the human population, has a concentration of 20 to 70 μg./ml. of serum in 80%, but can be as high as 400 μg./ml. Although genetic control of this variation has been suspected (Rowe *et al.*, 1968), it has not yet been demonstrated, probably because environmental factors influence the production of the rare classes and subclasses of antibodies much more than the major classes. The IgG subclass concentration of an individual depends on the genetic makeup of his IgG (Yount *et al.*, 1967; Morell *et al.*, 1972), indicating that, at least in part, genetic factors regulate the IgG subclass concentrations. In different mammalian species, immunoglobulin concentrations follow patterns similar to man's, although the absolute quantities vary, e.g., concentrations are lower in mice than in man with 3 to 5 mg. IgG/ml. in outbred mice and with great variations among inbred murine strains (Fahey and Barth, 1965). In guinea pigs and rats, immunoglobulin concentrations are also lower than man's, whereas other laboratory animals, such as rabbits, goats, sheep, and horses, usually have IgG concentrations similar to those in man.

B. Secretions

Immunoglobulin concentrations in external secretions, such as saliva, tears, bronchial secretions, colostrum, and intestinal fluid, are much lower than those in the serum; more importantly, however, the ratio among classes of immunoglobulins is significantly different. It has been well documented that IgA is the predominant class of immunoglobulins in secretions (Tomasi and Grey, 1972). The ratio of IgA to IgG is about 1:5 in the serum but 20:1 in the saliva and in other secretions. This ratio is probably even higher for IgA2 (Grey *et al.*, 1968a). Two factors appear to be responsible for the increased concentration of IgA in secretions. First, IgA combines with a special polypeptide chain, the secretory component, that is formed by the epithelial cells of the glands. Second, data from immunofluorescent

testing have shown that IgA is produced locally in lymphoid tissue around the glands, since many more plasma cells stain for IgA in these organs than in spleen or lymph nodes. Of the other immunoglobulin classes, only IgM has been shown capable of reacting with the secretory component (Mach, 1970). Moreover, only in the rare cases of patients who have agammaglobulinemias and lack IgG and IgA has IgM been found in significant concentrations in secretions, apparently as a compensation for the absent IgA (Tomasi and Grey, 1972). Although not proven to react with secretory component, IgE may also be more concentrated in secretions than in serum because IgE-producing plasma cells are more frequently found in lymphoid tissue around secretory glands than in lymph nodes and spleen (Ishizaka, 1970; Salmon, 1970). Because the amounts of IgE are so low, it has not been possible to prove this assumption.

C. Cerebrospinal Fluid

Immunoglobulins are generally believed to enter the cerebrospinal fluid by passive diffusion across the blood–brain carrier. Some, however, may also enter directly after synthesis by plasma cells in the central nervous tissue. In man, the IgG concentration is about 100 times less than in serum (2.5–7.5 μg./ml.) and represents about 12% of the cerebrospinal fluid protein (Laffin, 1970; Levin *et al.*, 1972) (Table II). Concentrations of IgA and IgM, similarly, are far less than in the serum, in fact, they are below the level of detection, as shown by Riddoch and Thompson (1970). No IgD and IgE have been detected in cerebrospinal fluid (Rowe *et al.*, 1968b; Ishizaka, 1970).

V. Distribution of Antibodies in Immunoglobulin Classes

Following administration of antigen, the recipient first synthesizes IgM antibodies but usually only for a limited time span. Subsequently IgG antibodies as well as IgA antibodies are formed and their production lasts for long periods. The secondary immune response consists primarily of IgG antibodies.

Relatively few studies have been made to quantitate the class and subclass distribution of antibodies to a specific antigen. Immunoglobulin G antibodies to protein antigens, such as tetanus toxoid, diphtheria toxoid, and thyroglobulin, are formed in quantities roughly similar to the normal IgG subclass distribution (World Health Organization Meeting on Subclasses, 1968, unpublished) (Table III). In contrast, carbohydrate antigens appear to elicit an immune response restricted to certain subclasses. Yount *et al.* (1968) reported that volunteers injected with dextran showed almost entirely only

TABLE III
DISTRIBUTION OF PURIFIED ANTIBODIES IN HUMAN IMMUNOGLOBULIN G SUBCLASSES[a]

Antibody	IgG1		IgG2	IgG3	IgG4
Antitetanus Toxoid	++		+	+	±
Antidiphtheria Toxoid	++		+	+	±
Antithyroglobulin	++		+	+	±
Anti-DNA	++	or	++	(+)	(+)
Anti-Rh	++		−	+	(+)
Anti-Factor VIII	−		−	−	+
Antidextran	−		+	−	−
Antiteichoic acid	−		+	−	−

[a] Symbols used: −, negative; (+), occasional positive reaction; ±, weakly positive; +, positive; ++, strongly positive; blank space, not tested.

IgG2 antibodies even after long periods of immunization. Similarly, antibodies to hemophilic Factor VIII appear to be restricted to the IgG4 subclass (Anderson and Terry, 1968; Shapiro and Carroll, 1968). In studies of anti-DNA antibodies, sera of patients with lupus erythematosus contained antibodies of many classes and subclasses. Individual patients, however, formed antibodies of predominantly one or another subclass of IgG, as semiquantitative analyses did not show a distribution of anti-DNA antibodies parallel to the normal subclass concentrations (Tojo *et al.*, 1970). Anti-Rh antibodies are predominantly of IgG1 and three subclasses (Natvig and Kunkel, 1968).

In rodents, immunization procedures influence the immune response with respect to γ1G and γ2G antibody production. When guinea pigs are injected with protein antigens incorporated into incomplete adjuvant, the result is formation of γ1G antibodies, whereas injection of antigen in complete adjuvant containing mycobacteria causes a predominant γ2G response (Benacerraf *et al.*, 1963; While *et al.*, 1963). Similarly, mice and hamsters form γ1G antibodies when injected with certain antigens in incomplete adjuvant (Coe, 1966, 1968).

The IgM responses are increased when protein antigens are coupled to particles such as erythrocytes or polystyrene beads; also, large protein antigens, such as keyhole limpet hemocyanin, produce prolonged IgM responses in rabbits and rats which usually form very small amounts of IgM to other protein antigens (Dixon *et al.*, 1966).

A predominant IgA response can be achieved under certain circumstances by intranasal immunization, probably because of a local IgA response in the respiratory passages and in the lung in the absence of a generalized immune response.

Immunoglobulin E antibodies can be elicited consistently with extracts or isolated antigens of helminth worms such as ascarids (Hus-

sain *et al.*, 1972). Although little is known about immune responses in the IgD class (Spiegelberg, 1972), IgD antibodies have been reported in sera having large quantities of antibovine serum proteins (Heiner and Rose, 1970), in sera from patients with lupus erythematosus (Kantor *et al.*, 1970), and in sera from patients with anti-insulin antibodies (Devey *et al.*, 1970). These IgD antibodies have, however, only been demonstrated by indirect methods.

Carbohydrate antigens of streptococcal and pneumococcal cell walls elicit a monoclonal type of antibody (Braun *et al.*, 1969; Pincus *et al.*, 1970) in a large percentage of rabbits. This restricted immune response appears to be related to the variable region of heavy and light chains, since no distinctions as to subclass have been shown in these rabbits. As mentioned, rabbit IgG consists almost entirely of one subclass and these monoclonal antibodies also belong to this subclass.

VI. Immunoglobulin Turnover

Whereas the quantity of immunoglobulin synthesized per day in an organism is dependent on the number of plasma cells, the rate at which immunoglobulins are catabolized is dependent in part on the structure of the constant region of the heavy chain. The Fc fragments of IgG are catabolized relatively slowly, similarly to the intact IgG, whereas Fab fragments and light chains are rapidly eliminated from the circulation and catabolized (Spiegelberg and Weigle, 1965, 1966a). The synthetic rates and average half-lives in days of human immunoglobulins are summarized in Table II: IgG has the highest synthetic rate and the longest half-life; turnovers of subclasses IgG1, IgG2, and IgG4 are similar to one another, whereas the IgG3 proteins are more rapidly catabolized (Spiegelberg *et al.*, 1968; Morrell *et al.*, 1970). In these studies performed with myeloma proteins, the half-lives varied considerably within a given subclass and the differences could not be related to the known genetic markers of myeloma proteins within that subclass (Table IV). Furthermore, such variations within a subclass and the more rapid turnover of IgG3 do not result from structural differences within the Fc fragment, since Fc fragments and heavy-chain disease proteins, which closely resemble Fc fragments in structure, are catabolized at the same rate regardless of immunoglobulin subclass or individual myeloma protein from which they are derived (Spiegelberg and Fishkin, 1972). Since IgG3 myeloma proteins from different individuals were catabolized more rapidly than other subclasses in two independent studies (Spiegelberg *et al.*, 1968; Morell *et al.*, 1970), rapid turnover is likely to be a characteristic of that subclass. However, since the frequencies of rheumatoid

TABLE IV

DIFFERENCES IN PLASMA HALF-LIVES OF PAIRED ^{131}I- AND ^{125}I-LABELED HUMAN IMMUNOGLOBULIN G MYELOMA PROTEINS AND HEAVY CHAIN DISEASE PROTEINS IN PATIENTS SUFFERING FROM NEOPLASIAS OTHER THAN MULTIPLE MYELOMA OR MACROGLOBULINEMIA[a]

Recipient	IgG subclass	MP[b]	*T*/2	IgG subclass	MP[b]	*T*/2	% Difference
R.L.	1	Cut	13.0	1	Cov	12.1	6.9
C.M.	1	Jon	18.4	1	Jac	14.0	24.0
M.H.	2	Tsc	23.0	2	Dah	23.0	0.0
L.B.	2	Lig	12.0	2	Dom	7.2	40.0
E.C.	3	Fra	10.0	3	Dep	9.7	3.0
V.H.	3	Fra	6.4	3	She	9.1	29.6
F.J.	4	Ger	9.2	4	Fer	9.7	5.2
T.J.	4	Heb	6.7	4	Fer	5.3	20.9
		HCD[b]			HCD[b]		
Bu	1	Cra	16.5	2	Gif	16.0	3.2
Pa	1	Cra	13.2	3	Zuc	13.2	0.0
Ba	1	Cra	19.6	3	Zuc	21.6	4.6

[a] Data from Spiegelberg *et al.* (1968) and Spiegelberg and Fishkin (1972).
[b] MP, myeloma proteins; HCD, heavy chain disease (proteins).

factor activity (Grey *et al.*, 1968b) and maybe of other, as yet unknown, antibody activities are increased in IgG3 myeloma proteins, turnover studies of normal IgG3 as compared to normal IgG1 will be necessary to prove that IgG3 is definitely different in catabolic rate from other IgG subclasses. In an unpublished series of experiments performed in collaboration with Drs. Grey and Fishkin, we found that IgA1 and IgA2 myeloma proteins, whether of monomeric or polymeric forms, are catabolized at similar rates in man. Only trace amounts, if any, of the injected radiolabeled myeloma proteins were excreted into the saliva, and in particular we observed no difference in excretion of IgA1 and IgA2. The half-life of IgM is similar to that of IgA, but IgD and IgE are more rapidly catabolized. Actually, the fractional turnover rate (percent immunoglobulin catabolized per day from the intravascular pool) is a better and more quantitative parameter of immunoglobulin catabolism than determination of the serum half-life (Waldman and Strober, 1969). As shown in Table II, the fractional turnover rate is increasingly greater for immunoglobulins in the order listed: IgG, IgA, IgM, IgD, and IgE.

In mice, immunoglobulins are catabolized faster than in man (Table V); the relative turnover rates among classes and subclasses

are, however, similar. Murine γ2aG and γ1G, which may correspond to IgG1 and IgG2 in man, respectively, have the longest half-lives (Fahey and Sell, 1965), but murine γ2bG, which might correspond to human IgG3, is more rapidly catabolized. In mice as in man, IgA and IgM are catabolized faster than IgG. The concentration of IgG controls, at least in part, the rate of catabolism in mice, as shown in the experiments of Fahey and Robinson (1963), who increased serum concentrations of IgG or its Fc fragment, thus specifically increasing IgG catabolism.

Elimination from the circulation of guinea pig γ1G and γ2G antibodies to bovine serum albumin indicated that γ1G (7.1 days) has a longer half-life than γ2G (5.7 days) (Lefever and Ishizaka, 1972).

The mechanism of immunoglobulin catabolism is presently not understood. An interesting hypothesis has been proposed by Brambell (1966), who assumes that IgG is pinocytized at a constant rate and that a fraction of the pinocytized IgG attaches to protective receptors and is returned to the circulation. In contrast, IgG that is not attached to the receptor is catabolized. Assuming a constant number of protective receptors, this theory would explain the increase of IgG catabolism at higher serum concentrations, since more IgG is pinocytized but not protected by the receptors. The IgG would attach to the receptor via Fc fragments, because it was shown that the rate of catabolism can be increased by injection of Fc fragments (Fahey and Robinson, 1963) and Fc fragments have a much longer half-life than Fab fragments (Spiegelberg and Weigle, 1965).

TABLE V
BIOLOGICAL ACTIVITIES OF MOUSE IMMUNOGLOBULINS[a]

Characteristics	γ2aG	γ2bG	γ1G	J606	IgA	IgM	IgE
Classic C	+		−	−	−	+	−
Alternate C					+		
Homologous passive cutaneous anaphylaxis	−	−	+	−	−	−	+
Heterologous passive cutaneous anaphylaxis	+	−	−	−	−	−	−
Half-life (days)	5	2	4	4	1	1	
Placental transfer	+	+	+	++	−	−	−
Staphylococcal A	+		−	+	−		
Macrophages	+		−				

[a] Symbols used: −, negative; (+), occasional positive reaction; ±, weakly positive; +, positive; ++, strongly positive; blank space, not tested.

VII. Placental and Gut Transfer

Brambell (1970) has extensively studied the transfer of immunoglobulins from mother to young. Immunoglobulins can be transferred to the offspring prenatally, postnatally, or both ways. The Fc fragment plays an important role in these transfers since isolated Fc fragments are passed but Fab fragments are not (Brambell *et al.*, 1960). In man and monkeys, prenatal transfer of immunoglobulin to the fetus appears to be the major route (Gitlin *et al.*, 1964), and only IgG is transferred (Table II). It appears that probably all IgG subclasses pass the placental barrier (Wang *et al.*, 1970; Virella *et al.*, 1972). Transfer of immunoglobulins through the gut does not appear to be important in man. Although human colostrum is rich in IgA (Tomasi and Grey, 1972), this IgA is probably not transferred but rather fulfills a function within the gastrointestinal tract. In most rodents, such as mice (Table V), rabbits, and rats, IgG but no other class of immunoglobulins is transferred via yolk sacs during pregnancy and additional IgG present in the colostrum is transferred to the young by intestinal absorption in the first 24 postnatal hours (Brambell, 1970). In guinea pigs, both γ1G and γ2G are known to be transmitted maternally (Caretti and Ovary, 1969).

The young of ruminants and pigs receive no prenatal immunoglobulin. Maternal colostrum contains large quantities of γ1G but no γ2G and the γ1G is absorbed in the gut of the newborn during early postnatal life. The sera of colostrum-deprived piglets contain small amounts of an immunoglobulin half-molecule (Franek and Rika, 1964), which might resemble human IgG half-molecules formed in patients with plasma cell tumors (Hobbs and Jacobs, 1969; Spiegelberg and Heath, 1973).

VIII. Activation of Complement

Immunoglobulins can activate complement by two different pathways (Fig. 2) (Müller-Eberhard, 1969; Götze and Müller-Eberhard, 1971; Spiegelberg and Götze, 1972). Activation of the classic pathway involves the complement components 1 to 9, reacting in the order: 1, 4, 2, 3, 5, 6, 7, 8, and 9. The alternate pathway of complement fixation is probably identical to the previously described properdin system (Pillemer *et al.*, 1954) and involves factors C3, 5, 6, 7, 8, and 9 of the classic system and additional serum proteins which at present are not fully characterized. It appears that properdin splits C3 (the hydrazine-sensitive Factor A of the properdin system) into C3a and C3b. The C3b together with an α-protein, C3 proactivator convertase (C3PAse) (Müller-Eberhard and Götze, 1972), and with magnesium ions convert

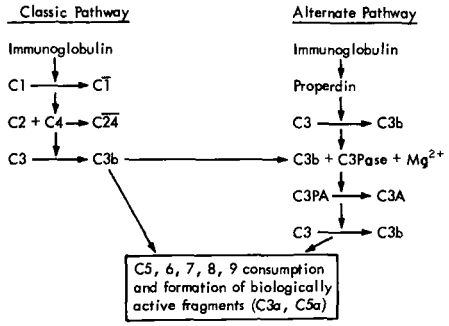

FIG. 2. Schematic diagram of the two pathways of complement activation. C3PAse = C3 proactivator convertase.

the β-globulin C3 proactivator (C3PA) (the heat-sensitive Factor B of the properdin system) to form the C3 activator (C3A) and this protein cleaves C3; this is followed by activation of late components of complement. There is a feedback mechanism between the classic and alternate pathway. The C3b generated by the classic pathway can also react with C3PAse and magnesium ions to convert C3PA to C3A. Therefore, all immunoglobulins that activate the classic pathway also cause cleavage of C3PA to create C3A. In order to determine which immunoglobulins activate the alternate pathway without activating the classic pathway, the conversion of C3PA was studied in C2-deficient serum (H. J. Müller-Eberhard and H. L. Spiegelberg, unpublished data). The activation of complement by human immunoglobulins is summarized in Table II. The classic pathway is activated by IgG1, 2, and 3 and IgM (Ishizaka *et al.*, 1967). There appear to be quantitative differences among and within subclasses when aggregated myeloma proteins are tested for their ability to fix classic components of complement (Table VI): IgG2 myeloma proteins are slightly less efficient than IgG1 and IgG3 proteins, and IgM macroglobulins are even more inadequate. The reasons for these differences are unknown. The Fc fragments of IgM fix CH50 units more efficiently than intact IgM, perhaps because not all sites on the Fc fragments of the five subunits of intact IgM molecules are available for reaction with complement (Plaut *et al.*, 1972). Variation within a subclass might be the result of differences in the degree to which myeloma proteins are aggregated by BDB. However, since unaggregated proteins within a single subclass are also catabolized at different rates (Table IV), these variations might reflect true differences within a subclass. Aggregated IgA, IgD, and IgE myeloma proteins do

TABLE VI
QUANTITIES (PROTEIN NITROGEN) OF REPRESENTATIVE SAMPLES OF AGGREGATED MYELOMA PROTEINS NECESSARY TO FIX 50% OF 100 CH50 COMPLEMENT UNITS[a]

Class, subclass	Myeloma protein	N (μg.)
IgG1	Ba	8
	Sa	31
	Pa	20
IgG2	Th	45
	Wa	35
IgG3	Vi	13
	Br	10
IgG4	Me	>800
	Du	>800
IgA	Br	390
	Cl	>800
	Lo	212
	Normal	>800
IgM	C	64
	Bo	115
	E	283
	Normal	110

[a] Data from Ishizaka *et al.* (1967).

not fix classic components of complement (Henney *et al.*, 1969; Ishizaka *et al.*, 1970).

Mouse γ2aG and IgM activate the classic pathway but not γ1G, J606, and IgA (Table V). In other species, only γ2G and IgM activate the classic pathway; however, since the subclasses of species without availability of myeloma proteins are not as well separated as those with them, antibodies of γ1G electrophoretic mobility may sometimes also activate classic complement components.

Two types of human IgM macroglobulins, one that fixes classic complement components and another that does not, have been reported (Mackenzie *et al.*, 1969). Similarly, two guinea pig IgM subpopulations differ in complement fixation capacity have been observed (Linscott and Hansen, 1969). These experiments suggest the presence of two IgM subclasses. However, they might also reflect differences within a subclass, as shown in Table VI, and, since no structural correlates in the constant region of the μ chain have been found which would prove the existence of IgM subclasses, the variation in the ability of IgM proteins to fix complement remains unexplained.

Activation of the alternate pathway of complement fixation by im-

munoglobulins has been studied by analyzing the conversion of C3PA to C3A in normal (Götze and Müller-Eberhard, 1971; Spiegelberg and Götze, 1972) and C2-deficient sera (H. J. Müller-Eberhard and H. L. Spiegelberg, unpublished data). As mentioned above, all immunoglobulins that activate classic complement components (IgG1, 2, 3, and IgM) also convert C3PA by formation of C3b, and, in addition, IgA1 and IgA2 also convert C3PA. However, only IgA1 and IgA2 but not IgG of all subclasses and IgM converted C3PA. The IgD myeloma proteins were weakly active, and IgE negative. In a study of fixation of individual complement components by IgE and its Fc fragment, Ishizaka *et al.* (1972) found that the Fc fragment was much more efficient than intact IgE in fixing late complement components. Similarly, C3PA was converted by aggregated IgD and IgE Fc fragments even though intact IgE was negative for C3PA conversion in our tests. The reason why Fc fragments of IgD and IgE are more efficient than the intact proteins is not understood.

Other than human immunoglobulins, only guinea pig, rabbit, and mouse immunoglobulins have been studied for their abilities to activate the alternate pathway. Guinea pig γ1G was the first immunoglobulin shown to activate late components of complement in the absence of C142 fixation (Osler *et al.*, 1969; Oliveira *et al.*, 1970; Sandberg *et al.*, 1971), and the $F(ab')_2$ fragment of guinea pig γ1G and γ2G had the site that stimulated the alternate pathway (Sandberg *et al.*, 1971). Similarly, rabbit IgG $F(ab')_2$ fragments were shown to convert C3PA (Spiegelberg and Götze, 1972). Mouse IgA myeloma proteins, particularly myeloma protein 315 which has anti-DNP activity, have been shown to convert human C3PA (Lambert *et al.*, 1973; O. Götze and H. L. Spiegelberg, unpublished). Mouse γ1G has not been studied for conversion of C3PA, but if it is the immunoglobulin analogous to guinea pig γ1G, it also should activate the alternate pathway. Man does not seem to have an IgG subclass analogous to γ1G of guinea pigs since none of the IgG myeloma proteins tested nor their F $(ab')_2$ fragments converted C3PA in the absence of activation of C142.

For lysis of cells, participation of all nine classic complement components appears to be necessary, since activation of the alternate pathway of complement fixation does not produce lysis of normal red cells (Götze and Müller-Eberhard, 1972). Other biological activities mediated by complement, however, do not involve all complement components. Phagocytosis and immune adherence appear to involve only components 1, 4, 2, and 3 (Nelson, 1962). Histamine release by complement-derived anaphylatoxin is mediated by split products

(C3a, C5a) of components C3 (Dias Da Silva and Lepow, 1967) and C5 (Jensen, 1967; Cochrane and Müller-Eberhard, 1968), and chemotaxis is mediated by C3a as well as by complexes of complement components 5, 6, and 7 (Ward *et al.*, 1965, 1966).

IX. Reaction with White Blood Cells—Cytophilic Antibodies

Of the many secondary functions of immunoglobulins, the so-called cytophilic property or the ability to bind to cell surface receptors has only recently been subjected to intensive investigation. Although cytophilic properties of IgG in relation to macrophages have been known for some time (Boyden and Sorkin, 1960), the finding that IgE antibodies cause allergies by binding to basophiles and mast cells and inducing release of vasoactive substances from these cells after the reaction with antigen (Ishizaka, 1970) has stimulated new research into the interaction between antibodies and different types of white blood cells. The data thus far obtained suggest that white cells have proteins in their membranes, called receptors, which specifically react with the Fc portion of certain immunoglobulins. These receptors appear to be distributed evenly over the cell surface and are normally occupied by cytophilic antibodies. When antigen reacts with cytophilic antibody, a signal is relayed to the cell in an as yet unknown manner which causes the cell to activate certain functions, such as the release of vasoactive amines or the phagocytosis of the antigens that have reacted with the cytophilic antibodies. The relay mechanism may be similar to the one proposed for immunoglobulin-bearing lymphocytes (Pernis *et al.*, 1970; Rebellino *et al.*, 1971; DePetris and Raff, 1973). It appears that following the reaction with antigen, immunoglobulin molecules on the cell surface cause aggregation of the receptor molecules into clumps which finally combine to form a conglomerate mass visible as a "cap" on the cell. Subsequently, the cap may be internalized, and this may provide the signal to the cell to perform its function. The study of antigen–antibody–cell receptor interactions leading to expression of specific cell functions will in the future certainly provide interesting and important data on cell membrane functions and on cell physiology.

The presence of receptors on white blood cells can be demonstrated by several different experimental approaches. Binding of immunoglobulins to white cells can be visualized when antibody-coated erythrocytes are added to the reaction mixture *in vitro* to form so-called rosettes (Fig. 3). In this reaction it is thought that the Fab fragments of the antierythrocyte antibody bind to the erythrocytes, and the Fc fragment is available to react with the receptor of the white

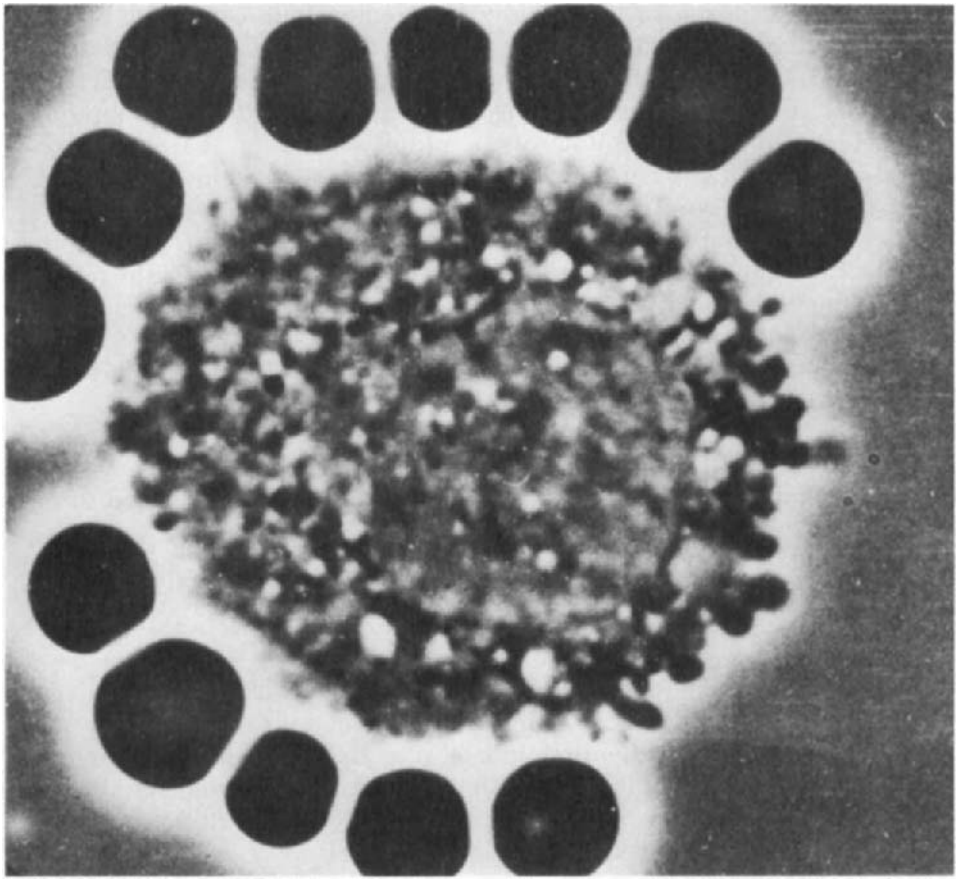

FIG. 3. Photomicrograph of rosette formed by mouse macrophage in center and of sheep erythrocytes that are coated with rabbit IgG antierythrocyte antibodies in outer circle.

cells. The erythrocytes, therefore, arrange themselves around the white cell until they look like a bouquet or "rosette." The specificity of a receptor on a white cell for a given immunoglobulin class can be tested in this system by inhibiting rosette formation with different immunoglobulins added to the culture medium. Repelling forces exist between certain white cells and erythrocytes, and the affinity of the immunoglobulins to the white cell receptors must overcome these forces. Since this affinity is not of great magnitude, it may often not be sufficient; therefore, a negative rosette phenomenon does not necessarily indicate the absence of receptors on the white cells and other

techniques must also be employed to demonstrate cytophilic properties. Binding of immunoglobulins to white cells has been determined by analyzing the uptake of ^{125}I-labeled antigen by antibody-coated cells, either by counting radioactivity (Boyden and Sorkin, 1960) or by demonstrating silver grains over cells (Tomioka and Ishizaka, 1971). Unfortunately, a nonspecific background that is usually present in this system makes it difficult to interpret the findings. A third method devised to study specific receptors is to gauge the effect of aggregated immunoglobulins on cell functions. Aggregated immunoglobulins such as purified myeloma proteins are added to cell suspensions, and the subsequent release of cell constituents, e.g., histamine and β-glucuronidase, or the phagocytosis of the aggregates is measured.

A. Macrophages and Monocytes

In man, monocytes were shown to have receptors for IgG1 and IgG3 when these two, but not IgG2 and IgG4, myeloma proteins inhibited rosette formation of IgG-coated red cells (Huber and Fudenberg, 1968; Lobuglio *et al.*, 1967). Whether or not macrophages have different receptors for IgG2 and IgG4 is still unknown. In the studies of monocyte receptors, the antibody used for coating the erythrocytes was probably of the IgG1 subclass; therefore, IgG1 could have preferentially inhibited rosette formation, and no information about receptors specific for other immunoglobulin classes could be obtained. Receptors for IgM on human monocytes were also studied, and it was found that rosettes would not form unless complement was added, suggesting that IgM is not highly cytophilic for monocytes (Huber *et al.*, 1968). Binding of human IgG myeloma proteins to guinea pig macrophages indicates that all IgG subclasses can bind to these heterologous cells, although great individual variations are found (Inchley *et al.*, 1970). In a recent study performed in our laboratory (Lawrence *et al.*, 1974) it was found that human monocytes bind unaggregated IgG1 and IgG3 myeloma proteins but not IgG2, IgG4, IgA, IgM, IgD, and IgE proteins. Following aggregation the monocytes also bind IgG2 and IgG4 proteins but none of the other classes.

In guinea pigs and mice it has clearly been shown that γ2G is cytophilic for macrophages and that γ1G is not (Boyden, 1964; Uhr, 1965; Berken and Benacerraf, 1966; Lay and Nussenzweig, 1969). The $F(ab')_2$ fragments are not cytophilic for macrophages, demonstrating the importance of the Fc fragment for the cytophilic properties of antibodies (Berken and Benacerraf, 1966; Inchley *et al.*, 1970). The receptors are specific for γ2G since they cannot be inhibited with γ1G

and IgA. The ability of radiolabeled antigen to bind to rabbit macrophages coated with IgG antibodies was the first demonstration of interaction of antibodies with white cells, and the term *cytophilic* was coined for this phenomenon (Boyden and Sorkin, 1960). Rabinovitch (1967) showed that rabbit IgM is not cytophilic for macrophages. In mice as in man, although IgM binds to macrophages when complement is present, the cytophilic property of γ2aG or of IgG1 and IgG3 immunoglobulins is independent of complement (Lay and Nussenzweig, 1969).

Since macrophages and monocytes are phagocytic cells, it is most likely that the cytophilic antibodies fulfill the function of opsonization. The opsonic role of the Fc fragment of IgG has been demonstrated *in vivo* by measuring antigen elimination from the circulation (immune elimination). In contrast to intact IgG, Fab fragments no longer cause immune elimination of bovine serum albumin (BSA) (Spiegelberg and Weigle, 1966b), in fact complexes formed between BSA and Fab fragments are eliminated at the same rate as the antigen alone. The $F(ab')_2$ fragments cause immune elimination of BSA only when antibody is in excess and, even then, much less efficiently than intact IgG. In certain instances, the opsonic function involves fixation of complement components, since erythrocytes and bacteria are more rapidly phagocytized by the reticuloendothelial system of liver and spleen in the presence of complement (Spiegelberg *et al.*, 1963).

B. Basophiles and Mast Cells

Antibodies that are cytophilic for basophiles or mast cells present in the skin, lung, etc., have been called *homocytotropic* antibodies. Even before detailed information on classes and subclasses of immunoglobulins was available, it was known that in certain species two types of homocytotropic antibodies existed (Table VII). One, called reaginic, is heat-labile, has a higher molecular weight than IgG, is in the serum in very small concentrations, and remains in the skin for relatively long periods during which a passive anaphylactic reaction can be induced. This type of homocytotropic antibody has now clearly been shown to belong to the IgE class (reviewed by Ishizaka, 1970). Immunoglobulin E is responsible for the passive anaphylactic reaction in the skin of an animal of the homologous species to the donor or in Prausnitz–Küstner reaction (Table II). The site on the IgE molecule that reacts with the basophiles is the Fc fragment, since the reaction can be blocked by isolated Fc fragments. Homocytotropic antibodies of the IgE class have been found in many species including rat (Stechschulte *et al.*, 1970), mouse (Prouvost-Danon *et al.*, 1966),

TABLE VII
PROPERTIES OF THE TWO TYPES OF ANAPHYLACTIC HOMOCYTOTROPIC ANTIBODIES

Characteristics	Type I, reaginic	Type II
Concentration in serum	Trace (0.1–1 μg./ml.)	High (1–2 mg./ml.)
Heat stability	Labile	Stable
Sedimentation rate	8 S	6.6 S
Molecular weight	200,000	150,000
Carbohydrate content	11%	3%
Classic complement	Negative	Negative
Alternate complement	Positive	Positive
Half-life in plasma	Short	Long
Half-life in skin	Long	Short
Class in man	IgE	?
Class in rodents	IgE	γ1G

rabbit (Zvaifler and Robinson, 1969), and guinea pig (Dobson *et al.*, 1971). The binding of IgE to mast cells is relatively strong, and the turnover determined in the skin is long with a half-life of 11 to 15 days (Cass and Anderson, 1968). In contrast, the half-life of IgE in plasma is very short as previously described.

The second type of homocytotropic antibodies is characterized by heat stability, the same sedimentation rate as IgG, and a half-life that is short in skin but long in plasma. In man this type of antibody appears to be rare, because reports of IgG homocytotropic antibodies are few (Malley and Perlman, 1966; Reid *et al.*, 1966) and the subclass is not established. The reported IgG skin-sensitizing antibodies do not seem to belong to the known four IgG subclasses, nor to the possible fifth subclass. In contrast, in guinea pigs (Ovary *et al.*, 1963), rats (Bach *et al.*, 1971) and mice (Ovary *et al.*, 1965), it has clearly been shown that the fast-moving γ1G is the second type of homocytotropic antibody. In rats, γ1G competes with IgE for the same cell receptors (Bach *et al.*, 1971); however, its affinity for the receptor is much lower than that of IgE. The γ1G homocytotropic antibody activity reported in rabbits (Zvaifler and Becker, 1966) was later shown to be the result of IgE in this serum fraction (Zvaifler and Robinson, 1969).

Human, rabbit, and mouse IgG, but not other immunoglobulin classes (Tables II and V), sensitize heterologous skin, as shown in the experimental system of reverse passive cutaneous anaphylaxis in the guinea pig (Ovary, 1960). In general, the IgG subclasses, such as IgG1, 3, and 4 of man, γ2aG of mouse and rat, and γ2G of rabbits, that do not sensitize homologous skin, have been shown to sensitize het-

erologous skin (Ovary *et al.*, 1965; Terry, 1966). The fact that human IgG2 neither sensitizes human skin nor heterologous skin remains unexplained. In quantity and electrophoretic mobility IgG2 could be the subclass analogous to guinea pig and mouse γ1G but has lost its ability to sensitize human basophiles and mast cells. Alternatively, homocytotropic IgG, analogous to the rodent's γ1G, is only rarely formed in man and only in small quantities.

C. NEUTROPHILES

Receptors on neutrophiles for human immunoglobulins have been shown by the rosette technique (Messmer and Jelinek, 1970) and by measuring the release of lysosomal constituents after addition of aggregated myeloma proteins (Henson *et al.*, 1972). When receptors for IgG on neutrophiles were sought by rosette formation and inhibition tests, the evidence suggested that the receptors were specific for IgG1 and IgG3, and no receptors for IgG2 and IgG4 were detected. Whether these two subclasses could react with a different receptor from that for IgG1 and IgG3 could not be determined by these methods, since the subclass of the antierythrocyte antibody was not known. In contrast, experiments in which lysosomal enzymes were released by aggregated myeloma proteins indicated that all IgG subclasses react with neutrophiles (Fig. 4). As observed in most experiments involving myeloma proteins, variations within a given subclass were found with respect to quantity of enzymes released from the

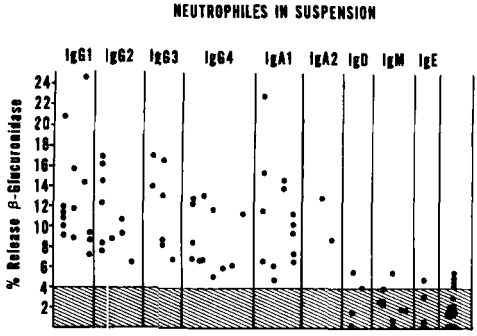

FIG. 4. Percent release of β-glucuronidase from human neutrophiles following incubation with bisdiazatized benzidine-aggregated myeloma proteins. Each column represents results with one myeloma protein and each point the mean of duplicate determinations with neutrophiles from a different donor. The shaded area indicates 1 standard deviation from the mean release from neutrophiles incubated alone, and the last column represents buffer control experiments. Reprinted from Henson *et al.* (1972), with permission of Williams & Wilkins Co., Baltimore, copyright owner.

neutrophiles. Significant differences among the subclasses were, however, not demonstrable. Aggregated IgA1 and IgA2 caused release of lysosomal enzymes to a similar extent as IgG, whereas IgM, IgD, and IgE were completely inactive. Addition of complement did not influence the reactivity, including that of IgM, suggesting that neutrophiles do not have a receptor for complement and IgM-like monocytes. When the activity of aggregated myeloma proteins that were bound to nonphagocytizable surfaces, such as Millipore membranes, was studied, the aggregates were particularly active, and much less aggregate was necessary to produce release of lysosomal enzymes from neutrophiles. This might indicate that antigen–antibody complexes adhering *in vivo* to membranes, such as glomerular basement membranes, synovial membranes, and blood vessel walls, might be especially potent in inducing release of enzymes from neutrophiles. Attempts to demonstrate the specificity of the receptors for subclasses of IgG or IgA by addition of unaggregated myeloma proteins to inhibit enzyme release were unsuccessful, apparently because the affinity of the aggregates to the neutrophiles was too strong to be satisfactorily inhibited by unaggregated immunoglobulins. Recent experiments on the binding of myeloma proteins on neutrophiles (Lawrence *et al.*, 1974) confirmed that IgG and IgA myeloma proteins are cytophilic for neutrophiles.

D. Platelets

It has been shown that platelets from different mammalian species react with antigen–antibody complexes or antigen–antibody–complement complexes. Platelets adhere to such complexes and subsequently release a portion of their content of vasoactive amines, such as serotonin (Mueller-Eckhardt and Lüscher, 1968; Henson, 1970). Experiments to elucidate the classes of immunoglobulin that react with platelets have been performed in two laboratories and have clearly shown that only IgG reacts with platelets (Pfueller and Lüscher, 1972; Henson and Spiegelberg, 1973). Aggregated myeloma proteins were added to platelets labeled with ^{3}H-serotonin, and the release of radioactivity was determined (Fig. 5). Again, considerable variation in activity of myeloma proteins within a given subclass was observed. Pfueller and Lüscher (1972) reported a quantitative difference between IgG1 and IgG3 as compared to IgG2 and IgG4; however, in the other study (Henson and Spiegelberg, 1973) involving a larger number of myeloma proteins, no significant differences were demonstrable among subclasses. The portion of the IgG molecule responsible for the stimulation of platelets appeared to be the Fc frag-

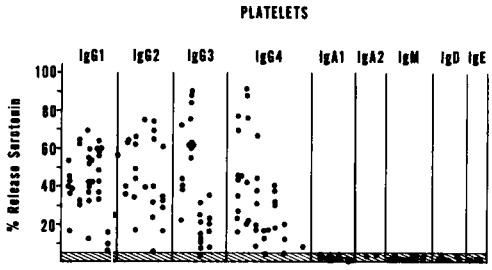

FIG. 5. Release of ^{3}H-serotonin from human platelets incubated with bisdiazatized benzidine-aggregated myeloma proteins. Each column represents one myeloma protein and each point the mean of duplicate determinations employing platelets of different donors. The platelets were incubated with 250 μg. of either insoluble (solid circles) or soluble (open circles) aggregates for 30 minutes at 37°C. The shaded area indicates 1 standard deviation from the mean release of platelets incubated alone Reprinted from Henson and Spiegelberg (1973), with permission of the Rockefeller University Press.

ment, since aggregated Fc fragments but not $F(ab')_2$ fragments induced release of serotonin.

Complement added to the medium inhibited release of serotonin produced by reaction of platelets with IgG1 and IgG3 but not with IgG2 and IgG4. Since inhibition of release of serotonin by complement paralleled the abilities of the subclasses to fix classic complement components, it appears likely that complement binds to the aggregates and interferes with the structure on the Fc fragment that reacts with the receptor on platelets.

Human and rabbit platelets differ in their reactions with aggregated immunoglobulins. Rabbit platelets adhere to aggregated immunoglobulins only after C3 becomes fixed (Sequeira and Nelson, 1961; Henson, 1969); furthermore, addition of neutrophiles enhances the release of serotonin from rabbit but not human platelets (Henson, 1970; Henson and Spiegelberg, 1973). As a result of the reaction with complement, rabbit platelets are lysed, a phenomenon that is not observed with human platelets. Apparently rabbit platelets have a receptor for complement, probably to the C3 component, and this receptor is not demonstrable on human platelets.

E. LYMPHOCYTES

Lymphocytes can be divided into two main categories according to their origin: T or thymus-derived lymphocytes and B or bone marrow-derived lymphocytes (Miller *et al.*, 1971). The B cells possess immunoglobulins on their surfaces. However, these immunoglobulins

are most likely synthesized by the lymphocyte and carried as antigen receptors; therefore they are not cytophilic immunoglobulins. The T cells have very small amounts of immunoglobulin, which may be IgM, on the surface (Marchalonis *et al.*, 1972; Lawrence *et al.*, 1973). Whether or not human B and T lymphocytes have receptors for Fc fragments for cytophilic immunoglobulins is presently unknown. A cytotoxic reaction is obtained by a combination of normal lymphocytes (probably B cells) and antibody, suggesting that there are receptors for IgG on lymphocytes (Perlman and Perlman, 1970). Moreover, in mice, evidence has recently been obtained that B cells may have receptors for γ1G (Basten *et al.*, 1972a,b). Although B cells bind antigen–antibody complexes involving γ1G, unaggregated γ1G is bound only weakly. The binding of γ1G complexes to B cells can be inhibited with γ2bG and IgM, but not with γ2aG, IgA, or light chains. In a recent study on the binding of myeloma proteins to human lymphocytes (Lawrence *et al.*, 1974) it was found that human lymphocytes bind unaggregated IgG1 and IgG3 proteins but no proteins of the other classes. In contrast, following aggregation, the lymphocytes bind IgG2 and IgG4 proteins in addition to IgG1 and IgG3 proteins.

X. Reaction with Staphylococcal A Protein

Staphylococcal A protein forms a precipitin reaction with certain immunoglobulins; however, unlike most antigen–antibody precipitates, not the Fab fragment but the Fc fragment reacts with the foreign protein (Forsgren and Sjöquist, 1966, 1967). The complexed staphylococcal A protein and immunoglobulin resemble, however, usual antigen–antibody complexes since they also fix complement (Sjöquist and Stalenheim, 1969). It has been shown that the IgG of all mammalian species except that of the American oppossum react with staphylococcal A protein, whereas IgG of lower species, reptiles, amphibians, birds (except *Rhea americana*), and fish, do not react (Kronvall *et al.*, 1970a). Of the four IgG subclasses in humans, IgG1, IgG2, and IgG4 react well (Table II), whereas neither IgG3 nor any other class or subclass reacts with staphylococcal A protein (Kronvall and Williams, 1969). Similarly, in mice, IgG subclasses γ2aG and J606 react with protein A (Table V), whereas γ1G and IgA do not (Grey *et al.*, 1971). The function of IgG in protecting an organism by this reaction from staphylococcal infection is not proven, but one would assume that it has such a protective capacity.

XI. Cystic Fibrosis Factor

Patients suffering from cystic fibrosis usually have in their serum a factor, called cystic fibrosis factor, that affects the movement of either

rabbit tracheal cilia (Spock *et al.*, 1967) or oyster cilia (Bowman *et al.*, 1970). Apparently the same factor is found in the fluid of skin fibroblast cultures established from patients with cystic fibrosis but not those from normal humans (Danes and Bearn, 1972). Thus, it has been concluded from these findings that cystic fibrosis cannot be regarded as a primary disorder of the exocrine glands. The relationship and role of the cystic fibrosis factor to the primary defect in cystic fibrosis, however, are presently not understood. The cystic fibrosis factor is heat- and acid-labile and of a low molecular weight, probably less than 5000 daltons, as judged by passage through a dialysis membrane. It binds to human IgG as demonstrated by elution of the factor either alone or mixed with IgG from Dowex-1-2X columns. When IgG is present, the cystic fibrosis factor elutes with it, whereas free cystic fibrosis factor elutes from the column with higher molarity buffers (Danes *et al.*, 1973). The reactivity of cystic fibrosis factor with different immunoglobulins was tested by addition of purified myeloma proteins to culture fluids of cystic fibrosis fibroblasts followed by precipitation of the immunoglobulin–cystic fibrosis factor complex with rabbit antisera specific for the immunoglobulin class. Removal of activity was observed only, and to a similar extent, with IgG1 and IgG2 myeloma proteins, whereas other classes and subclasses were completely inactive. Neither $F(ab')_2$, Fab, and Fc fragments nor free light chains complexed with cystic fibrosis factor. Isolated IgG1 heavy chains, in contrast, did form a complex. It appears from these studies that cystic fibrosis factor binds to the constant region of the $\gamma 1$ and $\gamma 2$ chains, possibly in the proximity of the inter-Fd–Fc (hinge) region which is partially destroyed by papain and pepsin digestion of IgG, and that binding of the factor to IgG is not the result of IgG antibody activity. When other serum proteins were tested, the cystic fibrosis factor was found to bind to β_2-microglobulin but not to haptoglobin or group-specific component (Danes *et al.*, 1973).

XII. Rheumatoid Factor

The rheumatoid factor is defined as an antibody to the Fc fragment of a particular immunoglobulin class or subclass. Immunoglobulins are, therefore, involved in this reaction both as antibodies and as antigens (Franklin *et al.*, 1957; Williams, 1964). Rheumatoid factor (antibody) activity has been shown to be associated with the three major immunoglobulin classes. Although careful quantitative studies have not been made, it appears that IgM rheumatoid factor is most common, and it has been studied most extensively (Kunkel and Tan, 1964). Immunoglobulin G rheumatoid factor is more difficult to demonstrate, since $F(ab')_2$ fragments often must be prepared from IgG–

anti-IgG complexes and the antibody activity of these fragments to normal IgG demonstrated (Schrohenloher, 1966). Rheumatoid factor belonging to the IgA class is also found but apparently on rarer occasions (Heimer and Levin, 1965; Torrigniani and Roitt, 1967), and IgD as well as IgE rheumatoid factors have as yet not been described. When IgG myeloma proteins were tested for rheumatoid factor activity, a relatively increased proportion of IgG3 myeloma proteins was found, as one would expect from the subclass distribution, and these IgG3 proteins had a strong activity (Grey *et al.*, 1968b). Immunoglobulin M macroglobulins found in Waldenström's disease can also have rheumatoid factor activity (Metzger, 1967). The affinity of both monoclonal and polyclonal rheumatoid factor for IgG is usually very low (Cerottini and Grey, 1969; Chavin and Franklin, 1969). Rheumatoid factor is found predominantly and in highest titer in patients with rheumatoid arthritis, yet much less in other collagen diseases and in hyperimmune states; its roles either in disease or in a normal immune response are as yet unknown.

In most cases, IgG is the antigen for rheumatoid factor. Of the different human IgG subclasses, IgG1 myeloma proteins react most strongly, IgG2 and IgG4 to a lesser extent, and IgG3 is negative (Normansell and Stanworth, 1968; Franklin and Frangione, 1971). Antibodies to other immunoglobulin classes, considered similar to classic rheumatoid factor, have been described, for example, to IgA in patients with ataxia telangiectasia (Strober *et al.*, 1968) and to IgM of certain macroglobulins found in patients with Waldenström's disease (Mackenzie *et al.*, 1969). Recently, Williams *et al.* (1972) described anti-IgE antibodies that appear to belong to the rheumatoid factor family of anti-immunoglobulins. Such anti-IgE antibodies were of IgM class and were found in sera of 8.5% of patients with miscellaneous diseases. In contrast, 53% of the patients with established allergic disorders, such as hay fever and extrinsic asthma, showed anti-IgE antibodies. These anti-IgE rheumatoid-factor-like antibodies neither caused release of histamine from basophiles nor inhibited the release by a rabbit anti-IgE antiserum. Like that of other rheumatoid factors, the biological role of these anti-IgE antibodies remains obscure.

XIII. Characterization of Submolecular Sites Related to Secondary Functions

Whenever immunoglobulin fragments have been studied for secondary biological activities, the Fc fragment, but not Fab or $F(ab')_2$ fragments, have had activity similar to that in the whole molecule.

The Fc fragment was shown to have a turnover rate similar to the intact protein (Spiegelberg and Weigle, 1965), to fix classic components of complement (Ishizaka *et al.*, 1962; Plaut *et al.*, 1972), to be transferred to placenta and gut (Brambell, 1970), and to block cytophilic (Berken and Benacerraf, 1966) and anaphylactic (Ishizaka, 1970) reactions. The only exceptions were activation of the alternate pathway of complement faxation by guinea pig γ1G and γ2G and rabbit IgG. In this reaction the $F(ab')_2$ fragment contained the site responsible for the activity (Sandberg *et al.*, 1971; Spiegelberg and Götze, 1972), but not the Fab fragment, suggesting that the site is localized in the portion of the inter-Fd–Fc region present on the $F(ab')_2$ but not on the Fab fragment. It is generally believed that most of the other activities are mediated by site(s) on the N-terminal half of the Fc fragment. One report on the ability to fix classic components of complement demonstrated that a large cyanogen bromide fragment, containing the second homology region or N-terminal half of the Fc portion of mouse γ chains, can fix complement (Kehoe and Fougerau, 1969). Furthermore, a fragment called Fc′, obtained after prolonged papain digestion of IgG and representing the C-terminal region of the Fc fragment, does not persist in the circulation, does not bind complement (H. L. Spiegelberg, unpublished), and also does not inhibit a passive cutaneous anaphylactic reaction (Minta and Painter, 1972).

Attempts to characterize the active sites on immunoglobulin molecules in more detail have not been very successful. Fragments smaller than the Fc fragment usually show no activity whatsoever. Whether large segments of the Fc fragment are required to form the sites or whether the procedures, such as enzymatic digestion or cyanogen bromide fragmentation, with which the smaller fragments are prepared destroy the sites is not known. The sites involved in conferring a secondary function may even vary slightly from molecule to molecule, probably influenced by the variable region of the light and heavy polypeptide chains where antibodies differ most strikingly. This conclusion was reached from the consistent variation in activities that is found among myeloma proteins within the same subclass (Tables IV and VI; Figs. 4 and 5). Half-lives of myeloma proteins of the same subclass differed significantly and reproducibly, and this variation could not be related to genetic markers, the only known structural difference among Fc fragments of different myeloma proteins in a single subclass (Spiegelberg *et al.*, 1968). Furthermore, there was no difference in the half-lives of two Fc fragments isolated from two immunoglobulin myeloma proteins which did behave differently (Spiegelberg and Fishkin, 1972). Therefore, one must assume that the

Fab fragment modulated the secondary function. In the case of elimination of myeloma proteins from the circulation, the variation could have also been the result of a weak autoantibody reaction to body constituents of the myeloma proteins and, depending on this antigen–antibody reaction, a faster or slower half-life might have resulted. Such autoantibody activity could, however, not account for the similar and reproducible variations seen when myeloma proteins were tested for complement fixation and for release of granular constituents from neutrophiles and serotonin from platelets. Since studies of small peptides derived from the Fc fragment and individual myeloma proteins did not help in further characterization of the active sites, perhaps better information on the active sites will be available when Fc fragments or portions thereof can be synthesized and analyzed for activity.

XIV. Conclusions

Of what significance and practical value to immunology is the finding that immunoglobulins of different classes and subclasses differ in their secondary biological activities? Perhaps divisions into classes evolved in order to provide the immune system with proteins that perform diverse functions. Whereas IgG antibodies protect an organism from invaders in the intra- and extravascular spaces, IgA antibodies secreted into the gastrointestinal tract, respiratory tract, etc., function at external areas of the body where invading agents attempt to enter. Immunoglobulin M may be the preformed antibody carried as receptors by lymphoid cells and released relatively quickly into the circulation before IgG and IgA are synthesized. Why then are there so many minor classes and subclasses, often with little known activity that is restricted to one or two known secondary functions, e.g., IgG4, IgD, and IgE? It is possible that minor components of the antibody population are, indeed, not very important and that evolutionary pressure has almost eliminated them, and, therefore, their concentrations are low.

Why certain antigens elicit antibody formation restricted to only one subclass is puzzling. It is presently assumed that antigens invading the body react with antibody-combining sites that are localized in the variable regions of the polypeptide chains in the Fab fragment and are probably present as receptors on lymphocytes. After the reaction of antigen with receptor antibody, it is believed that cellular differentiation and proliferation occur leading to mass antibody production by the stimulated cells. In this process, an as yet not understood switch from IgM to IgG or IgA as well as to other immunoglobulin classes appears to follow. How can an antigen, such as dextran (Yount

et al., 1968), direct the immune response in this process to form IgG2 antibodies only and therefore select the constant region of the polypeptide chains that is not reacting directly with antigen? One possible explanation of this phenomenon could be a restriction in the combination of variable- and constant-region assembly and the stimulation of only a few variable regions by carbohydrate antigens.

For a practical evaluation of immunity to a given antigen, it is important to know that antibodies differ in activities and, most importantly, that under certain circumstances an immune response can be restricted to one or another subclass. Unfortunately, very little quantitative information exists on immune responses according to classes and subclasses. It appears that a complex protein antigen such as tetanus toxoid elicits antibodies of most classes and subclasses; but other antigens, particularly carbohydrate antigens and some autoantigens, can elicit restricted responses (Table III). Therefore, in a test system that is independent of secondary functions, such as antigen binding or agglutination tests, antibodies can be demonstrated clearly, but if they belong to only one subclass they may not be capable of providing protection to the organism from the invading agents. Thus, many patients with recurrent infections in whom no apparent agamma- or hypogammaglobulinemia can be demonstrated could have immunities restricted to subclasses that do not fix complement or could fail to form IgA antibodies, etc. In tumor immunology, enhancing, blocking, and cytotoxic antibodies have been reported (Oettgen *et al.*, 1971); however, little is known about quantitative relationships among the different antibodies with regard to classes and subclasses. Determining amounts in each subclass of these antibodies might be rewarding in elucidating the role of antibody in tumor immunology. It has now been shown that antibodies of the various classes and subclasses differ in their biological activities, and, in the future, we should attempt to determine how frequently restricted immune responses occur and how important such restricted antibody formation is in immune deficiency syndromes.

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