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PREFACE

This volume contains four reviews that reflect both the broad spectrum and the interrelatedness of today's immunologic research. They range from the molecular characterization of the properdin system, to very critical analyses of some of the cell surface interactions which play important roles in the immune response and, finally, to a consideration of the vast amount of experimental data dealing with the effects of radiation on immunologic mechanisms. Today, the state of the immunologic jigsaw puzzle has progressed to the point where significant advances in one area almost immediately aid in the solution of problems in another, and the diverse reviews contained herein illustrate the cellular and molecular bases of this interrelationship.

Since its first description in 1954 the properdin system has been the center of much controversy. In the first article, Drs. Götze and Müller-Eberhard, leaders in the recent development of this field, define the properdin or alternative C pathway in molecular terms. The five plasma proteins constituting this pathway (initiating factor, proactivator, C3, proactivator convertase, and properdin) may be triggered by naturally occurring polysaccharides and lipopolysaccharides or by aggregates of IgA, after which they interact to form specific C3 and C5 convertases without participation of the classical components C1, C2, and C4. It now appears that properdin itself does not play the central role as was originally thought but rather is recruited late in the sequence and serves primarily to stabilize and protect the enzymes of the system against inactivators. The authors also review current knowledge of the biologically relevant interactions of this system, which apparently constitute a protective mechanism operative early during bacterial infection before effective antibody levels have been established.

Nowhere in the field of immunology has recent progress been greater than in our understanding of the events triggered in lymphocytes by ligand–surface receptor interactions. In the second review, Drs. Schreiner and Unanue draw on their own considerable experience in detailing and putting into broad biologic perspective the intricate sequence of events initiated in B cells by the interaction of their surface Ig with appropriate ligands. These events involving the cell surface, cytoplasm, and nucleus include redistribution of ligand-Ig complexes into polar caps, stimulation of cell movement with formation of uropods, endocytosis and elimination of ligand-Ig complexes and, finally, synthesis and replacement of Ig receptors, bringing the cell about full cycle. The free movement of recep-

tor–ligand complexes in the cell membrane, leading to the elimination of the ligand, has obvious implications for the immune response as does the stimulation of cell motion which would enhance any cell–cell interactions that might be involved. It already appears that failure of B cells to accomplish one or more of these responses may lead to tolerance rather than a response. Thus, these fundamental and probably relatively general cellular responses appear to be very integral elements of immunologic responsiveness.

Because the immune response involves a complex sequence of interactions among several different cell populations, the cell surface membranes via which these interactions are mediated are the object of much investigation. In the third article, Dr. Dickler, whose own research has made an important contribution to this field, discusses one of the most important elements of these cell surfaces, the Fc receptor, via which lymphoid cells can bind Ig and thus achieve a measure of immunologic specificity. Such Fc receptors are a property not only of a majority of B cells, but also of a substantial minority of T cells and many undefined lymphocytes. While the biological significance of such surface bound Ig is not as yet certain, it seems likely that it may play a role in antigen localization and is certainly required for antibody dependent cellular cytotoxicity.

Ionizing radiation has long been a potent tool in the study of immunologic responsiveness. However, much of this research was done prior to the development of our current understanding of cellular immunology and the recognition that subpopulations of lymphocytes exist and cooperate in immune responses. In the fourth article, Drs. Anderson and Warner review the critical earlier experiments dealing with the effects of radiation on immune responses and reexamine them in the light of our present knowledge of cellular immunology. They consider the effects of radiation on lymphoid cells *in situ* and *in vitro*, on antibody production, transplantation immunity and other forms of cellular immunity, and on tolerance, with the implied relationship to possible autoimmune responses. Finally, they outline ways in which radiation may be employed to study individual cellular components of the immune response by using the differential radiosensitivities of various lymphoid populations to help define their specific functions.

As always, the editors wish to thank the authors, who have given generously of their time and meticulous effort, and the publishers, who do much to ensure a volume of high quality.

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The Alternative Pathway of Complement Activation[1]

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I. Introduction

Two pathways for the recruitment of complement and its biological activities exist in plasma and serum. The first one, now termed the classi-

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cal pathway, is triggered by contact of C1 with immune complexes containing antibodies of the IgG or IgM class. Recognition of immune complexes by C1 is followed by the assembly of the activation unit (C2, C3, C4) and the membrane attack unit (C5, C6, C7, C8, C9). The second pathway, the alternative or properdin pathway, is activated by naturally occurring polysaccharides and lipopolysaccharides and by aggregates of IgA. It consists of at least five distinct proteins: the initiating factor, proactivator, C3, proactivator convertase, and properdin. The two pathways lead to the formation of their respective C5-activating enzymes (convertases), and the action of either C5 convertase on C5 results in the self-assembly of the C5b-C9 complex. A requirement for C3 is shared by both pathways: Native C3 is required for the formation of the initial C3 convertase in the alternative pathway, and its major fragment, C3b, is an essential subunit of the C5 convertases of the classic as well as of the alternative pathways. The alternative pathway of complement activation may then be defined as comprising those plasma proteins that, after a triggering event, interact to form specific C3 and C5 convertases without the participation of the classical components C1, C2, and C4.

Three reviews of the properdin system have been published, the first one 2 years after the description of the system (*109*). An extensive review and compilation of the earlier literature by Schmidt appeared in 1959 (*218*). A comprehensive account of the more recent work was given by Osler and Sandberg in 1973 (*180*). The present chapter reviews the current knowledge of the proteins of the properdin system, their biologically relevant interactions, and the modulation and regulation of these interactions. Emphasis will be placed on a novel comprehension of the entire properdin pathway which grew out of the most recent work of this laboratory. In essence this concept envisages a single enzyme to fulfill the various functions of the pathway by being modulated sequentially by different factors.

II. History

Two different experimental approaches have been used independently in virtually all explorations of the properdin system. The observations on which these two approaches are based were made early this century by several of the first investigators of the complement system. The first set of observations that established the anticomplementary activity of cobra venom (*37, 69, 178, 204, 264*) led to the isolation of the active principle from cobra venom (cobra venom factor or CVF) and to an analysis of its action on the third component of complement (C3). The other series

of experiments that demonstrated the anticomplementary activity of yeast cells and certain bacteria (*37, 53, 269*) were the basis for the studies of Pillemer and his collaborators on the mechanism of action of these substances on C3. This work resulted in the formulation in 1954 of a new concept of natural resistance to infections and the first description of the properdin system (*196*). Only recently has it become clear that both active principles, CVF and yeast cells (zymosan), utilize the same zymogen in serum (proactivator or Factor B) to activate and consume C3.

The findings of Whitehead *et al.* (*269*) on the action of yeast cell powder (zymin) on the heat-stable C3 were confirmed by Pillemer and Ecker (*190*) who prepared the active, insoluble, carbohydrate-rich material from yeast by tryptic digestion and extraction of the insoluble fraction with boiling water and ethyl alcohol. Studies on the action of this material, later termed *zymosan* by San Clemente and Ecker (*213*) to indicate its origin from yeast and its general carbohydrate character, led to the description by Pillemer of the new serum protein, *properdin.* The name is derived from the Latin and implies a capacity to destroy or eradicate. The properdin system was thought to be composed of properdin (P), complement, and magnesium ions (Mg^{2+}) (*189, 196*), because earlier investigations of the inactivation of C3 by zymosan had established a requirement for Mg^{2+} (*193*) as well as a hydrazine-sensitive and a heat-labile serum factor (*192*). The requirement for these serum factors was taken as an indication for the participation of C1, C2, and C4 (*189, 192*). The reaction between properdin and zymosan was shown to proceed at 15°–17°C in the presence of complement and Mg^{2+} and to result in the formation of a properdin–zymosan complex (PZ) that had the capacity to inactivate C3 when incubated with a source of this component at 37°C (*195*). Properdin could be recovered from the zymosan complex by elution with alkaline buffers of high ionic strength and could be further purified by precipitation at low pH and ionic strength (*195*). Properdin-depleted serum (RP) was found to be deficient in several biological activities, which could be restored upon addition of partially purified properdin. These activities included the killing of certain bacteria (*196*), the inactivation of susceptible viruses (*196*), the lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) (*196*), and the killing of the protozoan *Toxoplasma gondii* (*65, 94*). A large number of polysaccharides other than zymosan were reported to interact with the properdin system (*168, 194*) or to inhibit the formation of an active properdin–polysaccharide complex (*136*). Among these substances were cell wall constituents and endotoxins of *Escherichia coli,*

salmonellas, and pneumococci, certain glucans, dextrans, and levans as well as some preparations of gastric mucin. Strong inhibitory activity was found in several pyranosides but also in D-fructose and D-mannose.

The observations reported by Pillemer and his collaborators stimulated extensive investigations on the possible participation of properdin in various biological phenomena. Numerous publications dealt with the role of properdin in humoral defenses against bacterial and viral infections (*134, 179, 247, 259, 260, 262, 263*), lysis of PNH erythrocytes (*100, 101*), protection against the effects of ionizing radiation (*191, 205, 206*), sequelae of hemorrhagic and other types of shock (*68, 70, 139*), and resistance against cancer (*99, 208, 232*). The reports on the findings in these areas are too numerous and in part too uncritical to be all included in this review. Most of them are listed in Schmidt's monograph (*218*). We wish to emphasize, however, that the basic observations and conclusions of Pillemer and his collaborators published between 1954 and 1959, are born out by recent investigations. The flood of publications on the properdin system came to a virtual standstill in the years 1959–1960. This was due partly to the sudden death of Pillemer in August 1957 and partly to Nelson's proposal of an "alternative mechanism for the properdin system" in 1958 (*173*). Nelson's thesis was that the activities of the properdin system can be explained entirely by the presence in most sera of natural antibodies to zymosan that utilize C1, C2, and C4 for C3 fixation. This thesis was supported by the findings that (a) antigen–antibody complexes formed with limited amounts of antibody led to preferential fixation of C3 as did zymosan, (b) the rate of decay of PZ was similar to that of the EAC1,4,2 complex, (c) most sera, indeed, contained antibodies to zymosan, and (d) partially purified properdin, like antibody, agglutinated zymosan particles. Other investigators furnished additional evidence for the association of immunological specificity with the properdin system (*20, 45, 167, 179, 243, 261*). Today the controversy between Pillemer and Nelson is amply explained by the now established fact that, in human serum, both pathways are usually activated by zymosan (*231*).

More recently, a reevaluation of the concept of a second pathway of complement activation was undertaken. The presence of a second pathway of complement activation was firmly established by the work of (a) Gewurz *et al.* (*74*) who showed differential C3–C9 consumption by endotoxin and immune complexes, (b) Sandberg *et al.* (*214, 216*) who described the complement activating capacity of guinea pig γ_1 anti-DNP antibodies and their F(ab)'2 fragments, and (c) Frank and his collaborators (*55, 71*) who described a strain of genetically C4-deficient guinea pigs and the ability of C4-deficient serum to sustain complement activation. That complement-dependent reactions are not abrogated in C4-deficient guinea pig serum was consistent with results obtained by

Marcus *et al.* (*140*) indicating that the C3-cleaving activity found on lipopolysaccharide after interaction with guinea pig serum is not inhibited by an antiserum to C2.

It was at this time that the C3 proactivator had been isolated from human serum by us and characterized as the precursor of a C3-cleaving enzyme (*85, 86*). The discovery of the proactivator (PA) was a direct consequence of the study of the mechanism of action of CVF, which had previously been isolated and used to generate anaphylatoxin *in vitro* (*112*), to remove anaphylatoxinogen *in vivo* (*249*), and to inactivate C3 *in vitro* and *in vivo* (*38, 123, 174, 227, 265*). That the action of CVF on complement was not restricted to C3 but did result in the activation of the terminal complement components was subsequently reported by several laboratories (*14, 17, 84, 188*). It appears that the requirement of a heat-labile cofactor for the effect of cobra venom and bacteria on the third, heat-stable complement component was first pointed out by Nathan in 1917 (*171*). This report seems to have been forgotten. Fifty years later, it was found that highly purified CVF, a glycoprotein with a molecular weight of 140,000 daltons, does not affect isolated C3 but functions together with at least one normal serum protein, C3 proactivator, with which it forms an enzymatically active complex (*18, 157, 159, 166*).

Two modes of interaction of CVF with PA became apparent. The CVF and PA interact reversibly in the presence of Mg^{2+} to form a complex that has some C3- activating capacity (*41, 85*). The formation of a highly active stable complex is dependent on the proteolytic activation of PA by PA convertase (Factor D) (*41, 105, 251*). The recognition of PA as the precursor of a CVF-dependent C3 convertase prompted an exploration of its physiological role. It was found that inulin, a polyfructose that was used instead of zymosan (*148*) and that was known to generate anaphylatoxin in serum without significant consumption of early acting complement components (*182*), generated PA-cleaving activity in serum. The enzyme responsible for PA cleavage was termed C3PA convertase (PAse). This work led to the concept of the "C3 activator system," which included a heat-labile (PA) and a hydrazine-sensitive factor as well as PAse (*85*). Subsequent work resulted in the identification of C3 as the hydrazine-sensitive factor and in the description of the C3b-dependent feedback mechanism of the alternative pathway (*160*). Because of various similarities, a relationship of the C3 activator system to the properdin system appeared probable and was confirmed by showing the requirement of chromatographically isolated, highly purified properdin for full expression of the C3 activator system (*89*). The two previously recognized cofactors of properdin, the hydrazine-sensitive Factor A (*183*) and the heat-labile Factor B (*21*) could be equated with C3 (*78, 160*) and PA (*77, 85*), respectively. Finally, the very recent evi-

dence that CVF is, indeed, cobra C3b and is immunochemically related to human C3 (5) unifies the two described experimental approaches.

III. Nomenclature

The nomenclature of the properdin system has unfortunately been confusing because of the many synonyms used by different workers for the same activities or factors. Again, this is, in most part, due to the different approaches to the study of the problem, but it may also be attributed to the preference of some investigators to use historical symbols rather than terms reflecting the known function of the proteins involved. To date, a definitive nomenclature has not been adopted, although a provisional agreement has been reached to use, interchangeably, capital-letter symbols and descriptive, functional terms (Table I) (Resolution of the Complement Nomenclature Committee at the Second International Congress of Immunology, 1974, Brighton, U.K.).

IV. Proteins of the Alternative Pathway

A. Initiating Factor

Initiating factor (IF) is a heat-stable, 7 S pseudoglobulin that appears to consist of two identical polypeptide chains of 80,000–90,000 daltons each (222). It behaves very similarly to C3b inactivator on ion exchange and molecular sieve chromatography and electrophoresis. However, the two entities are physically distinct as evidenced by their different mobilities upon alkaline polyacrylamide gel electrophoresis. To date, the

TABLE I
Nomenclature of the Properdin System Proteins[a]

Component	Symbol	Scripps Clinic	Others
Initiating factor	IF	IF	—
Third component of complement	C3	C3	A
Proactivator	B	PA	GBG
Proactivator convertase	D	PAse	GBGase
Properdin	P	P	—
C3b inactivator	C3b INA	KAF	—
C3b inactivator accelerator	A-C3b INA	β_{1H}	—

[a] Activated components are generally denoted by the use of an overbar, e.g., $\bar{B}$ or $\bar{P}$ for activated Factor B or activated properdin.

final purification of IF has not been achieved. By physical and immunochemical properties, IF is distinct from immunoglobulins, from the C3b inactivator, and from other known proteins of normal serum.

The requirement for IF in the properdin system was recognized through work on an unusual serum protein that was found in the circulation of some patients with chronic hypocomplementemic glomerulonephritis (235), originally designated persistent hypocomplementemic glomerulonephritis (*266*) or progressive glomerulonephritis in the infant (*79*). This protein, termed C3 nephritic factor (NF), was shown to generate, in the presence of serum cofactors, a Mg^{2+}-dependent C3 convertase that, once formed, acted on C3 in the presence of ethylenediaminetetraacetic acid (EDTA) (*235, 245*). One of the cofactors, a heat-labile pseudoglobulin, was subsequently identified as PA (*212*), the others as native C3 and PAse (*224*). Initially it was suggested that NF might be related to IgG3 (*241, 242*). When NF was purified it was found to be a 7 S γ-globulin with a molecular weight of 150,000 daltons, being distinct from IgG (*246*). Nephritic factor is a glycoprotein with an isoelectric point of 8.75–8.95 (*47*). It is composed of two disulfide-linked polypeptide chains of 85,000 daltons each (*220*). An antiserum to NF was found to remove a factor from normal human serum that is essential for activation of the properdin system by zymosan, inulin (*152, 221, 222*), or rabbit erythrocytes (*197, 220*). This factor, which was termed IF, can be recovered in precursor form from the anti-NF immune absorbent column by elution with 0.2 *M* glycine–HCl, pH 2.2 (*221, 222*).

B. Proactivator

Proactivator was the first component of the properdin system to be recognized as an enzyme or, more specifically, the zymogen of a proteinase (*85, 86, 160, 166*). Proactivator is a β_1-glycoprotein with an isoelectric point of 6.6 (*59*). It consists of a single polypeptide chain of 93,000 daltons (*80, 81, 273*). The total carbohydrate content of PA was reported to be 10.6%, and the sedimentation coefficient $s_{20,w}$, 6.2 S (*24*). By sucrose density ultracentrifugation, its *s* rate was found to be 5.7–6 S (*41*). The protein exhibits genetic polymorphism with two common alleles, Bf F, (Gb^F) and Bf S (Gb^S) (*6, 7*), and it is genetically linked to the major histocompatibility locus in man (*3*) and monkey (*272*). The observed polymorphism was thought to be due to a tetrameric structure of PA (*4, 7*). The single polypeptide nature of the molecule necessitates revision of this concept.

Proteolytic activation of the molecule results in its cleavage into two fragments, Ba and Bb. The Bb fragment, previously called C3 activator (C3A) because it carries the active site of the enzyme (*85*), has a

molecular weight of 63,000 daltons, (*81*) and migrates as a γ-globulin (*24, 85*). The Ba fragment, which is inert, has a molecular weight of 30,000 daltons (*81*) and migrates as an α_2-globulin (*24, 85, 89*). The enzyme cleaves *N*-acetylglycyl-L-lysine methyl ester (AGLME) which is also a substrate for C2, the classical pathway counterpart of PA (*40, 42*). Diisopropylfluorphosphate (DFP) at a concentration of 10^{-3} to 10^{-2} *M* (*62, 81*), *p*-nitrophenyl *p'*-guanidine benzoate, an active site titrant for trypsin (*35*), and the trypsin inhibitors of bovine lung (Trasylol®), soybean, and lima bean as well as ovomucoid do not inhibit the action of the enzyme on C3 (O. Götze, unpublished). Recently, however, it was possible to demonstrate under modified conditions DFP inactivation of, and incorporation into, the zymogen and the activated form of Factor B and to establish that the enzyme constitutes a serine protease (*151a*).

A protein, apparently identical to Bb, was isolated from human plasma utilizng dextran sulfate and termed β_2-glycoprotein II by Haupt and Heide (*98*). These authors also observed electrophoretic heterogeneity of the isolated glycoprotein that could not be explained by differences in the neuraminic acid content of the two observed major bands. Genetic polymorphism as an explanation for these observations was, therefore, considered. Boenisch and Alper (*23*) purified the same protein, which they termed glycine-rich γ-glycoprotein (GGG) on the basis of its glycine content (8.35% of the peptide moiety). They observed an immunochemical relationship of GGG to a β-globulin that they subsequently purified and termed glycine-rich β-globulin (GBG) (*24*). Later, the identity of GBG with PA was established (*85*).

C. Components C3 and C3b

Component C3, a β_2-glycoprotein (*161, 163*), consists of two disulfide-linked polypeptide chains of 110,000 daltons (α-chain) and 70,000 daltons (β-chain) (*25, 177*). Proteolytic activation results in cleavage of the α-chain and the release of a basic 9000-dalton fragment, the anaphylatoxin C3a (*25, 26, 51, 102, 103*). The major fragment, C3b, is more negatively charged than its precursor and migrates as an α_2-globulin. Concurrent with the loss of C3a, the b fragment acquires a transiently available binding site for suitable acceptors (*162*). A second, stable binding site allows the bound or fluid phase molecule to interact with the immune adherence receptor (C3 receptor) on various mammalian cells (*54, 135, 172*). Component C3b is degraded by a serum enzyme, C3b inactivator (C3b INA), also called conglutinogen-activating factor (KAF), which cleaves C3b into C3c (140,000 daltons) and C3d (30,000 daltons) (*75, 209*). The C3b also contains a site with which it interacts with PA to form a reversible complex in the presence of Mg^{2+} (*149, 151, 175, 250,*

253). This complex, C3b,B is converted to a C3-converting enzyme ($\overline{C3b,B}$) upon proteolytic activation of PA by PA convertase (*149, 151, 160, 175, 250, 253*). Moreover, C3b also exhibits a binding site for activated properdin (*58, 224*).

D. Proactivator Convertase

Human PAse, the second recognized proteinase of the properdin system (*85, 160*), is a 2.5–3 S α-globulin with a molecular weight of 24,000 daltons consisting of a single polypeptide chain (*81*). Its isoelectric point as determined by isoelectric focusing was found to be 7.4 (*59*). The enzyme has also been purified from guinea pig serum (*29, 52*) and was found to have a molecular weight of 22,000 daltons, an *s* rate of 2.6 S, and an isoelectric point of 9.5 or 9.35, respectively. An antigenic relationship between guinea pig and human PAse has been demonstrated (*29b*). The reported isoelectric properties appear incompatible with the electrophortic behavior of PAse in fresh serum (*160*).

Human PAse was reported to exist both in an active and a zymogen form in fresh serum. The active enzyme can be generated by treatment of the precursor with trypsin (*62*). The reported effect of activated properdin on the zymogen (*63*) has been reinterpreted (*58*). Treatment with 5×10^{-3} *M* DFP completely inactivates the enzyme, whereas tosyl-L-lysine chloromethylketone (TLCK) at a concentration of 10^{-2} *M* has no effect (*62*). In another report 10^{-2} *M* DFP resulted in only partial inactivation of PAse. (*81*). The trypsin inhibitors from bovine lung (Trasylol®), soybean, lima bean, and ovomucoid have no effect on the active enzyme (O. Götze, unpublished). Further evidence for the serine esterase nature of PAse was obtained by showing that cyclohexylbutylphosphonofluoridate inhibits the enzyme noncompetitively at a concentration of 5×10^{-5} *M* and that *p*-tosyl-L-arginine methyl ester (TAME) inhibits it in a competitive fashion (*57*). Proactivator convertase does not act on casein, hemoglobin, fibrin, or elastin (*82*).

E. Properdin

Activated properdin ($\overline{P}$) is one of the most cationic proteins of human serum, having an isoelectric point of greater than 9.5 (*89*). It was first obtained in highly purified form by Pensky *et al.* (*184*) who determined its molecular weight (223,000 ± 7000 daltons) and sedimentation coefficient ($s_{20,w}$, 5.1–5.3). Minta and Lepow (*155*) analyzed the protein again and found its molecular weight to be 184,000 ± 12,000 daltons. The carbohydrate moiety (9.8%) of the protein is composed of hexose (including fucose), hexosamine, and sialic acid (*155*). Properdin is a tetramer that is dissociated into subunits of similar or identical molecular

weights by treatment with guanidine hydrochloride or sodium dodecyl sulfate (SDS). Its subunit molecular weight was found to be 45,000–46,000 daltons by Minta and Lepow (*155*) and 50,000–53,000 daltons by Götze (*81, 90*). After removal of the denaturing agent, the subunits tend to form dimers that possess about 50% of the functional activity and about 75% of the antigenic properties of the parent tetramers (*155*). The activity of $\overline{P}$ could not be inhibited by 5×10^{-3} *M* DFP or 10^{-2} *M* TLCK (*63*). Precursor properdin, which was recently isolated, has similar physicochemical properties (*90, 91*).

F. The C3b Inactivator

The C3b inactivator (C3b INA) (*129, 239*) is a 5.5–6.0 S β-pseudoglobulin that is resistant to heat (56°), hydrazine, iodoacetamide, DFP, and soybean trypsin inhibitor (*129*). It has an approximate molecular weight of 88,000 daltons and is composed of two nonidentical polypeptide chains (*223*). Moreover, C3b INA acts on bound (*129, 209*) and soluble C3b (*75, 209*) as an endopeptidase cleaving the molecule into the fragments C3c and C3d, thereby abrogating the hemolytic and immune adherence activities of bound C3b (*1, 129, 209, 239*). The C3d fragment of bound C3b remains affixed to the surface of the cell or particle, whereas C3c is released into the supernatant (*209*). With cleavage of C3b by C3b INA, the molecule acquires conglutinogen activity, that is, it is recognized by bovine conglutinin. It is for this reason that the enzyme is also called conglutinogen-activating factor or KAF (*129*). The C3b INA also impairs the function of C3b in the feedback mechanism of the properdin system as was reported in 1973 (*88, 176*). In addition, C3b INA has been claimed to act directly on PAse leading to inhibition of this enzyme (*9*). Information on the enzyme specificity of C3b INA is still not available. By itself, C3b INA is a sluggish enzyme. It is inhibited by ϵ-aminocaproic acid (EACA) (*254*) and its activity is destroyed by mercaptoethanol (*129*). Only lately has it been possible to isolate milligram amounts of the enzyme for structural studies (*223*). An additional effector, a 10 S protein (*170*) also termed C3b INA accelerator (*268*) is required for full expression of its activity. To exert its effect, the accelerator has to be present simultaneously with KAF. It is unlikely that this factor is identical to the tryptic enzyme postulated earlier (*75, 129*) to be required in trace amounts for optimal C3b INA activity because the accelerator has now been identified as β_{1H}-globulin (*177a, 268a*).

G. Additional Factors

A factor of rabbit serum (Factor X) has been purified and shown to interact directly with zymosan and dextran gels from which it could be

TABLE II
PROPERTIES OF PROTEINS OF THE HUMAN PROPERDIN SYSTEM

Proteins	Molecular weight	s rate (S)	Isoelectric point	Electrophoretic mobility	Approximate serum concentration (μg/ml)
IF	160–180,000	7	NA	β	20–50
C3	180,000	9	NA	β	1,104–1,373
PA	93,000	5–6	6.6	β	143–226
PAse	24,000	2.5–3.0	7.4	α[b]	1–5
P	212,000	5.4	>9.5	γ	25
C3b INA	100,000	5.5	NA	β	30–50
β_{1H}	150,000	5–6	NA	β	133

[a] NA, not available.
[b] In fresh serum.

eluted by 20% sucrose (*169, 230*). It has been proposed to be the C1 equivalent of the properdin system. Factor X differs from human IF in that it is exceedingly labile and of unusually high molecular weight. A human analog of rabbit Factor X has not yet been observed. A serum protein, termed properdin convertase, that is activated directly by contact with zymosan and interacts with properdin to form a C3 convertase was described by Stitzel and Spitzer (*234, 237*). Their findings have yet to be confirmed by other investigators. Table II summarizes the properties of the known components of the human properdin system.

V. Molecular Mechanisms of Action

A. Formation of the Initial C3 Convertase and Deposition of C3b

Initiation of the alternative pathway typically appears to require particulate activators. Experiments with serum immunochemically depleted of C4 and P have shown that an initial C3 convertase of low activity is formed upon the addition of particulate activators such as zymosan or inulin to serum. This C3 convertase requires for its generation the presence of precursor IF, C3, PA, PAse, and Mg^{2+}. In the absence of IF but in the presence of physiological amounts of properdin, C3, PA, PAse, and Mg^{+2}, the pathway is not triggered by zymosan or inulin. Addition of purified IF precursor to IF-depleted serum restores the pathway.

The inititial events seem to occur on the surface of the activating particle. It is presently assumed that, after the first contact of IF with essential, as yet undefined, structures of the activating particle, IF becomes active ($\overline{IF}$) and interacts with PA, PAse, and native C3 to generate the

first C3 convertase of the pathway. The enzyme deposits C3b onto the surface of the activating particle, thus generating the receptor for properdin. To reflect this essential role in the pathway the enzyme has also been termed properdin receptor-forming enzyme (PRFE) (*152*). The exact composition of this complex enzyme and the stoichiometry of its subunits are not known, although it is assumed that the enzyme constitutes an association product of at least three proteins—IF, PA, and C3. The question of the participation of an additional factor in the initiation of the pathway (*169, 230*) will only be solved when larger amounts of highly purified precursor IF become available. This question relates to the important function of recognition of activators by the properdin system. Whereas IF is clearly involved in the generation of the first enzyme of the pathway, its involvement in recognition has yet to be demonstrated.

Much has been learned about the probable mode of action of $\overline{IF}$ through the study of NF, which is thought to be closely related to IF. Nephritic factor forms a soluble C3 convertase by associating with PA and native C3 in presence of PAse and Mg^{2+} (*224*). Within this enzyme complex, PA and C3 function without undergoing enzymatic cleavage. The evidence for this is that PA can be recovered in intact form after it has functioned as a subunit of the enzyme and that C3b cannot substitute for native C3 in the formation of the enzyme. Utilization of PA as a subunit of a C3 convertase without cleavage of the molecule was first observed in a pathological serum (*48*) and subsequently was reported to occur under several other experimental conditions (*60, 175, 253*). The PAse requirement in the NF-dependent reactions indicates a second, nonenzymatic function of PAse (*224*). The requirement of native C3, instead of C3b, is biologically sound in as much as, prior to activation of complement, C3b is not available. Soluble C3 convertase acts only on C3 and not on C5. The NF-dependent enzyme has a half-life of 35 to 40 minutes at 37° (*47, 224*).

Daha *et al.* (*46, 47*) proposed that NF acts catalytically on the complex of C3 and PA conferring stability on it without incorporating itself. They were able to demonstrate the NF-stabilized C3 convertase upon sucrose density gradient ultracentrifugation and were unable to detect NF in the zone containing C3 convertase activity. On the other hand, the same authors showed that NF can stabilize the cell-bound, labile C3 convertase. As was found in this laboratory (*220, 220a*), NF stabilizes the soluble as well as the cell-bound C3 convertases by physically associating itself with these complexes. Similarly, $\overline{IF}$, formed by exposure of precursor IF to acidic pH, also stabilized the cell-bound, labile C3 convertase by physical association (222). On the basis of the observations with NF, it is

assumed that the $\overline{IF}$-dependent enzyme occurs primarily as soluble C3 convertase, although in loose association with the surface of activators and only secondarily in particle-bound form. The physiological significance of the latter form remains to be established.

Three other mechanisms of initiation have been proposed. Lachmann and Nicol (*130, 176*) envisage continuous, low-grade formation of C3b as a prerequisite of initiation. This mechanism would enable activators to capture nascent C3b and to bind and utilize it. Fearon and Austen (*60*) considered the initial C3 convertase to be composed of activated P, native C3, and uncleaved Factor B and to form and function without Factor D. Although it is conceivable, this proposal does not address itself to the required activation of properdin and is no longer compatible with experimental evidence. The same authors reported on the capacity of native C3 to form, together with Factor B and Factor D, a convertase that is capable of generating initial C3b (*59*). Such a mechanism might be of biological importance if it could be shown that sufficient amounts of activated Factor D are initially not available but are generated soon after the pathway is triggered.

B. Formation of Soluble, Labile C3 Convertase

Soluble C3b, although it fails to become deposited on the surface of activators of the alternative pathway, displays an Mg^{2+}-dependent affinity for PA (*149, 175, 250, 253*). In striking analogy to C4b and C2 (*164*), the two proteins form a reversible, enzymatically inactive bimolecular complex. In complex, C3b renders PA susceptible to PAse action. In this reaction, PAse can be replaced by other proteinases such as trypsin, plasmin, or pronase (*28*) and by the elastase and the chymotrypsin-like enzyme isolated from human granulocyte lysosomes (*83*). Following hydrolysis of a peptide bond, the 30,000-dalton Ba fragment is dissociated, whereas the 63,000-dalton Bb fragment is retained by the complex, a process that closely resembles the fate of C2 in the formation of the classical C3 convertase (*198, 238*). Complex $\overline{C3b,Bb}$ or simply $\overline{C3b,B}$ possesses C3-cleaving activity and represents the fluid-phase feedback mechanism of the properdin pathway, originally described in 1972 (*160*). The enzyme displays no measurable C5-cleaving activity (*152*).

C. Formation of Bound, Labile C3/C5 Convertase

The C3b deposited onto the surfaces of zymosan or cells (erythrocytes) serves as the site of attachment of PA that, after proteolytic activation by PAse, becomes more firmly bound to C3b to form the bound, labile C3 convertase, which is analogous to the fluid-phase feedback enzyme. The first stage of the generation of the solid-phase enzyme is the Mg^{2+}-depen-

dent binding of PA to C3b. The resulting protein–protein complex is fully dissociated by the addition of EDTA. The binding of PA to C3b exposes a bond in PA that is susceptible to the action of PAse (*250, 251, 253*). This action results in the cleavage of PA with release of the Ba fragment into the fluid phase. The enzyme undergoes decay/release with a half-life of approximately 2 minutes at 37°C in either Mg^{2+} or EDTA containing buffers (*149, 152*). Whereas the fluid-phase $\overline{C3b,B}$ enzyme acts on C3 but not on C5, the bound enzyme was shown to activate both C3 and C5 (*149, 152*). Moreover, the addition of native C3 is not a requirement for the activation of C5 by the assembled enzyme. This is seemingly in contrast to the structural requirements of the C5 convertase of the classic pathway that is generated from C3 convertase ($\overline{C4b,2a}$) by the incorporation of C3b ($\overline{C4b,2a,3b}$) (*43, 76, 158*).

The foregoing considerations and evidence presented below suggest that at least two closely spaced C3b molecules are necessary for the expression of C5 convertase activity by a $\overline{C3b,B}$ complex, which then would have the formula $\overline{C3b_n,B}$, where $n > 1$. If this turns out to be correct, the first C3b molecule fulfills a function analogous to that of C4b in the classic enzyme, whereas the second C3b molecule fulfills a function similar or identical to that of C3b in the $\overline{C4b,2a,3b}$ complex. This view is supported by the following experimental results. Sensitized erythrocytes bearing a low multiplicity of C3b molecules (EAC3b) allow formation of the C3 convertase, $\overline{C3b,B}$ upon addition of Factors B and D and Mg^{2+}, but when subsequently offered C5–C9, they do not lyse. If the same cells are first treated with C3 to increase the number of bound C3b, they will undergo lysis when afterwards offered C5–C9 (*151*). The simplest explanation of these data is that $\overline{C3b,B}$ converted itself to $\overline{C3b_n,B}$ by turning over substrate. Similarly, cells bearing C3b in random distribution may allow the generation of C3 but not of C5 convertase. Cells bearing the same number of C3b molecules in focal distribution allow the generation of C3 and C5 convertase activity without further addition of C3 (*151*). A difference in the composition of the C3 and the C5 convertase had previously been inferred from kinetic studies performed by Brade *et al.* (*29a*).

D. Activation of Properdin by Bound, Labile C5 Convertase

In the past, properdin was purified from serum and obtained in an active form ($\overline{P}$) (89 This form of the protein is characterized by two properties. It is able to form a soluble $\overline{P}$-dependent C3 convertase together with PA, PAse, native C3, and Mg^{2+} (89) and to combine directly with surface-bound C3b without a metal ion requirement (*58, 151, 152,*

224, 240a). In serum, however, properdin occurs in precursor form (P) that neither forms a fluid-phase convertase nor-binds directly to C3b.

For the purpose of a comparative analysis of P and $\overline{P}$, a modified method for the purification of the protein was used. This method employed, in succession, chromatography on QAE-Sephadex A50, Sepharose 6B, and an immune adsorbent column for the removal of contaminating proteins. The highly purified protein preparations obtained by this method displayed to a large extent the properties of P but were fully active when assayed in C4 and P-depleted serum in the presence of zymosan particles (*90, 91, 152*). Properdin labeled with ^{125}I by the chloramine T method (*145*) was bound by C3b-bearing erythrocytes (EC3b) only when PA, PAse, and Mg^{2+} were supplied as well. After formation of the $\overline{C3b,B}$ enzyme on the surface of the cells, P was equally well bound and activated in the presence of either Mg^{2+} or EDTA, indicating that conditions permitting continued formation of $\overline{C3b,B}$ are not required for the recruitment of P. Evidently, surface-bound C5 convertase (S-$\overline{C3b_n,B}$) is the properdin-activating principle of the alternative pathway. This is strongly suggested by the fact that an average of 50 bound C3b molecules are necessary for the binding–activation reaction of 1 properdin molecule. In this reaction only 1 or 2 bound Factor B molecules are required (*151, 152*). Furthermore, cells with a low multiplicity of bound C3b molecules are virtually unable to bind and activate precursor properdin. A plot of the formation of the properdin-stabilized C5 convertase vs the number of C3b molecules carried per cell yields a sigmoidal curve (*151*). Consistent with these observations and considerations is the report of Adam *et al.* (*2*) which shows that activation of the C3b-dependent feedback precedes activation of properdin. That pre-P has some affinity for bound C3b, although much less so than $\overline{P}$, has been demonstrated by binding of radio-labeled pre-P to EC3b at unphysiologically low ionic strength (*151*).

Although the S-$\overline{C3b_n,B}$ complex has the function of a proteinase, present evidence suggests that binding–activation of P is a nonenzymatic process. These conclusions were derived from experiments showing that neither activated nor irreversibly inactivated P accumulated in the fluid phase after interaction with S-$\overline{C3b_n,B}$, that loss of P from the fluid phase was totally accounted for by the amount of specifically bound properdin, and that maximally 1 molecule of radiolabeled P was bound per molecule of radiolabeled $\overline{B}$ (*90, 91, 152*). Finally, radiolabeled precursor properdin deposited onto zymosan particles after incubation with the required cofactors displayed an unchanged subunit molecular weight of 50,000 daltons, strongly indicating that a critical conformational change is responsible for the transition from P to $\overline{P}$ rather than limited proteolysis.

The concept of Minta (*153*) of a proteolytic activation of P to $\overline{P}$ is not supported by these results.

E. Soluble $\overline{P}$-Dependent C3 Convertase

Activated properdin when added to serum forms a fluid-phase C3 convertase that requires for its generation the presence of C3, PA, PAse, and Mg^{2+} (*89*). A more detailed analysis of the formation and function of this $\overline{P}$-dependent enzyme revealed that its generation is dependent on *native* C3 that could not be replaced by C3b and that PA is utilized in the enzyme complex without proteolytic cleavage despite a requirement for PAse (*224*). The soluble enzyme acts on C3 but not on C5 (*89*). The half-life of the $\overline{P}$-dependent C3 convertase was found to be 26 minutes at 37°C. Thus, the soluble $\overline{P}$- and the NF-dependent convertases are functional equivalents, although $\overline{P}$ interacts primarily with C3b (*224*) whereas NF displays an affinity predominantly for PA (*152*). Although these findings have shed light on the biologically relevant affinities of $\overline{P}$, C3b, and PA for each other, it is presently unclear whether soluble $\overline{P}$, which is thought to occur chiefly in particle-bound form, is ever generated under physiological conditions (*91, 151, 152*).

F. Properdin-Stabilized C5 Convertase

That properdin is part of an efficient form of the C5 convertase was first pointed out in 1974 when it was found that $\overline{P}$, although able to form an efficient fluid-phase C3 convertase, does not initiate C5 consumption unless assembly of the enzyme proceeds on the surface of zymosan particles. At that time it was proposed that $\overline{P}$ might stabilize the molecular interactions between several of the surface-bound factors involved (*89*). An affinity of properdin for C3 and its fragments, C3b and C3c, was subsequently demonstrated by Minta (*154*), Chapitis and Lepow (*34*), and Schreiber *et al.* (*224*). After binding–activation of properdin by S-$C\overline{3b}_n,\overline{B}$, the enzyme displayed a prolonged half-life of 8 minutes at 37°. It is envisaged that the properdin molecule is induced by the enzyme to arrange itself on the surface of the complex so that it is in physical association with both C3b and $\overline{B}$, thereby conferring stability on their association.

Retardation of the spontaneous decay of S-$\overline{3b,B}$ by properdin was first reported by Fearon and Austen (*58*) and was independently observed by Medicus *et al.* (*151, 152*). The stabilized enzyme acts on both C3 and C5, as does the labile enzyme, exhibiting a higher turnover for C3 than for C5 (*151, 152*). Its direct action on C5, in the absence of C3, was demonstrated by the formation of hemolytically stable C5b,6,7 sites

on erythrocytes and uptake of radiolabeled C5 upon incubation with highly purified C5, C6, and C7 (*151, 152*).

The stoichiometry of the erythrocyte-bound properdin-stabilized convertase was explored using radiolabeled components. In these experiments a probable ratio of 1 Factor B molecule to 1 P molecule to 2 or more C3b molecules was found (*151, 152*). The enzyme can undergo reverse assembly. After decay and dissociation of Factor B, the remaining surface-bound complex S-C3b,$\overline{P}$ can reacquire Factor B in the presence of Factor D and Mg^{2+} and regain full enzymatic activity. S-C3b may bind soluble $\overline{P}$ to form S-C3b,$\overline{P}$, which can then be converted to S-$\overline{C3b,P,B}$. The reincorporation of B into S-C3b,P appears to proceed analogously to the described formation of the labile S-$\overline{C3b,B}$ enzyme from S-C3b. Factor B is first bound in the presence of Mg^{2+} and then activated by Factor D through limited proteolysis with the concurrent release of the Ba fragment.

G. Regulation

Three modes of regulation of the properdin system are presently known. First, the C3/C5 convertases decay spontaneously due to loss of the subunit that carries the active site (Bb). Second, the P-stabilized C5 convertase is actively disassembled by soluble C3b with release of Bb. Native C3 has the same apparent effect, although it is not clear whether this is a property of C3 or of the C3b fragment produced by the enzyme. Soluble C5 has no effect. It is probable that the $\overline{IF}$-stabilized C3 convertase and the labile $\overline{C3b,B}$ enzyme are similarly regulated by soluble C3b. Third, a serum enzyme effects destruction of bound C3b and has been termed properdin receptor-destroying enzyme (PRDE) (*150, 152*). The enzyme acts on S-C3b as well as on S-C3b,$\overline{P}$.

In the first case, it destroys the receptor for properdin, and in the second case it releases $\overline{P}$, probably in association with a fragment of C3b. In contrast to these effects, the enzyme does not act on the stabilized C3 convertase but acts on the stabilized C5 convertase, indicating that C3b is protected in the C3 but not in the C5 convertase (*151*). Thus, PRDE controls the formation and function of $\overline{P}$–C5 convertase. It is probable that PRDE cleaves C3b into C3c and C3d and that it is identical with C3b INA. Its action on S-C3b,$\overline{P}$ which results in the release of properdin, might lead to the generation of soluble $\overline{P}$ (*152*). However, no information is as yet available indicating that fluid-phase $\overline{P}$ is formed as a result of the activation of the properdin system.

VI. Molecular Consequences of Activation of the Properdin System

The action of the alternative pathway convertases on C3 and C5 is indistinguishable from that of their classical counterparts. The anaphyla-

toxin C3a has the same NH_2- and COOH-terminal amino acid residues and the same biological activities regardless of whether it arose through the classical (*31*) or the alternative pathway (*102, 103*). It is anticipated that classical and alternative pathway C5a will be chemically and biologically identical. Chemotactic activity due to C5a and the C5b,6,7 complex can be generated by activators of the classical and the alternative pathways (*228, 256–258*) and can be produced in C4-deficient guinea pig and human C2-deficient sera (*36, 73, 217*) by CVF (*227*) and by activators of the properdin system (*36, 217*). In addition, chemotactic activity for human neutrophile polymorphonuclear leukocytes has been generated in mixtures of CVF and Factors B and D or C3b and Factors B and D (*210*). A major product of the properdin pathway is C3b, which is responsible for immune adherence and opsonization and is the nucleus of the feedback mechanism.

The assembly of the membrane attack mechanism, C5b–9, following proteolytic activation of C5 by the alternative pathway C5 convertase, can be detected in a variety of ways. Activation of the properdin system by inulin after addition to serum of the isolated, radiolabeled components of the membrane attack system has been shown to result in the incorporation of these components into the soluble C5b–9 complex (*126, 127*). The cytolytic potential of the properdin system was first demonstrated by the lysis of erythrocytes from patients with PNH (*84, 87, 100, 101*). In contrast to normal red cells, PNH erythrocytes and normal human erythrocytes treated with 2-aminoethylisothiouronium bromide or reduced glutathione (*87, 121, 229, 236*) are highly susceptible to "bystander cell" lysis that is, to the consequences of the activation of complement at a distance from the cells' surface. These conditions prevail in the acidified serum and the sucrose lysis tests for PNH and in the lytic reactions seen after complement activation by inulin or CVF (*84, 87, 96, 97, 120*).

The hemolytic inefficiency of fluid-phase activation of complement has in the past led to the erroneous impression that the alternative pathway has only a low cytolytic potential (*30, 55, 71, 142, 144*). More recent evidence indicates that efficient lysis does result when the properdin-system enzymes are assembled on the surfaces of susceptible cells rather than in the fluid phase. Fearon and Austen (*61*) showed that C3b, deposited on sheep red cells with the help of antibodies and cell-bound classic C3 convertase, can serve as the nucleus for the assembly of the properdin-system C5 convertase. Cells bearing C5 convertase will undergo lysis when incubated with C5–9. Lymphoblastoid cells derived from Burkitt lymphomas and kept in continuous culture undergo destruction when incubated with C3b and serum (*240*). Most likely, the reaction

proceeds via attachment of C3b to specific cell surface receptors ("complement receptors") followed by C5 convertase formation from components of the alternative pathway. Other recent examples of the utilization of the properdin system in cytolytic reactions are the lysis of human lymphocytes in the presence of certain anti-HLA antibodies (*66, 67*) and the killing of measles virus-infected cells after binding of antimeasles virus antibody to their surfaces (*119, 185*).

VII. Activators of the Properdin System

A. Immunoglobulins

That antibody is always required for initiation of the pathway is improbable. There are examples where specific antibody is clearly necessary as, for instance, in the destruction of measles virus-infected cells (*119, 185*). Furthermore, the heat-labile opsonins that depend on the pathway (*270, 271*) are also dependent on γ_2-immunoglobulin, which, however, may be obtained from a nonimmune animal. By contrast, zymosan, inulin, or endotoxins appear to function in the absence of specific immunoglobulin. This is supported by the observations of Verrouste *et al.* (*248*) who administered inulin suspensions to rabbits for periods up to several months without ever finding an antibody response to inulin. In addition, these substances function in human agammaglobulinemic sera (*110*). In whole human sera, almost all human myeloma proteins were found to initiate PA cleavage when aggregated by bisdiazotized benzidine (BDB) (*233*). However, in C2-deficient sera, which were used after the discovery of the C3b feedback to exclude classical pathway C3b production, only the BDB aggregated IgA1 and IgA2 myeloma proteins reproducibly activated PA and C3 (*165*). These findings are consistent with the observations of Boackle *et al.* (*22*) who found that "interfacially" aggregated secretory IgA (s-IgA) is an efficient activator of the alternative pathway. Kaplan *et al.* (*122*) and Knop *et al.* (*124*) also demonstrated the opsonizing capacity of s-IgA for human erythrocytes and *Escherichia coli*, whereas Colten and Bienenstock (*39*) did not detect activation of C3 and PA by human erythrocytes sensitized with blood group-specific s-IgA.

It has also been reported that the aggregated Fc piece of a human IgE myeloma protein caused C3–C9 consumption and that aggregates of the intact IgE or the $F(ab)'_2$ fragment were less active (*107, 108*). In a recent study using C4-deficient guinea pig and C2-deficient human sera and BDB aggregated human myeloma proteins, the conclusion was reached that all myeloma proteins tested (IgG1, 2, 3, and 4, IgA1, IgA2, IgM, and IgE) consume C3–C9 (*72*). However, the value of this study

was limited by the fact that only two of these proteins were obtained free of endotoxin. The activating capacity of guinea pig and rabbit IgG has been clearly demonstrated (*33, 181, 203, 215–217, 225*). Both guinea pig γ_1- and γ_2-immunoglobulins (*214, 215*) and rabbit IgG (*33, 203, 225*) activate the pathway via a site located on the $F(ab)'_2$ portion. It may be concluded that immunoglobulins, depending on their class and origin, can initiate the alternative pathway. Immunoglobulins, such as human IgA, are overtly endowed with this capacity, whereas immunoglobulins, such as human IgG, may acquire this function under exceptional circumstances.

B. Polysaccharides

The polysaccharides that are typical activators of the system are yeast cell walls, zymosan, inulin, and endotoxins from gram-negative bacteria. The structures on the endotoxin lipopolysaccharides (LPS) that are responsible for the activation of the pathway appear to be associated with the carbohydrate moiety of the LPS molecules rather than with the lipid A portion, which seems to activate the classical pathway (*131, 137, 156*).

C. Other Activating Substances

A variety of other substances have been reported to have alternative pathway-activating properties. Some of these are polyanionic in character, such as dextran sulfate (*32, 95, 138*), DNP–albumin (*128*), polyanethol sulfonate, and polyvinyl sulfate (*138*). Others are T-cell-independent antigens or B-cell mitogens, such as pneumococcal Type III polysaccharide, levan, polymerized flagellin, and pokeweed mitogen (*19*). Activation of PA was also reported to result from the addition to serum of autologous erythrocyte stroma (*44, 199*) but not of platelet stroma (*199*). The experimental design, however, did not allow the authors to distinguish between activation of the classical or the alternative pathways. Rabbit erythrocytes have been shown to activate the properdin system in human serum in an antibody-independent fashion (*197*), and this reaction was shown to require the IF in addition to PA and PAse (*222*).

No specific chemical structure common to the various activating substances has been identified. However, it is important to note that all of these substances are polymeric in nature or contain repeating subunits. The repeating structure must be a requirement for the engagement of the recognition mechanism of the pathway. This is exemplified by inulin, which is inactive in monomeric dissolved form ($\sim$5000 daltons) and is active in colloidal or particulate form. Such a structure might also be essential to provide closely spaced acceptors for nascent C3b to allow

binding–activation of properdin by the $\overline{C3b,B}$ complex. König *et al.* (*128*) observed a dependency of the activation of the pathway by DNP–human serum albumin (DNP–HSA) on the degree of DNP substitution. Complex DNP (15–19)-HSA had little activating activity, whereas DNP (32–36)–HSA was very active; DNP (56–60)–HSA was still more active but showed inhibition at higher concentrations. Similarly, the alternative pathway-activating capacity of dextran sulfate was dependent on a critical molecular size above which ($>5 \times 10^3$ daltons) only the degree of sulfation was found important for C3 consumption (*32*).

VIII. Clinical Manifestations Associated with Activation or Perturbation of the Properdin System

The properdin system has been implicated in the pathophysiology of a number of diseases. Among the first of the more recent observations were those of Spitzer *et al.* (*235*) on patients with hypocomplementemic membranoproliferative glomerulonephritis (MPGN). Sera from patients with MPGN were found to contain NF, which, together with normal serum components, mediated the proteolytic activation of C3. Nephritic factor was subsequently found to require PA for its action (*212*) and to generate a soluble C3 convertase in the presence of native C3, PA, PAse, and Mg^{2+} (*224*). This action of NF is similar to that of activated properdin. In addition, NF stabilizes the bound $\overline{C3b,B}$ enzyme (*47*) interacting primarily with activated Factor B (*220*). A report that NF is a degradation product of P to which it is immunochemically related (*46*) was not confirmed (*224*). Patients with NF-positive MPGN have very low levels of serum C3 presumably because of continuous consumption. The role of NF in the pathogenesis of MPGN is unclear. One hypothesis argues that patients who lack a functioning complement system become highly susceptible to infections, and that subsequent immune complex deposition in their glomeruli causes glomerulitis (*186*). However, a more direct role of NF in the pathogenesis of MPGN has also been considered, because renal biopsies of these patients may reveal deposits of properdin and C3 but often not of immunoglobulins (*147, 267*).

A pathogenetic involvement of the properdin system may be found in individuals with a genetic deficiency of one of the early acting, complement components. Some deficient individuals develop chronic glomerulonephritis or lupus nephritis, and despite total impairment of the classical activation mechanism, C3 is deposited in the glomerular basement membrane (GBM) in addition to properdin, IgA and, other immunoglobulins (*49*). In other types of glomerulonephritis, a similar role for the properdin system is suggested. In one case of progressive focal

glomerulonephritis, the patient's serum complement was activated in the cold. The reaction appeared to require IgA and PA which participated in the reaction without being proteolytically activated (*48, 50*). Several renal biopsies were obtained and showed deposition of properdin, C3, and IgA without detectable deposition of IgG, IgM, C1q, or C4. Recent evidence by Lambert *et al.* (*132, 133*) suggests that the properdin system might participate in local complement activation by altered GBM. It was found that a pronase digest of GBM contained glycopeptides that were able to activate complement. Intact GBM was devoid of such activity. The sequence of one of the active glycopeptides was found to be Glc-Gal-Hyl-Gly-Gln-Asp-Gly. Evidence for a participation of the properdin system has also been found in Henoch-Schönlein syndrome and in idiopathic focal nephritis (*56*) as well as in systemic lupus erythematous (SLE) (*106, 146, 200, 207*). The lowered heat-labile opsonic activity found in some lupus patients has been associated with a deficiency in the properdin system (*111*).

Evidence for the activation of the alternative pathway in several cutaneous diseases has been reported. In herpes gestationis (*200*), bullous pemphigoid (*116, 201*), pemphigus vulgaris (*115, 117, 118*), and dermatitis herpetiformis (*202, 226*), and SLE (*207, 219*), components of the alternative pathway and immunoglobulins were found deposited at the dermal–epidermal junction of the skin, as demonstrated by immunofluorescence (*114, 200, 219*). Other diseases and clinical situations in which the alternative pathway has been implicated include PNH (*87, 101*), rheumatoid arthritis (*92, 211*), dengue hemorrhagic shock (*27*), sickle cell disease (*113, 125*), and gram-negative bacteremia (*64*).

Two genetic deficiency states are known in which the properdin system is impaired, one involves C3 and the other C3b INA. So far only one individual with C3b INA deficiency has been described. The absence of C3 INA results in the unchecked activation and consumption of C3 via the C3b-dependent feedback mechanism. As a consequence, PA is absent from the patient's serum and most of the circulating C3 antigen is present as C3b (*1, 10, 11*). The patient suffers from repeated infections particularly by gram-negative organisms. Pathological *in vivo* and *in vitro* tests are restored to near normal upon infusion of fresh plasma or purified C3 INA (*10, 274*). The findings obtained by the study of this unique patient have established the important biological role of C3b INA as an inhibitor of the feedback mechanism of the alternative pathway. They also suggest that C3b is continuously generated *in vivo* by an as yet unknown mechanism.

A homozygous deficiency of C3 was first observed by Alper *et al.* in 1972 (*12, 13*). In the meantime two other cases of the complete absence

of C3 have been reported (*12, 15*). These patients suffer from recurring pyogenic infections similar in type to those observed in C3b INA deficiency. As expected, the C3-deficient sera did not activate PA upon the addition of polysaccharides.

Several recent observations have provided evidence for a role of the alternative pathway in platelet aggregation and release reactions *in vitro* (*141, 187, 275*) and for a participation of complement and the alternative pathway in the activation of mouse peritoneal macrophages *in vivo* and *in vitro* (*16*). Human platelets aggregate and release their granule contents when incubated in the presence of zymosan and citrated plasma or with zymosan particles washed after treatment with plasma (*187, 275*). Generation of active zymosan particles required Mg^{2+} as well as heat-labile and hydrazine-sensitive factors in addition to IgG (*187*) and fibrinogen or fibrinogen fragments (*187, 275, 276*). A role for C3, PA, and possibly properdin in these reactions was established in experiments using plasma genetically deficient or experimentally depleted in individual components (*276*). Similar results were obtained by Graff *et al.* (*93*) who showed that C3, C5, C6, and C7 are required for zymosan- or restocetin-induced aggregation and release reactions of human platelets. The two terminal components were not needed for these noncytolytic events. Solid evidence for a role of the alternative pathway in disease in the absence of classic pathway activation is difficult to obtain except in cases with genetic deficiencies of one of the early acting components. This difficulty may be explained by the fact that C3b generated through the classical pathway or through other tryptic proteinases can activate and bind PA and that the resulting $\overline{C3b,B}$ complex if deposited onto a surface can activate and bind properdin (*151, 152*).

IX. Reinterpretation of Some Earlier Observations

The conclusion (*233*) that all human immunoglobulin classes and subclasses activate the alternative pathway did not take into account the ability of C3b to initiate the feedback regardless of its mode of production. When C2-deficient serum was used, clear-cut activation of proactivator was observed only with aggregates of IgA1 and IgA2. Variable and low activity was obtained also with IgG4 (*165*). Therefore, primary activation of the pathway appears restricted to a limited number of human immunoglobulin classes with the exception of those IgG virus-specific antibodies that are in association with the virus-infected cell (*119, 185*). In the latter situation, it is the $F(ab)'_2$ portion of the IgG molecule that is critically involved in the reaction (*185*).

A number of factors have been described as novel constituents of the

properdin system, but they can now be identified with known entities: Factor C (*104*) was shown to be C3b inactivator; Factor E (*105*) was identified as C5 (*252*); and Factor F (*154*), in all probability, represents C3 or one of its products. That proactivator is the CVF-binding protein is now generally agreed upon. Until recently, an alternative view that invoked as CVF-binding protein a trace serum constituent entirely distinct from proactivator (*8*), was presented. With the description of CVF as snake C3b (*5*), this notion could no longer be maintained.

The reported activation of Factor D by $\overline{P}$ (*63*) has been reinterpreted (*58*). The physiological mechanism of activation of Factor D thus remains unknown. Limited proteolysis was proposed as the physiological mechanism of properdin activation (*153*). These results could not be confirmed in our laboratory; rather, evidence was obtained strongly indicating that activation is effected by a nonenzymatic physical process (*90, 91*). Totally unexplained remains the nature of properdin convertase (*234, 237*), an enzyme that is directly activated by contact with zymosan and that is proposed to catalyze the P to $\overline{P}$ conversion. The reported physical parameters of properdin convertase and its occurrence in soluble form, preclude its identity with the established properdin-activating principle (*152*). Also unexplicable remains the report by the same authors that a C3 convertase is formed upon interaction of properdin convertase and properdin in absence of PA.

That the alternative pathway is initiated by a $\overline{P}$-dependent C3 convertase (*60*) has been precluded by the observation that the pathway is initiated in the absence of properdin (*152, 222*). Until recently, properdin was generally believed to act early in the pathway (*89*), primarily because $\overline{P}$ can utilize native C3 in the PA- and PAse-dependent generation of a fluid-phase C3 convertase. The greatly diminished consumption of C3 and PA in properdin-depleted serum was initially interpreted as an indication for an absolute requirement of properdin for the early stage of the reaction (*89*). It is now clear that properdin is recruited in the final reaction of the pathway.

A C3 requirement for the hemolytic action of the cell-bound and stabilized convertase (*61*) could not be confirmed. Although this enzyme can act on C3, it also acts on C5 in the total absence of native C3 (*151, 152*). The proposed inhibition of PAse by C3b inactivator (*9*) could not be confirmed (*223*), and the observed effect in the published experiments can be fully explained by the known action of C3b inactivator on C3b.

The "C1-bypass activation pathway" of May and Frank (*143*) invokes C1 in the triggering of the properdin pathway by sheep erythrocytes heavily sensitized with rabbit antibody. Although these observations en-

vision participation of C1 under specific experimental conditions, they do not imply an essential role of C1 in the properdin pathway. By contrast, Volanakis *et al.* (*255*) presented data indicating an essential role of C1s in the activation of the properdin system. Addition of large amounts of isolated $C\overline{1s}$ to C2- or C4-deficient serum led to C3 consumption; and addition of zymosan or CVF to C1s-depleted serum failed to cause C3 consumption. The authors propose that C1s is involved in the activation of Factor D. However, the possibility of a concomitant depletion of $C\overline{1s}$ and Factor D by the antiserum was not excluded. In this laboratory, addition of highly purified $C\overline{1s}$ (*244*) to serum or depletion of serum of $C\overline{1s}$ did not affect the alternative pathway.

X. Synopsis

Due to the major advances in our understanding of the properdin system that occurred during the past year, it has now become possible to formulate a molecular concept of the entire properdin pathway.

The flow of events as presently envisaged is schematically depicted in Fig. 1. Throughout the pathway, the essential enzymatic site resides in Factor B, which initially is in complex with native C3 and IF and, later, with bound C3b and properdin. The initial events occur on the surface of the activating particle. It is envisioned that the precursor IF establishes contact with the activating particle (S_I) and then interacts with

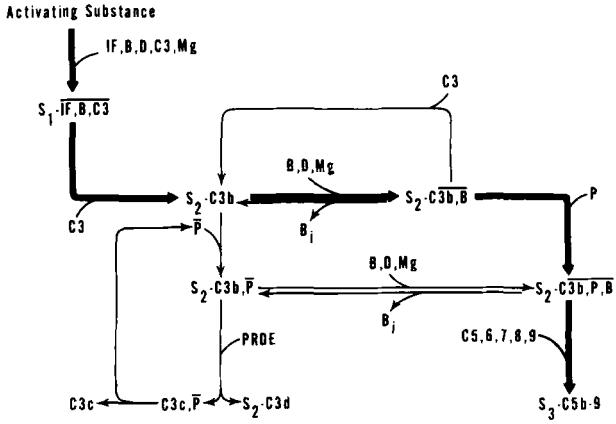

FIG. 1. Schematic representation of the molecular dynamics of the properdin pathway as proposed in the text. The S_I represents the site of initiation at which the properdin receptor-forming enzyme is generated; S_{II} denotes the site of attachment of C3b, which serves as properdin receptor, and also it is the site of formation of the feedback enzyme, of the properdin-activating principle, and of the C5 convertases. S_{III} refers to the site of binding of the C5b-9 membrane attack complex (151).

B, D, and native C3 to generate the initial C3 convertase. This enzyme is limited in its action by spontaneous decay and possibly by disassembly through soluble C3b. It deposits C3b at sites (S_{II}) in the vicinity of the enzyme. Properdin receptor function is thought to reside in at least two critically oriented and closely spaced C3b molecules. Binding of activated Factor B to the receptor results in generation of the labile C5 convertase, $\overline{C3b_n,B}$, that also acts on C3. This enzyme constitutes the properdin-activating principle. Upon collision of native properdin with the complex, properdin undergoes a transition to its bound form $\overline{P}$. The $\overline{P}$ confers an increased degree of stability on $\overline{C3b_n,B}$ converting it to the $\overline{C3b_n,P,B}$ enzyme. The labile and the $\overline{P}$-stabilized C5 convertases effect activation of C5 and self-assembly of the membrane attack complex C5b-9. Native C3 does not participate in this enzymatic reaction. When present, C3 is turned over, and the enzyme is disassembled at a rate greater than that characteristic for its spontaneous decay. Reverse assembly of the enzyme may commence with uptake of $\overline{P}$ by S_{II}-C3b and is completed after incorporation of $\overline{B}$ into the complex. Action of C3bINA on S_{II}-C3b abrogates its receptor function, and action of the enzyme on S_{II}-C3b,$\overline{P}$ releases properdin possibly in association with C3c.

Properdin itself does not play the central role in the pathway it had once been assigned. It is recruited late in the sequence rather than early as was previously assumed. Its sole function appears to be the stabilization and protection against C3b inactivator of the enzymes of the properdin system. Although *in vitro* the function of properdin would appear to be a nonessential one, its absence *in vivo* may well be detrimental to natural resistance to infections.

It is quite probable that the pathway constitutes a mechanism that provides protection during the early phase of bacterial invasion before effective antibody levels have been established. Clinical observation of patients deficient in one of the early acting classical components attests to this notion. That the alternative pathway can also participate in the inflammatory process has been pointed out by the diseases associated with some of the classical component deficiencies. A full appreciation of the biological functions of the properdin system will have to await the finding of various primary deficiencies of the system.

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Note Added in Proof

Although disassembly of the P-stabilized convertase has been reproducibly observed with several preparations of C3 and C3b, the effect was recently found to be due to the action of the plasma protein β1H [Nilsson, U. R. and Müller-Eberhard, H. (1965), *J. Exp. Med.* **122**, 277; Medicus, R. G., Pangburn, M., Götze, O., and Müller-Eberhard, H. (1976), Unpublished; Weiler, J. M., Daha, M. R., Austen, K. F., and Fearon, D. T. (1976), *Proc. Natl. Acad. Sci.* **73**, 3268], small amounts of which were detected in the C3 preparations used in the experiments.

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Membrane and Cytoplasmic Changes in B Lymphocytes Induced by Ligand–Surface Immunoglobulin Interaction

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I. Introduction

Periodically in science, a set of experiments in a particular discipline yields information sufficiently provocative that, even as researchers in that field endeavor to sort things out and define an ultimate significance, ideas and suggestions inescapably diffuse out to neighboring disciplines and interests. Eventually, those ideas return, accompanied by new ones, not only having made unexpected additions to the general body of knowledge but continuing to suggest new approaches to workers in the originating discipline. Such, we believe, has been the case with the studies on the binding of ligands to the lymphocyte membrane, particularly to the antigen receptor, some aspects of which form the topic of this review. With the recognition of antigen receptors as immunoglobulin (Ig) and the development of methods to probe this receptor cytologically and biochemically came the discoveries that the binding of ligand to these receptors initiates a sequence of early and late events that interweaves membrane, cytoplasm, and nucleus as components of the B lymphocytes' complicated response to an antigenic stimulus.

As plastic cells with remarkable functional potential, B lymphocytes, after exposure to antigen and modulatory signals from other cells, evolve through a programmed series of events integrated in yet unclear ways to achieve their ultimate goals of transformation, clonal expansion, and differentiation. Among these events are included some early ones discernible by microscopic examination. After the ligand has complexed with surface Ig receptors, the lymphocyte rapidly redistributes the complexes into polar caps and, as its membrane is being cleared, starts moving, concurrently synthesizing new receptors to insert into its surface. The reorganization of membrane and cytoplasm upon binding of ligand to surface antigen receptors is the initial sequence of that program and, as such, must be regarded as essential to any comprehension of lymphocyte activation overall.

The redistribution of surface Ig remains among the best studied activities of the system, with profound, general implications as a biological phenomenon for other areas of biology. Studies on the topographical modulations of Ig stand as a model to which the stimulated redistribution of components of other cell membranes must be compared. Research on Ig capping has had a major effect on current concepts of the structure and function of the cell membrane. In addition, Ig capping, operationally defined as an induced, large-scale segregation of certain membrane elements previously intermingled with other membrane elements, reflects not only the physical properties of the lymphocyte membrane but also the

apparent capacity of the cytoplasm, itself in the process of being altered, to establish order of some kind on a background popularly thought of as representing randomness. Thus, we have considered in some detail not only the distribution of proteins within the lymphocyte membrane (Sections II and IV) but also the question of transmembrane controls (Section V) in which both membrane and cytoplasm possess the reciprocal capacity to influence each other such that virtually any cell response to external stimulus must be represented as the outcome of these bidirectional controls. Studies on the lymphocyte, as well as on the fibroblast and red cell, have contributed greatly toward our understanding of this phenomenon.

We also have reviewed the subject of lymphocyte motility (Section III) in general as well as that which is specifically stimulated by antibody bound to surface Ig. Cell movement among lymphocytes has been recognized almost as long as neutrophil and monocyte motility and is potentially as important, yet understanding of its role in the initiation and development of immune responses and reactions has lagged far behind the progress made on the movement of nonlymphoid cells and its place in inflammation.

It should not be forgotten that comparable phenomena—some of them known in part previous to the discovery of lymphocyte capping and others examined subsequently in light of that discovery—take place in eukaryotic cells from all biological walks of life. The characteristics of the binding of various ligands, the patterns of surface redistribution, the extent, rate, and efficiency of endocytosis, the capacity to digest, and functional alterations all constitute variations on a theme of ligand binding and cell stimulation. These variations reflect cell structures and purposes, in accordance with particular ligands and particular differentiated states. Like cell motility, redistribution of membrane components possesses basic mechanisms but effects many ends. The professional phagocytes—monocytes and macrophages—are oriented to interact avidly with a number of ligands and to interiorize and digest them rapidly. In other cells these events may be integrated and subordinated to serve other more specialized functions. Protozoa employ these responses as a convenient way to alter the protein composition of their membranes (Nanney, 1968).

As an immune cell, the lymphocyte's response to the binding of its antigenic receptor appears to be the first step in its evolution toward transformation and differentiation. Analysis of this event has provided and will continue to provide information not only on the organization of the membrane, but also on the general biological and specifically immunological functions subserved by this mechanism.

II. Redistribution of Surface Ig by Antibody

Before describing the fate of anti-Ig–surface Ig complexes in B cells, it may be pertinent to review some characteristics of surface Ig, in particular, as they apply to the theme of this review.

A. Characteristics of Surface Ig

1. General Features

Surface Ig is the receptor for antigen molecules on B lymphocytes. Warner wrote an excellent and extensive review of the subject of Ig as an antigen receptor for Volume 19 of this series (Warner, 1974), to which the reader is referred. We consider in this section new information on surface Ig that has emerged since the time of his paper.

Surface Ig has been identified on the B-cell surface by using immunocytochemistry or by direct biochemical analysis. The first demonstration that any surface component could be visualized by immunocytochemistry was by Möller (1961) using murine living cells and a two-step antibody reaction, first with a murine alloantibody, then with a fluorescent anti-mouse globulin. Moller described the basic conditions and the different patterns of fluorescent staining. Dead or injured cells displayed diffuse cytoplasmic fluorescence, but healthy ones showed a pattern of discrete fluorescent spots on the cell surface. These cytochemical reactions were further explored by Cerottini and Brunner (1967) also for the demonstration of alloantigens. However, it was not until the report of Raff *et al.* in 1970 that Ig was identified cytochemically and recognized as being a component of the cell surface. Previous to this immunochemical analysis, the studies of Sell and Gell (1965a; Gell and Sell, 1965) had indicated that rabbit lymphocytes would transform into blasts upon exposure to anti-Ig antibodies, strongly suggesting that Ig was a component of the membrane (Section VIII).

Immunocytochemically, one employs anti-Ig antibodies tagged by various markers according to the method to be used: fluorescein or rhodamine isothiocyanate to be used for fluorescence microscopy; ^{125}I for autoradiography at the light or electron microscopy level; or ferritin, hemocyanin, enzymes, or viruses for ultrastructural analysis. Ig has also been identified by using mixed antiglobulin reactions with red cells as indicators (Coombs *et al.*, 1970). Various aspects of these techniques for demonstration of surface Ig were reviewed by Warner (1974) and Abbas *et al.* (1976b). Surface Ig has also been identified biochemically after extraction of the protein from the membrane with nonionic detergent. For this purpose, Ig (as well as other surface proteins) is labeled on the

intact cell either directly with ^{125}I by using the lactoperoxidase method introduced by Phillips and Morrison (1970) (e.g., Baur *et al.*, 1971; Vitetta *et al.*, 1971; Marchalonis *et al.*, 1972) or biosynthetically by feeding the cell labeled amino acids or sugars and then extracting the protein from the surface (Melchers and Andersson, 1973, 1974; Andersson *et al.*, 1974a). The labeled proteins are identified by the use of appropriate antibodies.

While it is easy to visualize or identify surface Ig by using the methods outlined above, this is not the case for specific antigen binding. For one thing, the number of cells binding a given antigen molecule is extremely sparse, usually no more than 50 per 100,000 cells, making it extremely laborious to carry out meaningful experiments. Workers in several laboratories have attempted to enrich for antigen-binding cells with some success. Undoubtedly, enrichment of the population of cells binding to a given antigen is a method to be followed in the future with better results as methodologies are improved. Studies of antigen binding to B cells have usually involved protein antigen labeled with ^{125}I (Naor and Sulitzeanu, 1967, 1968; Byrt and Ada, 1969; Humphrey and Keller, 1970; Warner, 1970; Davie and Paul, 1971, 1972a; Davie *et al.*, 1971a,b; Sulitzeanu, 1971; Unanue, 1971). Some investigators have also used fluoresceinated protein antigens (Sercarz and Modabber, 1968; DeLuca *et al.*, 1975; Nossal and Layton, 1976) or particulate antigens (Biozzi *et al.*, 1966; Wilson and Miller, 1971; Elliott and Haskill, 1973). By using autoradiography under the proper technical conditions, it is possible to identify protein antigens bound to the B cell membrane.

As a result of intensive work on antigen binding to B cells, the following statements can be made. The lymphocytes that bind antigen are of small or medium size (Byrt and Ada, 1969; Humphrey and Keller, 1970; Davie and Paul, 1971; Unanue, 1971). The binding of antigen is markedly decreased or abolished altogether by exposing B cells to anti-Ig antibodies, removing or covering the surface Ig, strongly indicating that antigen binding is indeed associated with surface Ig (Warner, 1970; Davie and Paul, 1971; Davie *et al.*, 1971a,b; Unanue, 1971; Ault and Unanue, 1974). Lymphocytes binding antigen are found prior to antigen stimulation and increase in number during the evolution of an immune response (Byrt and Ada, 1969; Warner, 1970; Davie *et al.*, 1971a). Not only does the number of antigen-binding cells increase during immunization, but also cells with surface Ig have a greater avidity for the antigen (Davie and Paul, 1972a,b). Finally, the lymphocytes that bind antigen are clearly essential to the expression of immunity inasmuch as their elimination results in an impairment of antibody production (Ada and Byrt, 1969; Wigzell and Anderson, 1969, 1971; Humphrey and Keller,

1970; Julius *et al.*, 1972). In summary, the experiments on antigen binding clearly support the clonal selection hypothesis of Burnet (1959), who postulated that the cellular basis of the response was the interaction of antigens with clones of lymphocytes bearing receptors of unique specificity. The response can be viewed as a selection by antigen of those cells that bear receptors of higher affinity (Eisen and Siskind, 1964; Siskind and Benacerraf, 1969).

Most of the surface Ig molecules on B cells are synthesized by the lymphocyte and somehow transported to the plasma membrane, where they are inserted. It is estimated that 80–90% of the Ig made by the unstimulated B cell is taken to the membrane to serve as an antigen receptor (Melchers and Andersson, 1974). Surface Ig can be removed almost completely by enzyme treatment of the cell or after redistribution by anti-Ig; upon culture, within a few hours, new molecules are reexpressed on the surface (Section VIII). In experiments with purified populations of small and medium-sized lymphocytes and internally labeled Ig molecules, the estimates of the half-life of Ig on the cell surface have ranged from 20 to 80 hours (Melchers and Andersson, 1973, 1974). The surface Ig in stimulated B lymphocytes already in an actively secretory stage, has a shorter half-life of about 4 hours (Andersson *et al.*, 1974a; Melchers and Andersson, 1974).

Although there are still arguments and unresolved questions concerning the class of surface Ig on the B-cell surface, the most convincing evidence, in our opinion, points to the following conclusions: (1) IgM and IgD, at least in man, are the major classes of Ig on the B cell membrane; (2) in many species the number of cells bearing IgG is variable, although usually the number tends to be low; (3) there are restrictions concerning the Ig found on the cell at a given time; IgM and IgD can coexist on the same cell; usually IgG or IgA are mutually exclusive (reviewed in Warner, 1974); all the surface Ig molecules have the same combining sites. A brief explanation of these points follows. There is a general agreement that IgM, in its monomeric form, represents one of the main Igs found on the B cell membrane (Baur *et al.*, 1971; Vitetta *et al.*, 1971; Marchalonis *et al.*, 1972; Marchalonis and Cone, 1973; Melchers and Andersson, 1974; Vitetta and Uhr, 1974). Recent studies, however, have indicated that IgD is also found on the cell surface; about three-quarters of peripheral blood B cells have both IgD and IgM on their membranes. Van Boxel *et al.* (1972), using immunofluorescence, were the first to report a high percentage of IgD-bearing cells among human peripheral blood lymphocytes. This observation was readily confirmed (Rowe *et al.*, 1973a,b; Kubo *et al.*, 1974; Fu *et al.*, 1974). The presence of IgD on the lymphocyte surface, serving as an antigen receptor, was

unexpected. IgD, discovered by Rowe and Fahey (1965) in a patient with multiple myeloma, is found in only trace amounts in normal serum (less than 0.04 mg/ml) (reviewed by Spiegelberg, 1972). The function of IgD was unknown until it was found on the B-cell surface, perhaps serving as an antigen receptor. IgD is made by the B cell and is not acquired from serum (Rowe *et al.*, 1973b). Although IgD molecules have not been identified serologically in other species, there is strong evidence that this molecule may be found on the surfaces of B cells from species other than man. This evidence has been obtained from biochemical analysis of radioiodinated surface proteins (Abney and Parkhouse, 1974; Melchers *et al.*, 1974). A substantial amount of Ig on the murine B cell possesses characteristics, including size, resembling those of an IgD type molecule. Definitive proof of this awaits the specific demonstration of IgD in the mouse.

It is well known that plasma cells secrete homogeneous Ig molecules of a single class, allotype, and specificity (Pernis *et al.*, 1965; Cebra *et al.*, 1966). The expression of Ig on the B cell membrane appears to be similarly restricted. The B cells can display molecules with μ or Δ chains, presumably having the same combining site. As the B cell is stimulated and differentiates, it may switch its production of heavy chains to γ or α (reviewed in Warner, 1974). The question of allelic exclusion was examined in the rabbit inasmuch as rabbit Ig have different groups of allelic markers in different sections of the molecule (reviewed by Mage *et al.*, 1973). The allotype markers are inherited as autosomal dominant traits. Early studies using antiallotype antibodies and assaying for blast transformation indicated the presence of two allelic forms within the same B cell (Gell and Sell, 1965). Similar results were obtained by using a mixed antiglobulin reaction (Wolf *et al.*, 1971). In contrast, immunocytochemical studies of Davie *et al.* (1971a) indicated that B cells from heterozygous rabbits can express one or the other allelic form but not both. The studies of Jones *et al.* (1973) are critical in this regard. They found that some of the Ig in rabbit B cells is not made by the cell but acquired from the serum. Studying the b allotype marker, which is localized to kappa light chains, Jones *et al.* (1973) found that heterozygote rabbits (b^5b^9) have a high percentage of B cells with both molecules. However, if surface Ig is stripped off by Pronase treatment, then the cell, upon culture, reexpresses only one of the allotype markers. Other experiments in the same study indicated that rabbit B cells can acquire Ig from the serum. Thus, although extracellular Ig can attach to B cells to a certain degree, the B cell itself appears to manifest allelic exclusion with respect to its internally synthesized Ig. Other studies in man also support allelic exclusion (Frøland and Natvig, 1973).

Finally, several lines of evidence agree that the surface Ig molecules all bind to a single antigen molecule. These include the results on antigen binding outlined before, as well as studies of redistribution of surface Ig, which are discussed later (Raff *et al.*, 1973; Nossal and Layton, 1976) (Section VII,A). Studies in man have indicated that lymphocytes from patients with chronic lymphatic leukemia bear only molecules having the same light chain and idiotype (Wernet *et al.*, 1972; Schroer *et al.*, 1974).

2. *The Fc Receptor Problem*

The question whether certain of the Ig molecules on the B cell membrane are passively acquired from the serum instead of being actively synthesized by the cell arose from several avenues of research, including studies on allelic exclusion in rabbit B cells, referred to above, and observed discrepancies in the number of cells bearing IgG molecules, particularly in man. Although many variations in number of B cells found in man can be attributed to using faulty or uncontrolled reagents, a great many others can be explained now by the fact that, under certain circumstances, some extracellular Ig can bind to a variety of cell types, including B lymphocytes via an Fc receptor. It is well known that mononuclear phagocytes—monocytes and macrophages—have a receptor on their membrane that specifically binds to the Fc fragment of IgG molecules (Boyden, 1964; Berken and Benacerraf, 1966; Uhr and Phillips, 1966; Huber *et al.*, 1969; Abramson *et al.*, 1970; etc.). Fc receptors are also found in most B cells as well as in some T cells and other unclassified lymphocytes by using methods more sensitive than those employed in macrophage studies (Basten *et al.*, 1972; Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972; Anderson and Grey, 1974). One of the most striking problems concerning Ig binding to Fc receptors has been in man, who has two classes of Ig-bearing cells (Kurnick and Grey, 1975; Winchester *et al.*, 1975; Frøland *et al.*, 1974; Lobo *et al.*, 1975). One class, true B cells, bears surface IgM and IgD which is maintained during culture and reexpressed if the cell is stripped of its Ig. The second class of Ig-bearing cells contains IgG on their membranes, but this Ig is rapidly eluted upon culture and not reexpressed. This last group of cells—null or K cells—has not been precisely identified but is thought to represent perhaps an early monocyte or a subgroup of B cells or an altogether new line of lymphocytes. The null cells are believed to be responsible for the phenomenon of antibody-dependent cell cytotoxicity described by Perlmann *et al.* (1972; Frøland and Natvig, 1973; Wisloff and Frøland, 1973). Although no precise data are available, it is quite apparent that depending upon the density of antibody molecules on the indicator par-

ticle (usually a red cell) one detects Fc receptors of variable affinities among the different leukocytes. The order of decreasing strength may go: macrophage > null cell > B cell > activated T cell. Thus, a red cell lightly coated with antibody binds to macrophages without detecting the Fc receptor on lymphocytes. However, with a highly coated red cell [or with a more sensitive test, such as the one used by Basten *et al.*, (1972)], the lymphocyte's Fc receptor is detectable. Thus, one must usually interpret the identification of IgG on the surfaces of lymphocytes with caution inasmuch as with sensitive methods, some serum IgG can be found bound loosely to Fc receptors. We shall discuss below the apparent differences in the redistribution of surface Ig between the two distinct populations of Ig-bearing cells in human peripheral blood.

Three further points concerning surface Ig are particularly important in the context of the redistribution phenomena that we are to analyze: the number of Ig molecules on the cell surface, their topographical distribution, and the mode of insertion of Ig into the membrane.

3. *Quantitation of Surface Ig*

The number of Ig molecules presumably serving as antigen receptors on the B cell surface is on the average of 10^5 molecules per B cell (Rabellino *et al.*, 1971; Ault and Unanue, 1974).

The amount of surface-bound Ig is large compared to the number of hormone receptors needed to trigger certain cellular responses. One could speculate that many of the surface Ig-antigen interactions result in nonfunctional events, therefore many receptors are needed; that the B cell requires such a high number of receptors in order to bind effectively antigen molecules of large size and variable epitope densities; or that a number of successive hits are needed in these cells to reach an effective threshold of stimulation.

The method used to quantitate Ig was based on a technique first described by Farr and associates (Smith *et al.*, 1970). Cell surface Ig was estimated by determining the extent to which B cells could block a standard reaction between radioiodinated Ig (or Fab or Fc fragment) and specific antibody. First, lymphocytes were incubated with a standard antibody for several hours, after which the cells were separated and the antibody was then incubated with nanogram amounts of ^{125}I-labeled Ig. It was found that the lymphocytes had bound a percentage of the anti-Ig, thereby producing a decreased binding of the labeled Ig (the antigen) in the standard test. By comparing this reduction in binding to a standard curve, using nonlabeled Ig, one could calculate the amount of Ig on the cell surface. This technique is extremely sensitive and requires close attention to various technical details (Lerner *et al.*, 1972). One potential

drawback is that surface Ig is quantitated by reference to soluble Ig, which may introduce some errors, inasmuch as both are not in the same physical state (the surface Ig is cell bound, the reference Ig is soluble). However, other estimates of surface Ig based on the amount of labeled anti-Ig bound by B cells are in close agreement with the average figure of 10^5 Ig molecules. Stobo *et al.* (1972b) calculated the amounts of bound ^{125}I-labeled anti-kappa to be equivalent to 70,000 to 80,000 molecules of antibody per cell. With polyvalent antibody, the figures have been about 1 to 2 $\times$ 10^5 molecules of antibody bound (Avrameas and Guilbert, 1971; Sidman and Unanue, 1975b).

All the above studies give average figures of surface Ig content but not the individual variability, which is large. A recent development for studying and quantitating surface Ig is the fluorescence-activated cell sorter (Bonner *et al.*, 1972). Cells labeled with a fluorescein-tagged antibody can be analyzed for the amount of fluorochrome emitted under light stimulation and, if need be, can be isolated. By using the sorter, it is possible to obtain profiles of fluoresceinated anti-Ig among B cells, and this may be useful in future studies of B cells and their receptor Ig content (Sher *et al.*, 1975).

4. Topography of Surface Ig

The distribution of surface Ig as well as of other surface macromolecules has been studied at the ultrastructural level by using various methods. One method was the ultrastructural analysis of regular thin section of cells exposed to antibodies conjugated to a large visible molecule (Aoki *et al.*, 1969, 1971). The first attempts to do two-dimensional topographical analysis came from Stackpole *et al.* (1971), who made composites of lymphocyte cell surfaces based on serial examination of thin sections. They incubated lymphoid cells with a hybrid antibody (Hämmerling *et al.*, 1968); one of the specificities was directed against the surface component and the other against a visual marker, in their case, a virus or ferritin. (Hybrid antibodies are made by treating antibody molecules of two different specificities with pepsin. They are then dissociated into half-molecules and oxidized; as the molecules reassociate, they form, at random, molecules containing both antibodies.) The main findings were that alloantigens were distributed in large irregular patches on the membrane. This method of Stackpole *et al.* is at present obsolete, being superseded by more direct and less laborious procedures. Their studies are, however, of important historical value since they represent the first efforts to visualize in two dimensions the surface components of lymphocytes. Also of historical value is the attempt of Boyse *et al.* (1968b) to map the antigenic features of the cell surface by functional means. If two

sites were close to each other then an antibody to one should block the binding of antibody to the second site. By doing this with several antigen systems, one could get an idea of how the different components were spaced. At the time, there was no information on the mobility of surface components.

There are several points to consider regarding topographical analysis of Ig or other surface molecules. First, the analysis should be two dimensional in order to obtain a direct appraisal of the relationship of molecules with each other. Thin sections of cells have been used to study distribution, but the information that they convey is of very limited value. The approach taken by Karnovsky in collaboration with us was to map Ig and other surface components by using freeze-etching procedures or by doing surface replicas of cells (Karnovsky *et al.*, 1972; Karnovsky and Unanue, 1973; Abbas *et al.*, 1975, 1976a). Lymphocytes exposed to the labeled ligand were fixed, quickly frozen in a solution usually of 20 to 25% glycerol, which served as a cryoprotectant, and processed for freeze-etching. Subsequent to cleavage, the preparation was eluted at high vacuum to sublime part of the ice table and to expose the outer surface of the cell, of which a replica was then made (Fig. 1). The method showed a flat, two-dimensional distribution of surface molecules. One defect of freeze-etching analysis is that the topographical distribution of macromolecules along surface projections such as microvilli cannot be deter-

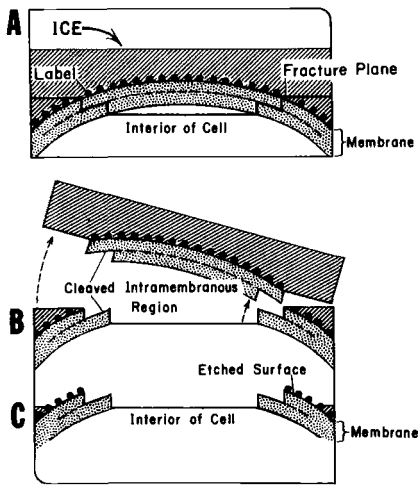

FIG. 1. The principles of freeze-etching and freeze-cleaving. The plane of fracture runs parallel to the surfaces of the cell and then dissects the membrane into two inner halves. By subliming the ice, the etched surface of the cell is exposed. Details are given in the text.

mined. Scanning electron microscopy, in this regard, seems to be the ideal method inasmuch as general topographical features are beautifully maintained. So far, high-resolution scanning studies have not been made. A problem common to topographical studies employing either thin-section or freeze-etching electron microscopy relates to the markers used to visualize the ligand, which, by necessity, have been of large size, introducing serious steric problems. Thus, the use of virus (Aoki *et al.*, 1971; Maher *et al.*, 1972; Hämmerling *et al.*, 1975; Kay, 1975) or large proteins, like hemocyanin (Karnovsky *et al.*, 1972), is satisfactory for identification of a given component on the membrane but inadequate in giving a precise topographical relationship, since their large size obscures many sites, especially those that are closely associated. Ferritin molecules about 100 Å in diameter are a satisfactory compromise.

A second general problem in topographical analysis is the redistribution produced by the ligands during the procedure, with a resultant distortion of the components' normal topography. In the next section, we shall discuss more fully the observations that, because of the fluid state of the plasma membrane, Ig and other membrane components can be *clustered* by cross-linking, multivalent ligands at warm temperatures. Therefore, precise topographical studies require conditions that prevent or minimize, as much as possible, a change from the normal distribution of membrane molecules as a result of cross-linking. Topographical studies made with multivalent ligands (i.e., regular antibodies) at ambient or 37°C temperatures are meaningless. The extent to which surface alterations take place at low temperatures (i.e., 4° to 10°C) has been debated (de Petris and Raff, 1972). De Petris and Raff (1972) did find evidence of receptor clustering at low temperatures, principally when a two-antibody method was used. In the studies of Karnovsky *et al.* (1972), using freeze-etching methods and incubating lymphocytes at 0°C to 4°C, there appeared to be minimal clustering of murine surface Ig but definite aggregation of murine histocompatibility (H-2) antigens. The distortion of H-2 sites on mouse cells is more marked when two antibodies are used, as in most of the studies. Such mapping studies have given the false impression that these H-2 sites are in very large, gross clusters, a point correctly criticized by Davis and associates (1971; Davis, 1972). Therefore, mapping is done best with monovalent antibodies and when sites cannot associate (de Petris and Raff, 1972) or, alternatively, with cells that are prefixed (Parr and Oei, 1974; Abbas *et al.*, 1975). With these reservations in mind, let us consider recent work on the distribution of surface Ig on the B-cell surface.

Analysis of thin sections of murine lymphocytes exposed to monovalent antibodies (de Petris and Raff, 1972) disclosed isolated molecules throughout the surface in no particular pattern. However, analysis of

molecular patterns on only one plane is not adequate to determine whether those patterns are random or organized. Also, regular transmission electron microscopy of thin sections of B cells exposed to peroxidase-labeled anti-Ig showed Ig distributed throughout the membrane including that of the small microvilli (Reyes *et al.*, 1975). Freeze-etching, which permits two-dimensional analysis was used by Abbas *et al.* (1975) to study Ig under conditions in which redistribution produced by the ligands appeared to be minimal. They employed a sandwich method in which the first antibody was a monovalent anti-Ig coupled to fluorescein isothiocyanate; this molecule was visualized by using a monovalent anti-fluorescein antibody coupled to ferritin. (Fluorescein is a strong immunogenic hapten.) Haptens coupled to antibodies have been employed by many (Hämmerling and Rajewsky, 1971; Lamm *et al.*, 1972; Wofsy *et al.*, 1974).

Surface Ig in murine B cells was distributed in a lacy discontinuous network with very small clusters—at the most four to eight ferritin grains. These clusters were often interconnected by strands of a few molecules (Figs. 2 and 3). The surface pattern was the same on cells exposed to the antibodies at low temperatures or at 20°C, or on cells previously fixed in paraformaldehyde. Although antibodies with a fluorochrome:protein ratio of 1 were employed, the same results were obtained by using antibodies with a wide range of fluorochrome:protein ratios. This last point eliminated the possibility that clustering was an artifact of variability in the degree of hapten substitution.

The patterns of surface Ig were subjected to a series of mathematical and statistical analyses in order to determine whether the distribution of Ig was that of molecules dispersed in an ordered fashion on the membrane or at random (Abbas *et al.*, 1975). Tracings of the freeze-etch analysis were compared to computer-generated tracings of randomly distributed points. The distribution of Ig deviated from a true pattern of randomness inasmuch as there was too much area of the membrane unoccupied by molecules implying that the sites tended to be clustered more than would be expected. A short-range organization of membrane molecules does not by any means negate the fluid mosaic model of membrane structure, a point clearly stressed by Singer (1974) (Section V). It is indeed possible to envision interactions of protein to protein on the membranes or particular association of the protein with the phospholipids. Alternatively, surface Ig molecules may be attached in variable numbers to a given anchoring structure. By extrapolation, one could interpret the nonrandom distribution observed by Abbas *et al.* (1975) as a reflection of some degree of restriction to the spontaneous, free diffusion of Ig on the membrane. In summary, surface Ig is found diffusely throughout the plasma membrane in a nonrandom distribution.

FIG. 2. A freeze-etch replica of mouse B cell labeled first with fluorescein-tagged Fab anti-Ig antibody and then with ferritin-tagged Fab antifluorescein antibody at 4°C. Ferritin grains are found in small clusters (×22,100). (From Abbas *et al.*, 1975.)

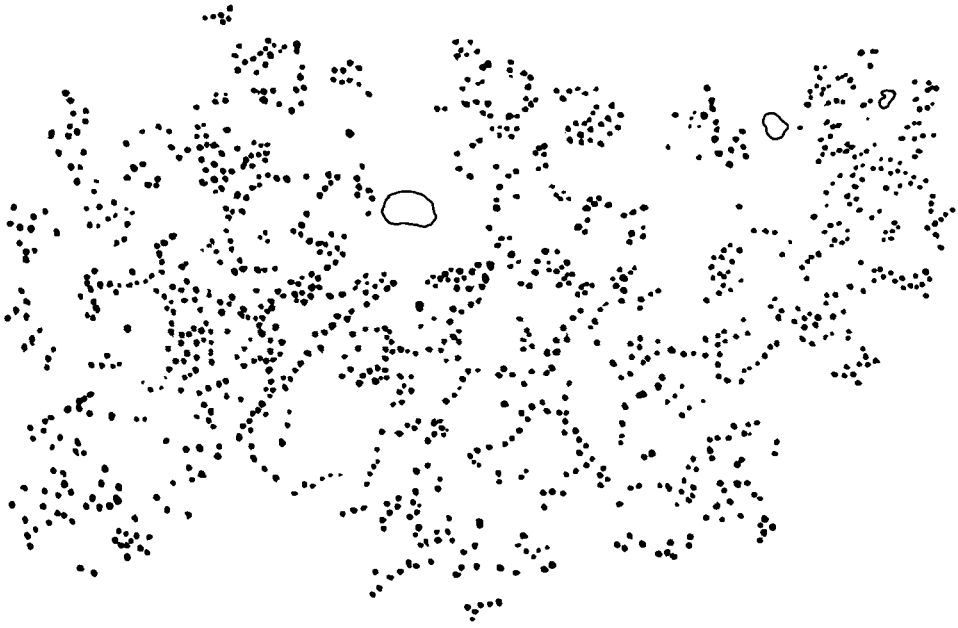

FIG. 3. A tracing of a freeze-etch replica similar to that of Fig. 2, showing the lacy pattern of small microclusters. (From Abbas *et al.*, 1975.)

Similar distribution of surface Ig has been described for human B cells. Although the initial study of Ault *et al.* (1974) disclosed surface Ig of human peripheral blood cells distributed, at 0° to 4°C, in prominent clusters separated by large spaces of bare membrane, these experiments were complicated by the use of bivalent antibody and by the more recent observation that, among the few Ig-bearing cells in peripheral blood, a substantial number have Ig acquired from the serum. Using monovalent antibodies, Abbas *et al.* (1975) observed diffusely distributed microclusters of surface Ig, with human cells differing from murine lymphocytes in their somewhat lowered density of determinants and a tendency in some cells for more extensive microclustering.

5. *Mode of Insertion of Surface Ig*

The manner in which Ig molecules are embedded in the plasma membrane is of considerable importance but has not yet been adequately explained. Ig molecules in serum are soluble, yet in the membrane they have the characteristics of integral membrane proteins (Section V); i.e., surface Ig molecules can be removed only by techniques that denature the membrane (Haustein *et al.*, 1974; Kennel and Lerner, 1973; Sherr *et al.*, 1972). In one experiment, after nine washes with large amounts of culture

medium the amount of Ig on the membrane of B cells was 86 ng per 10^7 cells as compared to 97 ng for untreated controls (Ault and Unanue, 1974). A great many of the radioiodinated Ig molecules extracted from cell surfaces, either IgM or IgD, have been found to be insoluble after removal of the nonionic detergent (Melchers *et al.*, 1975). In contrast, secreted IgM is perfectly soluble in aqueous medium. Some have speculated that surface Ig could be associated, noncovalently, to another structure that may be embedded in the hydrophobic portion of the lipids and serves as its anchor. Evidence for this putative structure has not been forthcoming. Milstein *et al* (1972). have reported a fifteen-amino acid peptide on Ig light chains isolated from myeloma plasma cells, which they think could serve to bind the chain to the membrane. Regardless of its mode of attachment, it is agreed that most of the Ig is exposed to the outside environment. The Fab portion of the molecules is obviously free and available to antigens; a great part of the Fc fragment reacts with antibodies and also can be radioiodinated. However, there is some immunocytochemical evidence that the Fc terminal portion of the molecule may be partially hidden. Frøland and Natvig (1973) found that the Gm(n) allotype, localized to the C terminal region of the Fc fragment, was not detected in human B cells. Fu and Kunkel (1974) reported very elegant experiments indicating that surface IgM and IgD do not absorb out all the antibodies made against *serum* IgM or IgD. Similar results were obtained by Jones *et al.* (1974), with IgA molecules on rabbit B cells. One logical interpretation of these results is that the C-terminal region is perhaps unfolded and bound within the membrane.

B. Patching and Capping of Surface Ig

While diverse methods for the visual demonstration of the Ig receptors generally resulted in agreement on the nature of the molecule, there was a consistent and puzzling disagreement in early studies as to the distribution of these receptors on the membrane of the B lymphocyte. Using fluorescein-conjugated antisera, many investigators visualized Ig, as well as other surface components, as occurring usually, but not always, in diffusely scattered patches. A fluorescent pattern resembling an interrupted or a beaded ring of patches was seen when the microscope was focused on the equatorial plane of the cell (Möller, 1961; Pernis *et al.*, 1970; Davie *et al.*, 1971a; Rabellino *et al.*, 1971). Significantly, some of these groups conducted their experiments at low temperatures and/or in the presence of metabolic inhibitors in order to minimize artifacts created by possible endocytosis of the label. Concurrently, in experiments carried out under somewhat different conditions, others described Ig as asymmetrically distributed in a shape resembling a crescent along part of the cell circumference (Johansson and Kelin, 1970; Raff *et al.*, 1970; Raff,

1970). However, in most of the early reports, it was clear that unexplained, mixed patterns of fluorescence were seen—rings, patches, caps. To cite one example, which in retrospect represents the first description of a cap, Möller in his 1961 study of alloantigens on various cells, described the ring pattern of fluorescence, but went on to say that this "reaction was often incomplete, however, with only part of the cell membrane stained, giving the appearance of fluorescent crescents" (Möller, 1961).

These discrepancies were resolved when results from two laboratories working independently were published together and comprised the first observations that the B lymphocyte, after the binding of antibody to its surface Ig, rapidly redistributes its receptors from a pattern of diffusely scattered sites to a "cap" at one pole of the cell for subsequent endocytosis (Fig. 4) (Taylor *et al.*, 1971). Thus, the phenomenon of capping and gross patching of fluorescence was placed in the correct perspective as resulting from redistribution of Ig easily movable within the plane of the membrane. Capping has been subsequently described in lymphocytes from every species examined.

1. Morphology

Although the early events that follow the interaction of ligand, whether antigen or anti-Ig, with B lymphocytes occur rapidly and continually, they

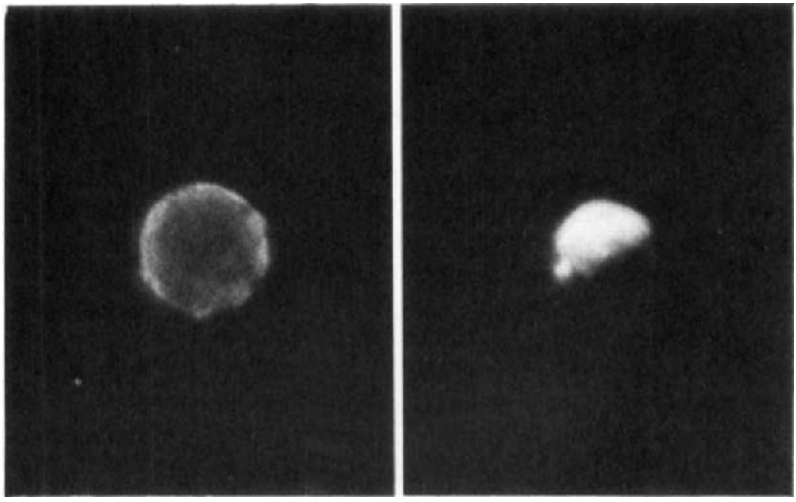

FIG. 4. Fluorescence micrographs of B cells exposed to fluorescent anti-Ig antibodies; at the left, the typical pattern of diffuse fluorescence after reaction in the cold; at the right is a typical cap that develops after incubation at 37°C.

can be separated into various stages, each of which is controlled or regulated by different mechanisms. These different stages have been studied best using anti-Ig antibodies and murine B cells. Technically, the procedure consists of adding appropriately tagged anti-Ig to B cells in culture and observing the effects directly under the microscope, as is best, or following fixation of the cells at appropriate time intervals (Fig. 5). After binding of anti-Ig to the B cell at 37°C, there is a brief stage of about 1–2 minutes, when the lattice of complexes thickens and aggregates in small, irregular clusters disseminated throughout the surface. This stage is followed by a rapid movement of the complexes in a highly coordinated fashion to one pole of the cell. The complexes readily condense into caps, which progressively occupy less and less of the cell surface. By 4 minutes at 37°C, over 90% of the cells have formed tight caps covering less than one-third of the cell surface.

After the complexes have moved into the cap, the rest of the membrane is devoid of any surface Ig receptors, whether examined by fluorescence or electron microscopy (Taylor *et al.*, 1971; Unanue *et al.*, 1972b). Calculations based on electron microscopic observations of ferritin-labeled anti-Ig in thin sections showed that the labeled receptors in the cap account for all the receptors previously distributed over the cell surface (de Petris and Raff, 1972).

During the very early stage, when the cap is forming, there is little change in the gross morphology of the lymphocyte, which remains round. After this initial phase, however, the lymphocyte's morphology rapidly

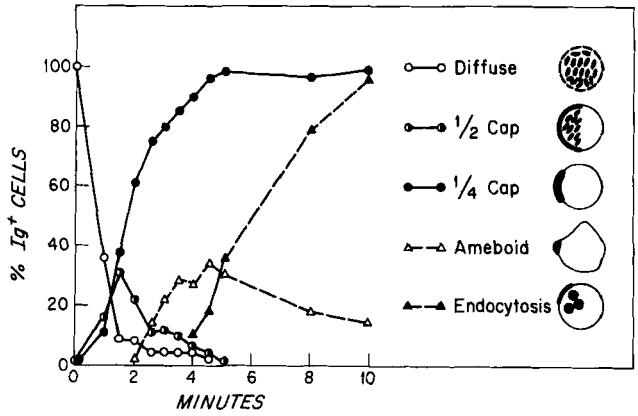

FIG. 5. The graph shows the different stages of redistribution of Ig–anti-Ig complexes. B cells were first incubated in the cold with anti-Ig antibodies, washed, and planted on culture dishes, and incubated at 37°C for the times indicated in the horizontal axis. The reaction was stopped by fixation with paraformaldehyde. (From Schreiner and Unanue, 1976a.)

alters. First, the B cell pushes out a small cytoplasmic projection always opposite the cap (Schreiner and Unanue, 1976a) (see also Fig. 8). This projection corresponds to actual ruffles when viewed by scanning electron micrographs (Karnovsky *et al.*, 1975, 1976). Second, once the cap is formed, a marked constriction rapidly develops in the area surrounding it, which forms into a uropod such that the lymphocyte acquires an ameboid or pear-shape configuration (Fig. 6). The lymphocyte then goes into a transitional stage of true translatory motion. In the cells that have moved subsequent to capping, one sees by electron microscopy that the complexes are all tightly clustered in the cap region, where numerous packed villous projections are found (Karnovsky *et al.*, 1972). Underneath the cap zone, in the constricted area of the cell, is a relatively clear area of cytoplasm containing a dense network of thin microfilaments (Fig. 7). This network separates the cytoplasmic organelles from the membrane containing the cap. Apposed to the constricted area is the perinuclear area with the Golgi zone (Taylor *et al.*, 1971; Unanue *et al.*, 1972b; de Petris and Raff, 1973a,b; Stackpole *et al.*, 1974b). The cell, therefore, is perfectly oriented: the cap at its back pole facing the perinuclear area with the Golgi zone, the pseudopod streaming forward in a direction always opposite the cap (Schreiner and Unanue, 1976a).

The last stage of the cycle is the cleaning of the complexes by endo-

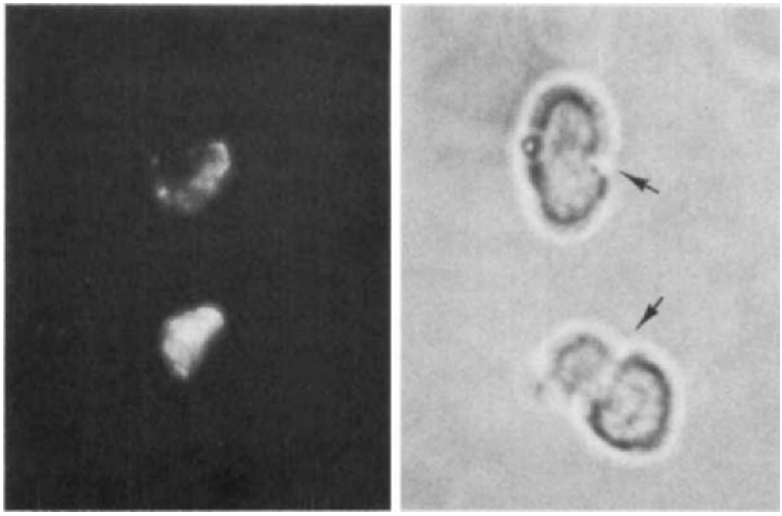

FIG. 6. Two B cells capping and exhibiting the morphological changes of movement. Left panel: fluorescent caps; right panel: the same cells by phase contrast. Arrows point to the constriction under cap area of each cell.

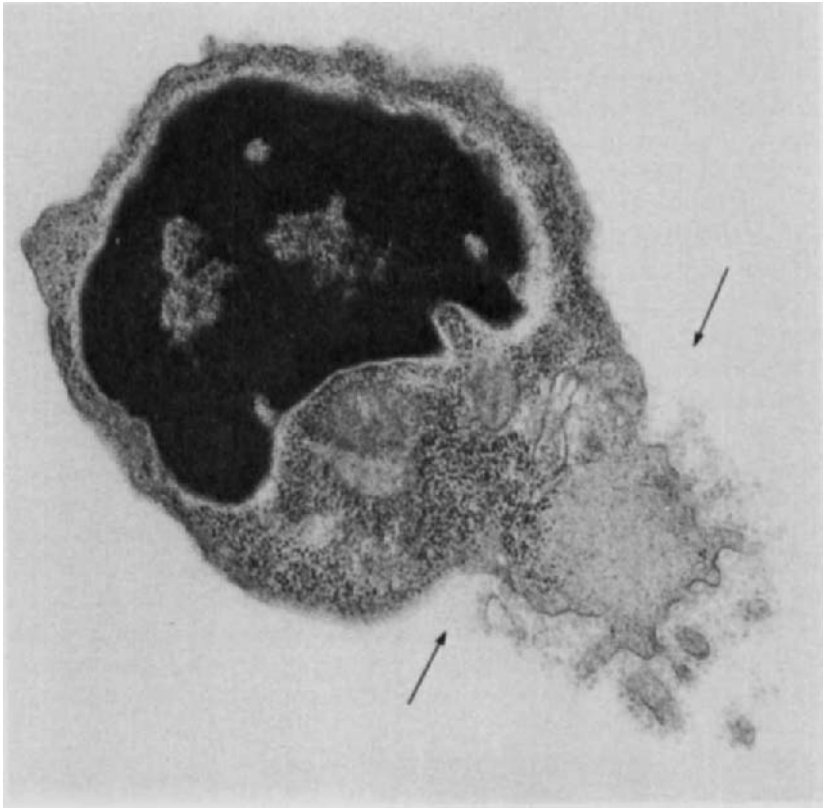

FIG. 7. An electron micrograph of a B cell capping hemocyanin-tagged Ig–anti-Ig complexes. All the label is in the cap area over the uropod (arrows). Note the marked changes in shape and the clear net of filaments that separates the cap zone from the rest of the cytoplasm. (Micrograph is from an unpublished study of M. J. Karnovsky.)

cytosis. Thus, the whole sequence can be divided, for practical purposes, into early clustering → capping → motility → endocytosis. This section describes the capping stage. Motility and endocytosis are discussed at length in Sections III and VI. In the section on motility we specifically analyze how the anti-Ig interaction serves as a stimulus for the cell to move and how the motile process in itself can be dissociated by several manipulations from the actual stage of capping.

During capping, B lymphocytes have been studied by scanning electron microscopy (Kay, 1975; Karnovsky *et al.*, 1975; Soni *et al.*, 1975). Before examining this point, first let us remark on the normal surface morphology of B and T cells. The surface of lymphocytes contains a series

of small stubs and fingerlike microvilli throughout. The general topography varies from cells with smooth surfaces, to others with many thin villi and yet others with intermediate features (Holt *et al.*, 1972; Lin *et al.*, 1973a; Polliack *et al.*, 1973; Linthicum *et al.*, 1974; Linthicum and Sell, 1975). In man there is a tendency for B cells to have many villi in contrast to the T cells, which tend to have smooth surfaces (Polliack *et al.*, 1973). [Similar conclusions have been drawn with thin-section, regular-transmission electron microscopy (Reyes *et al.*, 1975).] However, considerable overlap can be seen among both populations (Polliack and de Harven, 1975). The classification, therefore, of B and T cells just upon the basis of the number of villi must be made with caution and under highly standardized procedures. Indeed, the surface of the lymphocyte, as well as of other cells, is dynamic and can be altered depending on various factors, including temperature (Lin *et al.*, 1973b), contacts with surfaces of dishes (Linthicum and Sell, 1975) or of other cells (Rubin and Everhart, 1973), the nature of the culture medium (Kay, 1975), and stage of the cell cycle (Porter *et al.*, 1973) etc. The great incidence of microvilli on T cells that bind sheep red cells (Lin *et al.*, 1973a; Kay *et al.*, 1974) most likely results from the interaction with the red cell (Polliack *et al.*, 1974). It is of interest that most B and T lymphocytes examined *in situ* in lymph nodes have smooth-faced surfaces with only occasional villi. Only the cells in the blood bound to the endothelial surfaces of vessels have projections (M. J. Karnovsky, unpublished observations, 1975).

Karnovsky *et al.* (1975, 1976) developed a method for examining surfaces of cells during capping of fluorescent anti-Ig. B cells were incubated with fluorescent anti-Ig and then fixed at various intervals. The fixed cells were attached to a coverslip coated with poly-*l*-lysine, examined in the fluorescence microscope, photographed, and processed for scanning electron microscopy. The cells containing the complexes could be identified and correlated with the fluorescent pattern without altering the basic morphology by the use of anti-Ig bound to a large indicator particle. It was found that most B cells contained a variable, usually small, number of stubs and microvilli. During capping, the membrane first became smooth then typical ruffles developed, always opposite the cap; in the cap zone, at the time that the cell changed shape, numerous villi were seen (Fig. 8). The association of caps with microvilli was reported by Soni *et al.* (1975), also using scanning electron microscopy with fluorescent-labeled anti-Ig, and by Kay (1975) using anti-Ig conjugated to viruses or hemocyanin. The fluorescence in the former case was detected by cathodoluminescence. These results have been repeated often by using regular thin sections of cells (de Petris and Raff, 1972,

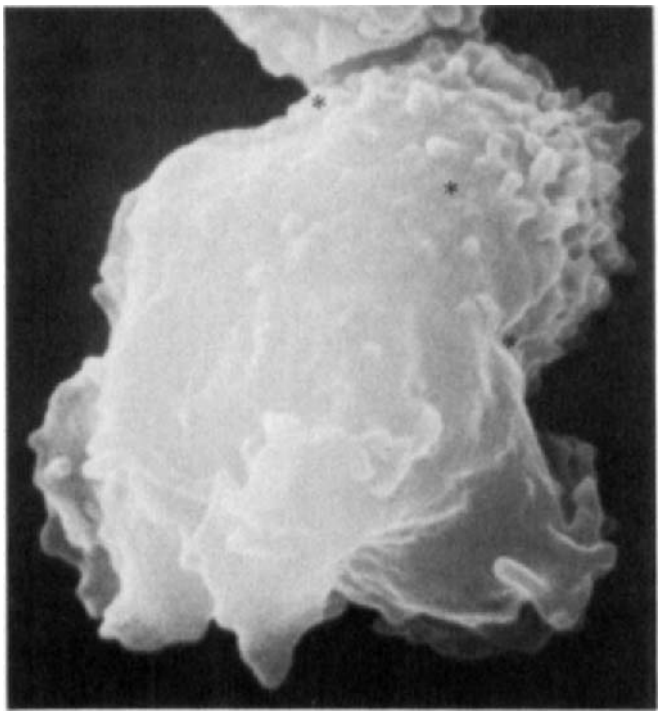

FIG. 8. Scanning electron micrograph of a B cell at the time of cap formation. The details are given in the text. The cap area is in the upper pole, in the zone distal to the asterisks. Note the microvilli associated with the cap and the ruffles in the cell area opposite the cap. The cell was tagged with fluorescent anti-Ig and identified first by fluorescence microscopy. (From Karnovsky *et al.*, 1976.)

1973a,b; Karnovsky *et al.*, 1972). In other studies, Karnovsky and associates (1976) showed that cells that have made caps in the presence of cytochalasin B, a drug that stops movement, have very few microvilli in the cap area.

2. *Requirements*

The formation of diffuse clusters of complexes—patching—always precedes capping (Loor *et al.*, 1972; de Petris and Raff, 1973a). Many factors influencing the rate and extent of patch formation affect cap formation in the same way. Thus, for example, both phenomena are dependent upon the valence of the ligand and, to a different extent, the temperature of the reaction. However, capping is not simply an amplified clustering of complexes, for it requires metabolic energy and the integrity of the cell's cytoskeletal system.

a. Temperature. The initial phase of cluster formation, or patching, can occur at, or slightly above 4°C. With fluorescent anti-Ig, small fluorescent patches cover the cell's surface. One can inhibit patch formation by lowering the temperature below 4°C, thereby presumably increasing the viscosity of the lipid membrane, although laboratories differ as to the point of inhibition. There does appear to be a temperature threshold in that a slight elevation of temperature from 0°C to 4°C has been reported to increase dramatically the rate and size of patch formation (de Petris and Raff, 1972, 1973a). De Petris and Raff (1972) did find some degree of clustering even at 0°C, although others have not called attention to this (Loor *et al.*, 1972; Unanue *et al.*, 1972b). No further redistribution occurs until the temperature of the reaction is raised to 20°–37°C, at which point the complexed receptors cap.

b. Metabolic Energy. A major difference between clustering and capping is the requirement for energy metabolism. Inhibitors of the respiratory chain (sodium azide, oligomycin, dinitrophenol, cyanide) or of glycolysis (fluoride, iodoacetamide, 2-deoxyglucose) have no effect on the initial clustering of Ig receptors. These agents completely prevent capping. Cells maintained for several hours at 37°C in the presence of metabolic inhibitors demonstrate patches randomly distributed on the cell surface in a kind of membrane-bound, two-dimensional form of a microprecipitation reaction (Loor *et al.*, 1972; Unanue *et al.*, 1973). The pattern of fluorescein-labeled Ig–anti-Ig complexes evolves from a fine reticulation to a coarsely beaded and still diffusely stained appearance. Thus, patch formation is a passive phenomenon arising from the diffusion of small complexes in a fluid, lipid matrix which, upon collision with one another become progressively larger (Taylor *et al.*, 1971; Loor *et al.*, 1972; de Petris and Raff, 1973b; Unanue *et al.*, 1973; Unanue and Karnovsky, 1973; Stackpole *et al.*, 1974a).

c. Multivalency. Ligand multivalency is a requirement for both patching and capping. Thus, papain-prepared Fab anti-Ig antibody conjugated with either fluorescein or ferritin upon binding to surface Ig does not induce either patching or capping. However, if these molecules are then cross-linked by divalent, antiligand antibody, patching readily occurs, followed by capping, in a manner identical to that seen when the first ligand is divalent. If the secondary antiserum is also monovalent and consequently unable to cross-link, there is no patching or capping of surface Ig (Taylor *et al.*, 1971; de Petris and Raff, 1972, 1973a; Loor *et al.*, 1972; Unanue *et al.*, 1972b; Antoine *et al.*, 1974; Stackpole *et al.*, 1974b). Using a hybrid antibody technique, Stackpole *et al.* (1974d) suggested that cross-linking may not be a strict requirement and aggregation might result from alterations in surface electrostatic charge. This

has not been further developed. In their experiments, it is difficult to rule out the possibility of anti-Ig-anti-Ig hybrid molecules contaminating the preparation. Certainly, alternative approaches have demonstrated a cross-linking requirement for reorganization of surface Ig. Protein A of *Staphylococcus aureus* is divalent and reacts specifically with the Fc region of the IgG molecule (Forsgren and Sjöquist, 1966). Ghetie *et al.* (1974) observed both patching and capping after binding of the fluorescent divalent molecule to guinea pig B lymphocytes bearing IgG. Neither occurred with its monovalent fragment, although it bound equally well. Experiments using haptens of varying densities on a carrier have also demonstrated the necessity for cross-linking ligands in order to redistribute surface Ig. This will be reviewed in Section IX.

Loor *et al.* (1972) have suggested that cross-linkage is no longer necessary after patch formation, based on experiments with $F(ab')_2$ fragments of anti-Ig and dithiothreitol, which reduces the pepsin fragment into monovalent fragments. $F(ab')_2$ anti-Ig induced patches and caps as well as a divalent ligand. However, addition of dithiothreitol after patch formation did not disperse the patches. There was no actual proof that the $F(ab')_2$ anti-Ig bound to the membrane was dissociated by dithiothreitol. If this assumption is correct, though, it is consistent with the possibility of some stabilization for formed patches by interaction with the cytoplasm.

d. Ligand Concentration. There is a minimum concentration of anti-Ig antibodies required for efficient capping. With fluorescence, Taylor *et al.* (1971) obesrved a minimum concentration of anti-Ig below which cap formation did not take place. Work in this laboratory (Unanue *et al.*, 1972b; Karnovsky *et al.*, 1972) demonstrated with electron microscopy that anti-Ig, at a concentration below that required to saturate all the surface Ig, is still able to induce some degree of clustering of the Ig receptors but does not stimulate visible capping. Similar observations were made by Stackpole *et al.* (1974a).

A very large concentration of anti-Ig has been postulated to inhibit capping (Taylor *et al.*, 1971; Loor *et al.*, 1972). This prozone phenomenon was interpreted to result from inhibition of an efficient lattice by the excess antibdy, since it occurs in some precipitation reactions. In the experiment of Taylor *et al.*, using purified anti-Ig antibodies, there was a progressive loss in the percentage of cells capping from about 75% with 40 μg per milliliter of antibody, to about 12% with 1 mg/ml. Other either have not observed this prozone effect (Unanue *et al.*, (1972b) or have described slight inhibition (Stackpole *et al.*, 1974a). Possible nonspecific effects of high concentrations of antisera remain to be excluded.

e. Membrane Fluidity. The inhibition of capping at low temperature

is thought to result from an increased viscosity of the lipid bilayer—as well as a reduction in metabolism. Attempts have been made to change the viscosity of the plasma membrane and to determine its effect on Ig redistribution. Loor *et al.* (1972) found that cells incubated with a high concentration of D-mannose or D-galactose do not cap well. They attributed this to a possible effect on membrane viscosity. No measurements of viscosity were made; furthermore, the concentrations of sugars that were used were highly unphysiological (0.5 *M*) and probably produced their effects by markedly increasing the osmolarity of the medium. A promising approach is that of Kosower *et al.* (1974), who treated rabbit lymphocytes with a series of cyclopropane unsaturated fatty acid esters that measurably increase membrane fluidity. Incubation of the lymphocytes for 1 hour with one of the compounds markedly increases the rate of Ig capping. This observation is of interest and should be extended. Finally, the poor capping of lymphoma cells discussed in the next section has been attributed to increased membrane viscosity.

3. *Variations in Capping*

Redistribution of surface Ig of B cells has been studied mostly in man and in mice, but also in rabbits (Linthicum and Sell, 1974) and guinea pigs (Rosenthal *et al.*, 1973). Some differences between species and within species are worth discussing. Recently, Fram *et al.* (1976) analyzed various mouse strains for their rate of capping and found marked differences among them. For example, the A strain, used in most of the experiments from our laboratory, had a fast rate of capping in contrast to the CBA strain. By 10 minutes at 20°C the number of B cells with caps in the A was about 75% whereas the CBA had 10 to 25%. With time, the CBA cells eventually capped to the same extent as the A cells. The differences were under genetic control—the low-rate CBA trait being dominant. The trait was not linked to the H-2 locus or to the sex chromosome and was probably accounted for by two gene differences.

Human B cells redistribute their receptor into caps in a manner analogous to that seen in the mouse (Preud'homme *et al.*, 1972; Rowe *et al.*, 1973b; Ault *et al.*, 1974; Huber *et al.*, 1974; Cohen, 1975). Ault *et al.* (1974) and Huber *et al.* (1974) found that the number of cells capped was only a small proportion of the total Ig-bearing cell population. However, others have found a much higher figure (Cohen, 1975). These discrepancies can be explained now that we know that the Ig-bearing cells comprise true B cells plus those null cells bearing IgG bound loosely to their Fc receptors (Section II,A,2). Indeed, the cells that bear IgG loosely bound (and which may represent 50% or more of the total

Ig-bearing cells) redistribute their complexes quite differently from the true B cells that bear μ and Δ chains. The IgG-antibody complexes are distributed into disseminated, large patches and are then cleared by endocytosis. In contrast, many, though not all, of the true B cells redistribute first into caps and then internalize (Kumagai *et al.*, 1975; Ault and Schreiner, 1976). The redistribution of complexes by the IgG—null—type of cell is very similar to that shown by macrophages which, in most cases, go from patching to endocytosis rather than from patching to capping to endocytosis.

Although no differences in capping have been seen between normal and LPS-transformed B cells (Kerbel *et al.*, 1975), chronic lymphocytic leukemia cells have been reported to cap Ig poorly (Huber *et al.*, 1974; Cohen, 1975). The same holds true for lectin binding sites (Inbar and Sachs, 1973; Inbar *et al.*, 1973; Ben Bassat *et al.*, 1974). Inbar *et al.* attribute this to a reflection of decreased membrane viscosity related to the malignant state, but other possibilities, including structural abnormalities, must be kept in mind (Section V). The increased viscosity of membranes of abnormal lymphocytes was reported by Inbar *et al.* (1974) and attributed to a higher ratio of cholesterol to phospholipid of the membrane (Inbar and Shinitzky, 1974; Vladavsky and Sachs, 1974).

Infection with Friend leukemia virus may prevent capping. Kateley *et al.* (1974) observed that soon after mice were injected with this virus, the B lymphocytes were unable to cap anti-Ig and clear the complexes. This interesting phenomenon appeared well before the appearance of leukemic cells.

Rabbit peripheral blood B cells have been reported to endocytose complexes very rapidly without much capping (Linthicum and Sell, 1974). A recent paper reports that B cells of the elasmobranch fish *Raya naevus* cap readily at 4°C, the temperature close to the environmental one experienced by the skate (Ellis and Parkhouse, 1975).

4. Pharmacology of Capping

Numerous drugs have been studied for their effects on capping, motility, and endocytosis in an attempt to gain an understanding of their mechanisms. Table I summarizes effects of various drugs on these three processes. We shall only outline the effects of various chemicals on capping now, leaving the interpretations of their effects until Section V.

Drugs that may affect the microfilaments or the microbulbular system have been tested extensively. In the first group are the cytochalasins, a series of fungal products that affect contractile functions of the cell (Wessells *et al.*, 1971). Cytochalasin B produces a slight to moderate inhibition of capping (Taylor *et al.*, 1971; Loor *et al.*, 1972; de Petris,

TABLE I
EFFECTS OF VARIOUS DRUGS OR EXPERIMENTAL MANIPULATIONS ON REDISTRIBUTION, MOTILITY, OR ENDOCYTOSIS OF IG

Experimental variable	Redistribution		Motility	Endocytosis	Key references[a]
	Patching	Capping			
Inhibitors of energy metabolism	No effect	Complete inhibition	Complete inhibition	Complete inhibition	1–3
Inhibitors of protein synthesis	No effect	No effect	No effect	No effect	1–3
Cytochalasin B	No effect	Partial inhibition at high doses	Complete inhibition	No effect	1, 2
Colchicine	No effect	No effect or slight enhancement	Some enhancement	No effect	1, 2
Cytochalasin and colchicine	No effect	Inhibition	Complete inhibition	Not studied	4, 5
Drugs that increase cyclic AMP	No effect	No effect	Inhibition	No effect	6
Drugs that increase cyclic GMP	No effect	No effect	Increase	No effect	6
Ca^{2+} deprivation	No effect	No effect	No effect	No effect	1, 7
Ca^{2+} ionophore	No effect	Complete inhibition	Complete inhibition	No effect	7
Local anesthetics	No effect	Complete inhibition	Complete inhibition	Complete inhibition	8
Diisopropyl-fluorophosphate	No effect	No effect	Partial inhibition	No effect	9
Cold temperature	Inhibition depends on temperature	Complete inhibition	Complete inhibition	Complete inhibition	10

[a] This is not a complete list of references, but includes only representative studies: 1. Taylor *et al.* (1971); 2. Loor *et al.* (1972); 3. Unanue *et al.* (1972b); 4. de Petris (1974, 1975); 5. Unanue and Karnovsky (1974); 6. Schreiner and Unanue (1975a); 7. Schreiner and Unanue (1976a); 8. Ryan *et al.* (1974a); 9. Unanue *et al.* (1974a); 10. de Petris and Raff (1973a).

1974). In our own experience, inhibitory effects are variable and usually slight; they require high doses of the drug and a temperature of 20°C, at which capping is slow. Cytochalasin B at low doses (i.e., 1 μg/ml) does not affect the extent of capping but reduces markedly the motile response that follows redistribution. Cytochalasin D, a much more potent drug that dramatically affects the cell's microfilament network, stops capping

altogether (at doses of 0.1 μg/ml) (E. R. Unanue and G. C. Godman, unpublished observations, 1975) (Section V).

Colchicine and vinblastine affect the cell's microtubular system, leading to an increased state of depolymerization. There is general agreement that lymphocytes treated with colchicine are not impaired in capping (Taylor *et al.*, 1971; Loor *et al.*, 1972; Unanue and Karnovsky, 1973, 1974). If anything, colchicine may enhance capping of some cells. For example, the B cells from the strains of mice found to have a slow rate of capping are readily brought to a faster rate by addition of colchicine but not of lumicolchicine, an inactive product of the drug (Fram *et al.*, 1976). [Some impairment of capping in human B cells has been reported (Cohen, 1975), but not always (Ault *et al.*, 1974).] Interestingly, the combination of cytochalasin B plus colchicine has a markedly synergistic inhibitory effect on capping (de Petris, 1974, 1975; Unanue and Karnovsky, 1974).

Drugs that are presumed to modulate the levels of cyclic AMP (cAMP) have no significant effect on capping (Unanue *et al.*, 1973; Schreiner and Unanue, 1975a). These include dibutyryl cAMP, phosphodiesterase inhibitors (theophylline, for example), a combination of both, or β-adrenergic agonists known to produce elevation of the cAMP level. Drugs that affect the cGMP level have no effect on capping, but increase motility (Section III,C).

Local anesthetics have a number of effects on cells. They increase membrane volume and fluidity, suppress membrane depolarization, and displace Ca^{2+} from the membrane (Seeman, 1974). Local anesthetics like lidocaine, chlorpromazine, and dibucaine stop capping reversibly (Ryan *et al.*, 1974a; Poste *et al.*, 1975a). The effects of chlorpromazine can be partially counteracted by an increase in extracellular Ca^{2+} (Schreiner and Unanue, 1976b).

While patching and capping are independent of extracellular Ca^{2+} (Taylor *et al.*, 1971; Schreiner and Unanue, 1976a), capping is profoundly inhibited by the Ca^{2+} ionophore (A-23187), which permits a passive influx of Ca^{2+} into the cell.

Inhibitors of protein synthesis (Taylor *et al.*, 1971; Loor *et al.*, 1972; Unanue *et al.*, 1973) have no effect on capping.

III. Motility Induced by Anti-Ig Antibodies

Soon after the discovery of capping, it became apparent that the redistribution of receptors was affecting not only the organization of the cell surface but cell activities involving cytoplasmic structures as well (Taylor *et al.*, 1971; Unanue *et al.*, 1972b). Indeed, one significant effect

of anti-Ig was to stimulate Ig-bearing lymphocytes to move (Unanue *et al.*, 1974a; Schreiner and Unanue, 1975a,b).

The movement of lymphocytes is particularly relevant to the cellular interactions of the immune response. The complexity of the B lymphocyte's interactions with other cells is becoming increasingly delineated; such interactions may lead to stimulation or inhibition influencing the defense of the entire host. Yet essential cell types are segregated within lymphoid tissue, necessitating as yet unknown mechanisms to assure that specific cells not only reach the antigen, but also reach each other. We believe that the directed movement of the spontaneously moving T lymphocyte and the stimulation of directed movement in the ordinarily sessile B lymphocyte will eventually be properly defined as important components of the immune mechanism. Therefore, before describing the ligand-induced motility of lymphocytes, we shall review the general features of this poorly understood aspect of lymphocyte activity.

A. History of Lymphocyte Motility

Although earlier investigators had observed the ameboid motility of leukocytes, primarily granulocytes (reviewed in Henderson, 1928; Harris, 1954), the first suggestion that lymphoid cells were motile was made by Ranvier in 1875 after watching thoracic duct cells from dogs and rabbits in culture. This concept received enthusiastic support form several workers, including Renaut (1881), who, in an effort to find some function of these cells, proposed in 1881 that they served to bring food and metabolites to immobile cells, which were unable to forage for themselves. Objecting on morphological grounds, Ehrlich made it clear that the lymphocyte was incapable of movement because it had "too little protoplasm surrounding the large nucleus, so that the small cell body could hardly push the voluminous nucleus along" (quoted from Harris, 1954). Investigators took sides on the question of whether lymphocytes moved, with some opposed to the concept, and others claiming to have observed it, a claim somewhat weakened by contemporary disagreements over cell classification (reviewed in Henderson, 1928; Harris, 1954).

The first clear demonstrations of lymphocyte motility came in 1921, when Lewis and Webster described lymph node cells in culture, and in 1923 when Florence Sabin used supravital staining to clearly distinguish the living lymphocyte. Both described the peculiar nature of its motility—its intermittent stops and starts and the constant presence of a trailing cytoplasmic tail. Lewis and Webster (1921) and McCutcheon (1924) placed the phenomenon on a semiquantitative basis; the former reported a wide range of rates of movement, the average speed of a lymphocyte being a respectable 20 μm per minute, and the latter described an aver-

age rate of 16 μm per minute. Both McCutcheon (1924) and Henderson (1928) observed poor motility at room temperature and a rapid increase in the number and rate of moving lymphocytes during the course of incubation of 37°C. McCutcheon described almost 100% of lymphocytes actively moving at about 25 μm per minute even after several hours of incubation.

B. Spontaneous Lymphocyte Motility

We owe the classical description of the morphology of moving lymphocytes to Lewis' (1931a,b) study of rat lymphocytes. Most investigators still use his technique. Basically, lymphocytes or lymphoid tissues are placed in a tissue culture dish, which is then warmed. Lewis covered the lymph node sections with a fibrin clot and observed lymphocytes crawling out over the fibrin. He described the motile lymphocyte as a polarized, asymmetric cell in a configuration resembling that of a hand mirror, with a thin, advancing pseudopod, a rounded area enclosing the anteriorly placed nucleus, and a trailing tail of cytoplasm whose invariability distinguished lymphocyte movement from that of other leukocytes. Nonmotile lymphocytes are always round. In lymphocytes initiating movement he observed a constriction which squeezes the nucleus foward and then forms a rigid, elongated cytoplasmic process at the tail of the cell; concomitant with the squeezing of the nucleus, a thin veil of cytoplasm flows forward, forming a pseudopod. Lewis believed that the tail provides a motive force pushing the cytoplasm forward. A contraction cycle lasts from seconds to a minute, after which the cell rounds and ceases moving for short periods of time before forming a new tail and sending out a new pseudopod, often in a different direction. Others described this ameboid, intermittent movement, both *in vitro* (Rich *et al.*, 1939; de Bruyn, 1944, 1946) and *in vivo* using tissue chambers in rabbit ears (Clark *et al.*, 1936; Ebert *et al.*, 1940). This phenomenon aroused interest as support for the sol–gel theory of cell movement (Lewis, 1942).

Lymphocyte motility did not arouse the interest of immunologists until much later when Humble *et al.* (1956) suggested that motile lymphocytes can enter the cytoplasm of living cells, a phenomenon termed "emperipolesis" and when Sharp and Burwell reported (1960) that lymphocytes in culture move preferentially around macrophages and form strong attachments to them. Prolonged contacts between lymphocytes and macrophages were, and have since been, described in antigenically stimulated cultures (Sharp and Burwell, 1960; Salvin *et al.*, 1971; Rosenstreich *et al.*, 1972; Rosenthal *et al.*, 1975). This work has assumed added significance with the accumulation of many data demonstrating the central role of the macrophage in the initiation of the immune

response (Unanue, 1972). The role of the ameboid lymphocyte was then further extended by the careful studies of McFarland and his associates (McFarland and Heilman, 1965; McFarland *et al.*, 1966; McFarland, 1969; McFarland and Schecter, 1969) on the cytoplasmic tail that Lewis had termed a distinguishing characteristic of the lymphocyte in motion. Terming this extension of cytoplasm a "uropod," they found it to be a highly specialized part of the cell, associated not only with movement but with a wide variety of lymphocyte interactions with the environment, including other cells. They discovered that lymphocyte motility and uropod formation became pronounced in human mixed leukocyte cultures and that the uropod was the part of the cell surface by which lymphocytes made contact with the tissue culture dish, debris, macrophages, and other cells. Indeed, McFarland and associates observed motile lymphocytes swinging their uropods laterally to make such contacts and even described a lymphocyte approaching a cell, turning around and backing up in order to adhere via the uropod (McFarland, 1969).

The uropod of a motile lymphocyte is differentiated on the surface by virtue of its end being studded with microvilli, or "microspikes," 125–2000 Å in diameter and by lengths varying up to 0.8 μm (Fig. 9). Internally, the uropods also possess a distinctive ultrastructure. As observed in lymphocytes from a large number of species, the uropod almost always contains the Golgi apparatus as well as mitochondria and, in the more activated cells, rough endoplasmic reticulum. Centrioles, microfilaments, and microtubules are very prominent (McFarland, 1969; Bessis and de Boisfleury, 1971; Biberfeld, 1971b; Rosenstreich *et al.*, 1972; Rosenthal and Rosenstreich, 1974). Mcirotubules are usually oriented longitudinally along the long axis of the moving lymphocyte (Hirsch and Fedorko, 1968). The uropod also appears to serve as an area specialized for endocytosis, as endocytic vesicles, lysosomal granules, and secondary lysosomes are localized in the uropod (McFarland, 1969; Bessis and de Boisfleury, 1971; Biberfeld, 1971a,b; Rosenthal and Rosenstreich, 1974). Endocytosis of ferritin, for example, occurs preferentially at the surface of the uropod (Biberfeld, 1971b). One of the effects of this concentration of organelles in the uropod may be to bring some of them, such as the Golgi apparatus, into very close apposition to the cell surface. Bessis and de Boisfleury (1971) have indicated that the Golgi apparatus appears to migrate from its juxtanuclear position out to the end of the uropod. In spite of its relative constancy in movement, uropod formation is reversible; a uropod can be formed and retracted in minutes, and it can continually vary in length and thickness. In contrast, the advancing edge of the lymphocyte, which was found by early investigators to be the part of the cell most adherent to the substrate, is much more irregular,

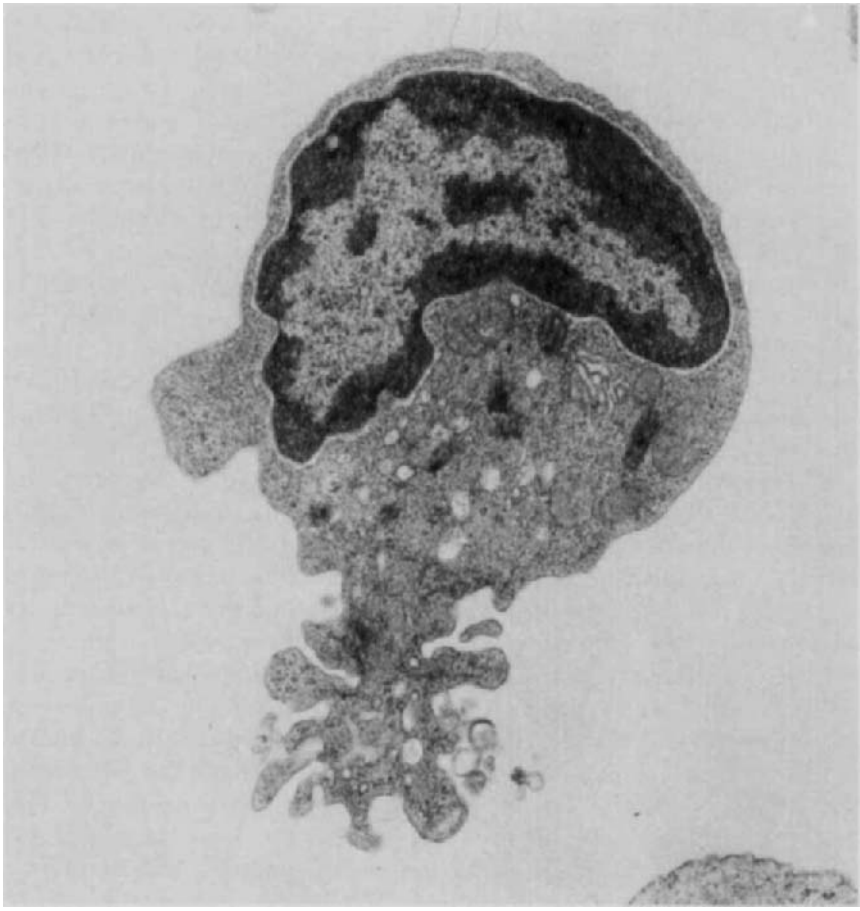

FIG. 9. Electron micrograph of a peritoneal exudate lymphocyte showing the typical uropod. The cells were harvested from the peritoneal cavity of guinea pigs and incubated at 37°C for 30 minutes. (From Rosenstreich *et al.*, 1972.)

appearing and disappearing in seconds; and it is generally devoid of major cytoplasmic organelles. It lacks the cytofibrils so prominent in the uropod, recalling Lewis' notion that the pseudopod is fluid and is pushed forward by the rigid, constricted cytoplasm of the uropod.

Most of the spontaneously moving lymphocytes in the early studies were cells from the lymph nodes or peripheral blood and thus predominantly T cells. Rosenstreich *et al.* (1972) found that all the guinea pig lymphocytes that formed uropods in tissue cultures were T cells. They

proposed that this structure was a characteristic unique to this lymphocyte class. Others have suggested a maturational requirement in that thymus cells do not manifest uropods to the same extent that lymph node cells do (Salvin *et al.*, 1971). Rosenstreich *et al.* (1972) and Rosenthal and Rosenstreich (1974) observed a correlation between uropod formation and lymphocyte activation, finding that not only is uropod formation enhanced by activation with antigen but also is enhanced in proportion to the degree of antigenic reactivity of lymphocytes from peritoneal exudate, thymus, or lymph node, as measured by thymidine incorporation. Uropod formation is clearly pronounced in activated T cells, such as those found in mixed-lymphocyte cultures and in cell-mediated cytotoxicity (Ax *et al.*, 1968; Biberfeld and Perlmann, 1970). Lymphocytes stimulated by phytohemagglutinin (PHA) form extensive uropods and move actively (Marshall and Roberts, 1965). In lymphocyte-mediated cytotoxicity reactions stimulated with PHA, lymphocytes move around target cells and then firmly adhere to them with associated, conspicuous uropod formation (Ax *et al.*, 1968; Biberfeld, 1971b; Biberfeld *et al.*, 1973). The uropod appears to be intimately involved in many aspects of inductive cellular interactions, whether stimulatory or cytotoxic. The attachment of lymphocytes to other cells appears to have dramatic effects on the lymphocytes themselves (McFarland *et al.*, 1966; Rosenthal *et al.*, 1975). For example, in mixed-lymphocyte cultures, lymphocytes adhering to macrophages by their uropod become blastic, accumulating vacuoles and granules in the uropod as they transform. Frequently, as the blast cells detach, they continue to be motile, and small lymphocytes immediately attach to the blast's uropods, the uropods on the two cells forming threadlike interconnections (McFarland *et al.*, 1966). Thus, the uropod appears to be a morphological structure associated with the activation and motility of lymphocytes, especially the thymus-derived population. It constitutes a specialized surface area for endocytosis or attachment and subserves a number of lymphocytic functions whose mechanisms are not fully understood. In contrast to T lymphocytes, only occasional B cells in culture move spontaneously and form uropods (Schreiner and Unanue, 1975a). Nonetheless, in the mouse the capacity to form a uropod is not unique to the thymus-derived population of lymphocytes. The B lymphocyte can be stimulated by anti-Ig to form a uropod and engage in translational motility.

C. Anti-Ig-Induced Motility

There is general agreement that the Ig–anti-Ig cap frequently forms on a pear-shaped lymphocyte. Structurally, the cap usually overlies a cytoplasmic projection resembling the uropod (Taylor *et al.*, 1971; de

Petris and Raff, 1972, 1973b; Unanue and Karnovsky, 1973) (see Fig. 7). Careful examination of the process of capping described previously revealed that formation of the uropod is secondary to formation of the cap, not vice versa. It is appropriate to summarize an experiment on this point; spleen cells were incubated at 0°C with fluorescent anti-Ig, then washed, allowed to settle on a dish, and warmed to 37°C. At short time intervals, the reaction was stopped by addition of a fixative and evaluated microscopically. The patches formed by anti-Ig rapidly and progressively flowed to one pole of the cell so that the percentage of tightly capped cells (i.e., with surface Ig covering less than 25% of the cell surface) rose to 50% after 2 minutes and was virtually complete at 90% after 4 minutes (Fig. 5). Under phase contrast microscopy, 30–60 seconds *after* a cell had formed a cap, an apparent constriction occurred—a contractile band seemed to encircle the shrinking cap along its outer circumference. As a result, the area under the cap was formed into a uropod, and the cytoplasm anteriorly was forced out into a pseudopod (Figs. 7 and 8). The direction of cytoplasmic flow into the pseudopod was away from the cap, so that the polarity induced by the positioning of the cap appeared to both stimulate and direct translational motility (Schreiner and Unanue, 1976a).

To study motility, we developed conditions whereby one could relate capping with the development of actual translatory motion (Unanue *et al.*, 1974a; Schreiner and Unanue, 1975a,b). Lymphocytes were allowed to settle in low density on a culture dish at 37°C, anti-Ig was added, and the reaction was stopped after various periods of time. We first found that untreated B cells would settle on the dish and remain round; very few would manifest a motile morphology. Addition of anti-Ig changed this completely in that up to about one-half of the B cells then became ameboid. Direct microscopic examination showed the lymphocytes moving, albeit slowly, and at random. Normal rabbit IgG or anti-Ig added to T cells did not stimulate motility. Thus, we concluded that the anti-Ig reaction in the membrane was the direct cause of the translatory motion of the B cells. The stimulation of movement was transient under the *in vitro* conditions, lasting between 15 and 25 minutes and occurring concomitantly with the endocytosis of the capped receptors.

Many of the requirements and conditions for capping and motility are identical. Both processes require bivalent antibodies and energy metabolism; both take place at a faster rate at 37°C than at 20°C or 24°C. Neither process requires extracellular Ca^{2+} (Taylor *et al.*, 1971; Unanue *et al.*, 1974a; Schreiner and Unanue, 1975a). Furthermore, colchicine enhances slightly anti-Ig-induced motility.

The stimulation of motility by a surface ligand reaction raised several

questions. Can one dissociate capping and translatory motility, or are both always associated? Does any surface–ligand interaction stimulate motility? Is it possible that anti-Ig, under appropriate circumstances, stimulates directional movement?

1. Relationship between Capping and Motility

Several manipulations clearly dissociated capping and translatory motion (de Petris and Raff, 1973b; Unanue *et al.*, 1974a; Schreiner and Unanue, 1975a). Lymphocytes cap equally well whether maintained in suspension or planted on a solid substrate. Yet motility is inhibited in the former situation. More conclusively, a series of chemicals were found to markedly reduce translatory motion without impairment of capping. These include cytochalasin B, diisopropylfluorophosphate (DFP), and drugs that modulate cAMP levels.

Incubation of B cells at 37°C with cytochalasin B at low doses (1–10 μg/ml) completely stops the motile response of the B cell, yet it affects the extent of capping very little. In cytochalasin B-treated cells the rate of capping is somewhat slower, the area of the surface occupied by the cap is larger, and the complexes in the cap zone are found in several dispersed aggregates (Unanue *et al.*, 1974a). It is possible that these changes are, in fact, a reflection of the effects of the translatory process itself on the cap, suggesting that, although motility per se is not causing capping, nevertheless it can influence its structure.

DFP, an irreversible inhibitor of serine esterases, has been used to examine whether several functions of cells may be related to enzyme activation. Degranulation of mast cells by antigen (Becker and Austen, 1966), the response of neutrophils to a chemotactic stimulus (Becker and Ward, 1967), and phagocytosis by macrophages (Pearlman *et al.*, 1969) can be markedly reduced by DFP (reviewed in Becker and Henson, 1973). In the mast cells, treatment with DFP does not alter the release of histamine produced by antigen if the inhibitor is washed away before exposure to antigen (Becker and Austen, 1966). This supports the hypothesis that the inhibitor is not toxic by itself but is acting by inhibiting an enzyme activated during the interaction with antigen. DFP does not inhibit Ig capping in B lymphocytes but reduces the stimulated motility (Unanue *et al.*, 1974a; Becker and Unanue, 1976). The following conclusions have been made: (1) treatment of B cells with DFP does not alter the motile response if the inhibitor is washed away prior to exposure to anti-Ig antibodies; (2) DFP inhibits the contractile response only if present during the time that anti-Ig is bound to the membrane, suggesting that the target of the inhibitor is an enzyme activated by the ligand; (3) the inhibition of the response is dependent upon the concen-

tration of DFP and time of contact of cells with it; (4) the effects of DFP on anti-Ig induced motility are counteracted by tosyl arginine methyl ester (TAME) and benzoyl arginine methyl ester (BAME), substrates for trypsin or trypsinlike enzymes. This suggests that these two esters are competing for the active site of the putative activated enzyme. Furthermore, TAME and BAME were found to be *reversible* inhibitors of the anti-Ig-induced motility. Last, when two phosphonate esters of similar chemical composition but different phosphorylating capacity were tested, only the phosphorylating compound inhibited anti-Ig-induced motility. Apparently there is a critical enzymic step in the lymphocyte needed to activate the cell for the motile response that follows capping.

The anti-Ig-stimulated increase in motility of B lymphocytes is extremely sensitive to modulation by cyclic nucleotides (Schreiner and Unanue, 1975a). Dibutyryl cAMP and theophylline exogenously applied or agents known to increase intracellular levels of cAMP, such as cholera enterotoxin or isoproterenol, a β-adrenergic agonist, completely prevent stimulated movement. These agents also inhibit the spontaneous motility of T lymphocytes (Schreiner and Unanue, 1975a; Unanue and Schreiner, 1975). Similar effects of cAMP on the motility of other mammalian cells have been observed (Johnson *et al.*, 1972; Pick, 1972; Rivkind and Becker, 1972). Conversely, exogenous cGMP or cholinergic drugs presumed to increase intracellular cGMP are excellent stimulators of motility for both T and B cells, as has been seen with other leukocytes (Estensen *et al.*, 1973). The suggested possibility of a role for cyclic nucleotides in the early response of the B cell to antigen remains to be proved. It is interesting that acetylcholine seems to stimulate B cells to move to the same extent that anti-Ig does and for virtually the same period of time, allowing for the possibility that the initial move of the capped lymphocyte is a preprogrammed "run." The effects of cholinergic drugs are seen with concentrations as low as 10^{-9} M and are inhibited by atropine, strongly suggesting that the drugs are acting by binding to a cholinergic receptor of the muscarinic type. Other effects of cholinergic drugs have been reported, such as a small enhancement of DNA synthesis (Hadden *et al.*, 1973; Strom *et al.*, 1973) or an increased activity of cytotoxic T cells (Strom *et al.*, 1974b). The latter effect appears to be muscarinic in its sensitivities, although inhibition by α-bungarotoxin, an inhibitor of nicotinic receptors, was also reported (Strom *et al.*, 1974b). We have not seen binding of α-bungarotoxin to B cells (Unanue and Schreiner, 1975). The cytoplasmic locus affected by cAMP appears to be the microtubular system, for colchicine is effective in counteracting its inhibitory effect on motility. This has suggested that increased stabilization of microtubules

may impede lymphocyte movement. Cyclic AMP has been shown to have a polymerizing effect of microtubules in other cell systems (Section V).

2. *Specificity of Anti-Ig-Induced Motility*

A second question concerned the specificity of the ligand-induced motile response. Anti-Ig antibodies are unique in inducing a motile response in contrast to most other ligand–membrane reactions. Antibodies to various alloantigens do not stimulate motility in B or T cells, nor does heterologous antilymphocyte antibody—a rabbit anti-mouse lymphocyte antibody (ALS) (Schreiner and Unanue, 1975b). ALS binds to the surface of B and T cells but does not cap unless a second antibody is added. Subsequent to ALS capping, there is no stimulation of a contractile response. Of great interest is the observation that B cells exposed to ALS are no longer able to show a motile response to anti-Ig antibodies. [Also, the normally spontaneous motility of T cells is suppressed by ALS.] To summarize one experiment, B cells were first incubated with ALS in the cold, washed, and then exposed to anti-Ig at 37°C. The anti-Ig bound to the B cells and capped, but the postcapping motile response was abrogated. The inhibitory effect of ALS was not seen in cells pretreated with colchicine, suggesting that its effect may be associated with increased microtubular activity (Schreiner and Unanue, 1975b) (Fig. 10). The effects of ALS

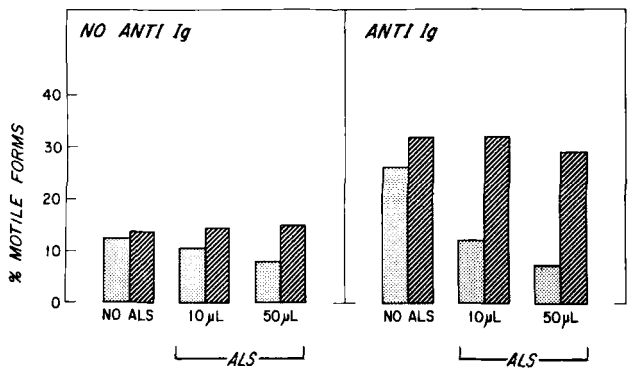

FIG. 10. Spleen lymphocytes were planted on a culture dish and incubated for 10 minutes with anti-Ig, antilymphocyte antibody (ALS), or both (total volume: 500 μl). The panel to the left shows that lymphocytes incubated with ALS do not develop motile changes. The panel to the right shows the combined effects of anti-Ig antibodies and ALS. Anti-Ig antibodies alone trigger a motile response which increases slightly after colchicine treatment; anti-Ig antibodies, however, do not trigger a response in ALS-treated cells unless colchicine is also added. ▩, no colchicine; ▨, colchicine. (From Schreiner and Unanue, 1975b.)

clearly indicate that ligand–receptor effects trigger different, and sometimes antagonizing, cytoplasmic responses.

Prior to these experiments, some reports had indicated that ALS-treated lymphocytes moved poorly. Biberfield *et al.* (1969) observed, in tissue culture experiments involving cytotoxic lymphocytes and target cells, a marked inhibition of motility of ALS-treated lymphocytes around the target cell. Lee *et al.* (1968) found that ALS inhibited the migration of lymphocytes out of a spleen fragment.

Unpublished experiments of G. F. Schreiner and A. K. Abbas (unpublished observations, 1975), showed that B cells could be triggered to move after interaction with soluble antigen–antibody complexes. A recent report of Alexander and Henkart (1976) demonstrated that human lymphocytes were stimulated to move by antigen–antibody complexes attached to dishes. This movement was inhibited by low temperatures, cytochalasin B, and chelating agents.

3. *Lymphocyte Chemotaxis*

Under appropriate circumstances, B cells are able to recognize a gradient of anti-Ig antibodies and move directionally toward it (Ward *et al.*, 1976; G. F. Schreiner and E. R. Unanue, unpublished observations, 1976). Before discussing this point, we shall briefly review lymphocyte chemotaxis.

Chemotaxis of lymphocytes has been a problem of interest equal to that of chemotaxis of other leukocytes. Yet far less progress has been made in determining whether or not a lymphocyte can be induced to move toward something, whether substance or cell, than progress in the impressive understanding we now have of many aspects of chemotaxis in other leukocytes, such as neutrophils, monocytes, and even eosinophils, extensively analyzed in two recent books (Sorkin, 1974; Wilkinson, 1974). Early attempts by Maximow, Schade and Mayer, Dixon and McCutcheon, and Harris (reviewed in Henderson, 1928; Harris, 1954, 1960) failed to show chemotactic movement of lymphocytes toward bacteria, starch grains, or partially digested tissues, when the movement of lymphocytes in tissue culture around a planted clump of material was observed directly.

Progress in studying leukocyte chemotaxis arose from the introduction of a new method by Boyden (1962). Boyden devised a chamber with two compartments separated by a filter through which cells can crawl. The putative chemotactic factor is placed in the lower chamber and the cells in the upper chamber, the diffusion of the factor creating a gradient. The migration of cells through the filter is evaluated microscopically. This technique, if properly applied, permits the differentiation between agents

that induce cells to move only when there is a concentration gradient, and are thus chemotactic, and agents that stimulate random movement (Zigmond and Hirsch, 1973). The latter is assayed by placing the substance on both sides of the filter at the same concentration, thus destroying any gradient, and then evaluating the movement of cells into the filter, and detectable increase being due solely to stimulated random motility. The Boyden method has proved successful in investigations of neutrophil and monocyte motility. Its potential remains to be realized with respect to lymphocyte chemotaxis.

One of the earliest reports on lymphocyte chemotaxis came from Ward *et al.* (1971). They showed direct migration of rat lymphocytes in response to a factor released by antigen-stimulated guinea pig lymph nodes. This has been confirmed by using purified T cells and active fluids from mixed lymphocyte reactions (Ward *et al.*, 1976). Chemotaxis of lymphocytes toward activated complement components or bacterial culture filtrates, as has been described for neutrophils, was not observed (Ward *et al.*, 1971). Higuchi *et al.* (1975) have reported that thoracic duct cells respond to a fragment of IgM cleaved by a neutrophil protease. This phenomenon has not, however, been differentiated from stimulation of random movement, nor have purified cell populations been studied. Russell *et al.* (1975) have made the very important observation that blast-transformed lymphocytes have much greater motility than normal lymphocytes. They used cells from human lymphoblastoid lines or murine lymphoblasts generated by the contact-sensitizing agent oxazolone. Human lymphoblasts increased their random background motility when exposed to endotoxin-activated plasma but also migrated chemotactically toward it. The population of sensitized murine cells showed increased motility and a poor chemotactic response. One must interpret the positive results with caution since these were from transformed cell lines, the behavior of which may not accurately reflect in all instances the cell source from which they were derived. This does, however, constitute a very promising approach.

Our first attempts to study chemotaxis with anti-Ig antibodies employed murine spleen cells and modified Boyden-type chambers (Schreiner and Unanue, 1975b). We found that, indeed, lymphocytes were stimulated to move though the filter by anti-Ig, albeit to a small extent, but that the movement was independent of gradients; that is to say, we found the same number of cells migrating into the filter whether anti-Ig was in the bottom compartment or in both the upper and lower ones. We have continued our studies using rat lymphocytes which appear to be better suited for chemotactic studies. A large amount of anti-Ig usually results in a small increase in migration whether or not a gradient is

created. However, using usually small, critical concentrations of anti-Ig, we have found true directional motility of rat B cells toward an anti-Ig gradient (Ward *et al.*, 1976). The extent of migration of the lymphocytes in the filter is limited, and seldom do the cells crawl entirely through, as do the nutrophils or macrophages.

This we believe is an important observation that raises the possibility that antigen molecules, in analogy to anti-Ig, may indeed act as chemoattractants for lymphocytes. Work along these lines is in progress.

There are several observations on the movement of lymphocytes *in vivo* that correlate quite well with the *in vitro* observations on T-cell and B-cell motility and the role of the uropod in the motile lymphocyte. In their description of the early stages of experimental allergic neuritis, Astrom *et al.* (1968) observed activated lymphocytes adhering by means of uropods to endothelial cells of blood vessels adjacent to nerve tissue; the lymphocytes proceeded to migrate through the endothelium and to invade the interstices of nerve fibers. Although this demonstrated a motile response of T cells to antigen depots, extensive work on the recirculation of lymphocytes has emphasized that B-cell motility may also constitute one of the early responses to the presence of foreign antigen in the host. The large majority of normally recirculating lymphocytes are T cells, which, when transferred to another animal intravenously, home first to the spleen and then to the peripheral lymph nodes, occupying the thymus-dependent areas (Born and Bradfield, 1968, Desousa and Parrot, 1968). Similarly, B cells home quite specifically to the follicular areas of spleen and lymph nodes normally populated by bone marrow-derived cells (Durkin *et al.*, 1972; Howard *et al.*, 1972). Sensitized B cells, however, preferentially migrate to lymph nodes draining the area in which the antigen to which they are sensitized has been injected (Durkin and Thorbecke, 1972). Their migration to the follicular areas of these nodes takes on added significance because antigen tends to localize in the follicles for prolonged periods of time (Nossal and Ada, 1971). B cells normally do not recirculate from blood to lymph to nearly the extent that T cells do (Strober and Law, 1971; Howard *et al.*, 1972; Sprent, 1973). Yet after antigen stimulation, specific, hapten-primed B cells appear to enter the lymph in great numbers, suggesting an antigen-stimulated movement of B cells into the circulation (Strober, 1972). It remains to be determined whether the mobilization of specific B cells and their migration into nodes containing that antigen reflect specific migration or passive retention of B lymphocytes stimulated by antigen to move randomly into general circulation. In either case, one must conclude that this constitutes an *in vivo* sequence resembling the activation observed in the chemotaxis chamber and apparently central to the organi-

zation—or reorganization—of lymphoid cells during the course of an immune response.

IV. Redistribution Induced by Other Ligands

A. Molecules of the Major Histocompatibility Complex (MHC)

Immunocytological studies have been done in man (Kourilsky *et al.*, 1972; Silvestre *et al.*, 1972; Menne and Flad, 1973) and in the mouse (Taylor *et al.*, 1971; Karnovsky *et al.*, 1972; Kourilsky *et al.*, 1972; Unanue *et al.*, 1972b; de Petris and Raff, 1974; Stackpole *et al.*, 1974a,b). We reproduce the description given by Kourilsky *et al.* (1972) on the redistribution of HL-A-anti-HL-A antibodies in human peripheral blood lymphocytes. This description is typical of the distribution pattern of most molecules of the MHC:

> Three stages of the evolution of the fluorescent pattern could be schematically distinguished.
>
> Stage A: When examined immediately after the staining procedure at 0°C, almost every lymphocyte showed a fluorescence dispersed all over the cell surface. The fluorescence was diffuse or reticular, or microgranular, with coarser dots in the case of indirect labeling, and had a ring-like appearance when the edges of the cells in suspension were tangentially seen.
>
> Stage B: Following incubation at 37°C, surface fluorescence gathered rapidly on most cells, forming large spots of fluorescence, with increased brightness and leaving zones of the cell surface unlabeled.
>
> Stage C: On a limited number of lymphocytes the clustering of fluorescent antibodies ended in the formation of a single crescent or cap at one pole of the cell.
>
> Following 2–3 h incubation of antibodies at 37°C, the membrane fluorescence staining tended to become faint and disappeared on some cells.

The same pattern has been observed in murine cells—thymus, peripheral T or B cells, i.e., some degree of clustering produced by the antibodies, a variable and limited capping which takes long periods of time to develop, and a relatively long persistence of some of the complexes on the membrane. In the studies of Kourilsky *et al.* referred to above, about 20% of cells developed caps, even after 2 hours of incubation. An approximately similar percentage of capped cells was found in the mouse, also after prolonged incubation (Unanue *et al.*, 1972b; Stackpole *et al.*, 1974a). The limited interiorization of these complexes will be further discussed in Section VI.

The redistribution of MHC molecules is enhanced by adding antibodies to the alloantibodies; i.e., by adding an anti-antibody. In this case, three major changes are noted. The first is an increase in the percentage of

cells capped or with large, disseminated aggregates; this increase is variable though not extensive. In man, for example, the addition of anti-Ig produces caps in about 45–50% of the cells, which is about double the amount found by adding only alloantibody—"but in no combination could complete polarization of fluorescence on all cells be observed" (Kourilsky *et al.*, 1972). In the mouse, figures are about the same or somewhat lower (Kourilsky *et al.*, 1972; Unanue *et al.*, 1972b; Stackpole *et al.*, 1974a). A second change is an increase in the rate of capping, which is usually complete by 30 minutes instead of 1–2 hours. Finally, there is a definite increase in the rate of disappearance of the complexes, much of it by endocytosis (Section VI).

Redistribution of MHC molecules either by direct ligand interaction or after a two-antibody addition is a poorly organized process. In contrast to Ig capping during which the entire complex readily flows into a single tight mass, the redistribution of MHC is irregular, many cells exhibiting few (sometimes three to four) large masses; occasionally, bipolar or tripolar caps are noticed. Thus, the process looks poorly coordinated, as though the complexes are pulled to various zones of the cell surface (Unanue, 1976).

Another characteristic of redistribution produced by antibodies to MHC molecules is that it is not accompanied by gross changes in cell shapes or by induced motility, either by using one or two antibodies (Schreiner and Unanue, 1975b), unlike the morphological changes seen in Ig-anti-Ig interactions. This is also true with other ligands (Section III). In our view, this failure to trigger a motile response is most likely responsible for the observation made by Stackpole *et al.* (1974b) that caps produced by anti-H-2 (with anti-Ig as a second layer) bear no relationship to the Golgi region of the cell. While Ig caps are always associated with the centrosphere area, H-2 (or TL or θ) caps are distributed at random and without any relationship to the perinuclear area of the cell.

The studies in man have involved antibodies against the serologically defined specificities. Those in the mouse incorporated antibodies reacting with all the H-2 determinants or with only the K or D end molecules. Very similar results have been obtained with antibodies to β_2-microglobulin, or to the I-region molecules of the H-2 complex of the mouse.

β_2-Microglobulin is a 12,000 molecular weight protein (Berggard and Bearn, 1968) found in abundance in the cell surfaces of lymphocytes and other cells. β_2-Microglobulin in the lymphocyte is, in part, associated with molecules of the MHC (Grey *et al.*, 1973; Nakamuro *et al.*, 1973; Creswell *et al.*, 1974; Peterson *et al.*, 1974). Anti-β_2-microglobulin antibodies are inefficient in redistributing β_2-microglobulins, which usually

require the addition of a second antibody (Neauport-Sautes *et al.*, 1974). Even with a second layer of antibody, redistribution is slow and irregular (i.e., into multiple large patches), typical caps forming in only about 40% of the cells, at the most. Patches or caps of antibody-β_2-microglobulin also contain HL-A antigens (Poulik *et al.*, 1973; Neauport-Sautes *et al.*, 1974; Solheim and Thorsby, 1974).

Redistribution of I-region-associated antigens in the mouse was studied by using antibodies made in appropriate congenic mice (Unanue *et al.*, 1974b). Ia antigens found mostly in B cells were bound diffusely throughout the cell surface and did not redistribute into caps, even after prolonged incubation at 37°C. The addition of anti-Ig antibodies produced capping in only about two-thirds of the cells. In order to study capping of anti-Ia by anti-Ig antibodies, the surface Ig of B cells had to be cleared first (if not, the anti-Ig would bind to both the surface Ig and anti-Ia antibodies). After treatment with anti-Ig, the B cells still maintained Ia antigens diffusely throughout the surface. These Ia antigens could be studied for redistribution.

The redistribution of murine TL antigens, which bear similarities to the H-2 molecules, will be described in the section on antigenic modulation (Section VI,C).

B. Fc Receptor

The redistribution of Fc receptors has not been studied extensively. For immunocytochemical studies, the Fc receptors have been detected by the use of fluoresceinated aggregated IgG (Dickler and Kunkel, 1972; Dickler, 1972) or with complexes of antibody with either fluorescent-labeled antigen (Dickler, 1974; Abbas and Unanue, 1975; Forni and Pernis, 1975) or radioiodinated antigen (Basten *et al.*, 1972; Eden *et al.*, 1973). Aggregated Ig or antigen–antibody complexes bound to murine or human B cells redistribute into large patches and caps at 37°C in a manner analogous to surface Ig (Anderson and Grey, 1974; Abbas and Unanue, 1975; Forni and Pernis, 1975). In one study in man, no capping was observed (Dickler, 1974) with Ig aggregates of very large sizes, which were found as irregular, large deposits binding to the cell membrane. Possibly the size and/or shape of the complexes may be critical for their redistribution; thus, one would predict that a certain size of the aggregates or complexes may be necessary to ensure effective cross-linking of the Fc sites. Complexes with very large polymers of treated IgG, in which there may be distortion of part of the Fc fragments, may interact to a limited extent with Fc receptors and produce limited capping. One point to note is a recent observation that fluoresceination of IgG produces a marked reduction in its binding to the Fc receptor

(Thrasher *et al.*, 1975). Unpublished observations of G. F. Schreiner and A. K. Abbas (1975) indicate that soluble antigen–antibody complexes trigger murine B cells to move. Human cells were stimulated to move when planted on dishes coated with antigen–antibody complexes (Alexander and Henkart, 1976).

C. C3 Receptor

Lymphocytes have receptor(s) for components of complement (C) (reviewed by Nussenzweig, 1974). Antigen–antibody complexes bound to the C3 receptor readily redistribute into caps (Eden *et al.*, 1973; Gormus and Shands, 1975). Gormus and Shands (1975) used live *Salmonella* coated with antibody and fresh serum. The bacteria-antibody-C initially bound diffusely all over the cell, capping by about 30–45 minutes. The bacterial complexes were then shed from the membrane.

D. Lectins

The evolution of research on the capping of lectin-binding sites in the lymphocyte membrane parallels that on Ig capping. The first investigators examining the distribution of Con A, the most intensively studied lectin, described it as a diffuse, random pattern of sites on the round lymphocyte but noted that fluorescein-labeled Con A in motile lymphocytes was concentrated over the uropod, the rest of the membrane being devoid of label (Osunkoya *et al.*, 1970; Smith and Hollers, 1970; Stobo *et al.*, 1972a). Some of these investigators concluded that the uropod—as a specialized part of the lymphocyte—possessed more glycoproteins, probably of higher affinity, with the resultant localization of the lectin there. With the observation of Taylor *et al.*, (1971) that the previously observed asymmetry of surface Ig distribution was the result of molecular movements, interest focused on the possibility that lectins, principally Con A, might similarly induce the redistribution of their binding sites. It soon became apparent that lectin-binding sites could patch and cap, that there were many similarities to, yet differences from, Ig capping.

Con A binds to most plasma membrane glycoproteins bearing α-mannopyranosyl molecules, including surface Ig (Hunt and Marchalonis, 1974). As one would expect, the glycoprotein-binding sites are heterogeneous with respect to affinity and other physical properties (Allan *et al.*, 1972). Labeled Con A binds to B and T lymphocytes equally efficiently, although only the latter respond mitogenically to the soluble lectin (reviewed in Greaves and Janossy, 1972). Con A binding sites are randomly dispersed on the lymphocyte surface (de Petris and Raff, 1974; de Petris, 1975). The addition of Con A to lymphocytes initially induces clustering or patching of the Con A binding sites. This patching requires a multivalent

ligand and is independent of metabolic energy (Unanue *et al.*, 1972b; Yahara and Edelman, 1972, 1973b; Loor, 1974; de Petris and Raff, 1974; de Petris, 1975). The clustering, as expected, requires receptor mobility, for it is prevented by prefixation (Inbar and Sachs, 1973). Similar results on patterns of distribution and redistribution have been observed on other cell types, including transformed fibroblasts and neutrophils (Nicolson, 1971, 1972, 1973; de Petris, *et al.*, 1973; Ukena *et al.*, 1974; Rosenblith *et al.*, 1973).

Depending upon experimental conditions, patching of Con A receptors in B or T lymphocytes is followed by capping. Capping of these lectin-binding sites is never as efficient as that of surface Ig. First, the percentage of caps usually ranges between 10 and 50%. Second, it occurs much more slowly than Ig capping—usually 30–60 minutes are required (Comoglio and Gugliemone, 1972; Yahara and Edelman, 1973b; Stackpole *et al.*, 1974b). As with Ig, there is a valency requirement. Succinylated Con A, which is bivalent, induces patches but does not cap well; normal, tetravalent Con A or divalent Con A cross-linked by antibody does cap (Gunther *et al.*, 1973). Capping of Con A may be enhanced by antisera to Con A (Inbar and Sachs, 1973; Stackpole *et al.*, 1974b). Con A receptors in lymphocytes do not cap at 4°C or in the presence of inhibitors of cell metabolism (Comoglio and Gugliemone, 1972; Sallstrom and Alm, 1972; Unanue *et al.*, 1972b, 1973; Yahara and Edelman, 1972; Loor, 1974; Rutishauser *et al.*, 1974; Stackpole *et al.*, 1974b). More distinctly than is the case with surface Ig, capping of Con A binding sites is critically dependent on the concentration of Con A. Low concentrations of Con A, even levels incapable of saturating all the binding sites, cap well (Unanue *et al.*, 1972b, 1973; Inbar *et al.*, 1973; Yahara and Edelman, 1973b; Loor, 1974). High concentrations—usually above 10–50 μg/ml—result in a progressive inhibition of capping (Greaves and Janossy, 1972; Greaves *et al.*, 1972; Gunther *et al.*, 1973; Yahara and Edelman, 1973b; Loor, 1974; de Petris, 1975). Con A caps are observed on lymphocytes in active movement (Loor, 1974; Unanue and Karnovsky, 1974) and are situated on the uropod trailing the ameboid lymphocyte (Osunkoya *et al.*, 1970; Smith and Hollers, 1970; Greaves *et al.*, 1972; Loor, 1974; Unanue and Karnovsky, 1974; de Petris, 1975). Once formed, the lymphocyte's Con A cap is not rapidly cleared from the membrane, unlike the rapid endocytosis of complexed surface Ig; pinocytosis occurs, but is relatively slow (Loor, 1974; de Petris, 1975). Metabolically active cap formation of Con A receptors has also been described in nonlymphoid cells, including neutrophils (Ryan *et al.*, 1974b; Oliver *et al.*, 1975), hamster ovary cells (Storrie, 1975), ovarian granulosa cells (Albertini and Clark, 1975), and amebocytes (Pinto da Silva and Martinez-Palomo, 1974).

There are some variations in capping among lymphoid cells. In the chicken, bursal B cells do not cap well, remaining diffusely stained after prolonged incubation with Con A in contrast to thymus cells (Sallstrom and Alm, 1972). Others have observed Con A capping in both T and B murine lymphocytes (Greaves *et al.*, 1972; Unanue *et al.*, 1972b; Loor, 1974); although thymus-derived cells in mice and humans usually seem to cap more readily (Sallstrom and Alm, 1972; Yahara and Edelman, 1973b). Malignant lymphocytes cap Con A poorly (Inbar and Sachs, 1973; Inbar *et al.*, 1973; Ben-Bassat *et al.*, 1974; Mintz and Sachs, 1975).

There is evidence for a role of the cytoskeletal system in the capping of Con A-binding sites. In striking contrast to Ig caps, capping of Con A is exquisitely sensitive to the inhibitory influence of cytochalasin B (de Petris, 1974, 1975; Unanue and Karnovsky, 1974), suggesting that microfilament activity might be of central importance. In an extension of Berlin's suggestion that colchicine-sensitive proteins may be important for the directed movement of membrane proteins (Berlin *et al.*, 1974) (see Section V), Edelman and his co-workers have implicated microtubule-like proteins as exerting a controlling influence on the redistribution of Con A receptors and other surface proteins (Yahara and Edelman, 1972, 1973a,b, 1974; Edelman *et al.*, 1973). They based this on the observation that high concentrations of Con A inhibit capping of Con A binding sites and also the capping of other receptors, including Ig (Yahara and Edelman, 1972, 1973a,b; Loor, 1974). This inhibition can be reversed by exposure of the cells to conditions or agents acting to depolymerize microtubules. Thus, preexposure of lymphocytes to a temperature of 4°C or to colchicine or vinblastine allows lymphocytes in the presence of high concentrations of Con A to cap their lectin-bound proteins and further permits the cocapping of surface alloantigens by antisera (Unanue *et al.*, 1972b; Edelman *et al.*, 1973; Yahara and Edelman, 1973a,b, 1975a,b; de Petris, 1974, 1975). The Con A inhibition of capping—whether of Con A receptors or other receptors—requires the tetravalent form of Con A; a succinylated divalent form does not cap and does not inhibit capping of other surface proteins unless cross-linked by anti-Con A antibodies (Edelman *et al.*, 1973; Gunther *et al.*, 1973). Although microtubules appear to play a restrictive role, the integrity of microfilaments is still essential inasmuch as colchicine-treated lymphocytes exposed to cytochalasin B are unable to cap (de Petris, 1974, 1975; Unanue and Karnovsky, 1974). These observations led to the suggestion that Con A stimulates the physical anchoring of its receptors and other membrane proteins to microtubules, which prevents redistribution (Edelman *et al.*, 1973). Ig capping by Con A was also prevented in lymphocytes bound to nylon wool fibers coated with Con A (Rutishauser *et al.*, 1974) or to Con A bound to latex beads or platelets (Yahara and Edel-

man, 1975b). A discussion of the mechanisms of Con A capping in comparison to Ig capping follows (Section V).

The relation between redistribution of Con A and its mitogenic property has been examined. Those concentrations of Con A that cap are precisely those within the mitogenic range; the dose of Con A that does not cap or that inhibits capping of other molecules also inhibits mitogenesis (Edelman *et al.*, 1973). However, other experiments clearly indicated that there was no relationship between the capping process per se and mitogenesis. Gunther *et al.* (1973) prepared a succinylated derivative of Con A that, at neutral pH, has half of the molecular size of the native molecule. Succinylated Con A retains its carbohydrate-combining capacity, binds to lymphocytes, and is as potent a mitogen as the native molecule but has two particular characteristics: (1) in contrast to the tetrameric molecule, the dose-response curve does not drop off as rapidly with increasing dose, thus, native and succinylated Con A both produce optimal mitogenesis at 3–6 μg/ml; at 10–25 μg/ml, native Con A stimulates very little, but succinylated Con A still stimulates at about the same level; and (2) succinylated Con A does not cap (Gunther *et al.*, 1973). The observation that B cells bind Con A but are not stimulated also indicates a lack of relationship between binding, redistribution, and activation.

E. Other Surface Molecules

Most human T lymphocytes bear receptors for sheep erythrocytes, the nature of which is not known. Sheep red cells bound to human T cells can redistribute, albeit slowly, into partial or complete caps and then dissociate from the membrane without being interiorized in vesicles (Yu, 1974). During redistribution, some of the lymphocytes adopt ameboid morphology.

Most of the ALS made in heterologous species did not redistribute into caps but remained diffuse throughout the cell surface with little patching and only slight endocytosis (Unanue *et al.*, 1972b). Addition of antibody to the ALS then induced cap formation, but the caps remained cell bound for long periods with only a slight degree of endocytosis. This is one clear example of a lack of relationship between capping and interiorization.

The relationship between ALS and changes in cell shape was referred to before (Section III). The θ antigen of thymocytes segregates into caps, but only when two antibodies are present (Taylor *et al.*, 1971; Stackpole *et al.*, 1974a,b; de Petris, 1975).

The binding of virus to membrane receptors on lymphocytes results

in the spontaneous capping of these receptors, in the absence of antibody, when the cells are warmed from 0°C to 37°C. Cap formation of these receptors is pH dependent as well as temperature dependent and reversibly inhibited by uncouplers of oxidative phosphorylation (Hinuma *et al.*, 1975). Similar capping of viruses or viral antigens, usually in the presence of specific antibody, has been reported for other cell types (Ehrnst and Sundquist, 1975; Rutter and Mannweiler, 1973; Phillips and Perdue, 1974; Joseph and Oldstone, 1974). The suggestion that capping of viral antigens enhances viral persistence and resistance to antiviral immune cytolysis raises the possibility that the membrane mobility of virus receptors or antigens may affect the *in vivo* interaction between viruses and the immune system (Joseph and Oldstone, 1974).

V. Mechanisms of Redistribution

We shall discuss the possible bases of the two systems, the capping of surface Ig and the capping of Con A binding sites. There are many similarities in the surface redistribution of both Ig and lectin-binding sites. There are also some surprising differences, sufficient to suggest that capping may not be a common property of all properly cross-linked surface molecules, but may actually consist of quite different mechanisms serving biological ends that may be similar in appearance but very disparate in consequence. An understanding of the mechanisms of capping is critical to any analytic approach to the subject of transmembrane controls. The importance of the interrelationship between membrane stimulation and cytoplasmic response extends beyond capping and the cellular immune responses; it is crucial to any future understanding of the most basic biological phenomena, including cellular interaction, mitogenesis, and differentiation.

The redistribution of Ig and other components of the lymphocyte membrane by ligands indicates that these components are free to diffuse within the plane of the plasma membrane. Indeed, the redistribution phenomenon, together with other observations, supports the fluid mosaic model of membrane structure proposed by Singer and Nicolson (Singer, 1971, 1974; Singer and Nicolson, 1972). The widely accepted fluid mosaic model describes the general constitution of the membrane proteins and lipids. It is based on the thermodynamic considerations that the proteins and lipids are organized in such a way in the membrane as to maximize their low energy state but yet carry out their functions. The phospholipids are organized as a bilayer (Davson and Danielli, 1952; Wilkins *et al.*, 1971) with the hydrophilic polar groups oriented to the aqueous environments of the cytoplasm and the external medium, and the hydro-

phobic fatty acids forming the internal, nonaqueous portion of the membrane. Interacting with the phospholipids are integral and peripheral proteins. Integral proteins are defined operationally as proteins extracted from the membrane only by materials that break hydrophobic interactions, such as organic solvents or detergents. Integral proteins are presumed to be amphipathic with a segment containing sufficient hydrophobic amino acids to be embedded within the internal core of the lipid layer and polar hydrophilic groups capable of interacting with aqueous elements outside or inside of the membrane. There are examples of integral proteins from the extensive biochemical studies of the red cell membrane (Bretscher, 1971; Marchesi *et al.*, 1972). In the lymphocyte, molecules from the MHC would be considered as integral protein, and so is surface Ig. Surface Ig is the special case of a protein that can be soluble in aqueous solution, but is in the membrane in such a form as to behave as an integral component (Section IIA). Peripheral proteins, on the other hand, are loosely bound to the membrane and can be removed from it by relatively mild treatment, such as altering pH or ion chelation. An example is spectrin on the inner side of the red cell membrane.

From the fluid mosaic model, one envisions that integral proteins are capable of translational motility within the plane of the fluid matrix and have no long-range pattern of organization. Restrictions of the model have been analyzed by the proponents. Evidence supporting the lateral diffusion of lipid and proteins in the membrane has emerged from several areas of research. By using electron spin resonance or nuclear magnetic resonance methods, it was shown that lipids display fast lateral motion (Kornberg and McConnell, 1971; Scandella *et al.*, 1972; Jost *et al.*, 1973; Lee *et al.*, 1973). Edidin (1974) recently reviewed this subject. The diffusion constants of lipid in natural membranes have been estimated to vary from about 0.5 to 11.0×10^{-8} cm^2 sec^{-1}. Accurate estimation of the lateral diffusion of membrane proteins in their natural state without the effect of ligands is difficult but has been done in various cells. We analyze four experiments in which diffusion rates of membrane proteins have been calculated.

In the first experiment Frye and Edidin (1970) used heterokaryons formed by fusion with Sendai virus of human and mouse lines. After fusion, aliquots of cells were stained with antibodies to mouse H-2 or to human antigens by the use of different fluorochromes. The pattern of protein distribution changed from an initial one in which each species' complement of antigen was localized in exclusive hemispheres to a complete mosaic (by 40 minutes) in which all proteins were totally mixed. Regardless of the effect of the virus on the system, these experiments gave an idea of the free mobility of membrane components. A

second approach for studying diffusion of membrane components consisted of applying a monovalent antibody fragment to a small spot on a long muscle fiber and observing its diffusion with time (Edidin and Fambrough, 1973). Third, Poo and Cone (1974) studied the lateral diffusion of rhodopsin in the disk outer segments of the frog. They bleached one area of the rod and estimated the time it took for the bleached area to recover. Since rhodopsin, once bleached, does not regenerate, the experiment was interpreted as an indication of the lateral fluidity of rhodopsin on the membrane. More recently, Edidin *et al.* (1976) estimated the diffusion constant of membrane proteins of L cells labeled with fluorescein isothiocyanate. A spot on the cell surface was bleached by light from a laser beam, and the time of recovery of fluorescence was estimated. The diffusion constants of the molecules in the four experiments were determined to be 0.2×10^{-9} cm^2 sec^{-1} in the heterokaryons, 1 to 2×10^{-9} cm^2 sec^{-1} in muscle, 3.5 to 3.9×10^{-9} cm^2 sec^{-1} for rhodopsin, and 2.6×10^{-10} cm^2 sec^{-1} in the L cells. All four estimates are about 10- to 100-fold lower than the values obtained with phospholipids and could be explained on the basis of size of the molecules. [Another experiment indicating rapid mixing of surface components was that of Gordon and Cohn (1970), who found mixing of surface ATPase in heterokaryons of macrophages and melanocytes.]

Lateral diffusion of lipids and proteins, as described above, is not itself sufficient to explain certain observations of topographical heterogeneity of cell surface proteins. Of course, some forms of membrane organization could result solely from inherent characteristics of membranes. Lipid–lipid interactions, for example, with the establishment of segregating domains accompanying lipid–protein interactions, could account for certain forms of, and changes in, membrane topography. Equally plausible are phenomena caused by aggregation of membrane components as a function of noncovalent forces, as probably occurs in some bacterial and mammalian membrane specializations, such as the formation of gap junctions (Singer, 1976).

It is clear that capping of Ig or other surface components cannot be explained solely in terms of passive diffusion of the complexes. To be sure, the phenomenon is indeed a manifestation of lateral diffusion in that it is temperature dependent and requires cross-linking ligands. Nevertheless, particular events require additional mechanisms. The first is the kinetic requirement. Capping of Ig occurs within a matter of 3 minutes at 37°C, at least with respect to Ig. Yet in Frye and Edidin's classic study (1970), the surface proteins of heterokaryons required 40 minutes at 37°C to passively diffuse over most of the membrane. In this regard, Kenneth A. Ault, in our laboratories, has made some esti-

mates of the diffusion constants of Ig molecules during capping. He estimated values on the order of 10^{-8} cm^2 sec^{-1}, which are 10- to 100-fold higher than those observed for passive diffusion of protein molecules analyzed before. Second, one does not observe patches of Ig receptors progressively increasing in size until several large patches of bound receptors coalesce into a cap, as one would expect if cross-linking were the principal mechanism. Instead, one sees the rapid coordinated migration of microclusters to one pole of the cell, with little interaction between clusters until joined in a cap. Most important, capping of both Ig and Con A receptors is a cellular process with extreme sensitivity to the modulation of metabolic energy. The implications of capping as a metabolically active process have led to an immediate examination of cytoplasmic structures that might be involved. Interest has naturally focused on the cytoskeletal system of the cell, composed of filaments and tubules, and seemingly responsible for cell form and deformation and for the motive force required for changes in membrane morphology.

Research in the cytoplasmic control of capping in lymphocytes has two approaches. One is the use of pharmacological agents that interact directly with these cytoplasmic structures and affect their integrity. Thus, the cytochalasins have been extensively exploited because of their postulated effects on the microfilaments; microtubules, in turn, have been studied by the use of drugs, such as colchicine or the *Vinca* alkaloids, that prevent the establishment of microtubular systems or disrupt those already established. An alternative approach has been to use agents whose effects are physiological rather than anatomical, agents such as Ca^{2+} or local anesthetics, which have served to establish relationships between the anatomical structures under study and their internal and external environments.

A brief and by no means comprehensive review of the structure and function of the microfilaments and microtubules of the cell cytoplasm and the drugs that directly affect them is in order before proceeding to a discussion of their postulated interaction with surface proteins and the cell membrane.

A. Microfilaments

Electron microscopy has demonstrated a variety of filamentous structures as constituents of cell cytoplasm. Microfilaments, 5–8 nm in diameter, have received a great deal of attention, for they have been implicated in a wide range of cellular activities. They generally appear as a mesh of filaments forming a randomly oriented network or reticulum that is most prominent in the cortical cytoplasm, proximal to the membrane. These microfilaments appear to have primary importance in ex-

tremely diverse types of cells for cytoplasmic streaming, cell motility, and other cell properties apparently requiring the generation of contractile motive force (Komnick *et al.*, 1973). Microfilaments can also be organized into thick bundles or sheaths extending through the cytoplasm and along cytoplasmic processes; these appear to fulfill a more structural role, although they may still be capable of contraction (Wessells *et al.*, 1971, 1973). The cortical microfilamentous network is apparently labile, assembling and disassembling without requiring protein synthesis (Goldman *et al.*, 1973). Filaments seem to attach to the plasma membrane (Allison *et al.*, 1971) and are prominent at loci of membrane stimulation, such as the membrane beneath a particle undergoing phagocytosis (Allison, 1973) or regions where plasma membrane adheres to substrate (Reaven and Axline, 1973).

The identification of microfilaments, which are almost universally important for membrane and cytoplasmic events requiring contractility, has been accompanied by their characterization as actinlike and (less clearly) myosinlike. Although largely circumstantial, suggestive evidence has steadily accumulated that the mechanism for developing shearing forces in striated muscle—with ATP hydrolysis, regulatory proteins, and regulatory divalent cations—may apply to contractility in nonmuscle cells.

Thus, proteins resembling actin in amino acid composition, molecular weight, and functional activities, e.g., interactions with purified myosin and troponin–tropomyosin, have been isolated from a variety of cells, including *Acanthamoeba*, sea urchin eggs, platelets, and fibroblasts (Allison, 1973; reviewed in Pollard and Weihing, 1974). Most interestingly, cytoplasmic filaments 5–7 nm in diameter have been shown to bind heavy meromysin—a specific property of actin. First demonstrated by Ishikawa *et al.* (1969) in chick embryo cells, filament binding of heavy meromyosin has since been observed in *Acanthamoeba*, platelets, macrophages, granulocytes, epithelial cells, and lymphocytes (reviewed in Miranda *et al.*, 1974a,b; Pollard and Weihing, 1974). These actinlike filaments have the same distribution as microfilaments. Recently, fluorescein-conjugated antiactin antiserum has demonstrated long bundles of actin filaments in fibroblasts; these bundles run along the cell's periphery and parallel to the long axis of the cell, appearing to converge with other fibers at occasional nodal points (Lazarides and Weber, 1974).

Precise identification of myosin in many microfilament systems has been more difficult. Myosin appears to be more variegated with repect to its composition, chemical properties, intracellular localization, and level of spontaneous and stimulated polymerization than actin (reviewed in Miranda *et al.*, 1974a,b; Pollard and Weihing, 1974). Nonetheless, anti-

myosin antibodies conjugated with fluorescein have demonstrated intense fluorescence not only in the smooth muscle, but in the cytoplasm of nonmuscle cells, such as fibroblasts, macrophages, lymphocytes, and neural tissue (Allison, 1973), with the stain most intense in the peripheral cytoplasm next to the membrane. Protein similar to myosin in structure and function has been identified in platelets, fibroblasts, amebas, brain cells, and granulocytes (reviewed in Pollard and Weihing, 1974).

In striated muscle, the interaction of actin and myosin is regulated by a complex of four proteins, the troponins and tropomyosin, which bind to actin. These proteins dissociate in the presence of Ca^{2+} at a certain concentration, allowing actin, in the presence of Mg^{2+}, to facilitate the hydrolysis of ATP by myosin, which, in turn, provides the energy for the filaments to slide past each other, generating a shear force (Huxley, 1969). Evidence for a similar regulatory system affecting contractility in nonmuscle cells is still fragmentary. Proteins resembling troponin–tropomysin have been described in platelets and slime mold. And, as in muscle, cytoplasmic myosin hydrolyzes ATP and is stimulated by purified actin. Threads of nonmuscle cytoplasmic "actomyosin" contract in the presence of ATP and Mg^{2+} (Pollard and Weihing, 1974).

Several observations indirectly support a role for Ca^{2+} in activating an actomyosin-like contractile system in nonmuscle cells. Intracellular sequestration of Ca^{2+} in vesicles perhaps analogous to sarcoplasmic reticulum have been described in several types. Amebas have granules that appear to release Ca^{2+} at the sites of pseudopod formation, and some investigators have isolated vesicles with calcium binding activity resembling sarcoplasmic reticulum from fibroblasts (reviewed by Pollard and Weihing, 1974). Cytoplasmic streaming in amebas is Ca^{2+} dependent, as is fibroblast locomotion (Gail *et al.*, 1973; Pollard and Weihing, 1974). Platelet actomyosin is stimulated by Ca^{2+} (Pollard and Weihing, 1974), and Ca^{2+} stimulates the superprecipitation of crude actomyosin from horse leukocytes (Shibata *et al.*, 1972). The complex interrelationship between Ca^{2+} and the mechanisms underlying capping of surface Ig will be discussed extensively.

Much of our understanding of the role of microfilaments arises from the use of the cytochalasins, especially cytochalasin B, that appear to act as moderately specific inhibitors of microfilament function. The cortical microfilaments, in particular, are affected. Cytochalasin B has been observed to disrupt the physical arrangement of these filaments, although not in all systems (Wessells *et al.*, 1971; Allison, 1973). The drug inhibits cytokinesis, cytoplasmic streaming, contracting (in muscle), and usually, though not always, secretion (Wessells *et al.*, 1971; Allison, 1973; Miranda *et al.*, 1974a,b). Cytochalasin B exerts a particularly in-

hibitory influence on movement of single cells, including neutrophils (Becker *et al.,* 1972; Zigmond and Hirsch, 1972), fibroblasts (Gail and Boone, 1971b), lymphocytes (Unanue *et al.,* 1974a), and a wide range of other cell types (Pollard and Weihing, 1974). Indeed, the contractile processes that underlie many membrane phenomena, including phagocytosis, membrane ruffling activity, and the initiation of membrane asymmetry, are all cytochalasin sensitive (reviewed in Allison, 1973; Miranda *et al.,* 1974a; Pollard and Weihing, 1974).

Cytochalasin B has been postulated to "disrupt" the architecture of the microfilaments, interfering with their interaction and thus inducing aggregation of clumps of dysfunctional protein (Wessells *et al.,* 1971). An alternative theory has arisen from the exhaustive studies of Miranda *et al.* (1974a,b) on its congener, cytochalasin D. They interpret the powerful effects of this drug as resulting from an initial *hypercontraction* of the filaments with dysfunction and clumping secondary to their unrestricted contraction. They have suggested that cytochalasin B may have a similar, weaker effect over a short time. This interpretation is significant with respect to the effects of these drugs on capping, discussed later. Finally, any consideration of the effects of the cytochalasins, principally in long-term exposures, must take into account their reported inhibition of transport of glucose, deoxyglucose, glucosamines, and nucleosides (reviewed in Allison, 1973; Pollard and Weihing, 1974). Despite these effects on metabolite transport, however, the cytochalasins do not seem to affect metabolically active processes nonspecifically; they have no effect on protein synthesis, for example.

B. Microtubules

Microtubules are cylindrical structures about 25 nm in diameter that constitute a principal part of the cell's cytoskeletal system. They compose the internal structure of cilia and flagella, the tubular elements in centrioles and basal bodies, and the mitotic apparatus of dividing cells. They also exist in rapidly changing arrays in the cell cytoplasm. They consist of globular protein subunits, tubulin, which can self-assemble into microtubules *in vitro* under the appropriate conditions. Microtubular structures in the cell vary markedly in stability; they are manifest in the cytoplasm in a continual state of labile equilibrium. The physiological variables responsible for shifts between tubulin's phases as soluble subunit or polymerized tubule are poorly understood, but certain physical and pharmacological manipulations have relatively clearly defined effects. *In vitro* the microtubules are extremely sensitive to Ca^{2+}, depolymerizing in the presence of low concentrations of Ca^{2+}, and spontaneously repolymerizing in its absence (Weisenberg, 1972). The attractive hypothesis

that Ca^{2+} is the regulator of microtubule assembly intracellularly has yet to be demonstrated (Taylor, 1975). Nonetheless, some investigators have observed an apparent inverse relationship between cytoplasmic Ca^{2+} and microtubular integrity (Byers *et al.*, 1973; Kirschner and Williams, 1974; Olmsted *et al.*, 1974; Timourian *et al.*, 1974; Poste *et al.*, 1975b). Microtubular inhibitors—colchicine, Colcemid, and vinblastine, for example—shift the intracellular equilibrium to the disassembled state. Colchicine acts by binding to free tubulin subunits and prevents assembly into tubules; vinblastine appears to precipitate microtubules, depositing tubulin in crystallike structures in the cytoplasm. Principally from the use of these drugs has come the postulated roles of microtubules in certain cell functions. Also, low temperature and high pressure induce depolymerization, whereas heavy water—D_2O—stabilizes microtubules (reviewed in Allison, 1973; Margulis, 1973; Burnside, 1975).

Microtubules perform a skeletal function in many cells. They are essential for the initiation of morphological asymmetry in many instances, including cell elongation and cytoplasmic projections, where they also seem to have a stabilizing effect (Goldman *et al.*, 1973; Burnside, 1975). Their absence may affect the polarity of movement of some, but not all, cells. Thus, fibroblasts exposed to colchicine round up and stop moving in a directed fashion (Gail and Boone, 1971a; Goldman *et al.*, 1973). Microtubules also frequently determine internal polarity as well. They are essential for the organized, nonsaltatory, intracellular movement of cytoplasmic elements, such as chromosomes, mitochondria, lysosomes, phagosomes, and pigment granules (reviewed in Burnside, 1975). Thus, HeLa cells exposed to microtubule-disrupting drugs manifest random distribution of lysosomal elements and Golgi elements, and fibroblasts similarly treated lose the organization of their granules. Last, loss of microtubules frequently, though not always, inhibits secretion (reviewed in Allison, 1973). It is uncertain whether they provide motive force directly or a guide along which motive force can be applied; current prejudice favors the latter.

Drugs like colchicine and vinblastine do not inhibit actomyosin–microfilament systems. Cytoplasmic projections, such as microvilli and microspikes, cytoplasmic streaming, and ameboid movement, are all independent of the integrity of the microtubular system (Allison, 1973; Margulis, 1973; Burnside, 1975).

C. Cytoskeletal Involvement in Surface Phenomena of Nonlymphoid Cells

Much of our current understanding of the role of the lymphocyte's cytoskeletal structures in effecting Ig cap formation is based upon ideas

derived from parallel research on the redistribution of membrane proteins, particularly glycoproteins, of nonlymphoid cells, especially fibroblasts, neutrophils, and red cells. It is not within the scope of this review to consider this literature in its entirety. Still, any examination of the possible mechanisms of Ig capping necessitates consideration of the recent work in this area, for patching and capping of certain membrane components can occur in fibroblasts and neutrophils, among other cell types. From studies on these cells came some of the first demonstrations that microtubules and microfilaments could possibly control events taking place on the exterior surface of the cell. Thus, we shall discuss first the effect that the modulation of microfilament and microtubules has on the topographical distribution of transport proteins and glycoproteins, specifically Con A receptors, in the plasma membranes of nonlymphoid cells before going on to glycoprotein patching and capping in lymphocyte membranes. We shall compare, then, Con A capping to Ig capping in order to determine which elements are common to both processes and which are unique to the capping of the Ig receptor.

One proviso should be made with respect to the following observations. Many of the effects of reagents on membrane events in the cells have been presumed to reflect those reagents' specific binding to microtubules or microfilaments. Colchicine, therefore, is presumed to bind to tubulin, and its effects are interpreted in terms of depolymerization of microtubules. This assumption is fair in light of the fact that many of these observations have been repeated under extremely differing conditions whose sole common property, frequently, is their effect on microtubules or microfilaments. Nonetheless, caution is still required because the nonspecific effects on the membrane caused by many of these reagents and conditions remain to be fully elucidated.

Experiments with inhibitors of microtubules were among the earliest clear demonstrations that cytoplasmic elements influenced the establishment and maintenance of topographical heterogeneity. Berlin and his co-workers showed that neutrophils phagocytosing latex particles appeared to discriminate among membrane proteins in the process of internalizing up to 50% of their plasma membrane; Con A binding sites were preferentially internalized, while proteins that transported amino acids and other metabolites appeared to move out of the membrane being endocytosed and thus were selectively left on the surface membrane. Exposure of phagocytosing cells to colchicine destroyed this discrimination, so that the cells internalized both transport proteins and Con A binding sites proportionally to the amount of membrane internalized, as though these proteins were now randomly distributed (Ukena and Berlin, 1972; Oliver *et al.*, 1974). They concluded from these and other experi-

ments that colchicine-binding proteins—either microtubules or their equivalent—are crucial in the directed redistribution of certain proteins (Berlin *et al.*, 1974; Berlin, 1975).

Cytoplasmic influence over properties of the cell surface has been postulated in recent experiments on the agglutination of normal and virally transformed cells—usually fibroblasts—by lectins, particularly Con A. This point has been exhaustively reviewed by Nicolson (1976), but several considerations with regard to patching and capping deserve to be mentioned here. In 1969, Inbar and Sachs observed that transformed cells are more agglutinable by Con A than are normal cells. This phenomenon seems to result not only from the increased number of lectin receptors or increased physical fluidity of the transformed membrane, as was originally thought, but also from changes in the cell's ultrastructural underlying the membrane with resultant alterations in the surface topography. The use of agents affecting this ultrastructure has produced some intriguing results.

Lectin-binding sites in transformed cells are more often clustered than in normal cells (Nicholson, 1971, 1972). Prefixation techniques have made it clear that transformed cells have randomly distributed Con A-binding sites on their surfaces, just as normal cells do, but the former sites more readily form many large clusters at lower concentrations of Con A than in normal cells. This cluster or patch formation (without capping) may then allow multiple Con A bridges to form between cells at contact points, enhancing their agglutination (Rosenblith *et al.*, 1973; Kaneko *et al.*, 1973; Inbar *et al.*, 1973; Nicolson, 1974, 1976; Poste and Reeve, 1974; Ukena *et al.*, 1974).

Many investigators have examined the ultrastructural differences between normal and transformed fibroblast cell lines. In general, normal cells are elongated with numerous cytoplasmic microtubules and abundant microfilaments frequently organized with respect to the cell periphery and the long axis of cytoplasmic projections and, as determined by ion-etching, orderly ridges of subsurface fibrils. Transformed cells, on the other hand, contain few microtubules, fewer and more disorganized microfilaments, a disordered distribution of fibrils in peripheral structures, such as pseudopods, and fewer and disorganized subsurface ridges of fibrils after ion-etching (Ambrose *et al.*, 1970; McNutt *et al.*, 1973; Vasiliev and Gelfand, 1973). Nicolson (1976) has suggested that there is a decrease in actin associated with the membrane after transformation in several cell lines and that the disorder of microfilaments and microtubules conceivably lessens or uncouples the associations between the cytoplasm and the membrane, resulting in an increased freedom for integral proteins. With respect to this point, it is interesting that Edidin

and Weiss (1974) observed slow intermixing of surface antigens when two normal cells were fused and rapid intermixing when two transformed cells or one transformed cell and one normal cell were fused, consistent with this lack of restraint.

A role for microtubules emerged from experiments showing that colchicine inhibits the Con A agglutination of transformed cells by permitting cap formation of the scattered patches. Capping inhibits agglutination by reducing the possibility of cell-to-cell contact over most of the membrane since Con A complexes concentrate in one place, the only place where cell–cell adhesions can form (Berlin and Ukena, 1972; Yin *et al.*, 1972; Ukena *et al.*, 1974; Poste *et al.*, 1975c). Yet capping of Con A binding sites in transformed cells is still dependent upon microfilaments. Colchicine-treated cells exposed to metabolic inhibitors or to cytochalasin B are unable to cap (Ukena *et al.*, 1974) but still manifest enhanced agglutination (Poste *et al.*, 1975c). Thus, although freedom from the postulated restrictive influence of microfilaments permits patching, capping probably does not simply follow decreased restrictions on the lateral mobility of molecules in the membrane but requires the active participation of cortical microfilaments, a participation whose expression is enhanced in the absence of microtubules.

One of the cyclic nucleotides, cAMP, appears to play a role in the cytoplasmic control of the cell surface. The cAMP generally prevents the enhanced agglutination of transformed fibroblasts by Con A. Nicolson (1976) has suggested that this is due to cAMP's ability to stimulate cytoskeletal organization. It has long been appreciated that transformed cells, which have lowered levels of cAMP (Abell and Monahan, 1973), and other cells can undergo striking changes in their morphology when exposed to cAMP and that this effect is suppressed by the presence of colchicine or Colcemid (Hsie *et al.*, 1971; Hsie and Puck, 1971; Johnson *et al.*, 1971; Puck *et al.*, 1972; Roisen and Murphy, 1973; Porter *et al.*, 1974). There is an inverse relationship between intracellular levels of cAMP and cell agglutinability (Willingham and Pastan, 1975). Two groups have correlated an increase in cytoplasmic microtubules with the presence of exogenously added cAMP (Porter *et al.*, 1974; Brinkley *et al.*, 1975). The inhibition of lymphocyte motility by cAMP, which is reversed by colchicine (Section III), may be a reflection of the putative polymerizing effect of this nucleotide on microtubules. This is by no means conclusive, since this microtubule-polymerizing effect is not accepted by everyone (Gillespie, 1975; Oliver *et al.*, 1975).

Although in normal fibroblasts the distribution of Con A-binding sites is little altered by Con A (Ukena *et al.*, 1974), normal cells of other types can spontaneously cluster or cap their Con A receptor sites, and

this redistribution can be modulated by the cytoskeletal system. One of the clearest demonstrations of a cytoplasmic system affecting the distribution of membrane constituents was in the erythrocyte membrane where cross-linking external glycoproteins altered the position of spectrin, the cytoplasmic protein found in close apposition to the membrane (Ji and Nicolson, 1974), and where cross-linking spectrin in the cell's interior led to aggregation of membrane glycoproteins (Nicolson and Painter, 1973). Nicolson and Yanagimachi (1974) observed that on the same nucleated cell, lectin-receptor sites have different mobilities and different clustering patterns, depending upon the region of the cell's surface on which they are located; this would not be expected were lateral mobility of glycoproteins simply an expression of a generalized membrane property. This suggestion of some form of transmembrane control in glycoprotein redistribution is supported by Ryan *et al.* (1974b), who found that, although neutrophils spontaneously formed Con A caps, cap formation in the presence of colchicine was completely inhibited after the addition of cytochalasin B.

The patching and capping of Con A on the normal lymphocyte is the most extensively studied of these systems. The results have yielded a great deal of information and a number of intriguing hypotheses: Con A capping stands as a model to which we have contrasted Ig capping and to which other forms of capping must eventually be compared.

D. Mechanisms of Con A Capping in Lymphocytes

Con A and Ig capping in lymphocytes, which were described in Sections IV and II,B, respectively, are compared in Table II. There are striking points concerning Con A capping that have been extensively analyzed and have served as a basis for formulation for various working hypotheses. First, Con A capping is strictly associated with true translatory motion of the lymphocyte; second, it is enhanced by treatment with colchicine; and, third, it is extremely sensitive to the inhibitory effects of cytochalasin B.

Virtually all the studies on Con A capping in lymphocytes have described localization of the bound lectin to the uropod, the trailing cytoplasmic projection of the motile lymphocyte (Smith and Hollers, 1970; Greaves *et al.*, 1972; Unanue and Karnovsky 1974). The association between Con A capping and lymphocyte motility was rendered unequivocal by the direct observations of Loor (1974) on lymphocytes exposed to high concentrations of Con A. Although the cells became progressively inhibited in their motility, he observed the initial capping of Con A as a reversible membrane flow phenomenon, secondary to the motile state of the cell. As the lymphocyte initiates movement, an unlabeled pseudopod

TABLE II
COMPARISON OF IG AND CON A CAPPING

Capping	Ig	Con A
Multivalency	Required	Required
Energy metabolism	Required	Required
Dose dependency	Some inhibition at high doses	Critical
Effects of capping of other molecules	No inhibition	Inhibition
Cytochalasin B	Some inhibition at high doses (10–30 $\mu g \times ml$)	Marked inhibition at low doses (1 $\mu g \times ml$)
Colchicine	No effect—some enhancement	Marked enhancement—permits capping at inhibitory doses
Association and motility	Capping precedes motility	Capping follows motility
Reversal of capping		
Spontaneous	No	Yes
Inhibitors of energy metabolism	Do not reverse capping	Reverse capping
Ca^{2+} ionophore	Reverse capping	Not studied
Cytochalasin B	Slight effect	Complete reversal
Colchicine	No effect	No effect
Cytochalasin and colchicine	Complete reversal	Complete reversal
Mechanism	Primary, active transport of patches	Secondary, passive transport of patches

flows forward, and the lattice of cross-linking receptor sites moves back toward the uropod, much as the particles on the dorsal surface of moving cells flow backward (Harris, 1973); when the cell stops its cycle of movement and rounds up, the capping Con A receptors flow forward again and cover the cell as the cap breaks apart. This can occur for many cycles (Fig. 11). de Petris (1975) made similar observations and also noted that this occurs in either cell elongation or actual motility.

A very critical experiment in interpreting the mechanisms of Con A capping involves the ability of the cell to maintain its Con A receptors in a cap. Since the work of Sallstrom and Alm (1972), it has been appreciated that metabolic inhibitors not only prevent Con A capping but cause previously formed caps to break apart and flow back over the membrane (Inbar and Sachs, 1973), much as Loor (1974) observed in a spontaneous sequence. de Petris confirmed this and also showed that cytochalasin B similarly causes Con A caps to break up. He concluded, correctly we believe, that both effects result from the suppression of movement with rounding of the cell and retraction of the uropod into

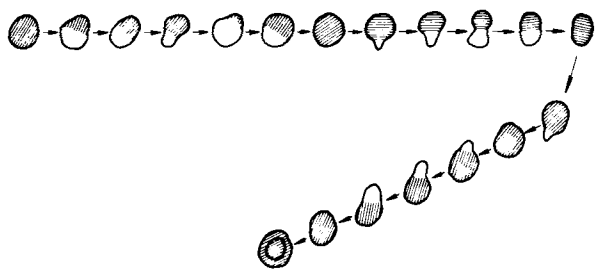

FIG. 11. Concanavalin A (Con A) capping in lymphocytes. The hatched areas correspond to the distribution of fluorescent Con A. Lymphocytes exhibit capping and spontaneous reversion of the cap associated with cell motility. Further explanation is given in the text. (From Loor, 1974.)

the cell, after which the labeled receptors previously localized to the uropod mix with the rest of the unlabeled membrane (de Petris, 1975). de Petris' description of Con A capping on lymphocytes is worth detailing: "The morphological characteristics of capping, in particular the observation that cell elongation is mainly due to forward displacement of unlabeled material, suggests that interaction of membrane with cytoplasmic structures is involved both in this forward displacement and in the 'anchoring' of the cross-linked patches which prevents them from following this movement. In the presence of cytochalasin B, no pseudopod is formed, the cell remains round, and the Con A cap does not form. If cytochalasin B is added after a cap has been allowed to form, the pseudopod retracts, the constriction, if present disappears, and the cell returns to a spherical shape, and the elements of the cap can mix again, at least partially, with the unlabeled membrane."

Finally, the observed differences in the lymphocyte's ability to cap Con A are clear with respect to the role of cell movement. As noted earlier, thymus-derived lymphocytes usually cap Con A more readily than do B cells and are the subpopulation of lymphocytes that spontaneously moves. B cells, in the absence of stimuli such as anti-Ig, are round, sessile, and inefficient in capping Con A.

The conclusion from these series of results is that Con A capping involves a countercurrent effect in which the whole cross-linked lattice of heterogeneous surface glycoproteins remains fixed, whereas the unbound part of the membrane actively displaces itself out of the fixed lattice by action of the cytoplasmic contractile elements. The whole process ends as a passive collapse of the lattice into a trailing cap as the uninvolved membrane and the cytoplasm stream forward.

How, then, does Con A at high concentrations inhibit capping of its

own binding sites and of other molecules, and how might colchicine release this inhibition? As noted earlier, Edelman and his co-workers have suggested that the binding of Con A to surface proteins provokes the independent attachment of Con A binding-proteins and other surface molecules, such as Ig, θ, and Fc receptor proteins, to submembranous assemblies of microtubules that effectively restrict their redistribution. This was based on their experiments in which colchicine treatment, or other conditions depolymerizing microtubules, removed the inhibition of large concentrations of Con A and permitted capping not only of the lectin-binding sites but also of other surface receptors bound by ligand (Edelman *et al.*, 1973; Yahara and Edelman, 1973a,b). They have argued against extracellular restrictions, such as external cross-linking, as an explanation because focally bound Con A appears to induce global changes in the membrane, interpreted as the anchoring of many surface proteins to microtubules. For example, lymphocytes bound to lectin-derivatized fibers or to large numbers of Con-A-coated beads or platelets are unable to cap surface Ig complexed with anti-Ig unless colchicine is present (Rutishauser *et al.*, 1974; Yahara and Edelman, 1975a,b). This latter point is particularly important but is complicated by the fact that bound anti-Ig would be expected to bind also to the Fc receptors of platelets, the nonspecific absorptive surface of the latex beads, and Con A itself. This would result in the functional cross-linking of Ig to the platelet-bound or bead-bound patches of lectin-binding sites. This cross-linking may be sufficient to inhibit the organized redistribution of Ig and thus must be ruled out. Finally, the lower spontaneously capping concentrations of Con A represents focal attachment of Con A to the lymphocyte surface, yet cells that have capped their Con A receptors in the absence of colchicine or vinblastine subsequently cap their surface Ig upon exposure to anti-Ig (Loor, 1974).

Recent data suggest an alternative explanation for the colchicine effect and that submembranous attachment of surface proteins to microtubules need not be invoked to explain the effect of Con A on the capping of glycoproteins or surface Ig. It now appears that Con A can restrict the mobility of surface Ig simply by binding to it and cross-linking it to the heterogeneous array of other surface glycoproteins similarly bound. Surface Ig subsequently behaves as an externally cross-linked glycoprotein, not as a free molecule. This problem was examined by de Petris, who reexamined the previous observation that Con A capping induces cocapping of surface Ig in the absence of any other ligand. With electron microscopy he demonstrated that Ig is clearly cross-linked with other Con A receptors on the surface, as is θ antigen, and that it composes part of the Con A patches. This explains why, whether in the presence

or in the absence of colchicine, the cap of Ig receptors exposed to Con A and then to anti-Ig behaves more like a Con A cap than an anti-Ig-induced cap with respect to its kinetics and its sensitivity to the pharmacological manipulations as shown in Table II. For example, capping of Ig by anti-Ig in the presence of Con A is completely suppressed by cytochalasin B. The rate of anti-Ig-stimulated endocytosis of surface Ig, in the presence of Con A, follows the slower kinetics of the pinocytosis observed for lectin-bound Con A receptors. In addition, increased concentrations of Con A seem to hinder sterically the binding of anti-Ig (de Petris, 1975). It is not surprising that surface Ig can act as a Con A binding site. It is a glycoprotein with carbohydrate side chains containing mannose, to which Con A binds (Andersson *et al.*, 1974a). Iodinated surface Ig has been shown to bind Con A when isolated *in vitro* (Hunt and Marchalonis, 1974).

We conclude that, at sufficiently high concentrations, Con A may simply cross-link a variety of glycoproteins into a rigid lattice on the lymphocyte surface such that these proteins, including Ig, would no longer be free to move with respect to other proteins when bound by specific antisera.

The interpretation of Con A's effects in terms of submembranous microtubule assembly or in terms of external cross-linkage is not definitive, nor are the theories mutually exclusive. Nonetheless, allowance for external modulation enables the effects on capping by Con A and colchicine to be understood within the same context of cytoplasmic streaming that we outlined earlier for spontaneously forming Con A caps. High doses of Con A retard and ultimately inhibit translational motility in lymphocytes and other cells and so would be expected to inhibit capping of any cross-linked receptors, including surface Ig (Friberg *et al.*, 1971; Loor, 1974; Unanue and Karnovsky, 1974). The effects of colchicine can also be readily understood in terms of lymphocyte motility. Colchicine stimulates cytoplasmic streaming and ameboid movement in macrophages (Bhisey and Freed, 1971a,b) and enhances macrophage migration (Pick and Abrahamer, 1973). It similarly enhances lymphocyte motility over a substrate (Unanue and Karnovsky, 1974; Schreiner and Unanue, 1975a) and through a Millipore filter (Russell *et al.*, 1975). It restores lymphocyte movement suppressed by cAMP and ALS (Schreiner and Unanue, 1975a,b). Thus, colchicine, by permitting or enhancing lymphocyte movement in the presence of the restrictive effect of Con A, would enhance the relative backflow of Con A receptors, including the cross-linked Ig, to the uropod at the posterior part of the cell, as has been observed (Unanue and Karnovsky, 1974). And since Con A capping is dependent upon lymphocyte movement, cytochalasin B would be expected to pre-

vent capping of surface Ig linked to other glycoproteins by Con A, even though anti-Ig is also present. We suggest that the transport of Con A patches on the lymphocyte's surface represents passive backward flow secondary to active cytoplasmic streaming. Cytoplasmic streaming occurs in cells at rest or actively motile; both situations require the generalized activation of microfilaments displacing surface and cytoplasmic material in the form of unified, bulk flow. Thus, Con A patches flow to the rear of forming pseudopods occasionally put out by adherent cells (Weller, 1974) and nonmotile lymphocytes (de Petris, 1975), and thus cytochalasin B can inhibit Con A capping in nonmotile epithelial cells (Storrie, 1975) or colchicine-treated lymphocytes unable to move because of being kept in suspension (Unanue and Karnovsky, 1974).

Given the complexity of the system, Con A might be retarding lymphocyte movement in any number of ways: altering calcium metabolism (Freedman *et al.*, 1975), preventing the establishment of a focusing point for filamentous contractions or enhancing microtubule stability, not on the membrane, but in the deep cytoplasm. This latter hypothesis is attractive because other manipulations that enhance microtubule formation, such as cAMP, retard lymphocyte movement, which is reversed with colchicine. Albertini and Clark (1975) have shown that microtubules are stimulated to form in the cytoplasm of ovarian granulosa cells by Con A. The observations that Con A can transiently increase intracellular concentrations of cAMP (Parker, 1974; Storrie, 1975) are consistent with a microtubule-stabilizing effect of this lectin serving to retard cytoplasmic streaming. Like high concentrations of Con A itself, exogenous cAMP can inhibit Con A capping, an inhibition that is reversed by colchicine (Storrie, 1975). The possibility that high concentrations of Con A increase cAMP, polymerize microtubules, secondarily inhibit cytoplasmic streaming, and thus suppress capping of Con A receptors and cross-linked surface Ig in lymphocytes should be investigated further.

E. Mechanisms of Ig Capping

The redistribution of the lymphocyte's antigen receptors stands in distinct contrast to that observed in Con A capping. One of the few surface molecules that is capped spontaneously by antisera without requiring a second cross-linking antibody, Ig is the most efficiently and rapidly redistributed protein in the cell surface. It differs from Con A with respect to the effects of cytoskeletal inhibitors (Table II). As noted in Section II,B, cytochalasin B has only a slight inhibitory effect, or none, on capping. Colchicine either has no effect or slightly enhances capping. Interestingly, the combination of cytochalasin B and microtubule-disrupt-

ing drugs profoundly inhibits cap formation, suggesting that an Ig cap does involve microfilaments and is dependent upon the influence of microtubules, but not upon cell movement (de Petris, 1974, 1975). We previously outlined several manipulations, including exposure to cAMP and DFP, demonstrating that capping of Ig is not dependent upon cell motility.

De Petris and Raff (1972, 1973b) and later de Petris (1975) have proposed mechanisms of Ig capping similar to that of Con A and other capped proteins. Because of the effects of cytochalasin and the association of the cap with the uropod, they have postulated that, since the Ig receptors are cross-linked in a lattice, they move back to the trailing edge of the motile lymphocyte to the uropod where they become functionally frozen as unlabeled membrane flows forward. Capping is then secondary to the bulk flow of cytoplasm and membrane whose coordinated displacement subserves cell motion. The difficulties inherent in regarding Ig capping as a manifestation of the same mechanism underlying Con A capping are apparent in the experiments on the formed Ig cap.

Unlike the Con A cap, the Ig cap is never spontaneously reversible, even at room temperature, which preferentially retards endocytosis (Schreiner and Unanue, 1976a). Ig caps on B cells placed in metabolic inhibitors do not flow back into unlabeled membrane but remain in place even though still on the surface of the lymphocyte (Schreiner and Unanue, 1976a,b). Nor do Ig caps reverse to the same great extent as do Con A caps when capped cells are placed in cytochalasin B (de Petris, 1974, 1975; Schreiner and Unanue, 1976a). Thus, Ig caps do not behave as though they were a product of the membrane-cytoplasmic flow of cell movement or cell elongation, for they would be expected to disperse as Con A caps do when that flow is blocked. In addition, careful time sequencing clearly demonstrates that Ig cap formation precedes the initiation of the motile process (Fig. 5).

Thus, the different susceptibilities of the Con A caps and the Ig caps to conditions altering microfilaments, microtubules, and metabolic energy, with respect to cap formation, maintenance, and reversibility, and the sequence of kinetics of Ig capping all suggest that an alternative basis for Ig receptor cap formation must be sought. De Petris (1975) has suggested that these differences in capping might result from differences in affinity, valence, accessibility, and stability of various surface molecules. But recent work on the role of Ca^{2+} in Ig redistribution, including the striking effects of anesthetics, has suggested to us that this model of membrane activity may be unique to the antigen receptor. We, therefore, describe in detail the effects of Ca^{2+} on B-cell capping and motility.

1. Ca^{2+} and Ig Capping

Neither the Ig cap formation nor stimulated motility in the B cell is dependent upon the presence of extracellular Ca^{2+} (Taylor *et al.*, 1971; Unanue and Karnovsky, 1973; Schreiner and Unanue, 1976a). However, the introduction of Ca^{2+} into the cytoplasm by a Ca^{2+} ionophore completely suppresses capping (Schreiner and Unanue, 1976a). If the ionophore-mediated Ca^{2+} influx occurs after cap formation, the cap is completely disrupted, with Ig receptors scattered in irregularly sized fragments across the plane of the membrane. While maintenance of the cap is not affected by the presence of metabolic inhibitors, the presence of such compounds completely suppreses the Ca^{2+}-stimulated reversal of the cap, indicating that cap disruption by Ca^{2+} is a metabolically active process. Cytochalasin D, a potent *activator* of microfilament contraction, whose effects also require metabolic energy (Miranda *et al.*, 1974a,b), similarly disrupts Ig caps. We have postulated that the effects of Ca^{2+} are due to the activation of the lymphocyte's contractile elements which, insofar as they constitute an actomyosin-like system, would be expected to be responsive to Ca^{2+} and manifest an ATP requirement for activation (Schreiner and Unanue, 1976a). That Ca^{2+} activation would not only prevent capping but also rapidly pull patches apart in an energy-consuming process suggests that surface Ig and cytoplasmic contractile elements are physically linked and that Ca^{2+} may be crucial both for activating those contractile elements and also for establishing the link between ligand-bound surface Ig and cytoplasmic microfilaments. In the erythrocyte membrane, Ca^{2+} certainly appears to act as a physical linkage, promoting the association of the actomyosin-like spectrin with the red cell membrane (Marchesi and Steers, 1968; Nicolson *et al.*, 1971), and Ca^{2+} physically binds spectrin to phospholipids on the cytoplasmic face of the erythrocyte membrane (Juliano *et al.*, 1971). It has also been long appreciated that Ca^{2+} is essential in all cell types for many membrane activities associated with microfilament activation, including phagocytosis, exocytosis (Durham, 1974), adhesiveness, generation of surface tension, and membrane deformation (Manery, 1969).

With respect to the lymphocyte, the effect of modifying Ca^{2+} associated with membrane has been examined by using tertiary amine anesthetics. This class of anesthetics, in addition to increasing membrane fluidity and volume and suppressing membrane depolarization, has the attribute of displacing Ca^{2+} by reversibly competing for Ca^{2+}-binding sites in the membrane (Papahadjopoulos, 1972; Seeman, 1974). Recent work has suggested that this Ca^{2+} displacement may sever the cytoplasmic mem-

brane linkages by which the cytoplasm controls the topography of surface Ig. For example, such anesthetics are effective inhibitors of Ig cap formation (Ryan *et al.*, 1974a; Poste *et al.*, 1975b; Schreiner and Unanue, 1976b). This effect is reversible; the cells cap when the anesthetic is washed out (Ryan *et al.*, 1974a). Further, its inhibitory effect can be partially overridden by increasing extracellular Ca^{2+} concentrations (Schreiner and Unanue, 1976b). However, the most direct manifestation of the importance of Ca^{2+} in capping lies in the effects of these anesthetics on the formed cap. Chlorpromazine or dibucaine completely disperse formed Ig caps (Poste *et al.*, 1975b; Schreiner and Unanue, 1976b). Metabolic inhibitors fail to protect the cap, as they do with the ionophore; instead they act synergistically with the anesthetics in reversing the caps. Increasing the concentration of extracellular Ca^{2+} almost completely suppresses the disruptive effect of the anesthetics, suggesting that the displacement of Ca^{2+} from the cytoplasmic face of the membrane frees the receptors from whatever directs them into caps and maintains them there. Subsequent endocytosis and stimulated movement are also prevented in the presence of anesthetics. However, the ligand-bound receptors retain the capacity to interact with the cytoplasm after dispersal by the anesthetics. After the anesthetics have been washed out, the receptors rapidly re-form into a cap and again stimulate motility and are ultimately endocytosed (Fig. 12) (Schreiner and Unanue, 1976b).

Anesthetics that are tertiary amines have comparable effects on other cell types; for example, they enhance the agglutination of fibroblasts by lectins (Poste *et al.*, 1975a, 1975c). This is not from a nonspecific effect on membrane fluidity but rather from the enhancement of patch formation by the cross-linking lectin, similar to that seen in transformed cells, as described earlier. This enhanced clustering is prevented by elevating the concentration of extracellular Ca^{2+} and is thus presumed to reflect the Ca^{2+}-displacing properties of these compounds (Poste *et al.*, 1975a, 1975c). The anesthetics mimic the effect of the cytochalasin in preventing the capping of lectin receptors on colchicine-treated transformed fibroblasts (Poste *et al.*, 1975c). This is consistent with their previously observed inhibition of both Con A and Ig capping (Ryan *et al.*, 1974a) and suggests that one effect of anesthetics is to interfere with microfilament integrity.

Some recent morphological studies support the concept that anesthetics can alter microfilaments and can displace them from the membrane by competing for Ca^{2+}-binding sites. Fibroblasts exposed to anesthetics manifest the dissociation of microfilament bundles from the membrane, the presence of filaments in the deeper cytoplasm, and the virtual absence

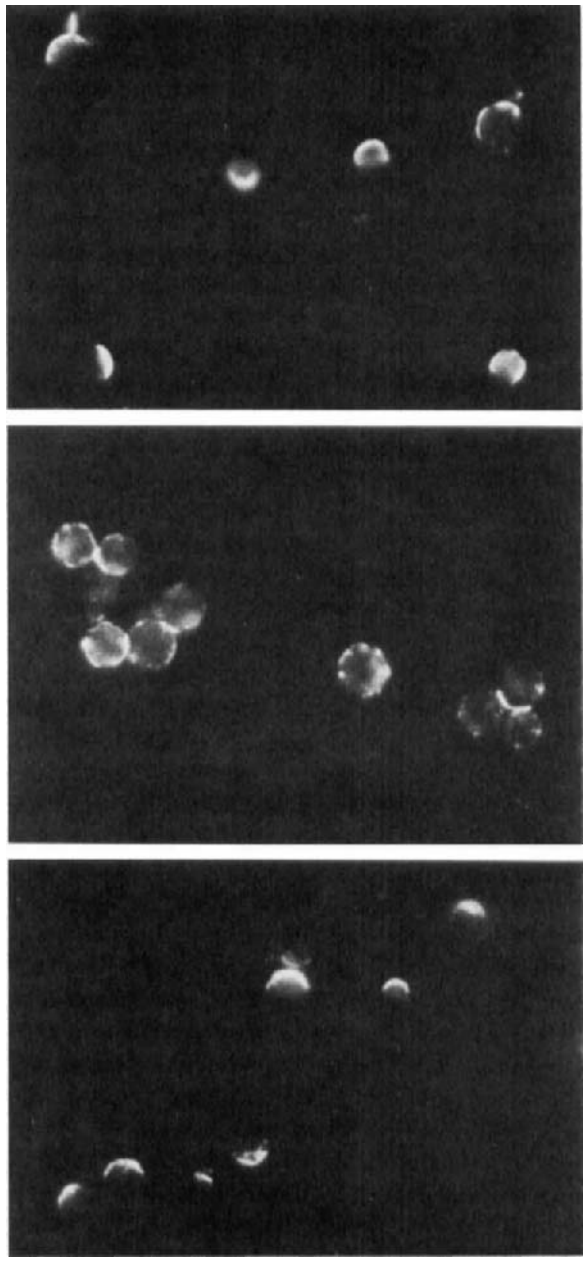

FIG. 12. Fluorescent micrographs of B cells with Ig–anti-Ig caps treated with chlorpromazine. The top panel shows the cells with caps (after 25 minutes' incubation at room temperature); the middle panel shows the same cells upon addition of chlorpromazine—the complexes now cover the surface; the lower panel shows that cells cap again after chlorpromazine is washed away. (From Schreiner and Unanue, 1976b.)

of microtubules (Poste *et al.*, 1975b; Nicolson, 1976; Nicolson *et al.*, 1976). The disorganizing effect of these anesthetics can perhaps be conceptualized as a functional displacement of membrane-associated microfilaments if Ca^{2+} truly does serve not only to activate actomyosin-like filaments, but also to link them to membrane sites in order to transfer generated force to the membrane or certain components of the membrane. Certainly, the structure of the membrane lends itself to the idea that Ca^{2+} may be important both as a linking element and as a releasable source for activation of the underlying cytoplasm. Lipids, for example, are asymmetrically distributed with the Ca^{2+}-binding sites, such as phosphatidylserine concentration on the cytoplasmic side of the bilayered membrane (reviewed in Nicolson, 1976). Tertiary amine anesthetics could displace Ca^{2+} from phospholipids (Papahadjopoulos *et al.*, 1975), Ca^{2+}-binding proteins, or domains of phospholipids preferentially associated with membrane proteins, whose dispersal by Ca^{2+} displacement could affect interaction of those proteins with other membrane elements or cytoplasmic structures (Poste *et al.*, 1975c). The apparent depolymerization of microtubules (Byers *et al.*, 1973; Haschke *et al.*, 1974) by anesthetics could result either from direct effects or, if the *in vitro* sensitivity of microtubules to Ca^{2+} is extendable to *in vivo* regulating mechanisms, from binding to Ca^{2+}-binding sites critical for microtubule integrity; alternatively, sufficient Ca^{2+} could be displaced from the membrane into the cytoplasm that microtubules spontaneously disassemble. Since colchicine or vinblastine have no inhibitory effect on Ig capping, an anesthetic-provoked depolymerization of microtubules is inadequate to explain the inhibition of cap formation and the stimulated cap disruption by tertiary amine anesthetics. This lends added strength to the notion that these anesthetics sever the cytoplasm membrane nexus by dissociating microfilaments from their membrane attachments. This effect may underlie not only their modulation of Ig capping but also their effects on other membrane-associated phenomena, such as adherence, motility, and phagocytosis (Kvarstein and Stormorker, 1971; Gail and Boone, 1972).

2. *A Hypothesis for Explaining Ig Capping*

In the case of the antigen receptor of the B lymphocyte, we propose that Ca^{2+} is the primary link in a form of stimulus–response coupling that results in the microfilament-dependent reorganization of the surface topography and the resultant cytoplasmic phases of stimulated cell movement and endocytosis. The stimulus for this response would be an inherent property of the patched Ig receptors and would be localized to the patches. In addition, we suggest that a Ca^{2+}-dependent bond between

antigen receptors and Ca^{2+}-responsive cytoplasmic microfilaments effects the transport of the receptors through the plane of the membrane without affecting other components of the membrane. This link between these particular receptors and the cytoplasm could be preformed or could occur subsequent to the stimulation of ligand complexing with the receptor. Singer and Nicolson's emphasis (1972) on the mobile characteristics of membrane-bound proteins and the lack of associated proteins on Ig isolated from the lymphocyte surface support the latter possibility. The ligand–receptor complex on the surface, linked to an assembled contractile network of the cell cortex, is then displaced as the latter contracts. Since the activation of the microfilaments would be focal to the patched receptors, adjacent filaments in this reticulum would be relaxed, allowing the net displacement of the complexed Ig receptors with respect to the rest of the membrane. One would then expect an area of activated microfilaments underlying the cap serving to stabilize it by means of its underlying attachments (and ultimately responsible for internalizing it), while the filament network underlying the rest of the membrane would be relaxed. This model does not require the coordinated displacement of cytoplasm and membrane that is required for cell motility, with the unified contraction of large areas of contractile filaments associated with the bulk flow of cell or membrane displacement. It is somewhat analogous to the Ca^{2+}-dependent, focal activation of microfilaments postulated as the mechanism of endocytosis (Allison, 1973). It allows the selective redistribution of stimulated antigen receptors.

This hypothesis explains a great many data obtained with the Ca^{2+} ionophore and the anesthetics. Thus, the introduction of Ca^{2+} before capping would provoke generalized contraction, functionally immobilizing the surface receptors by preventing the simultaneous development of areas of contraction and relaxation necessary for net displacement of complexes. Conversely, Ca^{2+} influx after a cap has formed would place tension on the activated microfilaments underlying the cap and still intercalated with the surrounding network of previously relaxed microfilaments; the sudden activation of the cortical microfilaments would presumably pull the cap in all directions in an energy-requiring mechanism. Not requiring energy, however, would be the effects of amine anesthetics which, by displacing Ca^{2+}, would sever these membrane-cytoplasmic linkages; the cap would then drift apart rather than be pulled apart (and this is supported by the synergistic action of metabolic inhibitors). The inherent ability of these receptors randomly diffusing through a membrane to generate interactions with the underlying contractile network is unaffected by dispersal because, after removal of the anesthetic, they are able to re-form rapidly into caps and restimulate movement, an ob-

servation that also suggests that lattice formation of Ig receptors may not be as important as was once thought (Taylor *et al.*, 1971).

A chain of localized microfilament contractions and relaxations moving a molecule within and through the membrane necessitates a sequence of activation running beneath the membrane along the path of the moving cluster of molecules. The possible nature of this focusing mechanism is unclear but may be related to experiments on the effect of focal membrane attachments on capping. Two investigators have found that, when lymphocytes are attached to either antigen-derivatized or lectin-derivatized fibers or beads, the cap induced by subsequent exposure to anti-Ig forms at the point of attachment (Kiefer, 1973; Loor, 1974), suggesting that prior membrane stimulation can act to direct the activated transport of receptor patches.

Several diverse pieces of evidence indirectly support the concept that antigen receptors cap and stimulate motility by means of direct interaction with the lymphocyte's actomyosin-like contractile filaments. First, de Petris (de Petris and Raff, 1973b; de Petris, 1975) has described a zone of dense, contracted filaments underlying the Ig cap, consistent with the hypothesis that patched Ig receptors can stimulate underlying filaments. This has not been observed in caps of other molecules. Second, Norberg (1971) has published interesting experiments on glycerinated lymphocytes, suggesting a role for an actomyosin-like system in lymphocyte activation. Long employed as an experimental technique in muscle physiology, glycerol extraction renders cell membranes permeable to electrolytes and ATP while not affecting actomyosin integrity; it has been used to demonstrate specifically stimulated contraction in muscle fibers, amebas, and fibroblasts. In glycerinated lymphocytes, the presence of ATP, Ca^{2+}, and Mg^{2+} induced irregular contractions that formed into a ring as a zone of constricting cytoplasm, pushed the nucleus forward in the protruding pseudopod of hyaline cytoplasm, and formed a uropod. As the dead cell assumed the classic hand-mirror shape, it went through one cycle of ameboid movement across fibrin fibers. Thus, the physiology of this contraction mimics that of striated muscle, although its effect is identical to that seen in a living lymphocyte stimulated to move by anti-Ig. Third, we have observed a peculiar phenomenon in lymphocytes allowed to cap and then depleted of ATP. In muscle contraction, ATP is required not only for the initiation of actomyosin filament contraction but also for its relaxation. If Ca^{2+} is not taken up into storage sites in an energy-dependent step, regulatory proteins are unable to bind actin and allow it to dissociate from myosin, which then induces relaxation. Contracted filaments stay contracted in the absence of ATP, and relaxed filaments are unable to contract. This is the basis of rigor mortis.

Lymphocytes with Ig caps that are then exposed to metabolic inhibitors such as azide or oligomycin do not suffer the reversal of their caps, as occurs with Con A caps. On the contrary, one frequently observes capped lymphocytes assuming an extraordinary morphology resembling that of an apple core. A tight constrictive band encircles the cell, at times almost severing it, while cytoplasm splays out in front (Fig. 13). Most important, this constriction is always associated with the Ig cap, as if this were a form of cellular rigor mortis with activated actomyosin filaments related to the cap unable to relax and relaxed filaments away from the cap unable to contract and thus streaming outward (Schreiner and Unanue, 1976a).

Last, unpublished observations in our laboratory support the idea that Ca^{2+} also serves to link the two systems of membrane receptors and cytoplasmic contractile proteins. Lymphocytes prevented from capping or with caps disrupted by the Ca^{2+} ionophore still internalize their ligand-bound Ig molecules, implying that, even if redistribution of these receptors across the membrane is not possible, the membrane-cytoplasm nexus is sufficiently intact to permit phagocytosis, a phenomenon reflecting a particular association of microfilaments with membrane (Allison, 1973). On the other hand, lymphocytes whose caps have been dispersed by local anesthetics are unable to phagocytose their antigen receptors, which remain on the surface for long periods of time, implying that the cytoplasm-membrane nexus has been broken by Ca^{2+} displacement since, after a subsequent increase in extracellular Ca^{2+}, the cells rapidly endocytose the Ig–anti-Ig complexes (Schreiner and Unanue, 1976b).

Since extracellular Ca^{2+} is not required for capping, we assume that intracellular Ca^{2+} is made available for capping by release from intracellular stores, such as vesicles or perhaps from certain Ca^{2+}-binding sites in the membrane. Ongoing experiments in this laboratory have demonstrated a triggered efflux of Ca^{2+} upon addition of anti-Ig, consistent with the supposition that release of Ca^{2+} at a local spot may be operative in activating the driving force for capping (Unanue and Schreiner, 1975).

The mediating role of Ca^{2+} also suggests a possible mechanism for the involvement of microtubules. If microtubule formation is regulated by the presence of free Ca^{2+} ion, compartmental shifts in Ca^{2+} would have a profound effect on microtubules but on an extremely localized level. For example, a shift in free Ca^{2+} toward patched receptors in the membrane would activate microfilaments underlying that patch and concomitantly depolymerize microtubules that might restrict the effectiveness of microfilament contraction. A simultaneous decrease in Ca^{2+} in the neighboring cytoplasm underlying other proteins in the membrane might

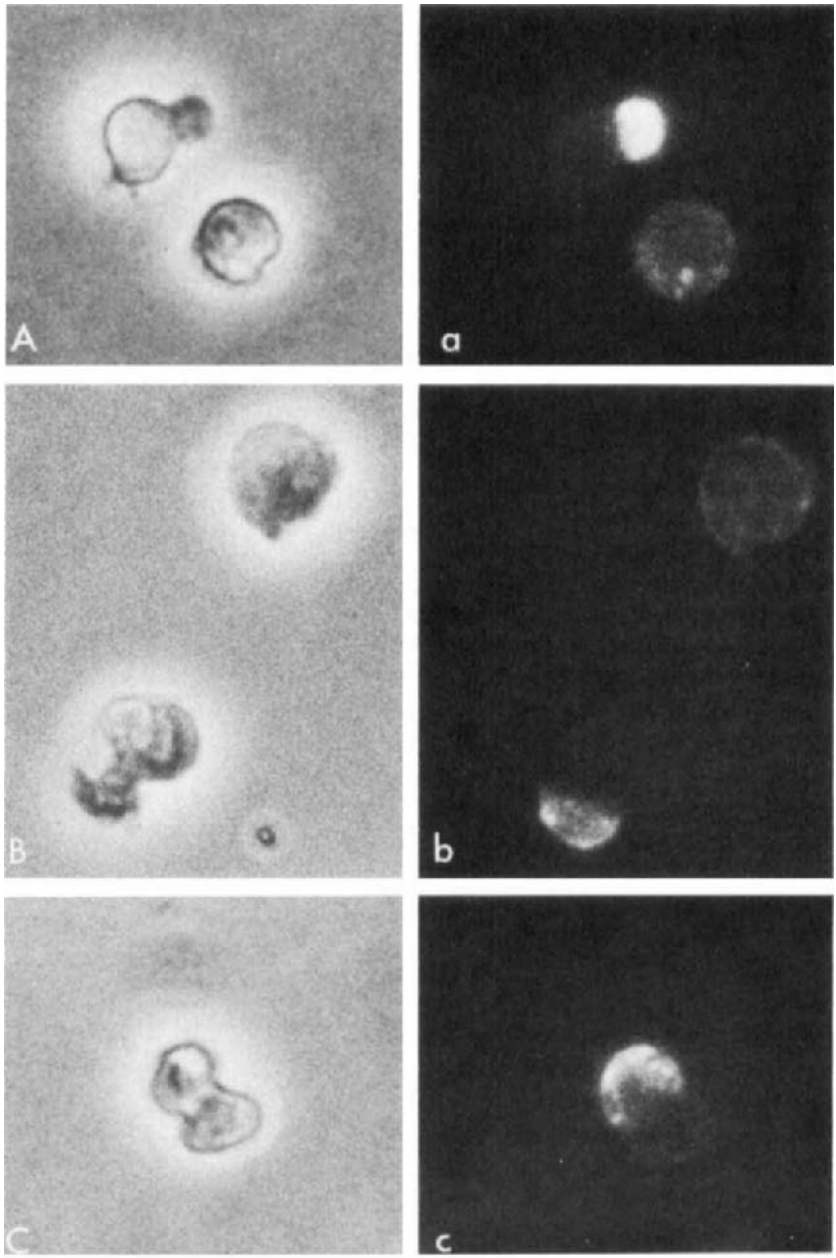

FIG. 13. Phase contrast and fluorescent micrographs of B cells showing caps of Ig–anti-Ig complexes. In the upper panel (A, a) is a representative cell showing a cap with the associated constriction and the ameboid morphology. The remaining panels show the effects of inhibitors of energy metabolism on the cells with caps. The constriction becomes an exaggerated band encircling the cell and separating the cap from the rest of the cell (B panel). (From Schreiner and Unanue, 1976a.)

stimulate the spontaneous polymerization of microtubules and relaxation of Ca^{2+}-sensitive filaments. This would constitute a channeling mechanism and restore to the microtubular network more of the directive role originally ascribed to it (Berlin, 1975).

In fact, many of the effects of the cytochalasins and the anesthetics can be properly understood in terms of the opposing functions of microfilaments and microtubules. As discussed earlier, cytochalasin D, a more potent congener of cytochalasin B, is extremely inhibitory to Ig cap formation and a powerful disrupter of the formed cap. Miranda *et al.* (1975a,b) have demonstrated that this agent provokes an ATP-dependent hypercontractile state in microfilaments which then contract into dysfunctional clumps. They suggested that cytochalasin B might have the same, but weaker, effect. This would explain the curious synergism observed between colchicine and cytochalasin B. Colchicine has no effect on the forming of the Ig cap; cytochalasin B is only marginally inhibitory. Yet both together completely suppress Ig capping (de Petris, 1974, 1975; Unanue and Karnovsky, 1974). More significantly, both agents together reverse Ig caps with the same efficiency as cytochalasin D alone or the Ca^{2+} ionophore alone (de Petris, 1974, 1975; Poste *et al.*, 1975b; Schreiner and Unanue, 1976a).

We suggest as an explanation that the power of the cortical system to contract and deform either membrane or cytoplasm is functionally opposed by the integrity of cytoplasmic microtubules. In many systems microtubules appear to restrict the range of microfilament activity, perhaps by defining domains. Once microtubules are depolymerized, specialized areas of microfilament activity expand. Thus, for example, fibroblasts in colchicine lose their elongated shape and the once-localized membrane ruffling at the advancing edge of the cell spreads over the cell surface. Macrophages exposed to colchicine lose their gliding fibroblast movement characterized by stable, intracellular architectural relationships and begin to manifest ameboid movement and cytoplasmic streaming (Bhisey and Freed, 1971a,b). Colchicine similarly induces oxygen-dependent contractions in granulation tissue composed of fibroblasts owing to the apparent release of microfilaments from the control exercised by microtubules (Van Den Brenk and Stone, 1974). Thus, we suggest that, although Ig capping is not normally susceptible to the weaker influence of cytochalasin B, these weaker effects are enhanced by the elimination of the restrictive influence of microtubules.

Nicolson (1976) has made a similar proposal for the opposing influence of microfilaments and microtubules in other cell types and suggested that these two systems could be linked to each other or to independent anchorage points. There is no way at present of deciding which.

F. Mechanisms of Capping of Other Surface Proteins

While the capping of surface Ig and of lectin-binding sites have been the two most extensively studied systems of redistribution on cells, virtually all the other proteins on the lymphocyte surface are redistributed to some extent by ligands, such as specific antisera (Section IV). Capping of various proteins—usually alloantigens, such as MHC determinants—differs distinctly from Ig capping. It is slower than Ig capping, occurs on fewer cells, and is inefficient, for it usually requires further cross-linking by secondary antisera in a sandwich-type technique. Alloantigen capping does not stimulate motility. It does resemble both lectin receptor and Ig receptor capping in that it is usually suppressed by metabolic inhibitors and is variably sensitive to modulation of microfilaments and microtubules (Unanue and Karnovsky, 1973; Stackpole *et al.*, 1974a,b,c; Schreiner and Unanue, 1975b; unpublished observations, 1976).

The poor spontaneous capping of most ligand-bound alloantigens has been previously postulated to reflect the pattern of their surface distribution. Proteins, such as H2 antigens or ALS-binding sites are separated by larger areas of bare membrane than is the case with Ig; it has been suggested that the additional cross-linking of a second layer of antibody is required to bridge these larger spaces (Karnovsky *et al.*, 1972; Unanue and Karnovsky, 1973). However, this may not be the entire mechanism, for even with the additional cross-linking, alloantigen capping remains very inefficient. Quite possibly this may prove to result from the relationship of the patched receptors with the cytoplasm. It remains to be seen whether alloantigen capping is similar to the Con A receptor model of passive transport of patches or represents an inexplicably poor variation of the active transport of patches, as seen with Ig, or is from still other cellular mechanisms. Essential to any definition of the basis and purpose, if any, of alloantigen capping will be studies on formation, maintenance, and reversibility of alloantigen caps as well as on the valency and heterogeneity of the surface proteins in the various systems.

Bretscher (1976) has just proposed that all capping is due to a rapid, continuous, oriented flow of lipid molecules in the plasma membrane. This flow would not affect single membrane proteins capable of rapid lateral diffusion but would be expected to sweep large, slowly diffusing molecules—such as cross-linked proteins—to the site of lipid resorption, forming a cap. He has further suggested that microfilaments are in some way essential to this lipid flow, thus explaining their role in cap formation. However, since this membrane streaming would actively involve lipids and only passively affect cross-linked proteins, one would expect all comparably cross-linked proteins to behave in the same way if such

a mechanism would be operative. From what we have been analyzing in the preceding section this is clearly not the case for all cross-linked surface molecules.

VI. Final Fate of Ligand–Receptor Complexes

The ligand–receptor complexes on the cell surface may: (1) be interiorized in vesicles; (2) remain on the cell surface for various periods of time; (3) be released from the cell into the extracellular environment; or (4) dissociate, leaving the antigen free on the cell surface. The fate of each complex varies with the antigen in question, its valence and density on the membrane, the nature and/or amounts of ligand, and the cell.

A. Fate of Ig–Anti-Ig Complexes

The interiorization in pinocytotic vesicles is the usual fate of many, but not all, antigen–antibody complexes on the lymphocyte surface. Most of the complexes involving surface Ig with ligands, anti-Ig (or antigens, Section IX) are removed by endocytosis after redistribution into patches and caps. The interiorization of Ig–anti-Ig complexes has been studied morphologically by using immunofluorescence (Taylor *et al.*, 1971; Unanue *et al.*, 1972a,b), autoradiography (Unanue *et al.*, 1972b; Antoine *et al.*, 1974), or electron microscopy (de Petris and Raff, 1972, 1973a; Karnovsky *et al.*, 1972; Unanue *et al.*, 1972b; Rosenthal *et al.*, 1973; Antoine *et al.*, 1974; Antoine and Avrameas, 1974; Linthicum and Sell, 1974) or by following the fate of radioiodinated anti-Ig antibodies (Ault *et al.*, 1973; Engers and Unanue, 1973; Knopf *et al.*, 1973). By immunofluorescence the endocytosed complexes are visualized as discrete, small, round beads that accumulate progressively in the perinuclear area. The progress of complexes from patches and caps on the cell surface to packets inside vesicles can be identified under immunofluorescence, although a superficial examination may easily lead to misinterpretation. In some reports, lymphocytes were examined long after the binding of anti-Ig (about 30 minutes at 37°C) at which times the complexes were all interiorized and grouped into many small vesicles in one area of the cytoplasm. Because the cytoplasmic mass of a lymphocyte is small, the interiorized mass of complexes may resemble caps. This problem is particularly striking in human or rabbit B cells where interiorization of complexes develops very fast (Ault *et al.*, 1973; Linthicum and Sell, 1974). Such problems obviously are not present in electron microscopic studies when one views antibodies that are radioiodinated or tagged with a visual marker or an enzyme.

Morphological studies have shown that the surface complexes of ligand-bound Ig are interiorized in vesicles that then move toward the Golgi region where they accumulate and fuse with lysosomes (Taylor *et al.*, 1971; Unanue *et al.*, 1972a,b; Santer, 1974; Stackpole *et al.*, 1974a,b). The interiorized complexes eventually disappear from the cell, presumably as a result of their catabolism by lysosomal enzymes. Interiorization is a very fast process that requires energy metabolism (Taylor *et al.*, 1971; Unanue *et al.*, 1972b, 1973). When cells are treated with inhibitors of energy metabolism, complexes of Ig–anti-Ig remain on the surface for long periods of time in irregular, large patches.

Endocytosis of Ig–anti-Ig complexes can follow capping of the complexes or take place from multiple sites on the cell surface. In murine B cells, for example, endocytosis is one of the events in the sequence rapidly taking place shortly after the cell has capped the complexes. However, anti-Ig, at doses too low to induce significant capping, is still internalized (Unanue *et al.*, 1972b). In human or rabbit B cells, endocytosis of many complexes develops as soon as multiple aggregates are formed, frequently without any prior formation of caps (Ault *et al.*, 1973; Linthicum and Sell, 1974). This is also true for other complexes not involving Ig. Thus, capping is not a required step for the internalization of surface Ig complexes.

The question of whether internalization requires cross-linking of two adjacent surface receptors has been considered in relation to monovalent antibodies. De Petris and Raff (1973) reported that monovalent antibodies, which could not cross-link, did not produce gross redistribution, such as patches or caps, but that some were eventually interiorized in small vesicles. The interiorization of rabbit Fab anti-Ig was seen after about 2 hours. When a bivalent antibody to rabbit Ig was added, cross-linking the sites, then capping developed with rapid and extensive endocytosis of complexes. Interiorization of Fab anti-Ig was also reported by Antoine *et al.* (1974) using peroxidase-labeled antibody. Our own experience has been along the same lines reported above in that some complexes of Fab anti-Ig do eventually leave the cell surface and disappear into endocytic vesicles.

In conclusion, endocytosis develops rapidly and extensively after interaction with a bivalent anti-Ig, irrespective of cap formation, but also takes place with monovalent antibodies, albeit less efficiently. One signal that favors the interiorization process involves the linking of two adjacent sites, this somehow triggering the mechanical event. The endocytosis that takes place with monovalent antibodies can reflect a change of the univalent ligand on the receptor, which may alter its conformation somehow, either favoring some clustering or allowing the receptor to interact

with key membrane components. In their original fluid mosaic model Singer and Nicolson (1972) stated that such effects of ligand and receptors could be predicted.

Endocytosis of Ig–ligand complexes is extensive and usually leads to the clearance of most, if not all, the Ig sites from the membrane. The fate of the complexes was studied by Engers and Unanue (1973) and Ault *et al.* (1974) using ^{125}I-labeled anti-Ig antibodies. B cells were incubated with ^{125}I antibodies in the cold, washed, and cultured for various periods of time; estimates were made periodically of cell-bound ^{125}I and ^{125}I in the culture medium. It was found that the cells progressively lose ^{125}I-anti-Ig (Fig. 14) into the culture fluid in two forms—as ^{125}I bound to tyrosine, indicating complete catabolism by the lymphocyte, and in macromolecular form. The macromolecular ^{125}I was of three sizes: the same size as monomeric IgG, various sizes smaller than IgG, or a much heavier polydispersed form. Most of the macromolecular material was released very early after incubation at 37°C. The heavy sedimenting material was thought to be anti-Ig complexed to cell surface Ig. There is also some morphological evidence of release of complexes by electron microscopy. Karnovsky *et al.* (1972) noticed small, tight masses of labeled anti-Ig peeling off the cell during patching and capping. However, final proof must be given that the surface Ig is indeed released with the anti-Ig before a definitive statement can be made on shedding of complexes.

The smaller-sized molecules were thought to be partially digested molecules. Precedence for release of partially digested molecules has been obtained with radiolabeled proteins or Ig in phagocytes where a definite, albeit small, percentage of the interiorized material was released very early (Cruchaud and Unanue, 1971; Calderon and Unanue, 1974). Perhaps, during the time when a large number of complexes are being digested, some of the endocytotic vesicles can flow back to the membrane to be released, pinocytosis in reverse; or alternatively, some of the vesicles where digestion is taking place may still be open to the outside, resulting in the spilling of part of their contents. A similar situation has been noted in neutrophils, from which lysosomal enzymes can be released shortly after pinocytosis (reviewed in Becker and Henson, 1973).

The release of ^{125}I-tyrosine clearly proved that the lymphocyte is capable of hydrolyzing and digesting the interiorized complexes. It increased progressively with time of culture—by the end of 6–10 hours of culture, 25–60% of the anti-Ig initially bound to the cell was eliminated, and that remaining was found intracellularly in the form of partially catabolized molecules. Studies similar to these but with murine myeloma cells were done by Knopf *et al.* (1973) with similar conclusions concerning the

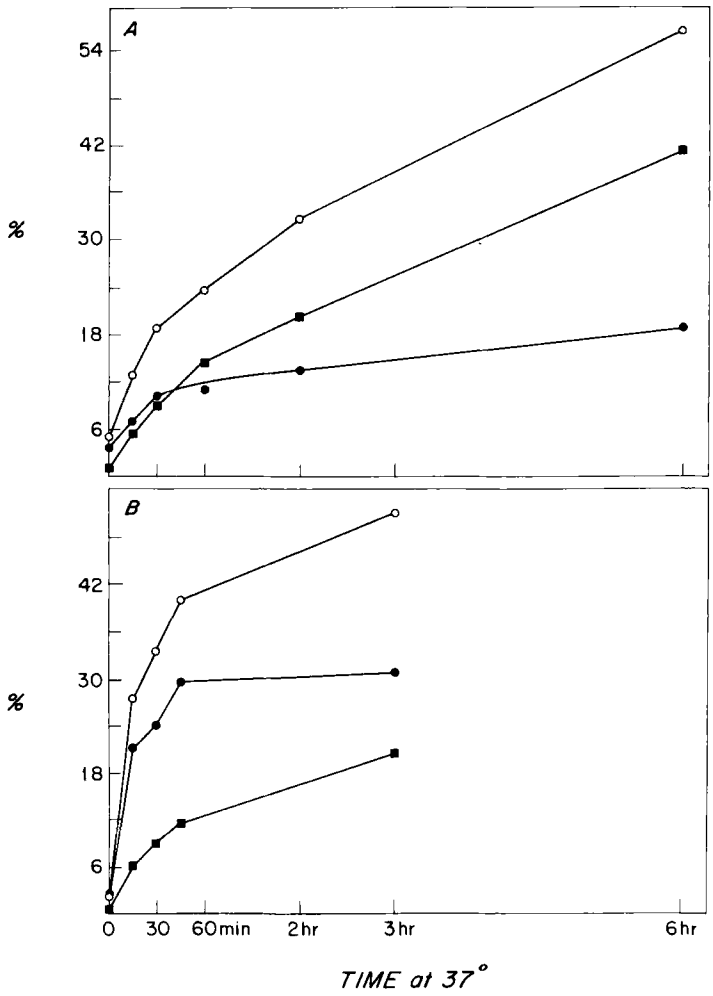

FIG. 14. Graphs show the elimination of ^{125}I from human B lymphocytes at the indicated times. Two different experiments, A and B, are indicated. Shown is the amount of ^{125}I released into the medium (○, total), bound to proteins (●, TCA precipitable), or to amino acids (■, TCA soluble). The explanation is in the text. (From Ault *et al.*, 1974.)

degradation of ^{125}I-labeled anti-Ig. Rapid elimination of labeled anti-Ig was also reported by Wilson *et al.* (1972).

Catabolism of complexes by the B cell results from interaction of the endocytotic vesicles with lysosomes in a manner analogous to that in other cells. Lysosomes in lymphocytes have been characterized by Bowers

and de Duve, who identified a series of about ten acid hydrolases in spleen cells (Bowers and de Duve, 1967; Bowers, 1970) and thoracic duct lymphocytes (Bowers, 1972). Many of the enzymes have optimal activity at an acid pH, sediment with a density corresponding to lysosomes, and show a latent period of activation.

Antigen molecules bound to the B lymphocytes' Ig receptor are likewise eliminated from the cell. In many instances, the antigen molecules have been visualized inside the cell, although precise quantitative and biochemical analyses have not been done because of the paucity of cells binding a given antigen molecule (Section IX). We should note here that some complexes involving surface Ig with large particulate antigens, such as red cells, are not interiorized as Ig–anti-Ig complexes are (Ashman, 1973), suggesting that the lymphocytes may be limited in this mechanical function (Section IX).

B. Fate of Other Ligands

The ultimate fate of other ligands on the lymphocyte surface has not been examined with as much detail as anti-Ig. It is apparent that not all ligand-surface molecules are eliminated as effectively as are Ig–anti-Ig complexes—some remain at the cell membrane for long periods of time.

The surface redistribution of molecules of the MHC was analyzed in Section IV. There we made the point that redistribution was slow, albeit more extensive when two layers of antibodies were used. Antibodies to MHC molecules are eliminated from the cell, although some of the surface complexes definitely persist. The ultimate fate of the complexes has been studied in various ways—directly by using morphological methods and following the fate of labeled antibody (Cullen *et al.*, 1973; Jacot-Guillarmod *et al.*, 1973), or indirectly by determining whether antibody-treated cells are killed by the addition of C (Amos *et al.*, 1970; Takahashi *et al.*, 1971; Cullen *et al.*, 1973). In this method cells are incubated with antibody, in the absence of fresh serum, and washed; at various times fresh serum is added. If immune complexes are still surface bound, C is fixed and the cell killed.

As viewed with immunofluorescence or autoradiography, the complexes of HL-A–anti-HL-A or H-2–anti-H-2 persist on lymphoid cells for a few hours, although the precise amounts have not been determined (Kourilsky *et al.*, 1972; Unanue *et al.*, 1972b; Menne and Flad, 1973). In one study, about half the cells retained the label after 7 hours of culture (Menne and Flad, 1973).

Antibodies radiolabeled with ^{125}I, after their binding to lymphocytes, elute from the cell with time. By 4 hours, about 40% of the antibodies are

lost; by 18 hours, about 70–80% are gone, with no further loss by 24–40 hours of culture (Cullen *et al.*, 1973; Jacot-Guillarmod *et al.*, 1973). Thus, the cell retains a small amount of radioactivity even after long periods of incubation. With the progressive loss of antibody, the cell becomes more and more resistant to lysis by the addition of C. For example, in the experiment of Cullen *et al.* (1973), 70% of the cells were still killed by addition of C *4 hours after* incubation with anti-HL-A; this figure dropped from 23 to 10% after 18 to 24 hours of incubation, respectively. Thus, from the studies with anti-HL-A antibody, some complexes clearly do persist, remaining surface bound, although this decreases with time.

What is the fate of the antibodies and the surface complexes? By immunofluorescence, some evidence of interiorization in vesicles was observed, but the extent was not significant (Unanue *et al.*, 1972b; Kourilsky *et al.*, 1972). This was also true when using electron microscopy and autoradiography with ^{125}I-labeled anti-H2 antibodies. After 2 hours most of the antibody remained cell associated with very little in endocytotic vesicles (Unanue *et al.*, 1972b). Of interest is the biochemical analysis of ^{125}I-labeled antibodies released into medium, a study done so far only in man. Seventy to 80% of the ^{125}I-labeled antibody released was the same size as the native molecule, the remaining being larger in molecular size (Jacot-Guillarmod *et al.*, 1973). This antibody was unable to bind to fresh cells (Cullen *et al.*, 1973), strongly suggesting that its combining site was occupied by antigen. However, this point is not clear inasmuch as there is no change in the size of the molecule unless the antigen bound to the combining site was a very small fragment. In other studies of tumor cells and alloantibodies, Amos *et al.* (1970) also found a dissociation of the antibody, but the released material retained its combining capacity. Although the postulate has been made that alloantibodies are shed from the membrane complexed to antigen (Miyajima *et al.*, 1972; Cullen *et al.*, 1973), further and stricter biochemical analyses are required to substantiate this point. A final point related to the dissociation of antibody is whether new sites are exposed with time, a point that appears to be clearly the case (Cullen *et al.*, 1972; Miyajima *et al.*, 1972). Whether these sites are newly reexpressed products or the "old" sites to which antibody bound has not been ascertained.

Finally, addition of a second antibody to the alloantibody–alloantigen complex, as mentioned before, increases the redistribution rates (Kourilsky *et al.*, 1972; Unanue *et al.*, 1972b; Menne and Flad, 1973; Stackpole *et al.*, 1974a), as well as the amount of complexes funneled into pinocytotic vesicles (Unanue *et al.*, 1972b; Karnovsky *et al.*, 1972; Menne and Flad, 1973), although precise quantitative figures are not available. There is a substantial loss of MHC molecules with time. This has been

ascertained by finding that the cells are not killed by addition of new antibody and C (Takahashi *et al.*, 1971; Cullen *et al.*, 1973).

Complexes of Con A or PHA with surface receptors are endocytosed (Smith and Hollers, 1970), but whether the interiorized complexes are digested is not known. In the macrophage, Edelson and Cohn (1974), reported that Con A bound to the macrophage membrane is interiorized in large vesicles, but these do not fuse with lysosomes. Somehow the normal fusion between primary lysosomes and an endocytotic vesicle coated with Con A is blocked. The lymphocyte transformed by exposure to the mitogen shows many endocytotic vesicles, often containing the lectin (Hirschhorn *et al.*, 1965; Packer *et al.*, 1965; Razavi, 1966; Robineaux *et al.*, 1969; Biberfeld, 1971a,b).

An interesting example of a surface complex that remains surface bound was found with ALS (Unanue *et al.*, 1972b). ALS caps poorly and only slowly disappears with some degree of interiorization. Cross-linking the ALS-surface sites produces immediate capping of the complexes, but these remain cell bound and are poorly endocytosed. The complexes slowly dissociate with time.

The fate of antigen–antibody–C complexes was studied by Nussenzweig and associates (Eden *et al.*, 1973). Their work was reviewed in a previous volume of this series (Nussenzweig, 1974). Murine lymphocytes were incubated with soluble complexes of ^{125}I-labeled bovine serum albumin (BSA)–antibody–mouse C, which bound to the cells' surfaces and redistributed into patches and caps. At various time intervals excess unlabeled antigen was added to the cells. It was found that the excess cold albumin "displaced" the ^{125}I-labeled BSA from the complex, which then appeared free in the culture fluid. After 2 or 3 hours, up to 70% of the cell-bound albumin could be readily removed. These studies leave little doubt that soluble antigen–antibody–C complexes at critical ratios remain cell bound for very long periods of time on the lymphocyte. However, if anti-mouse Ig is added to the cells, making a more extensive lattice, there is no "dissociation" of the labeled material by unlabeled antigen. Although no cytological details were given, it is possible that the persistent surface antigen–antibody–C complexes could have been interiorized by the addition of the second antibody. Gormus and Shands (1975), in studying the fate of live *Salmonella* opsonized with antibody and C, found that the bacterial complexes first redistributed into caps and then were readily shed from the cell without any evidence of interiorization. With the loss of the bacteria from the membrane, there was a concomitant loss of some of the C3 receptors.

In summary, there is no clear understanding of why some complexes are rapidly interiorized whereas others remain cell bound for long periods

of time, disappearing usually from a combination of dissociation of the antibody, shedding of the entire complex, and some degree of endocytosis. The complexes involving surface Ig and ligands are rapidly cleared by endocytosis, and so are those involving Fc receptors or lectins. In these instances, it is apparent that interiorization is favored by cross-linking, that it requires energy metabolism, and that the size of the ligand plays a role. In contrast, in situations involving the MHC or ALS, the lack of interiorization does not relate to cross-linking. With ALS, the complexes can be segregated into caps, and yet are not interiorized. Overall, one gets the impression that complexes involving two presumably crucial sites on the membrane—Ig and Fc receptor—favor rapid interiorization, whereas other complexes not involving these "immunological" components are not destined for rapid endocytosis.

C. Antigenic Modulation

Antigenic modulation is a phenomenon discovered by Boyse, Old, Stockert, and associates during the course of investigating murine TL antigens in thymocytes and thymic leukemias. The TL locus found in chromosome 17 of the mouse in close proximity to the H-2D locus codes for three allelic products (TL1, 2, and 3) occurring on the surfaces of thymocytes (Old *et al.*, 1963; Boyse *et al.*, 1964). TL antigens are lost as the thymocyte matures to a full competent lymphocyte and are absent in peripheral T cells. TL antigens are detected by specific antibodies made in strains of mice lacking the particular antigenic specificities (Boyse and Old, 1969). Boyse *et al.* (1963) discovered that TL antigens could be found in leukemic cells from mice of TL-negative strains. This raised the possibility that TL-negative strains of mice given TL-positive cells might be actively immunized against TL-positive leukemic cells. This exciting eventuality did not take place: the TL-positive leukemic cells transplanted into immunized TL mice making anti-TL antibodies (or into TL-positive mice repeatedly injected with anti-TL antibodies) grew normally. These TL-positive cells were no longer killed *in vitro* in a cytotoxic test involving anti-TL antibodies in the presence of guinea pig serum as a C source. In contrast, the control TL-positive leukemic cells not passaged in mice with anti-TL antibodies were readily killed. Later, Old *et al.* (1968) found that this apparent loss of TL antigens—termed "modulation"—occurred *in vitro* if the leukemic cells were incubated with antibodies. The leukemic cells were incubated first with antibody but not with C, usually for an hour or two, and afterward, in a cytotoxic system consisting of the antibody, but this time with guinea pig C. The leukemic cells lost their capacity to be killed by the antibody and C as a result of

incubation with the first ligand—the cell's TL antigens had been modulated. Thus, the system of modulation involved two incubations, the first with antibody alone (the modulating stage), the second with antibody and C (the cytotoxic indicator stage). Further observation disclosed that: (1) modulation was an active process taking place in cells incubated at 37°C or 22°C, but not at 0°C; (2) exposure of cells to actinomycin D or iodoacetamide stopped modulation; (3) modulation affected leukemic cells more than thymocytes; (4) modulation was accompanied by an increase in antigens coded by the H-2D locus; and (5) modulation varied, depending on the TL specificity under consideration. Thus, anti-TL3 modulated TL1 and 2, but, in contrast, anti-TL2 did not modulate TL2 and even inhibited somewhat the modulation produced by anti-TL1 or 3. The conclusion from all these experiments was to consider modulation "as an actual loss of TL components from the cell surface," not as a mere blocking of the sites by the first anti-TL antibody. [The relationship between the modulation by anti-TL and the increase in antigens coded by the H-2D locus remains unexplained. In this regard, Boyse *et al.* (1968a) have observed a relationship between the amounts of antigens of the H-2D region and the particular TL phenotype of the mouse. How the TL locus influences the expression of one particular set of membrane proteins has not been defined but could be of fundamental importance.]

At the time of discovery of TL modulation, there was little information on the fate of surface antigen–antibody complexes. Now, one can easily conceive of modulation (as defined operationally by Boyse *et al.*, i.e., the loss of the ability of a cytotoxic antibody to kill the cell after an early exposure to antibody) as resulting from the simple loss of the antigen–antibody complex from the cell membrane because of interiorization and/or shedding, as it occurs, for example, in the well-studied system of surface Ig of B cells. When the Ig–anti-Ig complexes are readily lost from B cells, one would expect that these anti-Ig-treated B cells would no longer be killed during a subsequent incubation by anti-Ig and C, which is indeed the case. Takahashi *et al.* (1971) performed protocols similar to those described above and found that anti-Ig and C would not kill B cells pretreated with anti-Ig. In this example, "modulation" clearly resulted from the active elimination of complexes by means of capping and endocytosis. Comparable antibody-mediated modulation of surface antigens, usually using a two-antibody layer, has been described for H-2 antigens (Amos *et al.*, 1970; Schlesinger and Chouat, 1972, 1973; Lengerova *et al.*, 1972; Lesley and Hyman, 1974), HLA (Sadeghee *et al.*, 1975), and viral antigens (Aoki and Johnson, 1972), and surface Ig (Takahashi *et al.*, 1971; Knopf *et al.*, 1973).

Are all modulation phenomena explained in this way, particularly in

the TL system where it was first found? There have been recent attempts to relate the surface redistribution of TL antigens with the process of modulation. Yu and Cohen (1974) studied TL modulation in a leukemic cell line and concluded that anti-TL antibodies produced a loss of TL antigens after 10 hours of incubation. During incubation with anti-TL, there was a progressive loss of cell-bound anti-TL antibodies (using fluoresceinated anti-mouse Ig) and a concomitant loss of TL antigens (as evidenced by the capacity of the cells to absorb TL antibodies). No evidence was obtained for undue shedding of TL antigen–antibody complexes, or for an interruption in the synthesis of new antigens within a 10- to 20-hour period of time. Precise quantitation of the loss of TL antigens internally labeled or radioiodinated was complicated by technicalities of the precipitating system.

Stackpole *et al.* (1974c) performed detailed cytological analyses of the fate of TL-antibody complexes. Using immunofluorescence and electron microscopy, they observed that the complexes were in large, irregular patches or caps, which took a rather long time to develop in contrast to Ig caps. At 1 hour only about one-fourth of the cells had capped. Some internalization also took place. Cytological analysis indicated that the complexes remained cell bound for long periods, a point also confirmed by Loor *et al.* (1975). The concentrations of antibodies used produced only weak immunofluorescence, and the results were not quantitative. Precise estimates have not been made of the amounts of TL antigens lost by internalization, blocked by antibodies, or free at the cell surface. Stackpole *et al.* (1974c) made two observations which indicate that some TL antigens are free on the membrane. First, they found that the source of C influenced the result of the cytotoxic test. Thus, anti-TL-treated leukemia cells—"modulated"—were not killed by anti-TL antbodies and guinea pig C but were lysed by anti-TL and *rabbit* C. Rabbit C, which is more cytolytic than guinea pig C enhances the cytotoxicity of anti-TL antibodies. Although no precise dose-response curve of anti-TL antibodies with both C sources was reported, this observation did indicate that some TL antigens must be free and available to fresh anti-TL, but somehow could not instigate an effective lytic interaction with guinea pig C. Second, it was shown that a heat-labile component of mouse serum was responsible for the modulation of anti-TL antibody with guinea pig C. Anti-TL antibodies, if heated at 56°C, lost their capacity to modulate, i.e., cells treated with heated anti-TL for 1 hour were subsequently killed in a cytotoxic assay using anti-TL and guinea pig C. In contrast, cells treated with unheated anti-TL were "modulated," not killed, in this cytotoxic system. The addition of fresh mouse serum now allowed the heated antibodies to modulate. Thus, a heat-labile component of mouse

serum in some way interfered with the cytolytic effects, but only of guinea pig C.

In trying to integrate the data, we conclude that after antibody binds, a great part of the TL-antibody complex must be lost from the cell surface as a result of interiorization and/or shedding, but that the loss is not complete and some complexes remain surface bound. A limited number of TL antigens become free with time and interact with fresh anti-TL, but lysis follows only with a very active C source present. A larger amount of TL antigens may dissociate from the complex if the initial antibody interacts with the TL antigen in the absence of mouse C (a poor lytic system), but not if mouse C is available; in other words, it would appear that mouse C glues the TL–anti-TL complex together for a longer period of time. Further quantitation of these points, in particular, of the amounts of antigen sites reactive with antibody during the cytotoxic tests, may make it possible to explain the phenomenon of TL modulation. Certainly, even slight alterations in the quantity of surface antigens can make a marked difference in C sensitivity. It has long been appreciated that surface antigen concentration correlates with cytotoxic sensitivity to antibody plus C (Klein *et al.*, 1966; Linscott, 1970). Removal of less than one-third of surface H-2 by endocytosis can result in complete loss of sensitivity to C-dependent lysis; this effect, in turn, is variably dependent on the type of C employed (Lesley and Hyman, 1974; Lesley *et al.*, 1974). Alternatively, steric interference with accessibility to C secondary to clustering of surface antigens has also been demonstrated (Lengerova *et al.*, 1972). Precise quantitation of the fate of TL antigens with time could perhaps reconcile the observation of Yu and Cohen, who used incubation periods of 10–20 hours, to those of Stackpole *et al.*, who used incubations lasting only up to 2 hours. It is likely that with time all of a cells' TL antigens are lost. Modulation in this case would imply loss of all surface antigens. Supporting the concept that the cell's genetic mechanisms controlling TL expression differ from the capacity of the cell to undergo modulation is an experiment by Liang and Cohen (1975) in which somatic hybrid cells formed by fusion of TL^+ leukemia cells and fibroblasts were perfectly capable of expressing TL antigens on their surface but were unable to undergo modulation in response to anti-TL antibody.

Finally, it should be noted that modulation has been produced by Fab-anti-TL antibodies, an unexpected finding inasmuch as these antibodies do not lead to significant redistribution (Lamm *et al.*, 1968). Stackpole *et al.* (1974c), in their cytological studies on modulation, indicated that Fab-anti-TL could be found in small aggregates after 2 hours. Thus, within the limitations of the immunofluorescence system, Fab

antibody appears to produce some redistribution of TL antigens, which may be associated with the subsequent inability of new antibody and C to lyse the cells.

VII. Relationship of Surface Components with Each Other and with Intramembranous Particles

A. Relationship between Two Surface Molecules

A critical question is: What is the relationship between two surface components on the cell membrane? Attempts to map two components by using electron microscopy and different visual markers have, in general, been unsatisfactory because of steric hindrance from these large markers. Furthermore, direct morphological approaches can only determine whether or not two components are close together, but cannot define any direct physical association. One approach that has been extremely fruitful in studies on lymphocyte membranes is that of differential redistribution, the principle of which is illustrated in Fig. 15. If one component is separated physically from a second component, then its patching, capping, or clearing by an antibody should not alter the distribution of the second component. On the other hand, if both components are linked, then both should redistribute simultaneously. Taylor *et al.* (1971) first observed this phenomenon, described in their now classical paper on capping. Treatment of murine B cells with anti-Ig led to the disappearance of surface Ig but did not change the distribution of H-2 antigens or components reactive with heterologous ALS. Studies of differential redis-

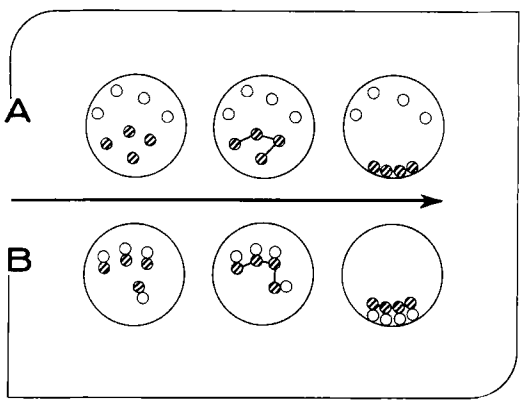

Fig. 15. This graph represents the principle of differential redistribution. In A, the molecules are not linked: capping of one does not interfere with the other. In B, both molecules are linked: capping of one cocaps the other.

tribution have been very important in outlining relationships between components of the lymphocyte surface. The most pertinent observations are summarized below.

The cytochemical methods used to study differential distribution of two membrane components are two: In the first method component *a* is redistributed by an antibody either at ambient temperature or at 37°C; the cells are then incubated in conditions where there is no further redistribution (at low temperature, after fixation, or in the presence of metabolic inhibitors) with antibodies to component *b*. Usually, the antibodies to each component are tagged by different fluorochromes. In the second method, component *a* is totally cleared from the cell surface by prolonged incubation at 37°C with antibody, usually unlabeled; then the cell is examined for the presence of component *b* using fluorescent-labeled antibody.

A third method, used by those interested in studying transplantation antigens, consists of determining whether the lysis of a cell produced by antibody to a surface antigen (plus C) is abrogated if the cell was previously incubated with antibodies to a different antigen (in the absence of C) (Cullen *et al.*, 1973; Hauptfeld *et al.*, 1975; Hauptfeld and Klein, 1975). First, component *a* is redistributed by incubation with antibody plus an anti-antibody (i.e., a two-layer reaction); then the cell is incubated with antibodies to component *b* in the presence of C.

1. Ig

The two Ig on human B cells, IgD and IgM, are localized independently (Knapp *et al.*, 1973; Rowe *et al.*, 1973b). Raff *et al.* (1973) used a differential redistribution method to determine whether all the surface Ig molecules in a B cell were directed to the same antigen. They incubated lymphocytes with polymerized flagellin, a large polymeric protein (Section IX). The few clonal cells bearing antiflagellin antibodies bound the antigen and capped. Then the cells were examined under noncapping conditions for surface Ig outside the cap area. Most of the Ig molecules were in the cap zone with the antigen, not diffusely localized on the cell surface. This experiment is probably the best demonstration that all Ig molecules on an individual B cell are, in fact, of a unique specificity. These results have now been confirmed by Nossal and Layton (1976), using cells primed to the dinitrophenyl (DNP) hapten.

A critical question is whether surface Ig is associated with other proteins on the plasma membrane. Surface Ig redistributes independently of C3 receptors (Abrahamsohn *et al.*, 1974; Nussenzweig, 1974; Parish and Hayward, 1974) and of the antigens of the MHC (Taylor *et al.*, 1971; Preud'homme *et al.*, 1972; Unanue *et al.*, 1974a). For example, capping

of surface Ig in man does not alter the distribution of HL-A antigens (Preud'homme *et al.*, 1972) and vice versa. Of particular interest has been the relationship, if any, between surface Ig and antigens coded by the I region of the MHC in the mouse. Ia antigens seem to be involved in a series of cellular interactions; hence, a possible association with Ig could be envisaged. Capping of surface Ig does not lead to redistribution of Ia molecules in murine B lymphocytes (Unanue *et al.*, 1974b; Abbas *et al.*, 1976a). Ia antigens were detected by immunofluorescence (Unanue *et al.*, 1974b) or by electron microscopy (Abbas *et al.*, 1976a). A similar conclusion was reached by Hauptfeld *et al.* (1975), using the serological method with antibody and C.

The relationship between Ig and Fc receptors is of particular interest. It has been found by some, but not all, that capping of surface Ig leads to redistribution of the Fc receptor (Abbas and Unanue, 1975; Forni and Pernis, 1975; Basten *et al.*, 1976). This was tested by capping Ig with anti-Ig and then looking for the Fc receptor with antigen–antibody complexes. The complexes localized to the region of the cap. However, if the reaction was done in the reverse sequence, it was found that capping of Fc receptors did not change the distribution of surface Ig. The conclusion drawn was that Fc receptors were, in fact, not associated with surface Ig but that an association developed when surface Ig became complexed to antibody. In two other reports, no association was found between Ig–anti-Ig complexes and Fc receptors (Parish and Hayward, 1974; Ramasamy and Lawson, 1975). In the studies described in these two reports, red cells coated with antibody were used as the indicator ligand for Fc receptors. In both experiments Ig was redistributed by anti-Ig first, then the cells were incubated under noncapping conditions with antibody-coated red cells. The number of cells forming rosettes or the number of red cells per cell did not change. The reason for this discrepancy between the two sets of observations is not clear at this time.

One obvious explanation for the redistribution of Fc receptors (detected by soluble complexes) together with Ig–anti-Ig complexes is that the Fc portion of the anti-Ig, which is free to move, binds to the Fc receptor and carries it into the patches and caps. This possibility was examined by using pepsin-treated anti-Ig antibodies. It was found that substantial coredistribution also took place with pepsin-digested anti-Ig antibodies, eliminating the explanation that it was caused by the intact anti-Ig molecule per se (Abbas and Unanue, 1975; Forni and Pernis, 1975; Basten *et al.*, 1976). There is no explanation at present for the association of ligand-bound surface Ig with the Fc receptor. We think that the surface Ig complexed to a ligand undergoes membrane changes that permit an association with the Fc receptor. Further studies with other

ligand–receptor complexes must be done to understand this phenomenon fully.

Concerning the Fc receptor on the lymphocyte surface, Dickler and Sachs (1974) observed that exposure of murine B lymphocytes to antibodies to histocompatibility antigens blocked the Fc receptor sites, as judged by the inability of fluorescein-tagged aggregated Ig complexes to bind to the cell. The authors concluded that: (1) the antibodies responsible for the inhibition were directed to I region antigens, not to K and D end molecules; (2) a Fab fragment of the antibodies could also inhibit the binding of aggregated Ig, therefore, ruling out the participation of the Fc end of the ligand in the phenomenon (see also Basten *et al.*, 1975, 1976); (3) antibodies to Ia specificities of one parental type could inhibit completely the binding of aggregates to an F_1 hybrid B cell. Thus, either the Fc receptor was identical to some Ia antigens or there was a very close association of both entities in the membrane.

Further studies have indicated that inhibition of Fc receptor binding by anti-cell surface components is more complex. For example, in a study reported by Schirrmacher *et al.* (1975), using antibody-coated red cells as indicators for Fc receptor binding, antibodies to various surface components inhibited Fc receptor binding. This inhibition was observed with anti-Ig antibodies, antibodies to K and D end molecules, as well as anti-Ia. Thus, the red cell indicator system appears, at face value, to be much more sensitive to inhibition by a number of surface antibodies than fluoresceinated aggregates, which are affected only by anti-Ia. However, Dickler *et al.* (1975) have inhibited Fc receptor binding of aggregates by using other alloantibodies, indicating that other membrane ligands can also produce the effects.

Cocapping of Fc receptors with anti-Ig sheds some light on the relationship between Ia, Fc receptors, and Ig. Thus, redistribution of anti-Ig leads to a similar coredistribution of Fc receptors but does not change the topography of Ia molecules, which remain diffuse (Unanue *et al.*, 1974b; Abbas and Unanue, 1975; Abbas *et al.*, 1976a). Likewise, capping with aggregates does not lead to any change in the distribution of Ia antigens (Basten *et al.*, 1976). Thus, a substantial number of, if not all, Ia molecules and Fc receptors are unrelated to each other. Recently, Rask *et al.* (1975) have isolated a 65,000-dalton protein from the surfaces of B cells thought to be the Fc receptor. This component lacked Ia determinants. This apparent lack of association between Fc receptors and Ia contrasts with the observations of Dickler and Sachs. Perhaps some membrane changes result from anti-Ia binding that reflect in the interaction of the Fc receptor with complexes.

A final comment on the differences between soluble complexes and

particulate complexes is pertinent. Three differences have been recorded: (1) treatment of cells with azide inhibits binding of particulate complexes (Parrish and Hayward, 1974) but not of soluble complexes; (2) low doses of anti-Ig inhibit the binding of particulate complexes to Fc receptors but not of soluble complexes; and (3) anti-Ig induces coredistribution of soluble complexes, but not of particulate complexes. The possibility either of two different structural components or of a single component but in two topographically different forms (clustered *versus* diffuse) must be considered.

2. *MHC*

The differential redistribution technique has been particularly useful in studying the interrelationships of molecules of the MHC. Studies from Neauport-Sautes and associates (1973) have indicated that antigenic specificities coded by H-2D and H-2K are on different molecules. Patching and capping of one do not alter the distribution of the other. Observations made on the HL-A system in man have demonstrated that the serological specificities of the three segregant loci are physically independent of each other (Neauport-Sautes *et al.*, 1972, 1973, 1974, 1975; Pierres *et al.*, 1975). Ia antigens in the mouse were found not to be associated with antigens of the H-2K and H-2D regions (Unanue *et al.*, 1974b). These series of observations have been confirmed biochemically (Cullen *et al.*, 1972). Also, TL antigens are independent of H-2 or of the alloantigen θ (Loor *et al.*, 1975).

The relationship between β_2-microglobulin and HL-A was shown both biochemically and cytologically, in the latter cases through coredistribution experiments. Thus, redistribution of β_2-microglobulin by antibodies produced patching and some degree of capping with concomitant redistribution of HL-A antigens (Bismuth *et al.*, 1973; Poulik *et al.*, 1973; Ostberg *et al.*, 1974; Solheim, 1974; Solheim and Thorsby, 1974). If the reaction was done in the other direction, not all β_2-microglobulin coredistributed with HL-A. Hence, all HL-A antigens are associated with β_2-microglobulin, but some of the β_2-microglobulin molecules are unrelated to HL-A molecules.

B. Relationship of Surface Molecules and Intramembranous Particles

The question has been raised whether some of the surface components of the lymphocytes are associated with the globular particles seen on freeze-cleaved surfaces. The principle of freeze-cleaving is illustrated in Fig. 1. During cleavage the membrane is fractured through its center and split into two faces, the concave outer face fraction and the com-

plementary inner face, both of which contain a variable number of small round masses (Branton, 1966; Pinto da Silva and Branton, 1970). The intramembranous particles, usually 70–80 Å in diameter, appear to represent the hydrophobic portion of integral amphipathic proteins intercalated within the phospholipid layer. In the red cell, for example, the particles are associated with the major glycoproteins of the membrane. This has been found by inducing clustering of various surface sites by cross-linking ligands and showing a similar clustering of the particles. Specifically, clustering of the antigen sites of blood group A (Pinto da Silva *et al.*, 1971), PHA binding sites (Tillack *et al.*, 1972), sites of influenza virus attachment (Tillack *et al.*, 1972), Con A binding sites (Pinto da Silva and Nicolson, 1974), and negatively charged groups (Nicolson, 1973) corresponds with clustering of the globular intramembranous particles. Intramembranous particles have been associated with calcium-sensitive ATPase in sarcoplasmic reticulum (Tillack *et al.*, 1974). In the particular case of rhodopsin it is actually possible to create the particles by incorporation of the purified protein into phosphatidylcholine layers (Hong and Hubbell, 1972).

In the lymphocyte, attempts have been made to relate the redistribution of a particular component to the distribution of the intramembranous structures. If the surface redistribution of one component is associated with a similar redistribution of the particles, then a case can be made that the surface component is indeed linked to a hydrophobic protein component within the membrane.

We shall review first the studies of cleaved surfaces of untreated lymphocytes. In lymphocytes the intramembranous particles are found mainly in the inner face fracture, usually in low number. The globular particles of lymphocytes are 85 Å in diameter (McIntyre *et al.*, 1974). The number of globular particles in the inner face is about $500/\mu^2$ (Scott and Marchesi, 1972), which is much less than the number seen in the cleaved surfaces of red cells ($4500/\mu^2$). It has been found that the number of particles is about 2- to 3-fold higher in lymphocytes exposed to PHA, or in continuously cultured lymphocyte lines (Scott and Marchesi, 1972). The increase in particles after exposure to PHA takes place as early as the 36th hr of culture.

The distribution of intramembranous particles in lymphocytes has been a matter of concern. Mandel (1972) reported that a number of T cells contained small aggregates of particles. However, subsequent studies have indicated that the intramembranous particles are distributed diffusely and that the clustering observed by Mandel can be explained by the technical procedure (McIntyre *et al.*, 1973, 1974). In the freeze-cleaving procedure, the cells are embedded in glycerol (20–25%) or di-

methyl sulfoxide, which act as cryoprotectants, not allowing ice formation during the procedure. Many lymphocytes exposed to these cryoprotectants without prior fixation show an aggregation of particles that depends on the length of exposure and the concentration of the cryoprotectants. Aggregation occurs within minutes and is reversible upon washing the cells. Most lymphocytes that are exposed to glycerol after fixation in glutaraldehyde or that are fixed in the absence of cryoprotectants do not show clustering of intramembranous particles. In the case of Mandel's experiments the phenomenon of clustering can be attributed to the glycerol effect, inasmuch as the lymphocytes were exposed to it prior to fixation. Clustering of intramembranous particles by glycerol has not been reported in nonlymphoid cells.

In the lymphocyte there has been no demonstrable association thus far between the surface redistribution of a number of surface components and intramembranous particles. This has been examined by interacting the cell with the appropriate ligand, allowing the complex to redistribute and then cleaving the membrane. The distribution of surface Ig, Con A binding sites, sites reactive with ALS, H2 antigens (Karnovsky and Unanue, 1973), θ alloantigens (Matter and Bonnet, 1974; M. J. Karnovsky and E. R. Unanue, unpublished observations, 1973), and PHA (Unanue and Karnovsky, 1973) is not reflected in any changes in intramembranous particles. Loor (1973) observed a minor degree of intramembranous particle clustering induced by PHA. However, in extensive studies, Karnovsky has been unable to demonstrate this to any significant extent.

VIII. Late Effects of Anti-Ig Antibodies

Anti-Ig antibodies have profound effects on B-cell functions aside from the surface and cytoplasmic events that already have been described. Overall, the effects of anti-Ig antibodies are similar, if not identical, to those produced by soluble protein antigens when bound to specific B cells in the absence of helper functions (Section IX).

A. Regeneration of Surface Ig

After capping and endocytosis of surface Ig, new receptors are expressed on the membranes of B cells (Loor *et al.*, 1972; Unanue, *et al.*, 1972a; Elson *et al.*, 1973; Ault and Unanue, 1974). Fluorescein or radioiodinated anti-Ig antibodies did not bind to B cells that had cleared their surface Ig by a brief exposure to unlabeled anti-Ig antibodies at 37°C. If these B cells were then cultured, binding of anti-Ig was measurable within 6–10 hours and increased progressively. By 24 hours, the number of cells binding anti-Ig antibodies was the same as in the untreated cell

population. Loor, *et al.* (1972) suggested that the number of surface Ig molecules was higher after regeneration inasmuch as the number and size of the fluorescent "spots" appeared larger in treated cells (i.e., those exposed briefly to anti-Ig 24 hours earlier) than in untreated ones. This apparent change was not seen by others (Unanue *et al.*, 1972a). Elson *et al.* (1973) and Ault and Unanue (1974) examined this important point directly by quantitating amounts of anti-Ig antibodies bound to B cells subsequent to a cycle of redistribution. Elson *et al.* quantitated the amount of ^{125}I-labeled anti-Ig bound and found, as expected, a drop immediately after exposure to unlabeled anti-Ig at 37°C, but a progressive increase with time, which reached a plateau at 24 hours. After 24 hours of culture, the amounts of [^{125}I-labeled anti-Ig bound to the treated cells was about three-fourths of that bound to untreated ones. Ault and Unanue quantitated surface Ig by using a modification of the method of Smith *et al.* (1970) (Rabellino *et al.*, 1971), described in Section II,A,1. The amounts of surface Ig declined after exposure to rabbit anti-mouse Ig but recovered to normal levels after 24 or 48 hours of culture—i.e., the content of Ig before incubation, at 24, and 48 hours of culture were 46, 45, and 52 ng per 10^7 cells, respectively. Furthermore, in that same experiment, the amounts on the membrane 1 day after a second exposure to anti-Ig antibodies (with a 24-hour interval) were the same as in control cells–56 ng per 10^7 cells. Prolonged exposure in culture to normal rabbit IgG did not change the amounts of surface Ig in B cells. Thus, after a cycle of redistribution and endocytosis, the surface Ig was reexpressed to about the same basal levels. Reexpression of surface Ig also occurred after treatment of B cells with proteolytic enzymes (Pernis *et al.*, 1971; Elson *et al.*, 1973).

Several other parameters of the process of reexpression have been studied: (1) As expected, the process required protein synthesis. Exposure of cells to inhibitors of protein synthesis stopped the reexpression after anti-Ig treatment (Unanue, 1974). (2) For reexpression to occur, one had to incubate the B cells in medium without anti-Ig for several hours (Loor *et al.*, 1972; Unanue, 1973). As long as anti-Ig was present, no free molecules could be detected, presumably because of continuous elimination of new surface Ig. Whether or not there was an exhaustion of reexpression after prolonged exposure was not determined. (3) Agents that interfered with microtubular function—e.g., colchicine—reduced the reexpression of surface Ig (Unanue, 1974). This observation may give some clue to how surface Ig is carried from the cytoplasm to the membrane. Colchicine is known to interfere with secretion in a number of cell types, suggesting that the microtubular system may participate in the release of packaged secretions (reviewed by Allison, 1973). The exact

manner in which Ig in the B lymphocyte is transported from the sites of synthesis in the cytoplasm to the membrane has not been studied. Uhr and Vitetta (1973) hypothesized from studies made in antibody-secreting cells that this process probably involves transport of Ig molecules in vesicles packaged in the Golgi region, into the surface membrane, a process akin to pinocytosis in reverse. No microscopic evidence has yet been obtained. (4) Finally, Loor *et al.* (1972) commented that the first Ig to be reexpressed appeared on one pole of the cell. In repeated observations at the light and ultrastructural level, we have been unable to confirm this in our laboratory; the first Ig molecules always appear diffusely throughout the membrane.

B. Anti-Ig-Induced Inactivation of Early B Cells

The reexpression of Ig was studied in B cells from spleens of very young mice with surprisingly different results from those in older mice (Sidman and Unanue, 1975a). Most of the immature B cells (i.e., Ig-positive cells) contain Fc receptors but do not have C3 receptors (Gelfand *et al.*, 1974; Sidman and Unanue, 1975b). Also, capping of surface Ig is a much slower process (Sidman and Unanue, 1975b). When a mouse is about 14 days of age, its B-cell population switches to the characteristics of the adult population. In experiments on the reexpression of cleared surface Ig, B cells obtained from young (before 14 days of age) and from adult mice were exposed to anti-Ig for 1–24 hours and then cultured. Although adult B cells reexpressed surface Ig, this was not the case with the young B cells which remained alive but incapable of making and/or inserting new Ig on their surfaces. The Ig-bearing cells of young mice were indeed true B cells because, upon removal of their surface Ig by treatment with Pronase, they resynthesized new surface Ig. Hence, the clearing of surface Ig by anti-Ig antibodies produced a unique, suppressive signal. Young B cells treated with anti-Ig and then Pronase were also inhibited in their reexpression. That the young B cells were still alive after the brief exposure was surmised since the cells bore another surface marker of B cells, the I region-associated antigen. Also, the young B cells treated with anti-Ig antibodies no longer responded to the mitogenic influence of *E. coli* lipopolysaccharide (LPS).

Results similar to those cited above were also obtained by using small explants of fetal liver tissue (Raff *et al.*, 1975). Treatment for over 48 hours with specific anti-μ chains irreversibly suppressed the appearance of Ig-bearing cells. Of interest was the observation that suppression could be obtained with Fab anti-Ig, albeit at a higher concentration.

These studies indicating that young B cells are extremely sensitive to a negative signal from anti-Ig may explain the high sensitivity of the

immature young mouse to tolerance induction—if we assume that anti-Ig and antigen behave the same way.

The inactivation of young B cells by a ligand is relevant to the observations on suppression of Ig production by anti-Ig antibodies *in vivo*. [This topic was well reviewed by Warner (1974) and is discussed only briefly here.] Allotype suppression, discovered by Dray (1962) in rabbits, is a phenomenon in which Ig synthesis of a particular allotype is lacking because the animal, soon after birth, was exposed to specific antiallotype antibodies (Mage and Dray, 1965). Allotype suppression in the rabbit is long-lived and associated with the disappearance of Ig cells bearing that allotype (Harrison *et al.*, 1973). Earlier, it had been found that lymphocytes from allotype-suppressed rabbits could not transform into blasts upon exposure to the antiallotype antibody *in vitro* (Sell, 1968). At the time of recovery, there were normal levels of B cells bearing the allotype on their membranes but synthesizing small amounts of Ig and responding poorly to mitogens (Harrison *et al.*, 1974). All these observations favor a central defect of the B cell after exposure to anti-Ig antibodies. Allotype suppression is also observed in the mouse in which it is a temporary phenomenon (Herzenberg *et al.*, 1967). In the SJL strain of mouse, the suppression is related to active participation of suppressor T cells (reviewed by Herzenberg and Herzenberg, 1974). Long-lasting suppression of Ig synthesis can also be produced in young animals by injections of heterologous class-specific antibodies (Kincade *et al.*, 1970; Lawton *et al.*, 1972; Manning and Jutila, 1972; Leslie and Martin, 1973) or idiotype-specific antibodies (i.e., antibodies reacting with the antibody-combining site) (Strayer *et al.*, 1975).

C. Stimulatory or Inhibitory Effects of Anti-Ig Antibodies

Anti-Ig antibodies may stimulate some degree of proliferation of lymphocytes, but there are important species variations. In no species does anti-Ig appear to stimulate differentiation of B cells to plasma cells; indeed, it may serve as a negative signal for differentiation unless it is accompanied or followed by some T-cell activity.

The first studies on the effects of anti-Ig antibodies were by Sell, Gell, and associates in the rabbit using rabbit antibodies to allotype markers (Gell and Sell, 1965; Sell and Gell, 1965a,b; Sell *et al.*, 1965) or heterologous antibodies to various classes or fragments of rabbit Ig (Sell, 1967a,b,c; Daguillard and Richter, 1969; Fanger *et al.*, 1970). A great number of rabbit peripheral blood lymphocytes, upon culture with the anti-Ig antibodies, transformed into blasts and synthesized DNA and protein. The stimulation with antiallotype serum did not result from interaction of the antibody with Ig acquired from serum. Lymphocytes

from a rabbit of a given allotype, if incubated with serum of a different allotype, did not respond upon incubation with antibodies directed against the allotype of the serum (Sell and Gell, 1965a). Conversely, lymphocytes obtained from newborn heterozygous rabbits at a time when only the maternal Ig was circulating could respond to an antiallotype directed to the paternal Ig (Gell and Sell, 1965). As mentioned, heterologous antibodies were also stimulatory and to the same extent, even antibodies directed to IgG or IgA. This point raises a serious consideration. It is known from immunocytochemistry that a substantial amount of rabbit blood lymphocytes bear IgM and have allelic exclusion (Section II,A,1). How, then, can we explain the fact that antibodies to various chains all induce a similar degree of proliferation? One could interpret the results of Sell and Gell along several lines: (1) The rabbit B cell carries on its membrane various Ig classes at some time; these Ig are not detected by immunofluorescence. The immunocytochemical work discussed in Section II,A,1 argues strongly against this point. (2) One is observing the results of various amplified reactions—one cell stimulating a second cell, etc. (3) Blast transformation in the rabbit can also take place if Ig–anti-Ig immune complexes are formed—these complexes can develop because some loose IgG is bound to the membrane of some cells bearing Fc receptors. There is good precedence for this last point. Regardless of the explanation, it does appear, mainly from the results of using antiallotype antibodies, that some rabbit B cells can respond by proliferating, but whether all the proliferation can be accounted for by the direct ligand–receptor interaction remains unclear to us.

The degree of proliferation to anti-Ig antibodies varies greatly from species to species. Chicken spleen cells respond well (Skamene and Ivanyi, 1969; Kirchener and Oppenheim, 1972) and so do pig lymphocytes (Maino *et al.*, 1975). Human B cells respond weakly (Holt *et al.*, 1966; Daguillard *et al.*, 1969; Oppenheim *et al.*, 1969; Greaves, 1970). It is interesting, reviewing Greaves' data of 1970—a time when immunocytochemical findings had not yet appeared—that anti-light chain antibodies were the most stimulatory, but some were better than others, that anti-IgM or anti-IgD were barely stimulatory, if at all, and that anti-IgG was stimulatory, albeit slightly. The obvious point is whether B cells were responding to the interaction of their antigen receptor Ig with the antibody or whether the proliferation resulted in some way from immune complexes formed with loosely bound IgG. In our own laboratory the experience of Ault has agreed with the early data of Greaves. Anti-μ or -Δ chain antibodies did not stimulate B cells. Cells from other species, like the mouse, the rat, or the guinea pig, proliferate poorly, if at all, upon exposure to anti-Ig antibodies.

A recent study concerning the mitogenic effect of anti-mouse Ig is pertinent. It has been the general experience that anti-Ig does not stimulate DNA synthesis in mouse B cells. Parker (1975) found that B cells would proliferate if incubated with anti-Ig antibodies bound, in large amounts, covalently to polyacrylamide beads. Previous reports had indicated that mitogens like Con A or PHA, which stimulate T cells in soluble form, could, if bound to insoluble beads, also stimulate B cells (Anderson *et al.*, 1972; Greaves and Bauminger, 1972). This information does suggest that repeated exposure of the B cell to a dense surface matrix of ligands is stimulatory. Curiously, in Parker's experiment, no differentiation to antibody-secreting cells took place.

In contrast with the proliferation that is observed by some lymphocytes upon exposure to anti-Ig antibodies (and which we interpret with caution as a reflection of a direct receptor-Ig ligand effect in all cases), no differentiation to plasma cells by anti-Ig has been observed. In some systems, as was discussed in the preceding section concerning young B cells, anti-Ig shuts off the cells for differentiation—or even proliferation induced by various mitogens. A brief analysis of the different systems follows.

Schuffler and Dray (1974a,b,c) developed an *in vitro* system in which the synthesis of Ig of a given allotype by rabbit spleen cells was measured after 4 days of culture. In this assay, the rabbit spleen cells were not stimulated by antigen or other substances except for the 20% fetal calf serum in the incubation medium. Exposure of the spleen cells to anti-a allotypes (marker of the Fd region of the heavy chain) or anti-b allotype (in kappa light chains) antisera or to anti-μ chain antibodies, but not anti-γ chain, markedly reduced the amounts of Ig produced. (The cells were incubated with the antibodies 24 hours before the standard 4 days of culture.) The reduction was clearly specific. In a heterozygous rabbit such as a b^4b^5, an anti-b^4 stopped the production of the b^4 allotype but not of b^5, and vice versa. Curiously, inhibition was produced by the whole IgG molecule or its $F(ab')_2$ or Fab fragment. Schuffler and Dray interpreted their results as meaning that the interaction with receptor Ig of the B cell stopped their differentiation to secretory cells.

Anti-Ig antibodies inhibit the proliferating and/or differentiating effects of polyclonal B cell stimulatory substances, like LPS (Elson *et al.*, 1973; Andersson *et al.* 1974b; Lonai and McDevitt, 1974; Schrader, 1975; Sidman, 1976). This has been studied only in mouse B cells. Andersson *et al.* (1974b) exposed spleen B cells to anti-μ chain or anti-κ chain antibody, and then cultured the cells for 2–3 days in the presence of LPS, purified protein derivative of tuberculin (PPD) (a B-cell stimulatory material), or just fetal calf serum. Cells treated with anti-Ig antibodies had a dra-

matic reduction in the number of hapten-specific plaque-forming cells (PFC). For example, in one experiment, the background PFC of 20 (per 10^6 cultured cells) rose to 410 after LPS exposure alone, but only to 61 if the cells were exposed to anti-Ig antibodies prior to LPS. Andersson *et al.* went on to establish that (1) The inhibition was indeed caused by the anti-Ig antibodies, specifically those having activities against light chains or μ chains. (2) A $F(ab')_2$ fragment was as inhibitory as an intact molecule, but (3) the Fab monovalent antibody was without effect. (4) The inhibition was best seen if cells were incubated with anti-Ig antibodies prior to LPS. (5) Enzyme treatment of the B cells, presumably affecting surface Ig, prevented the inhibitory action of anti-Ig.

In the hands of Andersson *et al.*, the inhibition of mitogenesis was slight, although some was noted when PPD was used. However, others (Elson *et al.*, 1973; Lonai and McDevitt, 1974; Schrader, 1975; Sidman, 1976) have found consistent inhibition of the mitogenic response to LPS. The inhibition was seen whether B cells were exposed to anti-Ig antibodies before or after LPS binding and most likely was not associated with an impairment of LPS binding produced by the disappearance of surface Ig (Schrader, 1975; Sidman, 1976). Sidman (1976) found that inhibition of LPS mitogenesis was best seen with intact, bivalent molecules and not with an $F(ab')_2$ fragment in contrast to the inhibition of differentiation noted by Andersson *et al.* Soluble immune complexes, however, had no negative effect on B cells. Two important points are worth emphasizing: (1) Although exposure to anti-Ig antibodies blocked mitogenesis and differentiation, it did not affect the reexpression of surface Ig *in the B cells from adult mice;* this contrasted with the higher susceptibility of the B cells from young mice, described before, in which the resynthesis of surface Ig also was affected. (2) The inhibition of mitogenesis produced by anti-Ig antibodies was not associated with capping: B cells were exposed to anti-Ig antibodies in the presence of sodium azide for 1 hour to prevent capping; the cells were washed well and then the complexes—in patches—were removed by treatment with pronase; the cells were then cultured with LPS. While control cells exposed to azide and Pronase made a good proliferative response, those exposed to anti-Ig and the same treatment did not.

The experiments with anti-Ig discussed before and those employing antigen Section IX) indicate that ligand-Ig interaction is usually not sufficient to trigger B cells to full differentiation and that, in fact, it may result in a powerful negative signal. Studies combining a ligand-receptor signal with the "helper" activity are illuminating. In a first attempt, Katz and Unanue (1972) tested *in vitro* whether murine B cells exposed to anti-Ig would differentiate into antibody-forming cells. This was not the

case. However, a brief exposure to anti-Ig antibodies followed by challenge with the antigen produced a marked enhancement of the antibody response.

Using spleen cells from rabbits immunized to DNP proteins, Kishimoto and Ishizaka (1975) developed a system in which the cells were first exposed to anti-Ig antibodies, then washed and cultured with supernatants from cultures of antigen and, presumably, activated T cells. Only upon sequential treatment with both materials did the rabbit spleen cells go on to make anti-DNP antibodies. The effects were obtained by using antibodies to Fab determinants or to γ chains, either intact or pepsin digested but not monovalent. The length of exposure time to anti-Ig was critical, about 24 hours. These results have been extended, also in the rabbit, by Kishimoto *et al.* (1975) and Watanabe *et al.* (1975). They have made the important observation that the incubation of cells with the soluble factor absorbs their activity, but only with cells previously exposed to anti-Ig antibodies. This suggests that the soluble enhancing factor interacts with a B-cell membrane which has been in some way affected by the anti-Ig antibodies. Overall, the evidence so far suggests that indeed ligand–receptor Ig interaction programs a brief series of events. These, if followed by helper activity, may lead to stimulation but, if not, may produce a state of relative inactivation.

IX. Antigen–Surface Ig Interaction

Analyses of the early surface and cytoplasmic events that follow interaction of B cells with antigen are not abundant, in contrast to the many studies done with anti-Ig antibodies. The reason for this is the paucity of specific antigen-binding B cells, which makes precise analysis difficult and laborious (Section II,A). Nevertheless, significant observations have been made, most of which agree with the findings with anti-Ig antibodies. The studies of antigen redistribution must be analyzed in the context of the immunogenicity of the particular antigen in question. With respect to their immunogenicity, two large groups of antigen molecules can be considered. First are those represented by foreign serum proteins that are weakly to moderately immunogenic and usually must interact with helper T cells and macrophages in order to trigger responses. These antigens tend to induce tolerance under appropriate circumstances, principally when they are introduced under conditions of poor T-cell helper activity. The second group of antigens stimulates B cells with very little, if any, activity from the helper cells, i.e., the so-called "thymic-independent antigens." Thymic-independent antigens stimulate mainly IgM responses, are immunogenic at very narrow dose ranges, and are able to

induce tolerance. Some, but not all, of the thymic-independent antigens stimulate B cells to proliferate, and are considered to be polyclonal mitogens (Coutinho and Moller, 1973). Included among the T-independent antigens are bacterial products like endotoxin, some carbohydrates with multiple repeated determinants, and nondigestible polymers. B-cell stimulation is a complex process involving multiple regulatory steps of which we have only fragmentary knowledge. In this context, we consider whether some of the early events described before—such as redistribution, motility, and endocytosis—have any particular relevance to the immunogenic or tolerogenic properties of an antigen.

Most lymphocytes, as expected, can redistribute and eliminate most antigen molecules quite effectively; however, the size and composition of the antigen play an important role.

A. Interaction of Foreign Serum or Other Metabolizable Proteins with B Cells

Most soluble protein antigens, after binding to surface Ig, redistribute into large aggregates and caps (Taylor *et al.*, 1971; Dunham *et al.*, 1972; Ault and Unanue, 1974). The surface-Ig antigen complexes are then rapidly eliminated from the cell. Subsequent to their elimination, the lymphocyte membrane is left bare of receptors, but new receptors are reexpressed by new protein synthesis within a few hours (Ault and Unanue, 1974). Wilson and Feldmann (1972) exposed lymphocytes from mice immunized to dinitrophenylated human IgG (DNP-HGG) at 4°C for 0.5 hour, then washed away the excess unbound antigen, and cultured the cells for several hours. Immediately after the binding to DNP-HGG, all the surface Ig receptors were occupied; hence, no binding to DNP-conjugated red cells took place. However, free receptors were rapidly reexpressed; by 3 hours, the number of B cells rosetting DNP-red cells was back to normal levels. In contrast, a monovalent hapten, DNP-lysine, rapidly dissociated from the cell, freeing the anti-DNP receptors.

Ault and Unanue (1974) explored this point, specifically the disappearance of the bound antigens as well as the reappearance of new binding sites. Some of the results are illustrated in Fig. 16. They incubated cells from mice immunized to DNP-KLH with a highly radioiodinated DNP conjugated protein, the protein being unrelated to KLH so as not to stimulate T cells. DNP_{12}-GPA (i.e., 12 moles of DNP per mole of protein) was rapidly lost after binding to the B cells receptors so that by 1 hour no labeled cells could be detected. At this time the cells would not relabel with fresh antigen, indicating that the receptors were lost at the same time that the antigen was eliminated. By 4 hours of culture, some cells did relabel; and this increased with time. Similar results were

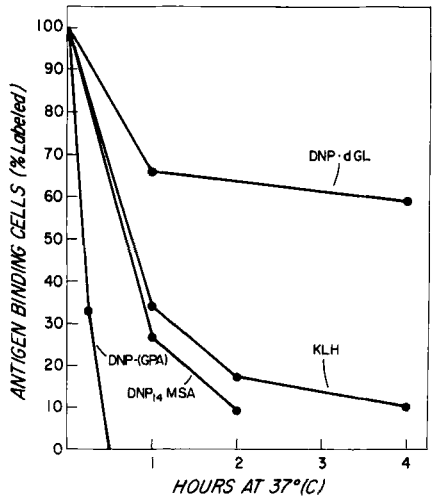

FIG. 16. The elimination of radioactive antigens from specific B cells with time. Primed B cells were incubated first with the antigens [DNP-(GPA) = dinitrophenylated guinea pig albumin; $DNP_{14}MSA$ = dinitrophenylated mouse albumin; KLH = keyhole limpet hemocyanin; DNP•dGL = dinitrophenylated polymer of *d*-glutamic acid lysine], then washed, and placed in culture, 100% refers to the number of cells bearing the antigen (estimated by autoradiography) immediately after labeling.

obtained with another protein, DNP-MSA. Thus, the cell passed through a cycle of redistribution and clearing of receptors and replacement with new surface receptors in a manner identical to the phenomenon observed with anti-Ig antibodies discussed before. In contrast, albumin molecules with low density of DNP ($DNP_{1.5}GPA$, for practical purposes a monovalent hapten) were immediately lost from the cell, free receptors immediately reappearing. In this case the receptors were not lost with the antigen. Presumably, antigen was lost not as a result of pinocytosis and/or shedding, but rather because of dissociation from the surface Ig.

Clearly, a multivalent antigen binds with a greater avidity to surface antibody, making the complex very stable, thus allowing for the redistribution events to take place—in practical terms, the complex is irreversible (Bystryn *et al.*, 1973). Bystryn *et al.* (1973, 1975) examined this point directly with myeloma tumor cells that secreted anti-DNP antibodies. The malignant plasma cells also had anti-DNP antibodies on their surfaces. Their experiments consisted of incubating the myeloma cells with variable amounts of radioiodinated DNP-conjugated proteins and then estimating association constants, number of binding sites, and dissociation rates. The binding affinity of the cell for a multivalent conjugate was about 100 to 300 times greater than for a univalent ligand. In other studies the avidity

of immunized B cells for DNP-conjugated proteins increased with a higher density of the hapten on the carrier protein (Davie and Paul, 1972a). The increased binding strength of surface Ig to a multivalent ligand results most likely from cooperation among a large number of binding sites.

The cycle induced with garden variety protein antigens clearly does not stimulate a full response by the B-cell—in the absence of the cooperative cellular interactions. Furthermore, most of the protein antigens examined, such as those employed by Ault and Unanue, produce tolerance provided that they are administered to an individual under circumstances whereby no T-cell helper activity is brought into play. Several systems of tolerance to protein antigens have been developed, some of which suggest that B cells exposed to antigen molecules directly become inactivated, at least for a time: (1) Administration of haptens in nonimmunogenic carrier proteins, usually autologous Ig, into mice produces a state of hapten-specific B-cell tolerance (among many, see Havas, 1969; Borel, 1971; Golan and Borel, 1971; Fidler and Golub, 1973; Hamilton and Miller, 1973; Taussig, 1973). Spleen cells from these mice cannot, upon transfer, mount an antibody response. (2) The same effects are obtained by administration of haptens bound to nonimmunogenic synthetic polymers (Katz *et al.*, 1971, 1972, 1974) or to polysaccharides (Mitchell *et al.*, 1972). (3) Prolonged exposure of B cells to DNP-proteins *in vitro* produces a relative state of unresponsiveness when these cells are tested *in vitro* or *in vivo* (Katz *et al.*, 1974; Nossal and Pike, 1975).

Although the relationship in these systems between the presumed functional state of the B cell with the cycle studied *in vitro* is not yet apparent, certain points are worth signaling. The nature of the unresponsive state of the B cell appears to be unrelated to whether or not the metabolizable antigen can be cleared from the membrane. Indeed, B cells cleared the complexes of protein antigen–surface Ig rapidly with kinetics roughly identical to those produced with anti-Ig. This clearing of surface complexes from the B cell is an intrinsic property of the cells, taking place in the absence of any cooperative interactions (Ault and Unanue, 1974). A hypothesis has been advanced that T cells "help" the B cell to get rid of the surface complexes. If absent, the B cells cannot clear their excess surface antigen and become unresponsive (Mitchell, 1975). This hypothesis really has no experimental proof in favor of it and many points against it. Other explanations should be sought for this form of tolerance. Aldo-Benson and Borel (1974, 1976) claim that murine B cells made tolerant to DNP by *in vivo* injection of DNP-autologous IgG retain some antigen on their membranes and conclude that the B cells have a "receptor blockade." The conclusion is based on very weak experimental

results. DNP-IgG persists in tissues for a long time and binds—very loosely—to many cells with Fc receptors, including B cells. Therefore, there is no way of establishing whether the small amount of material presumed to be on the surfaces of the spleen cells has anything to do with the antigen encountered in the putatively tolerant B cells.

Arguments can be raised that the continuous exposure of B cells to antigen, repeating the cycle of redistribution, clearing, and synthesis, ultimately leads to a nonfunctional state of the cell. Direct proof of this point must be obtained, but some scattered pieces of evidence favor it. Unpublished results of K. A. Ault and E. R. Unanue support this statement in that repeated exposure of B cells to some DNP-conjugated proteins eventually led to an exhaustion in the reexpression of new receptors. The many effects of exposure of B cells to anti-Ig are outlined in Section VIII. The most pertinent observation is that, under appropriate conditions, exposure of murine B cells to anti-Ig antibodies produces a transitory state of unresponsiveness marked by a low synthesis of DNA or poor differentiation to plasma cells. The B cells under these circumstances, although unable to respond functionally, cleared their membranes of the Ig–anti-Ig complexes and, in many cases, went on to resynthesize new Ig receptors.

A further point concerning the problem of inactivation of B cells upon direct exposure to antigen (or anti-Ig) concerns the functional state of the cell. Nossal and Pike (1975) found that direct exposure of bone-marrow cells to $DNP_{1.0}$ human IgG at a concentration as low as 10^{-8} M for at least 24 hours of culture inactivated the cells. The bone marrow B cells, upon transfer to X-irradiated recipients and challenge by DNP-flagellin (a T-independent antigen), were unable to differentiate to antibody-secreting cells. Mature spleen cells, on the other hand, were not inactivated under the same set of circumstances. They postulated a special sensitivity of the early B cell from direct exposure to a ligand, analogous to the sensitivity of immature B cells to interaction with anti-Ig antibodies discussed previously. No data have yet been presented on the early surface and cytoplasmic events.

B. Interaction of Highly Tolerogenic Antigens with B Cells

Two antigens of particular interest because of their immunogenic/tolerogenic properties are the D-amino acid copolymer of glutamic acid and lysine (D-GL) and polymerized flagellin.

D-GL is a nonmetabolizable copolymer of about 60,000 daltons which by itself is poorly immunogenic (Katz *et al.*, 1971, 1972). The dinitrophenylated conjugate of D-GL induced a profound state of tolerance in DNP-specific B cells. Administration *in vivo* of DNP-D-GL prevented an

anti-DNP response to challenge with an immunogenic form of DNP (Katz *et al.*, 1971; Davie *et al.*, 1972). Furthermore, lymphocytes exposed *in vitro* were rendered irreversibly tolerant (Katz *et al.*, 1972). This led Ault *et al.* (1974) to expose lymphocytes to radioiodinated DNP-D-GL, or DNP bound to a similar polymer but made of L-amino acids DNP-L-GL, and to follow their fates by autoradiography. DNP-L-GL, as with the DNP conjugated proteins studied before, was rapidly eliminated by B cells after redistribution of their receptors (i.e., the number of binding cells after a brief exposure in the cold was 179 per 10^5; this number progressively declined to less than 5 per 10^5 by 4 hours). In contrast, the B cells eliminated the complexes of DNP-D-GL very poorly (Fig. 16). Two other interesting points emerged from this study. Using purified anti-DNP, it was possible to show that many of the DNP-D-GL complexes remained cell associated even after prolonged incubation. How much remained surface bound or interiorized could not be determined. Thus, while the DNP-L-GL had indeed been cleared off the membrane, this was not the case with the D-compound, where at least some molecules appeared to persist on the surface. Furthermore, it was possible to show that after brief exposures to DNP-D-GL the appearance of new receptors was impaired. Indeed, cells exposed for just 30 minutes never regained their ability to interact with new radiolabeled DNP-conjugated protein.

The study of Ault *et al.* (1974) raises a number of important questions. Why are DNP-D-GL complexes surface bound for long periods? Is the profound functional inactivation of B cells produced by brief exposure to DNP-D-GL related to its inability to leave the cell surface? One consideration in this study is whether the DNP-D-GL found on the membrane is the original compound bound to the B cells or the result of a constant recycling of material that goes inside the cell and is then released back to the membrane of the same cell or others. Although this last possibility is unlikely, it must be considered, inasmuch as DNP-D-GL is a poorly digestible material that remains in the culture for long periods of time. If indeed some of the complexes remain cell bound, possibly endocytosis in the lymphocyte may require some biochemical-enzymic step that cannot take place with D-compounds. No precedence for this exists in other cells like macrophages, which can endocytose D- or L-polymers readily.

The immune response to polymerized flagellin from *Salmonella* organisms has been studied extensively by Nossal and Ada (1971) at the Walter and Eliza Hall Institute in Australia. Flagellin of about 40,000 daltons is the unit protein of the flagella and contains the H system of antigens. In its polymerized form (of about 10^7 daltons or more), de-

pending on the dose, flagellin can either stimulate an antibody response of the IgM class in the absence of helper T cells (i.e., it behaves as a thymic-dependent antigen) or produce a long-lasting state of B-cell unresponsiveness (Diener and Feldmann, 1972; Feldmann, 1972). B cells' handling of polymerized flagellin has been studied by using radiolabeled polymer (Diener and Paetkau, 1972) or haptens like DNP or NIP (4-hydroxy-3-iodo-5-nitrophenacetyl), then visualizing the compound with fluorescein (Nossal and Layton, 1976). The study of Nossal and Layton (1976) is of particular interest because it contained a series of manipulations that clearly indicated the fate of this antigen, related to its immunogenic status. Their study employed populations of cells markedly enriched for the specific antigenic determinant (Haas and Layton, 1975). They used B cells exposed to hapten-polymerized flagellin either briefly or for long periods of time. Interpretations were complicated by the presence of many large blast cells, which often were unrelated to the specific cell. Flagellin is a potent adjuvant and tends to bind nonspecifically to cells. Both polymerized flagellin or hapten-derivatized flagellin, upon binding to the B cells, readily redistributed into caps. Within a relatively short period of time, a great many of the surface complexes were lost from the cell, a substantial amount by endocytosis.

Two important points emerge from Nossal and Layton's precise analysis. First, elimination of the surface complex was incomplete in that after 24 hours following a brief exposure to the antigen about one-fifth or so of the cells still contained some residual material on their surfaces in the form of a small, irregular cap. This point was verified by using specific antibody. Although the residual amounts could not be quantitated, they appeared to be only a small portion of the amount bound initially. A second point concerned the relationship between handling and immunogenicity. B cells exposed briefly to a small, immunogenic amount of hapten-flagellin cleared their membranes of most of the complex. The immunogenic doses always left a large number of free receptors on the surface; in other words, it was a dose that did not saturate the 10^6 surface Ig molecules. In contrast, *tolerogenic* doses of flagellin saturated all the surface Ig-binding sites. The complexes in this case were also cleared from the surface, leaving a membrane naked of free receptors. Of great interest is the observation that in a substantial number of these cells no new free binding sites appeared after 24 hours of culture, a situation analogous to that found by Ault *et al.* (1974), using D-GL. Diener and Paetkau (1972) claimed that B cells exposed *continuously* to polymerized flagellin expressed an increased number of binding sites very rapidly. At this stage the B cells capped polymerized flagellin poorly. Nossal and Layton pointed out the difficulties in interpreting Diener and

Paetkau's data. Indeed, it was difficult to determine by autoradiography and with continuous 6-hour exposure whether the complexes were on the membrane or inside the cell. The data of Nossal and Layton convincingly showed that most of the complexes of tolerogenic amounts of polymerized flagellin could be capped and suggest that the cell suffered an irreversible metabolic change.

In summary, the overall evidence with B cell–antigen interaction goes along the same lines as results obtained with anti-Ig antibodies—that the overall encounter is usually ineffective and may result in a negative signal. Exception must be made of the T-independent antigens which perhaps have some particular structural component that, in itself, contains the "second" signal, i.e., LPS, flagellin. The explanation of unresponsiveness by direct exposure of B cells to antigen suggests a metabolic mechanism involving some kind of negative signal. Of particular importance is the behavior of D-GL and flagellin, both of which may have the capacity to tolerize and to remain cell associated. Further progress requires the careful evaluation of immunogens and tolerogens.

C. Interaction of Red Cells with B Cells

Redistribution of particulate antigens has been limited to red cells (Ashman, 1973). Ashman obtained spleen cells from mice immunized with sheep red cells, incubated them with the red cells, and studied their redistribution. Figure 17 is a diagram from his paper illustrating the progression of capping from a partial cap, to horseshoe stage, to tight cap. There are several points of interest in this study. First, capping was a very slow progress in comparison to the rate found with anti-Ig. Thus, the peak number of caps occurred after 30 minutes at 37°C and after 3–7 hours at 23°C. Also, the total percentage of cells that bound sheep red cells and formed caps was rather small, usually not exceeding 30%. This figure might, in fact, be misleading inasmuch as direct microscopic observations indicated that the kinetics were markedly heterogeneous—"rosettes may wait different lengths of time before beginning receptor movements, once

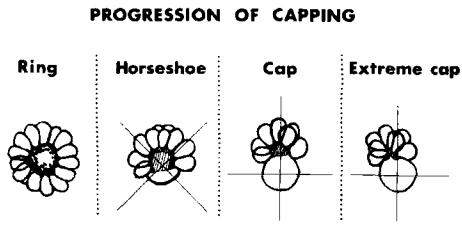

Fig. 17. The sequence of redistribution of sheep red cells on specific, murine lymphocytes. The explanation is in the text. (From Ashman, 1973.)

begun, these movements achieve the final horseshoe or cap configuration within ten minutes even at 23°C" (Ashman, 1973). This slow rate of capping could result from the large size of the antigen, which is difficult for the cell to carry to one pole. Some evidence for this came from unpublished experiments of E. R. Unanue and M. J. Karnovsky and their associates studying capping of anti-Ig antibodies bound to various markers. Whereas capping of fluoresceinated anti-Ig was extremely fast, capping of ferritin-tagged anti-Ig was much slower; strikingly, KLH-tagged anti-Ig capped at a very low rate. KLH is a very large protein of about 7 to 9 $\times$ 10^6 daltons. (Note also in Ault and Unanue's experiments shown in Fig. 16 that the rate of disappearance of KLH from specific B cells was slower than for albumins.)

Ashman further indicated that redistribution was accompanied by a "dramatic change in shape" of the B cell. Capping of the red cells was not dependent on motility inasmuch as it took place in the presence of cytochalasin B or in B cells in suspension. However, the lymphocyte was unable to eliminate the red cells by endocytosis. The bound red cells were progressively shed from the cell surface so that about one-half of the B cells totally lost the antigen within 3–4 hours. The failure of lymphocytes to interiorize red cells may imply difficulties in phagocytizing such a relatively large particle.

D. The Question of Antigen in Antibody-Secreting Cells

It is interesting at this point to recall the attempts to visualize antigen in plasma cells during the middle 1960s at a time when it became important to establish whether or not antigen was needed as a template for the formation of antibody. Initial studies of Wellensiek and Coons (1964) and later studies by Han and Johnson (1966) disclosed the presence of ferritin molecules in antiferritin forming blasts and plasma cells. This was not surprising considering the manner in which the experiment was done and since we now know that antibody-forming cells have surface Ig. A rather large amount of soluble ferritin was injected into primed animals, and the cells were examined very shortly thereafter. It was expected that the excess ferritin would interact with the surface Ig of secretory B cells (plasma cells) and would be interiorized in vesicles. In contrast, McDevitt *et al.* (1966) and Nossal *et al.* (1965) examined antigen in plasma cells after conventional immunizations with small amounts of antigen. By autoradiography neither group detected any antigen in plasma cells. One can envision that the B cells disposed of the antigen molecules readily by the pathways described herein involving endocytosis and catabolism with some shedding, leaving a few residual molecules that were diluted as the cell divided to expand the clone. Thus, it is not surprising from the information now available that the B cell, by

the time it reaches its fully differentiated state, contains no antigen molecules.

E. Interaction of Antigen with Antibody-Secreting Cells

Schrader and Nossal (1974) reported that the interaction of antibody-secreting cells with antigen reduces markedly the rate of secretions of antibodies. The phenomenon was produced *in vitro* by incubating DNP-specific immune cells with various DNP conjugates and then testing for secretion of antibodies by a plaque method. Incubation with the DNP-protein reduced the rate of plaque formation. Also, the plaques were small and fuzzy, indicating small amounts of secreted antibody. (Incubation with a monovalent ligand, DNP lysine, or with unrelated conjugates had no untoward effects.) Similar results were found after injection of large amounts of antigen *in vivo* by testing the spleen cells for PFC immediately. In retrospect, similar observations were found by those working with polysaccharide antigens (which remain in tissues for a long time) when, after exposure to the antigen, PFC were reduced in number and in size (Baker *et al.*, 1971; Klaus and Humphrey, 1974). This phenomenon, termed effector cell blockade, has not yet been explained. It is interesting to note that similar effects were also seen with anti-Ig (Nossal and Pike, 1974: note comment on p. 370). In this regard, Antoine *et al.* (1974) reported an interesting observation when plasma cells were incubated with anti-Ig. The complexes were interiorized and localized to the cisternae of the Golgi apparatus. This localization is peculiar and has not been found in other cells except for nerve cells after interactions with ricin, which binds to galactose-containing glycoproteins (Gonatas *et al.*, 1975). It is possible that the funneling of ligand–receptor complexes into the Golgi somehow shuts off secretion in the plasma cells.

X. Conclusions

The sequence of ligand binding to Ig receptors in the B lymphocyte membrane followed by the redistribution of these receptors into patches and then caps, with the concomitant stimulation of cell movement and endocytosis, and then synthesis and replacement of receptors, constitutes a closed cycle. At the end of this cycle lies a cell apparently little different from one that has never contacted antigen; addition of ligands to a B cell that has regenerated its antigen receptors initiates the same sequence. Yet we know that the B cell can evolve into two other functional states *in vivo*. The first is very well known: after encountering antigen in the presence of other immune cell types, the B cell divides and forms a clone of cells of the same antigen specificity, some of which differentiate into the antibody-secreting plasma cell. The second functional state is much less understood: the cell becomes functionally paralyzed, unable

to differentiate and to respond to subsequent exposure to antigen. Both fates necessitate additional external or internal signals that force the capping B cell off the closed track that leads back to its precapping self and on to diverging pathways. The precise nature of those signals and the point at which they enter the capping cycle are unknown. There is today a large gap between our understanding of the factors affecting the workings of immunological cells considered as an integrated system and our knowledge of the functions of particular cell types considered in isolation. Evidence linking the capping cycle to the ultimate stimulation or inhibition of antibody production is still too fragmentary to support definite conclusions. Nonetheless, it is sufficiently suggestive to underline this area as important for any ultimate undertanding of the bases of the immune response.

We have tried, to the limited extent currently possible, to place the ligand-induced cycle of the B lymphocyte within two contexts. The first is its position in the host's immunological response; the second is its similarities, if any, between mechanisms underlying the specific response of the B lymphocyte and the responses of cells in other systems whose specificity is also determined by the interaction between signal and membrane receptor. With respect to the former, the potential significance for immunology of the B cell's handling of antigen is obvious; unfortunately, exact correlations are not, given our present state of knowledge. Accordingly, we shall only briefly touch upon events on the single-cell level and their possible relationship to the eventual outcome of the immune response to antigenic stimulus. As to the latter, virtually nothing is known. Nonetheless, we expect that the ultimate significance of the capping phenomenon may rest upon extrapolations made from its mechanisms and extended to other systems in which cells reorganize both membrane and cytoplasm in response to a receptor-mediated, transmembrane stimulus.

We believe that redistribution of ligand–receptor complexes is important because: (1) it is critical in the transmission of signals from the membrane to the cytoplasm; (2) it favors strong multipoint binding of ligands, thus forming stable complexes; (3) it serves as an efficient safety valve to eliminate stimulatory surface complexes; and (4) it produces the polarization of the cell required for directional movement.

The importance of membrane fluidity and redistribution for transmission of signals across the membrane boundary is clear. The redistribution of the complexes can be visualized as a means to bring together component parts of the membrane that together constitute a functional unit.

Such an activated surface aggregate might generate second messengers or affect transport of ions or metabolites that could profoundly alter

the activity of the underlying cytoplasm. Indeed, the directed translational motility of molecules in the membrane might constitute the rate-limiting step in the interaction between enzyme and substrate, both of which might be freely floating membrane components (Singer, 1976). In this regard, the association between surface Ig and the Fc receptor stimulated by the binding of ligand to the surface Ig may turn out to be an important model for the study of the result of membrane-limited molecular interaction. A similar model has been outlined by Cuatrecasas to explain the stimulation of cells by hormone–receptor complexes (Cuatrecasas, 1974). Such membrane associations also presumably underlie the cross-linking requirement for lectin-stimulated mitogenesis (Gunther *et al.*, 1973).

We have described a particular set of events subsequent to the intramembranous linking of receptors in which a calcium-sensitive system was responsible for the activation of cytoskeletal elements. This activation affected the reorganization of the membrane topography so as to modulate both the ongoing cellular response and any subsequent cellular reactions to the same ligand. We suggest that these cytoplasmic elements play the same active role in altering the organization of the membrane as the membrane does in maintaining or changing the internal organization of the cell's cytoplasm.

The free mobility of complexes in the plane of the membrane undoubtedly increases the efficiency of the cell in binding multivalent antigens as a result of cooperation among a larger number of binding sites. This relative stabilization of the antigen–receptor complexes on the membrane for a finite period could be essential for the subsequent response of the cell. Redistribution may not only promote cell–antigen interaction but may enhance cell-to-cell association. It is conceivable that macrophages, or other cells, may interact best with lymphocytes that have antigens concentrated on their membranes. Whatever the precise mechanism, we suggest that the enhanced antibody response that multivalent antigens elicit when compared to monovalent forms of the same antigen is a reflection of the superior efficiency of multivalent ligands in binding and capping on the lymphocyte's surface.

Subsequent stages in antigen handling by the cell may also prove to be critically important to the overall immune response. Full elimination of the complexed antigen receptors is striking in the B cell. That clearing the membrane, at least temporarily, of receptors is important to the cells has been suggested by recent experiments using a nondegradable haptenated antigen, the D-amino acid copolymer, D-GL, which binds and caps efficiently but which appears to block the lymphocyte's capacity to endocytose and degrade it. As a result, the complexed receptors remain

on the cell surface. Significantly, this copolymer, when haptenated, is highly tolerogenic, specifically suppressing immune responses to the attached hapten (Section IX). Whether nondegradability of the antigen is the mechanism of the cell's paralysis remains to be proved. It does suggest, however, that clearing the membrane of receptors may constitute a form of safety valve, acting both to eliminate suppressive signals and perhaps to prevent an incorrect positive response. For a B cell stripped of its receptors is a cell that is tolerant until the replacement of those receptors. This may constitute a built-in check whereby a B cell exposed to the stimulation of antigen binding in the absence of another signal from a neighboring T lymphocyte or macrophage eliminates that stimulus and so prevents a self-triggered transformation. Certainly, the B cell seems particularly susceptible to persistent stimulation at its surface. Certain soluble T-cell mitogens have no effect on B cells until they are rendered insoluble. B cells become blastogenic in the presence of these insoluble compounds, perhaps because of the cells' inability to clear a stimulating signal from their membranes (Section VIII).

The functional significance of stripping the membrane of receptors extends to all stages of the life cycle of the B lymphocyte. For reasons poorly understood, immature B cells exposed to anti-Ig antibodies are unable to regenerate new receptors and place them back on their surfaces, preventing those cells from recognizing and responding to subsequent exposure to that ligand. This not only supports the hypothesis that one form of tolerance depends upon the maturational stage at which the immune system is exposed to antigen, but points to the B lymphocyte as critical to the establishment and maintenance of this type of tolerance. Research on mature B cells possessing specificity for flagellin has demonstrated a correspondence between the extent of receptor elimination from the B-cell surface and the ultimate immunogenicity or tolerogenicity of the antigen inducing the receptor elimination (Section IX). At the far end of the B cell's differentiation lies the extreme sensitivity of secreting plasma cells (which cap and endocytose their few surface Ig receptors poorly) to the inhibitory effect of the same antigenic stimulus that induced them to transform, which may represent a homeostatic response to prolonged stimulation.

Another component of the capping cycle, the stimulation of movement and the formation of uropods in the B lymphocyte, remains to be adequately defined with respect to its place in the immune response (Section III). The phenomenon of lymphocyte motility has been recognized for a century, but its role in immune function is not much clearer than when it was first described. Yet the import of this property is obvious. Well-defined pathological lesions exist in which the lymphocyte constitutes the

predominant cell type, yet the mechanism of the infiltration is completely unknown. Perhaps more significantly, lymphocytes possess a profound requirement for interaction with other cells in order to effect their functions, yet the lymphoid tissue is compartmentalized with B cells in the outer cortex of the lymph node and T cells in the deep cortex. In a biological system containing numerous ongoing reactions to antigen, the cellular interactions, whether mediated by direct contact or chemical factors, require a close juxtaposition of cells in order to maintain specificity. This juxtaposition may necessitate some form of directed motion in order to bring together antigen and diverse cells that possess a common recognition of antigen. Thus, discussion of the stimulation of lymphocyte motility by ligand binding to antigen receptor must be placed not only within the narrow context of capping receptors but in the much broader one of the mechanisms of lymphocyte function. Ongoing research on lymphocyte motility promises to add a great deal to our understanding of these matters.

As we noted at the beginning of this review, the implication of the lymphocyte's capping and endocytosis of its ligand-bound surface receptors extends beyond strictly immunological concerns. The phenomena of cell recognition, attachment, and aggregation, the development of embryonic tissue, the modulation of endocrine cell activity, and the sequential changes in cell surface proteins accompanying cell differentiation all may involve, to some extent, the ability of a particular ligand to bind to a particular membrane receptor, induce the transmission of the appropriate signal across the membrane boundary, and evoke, in return, a particular cell response.

Whatever the nature of the response, it is clear that we are only beginning to understand that the cell is not a passive responder to signals emerging from its outer space. It has the capacity to react, to be sure; but it also possesses the means to alter its reaction by modifying its reception of that signal. The cell's means of altering the signals of its environment have been used by nature for many purposes, not the least of which is the establishment of an immune system capable of acquiring the discrimination of self from nonself in order to maintain the integrity of the former at the expense of the latter.

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Lymphocyte Receptors for Immunoglobulin

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I. Introduction

The immune response is a complex process involving interactions among several different immunocompetent cell populations and their products. It seems reasonable to assume that molecules on the surfaces of these cells mediate some of the interactions, and considerable investigation has been focused on components of the lymphocyte surface membrane. Among these components are sites that bind immunoglobulin (Ig) mole-

cules. In view of the obvious importance of antibody, such sites may themselves play a role in the immune response.

As early as 1967, the observation was made that "certain" lymphocytes could bind immunoglobulin complexed to antigen by a mechanism that did not involve serum complement (LoBuglio *et al.*, 1967). However, the binding of immunoglobulin to lymphocytes was not carefully characterized until 1972, when studies were published demonstrating such binding by murine B cells (Basten *et al.*, 1972a,b,c; Cline *et al.*, 1972; Paraskevas *et al.*, 1972a,b), murine T cells (Grey *et al.*, 1972; Lee and Paraskevas, 1972; Yoshida and Andersson, 1972), and human B cells (Dickler and Kunkel, 1972). Since the Fc portion of the Ig molecule is required for this binding (see Section IV,A) the membrane sites are referred to as Fc receptors, a term first suggested by Paraskevas (Paraskevas *et al.*, 1972a). This publication will critically review the information available on lymphocyte receptors for immunoglobulin with emphasis on those areas that are controversial.

II. Methods of Detection

The methods used to detect lymphocyte receptors for Ig vary widely both as to the type of complex utilized and the technique for measuring binding. In this section the various techniques available will be described briefly without attempting to consider all studies that have used each method. Further, the nature of the lymphocytes that bind complexed Ig will be considered wih reference to the sensitivity and/or specificity of the various methods. However, a detailed consideration of lymphoid cell subpopulations—including the criteria for defining B lymphocytes, T lymphocytes, and undefined lymphocyte-like (UL) cells, will be deferred until Section III.

Any useful Fc receptor assay system should meet certain basic criteria: (1) The percentage of lymphocytes binding Ig should be titratable to a plateau value. This assumes a discontinuous distribution of receptors on different subpopulations, an assumption that appears to be supported by the available data (see Sections III and IV,B). The ability to define a plateau will ensure reproducibility and increase the probability that a particular assay will detect defined subpopulations. (2) The Fc dependence of binding should be demonstrated. Since the binding of Ig is dependent on the Fc portion of the molecule (see Section IV,A), this will help ensure that different assays are detecting similar receptors. (3) Cells, other than lymphocytes, that bear Fc receptors should be either identified and excluded from counts or removed from the population being tested. Such cells include monocytes (Huber and Fudenberg, 1968),

macrophages (Howard and Benacerraf, 1966), neutrophils (Lay and Nussenzweig, 1968), and basophils (Ishizaka *et al.*, 1970). For further recommendations on this point, see Auti *et al.* (1974). (4) The subpopulations of lymphocytes that bind Ig should be defined simultaneously on individual cells by the use of other lymphocyte markers and/or by population studies.

A. Molecular Antigen–Antibody Complexes

Complexes composed of specific antibody bound to molecular antigen occur naturally under a variety of circumstances. Moreover, molecular antigens and antibodies to them are readily available in the laboratory. The binding of such complexes to lymphocytes has been detected by a variety of techniques.

1. Radioautography

This method was first described in detail by Basten and associates (1972b). It involves the sequential incubation of lymphocytes with antibody and then antigen, the latter having been labeled with radioactive iodine. The cells are then washed and radioautographs prepared. This technique detects binding of Ig complexes to B cells and some splenic T lymphocytes, but not to thymocytes (Basten *et al.*, 1972b, 1975b). UL cells have not been examined. Studies with this assay which also involve antibodies directed against lymphocyte surface antigens require the use of $F(ab')_2$ fragments to avoid nonspecific inhibition of the binding of complexed Ig to lymphocytes (Basten *et al.*, 1975a).

2. Fluorescence

Direct fluorescence utilizes complexes in which either the antibody (Forni and Pernis, 1975) or the antigen (Dickler, 1974; Abbas and Unanue, 1975; Forni and Pernis, 1975) or both (Winchester *et al.*, 1975b) are conjugated with a fluorescent reagent. The complexes and lymphocytes are incubated together, following which the cells are washed and mounted on slides, and analyzed by fluorescence microscopy. Only certain protein antigens have been used successfully with this method. The antigen utilized was either large, such as keyhole limpet hemocyanin (KLH) (Dickler, 1974; Abbas and Unanue, 1975), or was immunoglobulin (Forni and Pernis, 1975; Winchester *et al.*, 1975b). In the latter cases, the immunoglobulin used as antigen was chosen to avoid cross-reactions between lymphocyte surface Ig and the immunoglobulin used to make the reagent complexes. This technique apparently detects binding of complexes to B cells but not to T lymphocytes (Dickler, 1974; Abbas and Unanue, 1975; Forni and Pernis, 1975; Winchester *et al.*,

1975b). It can be inferred that binding to UL cells in human blood is detected by this method (see Section III,C), but this has not been directly demonstrated.

A variation of the direct fluorescent technique involves the use of the fluorescence activated cell sorter (FACS) instead of microscopy to analyze the lymphocytes (Stout and Herzenberg, 1975a). Using this highly sensitive instrumentation and very large complexes, the binding of Ig complexes has been detected on both B cells and some T cells from the spleen and thymus. UL cells have not yet been examined.

The indirect fluorescence technique (Arbeit *et al.*, 1976) is similar to the direct technique except that the complexes themselves are not labeled. Instead, the lymphocytes, after incubation with complexes, are washed and then stained with a fluorescent antibody specific for the antibody of the complex and not reactive with the lymphocyte surface Ig. This method appears to have great potential since it utilizes soluble complexes and can be titrated to detect different lymphocyte subpopulations. Thus, depending on the concentration of complexes used, binding can be detected to either B but not T lymphocytes, or to both B and some T lymphocytes in the spleen and thymus. UL cells have not been studied.

3. *Inhibition of Reverse Immune Cytoadherence (RICA)*

The RICA test involves the bridging of lymphocyte surface Ig and antigen-coated erythrocytes by hybrid antibody with specificity for both Ig and the antigen (i.e., it detects lymphocyte surface Ig). Fc-dependent binding of complexes is detected by their ability, for reasons unknown, to inhibit RICA (Paraskevas *et al.*, 1972a). This technique is limited by being an indirect assay and by being able to detect binding only on cells which bear membrane Ig.

B. Aggregated Immunoglobulin

Aggregated immunoglobulin possesses many of the properties of antigen–antibody complexes (Ishizaka and Ishizaka, 1960), and has been used to study the interaction between complexed Ig and lymphocytes. Aggregated Ig and antigen–antibody complexes appear to bind to the same sites on the lymphocyte membrane based on inhibition experiments (Eden *et al.*, 1973; Hallberg *et al.*, 1973; Dickler, 1974; Dickler and Sachs, 1974; Kedar *et al.*, 1974b; Abbas and Unanue, 1975; Basten *et al.*, 1975a; Revillard *et al.*, 1975; Warner *et al.*, 1975).

Complexed Ig appears to have greater avidity for lymphocyte Fc receptors than monomeric Ig (see Section IV,B). It is not known for either antigen–antibody complexes or aggregated Ig whether this increased affinity is due to alterations in the Fc portion of the Ig molecule

or to the presence of multiple Fc pieces in close proximity in the complex. However, aggregates prepared by different means (heat or chemical aggregation) appear to behave similarly with respect to lymphocyte-binding capacity (Anderson and Grey, 1974).

Since aggregates are prepared from immunoglobulin with undefined antigen specificity, it is necessary to check the immunoglobulin for hetero- or alloantibodies specific for the population of lymphocytes being tested. Such antibodies must be removed by absorption before the immunoglobulin is aggregated to ensure that binding of the aggregates to lymphocytes is not due to such antibody activity.

1. Radioautography

This method was first described by Anderson and Grey (1974) and involves the labeling of aggregated Ig with radioactive iodine. Labeled aggregates and lymphocytes are incubated together, the latter washed, and radioautographs prepared. This technique detects binding of Ig complexes to B cells and some T lymphocytes in spleen and thymus (Anderson and Grey, 1974; Basten *et al.*, 1975b). UL cells have not been examined.

2. Fluorescence

The direct fluorescence detection of lymphocyte binding of aggregated Ig was first described by Dickler and Kunkel (1972; for review of this method, see Dickler, 1976a). Immunoglobulin is fluorescent reagent conjugated and heat-aggregated. Aggregates and lymphocytes are incubated together, the latter washed, mounted on slides, and analyzed by fluorescence microscopy. This method detects binding of aggregates to B lymphocytes (Dickler and Kunkel, 1972; Preud'homme and Seligmann, 1972; Brown and Greaves, 1974; Dickler and Sachs, 1974; Frøland *et al.*, 1974b; Sidman and Unanue, 1975), but not to T cells in spleen or thymus (Dickler *et al.*, 1973; Dickler and Sachs, 1974; Sidman and Unanue, 1975). A small number of T lymphocytes in human blood also bind these complexes (Bentwich *et al.*, 1973b; Brown and Greaves, 1974; Dickler *et al.*, 1974a). It can be inferred that binding of aggregates to UL cells in human blood is detected by this method (see Section III,C), but this has not been directly studied. Binding of Ig complexes by UL cells in mouse spleen appears to be detected by this method (Dickler *et al.*, 1976b). In general, the direct fluorescence-aggregated Ig method appears to detect fewer lymphocytes binding complexed Ig than some other methods (see Sections III,A,2 and B,1).

Indirect fluorescence detection of aggregate binding is similar to the direct method except that the aggregates are unlabeled and are detected

on the lymphocyte surface by subsequent staining with fluorescent reagent conjugated antibody specific for the aggregated Ig (Dickler and Kunkel, 1972; Van Boxel and Rosenstreich, 1974; Basten *et al.*, 1975a,b). This method is not ideal for the evaluation of B lymphocytes in the homologous situation where the fluorescent reagent conjugated antibody detects both bound aggregates and lymphocyte surface Ig, since subjective evaluation is then required to differentiate between the two (Dickler and Kunkel, 1972). Use of this method is best limited either to cell populations that lack easily detectable surface Ig (Dickler *et al.*, 1973; Van Boxel and Rosenstreich, 1974), or to situations where the antibody used for detection of aggregates does not react with surface Ig (Basten *et al.*, 1975a,b).

Aggregates are heterogeneous in size and those with sedimentation properties similar to cells but not bound to them can be difficult to remove from the lymphocyte suspension by washing (H. B. Dickler, unpublished observation). This creates the potential for artifact if the indirect technique is used to study specificity of binding. Thus, unbound aggregates remaining in suspension with lymphocytes could become bound to them via the Fc of the antibody used for detection of the complexes.

This technique detects binding to B lymphocytes (Dickler and Kunkel, 1972; Dickler *et al.*, 1973; Basten *et al.*, 1975a). Binding to T lymphocytes in thymus and spleen has not been observed by this method (Van Boxel and Rosenstreich, 1974; Basten *et al.*, 1975a), but recent experiments have provided evidence that this technique will detect such binding provided high concentrations of aggregates are used (H. B. Dickler, unpublished observation). UL cells have not been directly examined, but it can be inferred that binding of complexed Ig by those in human blood are detected by this method (see Section III,C).

C. Cellular Antigen–Antibody Complexes

Cellular antigen–antibody complexes are "physiologic" in that such complexes occur *in vivo* in a variety of circumstances including viral and bacterial infections, pregnancy, and transplantation. Two main methods have been used to evaluate the interaction between lymphocytes and antibody-coated cells: rosette formation and cytotoxicity.

1. Rosette Formation

The binding of antibody-coated erythrocytes to some lymphocytes by a mechanism not involving complement was first reported by LoBuglio and associates (1967) and was described in detail in 1972 (Cline *et al.*, 1972; Yoshida and Andersson, 1972). Erythrocytes are used as a model for cellular antigens, and those of several species have been utilized, includ-

ing sheep (Cline *et al.*, 1972; Yoshida and Andersson, 1972), chicken (Halloran *et al.*, 1975; Revillard *et al.*, 1975), bovine (Hallberg *et al.*, 1973; Ramasamy and Munro, 1974), and human (Brain and Marston, 1973; Frøland and Natvig, 1973). The method involves coating the erythrocytes with a subagglutinating concentration of specific antibody. Lymphocytes are incubated with the sensitized erythrocytes (EA), suspended in solution, mounted onto slides, and viewed microscopically for the percentage of lymphocytes that have bound erythrocytes (rosettes).

Most types of antibody-sensitized erythrocytes bind to B lymphocytes, but with two exceptions (Yoshida and Andersson, 1972; Soteriades-Vlachos *et al.*, 1974) do not bind to T cells from the spleen or thymus (Cline *et al.*, 1972; Brain and Marston, 1973; Hallberg *et al.*, 1973; Fridman and Golstein, 1974; Jondal, 1974; Kedar *et al.*, 1974b; Möller, 1974; Parish and Hayward, 1974b; Ferrarini *et al.*, 1975b; Halloran *et al.*, 1975; Krammer *et al.*, 1975; Ramasamy and Lawson, 1975; Revillard *et al.*, 1975). Some T cell marker-positive cells in human blood bind these complexes (Hallberg *et al.*, 1973; Ferrarini *et al.*, 1975a; Moretta *et al.*, 1975; Revillard *et al.*, 1975). Human erythrocytes sensitized with Ripley anti-CD antibody appear to bind to UL cells in human blood, but not to B or T lymphocytes (Frøland *et al.*, 1974a; Kurnick and Grey, 1975). In general, the rosette method appears to detect fewer lymphocytes binding complexed Ig than do some other methods (see Sections III,A,2 and B,1).

The rosette method seems to have a number of characteristics that are not shared with other methods of detecting Fc receptors. (1) Several laboratories have reported inhibition of EA rosette formation by antibodies with specificity for surface Ig (Hallberg *et al.*, 1973; Kedar *et al.*, 1974b; Ramasamy and Lawson, 1975; Schirrmacher *et al.*, 1975a; Soulillou *et al.*, 1976). Anti-Ig antibodies do not inhibit binding to Fc receptors as measured by other techniques (Basten *et al.*, 1972c; Dickler and Kunkel, 1972; Dickler and Sachs, 1974; Abbas and Unanue, 1975; Forni and Pernis, 1975). In several of these studies with other techniques the complexes were chosen or controls were performed to rule out the possibility of interaction between the anti-Ig reagent and the complexes. In some cases (Parish and Hayward, 1974a; Ramasamy and Lawson, 1975; Krammer and Pernis, 1976b), but not others (Schirrmacher *et al.*, 1975a; Soulillou *et al.*, 1976), this inhibition was avoided by the use of $F(ab')_2$ fragments of the anti-Ig. (2) Some laboratories have found EA rosette formation to be temperature dependent (Parish and Hayward, 1974a; Warner *et al.*, 1975). It appears that this temperature dependence can be overcome by bringing about good contact between the lymphocytes and the EA either by prolonged stationary incubation (Yoshida and Andersson, 1972; Soteriades-Vlachos *et al.*, 1974) or by centrifugation (Frøland *et al.*,

1974a; Jondal, 1974; Kedar *et al.*, 1974b; Möller, 1974; Halloran *et al.*, 1975; Krammer *et al.*, 1975). (3) The metabolic inhibitor sodium azide has been shown to inhibit rosette formation in some laboratories (Parish and Hayward, 1974a; Krammer and Pernis, 1976b) but not all (Ramasamy and Lawson, 1975). (4) Some groups recommend gentle resuspension of rosettes (Frøland *et al.*, 1974a; Kedar *et al.*, 1974b; Ramasamy and Munro, 1974; Schirrmacher *et al.*, 1975a) suggesting that vigorous handling can disrupt the binding of EA to lymphocytes.

There are at least two possible explanations for the inability of the rosette method to detect some lymphocytes which bind immunoglobulin and for the characteristics described above. First, it is necessary to avoid hemagglutination of the sensitized erythrocytes by the use of subagglutinating concentrations of antibody. Limitation on the amount of antibody in the complex may in turn lead to lower avidity of binding. Bovine erythrocytes, which are relatively nonagglutinating (Uhlenbruck *et al.*, 1967), may be less subject to this limitation. Second, both binding to Fc receptors and a metabolic step may be involved in stable rosette formation. This latter possibility is suggested by some of the characteristics mentioned previously and by experiments which appeared to indicate that rosette formation might be a two-step process (contact and metabolic) in that rosette formation at 37°C was more rapid if lymphocytes and EA had prior contact at 4°C (Warner *et al.*, 1975). This metabolic step could involve either a change in the shape of the lymphocyte membrane or the formation of clusters of the receptors on the cell surface. Morphologic alterations of lymphocytes binding to Ig complex coated surfaces (Alexander and Henkart, 1976) and aggregation of C3 receptors (Dierich and Reisfeld, 1975) have been described. Cytochalasin B, which inhibits microtubule formation, affects EA formation to a greater extent than aggregate binding (Gutierrez *et al.*, 1976).

Although rosette methods may detect a limited number of lymphocytes which bind Ig complexes, this is not necessarily a disadvantage. Indeed, where a particular subpopulation is detected (Frøland and Natvig, 1973), it is probably the method of choice. If a metabolic step is required for stable rosette formation it seems possible that various experimental manipulations might affect the metabolic process rather than Fc receptors. If this is the case this method would seem to be particularly suited to the study of cell–cell interactions mediated by Fc receptors.

2. *Antibody-Dependent Cellular Cytotoxicity (ADCC)*

Cytotoxic activity against antibody-coated target cells by nonimmune lymphocytes was first described in 1965 (Möller, 1965) and has since been studied by a large number of laboratories (reviewed by MacLennan, 1972; Perlmann *et al.*, 1972b; Cerottini and Brunner, 1974). The assay in-

volves the incubation of lymphocytes either with target cells pretreated with antibody or with target cells in the presence of antiserum for various lengths of time and then assaying for target cell damage. Target cell damage is usually assessed by labeling the target cell with radioactive chromium and measuring its release compared to various controls. The process of ADCC appears to require (and can therefore be used to assay) effector cell Fc receptors. This conclusion is based on the observations that (1) $F(ab')_2$ fragments used to sensitize the target cells will not mediate ADCC (Larsson and Perlmann, 1972; Möller and Svehag, 1972; Hallberg, 1974; MacLennan *et al.*, 1974; Michaelsen *et al.*, 1975); and (2) immunoglobulin mixed with the lymphocytes and target cells (Larsson *et al.*, 1973; MacLennan *et al.*, 1973; Hallberg, 1974; Wisløff *et al.*, 1974a) or bound to the lymphocytes (Dickler, 1974) inhibits ADCC whereas $F(ab')_2$ fragments do not (Larsson *et al.*, 1973; Hallberg, 1974; Wisløff *et al.*, 1974a).

The use of ADCC as an assay for Fc receptors is somewhat limited by the fact that the cytotoxic mechanism is a complex process that appears to require metabolic activity by the cytotoxic cell since ADCC can be inhibited by various antimetabolites (reviewed by Perlmann *et al.*, 1972b; Cerottini and Brunner, 1974). Thus, various experimental manipulations may inhibit ADCC without having an effect on the receptors for immunoglobulin. However, ADCC, like the rosette method, may provide a useful method for study of cell–cell interactions mediated by Fc receptors.

A second limitation of the usefulness of ADCC as an assay for Fc receptors concerns the fact that it measures the activity of cell populations rather than individual cells. Thus, if more than one cell subpopulation mediates ADCC it is necessary to isolate these populations in order to use ADCC to characterize their Fc receptors. While the question of which lymphocyte subpopulations mediate ADCC remains unresolved, the majority of reports at the moment would suggest that most ADCC activity is expressed by UL cells, not by B or T cells, provided that macrophages have been excluded (Greenberg *et al.*, 1973a; Wisløff and Frøland, 1973; Brier *et al.*, 1975; Nelson *et al.*, 1976; Ramshaw and Parish, 1976). Further, there may be more than one subpopulation capable of mediating ADCC among UL cells (Greenberg *et al.*, 1973b, 1975a; Sanderson *et al.*, 1975). Bearing these reservations in mind, results with ADCC assays can be taken to reflect the Fc receptor activity of UL cells.

III. Lymphocyte Subpopulations That Bind Immunoglobulin

In order to understand what role Fc receptors may play in the immune response, it is important to know which lymphocyte populations bear these receptors. This question necessitates the adoption of practical (al-

though not necessarily ideal) criteria for the identification of different lymphocyte populations. B cells will be defined as those lymphocytes that bear readily detectable Ig of presumed endogenous origin on their surface membranes (Raff, 1970; Unanue *et al.*, 1971). T cells will be defined by the presence on their surface membranes of various markers. In the mouse these include the alloantigens Thy 1 (θ) (Reif and Allen, 1963; reviewed by Raff, 1971), and the heteroantigens defined by raising antisera in other species against mouse brain and absorbing with RBC and liver (Golub, 1971) or against mouse thymocytes and absorbing with bone marrow cells (Sarker *et al.*, 1973). In humans, T cell markers include the ability to form spontaneous rosettes with sheep erythrocytes (Coombs *et al.*, 1970; Lay *et al.*, 1971; Frøland, 1972; Jondal *et al.*, 1972). In addition, T cell heteroantigens have been identified by raising antisera in other species against thymocytes (Smith *et al.*, 1973; Williams *et al.*, 1973) or brain (Brown and Greaves, 1974), which were then absorbed to render them T-cell specific. In the guinea pig, the formation of spontaneous rosettes with rabbit erythrocytes appears to be a T-cell marker (Wilson and Coombs, 1973; Van Boxel and Rosenstreich, 1974).

In addition to B and T lymphocytes, there are mononuclear cells that are morphologically identical to lymphocytes, are nonphagocytic, and lack B- and T-cell markers. These cells will be referred to as undefined lymphocytelike (UL) cells. This term has been adopted for this review in preference to the names non-B, non-T cells, null cells (Greenberg *et al.*, 1973a; Stobo *et al.*, 1973), third population cells (Frøland and Natvig, 1973), and K cells (Perlmann *et al.*, 1975b) for several reasons. (1) There may be more than one population of cells that meets these criteria (Greenberg *et al.*, 1973b, 1975a; Sanderson *et al.*, 1975). (2) It is not clear that all such cells are active in ADCC. (3) Many such cells are not null in that they do bear receptors for Ig and possibly C3 (see Section III,C). (4) The lineage of such cells is not clear. Thus there is some evidence that some of these cells may be monocyte–macrophage related (Greenberg *et al.*, 1973b; Hayward and Greaves, 1975b), and other evidence suggesting that some are B-related (Wernet *et al.*, 1974; Chess *et al.*, 1975; MacDermott *et al.*, 1975). The term UL cells allows for these unresolved questions.

A. B Lymphocytes

A considerable body of evidence indicates that B lymphocytes bear on their surface membranes receptors for immunoglobulin. (1) With most techniques, the number of lymphocytes in normal tissues that bind Ig approximately equals the number of B cells as assessed by an inverse correlation with T-cell markers (Basten *et al.*, 1972b; Brown and Greaves,

1974; Dickler *et al.*, 1974a; Ferrarini *et al.*, 1975a), or by a direct correlation with the number of cells bearing surface Ig in the mouse (Dickler and Sachs, 1974; Abbas and Unanue, 1975; Parish, 1975), rat (Parish and Hayward, 1974b), and man (Dickler and Kunkel, 1972; Brown and Greaves, 1974; Dickler *et al.*, 1974a; Ferrarini *et al.*, 1975b). The studies in man must be interpreted with caution since the percentage of lymphocytes detected bearing surface Ig was probably artifactually high (Kumagai *et al.*, 1975; Lobo *et al.*, 1975; Winchester *et al.*, 1975a; this will be discussed in detail in Section III,C). (2) Conditions or procedures that result in an increased percentage of B cells lead to an increased percentage of lymphocytes that bind Ig. These include: (a) depletion of T lymphocytes by treatment with anti-Thy 1 antiserum and complement (Basten *et al.*, 1972b, 1975a; Rask *et al.*, 1975), thymectomy (Basten *et al.*, 1972b, 1975a; Cline *et al.*, 1972), or depletion of spontaneous E rosettes in humans (Greaves and Brown, 1974); (b) nude mice (Basten *et al.*, 1972b, 1975a; Cline *et al.*, 1972; Möller, 1974); and (c) human chronic lymphocytic leukemia (Dickler *et al.*, 1973; Hallberg *et al.*, 1973; Frøland *et al.*, 1974b; Ferrarini *et al.*, 1975b). (3) Procedures that deplete B cells diminish the percentage of Fc receptor-positive lymphocytes including: (a) irradiation and reconstitution with thymocytes (Basten *et al.*, 1972b; Cline *et al.*, 1972); (b) bursectomy of chickens (Basten *et al.*, 1972b); and (c) removal of Ig-positive lymphocytes by nylon fiber columns (Frøland *et al.*, 1974b; Greaves and Brown, 1974), anti-Ig columns (Brown and Greaves, 1974; Krammer *et al.*, 1975), or rosetting (Parish and Hayward, 1974b). The reciprocal experiment of depleting Fc receptors also diminishes Ig-bearing lymphocytes (Jondal, 1974; Parish and Hayward, 1974b). (4) Double labeling of lymphocytes for surface Ig and Fc receptors has shown that the vast majority of Ig-positive cells also bear Fc receptors (Dickler and Kunkel, 1972; Preud'homme and Seligmann, 1972; Brain and Marston, 1973; Anderson and Grey, 1974; Forni and Pernis, 1975; Ramasamy and Lawson, 1975; Basten *et al.*, 1976; Dickler, 1976a; Dickler *et al.*, 1976b; Nelson *et al.*, 1976). In most of these studies the complexes were chosen or controls were performed to rule out the possibility that the anti-Ig reagent used to detect surface Ig reacted with Ig complexes. Again, the interpretation of some of the human studies is complicated by the fact that the anti-Ig reagent was detecting UL cells as well as B cells (Kumagai *et al.*, 1975; Lobo *et al.*, 1975; Winchester *et al.*, 1975a). Nevertheless, it is apparent that the true B cells among those stained by the anti-Ig were also binding complexed Ig. Detection of binding of Ig complexes by inhibition of RICA (Paraskevas *et al.*, 1972a) can be interpreted as a double-marker study since the technique requires the presence of lymphocyte surface Ig.

The Fc receptors of B lymphocytes are apparently synthesized by the cells on which they are detected. Thus, lymphocytes depleted of their Fc receptor by either capping with complexes (Abbas and Unanue, 1975) or by enzyme treatment (Basten *et al.*, 1976) regenerated the capacity to bind complexed Ig in short-term culture.

While most B lymphocytes bear Fc receptors, it appears that not all do. Thus, various double marker studies in mouse and man have shown that 96% (Dickler and Kunkel, 1972), 80% (Anderson and Grey, 1974), 64–95% (Forni and Pernis, 1975), 81–95% (Ramasamy and Lawson, 1975), 91% (Basten *et al.*, 1976), and 89% (Dickler *et al.*, 1976b) of surface Ig-positive lymphocytes bind Ig complexes. These figures should probably be regarded as minimums, since more sensitive methods of detection may show higher percentages of B cells bearing Fc receptors.

If not all B lymphocytes bear Fc receptors, the question arises whether functional B cells (those capable of producing antibody) as well as their precursors and progeny bear these receptors.

Antigen–antibody columns have been shown to deplete indirect plaque-forming cells (PFC) from primed spleen suspensions (Basten *et al.*, 1972a). The possibility that removal of B cells by this column was due to antihuman Ig antibodies (used in the complex) cross-reacting with mouse Ig was not investigated. However, later studies using different techniques have confirmed this result. Depletion of Fc receptor-bearing spleen cells by rosette formation and Isopaque/Ficoll separation markedly diminished both primary and secondary PFC (Parish and Hayward, 1974b; Parish, 1975). Suicide experiments using ^{125}I labeled aggregated Ig also markedly depleted direct and indirect PFC (Basten *et al.*, 1975a).

B lymphocytes that respond to mitogens also appear to bear Fc receptors. In the mouse most cells which responded to LPS bore Fc receptors (Möller, 1974). In humans, B cells that responded to pokeweed mitogen were found to bind Ig by one laboratory (Greaves *et al.*, 1974) but not another (Jondal, 1974). The reason for this discrepancy is not clear. Lymphocytes cultured in the presence of insolubilized antigen–antibody complexes were markedly inhibited in their response to B-cell mitogens (Ryan *et al.*, 1975). In related experiments, but not using mitogen, S-phase lymphocytes from the rat thoracic duct were found to be ~60% Ig positive but <5% Fc receptor positive (Parish and Hayward, 1974b).

Stem cell precursors of lymphocytes do not appear to bear receptors for Ig. Thus, antigen–antibody columns do not deplete stem cells as assessed by a colony-forming assay (Basten *et al.*, 1972c). Conflicting data exist as to the ontogeny of Fc receptor-bearing lymphocytes. Hayward and Greaves (1975a) found that 18–33% of human fetal spleen

lymphocytes bound Ig compared to 30–44% bearing surface Ig and 46–68% staining with anti-B cell antiserum. Sidman and Unanue (1975) reported that the percentage of lymphocytes binding Ig was low in neonatal mouse spleens and approximately equal to the percentage that bore surface Ig. In contrast, Forni and Pernis (1975) have observed percentages of Fc receptor-positive lymphocytes in both human cord blood and neonatal murine spleens which approximated those found in the adult, and few of these cells bore surface Ig. Further experiments are needed to clarify the time course of the appearance of Fc receptors on B lymphocytes.

Studies of plasmacytomas have provided evidence that plasma cells probably do not bind immunoglobulin since most of these tumors did not bind Ig (Basten *et al.*, 1972c; Cline *et al.*, 1972; Ramasamy *et al.*, 1974; Warner *et al.*, 1975). However, variants that do not secrete Ig or only secrete partial Ig molecules have been observed to possess Fc receptor activity. This has led to a theory as to the possible function of Fc receptors (Ramasamy *et al.*, 1974; see Section VII,D).

B. T Lymphocytes

Most early studies of the binding of immunoglobulin to lymphocytes failed to demonstrate such binding to T cells from normal tissues. However, evidence has accumulated that at least a substantial minority of normal T lymphocytes bear receptors for Ig.

In the mouse, the demonstration of receptors for immunoglobulin on normal T cells has generally required the use of large complexes and/or sensitive assays (Anderson and Grey, 1974; Basten *et al.*, 1975b; Stout and Herzenberg, 1975a; Arbeit *et al.*, 1976). In addition, certain studies with other techniques, although detecting a lower percentage of lymphocytes bearing Fc receptors, have nevertheless been able to demonstrate binding of Ig to T cells from normal tissues (Lee and Paraskevas, 1972; Yoshida and Andersson, 1972; Orr and Paraskevas, 1973; Soteriades-Vlachos *et al.*, 1974). Both population and double-marker studies indicate that these are indeed T cells. (1) A substantial percentage of normal thymocytes bind Ig, although the percentages observed vary in different laboratories: 5–25% (Yoshida and Andersson, 1972), 20–45% (Anderson and Grey, 1974), 25% (Basten *et al.*, 1975b), 10% (Stout and Herzenberg, 1975a), and 10–20% (Arbeit *et al.*, 1976). In one study (Santana and Turk, 1975), nearly all thymocytes (as well as most spleen and lymph node lymphocytes) were observed to bind Ig. However, the possible presence of heteroantibodies in the complexes was not excluded. (2) Certain techniques detect binding of Ig to a greater percentage of spleen lymphocytes than can be accounted for by B cells: 70–80% (Anderson and Grey,

1974), 65% (Stout and Herzenberg, 1975a), and 65–80% (Arbeit *et al.*, 1976). (3) Double-marker studies with T cell-specific antisera (anti-Thy 1 or heteroanti-T) show that some cells from spleen defined by this criteria bind Ig: 30% (Anderson and Grey, 1974), 10% (Soteriades-Vlachos *et al.*, 1974), 25% (Basten *et al.*, 1975b; Stout and Herzenberg, 1975a), and 50% (Arbeit *et al.*, 1976). (4) Treatment with anti-Thy 1 plus complement eliminated binding of Ig to cells lacking surface Ig (Lee and Paraskevas, 1972). (5) In general, the percentage of T cells from normal tissues which bind Ig correlates with the percentage of murine T cell lymphomas which have been found to bind Ig (Grey *et al.*, 1972; Harris *et al.*, 1973; Ramasamy and Munro, 1974; Warner *et al.*, 1975; Krammer *et al.*, 1976a).

In the human, a small but significant percentage of peripheral blood lymphocytes form spontaneous rosettes with sheep erythrocytes and also bind Ig (Bentwich *et al.*, 1973b; Greaves and Brown, 1973; Hallberg *et al.*, 1973; Brown and Greaves, 1974; Dickler *et al.*, 1974a; Jondal, 1974; Ferrarini *et al.*, 1975a; Moretta *et al.*, 1975; Revillard *et al.*, 1975). In addition, one laboratory (Moretta *et al*, 1975) has observed that more than 50% of human peripheral blood T cells will bind Ig of the μ class after overnight incubation (see also Section IV,C).

Some T lymphocytes that have been activated by antigen bind Ig, and this binding has been demonstrated by a variety of techniques (Yoshida and Andersson, 1972; Anderson and Grey, 1974; Fridman and Golstein, 1974; Van Boxel and Rosenstreich, 1974; Basten *et al.*, 1975b; Gyöngyössy *et al.*, 1975; Krammer *et al.*, 1975; Neauport-Sautes *et al.*, 1975). Not all activated T lymphocytes bear Fc receptors. Thus, while activated T lymphocytes from spleen, lymph node, and peritoneal exudate can bind Ig, those from the thoracic duct do not (Basten *et al.*, 1975b; Krammer *et al.*, 1975). The relative ease of detection of the binding of Ig to activated T cells as compared to those found in normal lymphoid organs appears to be related to both an increase in the amount of Ig bound per cell and an increase in the number of cells binding Ig. Thus, a direct comparison of the amount of Ig bound has shown that activated T cells bind more than those from normal lymphoid organs (Anderson and Grey, 1974). Further, T cells with Fc receptors bind an increased amount of Ig after being cultured on allogeneic fibroblast monolayers, and some T cells that did not bind Ig become capable of doing so after such exposure (Stout *et al.*, 1976a). These latter experiments, as well as the regeneration in culture following enzyme treatment of the capacity of T cells to bind Ig (Moretta *et al.*, 1975), provide evidence that T cells synthesize these receptors. It should also be noted that some of the T cells found in normal tissues that bind Ig may have been activated by natural exposure to

endogenous or environmental antigens. Brown and Greaves (1974) and Stout and Herzenberg (1975a) observed that some of the T cells from normal tissues that bound Ig were quite large.

Since the majority of T lymphocytes appear not to bear Fc receptors and since there appears to be functional heterogeneity among T cells, it is of interest to know which functional subpopulations, if any, bear Fc receptors. These studies are usually performed by separating the Fc receptor-positive and -negative populations and assaying for a particular function. The interpretation of these experiments is dependent on both the efficiency of separation and on the sensitivity of the method used to detect the Fc receptors in the separated populations. In addition, testing of the Fc receptor-positive population (Krammer *et al.*, 1975; Stout and Herzenberg, 1975a,b; Stout *et al.*, 1976a) is subject to the artifact caused by any residual bound Ig, and column methods of separation (Golstein *et al.*, 1972a,b; Rubin, 1975; Rubin and Hertel-Wulff, 1975) are subject to the possible artifact of Fc receptor-positive T cells contaminating the Fc receptor-negative population, but which are not detectable owing to coating by Ig from the column (Karpf *et al.*, 1975). These considerations limit the interpretation of the data that have been obtained. T cells that can cooperate with B cells in the humoral immune response (helper T cells) appear to lack the capacity to bind Ig (Rubin and Hertel-Wulff, 1975; Stout and Herzenberg, 1975a), and the same is true for the precursors of cytotoxic T cells (Golstein *et al.*, 1972b; Stout *et al.*, 1976a). In contrast, T cells that can amplify the cellular cytotoxic response appear to be Fc receptor positive (Stout *et al.*, 1976a), as are those T cells that respond directly to the mitogen conconavalin A (Ryan *et al.*, 1975; Stout and Herzenberg, 1975b). Some T cells that bind Ig are Ly 1 positive and others are Ly 2 positive (Stout *et al.*, 1976a). Direct examination of suppressor T cells for the capacity to bind Ig has not been reported. There is conflicting evidence as to whether cytotoxic T cells bear Fc receptors. They have been reported to be Fc receptor negative (Golstein *et al.*, 1972a; Rubin, 1975), both negative and positive (Krammer *et al.*, 1976b), and almost entirely Fc receptor positive (Stout *et al.*, 1976a). The reasons for these discrepant results are not totally clear but may be technical (see above). Further studies are needed to clarify this area.

C. Undefined Lymphocyte-like (UL) Cells

Binding of Ig to individual human UL cells was first observed using human Rh-positive erythrocytes sensitized with Ripley anti-CD antibody (EA) (Frøland *et al.*, 1974a). Cells binding these complexes appeared not to be T cells in that they did not form spontaneous rosettes with sheep erythrocytes (E) in double-marker studies, and depletion of E

rosette-forming cells increased the number of EA binding cells. Also, these cells did not appear to be B cells in that depletion of Ig-bearing cells did not reduce the number of cells binding these complexes and vice versa. Further, there was no apparent correlation between the number of cells binding these complexes and the number bearing Ig in normal tissues and disease states. Thus, cells binding these complexes were found in patients with agammaglobulinemia who lacked Ig-bearing cells (see also Dickler *et al.*, 1974b; Wernet *et al.*, 1974; Hayward and Greaves, 1975a,b) and were decreased in patients with chronic lymphocytic leukemia who had high percentages of B cells (see also Kurnick and Grey, 1975).

These findings using Ripley EA seemed to conflict with studies using other techniques (Dickler and Kunkel, 1972; Preud'homme and Seligmann, 1972; Brain and Marston, 1973; Hallberg *et al.*, 1973; Brown and Greaves, 1974; Dickler *et al.*, 1974a) where the lymphocytes binding Ig appeared to correlate with those bearing surface Ig (see Section III,A). This discrepancy has apparently been resolved. Many of these latter studies stained lymphocyte surface Ig using methodology which probably detected both B cells (primarily IgM- and/or IgD-bearing cells) as well as a population of cells that bore exogenous IgG (Kumagai *et al.*, 1975; Lobo *et al.*, 1975; Winchester *et al.*, 1975a), while the studies of Frøland and colleagues were less subject to this phenomenon in that they detected relatively few IgG-bearing cells (Frøland and Natvig, 1973). Moreover, Ripley EA appear to form rosettes almost entirely with those cells that bear exogenous IgG (Kurnick and Grey, 1975), and these cells for the most part do not form spontaneous rosettes with sheep erythrocytes (Lobo *et al.*, 1975) although there is some controversy on this point (Winchester *et al.*, 1975a). It seems reasonable to conclude that the population that stains for and binds exogenous Ig (Horwitz and Lobo, 1975) is the same population characterized by Frøland and associates. Further, others have observed the binding of antibody-sensitized erythrocytes to cells that lack Ig and do not form E rosettes (Revillard *et al.*, 1975) while comparisons of IgM- and/or IgD-bearing populations and Ig binding as assayed by aggregated Ig or antigen–antibody complexes indicate that both B cells and cells which do not bear Ig bind complexed Ig (Forni and Pernis, 1975; Winchester *et al.*, 1975a,b). Thus, the evidence favors the view that Ripley EA bind preferentially to UL cells while other forms of complexes bind to both B cells and UL cells.

There are relatively few studies of the binding of Ig to individual cells in the mouse which lack both Ig and T-cell markers. However, in both double-marker studies (Anderson and Grey, 1974) and depletion studies (Parish, 1975; Dickler *et al.*, 1976b), such cells binding Ig have been

observed. Studies with Ripley EA which bind primarily to UL cells in human have shown that these bind to 4–10% of cells in mouse peripheral blood and spleen but <1% in lymph nodes (Grey *et al.*, 1975).

Further evidence for the existence of Fc receptors on UL cells comes from studies of the nature of the population(s) which mediates antibody-dependent cellular cytotoxicity (ADCC), a process which, as noted earlier (Section II,C), requires receptors for Ig. Studies utilizing populations either lacking or depleted of B cells, T cells, or both, have indicated that the cells which most actively mediate ADCC lack both B- and T-cell markers (Van Boxel *et al.*, 1972; Greenberg *et al.*, 1973a,b 1975a; Wisløff and Frøland, 1973; Wisløff *et al.*, 1974b; Brier *et al.*, 1975; McDermott *et al.*, 1975; Nelson *et al.*, 1976; Ramshaw and Parish, 1976).

There is evidence that at least some UL cells that bear receptors for Ig synthesize them. Thus, the ability of cells lacking B- and T-cell markers to bind Ig was lost after Pronase treatment but regenerated in subsequent culture (Horowitz and Lobo, 1975).

While the evidence is considered that some UL cells bear Fc receptors, this does not appear to be true for all such cells. Using double-marker studies, Anderson and Grey (1974) found 55% of murine splenic lymphocytes lacking Ig and Thy 1 bound complexed Ig. Roughly similar percentages (25–50%) were obtained in human PBL lacking B and T cell markers utilizing depletion techniques (Revillard *et al.*, 1975).

It is not clear how many subpopulations of Fc receptor bearing UL cells exist. Some such cells may be adherent to glass or plastic (Greenberg *et al.*, 1973b) whereas others are not (Frøland *et al.*, 1974a; Kedar *et al.*, 1974b; Brier *et al.*, 1975; Greenberg *et al.*, 1975a; Horwitz and Lobo, 1975). Based on depletion studies, some of these cells apparently have receptors for the third component of complement (Brier *et al.*, 1975; Greenberg *et al.*, 1975a; Perlmann *et al.*, 1975a), whereas others do not (Horwitz and Lobo, 1975; Ramshaw and Parish, 1976). There is evidence of a functional nature that some UL cells with Fc receptors can be B cell precursors (Wernet *et al.*, 1974; Brier *et al.*, 1975; Chess *et al.*, 1975) whereas other UL cells with receptors for Ig may be more closely related to monocytes (Greenberg *et al.*, 1973b; Hayward and Greaves, 1975a,b), based on adherence and marker studies. A variety of approaches have indicated that the number of UL cells which mediate ADCC is probably very small (Perlmann *et al.*, 1975b; Revillard *et al.*, 1975; Ramshaw and Parish, 1976). For example, Revillard and co-workers (Revillard *et al.*, 1975) depleted less than 1% of human PBL by the formation of EA rosettes formed with very low concentrations of sensitizing antibody and yet inhibited ADCC activity >90%. Studies that combine several of the above criteria will be necessary to establish the number of subpopulations

of UL cells bearing Fc receptors and to ascertain whether they are functionally distinct.

IV. Specificity of Binding

Specificity of binding of immunoglobulin to lymphocytes is relevant to an understanding of immunologic function of Fc receptors. In addition, studies of specificity provide an indication as to whether the receptors for Ig on various lymphocyte subpopulations are similar or different, and whether more than one type of receptor for Ig can exist on a single cell. Finally, such studies may provide insight into the requirements and mechanism of binding.

A. Requirement for the Fc Portion of the Immunoglobulin Molecule

The binding of immunoglobulin to lymphocytes is specific in that other proteins, such as human or bovine albumin, transferrin, and ferritin, do not bind to the same degree as Ig as assayed either directly or indirectly by inhibition of Ig binding. This has been shown for B cells (Paraskevas *et al.*, 1972a; Anderson and Grey, 1974; Dickler, 1974; Lawrence *et al.*, 1975), T cells (Yoshida and Andersson, 1972; Anderson and Grey, 1974; Fridman and Golstein, 1974), and UL cells (Hallberg, 1974).

The evidence overwhelmingly indicates that the binding of immunoglobulin is mediated by sites on the lymphocyte membrane that specifically recognize the Fc portion of the Ig molecule, irrespective of the type of complex employed. (1) Light chains, Fab, and $F(ab')_2$ fragments and complexes thereof do not bind. This has been directly demonstrated for B cells (Basten *et al.*, 1972c; Cline *et al.*, 1972; Paraskevas *et al.*, 1972a; Dickler, 1974; Abbas and Unanue, 1975; Halloran *et al.*, 1975; Revillard *et al.*, 1975; Arbeit *et al.*, 1976), T cells (Fridman and Golstein, 1974; Neauport-Sautes *et al.*, 1975; Revillard *et al.*, 1975; Santana and Turk, 1975; Stout and Herzenberg, 1975a; Arbeit *et al.*, 1976), and UL cells (Larsson and Perlmann, 1972; Möller and Svehag, 1972; Frøland *et al.*, 1974c; Hallberg, 1974; Revillard *et al.*, 1975). These portions of the Ig molecule also do not inhibit the binding of intact Ig to B cells (Basten *et al.*, 1972c; Cline *et al.*, 1972; Anderson and Grey, 1974; Lawrence *et al.*, 1975), T cells (Harris *et al.*, 1973; Anderson and Grey, 1974; Neauport-Sautes *et al.*, 1975), or UL cells (Greenberg *et al.*, 1973b; Larsson *et al.*, 1973; Frøland *et al.*, 1974c; Hallberg, 1974; Wisløff *et al.*, 1974a; Brier *et al.*, 1975). (2) The Fc fragment has been shown to bind to B lymphocytes directly (Paraskevas *et al.*, 1972a) and indirectly by inhibition studies to bind to B cells (Lawrence *et al.*, 1975), T cells (Neauport-

Sautes *et al.*, 1975), and UL cells (Frøland *et al.*, 1974c; Wisløff *et al.*, 1974a).

In contrast to most of the evidence, the binding of Fab or $F(ab')_2$ fragments and complexes thereof has been reported by three laboratories (Frøland *et al.*, 1974b; Ramasamy and Munro, 1974; Johnson *et al.*, 1974). In each of these cases the binding of the fragments was assayed indirectly using another antibody. The possibility exists that the second antibody (which was intact) contributed to the observed binding via its own Fc. Although not studied, this possibility could have been excluded by using $F(ab')_2$ fragments of the second antibody. Further, in one of these studies the possible presence of heteroantibodies was not excluded (Johnson *et al.*, 1975) and in one study (Ramasamy and Munro, 1974) binding of Fab was observed only on a single lymphoma, which may not reflect the characteristics of normal lymphocytes.

The region on the Fc fragment involved in binding to lymphocytes is not definitely established. Reduction and alkylation markedly inhibits the ability of Ig to bind to both B cells (Dickler, 1974; Lawrence *et al.*, 1975) and UL cells (Denk *et al.*, 1974; Frøland *et al.*, 1974c; Wisløff *et al.*, 1974a; Michaelsen *et al.*, 1975). In several studies, this could not have been due to lack of aggregation of the Ig caused by reduction and alkylation. T cells have not been studied in this regard. In two of these cases, the reduction and alkylation was partial and only affected the interchain disulfide bridges and was also shown to specifically affect Fc fragments (Frøland *et al.*, 1974c; Wisløff *et al.*, 1974a). Based on the fact that such a procedure affects the N-terminal part of the Fc piece as well as the fact that the pFc′ fragment (which roughly corresponds to the C_H3 region) did not bind, these workers suggested that the site recognized by lymphocytes was in the C_H2 region. However, they appropriately noted that the interaction site might be located elsewhere on the Fc piece and that the C_H2 region might serve to maintain the structural integrity of the Fc fragment. Also, they could not exclude the possibility that a site in the C-terminal half of Fc might have been destroyed during preparation of the pFc′ fragment or that aggregation of pFc′ is required for binding. Consistent with this latter possibility is the fact that the other fragment obtained when preparing pFc′, namely Facb, also does not bind (MacLennan *et al.*, 1974; Michaelsen *et al.*, 1975). Other data suggest that the site recognized by lymphocytes is in the C_H3 region. IgG molecules with deletions of this region produced by mutant myeloma cell lines were unable to bind to B lymphocytes (Ramasamy *et al.*, 1975). However, an effect on the C_H2 region in these IgG molecules could not be excluded. Perhaps the strongest data relate to the observation that the pep III fragment (C-terminal 114 amino acids) of rabbit IgG inhibited the

binding of intact IgG to activated T cells (Neauport-Sautes *et al.*, 1975). It will be of interest to test this fragment on both B cells and UL cells.

B. Affinity of Binding of Immunoglobulin to Lymphocytes

The functional implications of the binding of Ig to lymphocytes would be markedly different if monomeric Ig binds with high affinity as opposed to the possibility that Ig binds with high affinity only when complexed either to antigen or by aggregation. While no precise studies of affinity have yet been reported, some information bearing on this point is available. A large number of laboratories have either not detected binding of monomeric Ig or have detected binding that was relatively weaker than the binding of complexed Ig. This has been reported using both direct and indirect (inhibition) assays for B cells (Basten *et al.*, 1972b; Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972a; Preud'homme and Seligmann, 1972; Yoshida and Andersson, 1972; Dickler *et al.*, 1973; Anderson and Grey, 1974; Dickler, 1974; Dickler and Sachs, 1974; Parish and Hayward, 1974a; Lawrence *et al.*, 1975; Revillard *et al.*, 1975; Arbeit *et al.*, 1976), T cells (Yoshida and Andersson, 1972; Anderson and Grey, 1974; Revillard *et al.*, 1975; Arbeit *et al.*, 1976), and UL cells (Larsson *et al.*, 1973; McLennan *et al.*, 1973; Hallberg, 1974; Wisløff *et al.*, 1974a; Greenberg *et al.*, 1975a; Horwitz and Lobo, 1975; Revillard *et al.*, 1975). However, these studies do not exclude the possibility that monomer binds with high affinity. In direct binding assays, individual Ig molecules might not carry a sufficient quantity of label to be detected even when bound. In inhibition studies, monomer might bind with high affinity but be displayed by complexes of Ig that have an even higher affinity.

In contrast to the above studies, several laboratories report the binding of noncomplexed Ig to B cells (Basten *et al.*, 1972b; Modabber and Coons, 1972; Yoshida and Andersson, 1972; Lawrence *et al.*, 1975; Revillard *et al.*, 1975) T cells (Modabber and Coons, 1972; Fridman and Golstein, 1974; Moretta *et al.*, 1975; Neauport-Sautes *et al.*, 1975; Revillard *et al.*, 1975) and UL cells (Larsson *et al.*, 1973; Frøland *et al.*, 1974c; Hallberg, 1974; Wisløff *et al.*, 1974a; Brier *et al.*, 1975; Greenberg *et al.*, 1975a; Revillard *et al.*, 1975). However, none of these studies reported stringent analysis of their Ig preparations in order to exclude the possible presence of small aggregates and/or altered monomer. Storage and/or methods of preparation can produce such changes. For example, MacLennan and associates (1973) reported that neither normal serum nor the 7 S fraction from a G-200 column inhibited ADCC whereas Ig which had been precipitated or passed on a DEAE column did inhibit. Further studies utilizing carefully defined Ig preparations (e.g., analytical ultracentrif-

ugation, polyacrylamide gel electrophoresis, complement fixation, *in vivo* half-life) will be required to establish the affinity of binding of monomeric Ig to the various lymphocyte subpopulations. Regardless of the affinity of binding of monomeric Ig to lymphocytes, it will be of interest to determine whether this affinity increases when individual Ig molecules bind antigen but do not form complexes. If this is the case, it would provide evidence for alterations in the Fc portion of the Ig molecule produced by binding of antigen at the combining site.

There is some evidence to suggest that the different lymphocyte subpopulations differ in their relative avidity for Ig. In studies in the mouse, it has been shown that activated T lymphocytes bound more Ig than spleen lymphocytes, which in turn bound more Ig than thymocytes Anderson and Grey, 1974). The possibility that B lymphocytes have a higher avidity for Ig than T lymphocytes form normal tissues is suggested by the apparent requirement for sensitive assays and/or large complexes to detect binding to the latter (see Section III,B). In addition, UL cells may have a higher avidity than either B or T cells. UL cells are detected preferentially by certain techniques and, under certain circumstances in the human, bear exogenous IgG whereas B and T cells do not (see Sections II,C and III,C). It is not known whether these apparent differences in avidity of the various lymphocyte subpopulations are due to differences in the number of receptors per cell, the affinity of individual receptors or the topographical distribution of receptors on the cell surface.

C. Class and Subclass Specificity

If different classes and subclasses of antibody serve different functions in the immune response, and if Fc receptors are involved with some of these functions, then one might expect to observe receptors that bind specifically one class or subclass of Ig. Ideally, studies related to this point would utilize a single antigen complexed to purified antibody of the various classes and subclasses. The complexes would be of defined size, and the antibody would be unaltered by the methods used in purification. In addition, the binding of B cell surface Ig and possibly other lymphocyte factors could be evaluated. Unfortunately, technical limitations have prevented such studies. Thus, interpretation of the studies which have been performed is subject to some limitations. (1) The most common approach has been to examine the binding of purified myeloma proteins of the various classes and subclasses either directly or by inhibition studies. Individual myeloma proteins appear to vary in the degree to which they will aggregate, either spontaneously during preparation or storage or purposefully (e.g., by heating). Since the degree of

aggregation may affect binding or the detection of binding (see Section IV,B), differences in the ability of individual myeloma proteins to aggregate could affect the results. (2) Failure to detect binding by direct techniques is relative and dependent on the sensitivity of the assay. (3) In many inhibition studies, the class or subclass of antibody being inhibited is often not known. Thus, failure to inhibit binding with a particular Ig protein does not exclude the possibility that that protein could bind to another receptor. Conversely, in cases where the binding of antibody is characterized, inhibition by an Ig of a different class or subclass implies binding to the same membrane site. Similar inferences can be drawn from studies where immunoglobulins of different classes or subclasses inhibit the majority of binding of Ig even if the latter is not characterized. In general, each of the above limitations necessitates the view that positive results are more meaningful than those that are negative.

1. B Lymphocytes

There is universal agreement that antibodies of the IgG class bind to B lymphocytes, and this was shown by the earliest studies (Basten *et al.*, 1972c; Cline *et al.*, 1972; Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972a). While there is evidence which suggests that each of the IgG subclasses can bind, there is some controversy as to the relative efficiency of binding of the various subclasses. In the mouse, one laboratory observed approximately equal binding of IgGl, IgG2a, and IgG2b (Cline *et al.*, 1972); one laboratory found the most binding with IgG1 (Basten *et al.*, 1972c); and others have found that IgG2a or IgG2b bound better than IgG1 (Anderson and Grey, 1974; Soteriades-Vlachos *et al.*, 1974; Gyöngyössy *et al.*, 1975). Even though different techniques were utilized, the reasons for this discrepancy are unclear. In man, IgG1 and IgG3 apparently bound more avidly than IgG2 and IgG4 although binding of the latter could be shown (Lawrence *et al.*, 1975). This last study did not differentiate the various lymphocyte subpopulations. Some inhibition studies indicate that the various IgG subclasses are probably binding to the same receptor (Basten *et al.*, 1972c; Anderson and Grey, 1974; Soteriades-Vlachos *et al.*, 1974; Lawrence *et al.*, 1975).

One laboratory has reported the binding of IgM to some B cells (Basten *et al.*, 1972c). However, other laboratories have not been able to confirm this observation (Cline *et al.*, 1972; Soteriades-Vlachos *et al.*, 1974; Gyöngyössy *et al.*, 1975; Lawrence *et al.*, 1975; Revillard *et al.*, 1975). Binding has not been observed with IgA (Basten *et al.*, 1972c; Cline *et al.*, 1972; Anderson and Grey, 1974; Soteriades-Vlachos *et al.*, 1974; Lawrence *et al.*, 1975) or IgD (Lawrence *et al.*, 1975). Some

binding of IgE has been reported (Lawrence *et al.*, 1975), but the possible presence of some contaminating basophils in the cell population was not excluded.

2. *UL Cells*

It is clear that UL cells bind IgG (Carlsson *et al.*, 1971; Larsson *et al.*, 1973; MacLennan *et al.*, 1973; Frøland *et al.*, 1974c). Although there is evidence that all of the IgG subclasses can bind, the relative avidity of some subclasses appears to be higher than others. In the mouse, IgG2b and IgG2a appeared to bind more than IgG1 (Greenberg *et al.*, 1973b, 1975a). In the human, all laboratories have observed binding of IgG1 and IgG3 (Larsson *et al.*, 1973; MacLennan *et al.*, 1973; Frøland *et al.*, 1974c; Wisløff *et al.*, 1974a). Less avid binding of IgG2 has been reported (Larsson *et al.*, 1973; MacLennan *et al.*, 1973; Wisløff *et al.*, 1974a) but not by all workers (Frøland *et al.*, 1974c). Binding of IgG4 has been reported by one laboratory (MacLennan *et al.*, 1973) but not by others (Larsson *et al.*, 1973; Frøland *et al.*, 1974c; Wisløff *et al.*, 1974a). It can be inferred from many of these studies that the various subclasses are probably binding to the same receptor. No binding of IgM, IgA, IgD, or IgE has been reported for UL cells (Carlssen *et al.*, 1971; Greenberg *et al.*, 1973b; Frøland *et al.*, 1974c; Wisløff *et al.*, 1974a; Greenberg *et al.*, 1975a; Horwitz and Lobo, 1975; Revillard *et al.*, 1975).

3. *T Lymphocytes*

Beginning with the earliest reports, there is general agreement that IgG can bind to some T lymphocytes in the mouse (Grey *et al.*, 1972; Lee and Paraskevas, 1972; Yoshida and Andersson, 1972; Harris *et al.*, 1973; Anderson and Grey, 1974; Fridman and Golstein, 1974; Ramasamy and Munro, 1974; Soteriades-Vlachos *et al.*, 1974) and to some lymphocytes bearing T-cell markers in the human (Bentwich *et al.*, 1973b; Hallberg *et al.*, 1973; Brown and Greaves, 1974; Dickler *et al.*, 1974a) and guinea pig (Van Boxel and Rosenstreich, 1974). In the mouse there is evidence that all the subclasses tested can bind to some T lymphocytes, but there appear to be differences in the relative avidity. Studies with both T lymphocytes from normal tissues and activated T lymphocytes suggest that IgG2a or IgG2b are bound more avidly than IgG1 (Lee and Paraskevas, 1972; Anderson and Grey, 1974; Soteriades-Vlachos *et al.*, 1974; Basten *et al.*, 1975b; Krammer *et al.*, 1975). There are conflicting data as to whether activated T lymphocytes bind IgG2a or IgG2b more avidly (Soteriades-Vlachos *et al.*, 1974; Krammer *et al.*, 1975). T-cell lymphomas are reported by some laboratories to bind IgG1 more avidly

than IgG2a or IgG2b (Harris *et al.*, 1973; Basten *et al.*, 1975; Warner *et al.*, 1975), whereas the reverse is reported by others (Ramasamy and Munro, 1974; Krammer *et al.*, 1976a). Subclass specificity of human T lymphocytes has not been examined. It would appear from the relative degree of inhibition obtained in many of the above studies that the various subclasses are probably binding to the same receptor.

The binding of IgM to T lymphocytes is highly controversial. A large number of laboratories have failed to detect the binding of IgM to T lymphocytes (Yoshida and Andersson, 1972; Harris *et al.*, 1973; Fridman and Golstein, 1974; Ramasamy and Munro, 1974; Soteriades-Vlachos *et al.*, 1974; Gyöngyössy *et al.*, 1975; Krammer *et al.*, 1975, 1976a; Revillard *et al.*, 1975; Stout and Herzenberg, 1975a). In contrast, other laboratories have detected the presence of exogenous IgM on the surface of T lymphocytes (Hudson *et al.*, 1974; Hunt and Williams, 1974), as well as the ability of exogenous IgM to augment antigen binding by T cells (Webb and Cooper, 1973) and T cell cooperation in a humoral immune response (Playfair *et al.*, 1974). Also it has been reported that T cells can mediate ADCC via IgM antibody (Lamon *et al.*, 1975a,b). However, in some cases (allogeneic stimulation) it appears that exogenous IgM might be bound to T cells via antigen itself. Purified allogeneically activated T cells that were IgM negative became IgM positive if incubated in alloantiserum specific for the same determinants that the cells were activated against, but not if incubated in unrelated alloantiserum (Hudson and Sprent, 1976). Similarly, when T cells activated against a mixture of two different allogeneic cell populations were incubated with alloantiserum directed against one stimulator population, only approximately half the cells became Ig positive whereas nearly all became positive if incubated in alloantisera with activity against both stimulator populations (Nagy *et al.*, 1976). While these experiments suggest a mechanism for binding of IgM to T cells without involving an Fc receptor, it is not clear that all binding of IgM to T cells is via such a mechanism. One laboratory (Moretta *et al.*, 1975) has recently reported that more than half of human peripheral blood T lymphocytes incubated at 37°C in the absence of human serum, in contrast to those freshly isolated, are able to bind IgM antibody-sensitized erythrocytes. This binding was inhibited by IgM but not IgG. This result suggests that many T lymphocytes have at least some receptors for IgM, but under normal circumstances these are occupied, and therefore not detected in binding assays. Moreover, such receptors would be distinct from those that bind IgG. Further experimentation is required to resolve the controversy of the existence of T-cell receptors for IgM.

Several studies have failed to detect binding of IgA to T cells (Harris

et al., 1973; Anderson and Grey, 1974; Soteriades-Vlachos *et al.*, 1974; Krammer *et al.*, 1975, 1976a; Warner *et al.*, 1975). No studies of the possible binding of IgD or IgE have been reported.

D. Species Specificity

In contrast to the class specificity of lymphocyte Ig binding, relatively little species specificity has been observed. This has practical significance since it allows flexibility in the design of experiments especially with regard to avoidance of cross-reactions with endogenous membrane Ig. In addition, the lack of species specificity is of theoretical interest, since it indicates that the recognition mechanism, as well as the site on the immunoglobulin molecule recognized by lymphocytes were conserved during evolution, which might be interpreted as indicating a functional role for Fc receptors.

Direct binding studies with B cells have shown no evidence for species specificity. B lymphocytes from the mouse has been shown to bind Ig from rabbit (Basten *et al.*, 1972b; Modabber and Coons, 1972; Paraskevas *et al.*, 1972a; Möller, 1974; Ramasamy and Munro, 1974; Abbas and Unanue, 1975; Halloran *et al.*, 1975; Warner *et al.*, 1975; Arbeit *et al.*, 1976), human (Anderson and Grey, 1974; Dickler and Sachs, 1974; Basten *et al.*, 1975a; Rask *et al.*, 1975; Sidman and Unanue, 1975; Warner *et al.*, 1975; Nelson *et al.*, 1976), rat (Modabber and Coons, 1972), and chicken (Basten *et al.*, 1972b). One laboratory was unable to demonstrate the binding of chicken Ig to mouse lymphocytes (Anderson and Grey, 1974). B cells in the rat have been shown to bind rabbit (Kedar *et al.*, 1974b; Clancy *et al.*, 1976) and human (Dickler, 1976a) Ig. Human B cells bind rabbit Ig (Dickler, 1974; Jondal, 1974; Ferrarini *et al.*, 1975a; Forni and Pernis, 1975; Revillard *et al.*, 1975; Wernet *et al.*, 1975; Winchester *et al.*, 1975b). Guinea pig and monkey B cells bind human Ig (Dickler, 1976a). In addition to direct binding studies, inhibition studies indicate that the immunoglobulins of the various species bind to the same sites on the B cell membrane (Eden *et al.*, 1973; Dickler, 1974; Dickler and Sachs, 1974; Kedar *et al.*, 1974b; Abbas and Unanue, 1975; Basten *et al.*, 1975; Krammer and Pernis, 1976a).

UL cells also fail to demonstrate species specificity. Human UL cells bind rabbit Ig (Carlsson *et al.*, 1971; Larsson and Perlmann, 1972; Perlmann, *et al.*, 1972a; Denk *et al.*, 1974; Hallberg, 1974; MacLennan *et al.*, 1974; Wisløff *et al.*, 1974a; Brier *et al.*, 1975; Revillard *et al.*, 1975). Mouse UL cells have been shown to bind Ig from rabbit (Greenberg *et al.*, 1973a,b, 1975a,b; Nelson *et al.*, 1976), guinea pig (Nelson *et al.*, 1976), and human (Dickler *et al.*, 1976b; Nelson *et al.*, 1976); and those in the rat bind rabbit Ig (Kedar *et al.*, 1974b). Inhibition studies in-

dicate that the various immunoglobulins are bound to the same receptors (Greenberg *et al.*, 1973b, 1975a; Frøland *et al.*, 1974c; Hallberg, 1974; Kedar *et al.*, 1974b).

T lymphocytes are similar to B and UL cells in their lack of species specificity. Direct binding studies have shown that murine T cells bind Ig from rabbit (Yoshida and Andersson, 1972; Fridman and Golstein, 1974; Krammer *et al.*, 1975; Neauport-Sautes *et al.*, 1975; Warner *et al.*, 1975; Arbeit *et al.*, 1976), human (Basten *et al.*, 1975b; Neauport-Sautes *et al.*, 1975; Santana and Turk, 1975; Warner *et al.*, 1975), and goat (Warner *et al.*, 1975). Human T cells bind rabbit Ig (Ferrarini *et al.*, 1975a; Moretta *et al.*, 1975; Revillard *et al.*, 1975). In the case of T cells, inhibition studies again indicate that the same receptors are responsible for binding Ig of various species (Neauport-Sautes *et al.*, 1975; Warner *et al.*, 1975).

While no species specificity has been observed, the possibility of differences in affinity of binding of the various species has not been ruled out. Information on this point might be obtained from comparative dose-response inhibition studies.

E. Lack of Other Requirements for Binding

Studies of the binding of immunoglobulin to B lymphocytes have indicated that a variety of other factors are not required for binding. The fact that binding can take place in the cold (Dickler and Kunkel, 1972; Modabber and Coons, 1972; Hallberg *et al.*, 1973; Möller, 1974; Abbas and Unanue, 1975; Forni and Pernis, 1975; Halloran *et al.*, 1975; Lawrence *et al.*, 1975; Sidman and Unanue, 1975; Krammer and Pernis, 1976b) and in the presence of sodium azide (Basten *et al.*, 1972b; Dickler and Kunkel, 1972; Ramasamy and Lawson, 1975) indicates that the binding process does not require metabolic activity by the B cell. In addition, neither protein in the incubation medium (Dickler and Kunkel, 1972; Revillard *et al.*, 1975) or divalent cations (Basten *et al.*, 1972b; Dickler and Kunkel, 1972; Siegel, 1972; Lawrence *et al.*, 1975; Revillard *et al.*, 1975) are required. Finally, there is no requirement for complement components. Thus, neither the absence of complement nor the presence of cobra venom factor prevented binding (Basten *et al.*, 1972c; Cline *et al.*, 1972; Dickler and Kunkel, 1972; Paraskevas *et al.*, 1972a; Eden *et al.*, 1973). It should be noted that, although the above factors are not required for binding, this does not exclude the possibility that their presence may influence the process. For example, the binding of complement to complexes can interfere with binding to Fc receptors (see Section VII,A).

Binding of immunoglobulin to UL cells does not require the presence of complement (see reviews by MacLennan, 1972; Perlmann *et al.*, 1972b; Frøland and Natvig, 1973; Cerottini and Brunner, 1974). The other fac-

tors have not been directly studied by binding assays to UL cells, which will be necessary since a requirement for any of these factors in ADCC may not reflect Fc receptor binding (see Section II,C,2). However, it might be inferred that binding of Ig to UL cells will not require these factors, since some of the studies which demonstrated that these factors were not needed were performed on human peripheral blood lymphocytes (Dickler and Kunkel, 1972; Hallberg *et al.*, 1973; Lawrence *et al.*, 1975; Revillard *et al.*, 1975) and probably detected binding to UL cells as well as to B cells (see Section III,C).

The binding of Ig to activated T lymphocytes or T-cell lymphomas can take place in the cold (Yoshida and Andersson, 1972; Ramasamy and Munro, 1974; Van Boxel and Rosenstreich, 1974; Krammer *et al.*, 1975, 1976a), whereas binding to T cells from normal tissues is less efficient in the cold (Anderson and Grey, 1974). However, binding to both activated T cells (Van Boxel and Rosenstreich, 1974) and those from normal tissues (Arbeit *et al.*, 1976) can take place in the presence of sodium azide. The presence of complement is not required for binding to either activated T cells (Yoshida and Andersson, 1972; Anderson and Grey, 1974; Van Boxel and Rosenstreich, 1974; Basten *et al.*, 1975b; Krammer *et al.*, 1975) or those from normal tissues (Anderson and Grey, 1974; Basten *et al.*, 1975b; Stout and Herzenberg, 1975a; Arbeit *et al.*, 1976). Divalent cations also do not seem to be required (Arbeit *et al.*, 1976). Again, the possibility is not excluded that these factors, although not required, may nevertheless affect binding.

V. Nature of the Receptors

A. B Lymphocytes

The first indication of the nature of B lymphocyte Fc receptors came from studies that evaluated the ability of B cells which had been treated with various enzymes to bind Ig. No inhibition of binding to B cell Fc receptors was seen following treatment with trypsin (Cline *et al.*, 1972; Eden *et al.*, 1973; Dickler, 1974; Jondal, 1974; Parish and Hayward, 1974a; Theofilopoulos *et al.*, 1974; Schirrmacher and Halloran, 1975; Basten *et al.*, 1976), neuraminidase (Bentwich *et al.*, 1973a; Jondal, 1974; Kedar *et al.*, 1974b; Basten *et al.*, 1976), papain (Jondal, 1974; Kedar *et al.*, 1974b), chymotrypsin, and phospholipase A (Basten *et al.*, 1976). One laboratory (Kedar *et al.*, 1974b) has observed an inhibitory effect of trypsin. However, the possibility of contaminating enzymes was not excluded. In contrast, pronase (Dickler, 1974; Kedar *et al.*, 1974b; Basten *et al.*, 1976) treatment of the B cells inhibited their ability to bind Ig.

This result suggested that the receptor was, at least in part, protein. Two laboratories (Kedar *et al.*, 1974b; Basten *et al.*, 1976) have reported that B cells do not bind Ig as well after treatment with phospholipase C. The possibility that the effect was on the cell membrane, not on the receptor for Ig, was not ruled out. In fact, the latter may be the case since this enzyme also diminished surface Ig (Basten *et al.*, 1976), which does not have a lipid component.

The receptor for immunoglobulin on B cells appears to be distinct from certain other molecules which are expressed predominantly on B lymphocytes. Thus, Fc receptors differ from surface immunoglobulin in that: (1) anti-Ig antibodies (or $F(ab')_2$ fragments thereof) do not inhibit binding of Ig to Fc receptors (Basten *et al.*, 1972c; Dickler and Kunkel, 1972; Dickler and Sachs, 1974; Parish and Hayward, 1974a; Abbas and Unanue, 1975; Forni and Pernis, 1975; Ramasamy and Lawson, 1975; Krammer and Pernis, 1975b). In many of these studies the possibility of cross-reactions between the anti-Ig reagent and the complexes was excluded. While there is some conflicting evidence on this point, this may be due to differences in technique (see Section II,C,1). (2) Surface Ig is susceptible to treatment with trypsin in the human (Preud'homme and Seligmann, 1972; Dickler, 1974) and chymotrypsin in the mouse (Basten *et al.*, 1976), both of which do not affect Fc receptors (see above). (3) Capping of Fc receptors does not lead to redistribution of surface Ig (Abbas and Unanue, 1975; Forni and Pernis, 1975; Basten *et al.*, 1976; Dickler, 1976b). The reverse situation is controversial (see Section VI,A). (4) Some B lymphocytes can be observed that bear Ig but not detectable Fc receptors (Dickler and Kunkel, 1972; Anderson and Grey, 1974; Forni and Pernis, 1975; Ramasamy and Lawson, 1975; Basten *et al.*, 1976; Dickler, 1976b). The reverse situation exists but is more difficult to interpret since such cells may not be B cells. However, in two reports where cells bearing Fc receptors but not detectable Ig were observed, there was some evidence that such cells were B lymphocytes (Dickler *et al.*, 1973; Wernet *et al.*, 1974).

B lymphocyte Fc receptors also appear to be distinct from receptors for complement components: (1) Receptors for C3 are sensitive to trypsin (Eden *et al.*, 1973; Parish and Hayward, 1974a; Theofilopoulos *et al.*, 1974) whereas Fc receptors are not (see above). (2) Complexed Ig does not inhibit binding to complement receptors (Eden *et al.*, 1973; Parish and Hayward, 1974; Theofilopoulos *et al.*, 1974), and complement components do not inhibit binding of complexed Ig (Theofilopoulos *et al.*, 1974). (3) C3 receptors but not Fc receptors decline after stimulation of B cells with mitogens (Möller, 1974). (4) Not all cells that bear Fc receptors bear C3 receptors (Möller, 1974; Horowitz and Lobo, 1975; Ramshaw and Parish, 1976), but it is not clear that such cells are B

lymphocytes. (5) Antibodies with specificity for Ir gene associated (Ia) antigens inhibit binding to Fc receptors but not C3 receptors (Dickler and Sachs, 1974; Krammer and Pernis, 1976a (see also Section VI,B).

Although Fc receptors and Ia antigens appear to be associated (see Section VI,B), there is evidence which suggests that they are distinct molecules: (1) Capping of Fc receptors does not appear to redistribute the majority of Ia antigens (Basten *et al.* 1976; Dickler, 1976b). In addition, a human B cell tissue culture line has been observed to bear "Ia-like" antigens but not detectable Fc receptors (Wernet *et al.*, 1975). Both these lines of evidence do not exclude the possibilities that some Ia antigens but not others are Fc receptors, or that Ia antigens act as Fc receptors only when clustered together. (2) A molecule has been isolated that may be the B cell Fc receptor (Rask *et al.*, 1975; see below). This molecule had a different molecular weight than Ia antigens and an antiserum with specificity for this molecule did not inhibit detection of Ia antigens.

Recently, isolation of molecules that may be B-cell Fc receptors has been reported (Rask *et al.*, 1975; Wernet and Kunkel, 1975). One laboratory (Rask *et al.*, 1975) incubated crude membrane fractions from murine spleen cells in buffer containing EDTA and β-mercaptoethanol. Macromolecules solubilized in this manner were subjected to affinity chromatography on a complexed Ig column. Molecules bound to this column and subsequently eluted were labeled with radioactive iodine and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Three types of polypeptide chains of apparent molecular weight 65,000, 18,000, and 15,000 were distinguished. Experiments utilizing thermolysin digestion suggested that the two smaller components were proteolytic breakdown products of the MW 65,000 protein. Extensive reduction and alkylation did not alter the apparent size of the MW 65,000 molecule, suggesting that it is a single polypeptide chain. Labeling with radioactive sodium borohydride provided evidence for the presence of sialic acid on the three components. Isolated "Fc-receptor" components could be shown to bind again to complexed Ig after desorption from the column. Finally, antisera, and $F(ab')_2$ fragments thereof, raised in rabbits against either the MW 65,000 molecule or the MW 18,000, 15,000 mixture, bound primarily to B lymphocytes and inhibited the binding of Ig to these cells. Thus, these studies provided evidence that the Fc receptors of B cells are MW 65,000 glycoproteins. The possibility was not ruled out that other molecules (? also Fc receptors) bound to the complexed Ig column but were not eluted. In addition, specificity for the Fc portion of the molecule was not demonstrated.

Another laboratory (Wernet and Kunkel, 1975) incubated either serum (centrifuged or not), aggregated IgG, or IgG Fc fragments with the

detergent (NP40)-soluble fraction of a human B cell (Fc receptor positive) tissue culture line which had been labeled with radioactive iodine via the lactoperoxidase method. After precipitation with anti-Ig, the precipitates were analyzed by polyacrylamide gel electrophoresis. A protein peak of MW ~70,000 was observed if the soluble cell fraction was incubated with aggregated IgG, IgG Fc, or serum that had not been centrifuged. This peak was not observed if cells not bearing Fc receptors were analyzed or if centrifuged serum was used. The reasons for this latter result are unclear since complexes would have been present during the precipitation step. In contrast to the possible Fc receptor isolated from murine spleen cells (see above), the protein isolated from human cells was MW 30,000–35,000 after reduction and alkylation. Those studies provide evidence that the Fc receptor of human B cells is a protein of MW ~70,000 with a possible dimeric configuration. However, although binding to Fc was demonstrated, specificity for Fc, i.e., failure of $F(ab')_2$ to bind, was not. In addition, identity between this protein and the cell surface structure which binds Ig (e.g., by the use of a specific antiserum) must still be shown.

B. UL Cells

While there are no reports of the isolation of Fc receptors from UL cells, some information as to the nature of this receptor is available from enzyme studies. Binding of complexed Ig to UL cells was not inhibited if the cells were treated with trypsin (Frøland and Natvig, 1973; Horwitz and Lobo, 1975) or neuraminidase (Frøland and Natvig, 1973), but was inhibited by pronase treatment (Horwitz and Lobo, 1975), suggesting that the receptor is, at least in part, protein. In addition, the phospholipases A and C inhibited binding (Frøland and Natvig, 1973). The possibility that these latter enzymes had an effect on the cell membrane as opposed to the receptor itself was not excluded. The effect of enzymic treatment of cells on ADCC has also been evaluated. Hwever, it is difficult to interpret these studies in terms of Fc receptors, since such alterations can affect cell-mediated lympholysis not involving Fc receptors (Kedar *et al.*, 1974a).

C. T Lymphocytes

T lymphocytes appear to be similar to both B and UL cells in enzyme studies. Thus, T-cell binding of Ig is not inhibited by treatment of the cells with trypsin (Neauport-Sautes *et al.*, 1975; Warner *et al.*, 1975) but is inhibited by pronase (Moretta *et al.*, 1975; Warner *et al.*, 1975). Again, this provides evidence for the T-cell receptors for Ig being proteins. Re-

cently, Fridman and his associates (Fridman and Golstein, 1974; Fridman *et al.*, 1974, 1975; Neauport-Sautes *et al.*, 1975; Guimezanes *et al.*, 1976) have described a soluble factor produced by educated T lymphocytes from rat or mouse which binds to Ig (immunoglobulin-binding factor-IBF) and may be identical to the Fc receptors of these cells. When activated T cells are incubated in medium at 37°C, the supernatant contains a factor that induces hemagglutination of IgG-sensitized erythrocytes and protects the latter from complement-induced hemolysis. Using column fractionation techniques, rat IBF activity was found in fractions migrating with an MW of ~150,000 by gel filtration and eluting at a pH of ~6.3 upon ion-exchange chromatography. The possibility was not excluded that these fractions contained activity because IBF was already bound to Ig. The evidence that IBF and T cell Fc receptors may be identical is as follows: (1) IBF binds to IgG but not to IgG $F(ab')_2$, IgM, or BSA. This specificity pattern is similar to T-cell binding of Ig (see Section IV,A and C). (2) Incubation of activated T lymphocytes at 37°C results in production of IBF and a decrease in detectable Fc receptors on these cells. Further, both events are abrogated by inhibitors of protein synthesis. (3) Complexed Ig columns retain both IBF producing cells and cells which bear Fc receptors. Demonstration of identity between IBF and Fc receptors will require either chemical characterization and/or use of specific antisera.

VI. Relationship of Fc Receptors to Certain Other Lymphocyte Surface Molecules

Interactions or associations between Fc receptors and other molecules on the lymphocyte surface might have important functional implications. Such relationships have been described for both the antigen receptor of B cells, immunoglobulin, and for antigens determined by genes associated with immune response genes (*Ir* genes) which map in the major histocompatibility complex of the mouse (Ia antigens; for reviews, see Shreffler and David, 1975; Sachs, 1976).

A. Lymphocyte Membrane Ig

Several lines of evidence have indicated that the surface Ig and Fc receptors of B lymphocytes are distinct moieties, and early studies failed to provide evidence for a relationship between the two (see Section V,A). Thus, incubation of B cells with anti-Ig did not inhibit binding to their Fc receptors, indicating that the two entities were probably not in close proximity on the cell surface. Further, the movement of surface Ig to one pole of the cell observed at 37°C following cross-linking with anti-Ig, a

process referred to as capping (Taylor *et al.*, 1971), was reported not to induce similar movement, or cocapping, of Fc receptors in the human (Preud'homme and Seligmann, 1972; Dickler, 1974), rat (Parish and Hayward, 1974a), or mouse (Ramasamy and Lawson, 1975).

In contrast to these studies, capping of surface Ig has recently been reported by several laboratories to induce cocapping of Fc receptors in both human (Forni and Pernis, 1975) and mouse (Abbas and Unanue, 1975; Forni and Pernis, 1975; Unanue and Abbas, 1975; Basten *et al.*, 1976; Krammer and Pernis, 1976b). In many cases the cocapping occurred even when $F(ab')_2$ anti-Ig was used (Abbas and Unanue, 1975; Forni and Pernis, 1975; Unanue and Abbas, 1975; Krammer and Pernis, 1976b) indicating that the Fc portion of the anti-Ig molecule was not required. However, in one instance (Abbas and Unanue, 1975) the degree of cocapping was less. Moreover, the interaction between surface Ig and Fc receptors could occur when a monovalent ligand was attached to the Ig. Thus, if Fab anti-Ig was bound to the surface Ig and then the Fc receptors were capped using complexes, the surface Ig would cocap, whereas it would not if the Fab was not attached (Unanue and Abbas, 1975). The human situation is particularly interesting since anti-μ caused cocapping but anti-δ did not (Forni and Pernis, 1975). This would indicate that the interaction takes place with one class of surface Ig but not the other. However, the experiments in humans are more difficult to evaluate than those in the mouse since clear polar capping often did not occur (Forni and Pernis, 1975).

It is not readily apparent why some laboratories observed cocapping and others did not. One possibility is suggested by recent studies which indicate that when Fc receptors are capped and then immediately reevaluated with a second label, all Fc receptors are not present in the cap (Basten *et al.*, 1976; Dickler, 1976b). If capping of Ig similarly cocaps most but not all Fc receptors, then some techniques might have detected the Fc receptors outside the cap whereas others might not. While further experiments will be needed, the weight of evidence at present favors the concept that redistribution of surface Ig affects the distribution of many Fc receptors.

The nature of the interaction between Fc receptors and surface Ig when a ligand is bound to the latter is unknown. Since the redistributed Fc receptors are still detectable, the surface Ig is presumably interacting with a site on Fc receptors different from that which interacts with exogenous Ig. However, the possibility that the exogenous Ig successfully competes for the same site cannot be excluded. Moreover, surface Ig is primarily IgM and IgD while the class of exogenous Ig which binds is primarily IgG (see Section IV,C). This suggests that relevant differences

exist between the IgM (and ? IgD) molecules on the membrane and those in the serum, or that sites on the Fc receptor are available to Ig in the membrane which are not available to exogenous Ig. Whatever the nature of the interaction, it will be of interest to determine whether antigen bound to surface Ig can also effect an interaction between the latter and Fc receptors. It has been proposed that such an interaction plays a role in B lymphocyte triggering and regulation (Forni and Pernis, 1975).

B. Ia Antigens

It appears that antigens determined by the *I* (immune response) region of the *H-2* complex in the mouse (Ia antigens) and antigens in other species that have some of the characteristics of Ia antigens but are determined by genes not yet genetically mapped (Ia-like antigens) are associated on the B lymphocyte surface with Fc receptors. This conclusion is based on the observation that B lymphocytes pretreated with antibodies against Ia or Ia-like antigens are markedly inhibited in their ability to bind Ig. This has been shown in the mouse (Dickler and Sachs, 1974; Basten *et al.*, 1975a, 1976. Dickler *et al.*, 1975a; Halloran *et al.*, 1975; Schirrmacher and Halloran, 1975; Schirrmacher *et al.*, 1975; Krammer and Pernis, 1976a), human (Arbeit *et al.*, 1975; Wernet *et al.*, 1975), and rat (Soulillou *et al.*, 1976). In addition, B lymphocyte binding of Ig can inhibit binding of anti-Ia antibodies, although only partially (Basten *et al.*, 1976).

The inhibition of binding of Ig to B cells appears to be specific for antibodies directed against Ia or Ia-like antigens in that: (1) $F(ab')_2$ or Fab fragments of antibodies directed against Ia or Ia-like antigens also inhibit binding to Fc receptors (Dickler and Sachs, 1974; Basten *et al.*, 1975a; Dickler *et al.*, 1975a; Halloran *et al.*, 1975; Schirrmacher and Halloran, 1975; Schirrmacher *et al.*, 1975a; Wernet *et al.*, 1975; Kramer and Pernis, 1976a; Soulillou *et al.*, 1976). This indicates that the blocking was not due to the Fc portions of the anti-Ia antibodies. (2) Antibodies directed against other cell surface antigens did not inhibit binding of Ig to B cell Fc receptors. These antigens include surface Ig (see Section V,A), antigens determined by the *K* and *D* regions of the murine *H-2* complex (Dickler and Sachs, 1974; Dickler *et al.*, 1975a; Krammer and Pernis, 1976a), the classical "serologically defined" antigens of the major histocompatibility complex of man (Arbeit *et al.*, 1975) and rat (Soulillou *et al.*, 1976), and β-2 microglobulin (Rask *et al.*, 1975; Revillard *et al.*, 1975). One laboratory has seen partial (25–50%) inhibition of Fc receptors by anti-β_2 microglobulin (Grey *et al.*, 1975). In addition, other ligands, such as the mitogens phytohemagglutinen and concanavalin A, do not inhibit Fc receptors (Ryan *et al.*, 1975). In contrast to these findings, one group of

workers (Halloran *et al.*, 1975; Schirrmacher and Halloran, 1975; Schirrmacher *et al.*, 1975a) found that any antibody, and $F(ab')_2$ fragments thereof, bound to the B lymphocyte surface (including anti-Ig) inhibited binding to Fc receptors. The reasons for the discrepancy between these results and those of others (see above) are unclear. One possible explanation is that the affinity of binding of their complexes (chicken erythrocytes sensitized with antibody) may be lower than other forms of complexed Ig, such that any antibody bound to the cell surface can produce enough of an alteration to interfere with binding (see also Section II,C). (3) The same anti-Ia antibodies that inhibit binding to Fc receptors do not affect other cell surface molecules, such as Ig (Dickler and Sachs, 1974; Unanue *et al.*, 1974; Hauptfeld *et al.*, 1975) or C3 receptors (Dickler and Sachs, 1974; Krammer and Pernis, 1976a).

Thus, the weight of evidence from studies on the inhibition of binding of Ig to B-cell Fc receptors by anti-Ia antibodies indicates a specific association between the two moieties on the cell surface. The nature of this association is unclear, although there is some evidence which suggests that the two are not identical (see Section V,A). The observations that antibodies against individual *I* subregions produce inhibition equivalent to antibodies against the whole *I* region (Dickler *et al.*, 1975a; Krammer and Pernis, 1976a), and that reciprocal parental H-2 antisera could each produce maximal inhibition of binding to Fc receptors when tested on F_1 B cells (Dickler and Sachs, 1974; Krammer and Pernis, 1976a), suggest that individual Ia antigens are grouped into patches or clusters on the B cell surface. The simplest concept of the Fc receptor-Ia antigen association is that Fc receptors are located within or adjacent to clusters of Ia antigens. With the exception of one study, which must still be confirmed (Rask *et al.*, 1975), the possibility that such a patch of Ia antigens itself can function as an Fc receptor has not been excluded. It has been hypothesized that the association of Fc receptors and Ia antigens has functional significance (Sachs and Dickler, 1975).

The binding of Ig to the Fc receptors of B cells is also inhibited by antibodies against antigens determined by a single non-H-2 locus (Dickler *et al.*, 1975b). The genetic mapping of this locus as well as the nature of these antigens remains to be determined. The possibilities exist that such antigens are Ia-like but associated with non-H-2 Ir genes, or that such antigens might themselves be Fc receptors.

UL cell Fc receptor binding of Ig is not inhibited by anti-Ia antibodies. Thus, $F(ab')_2$ anti-Ia antibodies failed to inhibit ADCC (Schirrmacher and Halloran, 1975; Schirrmacher *et al.*, 1975a). While whole anti-Ia antibodies did inhibit ADCC, this was probably due to the creation of third-party target cells, a phenomenon recently described (Halloran *et al.*,

1974; Schirrmacher *et al.*, 1975b). In addition, it has been recently shown in direct binding studies that UL cell Fc receptors are not inhibited by anti-Ia antibodies (Dickler *et al.*, 1976b). These studies provide evidence that the Fc receptors of UL cells are different from those of B cells.

The subpopulation of T cells from thymus which bear Fc receptors are partially inhibited (50–70%) in their ability to bind Ig by anti-Ia antibodies (Dickler *et al.*, 1976a). Again, this inhibition is specific in that $F(ab')_2$ fragments can also produce this inhibition, and other antibodies that bind to T lymphocytes (anti-Thy 1, anti-H-2 K region, and anti-H-2 D region) did not produce inhibition. Similar results have been obtained with T cells from spleen (Stout *et al.*, 1976b). Thus, an association between Ia antigens and Fc receptors on some T cells appears to exist. As in the case of B lymphocytes, the nature of this association and its functional significance remain to be elucidated.

VII. Possible Functions of Fc Receptors

With the exception of specific antigen receptors, the function(s) of the various molecules found on the lymphocyte surface, including Fc receptors, is largely unknown. Nevertheless, it seems useful to consider the possible functions of lmyphocyte receptors for immunoglobulin.

A. Theoretical Limitations

While it is clear that *in vitro* Ig binds to lymphocytes, *in vivo* there are additional factors that may limit such interactions. Thus, other cells that bind Ig (see Section I) may do so preferentially, thus preempting binding to lymphocytes. For example, some effects of antibody on the immune response (see Section VII,C) appear to be mediated by macrophages alone (Abrahams *et al.*, 1973). Conversely, it seems possible that other cells, particularly macrophages, may actually present complexed Ig to lymphocytes. A second possible limitation is the complement system, as there is evidence that binding of complement to complexed Ig prevents uptake of the latter by Fc receptors. It has been observed that the binding of complexed Ig to cells which had Fc receptors but not C3 receptors was inhibited by the presence of complement (Eden *et al.*, 1973; Theofilopoulos *et al.*, 1974). Therefore, it seems likely that complexed Ig would interact with lymphocyte Fc receptors *in vivo* only under certain circumstances. (1) Complexed Ig of certain classes or subclasses which do not fix complement might bind to Fc receptors. In mouse, IgG1 binds to lymphocytes (see Section IV,C) but does not fix complement (see review by Spiegelberg, 1974). However, in man the same classes and subclasses that bind to lymphocytes also fix complement with the possible exception of IgG4 (see Section IV,C and Spiegel-

berg, 1974). (2) Ig complexes of certain sizes might have a higher affinity for lymphocytes than for complement. No information is available on this point. (3) Fc receptors might be able to interact with a site on the Fc portion of Ig that has been fixed to the cell via complement. This possibility is not excluded by the inhibition studies mentioned above. (4) A complex formed on the surface of the lymphocyte by the sequential binding of antigen (to the specific antigen receptor) followed by antibody binding to the antigen (presumably to a second site) might interact with an Fc receptor before complement could become fixed. This last possibility is particularly attractive since it would provide a mechanism for antigen-specific interaction between immune complexes and lymphocytes. In any case, these theoretical restrictions on the binding of complexed Ig to lymphocytes *in vivo* should be kept in mind when considering possible functions of Fc receptors.

B. Antibody-Dependent Cellular Cytotoxicity (ADCC)

Evidence strongly supports the conclusion that the process of *in vitro* target cell damage by nonimmune lymphocytes which is dependent on antibody (ADCC) requires lymphocyte Fc receptors (see Section II,C,2). The relevance of this function of Fc receptors is, in turn, dependent on the demonstration that ADCC plays a significant role in either physiologic or pathologic situations *in vivo*. Unfortunately, no definitive evidence on this point exists. Criteria for establishing a direct relationship between the *in vitro* model and the *in vivo* situation with regard to the effector cell have been put forward (Cerottini and Brunner, 1974). In addition, the requirement for Fc receptors *in vivo* must also be established (see Section VII,C) and possible effects by complement excluded (see Section VII,A). Finally, since ADCC is primarily mediated by UL cells (see Section II,C,2), it seems apparent that this process may not be a significant function of the Fc receptors of B or T lymphocytes.

C. Regulation of the Immune Response

It is clear that antibody can have a variety of effects on the immune response. This fact together with the observation that some of these effects appear to require an intact Fc portion of the immunoglobulin molecule suggest that Fc receptors may play a role in the regulation of the immune response. Experiments consistent with this possibility include (1) Antibody, or molecules that may be antibody, can interfere with the function of cytotoxic lymphocytes and/or enhance tumor growth (Irvin *et al.*, 1967; Klein, 1971; Sjögren *et al.*, 1971; Baldwin

et al., 1972; Cruse *et al.*, 1972; Wright *et al.*, 1973). In some experiments, class and subclass specificity have been shown (Irvin *et al.*, 1967) and in others the Fc portion of the molecule appeared to be important (Cruse *et al.*, 1972). (2) Antibody can both inhibit and augment the humoral immune response (reviewed by Uhr and Möller, 1968). Different effects can be mediated by different classes (Henry and Jerne, 1968; Dennert, 1971) and subclasses (Murgita and Vas, 1972) of antibody, and in a number of studies, an intact Fc portion of the Ig molecule was important (Sinclair, 1969; Chan and Sinclair, 1971; Kappler *et al.*, 1973; Lees and Sinclair, 1973; Wason and Fitch, 1973; Gordon and Murgita, 1975). With T-independent antigens, the Fc portion of immunoglobulin may not be required (Diener and Feldmann, 1972). (3) Antibody has been shown to augment (Playfair *et al.*, 1974; Janeway *et al.*, 1975) but not suppress (Kappler *et al.*, 1971) the helper effect of T lymphocytes and has been implicated in suppressor T-cell activity (Gershon *et al.*, 1974; Gorczynski, 1974; Gorczynski *et al.*, 1975). Moreover, different subclasses of guinea pig anti-idiotypic antibody can augment the activity of either murine suppressor (IgG2) or helper (IgG1) T lymphocytes (Eichmann, 1975; Eichman and Rajewsky, 1975). (4) Immunoglobulin can affect the rate of DNA synthesis of lymphocytes. Thus, antigen–antibody complexes have been shown to increase DNA synthesis in the lymphocytes of humans (Möller, 1969) although not those of mice (Möller and Coutinho, 1975). Insolubilized or large complexes have been shown to inhibit DNA synthesis in response to B cell mitogens and concanavalin A (Ryan *et al.*, 1975; Stout and Herzenberg, 1975b) whereas soluble complexes do not (Möller and Coutinho, 1975; Ryan *et al.*, 1975). In addition, antibody suppression of the DNA response to viral antigen and phytohemagglutinin was class specific (Lee and Sigel, 1974), and inhibition of DNA synthesis in response to tumor cells was inhibited by molecules which may be antibody (Hattler and Soehllen, 1974).

Although there are numerous data consistent with the concept that Fc receptors play a role in regulation of the immune response, there is no definitive evidence. Several criteria should be fulfilled in order to establish a direct relationship between lymphocyte Fc receptors and immunoregulation: (1) identification of the lymphocyte population involved through the use of cell surface markers, depletion and add-back experiments; (2) demonstration that this cell population bears Fc receptors; (3) demonstration that the regulatory molecules (immunoglobulin or other lymphocyte factors) bind to the Fc receptors; (4) absence of the effect after specific removal or inhibition of the Fc receptors (regeneration of Fc receptors must also be excluded.); (5) demonstration that the presence of complement or of other cells bearing Fc receptors does not

interfere with the effect (see also Section VII,A). It is obvious that some of these requirements are still difficult to achieve, but they may become feasible in the near future in view of the recent improvements in cell separation techniques and methods for detection of Fc receptors. Nevertheless, it seems worthwhile to consider some aspects of the manner in which Fc receptors might regulate the immune response.

The nature of the lymphocyte subpopulations whose function might be affected by Fc receptor interactions remains unknown. It has been suggested that Fc receptor function may affect B lymphocytes (Sinclair and Chan, 1971; Kerbel and Davies, 1974; Forni and Pernis, 1975) T lymphocytes with either helper of suppressor activity (Gorczynski *et al.*, 1974; Kerbel and Davies, 1974; Playfair, 1974) or interactions between different cell populations or their products (Hoffman *et al.*, 1974; Sachs and Dickler, 1975). It is important to note that although all these cell populations (e.g., helper T lymphocytes) have not been demonstrated to bear Fc receptors (see Section III,B), this does not exclude the possible presence of Fc receptors, either present in low numbers, or that have specificities which may not have been adequately evaluated (see Section IV,C).

Antibody can have opposite effects (augmentation vs inhibition) on the immune response (see above). There are several possible ways in which this could occur. Thus, antibody of different classes or subclasses might have opposing effects on a single lymphocyte either via a single receptor or via different receptors. Alternatively there could be a single mechanism for all lymphocyte subpopulations, but the different effects could be generated according to the function of a particular subpopulation. Moreover, the outcome of the interaction with Fc receptors may in turn be part of a more complex mechanism involving other cell surface molecules. This possibility is suggested by the association or interaction of Fc receptors with Ia antigens and surface Ig (see Section VI). Finally, the possibility should be considered that Fc receptors themselves can act as soluble mediators in the immune response. This latter concept is suggested by experiments in which T-cell supernatants containing immunoglobulin binding factor, which may be Fc receptors (see Section V,C), inhibit the humoral immune response (Gisler and Fridman, 1975). It is hoped that further experimentation will clarify the still hypothetical role of Fc receptors in immunoregulation.

D. Other Possible Functions

The possibility that Fc receptors on lymphocytes play a role in antigen localization was suggested by experiments which showed that lymphocytes could transport aggregated Ig into the spleen (Brown *et al.*, 1970)

and that antibody-coated B cells could carry antigen into the spleen (Miller *et al.*, 1971). This particular function of Fc receptors seems to be particularly susceptible to the limitations on interaction between Ig and lymphocyte Fc receptors present in the *in vivo* environment (see Section VII,A). In fact, there is evidence that mice depleted of complement by cobra venom factor can no longer localize aggregated Ig in the spleen (Papamichail *et al.*, 1975). However, chickens treated similarly could still do so (White *et al.*, 1975). The reasons for this discrepancy are unclear and will require clarification, since such experiments may exclude a role for Fc receptors in antigen localization.

It has been suggested that Fc receptors on B lymphocytes function as receptors for the endogenous membrane Ig, which serves as antigen receptors for these cells (Ramasamy *et al.*, 1974). This proposal (termed proreceptor function) was based on studies of mutant myeloma cell lines. In general, those lines that secreted Ig and bore surface Ig did not have detectable Fc receptors, and, conversely, those that did not secrete or bear Ig did have Fc receptors. Thus, it was possible that the Fc receptors of such cells were occupied by endogenous Ig produced by the cell. This possible function of Fc receptors is not excluded by the fact that, on B cells, most membrane Ig is either IgM or IgD, classes that, in the majority of studies, do not bind to B-cell Fc receptors (see Section IV,C), since membrane Ig and secreted Ig may differ in their ability to bind. It is also not excluded by studies which demonstrate that capping of Fc receptors does not alter the distribution of surface Ig (see Section V,A), since those occupied by membrane Ig would not be expected to be available to the complexes used for capping. Definitive studies on this point will require the ability to quantitate Fc receptors before and after selective removal of surface Ig.

VIII. Conclusions

Considerable information has been generated concerning the interaction of immunoglobulin and lymphocytes. A variety of methods have been developed to assess this interaction, and these techniques differ considerably in their ability to detect binding of Ig to lymphocytes both as to the total percentage of lymphocytes and the nature of the lymphocyte subpopulations involved. While differences in sensitivity may in part account for these discrepant observations, other factors, such as variations in the avidity of Ig for different subpopulations, nature of the Ig utilized, or aspects as yet undefined, are probably involved.

It is clear that the vast majority of B lymphocytes, a substantial minority of T lymphocytes, and many undefined lymphocyte-like (UL)

cells bind Ig. The B lymphocytes that bind Ig include the precursors of antibody-forming cells. Among the UL cells with this capacity are those which mediate antibody dependent cellular cytotoxicity (ADCC). While many activated T lymphocytes bind Ig the functional category of Ig binding T cells is controversial at present.

Certain aspects of the specificity of binding of Ig to lymphocytes appear to be established. The site on the immunoglobulin molecule recognized by the lymphocyte receptor is located on the Fc piece (hence the term Fc receptors) and the integrity of this site is dependent on intact disulfide bonds. A number of factors (metabolic activity, divalent cations, complement) are not required for binding, but may, under certain circumstances, affect binding (e.g., complement fixed to complexed Ig can inhibit binding to Fc receptors). Little, if any species specificity has been demonstrated. In contrast, other aspects of specificity have not been fully delineated owing to technical limitations. Fc receptors of all lymphocyte subpopulations bind IgG but there is conflicting evidence as to the relative avidity of the different subclasses. In addition, there are preliminary data which suggest the presence on some T lymphocytes of a distinct receptor for IgM. Although binding of the other Ig classes has not been demonstrated, these studies have not excluded the possibility of distinct receptors for these classes. Interactions of lymphocyte membrane Ig and other lymphocyte factors with Fc receptors has not been studied. Neither the affinity of Fc receptors of different lymphocyte subpopulations for monomeric Ig and Ig complexes of defined size nor the number of receptors per cell has been conclusively established.

Information as to the chemical composition and configuration of Fc receptors is neither extensive nor conclusive. The available data indicate that the receptors which bind to IgG are distinct from other known surface molecules and are, at least in part, protein. There are conflicting data as to molecular weight and whether such receptors are single chains or are dimeric. These conflicts may reflect the possibility that Fc receptors of different subpopulations, despite having similar specificities, are distinct molecules.

Fc receptors appear to lie adjacent to antigens determined by genes associated with immune response genes (Ia antigens) on the surfaces of B lymphocytes, and some T lymphocytes, but not UL cells. In addition, Fc receptors appear to interact with surface Ig when ligands are bound to the latter molecules. These relationships with other lymphocyte surface molecules may have functional significance, but this remains to be determined.

With regard to function, it is clear that Fc receptors are required for the process of ADCC. However, the physiologic significance of this

mechanism for cellular damage remains an open question. While there are data consistent with the suggestions that Fc receptors may be involved in immunoregulation, antigen localization, and as prereceptors for surface Ig, direct evidence to support these hypotheses is still lacking.

Much has been learned about lymphocyte receptors for Ig, but it is obvious that many important questions remain. With the advent of more sensitive methods of detection of these receptors, improved characterization of reagents, and better cell separation techniques, answers to these questions should be forthcoming in the near future.

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Ionizing Radiation and the Immune Response[1,2]

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[2] Abbreviations used: ALS, antilymphocyte serum; ATC, activated thymus cells; ATx, adult thymectomy; ATxXBM, adult thymectomy—lethal irradiation—restoration with syngeneic bone marrow; B cells, bone marrow-derived lymphocytes; BGG, bovine γ-globulin; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CML, cell-mediated lympholysis; CNS, central nervous system; Con A, concanavalin A; DNP, 2,4-dinitrophenyl; DTH, delayed-type hypersensitivity; EAE, experimental allergic encephalitis; ECI, extracorporeal irradiation; FLA, *Salmonella* flagella; FγG, fowl immunoglobulin; GVH, graft versus host; [^{3}H]TdR, tritiated thymidine; Ig, immunoglobulin; LET, linear energy transfer; LPS, lipopolysaccharide; MIF, migration-inhibition factor; MLC, mixed lymphocyte culture; *n*, extrapolation number or the intercept of the exponential portion of a survival curve extrapolated back to 0; NNP, 4-hydroxy-3,5-dinitrophenylacetic acid; nTx, neonatal thymectomy; OVA, ovalbumin; PCA, passive cutaneous anaphylaxis; PFC, plaque-forming cells; 7 S PFC, indirect plaque-forming cells; PHA, phytohemagglutinin; r, rads; R, roentgens; RRBC, rat erythrocytes; SEM, scanning electron microscopy; SRBC, sheep erythrocytes; T cells, thymus-derived lymphocytes; TDL, thoracic duct lymphocytes.

I. Introduction

The effects of radiation on the immune response have been extensively investigated during the past several decades, and this work forms the basis of a number of excellent monographs (Taliaferro *et al.*, 1964; Micklem and Loutit, 1966). However, many of the attendant experiments were performed and interpreted prior to the development of concepts that underlie current thinking with respect to cellular immunology, including the definition of subpopulations of lymphocytes and their known cooperation in immune responses. Hence, many of the dose response estimates based on these results may primarily reflect injury of the most radiosensitive lymphocyte subpopulation involved in the particular immune response. One of the primary purposes of this presentation, therefore, is to review some of the earlier experiments that deal with radiation and the immune response and to reexamine the data in light of the recent advances in cellular immunology. Particular attention will be devoted to subpopulations of cells, particularly lymphocytes, and possible differences in the radiosensitivity of these subpopulations.

A second major purpose of this review is to illustrate ways in which radiation may be employed to dissect some of the individual cellular components of the immune response, and in this manner also obtain data on the actual radiosensitivity of the various subpopulations of lymphocytes.

This review will consider the interrelationships between radiation and the various components of the immune response from three perspectives: (1) The effect of irradiation on normal lymphoid tissues and on isolated lymphocytes. (2) The effect of irradiation on antibody production, transplantation immunity, and other forms of cellular immunity. (3) The

effect of irradiation upon tolerance with specific references to putative autoimmune consequences after radiation-induced alterations in normal immunological homeostasis.

Before considering these data in detail, it may be of value to cite briefly our general understanding of lymphocyte heterogeneity and select relevant radiobiological concepts.

A. Lymphocyte Heterogeneity

In recent years, a wide variety of approaches has become available to characterize and define distinct populations of lymphocytes. These include biophysical and functional methods and characterization of antigenic and cell surface receptor components. Many of these aspects have been reviewed elsewhere (Greaves *et al.*, 1974; Warner, 1974; Warner and McKenzie, 1976; *Transplantation Reviews*, 1971, 1973, 1975) and will not be detailed herein. However, for the purpose of the present review, it is important to stress the increasing recognition of lymphocyte heterogeneity. Many of the subpopulations of lymphocytes that have been defined are morphologically similar, if not identical, and studies on the radiosensitivity of lymphocytes must consider the precise character of the cellular population under investigation. For example, the use of lymphoid cell populations by radiobiologists concerned with biochemical aspects of radiation injury could lead to erroneous conclusions if the particular cell population studied was heterogeneous and contained a subpopulation of radioresistant cells.

The original classifiers of immunity into cell-mediated and humor responses found a cellular basis from studies with birds, in which separate thymus-dependent and bursa-dependent immune responses were described (Warner *et al.*, 1962; Cooper *et al.*, 1966). However, since the recognition of cellular cooperation between T and B cells (Claman and Chaperon, 1969; Miller and Mitchell, 1969a; Davies, 1969), it has become increasingly evident that in any one type of immune response several different cell types may participate. Although the definition of these subpopulations is still under active investigation, a provisional listing of many of the involved cell types can be given (Table I). The B-cell series includes various distinct stages both pre- and post-antigen activation and B memory cells, in addition to possibly heterogeneous precursors for cell lines eventually secreting different Ig classes. The T-cell series similarly contains various stages in a given differentiation sequence, but may also contain several distinct subpopulations that involve different differentiation pathways. Some of the categories listed in Table I may eventually be shown to represent different functional activities of the same cell type, although in several cases it is now clear from surface alloantigen studies

TABLE I
CELLULAR ELEMENTS INVOLVED IN IMMUNE RESPONSES

B-cell lineage	T-cell lineage	Nonlymphoid lineage
Pre-B cells	Pre-T cells	Macrophages and monocytes (?several subpopulations)
Immunocompetent B cells (antibody-forming precursor cells)	Immunocompetent T cells (precursors of DTH effector cells, and of cytotoxic lymphocytes)	K cells
Several types for different Ig class expression (?)	Helper-T cells	N cells
Activated B cells	Memory T cells	Granulocytes
Memory B cells	Cytotoxic lymphocytes	Eosinophils
Plasma cells	DTH effector cells	Mast cells
	Suppressor T cells	

that distinctly different cell types are involved (Cantor and Boyse, 1975). The induction and expression of an immune response can also involve functional activity of nonlymphoid cells, and these cell types must also be considered in any study of the radiosensitivity of an immune response.

B. General Radiobiology

Much of our understanding of the mechanisms of radiation effects in biological systems, particularly in man, has been derived from experiments utilizing cells exposed *in vitro* and maintained in tissue culture. Such cells can be examined for: (1) loss of viability; (2) alterations in biophysical structure; (3) loss of functional capabilities; (4) biochemical changes; and (5) evidence of injury to subcellular components.

Loss of the ability to divide in an unlimited fashion is referred to as reproductive death and is observed with all dividing cell types. Some lymphocytes are unusual in that small amounts of radiation can also cause immediate cell death, termed interphase death, which occurs prior to any cell division. Despite intensive investigation, the mechanisms involved in interphase death are not clearly known, although various biochemical changes have been observed (see Section II,A).

In common with other biological systems, radiation-induced injury of the various cell types involved in the immune response, occurs via a variety of mechanisms, both direct and indirect. The relative importance of each mechanism depends upon the response under consideration and a variety of other factors, the most important of which are the three listed below.

1. Physical factors—the character of the radiation (e.g., neutron, γ, or X-irradiation); the total amount administered; the time frame during which radiation is given as a single dose (dose rate); whether the radiation is given in several separate irradiations (fractionated dose); and whether the radiation is presented *in vivo* from an external source (e.g., X-ray machine) or from an internal radioisotopic emitter, or *in vitro* to a cell suspension.

2. Chemical factors—chemical factors may either exert a protective effect or potentiate the effects of radiation. An important substance in this regard is molecular oxygen, which potentiates many of the effects of radiation, perhaps by enhancing the formation of free radicals. In contrast many sulfhydryl reagents tend to depress the biological effects of radiation.

3. Biological factors—such as the timing of the irradiation with respect to the stage of cell cycle and the repair of sublethal injury. In immunity, the time relationship between irradiation and antigen challenge can also be a critical factor in determining the subsequent effect.

In order to compare the radiation sensitivity of different cell types quantitatively, dose-response curves must be obtained that relate a given functional or other parameter to the radiation dose absorbed.

In describing such dose-response curves for radiation-induced injury several pieces of information are required:

a. The reciprocal of the slope of the exponential portion of the curve of the dose to effect or, more commonly, the dose that results in 37% survival of the given measured parameter. This value is referred to as the D_{37} or, less commonly, the mean lethal dose or the D_0. It is important to emphasize that these calculations are made on the linear portion of the curve when the surviving fraction is plotted on a logarithmic scale against dose on a linear scale.

b. The intercept of the exponential portion of the curve extrapolated to zero dose. The number is the extrapolation number, n, and reflects, in part, the breadth of the nonlinear portion of the dose-response curve at low dose levels. This portion of the curve is also known as the shoulder, and is thought to reflect the degree of radiation repair that has occurred at the low doses. The higher the n value, the greater the degree of repair.

II. Lymphocytes, Lymphoid Tissues, and Radiation

A. General

Organized lymphoid tissues and individual recirculating small lymphocytes are exquisitely radiosensitive. Thus, significant depression of the

absolute lymphocyte count is noted after whole-body exposure to doses as low as 25 r (Suter, 1947) and 100 R reduces the peripheral lymphocyte count to 25% of normal values within 4 hours of exposure in rats 4–7 months old (Schrek, 1961). In addition to reduced numbers, circulating small lymphocytes demonstrate numerous morphological abnormalities after irradiation. However, even after exposure to large doses, varying numbers of lymphocytes remain, and in the following sections the possible significance and nature of radioresistant lymphocytes will be considered.

Experiments conducted *in vitro* also document the marked radiosensitivity of the small lymphocyte. Normal lymphocytes obtained from human peripheral blood show statistically significant alterations in motility and morphology following exposure to 2 and 5 R (Stefani and Schrek, 1964). It should be noted, however, that the extreme sensitivity of lymphocytes to these small doses was observed only with cells irradiated *in vitro* and maintained in tissue culture. There is some question, therefore, as to whether the results are confounded by the suboptimal environment afforded by the tissue culture setting. Despite these qualifications, the lymphocyte is clearly one of the most radiosensitive of mammalian cells.

Lymphocytes are also unusual with respect to the timing of radiation-induced cell death. Whereas most cells exposed to moderate doses of radiation (50–1000 r) do not die until the first or second postirradiation mitosis (mitotic death), small lymphocytes can be killed without entering the mitotic cycle (interphase death). Thus, within 1 hour (Cronkite *et al.*, 1974) or less (Bari and Sorenson, 1964; Jordan, 1967) after exposure to an $LD_{50(30)}$ dose of X-rays, necrosis of the various lymphoid tissues is evident, and the number of recirculating small lymphocytes is decreased. Phagocytosis of necrotic lymphocytes and nuclear debris also proceeds rapidly and reaches a maximum approximately 3 hours after irradiation (Jordan, 1967). Within a few days, the lymphoid tissues are almost, but not completely, devoid of lymphocytes. It was noted by Trowell (1952) that significant numbers of intact, presumably viable lymphocytes, remained scattered diffusely throughout the villi of the small intestine even after large doses of radiation. In this connection, it is of interest to note that antigen-activated recirculating lymphocytes appear to home preferentially to the gastrointestinal tract (Sprent and Miller, 1972), and such cells are much more resistant to the effects of ionizing radiation than are nonactivated small lymphocytes (see below).

Radiation-induced depletion of lymphocytes in the various lymphoreticular tissues is roughly dose-dependent up to the beginning of the lethal range (Cronkite *et al.*, 1974) provided that the administration of the dose is not protracted. Such depletion is near maximal within the lethal range. The time required for the physical restoration of the in-

volved tissues is also proportionate to the dose. The regeneration of lymphatic tissues usually involves the reappearance of the parenchymal elements in the same order as that noted in ontogenic development; that is, collections of cortical lymphocytes appear first and are followed by germinal center formation. The presence of an intact thymus is required for restoration of immune reactivity in sublethally irradiated mice (Miller, 1962; Globerson *et al.*, 1962; Miller *et al.*, 1963). Restoration is more rapid after local irradiation of lymph nodes and spleen than after whole-body exposure (Benninghoff *et al.*, 1969). In the former situation, restoration is apparently accelerated by the normal traffic of nonirradiated recirculating small lymphocytes, and perhaps stem cells, through the involved tissue.

When comparable doses are delivered over prolonged intervals, less pronounced effects are apparent. Thus, in two studies (Courtenay, 1963; Gengozian, 1964) on transplantation of foreign bone marrow, dose rates of 1–4 r per minute were much less effective in immune depression than dose rates of 29–54 r per minute, although the same cumulative dose was given. Dose rates in the range of 1.1–1.8 r for 8 hours per day elicited only moderate morphological changes in lymphatic tissue, and it often took several months to produce discernible changes. As will be discussed subsequently, these dose rate-related differences are thought to relate to repair of radiation-induced injury that occurs during prolonged or fractionated exposures.

As discussed later in relation to immunological function, at least some types of T lymphocytes appear to function even after high doses of radiation. Similar observations of radioresistant lymphocytes have been made in various histological and related studies. Thus, 5–10% of T cells (compared to 0% of B cells) survive in tissue culture after exposure to doses as high as 1000 r (Anderson *et al.*, 1974). In addition, a small number of thoracic duct lymphocytes (TDL) can be collected from mice 4 days after exposure to 800 r of whole-body irradiation (Sprent *et al.*, 1974) and 5 days after comparable exposure of rats to 520 r (Lamberg and Schwartz, 1971). These cells are predominantly T cells (Sprent *et al.*, 1974), but, although viable, are unable to proliferate when exposed to antigen *in vivo* (Sprent *et al.*, 1974) or *in vitro* (Lamberg and Schwartz, 1971). These data suggest the existence of a radioresistant subpopulation of T cells which are able to survive interphase death despite exposure to large amounts of radiation but, when stimulated to proliferate, appear to die soon after entering mitosis, presumably as a consequence of severe chromosomal damage.

Antigen- and mitogen-activated T cells are less sensitive to radiation-induced interphase death than are nonactivated cells (Conard, 1969;

Rickinson and Ilbery, 1971; Sprent *et al.*, 1974; Nichols *et al.*, 1975). The mechanisms responsible for this protective influence have not been defined, but one suggestion is that the heightened metabolic activity associated with such stimulation serves to activate repair enzymes. It is important to note, however, that no data exist to indicate that metabolically active lymphocytes are protected from radiation-induced mitotic death. To the contrary, PHA-stimulated lymphocytes, although able to survive after exposure to doses of radiation that kill their nonstimulated counterparts, demonstrate a markedly restricted capacity to proliferate (Conard, 1969; Rickinson and Ilbery, 1971).

Even within populations of relatively radioresistant mitogen-responsive T lymphocytes, further subpopulations may be defined. The differential responsiveness of T cells to phytohemagglutinin (PHA) and concanavalin A (Con A) has been employed to define two subpopulations of T cells (Stobo and Paul, 1973; Stobo *et al.*, 1973), originally termed T_1 and T_2 (Greaves *et al.*, 1974). T_2 cells are preferentially responsive to Con A, and T_1 cells equally responsive to PHA and Con A. These subpopulations differ in relative radiosensitivity, as shown in Table II. The dose-related shift in the PHA-Con A ratio indicates that the T_1 population is more radiosensitive than the T_2 population.

TABLE II

PHYTOHEMAGGLUTIN (PHA):CONCANAVALIN A (CON A) STIMULATORY RATIOS OF RESIDUAL SPLEEN CELLS AFTER VARYING DOSES OF WHOLE-BODY IRRADIATION[a]

Expt. No.	Radiation dose	Δ Cpm		PHA:Con A	θ Positive cells (%)
		PHA	Con A		
I[b]	0 r	45,595	183,086	0.25	39
	5 r	54,890	155,825	0.35	31
	50 r	53,255	187,637	0.28	23
	500 r	5,572	62,273	0.09	16
II[c]	0 R	69,133	100,342	0.69	38
	250 R	27,837	51,768	0.64	40
	300 R	10,011	29,543	0.34	35
	350 R	5,085	23,814	0.21	38

[a] Spleen cells obtained from CBA (Expt. I) or BALB/c (Expt. II) mice irradiated 24 hours (Expt. I) or 48 hours (Expt. II) prior to sacrifice were cultured with medium or with PHA or Con A. Reactivity was measured by the incorporation of [^{3}H]TdR, and the results are expressed as the difference between stimulated and nonstimulated cultures (Δ cpm).

[b] After R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results (1976).

[c] After Stobo and Paul (1973).

Direct studies attempting to assess the relative radiosensitivities of T lymphocytes and B lymphocytes have primarily concentrated on assessing residual functional activities, and have led to the general conclusion that B cells are more radiosensitive than T cells. However, a variety of other approaches have been made to compare T and B cell radiosensitivity, and include the following observations.

At a variety of doses, irradiated B cells (nu/nu TDL) survive considerably less well than irradiated T cells (CBA TDL depleted of B cells) when placed in tissue culture and followed for viable cell counts (Anderson *et al.*, 1974). Similarly, after whole-body irradiation, a much greater reduction in numbers of splenic B cells than T cells was observed when cell suspensions were prepared and examined by immunofluorescence for residual B and T cells (Nossal and Pike, 1973; M. C. Raff, unpublished observations, 1974, cited in Greaves *et al.*, 1974; Kataoka and Sado, 1975). As will be discussed elsewhere, direct histological examination supports these observations in that B cell-dependent areas of spleen and mesenteric lymph nodes are depleted more extensively than T cell-dependent areas (Keuning *et al.*, 1963; Durkin and Thorbecke, 1972; R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished observations, 1976). Few attempts have been made to assess directly radiobiological parameters in this regard, principally because of the problems of cell heterogeneity. However, one report has defined a D_0 of 200 R for splenic B_γ cells (Kataoka and Sado, 1975). This value is rather high in relation to other studies on the radiosensitivity of precursors of antibody or Ig-forming cells (see Section III) which generally yield D_0 values in the range of 60–120 r. Whether this represents a greater radioresistance of B_γ cells as compared to B_μ cells, perhaps analogous to the increased radioresistance of antigenically activated T cells, remains to be determined, although functional studies (see Section III) do not support this conclusion.

After whole-body irradiation with 800 r, a proportionately greater number of T cells than B cells are mobilizable via thoracic duct cannulation (Table III). Although viable, these radioresistant T cells are unable to proliferate in response to alloantigens on transfer to F_1 hybrids (Sprent *et al.*, 1974). In a related study on cell traffic, radiation-induced inhibition of the primary and secondary migration of B cells to spleen and lymph nodes was noted at lower doses in comparison with T cells (Anderson *et al.*, 1974).

Studies with indirect immunofluorescence on the radiosensitivity of splenic T cells (Kataoka and Sado, 1975) have clearly shown that two distinct subpopulations are present, with a minority population of approximately 8% being extremely radioresistant. The majority population had a

TABLE III

PROPORTIONS OF T AND B LYMPHOCYTES IN THORACIC DUCT LYMPHOCYTES (TDL) FROM CBA MICE GIVEN 800 r WHOLE-BODY RADIATION 4 DAYS PRIOR TO CANNULATION[a]

Mice cannulated	Number of mice per group	Number of TDL collected during initial 12 hours of drainage ($\times 10^{-6}$)	Percent T cells[b]	Percent B cells[c]
Normal CBA	5	75.4 ± 3.4	74	24
Irradiated CBA	16	0.9 ± 0.1	96	1

[a] After Sprent *et al.* (1974).

[b] T cells determined by cytotoxicity with anti-θ serum.

[c] B cells determined by incubating cells *in vitro* with rhodamine-labeled polyvalent rabbit anti-mouse Ig antibody.

D_0 value of 195 R. Further considerations on the possible functional activities of the radioresistant population appear in Section III,C.

With the recent introduction of sophisticated technological approaches to quantitate assessment of cell morphology, evidence has been obtained to confirm that B lymphocytes show more acute and rapid evidence of radiation damage than do T lymphocytes. Computer-assisted cytometric analysis of murine lymphocytes has revealed the existence of various descriptor parameters that can differentiate T and B lymphocytes (Olson *et al.*, 1974). Using this system, radiation-induced abnormalities of the nuclei of T and B cells can be seen immediately after exposure. These changes are especially pronounced with B cells and include redistribution of the nuclear chromatin, which becomes more homogeneous. T cells, on the other hand, show less pronounced alterations at low dose levels but marked clumping of the nuclear chromatin at 2000 r. Figure 1 summarizes these observations and shows the distribution of optical density values in digitized form obtained from the automated scanning of Feulgen-stained T and B cells exposed to several radiation doses. Each histogram depicts the relative frequency of occurrence of arbitrarily selected optical density intervals (Anderson *et al.*, 1975). By utilizing transmission electron microscopy, rearrangement of the nuclear chromatin has also been noted 30–60 minutes after irradiation (Smith *et al.*, 1967).

The general conclusion that might be reached from many of these above-mentioned studies is that B lymphocytes are exquisitely radiosensitive relative to T lymphocytes, particularly with respect to interphase death. However, some caution should be introduced at this stage in regard to possible B-cell heterogeneity. We have emphasized that T-lymphocyte subpopulations probably differ in relative radiosensitivity.

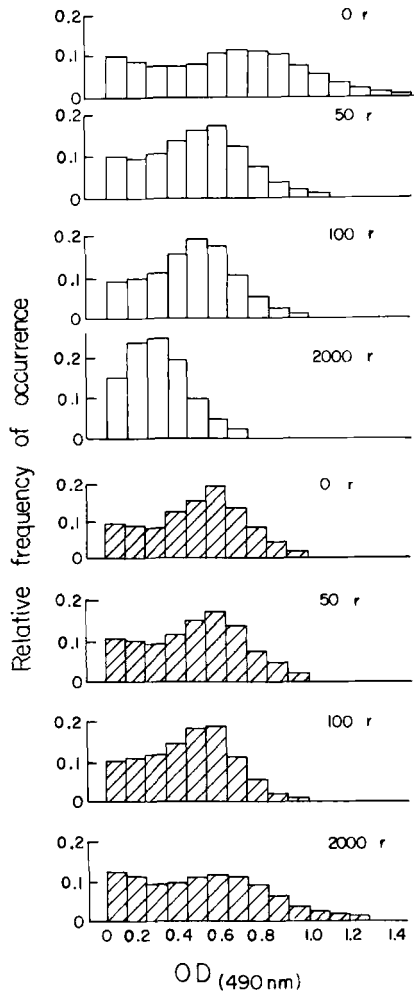


FIG. 1. Distribution of optical density values as a function of dose for irradiated T (▨) and B (□) cells. (After Anderson *et al.*, 1975.)

This may also be true for B lymphocytes, particularly with respect to activated cells. Drewinko *et al.* (1972) have studied the effect of radiation on a long-term culture of human immunoglobulin (Ig)-producing lymphoid cells by the colony-forming method and found that these cells were not extremely radiosensitive. Similarly, in a comparative study of the effects of irradiation upon human cultured T and B lymphoid cell lines, a higher percentage inhibition of [^{3}H]TdR incorporation and a greater reduction in cell numbers was observed for a T-cell line than for B cells

(Han *et al.*, 1974). At a dose of 100 r, a significant reduction in the number of T cells occurred without any significant change noted for the B cells. These studies would seem to indicate that at least some types of B cells are not exquisitely sensitive to radiation-induced interphase death, whereas the T cells used in this experiment are comparatively quite radiosensitive. Since it is well recognized that mature B cells, i.e., plasma cells, are extremely radioresistant (see Section III), a change in radiosensitivity clearly occurs with B-cell maturation. We have also observed, using the *in vitro* agar cloning technique, that murine T lymphomas are quite radiosensitive whereas murine plasmacytoma cell lines are usually more resistant (D_{37} values, respectively, of approximately 70 r and 120 r) (N. L. Warner, R. E. Anderson, and A. W. Harris, unpublished results, 1975). It may be that the particular human B cell lines used by Han *et al.* (1974), represent a relatively late stage of B-cell differentation that has progressed toward the plasma cell stage, resulting, for some unknown biochemical reason, in a relative insensitivity to radiation-induced interphase death.

Biochemical consequences of the irradiation of lymphocytes have been studied in some detail (Okada, 1969; Altman and Gerber, 1970). Most, if not all, of the alterations described to date appear to be secondary phenomena occurring subsequent to some, as yet undefined, primary event. Suspected causes include: (1) interruption of the normal metabolic pathways that provide energy to the cell (Bacq and Alexander, 1961); (2) activation of ATPase-like enzymes that subvert normal metabolism; (3) injury to the plasma membrane; (4) interference with the biosynthesis of nucleic acids; and (5) chromosome breakage. Inhibition or delay of DNA synthesis are generally acknowledged to represent critical consequences of radiation injury of all dividing cells including lymphocytes. However, based upon presently available evidence, it is difficult to see how such injury could account for interphase death and, in particular, for the apparent differences in the radiosensitivity of subpopulations of lymphocytes. At the moment, it is tempting to relate the remarkable radiosensitivity of the lymphocyte, and especially the phenomenon of interphase cell death, to as yet undefined characteristics of the plasma membrane, characteristics that are known to differ appreciably among immunologically distinct subpopulations (see Section II).

As noted previously, interpretation of the data from comparative studies of irradiated and control lymphocytes may be confounded by different proportions of various lymphocyte subpopulations, each with its own D_{37}. This problem is not confined to immunological studies but relates to the interpretation of biochemical data as well. For example, the basal adenylate cyclase activity of lymphocytes recovered from 800 R-treated

mice was similar to that of untreated controls. However, the ability of the cyclase of the irradiated lymphocytes to respond to stimulation by epinephrine was greatly diminished (Kemp and Duquesnoy, 1975). This might suggest that the "more mature" radioresistant lymphocytes are lacking in functional catecholamine receptors. This may well reflect that in the original untreated population, there exist at least two subpopulations—one very radiosensitive and possessing such receptors, and the other radioresistant and without such receptors.

In common with other cell types, anoxia protects lymphocytes from radiation injury (Vos, 1967). Utilizing a modified graft-versus-host (GVH) reaction, Vos (1967) documented a D_0 of 230 R for lymph node cells from anoxic mice in contrast to 85 R for cells from normal donors (see Fig. 2) which yields an oxygen enhancement ratio of 2.7. Employing a similar experimental approach, Blackett (1965) obtained values of 75 and 195 r, respectively, for spleen cells from normal and anoxic rats for an oxygen enhancement ratio of 2.6.

Vos (1967) also showed less loss of GVH activity when the lymph node cells were exposed to 2 doses of radiation separated by 1–12 hours as compared to the same dose administered in a single exposure. These split-dose experiments imply that repair of radiation-induced injury took place during the interval between exposures. However, the results may be confounded by the subsequent observation that the magnitude of the

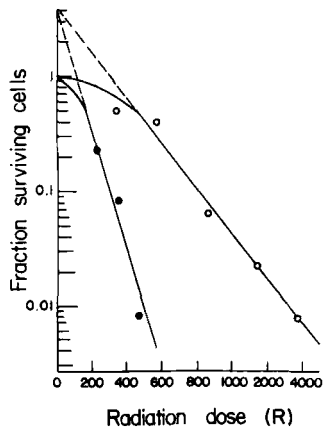


FIG. 2. Radiosensivity of lymph node cells under normal (●——●) and anoxic (○——○) conditions. Lymph node suspensions obtained from 10-week-old CBA male mice and irradiated *in vitro*. Lymph node cells were considered anoxic 10 minutes after decapitation of the mice. Recipients were lethally irradiated 10–12-week-old (CBA × C57BL)F_1 males. Each experimental result represents data from at least 80 mice. (After Vos, 1967.)

GVH reactions was modulated by subpopulations of T cells (Asofsky *et al.*, 1971) and the suggestion that these subpopulations might differ in relative radiosensitivity (see Section IV), although Vos obtained comparable results with two other cell populations (peripheral blood lymphocytes and spleen cells). Thymus cells could not be evaluated in this system for technical reasons. Utilizing a much different experimental approach, Jackson and co-workers (1969; Jackson and Christensen, 1972) concluded that thymocytes irradiated *in vivo* or *in vitro* are not able to repair the component of radiation injury that leads to interphase death.

Looking at DNA repair in a more specific fashion, lymphoma cells grown in tissue culture efficiently repair radiation-induced breaks of DNA strands (Lett *et al.*, 1967; Ormerod and Stevens, 1971). Normal human lymphocytes are stimulated by radiation to incorporate [^{3}H]TdR into their DNA. Such stimulation occurs in the absence of ligands or related substances. This process, which persists in the presence of concentrations of hydroxyurea known to inhibit normal DNA synthesis, involves about 90% of irradiated peripheral blood lymphocytes (Evans and Norman, 1968). This unscheduled DNA synthesis is believed to reflect repair (Painter and Cleaver, 1967; Prempree and Merz, 1969; Cleaver, 1969) and occurs after exposure to both ultraviolet and ionizing radiation (Evans and Norman, 1968). In the presence of fresh media, cells that had been stimulated by radiation to incorporate [^{3}H]TdR could respond to PHA by transformation and cell division (Evans and Norman, 1968).

On the basis of the above comments, it might be inferred that lymphocytes possess a fully operative enzymic mechanism with which to repair radiation-damaged DNA. To the contrary, very low levels of DNA polymerase and ligase, two of the enzymes implicated in repair (Regan *et al.*, 1971; Town *et al.*, 1971), are found in peripheral blood lymphocytes (Loeb *et al.*, 1968; Lindahl and Edelman, 1968; Pedrini *et al.*, 1971). Thymocytes also contain low amounts of ligase and polymerase activity but can repair single-stranded DNA breaks produced by X-irradiation (Scaife, 1972). However, in contrast with peripheral blood lymphocytes, thymocytes exhibited a limited capacity to carry out unscheduled DNA synthesis after ultraviolet exposure, a maneuver known to produce base damage without strand breakage.

Alterations in repair mechanisms may also explain some of the observed differences in radiosensitivity of normal versus transformed cells. As previously noted, PHA-stimulated cells are more radioresistant than nonstimulated cells to interphase death. It has been reported that PHA-stimulated peripheral blood lymphocytes contain markedly elevated DNA polymerase and ligase activity (Loeb *et al.*, 1968; Lindahl and Edelman, 1968; Pedrini *et al.*, 1971), and that despite an approximate 2-fold greater

increase in frequency of single-strand breaks of DNA per rad, the rate of rejoining was about 10 times that of nontransformed lymphocytes (Hashimoto *et al.*, 1975). It appears that most G_0 cells, although unable to synthesize DNA, are able to repair single-strand scissions of cellular DNA and that this repair mechanism is augmented on lymphocyte activation.

Consideration of repair mechanisms in lymphocytes is confused by uncertainties as to the mechanism(s) involved in interphase death. For example, the observation that PHA-stimulated lymphocytes, known to be moderately radioresistant, contain increased ligase and polymerase activity is relevant to interphase cell death only if DNA damage is involved in this form of lymphocyte injury. As indicated above, inferential evidence suggests that such is not the case. For reasons such as this, it is probably important not to draw too many conclusions from experiments directed toward repair of interphase death until more is known with respect to the mechanisms involved in this form of injury.

B. Thymus

Heineke (1903) and Rudberg (1907) first described the effects of X-irradiation on the thymus and noted the marked radiosensitivity of the lymphoid component. Thus, morphological and possible functional alterations are present after exposure to doses as small as 5 r (R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976). The marked sensitivity of thymic lymphocytes is in contrast to the radioresistance of the reticulum cell component which shows few, if any, morphological alterations after exposures up to 5000 r (Trowell, 1961).

In the mouse, physical restoration of the thymus is complex, and the relative contribution of the several involved cell types appears to be dose-dependent. Thus, at low doses of whole-body radiation (about 150–200 r), recovery begins on day 5–7 and normal thymus weight is approximated by day 12. After intermediate doses (400–500 R), however, the kinetics of thymic regeneration follow a biphasic pattern as measured by changes in weight and mitotic index (Takada *et al.*, 1969). As shown in Fig. 3, an acute decrease in thymic weight occurs during the initial 24 hours after irradiation, followed by a gradual recovery between days 5 and 12 to near control values. This period of early recovery is followed by a second, apparently spontaneous, decrease in thymic weight. A second recovery phase then begins about 20 days after irradiation. Takada *et al.* (1969) also found that, although the second drop in weight could be prevented by shielding the hind limbs or by the intravenous injection of syngeneic bone marrow administered at the time of irradiation, the initial drop in thymic weight was unaffected by these maneuvers (Fig. 4).

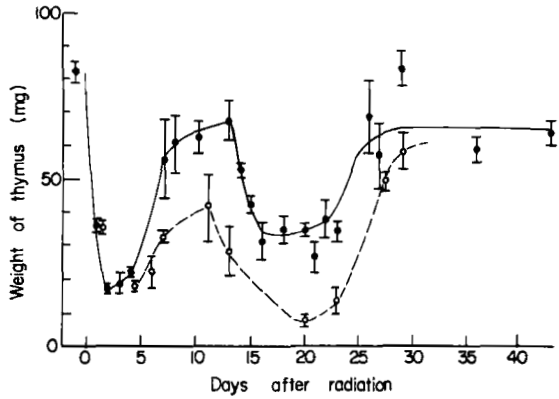


FIG. 3. Regeneration of thymic weight as a function of time after whole-body exposure to 400 (●——●) or 500 (○---○) R. Results are expressed as mean values ± SE per experimental group, each group consisting of 10 male 6-week-old ICR/Ha mice. (After Takada *et al.*, 1969.)

In a careful cytogenetic analysis of this latter aspect, Takada and Takada (1973) have found that the delay in bone marrow-induced thymic restoration is due, at least in part, to the need for the transferred bone marrow cells first to proliferate in host bone marrow or splenic sites prior to their move to host thymus. This observation suggests that the acquisi-

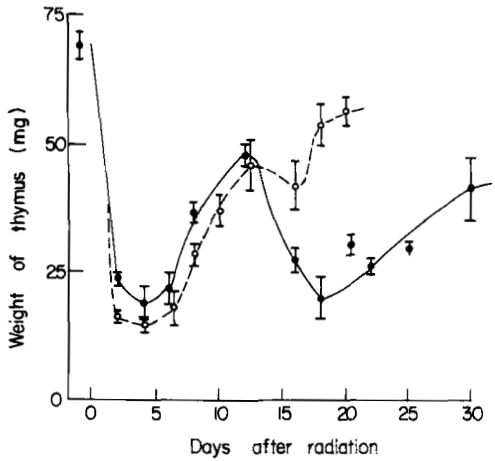


FIG. 4. Influence of shielding of hind legs on regeneration of thymus after whole-body exposure to 400 R. Results are expressed as mean values ± SE per experimental group, each group consisting of 10 male 10–12-week-old (C57BL/6 × DBA/2) F_1 mice. Exposure with (○---○) and without (●——●) leg shielding. (After Takada *et al.*, 1969.)

tion of certain membrane properties are required before the cells can settle and repopulate the thymus.

Utilizing other techniques, Kadish and Basch (1975) have supported the hypothesis of Takada *et al.* (1969) that the initial recovery phase is due to the proliferation of a relatively radioresistant subpopulation of intrathymic T-cell precursors. Parenthetically, however, it might be noted that no evidence of such a population was previously found in thymus graft studies in which irradiation of the graft with 2000 R prior to syngeneic transplantation totally failed to result in evidence of proliferating cells of donor type (Dukor *et al.*, 1965). The relative radiosensitivity of normal thymocytes and the cells responsible for the initial recovery phase as determined by Kadish and Bash (1975) is shown in Fig. 5. The radiosensitivity of normal thymocytes was evaluated by measuring [^{3}H]TdR incorporation by the gland 24 hours after irradiation. The radiosensitivity of the subpopulation responsible for the initial recovery phase was assessed by determining [^{3}H]TdR incorporation by the gland 8 days after irradiation. Since there appears to be no influx of stem cells during this period of time, the latter data presumably reflect the radiosensitivity of endogenous thymocytes responsible for the initial phase of regeneration.

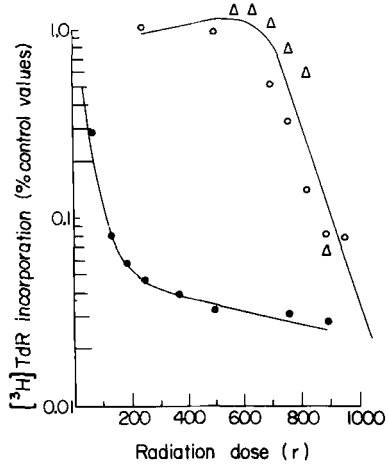


FIG. 5. Radiosensivity of normal thymocytes (●) and the subpopulation of cells responsible for thymic regeneration (○, △—triangles and circles represent separate experiments). Radiosensitivity of thymocytes was measured directly by determining [^{3}H]TdR incorporation by gland 24 hours after irradiation. Radiosensitivity of subpopulation responsible for regeneration measured indirectly by determining [^{3}H]TdR incorporation 8 days after irradiation. Ordinate: Ratio of [^{3}H]TdR incorporation in irradiated thymus to [^{3}H]TdR incorporation in nonirradiated thymus. (After Kadish and Basch, 1975.)

The broad plateau of the curve reflects the radioresistance of this subpopulation. In this context, note also the presence of a subpopulation of relatively radioresistant cells among the normal thymocytes assayed at 24 hours. Presumably this radioresistant subpopulation represents the progenitors of the actively dividing cells responsible for the initial phase of regeneration. In this connection, several observers have noted an apparent discrepancy in the radiosensitivity of cortical and medullary thymocytes, the latter appearing to be less sensitive (Murray, 1948; Trowell, 1961; Blomgren and Andersson, 1971). Thus, the medulla may house the radioresistant subpopulation responsible for the initial phase of regeneration. Two observations may relate to this suggestion. First, thymocytes appear to migrate from the cortex to the medulla as a part of their maturational process. Thus, medullary thymocytes are H-2 rich, cortisone resistant, and smaller than cortical thymocytes. Second, among tissues requiring continuous rapid renewal (bone marrow, mucosa of the gastrointestinal tract, urinary bladder, and skin), radiosensitivity is inversely proportional to the degree of maturation. Therefore, the differential radiosensitivity of the cortex and medulla of the thymus may relate primarily to differences in the degree of maturation of the lymphocytes from these two sites.

Takada *et al.* (1969) suggested that the second decrease in thymus weight (peak between days 18 and 22) is due to an inadequate influx of stem cells from the radiation-depleted bone marrow presumably accompanied by the release of a significant number of mature T cells derived from the residual radioresistant cell population. After small doses (50–200 r), this second phase is not noted presumably because injury to the bone marrow is of insufficient magnitude to interfere with stem cell traffic to the thymus. These authors attribute the second regenerative phase to regeneration of the bone marrow and release of adequate numbers of stem cells to repopulate the thymus. Two to three weeks are required to complete this process (Micklem and Loutit, 1966; Micklem *et al.*, 1966).

A similar conclusion that radiation-resistant T-cell precursors exist resulted from *in vitro* studies on T-cell differentiation (Kubai and Auerbach, 1973). Thymus rudiments obtained from 12–13-day mouse embryos were irradiated with doses up to 1000 r and, although the total growth of the rudiments was markedly inhibited, differentiation of lymphoid cells still occurred. Whether these thymus lymphocytes derived by differentiation from a radiation resistant intrathymic precursor cell are different functionally from those derived from circulating bone marrow-derived stem cells remains to be determined.

Several facets of these experiments involving thymus irradiation have

particular relevance to immunologists. In the first place, the presence of a radioresistant precursor T-cell subpopulation that proliferates during the initial phase of thymic regeneration makes it evident that lethally irradiated mice are not totally thymoprivic (Kadish and Basch, 1975). Furthermore, if the cell line derived from radioresistant endogenous precursor cells is functionally distinct from thymic lymphocytes derived from bone marrow stem cells, this former line may have unique immunological potential, and characterization of the possible helper or suppressor properties of these cells would be of considerable interest. Second, experiments designed to isolate the "pre-T cell" of bone marrow origin from the thymuses of lethally irradiated mice will be confounded by unknown numbers of residual radioresistant thymocytes. Finally, the above experiments join an increasing reservoir of evidence that subpopulations of thymocytes may be defined on the basis of differences in radiosensitivity.

Further evidence to support the concept that subpopulations of thymocytes differ in radiosensitivity comes from several types of experiments. Utilizing the cellular transfer of Ig production by the use of allotype congenic mouse strains, Warner and Anderson (1975) have shown both augmentation and suppression of Ig transfer by added thymus cells syngeneic to the host. Irradiation (400 r) of the thymus uniformly resulted in augmented transfer often in excess of that associated with nonirradiated thymus.

In an investigation of the effect of cortisone treatment on the mouse thymus, Blomgren and Andersson (1971) demonstrated that the cortisone-resistant population was also relatively radioresistant. Thus, after 2 days of cortisone treatment, when the total cell numbers in the thymus had dropped to 3% of control values, the immunological reactivity (as measured by GVH activity and capacity to restore a humoral antibody response in ATxXBM mice) was not reduced, and the proportion of radioresistant cells was 50% as compared with 4% in untreated control mice.

Konda *et al.* (1973) utilized a different experimental approach to the same general problem. These investigators divided the thymus into subpopulations on the basis of membrane antigen representation and average buoyant density. The major cell population, which comprised 80–90% of thymic lymphocytes, was of high relative density in BSA gradients and was rich in θ, TL, G_{ix}, Ly-A, Ly-B, and Ly-C but contained little, if any, H-2. This population, which did not respond to PHA and did not exhibit primary alloantigen recognition (Konda *et al.*, 1972), was markedly reduced 10 days after whole-body exposure to 880 r and was also cortisone sensitive. In contrast, the minor subpopulation ($<10\%$) was characterized by: low relative density, low thymus antigen content, and large amounts of H-2. The minor population also contained most or all of the

properties of primary alloantigen recognition and PHA reactivity present in whole thymus (Konda *et al.*, 1972, 1973).

The foregoing has focused upon the acute (e.g., initial 30 days) effects of whole-body exposure upon the thymus of the mouse. Based upon the evaluation of persons accidentally and therapeutically irradiated, the majority of human thymocytes also appear to be extremely radiosensitive (Liebow *et al.*, 1949; Andrews, 1962). In addition, localized radiation of the human thymus predisposes to the development of a variety of untoward late effects. Recognition of many of these late effects has evolved from an ongoing evaluation by Hempelmann and co-workers of individuals irradiated in infancy for an allegedly enlarged thymus as prophylaxis against the development of "status thymolymphaticus." The tumorigenic effects of such exposure are well known (Hempelmann *et al.*, 1967). It is not clear, however, to what extent these radiation-induced neoplasms reflect the general carcinogenic effects of irradiation as opposed to the possible role of the thymus in immune surveillance (see Section VI). Of greater interest in the context of this report, however, is the increased frequency of asthma and a variety of uncommon diseases with immunological features (collagen diseases, sarcoid, regional enteritis, thyroiditis, etc.) among the irradiated individuals in comparison with their untreated siblings (Hempelmann and Grossman, 1974). Immunological evaluation of the irradiated group for delayed-type hypersensitivity (DTH) reactivity demonstrated no abnormalities (Bakemeier and Hempelmann, 1965), although there was a persistent depression of T cells (L. H. Hempelmann, personal communication, 1975). Similar T-cell depression was noted among patients who received unilateral parasternal irradiation after surgery for carcinoma of the breast (Stjernswärd *et al.*, 1972). One-half of the thymus was included in the exposure field and received an estimated 2000–2400 r. The ability of the lymphocytes of 15 of the 19 patients evaluated to respond to PHA was also reduced significantly. Following a similar line of thought, Hamburger *et al.* (1962) administered 1500 r to the thymus of 24 patients prior to the transplantation of renal allografts, but no subsequent graft prolongation was observed.

C. Spleen

In contrast with the thymus, only about 60–70% of the volume of the normal mouse spleen is composed of lymphocytes. Many of the other elements (macrophages, plasma cells, hematopoietic cells of varying degrees of differentiation) are less radiosensitive than lymphocytes. Therefore, the loss in spleen mass that follows whole-body exposure is less pronounced than that associated with the thymus. Splenic weight as a

function of magnitude of dose and time after exposure is shown in Fig. 6. The sequence of histological and ultrastructural changes of the spleen that follow whole-body exposure has been studied by Jordan (1967) and is summarized in Table IV. At low dose levels, necrosis and cell loss are more pronounced in B-dependent areas than in T-dependent areas (R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976). As noted previously, evaluation of irradiated spleen cell suspensions with specific antisera suggests that A and B cells differ with respect to the kinetics of cell death. This is shown in Fig. 7, where the relative numbers of residual T and B cells are plotted for the initial 30 days after whole-body exposure to 5, 50, and 500 r. In general, B-cell loss occurs earlier and is more pronounced than T-cell loss. At higher dose levels, these differences are even more pronounced. Thus, one day after whole-body exposure to 800 r, the number of B cells fell by a factor of over 200 while the total cellularity decreased by only a factor of 10 (Nossal and Pike, 1973).

One of the most radiosensitive structural components in spleen and lymph nodes is the germinal center (Keuning *et al.*, 1963; Jordan, 1967; Durkin and Thorbecke, 1972). After irradiation, widespread necrosis commences in the germinal centers and remains most marked in the nonthymic dependent follicular (B) areas of the outer lymph node cortex and spleen, with marginal zone cells disappearing within 12–24 hours.

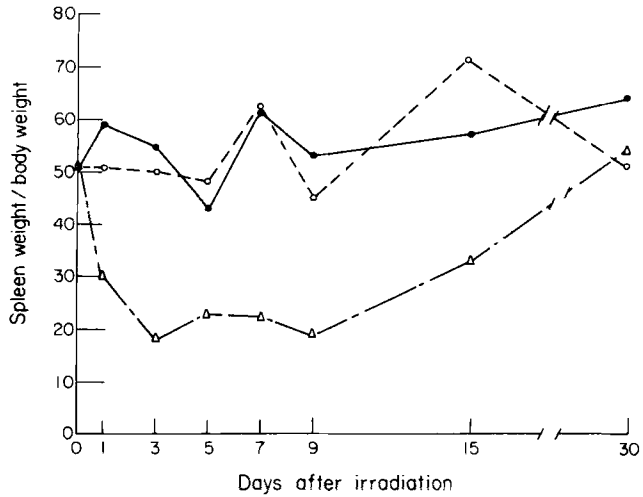


FIG. 6. Spleen weight as a function of dose and time after irradiation. Results are expressed as percent of control values, 9–10 female 10-week-old CBA mice per experimental group. ●, 5 r; ○, 50 r; △, 500 r. (After R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1967.)

TABLE IV
ULTRASTRUCTURAL ALTERATIONS IN MOUSE SPLEEN AS A FUNCTION OF TIME AFTER 800 r WHOLE-BODY IRRADIATION (SEE JORDAN, 1967)

Time after irradiation	Cell type	Observations
45 Min	Lymphocytes	Marked shrinkage of nucleus and cytoplasm of small lymphocytes with concomitant increase in electron density; clumping of nuclear chromatin of large lymphocytes; focal disruption of nuclear membrane
	Reticular macrophages	Phagocytosis of intact but shrunken lymphocytes; phagocytosis of nuclear debris
	Plasma cells, fibroblasts, megakaryocytes	No change
3 Hr	Lymphocytes	Marked necrosis
	Reticular macrophages	Maximum phagocytosis of condensed intact lymphocytes and cell fragments
	Plasma cells, fibroblasts, megakaryocytes	—
8 Hr	Lymphocytes	Moderate necrosis
	Reticular macrophages	Moderate to marked increase in phagocytized debris including intact lymphocytes
	Plasma cells, fibroblasts, macrophages	Moderate dilatation of endoplasmic reticulum and slight disorganization of rough ER of plasma cells
24 Hr	Lymphocytes	Moderate necrosis
	Reticular macrophages	Moderate increase in phagocytic debris
	Plasma cells, fibroblasts, megakaryocytes	Similar to findings at 8 hr
3 Days	Lymphocytes	Markedly reduced in absolute numbers
	Reticular macrophages	Slight increase in phagocytized material
	Plasma cells, fibroblasts, megakaryocytes	Reduced numbers of megakaryocytes
6 Days	Lymphocytes	Markedly reduced in absolute numbers; increased numbers of immature cells with large multiple nucleoli and moderate amounts of cytoplasm with prominent mitochondria
	Reticular macrophages	Large amounts of partially digested phagocytized material
	Plasma cells, fibroblasts, megakaryocytes	Increased numbers of plasmoblasts

The recovery of germinal centers is also quite rapid. The relative effects of radiation given at various times after antigen on germinal center activity and immunological memory are considered in Section III.

Similar observations of a relatively greater radiosensitivity of the B-cell predominant areas were made on rabbit appendix (Blythman and Waks-

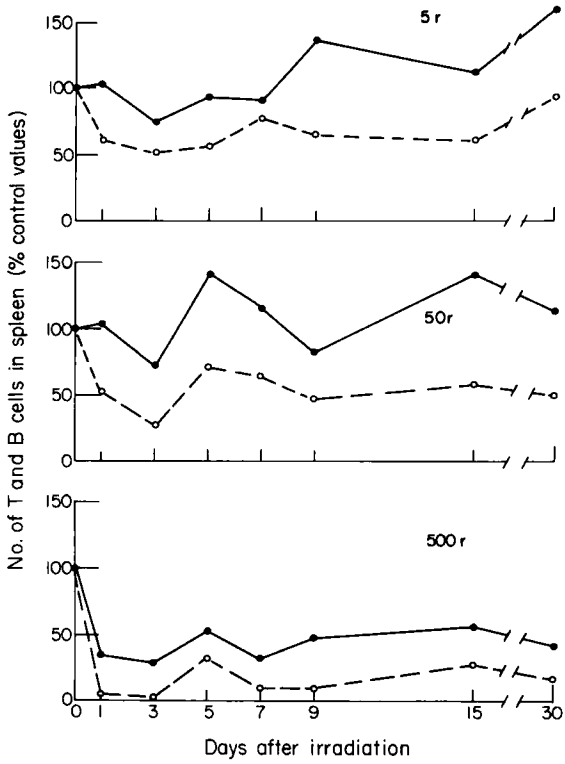


FIG. 7. Relative numbers of residual T (●) and B (○) cells as a function of time in CBA spleen after whole-body exposure to 0, 5, 50, and 500 r. Results are expressed as a percentage of control data and represent arithmetic means from 9–10 female 10-week-old mice per data point with cell counts and immunofluorescence performed in triplicate. (After R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976.)

man, 1973). After 450 R, the thymus-dependent areas showed considerably less depletion and necrosis than the dome and follicular lymphoid areas. Seven days after irradiation, PHA treatment of lymphoid cells derived from the irradiated appendix gave a greater degree of stimulation than controls. Both of these observations are consistent with a relative depletion of B lymphocytes.

After whole-body exposure, restoration occurs earlier in the spleen than in the thymus. The return of DNA synthesis in the spleen of lethally irradiated mice occurs on day 6 (Gershon and Hencin, 1971). Splenic T cells appear to recover more quickly than B cells (Nossal and Pike, 1973; R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976). Nossal and Pike (1973) noted that B

cells reached normal levels in the spleen 22 days after lethal irradiation and stem cell (fetal liver) restoration. This slow emergence was paralleled by a laggardly recovery of responsiveness to a B-cell antigen (2,4-dinitrophenyl *Salmonella* flagella, DNP–FLA). Restoration is mediated by hematopoietic stem cells of bone marrow and splenic origin. In contrast with the thymus, injection of exogenous bone marrow accelerates splenic recovery by providing a greater source of readily usable stem cells.

The microenvironmental stimulus provided by the splenic stroma for proliferation and differentiation of exogenous stem cells is itself extremely radioresistant. Tavassoli *et al.* (1975) observed that *in vitro* irradiation of splenic fragments with 1500 r did not prevent subsequent regeneration of autoimplants in splenectomized rats. Thus, it appears to be a general observation for hematopoietic tissues that the stromal elements are considerably more radioresistant than the hematopoietic cells (Trowell, 1961; Maniatis *et al.*, 1971).

Shielding of the spleen during exposure protects experimental animals and possibly humans (Gregory *et al.*, 1968) from acute mortality. Thus, the $LD_{50(28)}$ for control versus spleen-shielded 10–12-week-old CF-1 female mice is less than 600 r versus approximately 975 r, respectively (Jacobson *et al.*, 1949). The magnitude of protection appears to be age related (Simmons *et al.*, 1951–1952). Splenectomy does not, however, result in a pronounced increase in acute mortality; the slight increase observed perhaps relates to the loss of a significant source of hematopoietic elements (Raventos, 1954; Anderson *et al.*, 1971).

D. Lymph Nodes

Since lymph nodes contain a smaller proportion of radiosensitive T_1 cells than does the spleen, the lymphoid component of the former would be expected to be less susceptible to radiation injury than the latter. The striking effect of a single lethal whole-body dose of X-rays on the mouse lymph node is indicated in Fig. 8. Most of the cortical lymphocytes and the dividing cells in the germinal centers of the lymph node are destroyed, leaving intact the stroma, blood vessels, mature plasma cells, and reticuloendothelial cells (De Bruyne, 1948; Congdon, 1966).

A few viable lymphocytes persist in the lymph nodes of animals given whole-body irradiation in the lethal dose range. These cells may represent either a random fraction or a specific subpopulation of more radioresistant lymphocytes. Since some small lymphocytes are known to live for at least a year (Miller, 1964), and some of these may be responsible for immunological memory (Nowell, 1965; Gowans and Uhr, 1966), several studies have been carried out to determine whether there is a difference in the radioresistance of the long-lived and short-lived lymphocyte. In

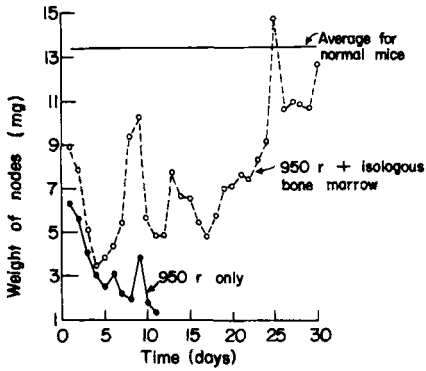


FIG. 8. Weight changes in peripheral nodes as a function of time in mice exposed to 950 r in whole-body fashion. Also shown are weight changes in mice similarly irradiated but given isologous bone marrow. (After Congdon, 1966.)

two reports (Everett *et al.*, 1964; Whitelaw, 1965), no change in the proportion of long-lived to short-lived lymphocytes was found in blood lymphocytes or TDL after 215 or 300 r. In another study (Miller and Cole, 1967), after doses of 500–800 r were used, lymphocytes were examined in the local lymph nodes draining an antigen-injection site. Despite a marked generalized destruction of lymphocytes, the nodes examined contained significantly higher proportions of the long-lived lymphocytes (identified by [^{3}H]TdR introduced at the time of antigen stimulation 1 month prior to irradiation). It was felt that these cells were probably not part of the circulating pool of small lymphocytes, and the results therefore do not necessarily contradict the other two reports which are concerned with the recirculating pool that may contain a different proportion of T_1:T_2 cells. In any event, it has been proposed that at least some types of long-lived lymphocytes are relatively resistant to quite high doses of X-rays (Miller and Cole, 1967).

Regeneration of lymph nodes after whole-body exposure proceeds slightly less vigorously than with spleen and is accelerated by injection of hematopoietic stem cells. Regeneration of lymph nodes after local, rather than whole body, irradiation is extremely rapid, probably owing to the influx of normal cells from nonirradiated tissues and the recirculating pool of small lymphocytes (Congdon *et al.*, 1958; Hall and Morris, 1964; Benninghoff *et al.*, 1969). However, a high local dose (e.g., 3000 r) results in vascular damage, destruction of the stroma, and extreme atrophy (Engeset, 1964).

The function of lymph nodes as the primary site of an immune response to a local antigen deposit, and the role in dispersing the subsequent immune lymphocyte population, is clearly seen in local lymph node irradia-

tion studies. Repeated irradiation of local draining nodes prevents both local and distant development of antibody-forming cells (Weissman *et al.*, 1973). This can even be seen with respect to subsets of immunocytes in that when the local node is the sole site of IgG antibody-producing cells, local lymph node irradiation prevents any subsequent IgG formation at distant sites.

E. Cell Traffic

Irradiation greatly modifies the capacity of lymphocytes to traffic in normal fashion. *In vitro* exposure followed by reintroduction into a syngeneic host is associated with impaired homing abilities, whereas whole-body irradiation results in a precipitous drop in the numbers of recirculating small lymphocytes mobilizable via thoracic duct cannulation (R. E. Anderson, J. Sprent, and J. F. A. P. Miller, unpublished results, 1971) and an impaired capacity of these cells to traffic normally.

Whole-body irradiation is associated with an acute drop in the number of lymphocytes found in the peripheral blood. As shown in Fig. 9, the decrement is dose dependent and has been employed to estimate the magnitude of exposure in reactor accidents. As shown in Table III, the numbers of TDL obtainable by cannulation of mice 4 days after 800 r whole-body irradiation are reduced by more than 50-fold (Sprent and Miller, 1972; Sprent *et al.*, 1974).

Recovery of the recirculating lymphocyte population following whole-body exposure has not been well studied. As implied in Fig. 8, recovery proceeds slowly and, in man, years may be required for normal values to be approximated. T cells recover more quickly than B cells and exhibit a biphasic response. The putative asynchronous recovery of T-cell subpopulations has not been investigated.

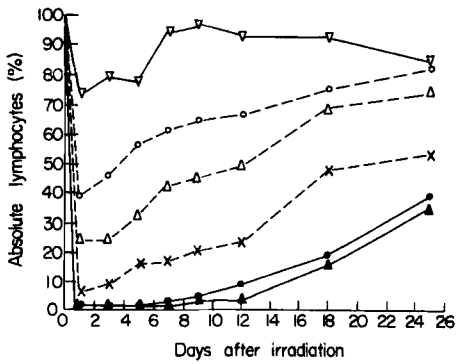


Fig. 9. Absolute lymphocyte count in the rat as a function of radiation dose and time after exposure. Results are expressed as percent of control values. Radiation dose (r): ▽, 25; ○, 50; △, 100; ×, 200; ●, 500; ▲, 600. (After Suter, 1947.)

When injected intravenously, lymphocyte home primarily to the spleen and lymph nodes (Lance and Taub, 1969; Sprent, 1973). In the absence of a strong antigenic stimulus, a portion of the transfused cells appear to be trapped nonspecifically in the spleen, presumably because of the absence of direct connections between arteries and veins (Weiss, 1972). Homing of transfused cells to lymph nodes appears to relate to unique properties of the lymphocyte membrane (Woodruff and Gesner, 1968). If membrane damage is a consequence of irradiation, a reduction in the lymph node-seeking capacity of the transfused cells would be expected. In a series of experiments addressed to this question, it was observed that exposure of T cells to large doses of radiation (e.g., 1000 r) did not impair their initial capacity to migrate to spleen and lymph nodes provided the cells were injected immediately after exposure. Cell traffic at later stages, however, was inhibited by even small doses of radiation. Thus, whereas redistribution of a large number of nonirradiated cells from spleen to lymph nodes was observed between 4 and 24 hours after injection, this redistribution was markedly inhibited by irradiation doses as low as 50 r. These observations suggest that radiation damages the membrane of lymphocytes, either directly or indirectly, but that the damage is manifest only after a few hours (see Section VII). This hypothesis is supported by the observation that lymph node homing of T cells is virtually abolished by incubating irradiated cells *in vitro* for 7 hours at 37°C prior to injection. By utilizing a similar approach, B cells were shown to be much more sensitive to radiation injury than T cells. Thus, initial localization to spleen and lymph nodes was abolished at lower dose levels, as was redistribution.

Considerations of the possible effects of radiation on cell traffic are particularly relevant to studies of radiation-induced loss of function in cell-transfer experiments in which lymphocytes are required to home to an appropriate location in order to carry out the function under evaluation. Several aspects of this problem, especially in relation to the radiosensitivity of T-cell helper activity, will be discussed elsewhere (Section III,C), but the cell "traffic" aspect will be considered at this time.

Primed irradiated spleen cells from guinea pigs (Asherson and Loewi, 1967) and rats (Feldman, 1968) are able to transfer DTH into normal recipients. Similarly, irradiated primed guinea pig spleen cells can transfer helper activity into normal recipients (Katz *et al.*, 1970). In mice, however, DTH against SRBC can be transferred to normal mice *only* when the irradiated primed spleen cells are injected with antigen directly into the footpad. Thus, irradiated primed spleen cells injected intravenously, fail to sensitize normal mice, presumably on the basis of inhibition of normal cell traffic (Kettman and Mathews, 1975). Similarly, the ability of carrier-primed T cells to collaborate with hapten-primed B cells

in vivo is abolished by exposure to 1000 r, the latter administered *in vitro* (Anderson *et al.*, 1972) (see below). The latter results, also in mice, cannot be attributed to a gross interference with the spleen-seeking properties of the irradiated cells since such homing appeared normal or slightly increased, but subtle homing differences might nevertheless occur (Anderson *et al.*, 1974).

The basis for the above discrepancies is difficult to define. The inability of irradiated lymphocytes to traffic in normal fashion is undoubtedly a factor. Even in experiments that document the presence of irradiated lymphocytes in the appropriate organ, there is no assurance that such cells home normally to the proper microenvironment where cell–cell collaboration transpires. On the other hand, it appears clear that sufficient numbers of primed lymphocytes, placed in an appropriate environment, can affect select responses despite exposure to large doses of radiation, particularly when such responses do not require cell division (see Section III,C).

Recently, Zatz and Gershon (1975) have investigated the effect of radiation on the sequestration of lymphocytes in lymphoid organs after antigenic challenge. Increased sequestration, or lymphocyte trapping (Zatz and Lance, 1971), is mediated in part by T cells (Frost and Lance, 1973; Zatz and Gershon, 1974) and is thought to provide a mechanism for the accumulation of antigen-reactive cells at the site of antigen depot (Zatz and Lance, 1971). Regulation of lymphocyte traffic through the spleen after antigen challenge appears to be a complex process that has both positive and negative elements (Zatz and Gershon, 1975). In the GVH reaction, parental thymocytes exposed to 850 r are competent to induce lymphocyte trapping but are incapable of producing the subsequent "negative trap" (Fig. 10). In other words, a radiosensitive subpopulation exists which suppresses the positive trap and augments the negative trap in the spleen. Subsequent experiments (M. M. Zatz, unpublished results, 1975) have shown that this radiosensitive subpopulation is also capable of amplifying the magnitude of the positive trap in lymph nodes.

The normal traffic pattern of the recirculating small lymphocyte (Ford and Gowans, 1967) suggests that local continuous irradiation of the spleen would lead to a marked reduction in the recirculating pool and therefore alter the immune status of the host (Gowans *et al.*, 1962). This has been studied by attaching a ^{32}P-impregnated polythene strip to the antihilar surface of the rat spleen (Ford, 1968). This approach resulted in a profound drop (to 15% in 4 days) in the output of small lymphocytes from a thoracic duct fistula. No other type of blood cell was affected. It appears that the lymphopenia was brought about by radiation-

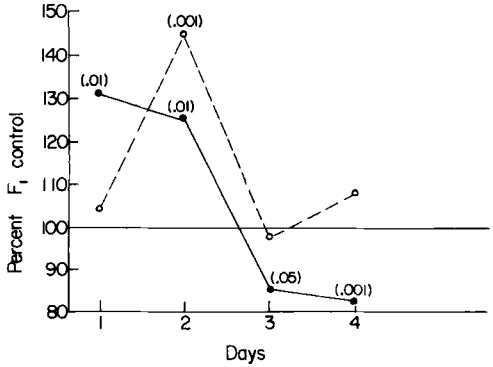


FIG. 10. Trapping of irradiated and nonirradiated thymocytes in spleen as a function of time after injection. Lethally irradiated CDF_1 mice were injected with 10^8 nonirradiated (●——●) or irradiated (○— —○) C3H thymocytes on day 0. Splenic trapping was assessed on subsequent 4 days. Significant differences between the experimental and F_1 control groups are shown by p values in parentheses. (After Zatz and Gershon, 1975.)

induced interphase death of small lymphocytes passing through the spleen from the blood. Other studies (Hall and Morris, 1964) on isolated lymph nodes had previously shown that large acute doses of radiation did not impair the organ structures essential for the recirculation of lymphocytes, at least in the immediate postirradiation period although later deleterious effects have been noted. The profound lymphopenia induced by the ^{32}P strip application to spleen was also associated with marked immunosuppressive effects. Using a strong immunogen presented in adjuvant to local draining nodes, a marked inhibition of both cellular and humoral responsiveness was shown (Yoshida *et al.*, 1970). Clearly T-cell immunosuppression occurred, and presumably B-cell suppression also, although the humoral system studied involved a T cell-dependent response.

Lymphopenia has also been produced by chronic extracorporeal irradiation (ECI) of the blood (Cronkite *et al.*, 1962), by intraatrial implantation of a β-emitting source (Barnes *et al.*, 1964), and by intralymphatic infusions of radioisotope-labeled agents (Tilak and Howard, 1964; Wheeler *et al.*, 1965; Edwards *et al.*, 1967). In this latter instance, studies in man with intralymphatic infusion of [^{131}I]lipidol have shown that even with a unilateral lower-limb infusion an appreciable volume of lymphoid tissue is irradiated, and histological examination of lymph nodes reveals widespread destruction. Although some workers have proposed that depression of lymphopoiesis accounts for the lymphocytopenic state, in the experiments with the ^{32}P-soaked strip (Ford, 1968) the lympho-

penia occurred far too rapidly (50% fall in 1 day) to be accounted for by depressed lymphopoiesis. A direct radiation death of the recirculating small lymphocytes seems far more likely. Leukemic lymphocytes also appear to be markedly radiosensitive, and accordingly, chronic ECI of blood was once considered as a means of removing leukemic cells. ECI of blood has been shown to be moderately successful in prolonging skin and kidney allografts in animals (Cronkite *et al.*, 1974). The topic of ECI of blood or lymph is quite extensive and has been reviewed in detail elsewhere (Cronkite *et al.*, 1964, 1974; Cronkite, 1968; Chanana *et al.*, 1971; Andersen *et al.*, 1974).

F. Susceptibility to Infection

A vast body of literature documents the increased susceptibility of experimental animals and man to infection after whole-body exposure to doses of radiation in the low- to mid-lethal range. The infectious agents may be of either endogenous (normal flora) or exogenous origin. Bacteria, viruses, protozoa, rickettsia, and fungi have each been implicated in this regard. It is beyond the scope of this presentation to attempt to review the 1000 or more independent observations that concern the relationship between infection and irradiation, and the reader is referred to several excellent reviews on this subject for more extensive data (Talmage, 1955; Donaldson and Marcus, 1956; Miller, 1956; Bond, 1957; Petrov, 1958, 1964; Sumnicht, 1958; Benacerraf, 1960; Draper, 1962; Hoptman, 1962; Troitsky, 1962; Smith, 1963; Stoner *et al.*, 1965). Primary attention in this section will be devoted to an examination of the variables that influence the increased susceptibility to infection associated with irradiation. This enhanced susceptibility is caused primarily by a decrease in the immune responsiveness of the host. As such, the prevalence of spontaneous infections is often employed as an indirect measure of radiation-related depression of the immune response. Other experimental approaches to this relationship include the assessment of radiation-related infection occasioned by challenge with either known pathogens or conditionally pathogenic agents (normal flora).

Death after whole-body exposure to doses injurious to hematopoietic and gastrointestinal tissue is often caused by infection. Leukopenia in association with the loss of physical integrity of mucous membranes, especially those of the gastrointestinal tract, appears to be of prime importance in this regard. Susceptibility is increased not only toward pathogenic agents, but also to bacteria that are part of the normal flora. Thus, the $LD_{50(30)}$ of germfree mice is considerably higher than for their conventional counterparts (Anderson *et al.*, 1968). Susceptibility to known and

conditionally pathogenic agents will now be considered, followed by an examination of several of the attendant variables, such as the relative timing of the infectious challenge and radiation exposure.

For more than 50 years (Coyter and Chovey, 1920), investigators have studied the sensitivities of irradiated animals to various pathogens. For example, in a study by Yakovleva *et al.*, (1957), only 1 of 14 monkeys given approximately 4×10^{10} paratyphoid B organisms orally, died of paratyphoid. However, in monkeys also given 300 R (in itself nonlethal to monkeys), four-fifths died with paratyphoid 5 days later. In other studies of this type, susceptibility to hemolytic streptococci increased 5 times (Troitsky *et al.*, 1965), and susceptibility to *Salmonella enteritidis* increased hundreds of times (Schechmeister *et al.*, 1952) in mice exposed to 350 R. A sharp drop in resistance to influenza virus was noted in experiments with irradiated mice and rats (Smorodintsev *et al.*, 1957). Similar increases in sensitivity to the organisms responsible for gas gangrene and tetanus icterohemorrhagic leptospirosis (Petrov, 1962) and to tularemia (Shevelev, 1958) were observed in sublethally irradiated mice. It is essential to note that any measure of increased sensitivity to an infectious agent after irradiation is accurate only for the given host, pathogen, and irradiation conditions. The general rule, however, is clear: whole-body irradiation in the low to mid-lethal range increases susceptibility to pathogenic agents.

In several studies with continuous exposure to low-dose γ radiation, increased susceptibility to chronic infections was also observed. In studies of mice with *Listeria monocytogenes*, and using ^{60}Co γ-radiation at a dose rate of 1.0–1.5 r per hour, Silverman *et al.*, (1965) found that the greater the total dose of radiation administered, the greater became the susceptibility. Mice receiving 500 r were 3 times as susceptible as nonirradiated mice, while those exposed to 2500 r were approximately 30 times as susceptible.

In irradiated animals, conditionally pathogenic microorganisms often become pathogenic. For example, doses of *Bacillus proteus*, which are nonlethal in control mice, resulted in bacteremia and eventual death in mice given 400 R 3 days previously (Hatch *et al.*, 1952). Similar observations have also been made with other bacteria that are usually not pathogenic for normal animals (colon and paracolon bacilli, *Pseudomonas aeruginosa*, and type IV pneumococci).

In some species, resistance to uncharacteristic infectious agents (innate resistance) appears to persist even after irradiation. Thus, Kolmer *et al.* (1937), were unable to overcome the innate resistance of rabbits, guinea pigs, rats, and ferrets to poliomyelitis virus, despite the fact that the animals were twice irradiated. Many other examples of this type are

documented in the review of Petrov (1962) and include the agents for anthrax, tularemia, diphtheria, typhus, dysentery, typhoid, and leptospirosis. It therefore appears that there is a high degree of stability of the animals' innate resistance to the effects of ionizing radiation. Possibly this type of resistance is particularly dependent on the phagocytic activities of various cell types—a mechanism that is quite resistant to irradiation (see Section III,A).

Although irradiated animals are severely compromised in their ability to undergo active immunization against bacteria and bacterial toxins, they can be protected satisfactorily by the use of passive immunization with antisera. This has been shown with diphtheria, tetanus, and gas gangrene infections (Kiselev and Karpova, 1956; Petrov, 1957; Klemparskaia *et al.*, 1958; Troitsky, 1958). One problem to be considered here, however, is the persistence of such transferred antibodies. In view of other observations on the loss of IgG and IgA through the irradiated gut wall (Bazin *et al.*, 1970), it might be expected that some loss of passive antibody would occur. Indeed it has been shown that, to obtain equal antitoxic effects in normal and irradiated recipients given passive antisera, 3–5 times more serum must be given to the irradiated recipients than to the controls (Petrov, 1957; Klemparskaia *et al.*, 1958). In addition, an increased sensitivity to toxin–antitoxin complexes has been observed in irradiated animals (Troitsky *et al.*, 1965).

Increased susceptibility to viral infections after irradiation is also observed frequently with many types of viral diseases, including influenza, smallpox, ornithosis, mouse encephalomyelitis, and mouse hepatitis. This enhanced susceptibility is often manifest as a shorter incubation period, increased virus proliferation, or more virus-induced pathogenic lesions, and is observed with sublethal doses of 200–500 r.

In some situations, however, the opposite result is noted, namely, a reduction in the severity of the disease. On general grounds, this might be expected on the premise that cell metabolism is markedly disturbed after irradiation and intracellular virus proliferation may thereby be inhibited. Several examples from the earlier literature (Goldberg *et al.*, 1934; Portmann and Laigh, 1945) concern encephalitis in man and show alleviation of symptoms by irradiation, possibly as a result of lymphocyte destruction. Similar results are also observed in studies of lymphocytic choriomeningitis in mice. Mice exposed to 500 R 24 hours prior to virus inoculation are protected for 48 days (Hotchin and Wergan, 1961; Hotchin, 1962), the depression of disease presumably being caused by inhibition of the proliferation of the pathogenic lymphocytes.

Studies concerned with the relative timing of irradiation and challenge with an infectious agent show two distinct patterns: (1) agents associ-

ated with an increased sensitivity on the part of the host during or immediately after irradiation; (2) agents associated with an increased sensitivity only after several days, generally 3 days, after irradiation. In the first group, an increased sensitivity to infection when the organism is given simultaneously with the radiation has been shown for trypanosomes, plasmodia, influenza, yellow fever, and tuberculosis (Petrov, 1962). On the other hand, in a number of cases in which increased sensitivity to infection could be clearly demonstrated, if the infectious challenge was given several days after radiation, no increased susceptibility occurred with simultaneous challenge. This includes studies with hemolytic streptococci, pneumococci, staphylococci, and colon bacilli. The distinction between these two groups seems to relate to the duration of the infectious process. Thus, those instances in which simultaneous challenge leads to increased sensitivity all involve chronic infections, whereas the second group are acute infections.

To summarize, several points derived from a large number of individual reports in this general area might be stressed: (1) radiation leads to increased susceptibility not only to pathogenic organisms, but also to conditionally pathogenic bacteria; (2) species resistance to infections that are not characteristic of that species is usually maintained after irradiation; (3) increased susceptibility to viral infections also results from radiation exposure, except in those cases where the cellular immune process constitutes an integral part of the disease (i.e., lymphocytic choriomeningitis); (4) increased sensitivity to acute infections is only manifest if challenge is made at least several days after irradiation, whereas simultaneous irradiation and challenge or irradiation after challenge are effective with chronic infections; and (5) the majority of these consequences are mediated through the effect of radiation on the immune response. Accordingly, the duration of the period of reduced resistance to pathogens follows the period of immune depression and depends on many factors, such as the dose of radiation, the dose rate, the animal species and its individual sensitivity to the particular infectious agents.

The delayed consequences of radiation in man with respect to infection are not clearly defined at present. Considerable effort in this regard has been expended at the Atomic Bomb Casualty Commission, yet no general relationship between a variety of infectious diseases and radiation has been documented. An analysis of mortality data among members of the Life Span Study Sample in Hiroshima and Nagasaki during the period 1950–1960 showed elevated ratios for all causes of death, all natural causes, leukemia and other malignant neoplasms for persons located 0–1399 meters from the hypocenter (Jablon *et al.*, 1963). Males in Hiroshima so located demonstrated a significant excess of deaths due

to tuberculosis, while females in Hiroshima showed an increased frequency of deaths attributable to infectious or parasitic diseases other than tuberculosis. These discrepancies were particularly marked during 1951–1952 and seemed to disappear thereafter. Periodic evaluations of the Adult Health Study Sample have shown no clinical, radiographic, or laboratory evidence of radiation-related infectious disease. Komatsu *et al.* (1963) found no relation between absence from work and exposure dose in a group of male shipyard workers. A review of the Atomic Bomb Casualty Commission autopsy experience also failed to document a consistent relationship between exposure status and infectious disease (Angevine *et al.*, 1963).

Finally, it must also be stressed that immune depression is not the sole mediator of radiation-induced increased susceptibility to infection. It is almost certainly the major factor, but other components play a role. Increased permeability of biological barriers has been demonstrated for the skin, the gastrointestinal tract, and the blood–tissue barrier. Shortly after irradiation, and even before the development of the acute radiation syndrome, there is a depression of the bactericidal properties of the skin with respect to intestinal bacilli and other microbes applied to it (Klemparskaia *et al.*, 1958). Decreases in serum levels of complement and properdin have also been noted, and these nonspecific aspects have been discussed in more detail elsewhere (Petrov, 1962).

III. Effects of Radiation on Antibody Formation

The suppressive effect of irradiation on the humoral antibody response has been recognized for many years (e.g., Benjamin and Sluka, 1908; Hektoen, 1915; Taliaferro and Taliaferro, 1951; Dixon *et al.*, 1952; Silverman and Chin, 1954; Celada and Carter, 1962; Makinodan *et al.*, 1962; Petrov, 1962; Stoner and Hale, 1962; Taliaferro *et al.*, 1964), and considerable attention, especially with respect to the involved mechanisms, has been focused upon radiation-induced injury to lymphocytes. As stressed previously, however, the induction and continued expression of an antibody response can involve a variety of cell types including macrophages (Roseman, 1969), T and B lymphocytes (Claman and Chaperon, 1969; Miller and Mitchell, 1969b), and possibly even other unidentified cell types (Talmage and Hemmingsen, 1973). In this section we consider individually the possible influence of radiation on the afferent limb of the immune response (primarily macrophages), on the primary and secondary antibody response *in vivo*, and on the separate subpopulations of lymphocytes.

A. The Afferent Limb of Antibody Formation

The afferent limb of the immune response involves the initial events following antigen injection that lead to effective immunogen presentation to the various immunocompetent lymphoid cell types. Many studies have shown that the amount of injected antigen that survives initial degradation within macrophages and reaches the appropriate lymphoid cells is a very small fraction of the original injected amount (Ada *et al.*, 1964; Nossal *et al.*, 1964). The actual form of the macrophage-processed or macrophage-associated antigen may be quite critical for effective immunogenicity (Unanue, 1972; Erb and Feldmann, 1975) and may essentially depend on the ability of the macrophage cell type to carry out certain metabolic processes. Accordingly, if radiation were capable of interfering with certain crucial functional capacities of the macrophage, then ineffective antigen presentation and resulting immunosuppression might occur.

In studying the radiosensitivity of accessory cell types that may play a role in the afferent stages of the immune response, several points need to be considered: (1) Possible heterogeneity of macrophage subpopulations. Radiation studies involving macrophages obtained from a variety of sources may be confounded by variable numbers of putative macrophage subpopulations. For example, radiation studies on macrophage preparations from peritoneal exudate cells may not necessarily relate directly to the radiosensitivity of accessory cells that are involved in primary antibody formation, since these cells may only represent a minority subpopulation in such preparations. (2) Examination of the radiosensitivity of some macrophage functions, such as phagocytic activity, need not necessarily be relevant to the actual function of select macrophage subpopulations in appropriately processing antigen for optimal immunogenicity. (3) Elicitation of immune responses to some antigen may not require macrophage processing of antigen (Shortman *et al.*, 1970), and, accordingly, radiation depression of immunity to these antigens would be due solely to lymphocyte effects.

Following the diffuse spread of injected antigen throughout tissues, antigen can be taken up by various cell types including polymorphs, follicular reticular cells and macrophages. An overall statement of the radiosensitivity of the afferent limb should thus consider all three cell types.

1. Polymorphonuclear Leukocytes

Direct irradiation of polymorphs *in vitro* (Selvaraj and Sbarra, 1966) or irradiation of whole animals (Smith *et al.*, 1963) appear to have no

effect on the ability of polymorphs to phagocytose bacteria. However, the bactericidal activity of polymorphs can be increased by irradiation given at the time of or after phagocytosis (Mukherjee *et al.*, 1967, 1968; Mukherjee and Sbarra, 1968), or decreased by irradiation given prior to phagocytosis (Paul *et al.*, 1968). In the latter case, it was suggested that the decreased activity may be due to a specific decrease in particle associated (? lysosome) metabolic H_2O_2 possibly as a result of radiation-induced depression in the production of H_2O_2 through the hexose monophosphate shunt. Therefore, this decreased bactericidal activity might be a factor in the increased susceptibility to infection noted after low-dose exposures.

Irradiation also causes a profound depression in the production of polymorphs in the bone marrow by virtue of the destruction of the hematopoietic stem cells which are extremely radiosensitive with D_{37} of about 90 r (McCulloch and Till, 1962; Siminovitch *et al.*, 1965). Similarly, the *in vitro* colony-forming cells, which are the direct precursors of the macrophage and granulocytic cells, show a D_{37} of approximately 85 r (Robinson *et al.*, 1966; Chen and Schooley, 1970). Regeneration of normal numbers of *in vitro* colony-forming cells in the bone marrow takes about 16 days after 250 r (Hall, 1969).

2. *Follicular Localization of Antigen*

Primary lymphoid follicles in both spleen and lymph nodes represent densely packed collections of lymphocytes in close relationship to a web of cytoplasm derived from specialized dendritic reticular cells (Miller and Nossal, 1964). These dendritic cells have few free ribosomes and an almost complete absence of lysosomes and of phagocytic inclusions (Milanesi, 1965). The handling of injected antigen by these cells differs markedly from that of medullary macrophages. A substantial proportion of antigen localized in follicles is not phagocytosed, but is retained on the surface of the long dendritic processes of these specialized reticular cells (Mitchell and Abbot, 1964; Maruyama and Masuda, 1965). The retention of antigen in follicles can be profoundly affected by sublethal whole-body X-irradiation. Whereas the cytoplasmic dendritic web is extremely radioresistant, requiring doses of over 1250 r for significant damaging effects, the process of antigen localization and retention was inhibited with exposures of 450 R (Jaroslow and Nossal, 1966) and 800 R (Williams, 1966). This inhibition could be overcome somewhat by the injection of either specific immune serum or relatively large amounts of normal isologous serum (Williams, 1966). These observations suggest that a decline in serum levels of natural opsonic materials produced by radiosensitive cells might be responsible. However, several studies have

not shown any reduction in levels of serum opsonins after irradiation (Solomon, 1966; Saba and DiLuzio, 1969), and serum IgM levels do not show significant change after irradiation (Bazin and Malet, 1969). Reduction in serum levels of IgG and IgA appears to result from increased Ig secretion through the radiation-damaged intestinal epithelium (Bazin *et al.*, 1970).

In another study of radiation damage (600 r) to antigen capture and retention by the germinal center stroma (Nettesheim and Hanna, 1969), maximum change was observed 2 weeks after irradiation, with recovery taking several weeks. Electron microscopic examination indicated that the defect in trapping might be due to direct damage of the antigen-capturing cells.

Complete resolution of the possible mechanisms of radiation damage will probably require specific examination of the effect of radiation on the functional integrity of Fc receptors, which play an essential role in mediating antigen capture through natural or induced antibody binding (Berken and Benacerraf, 1966; Herd and Ada, 1969).

What role this impaired antigen trapping plays in the primary immune deficiency of irradiated animals is not clear, particularly since at least some antibody responses can be initiated in the total absence of follicular antigen localization (Lang and Ada, 1967). Follicular localization may be of greater importance in the development of immunological memory (Thorbecke *et al.*, 1967) and thus more important in radiation-induced depression of secondary responses (see Section III,B).

3. *Macrophages*

In considering the potential effect of radiation damage on macrophage function with respect to immune induction, several aspects need to be distinguished, namely, the influence on phagocytosis and the catabolism and degradation of antigen as well as possible effects on the actual immunogenicity of the macrophage-processed antigens (Table V).

It is generally considered that the phagocytic function of macrophages is quite resistant to irradiation. Direct histological studies have not shown evidence of damage (Bloom, 1948; Brecher *et al.*, 1948), and macrophages in irradiated lymphoid tissues have been noted to be very active in phagocytosing the debris of dead cells (Brecher *et al.*, 1948; Smith *et al.*, 1967). The migratory activity of macrophages is also quite radioresistant (Muramatsu *et al.*, 1966), although the capacity of phagocytes to replicate is as radiosensitive as that of any other cell population (Gadeberg, *et al.*, 1975). The ability of macrophages to phagocytose a wide variety of other agents is also quite resistant to irradiation. This includes the ability of the reticuloendothelial system to clear bacteria

TABLE V
EFFECTS OF RADIATION ON MACROPHAGE ACTIVITIES

Macrophage function or activity	Effect of radiation
1. Morphology	No change
2. Migratory activity	No change
3. Phagocytosis of antigens, particles, etc.	No change
4. Catabolism of ingested antigens	Variably reduced, particularly in kilorad range
5. Intracellular biochemical activities (lysosomal enzyme levels, etc.)	Variable increase
6. Membrane-bound antigen	Increased levels
7. Immunogenicity of macrophage-associated antigen	Most protein and erythrocyte antigens—no change; *Shigella* antigen—marked reduction

from the blood of various animals (Gordon *et al.*, 1955; Benacerraf, 1960; Geiger and Gallily, 1974), the clearance of colloidal carbon (Benacerraf *et al.*, 1959), the *in vitro* capacity of mouse peritoneal phagocytes to engulf SRBC (Perkins *et al.*, 1966), and the *in vitro* phagocytosis of radioiodinated protein antigens (Schmidtke and Dixon, 1972). In these various studies, doses of irradiation of around 600–800 r or in the kilorad range of up to 10,000 r have been used without any demonstrable reduction in phagocytic activity. Several reports have indicated, however, that a discrepant tissue distribution of injected material may occur following radiation, which may not be reflected in the overall phagocytic removal or rate of clearance (Gabrieli and Auskaps, 1953; DiLuzio, 1955; Saba and DiLuzio, 1969).

Some controversy, however, exists concerning the radiosensitivity of several post-phagocytic macrophage functions—including the ability to catabolize, degrade, or retain antigen. Donaldson *et al.* (1956) and Kakurin (1959) found that macrophages from irradiated animals had a depressed ability to digest intracellular material, and Gordon *et al.* (1955) observed the reappearance of live organisms in the blood of irradiated rabbits after a period of normal clearance. In contrast, Benacerraf *et al.* (1959) observed normal catabolism and release of an iodinated protein antigen from the Kupffer cells of irradiated mice. Perkins *et al.* (1966) observed no impairment to the digestion of SRBC by heavily irradiated macrophages, and Schmidtke and Dixon (1973a) found that *in vivo* irradiation (660 R) of mice 1–3 days before harvest of macrophages did not significantly affect the subsequent uptake or handling of iodinated hemocyanin *in vitro* by these cells. *In vitro* irradiation of macrophages with 10,000 R (but not with 3000 R) did, however,

result in some decrease of hemocyanin catabolism (Schmidtke and Dixon, 1972). The intracellular location of degraded antigen may also be altered by irradiation, in that less material is found in the dense storage compartments of irradiated than of normal cells (Kolsch and Mitchison, 1968). Thus, although irradiation does not affect the uptake of antigens by macrophages, some alterations in the intracellular processing of antigens can be detected.

Analysis of various biochemical properties of macrophages has in fact demonstrated radiation-induced changes. DNA synthesis in irradiated macrophages was slightly higher; RNA synthesis was 6 times higher than in nonirradiated controls, and choline uptake was significantly enhanced (Geiger and Gallily, 1974). Macrophages irradiated *in vivo* also exhibit an enhanced capacity to release lymphocyte-activating factors (Geiger *et al.*, 1973). Macrophages derived from animals irradiated several days previously show evidence of increased activation in that levels of lysosomal enzymes, such as acid phosphatase, cathepsin, and β-glucuronidase are significantly greater than in controls (Meyer and Dannenburg, 1970; Schmidtke and Dixon, 1973a; Geiger and Gallily, 1974).

These various reports have primarily dealt with the problem of the handling of antigens by irradiated macrophages. The further question is whether irradiation interferes with the crucial process of immunogenic presentation of antigen by macrophages. At the present time, it appears that differences exist in this regard among specific antigens. In one series of observations irradiated macrophages appear to be more efficient in presenting antigen to lymphocytes, while in another series of experiments they appear less efficient. Approaches to this problem have primarily involved cell transfer studies.

Using radiation doses that significantly suppress antibody formation in the whole animal, several groups have investigated whether normal macrophages restore antibody-forming capacity. Although this has not been observed with the protein antigens HGG, hemocyanin, or BSA (Spitznagel and Allison, 1970; Schmidtke and Dixon, 1972), several studies with the bacterial antigen *Shigella* have shown that the immunodepression in the recipient can be restored by transferred macrophages (Gallily and Feldman, 1967). Conversely, macrophages derived from irradiated mice were shown to have lost the capacity to induce an immune response to *Shigella* (Gallily and Feldman, 1967; Geiger and Gallily, 1974). Exposure to 450 R was fully effective with some inhibition observed after 150 R. Similarly, Pribnow and Silverman (1967) and Mitchison (1969) have shown a diminished capacity of irradiated antigen-containing macrophages to transfer an immune response *in vivo*.

In distinction, the immunogenic function of macrophages in the immune

response to sheep erythrocytes appears to be quite radioresistant (Ellis *et al.*, 1967; Roseman, 1969; Goldie and Osoba, 1970; Cosenza *et al.*, 1971; Gershon and Feldman, 1968). With protein antigens, an increased immunogenicity of irradiated macrophages has been observed. Quantitative analysis of membrane-bound (trypsin removable) iodinated protein antigens has shown that macrophages irradiated *in vitro* (Schmidtke and Dixon, 1972), but not *in vivo* (Schmidtke and Dixon, 1973a), have a greater resultant surface binding of antigen. Since the immunogenicity of macrophage-bound antigen has been primarily attributed to the small amounts of antigen bound to cell membranes (Schmidtke and Unanue, 1971; Unanue, 1972), irradiated macrophages would be expected to be more efficient in this regard, and this was observed by Schmidtke and Dixon (1972).

It therefore appears that with most antigens the immune depression after irradiation with doses below the kilorad level is not primarily due to interference with macrophage functions (Table V). Whether the macrophage depression clearly observed in the *Shigella* system will be applicable to immunity to other bacterial antigens remains to be elucidated.

B. Radiosensitivity of Antibody Formation *in Vivo*

Although many of the earlier studies on the radiosensitivity of antibody formation have clearly focused on lymphoid cells as the prime target cell, it is only relatively recently that specific considerations of differential radiosensitivities of subpopulations have emerged. Accordingly, this section will primarily concentrate on the overall radiosensitivity of the immune response, both in the inductive and productive phases, and the next section will consider the various cell types separately.

1. The Inductive Phase of the Antibody Response

a. Primary Response. In considering the possible effects of radiation on the induction of a primary humoral antibody response, it is to be stressed at the outset that the detailed process of differentiation of immunocompetent cells, although not yet fully elucidated, clearly involves several distinct stages. Some of the problems still to be resolved in this area have been considered elsewhere (Warner, 1974, 1976), and briefly concern the number of distinct stages in differentiation from the pluripotential hematopoietic stem cell to the immunocompetent B cell capable of responding to antigenic challenge. It is probable that all stages in early differentiation are quite radiosensitive, with D_{37} values of the order of 80–90 r, like that of the pluripotential hematopoietic stem cell

(McCullough and Till, 1962; Siminovitch *et al.*, 1965). It is unlikely, however, that this acute radiosensitivity of cells in the early differentiation stages, plays any significant role in the immunodepression of antibody formation noted in the period immediately following a single whole body dose of radiation. If all hematopoietic stem cells were to be inactivated by radiation, all activities of the immune system which are dependent on a continual input of differentiating cells would eventually fail. However, the reserve of stem cells appears to be such as to outweigh any possibility of its complete eradication with moderate doses of irradiation. After a dose of 150 r, all parameters of hematopoiesis recover to normal values by 7–8 days, and on a daily schedule of 50 r after an initial 150 r, it requires a further 250 r to reduce stem cell repopulating activity to 5% of control values, which still represents a massive reserve of hematopoietic elements (Draper, 1962).

Accordingly, the main focus of attention on radiation-induced depression of primary antibody formation concerns the lymphoid cells that proliferate in response to antigen presentation. From many early studies (see reviews, Taliaferro and Taliaferro, 1951; Taliaferro *et al.*, 1964; Makinodan and Gengozian, 1960; Nossal, 1967), the general conclusion was drawn that the early phases of induction of antibody formation are quite radiosensitive, and that the D_{37} values observed indicate that cell proliferation is an essential feature of the early immune response (Makinodan *et al.*, 1962). In the light of more recent studies (see Section III,C) indicating that B lymphocytes are perhaps particularly sensitive to interphase death, this statement may require some modification.

The existence of an early radiosensitive phase that rapidly moves to a radioresistant phase was clearly shown by Dixon *et al.* (1952), who irradiated rabbits 2 days prior to the injection of iodinated bovine (BGG). A slight inhibition of antibody formation was observed with 75 R and 125 R, which resulted in considerable depression, and 200–300 R was almost totally immunosuppressive. In order to determine more quantitative parameters of radiosensitivity, Makinodan *et al.* (1962) tested the ability of spleen cells transplanted into lethally irradiated recipients to produce hemagglutinins against SRBC when the donor cells were derived from mice that had been subjected to varying doses of radiation 3 hours previously. These and similar studies by Simic *et al.* (1965) are shown in Fig. 11. Based on the linear portion of the inactivation curve, a D_0 value of approximately 70 r is observed. Using a similar approach, Celada and Carter (1962) obtained a value of approximately 50 r.

Many of the early studies relevant to this discussion involved estimations of the amount of specific antibody in the serum of the irradiated

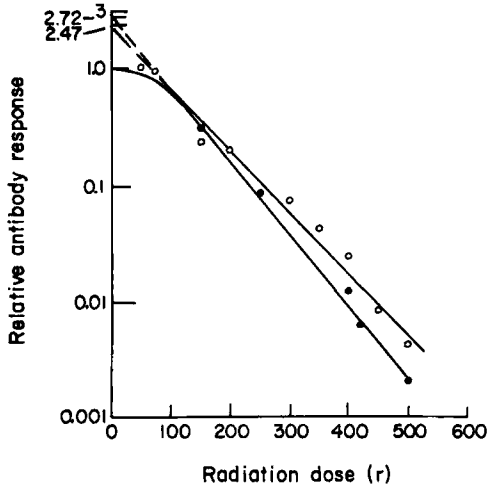


FIG. 11. Radiation-induced inactivation of the immune response in rats (○——○) and mice (●——●). Spleen cell suspensions were exposed to varying doses of radiation and transferred to lethally irradiated syngeneic recipients together with antigen. The attendant immune response is plotted relative to that given by non-irradiated controls. (After Makinodan *et al.*, 1962; Simic *et al.*, 1965.)

host. However, it is not at all certain that serum antibody levels are directly proportional to the number of surviving cells producing the antibody. It would need to be shown that the doses of radiation employed had an all-or-none effect on antibody production by single cells, and that irradiation affects neither the rate of removal of antibody from the circulation nor the concentration of various serum factors that might alter the sensitivity of the assay. These objections apply mainly to whole-body irradiation studies rather than to the cell transfer systems as shown in Fig. 11. However, a more direct assessment is obtained when assays for measuring the number of antibody-producing cells are used. This system was employed by Kennedy *et al.* (1965) in studying the radiosensitivity of primary antibody formation to SRBC. The results, depicted in Fig. 12, show a D_{37} value of approximately 80 r. The parameters of the curve were similar for two different doses of antigen and two different times of assay.

Various investigations have compared the radiosensitivity of the IgM versus the IgG humoral response. As will be discussed in more detail in the next section, this must be viewed in the light of the various cell components involved in the response. Many studies in athymic nude mice clearly indicate the importance of T-cell involvement in controlling IgG responses (Wortis, 1974; Mitchell, 1974). Accordingly, the radiosensitivity

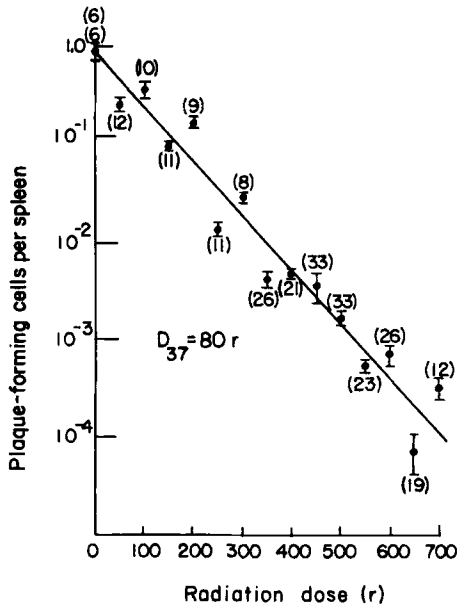


FIG. 12. Relationship between magnitude of irradiation and plaque-forming capacity of mice. SRBC, 4×10^8, were given 10 days after whole-body irradiation, and assays for plaque-forming spleen cells were performed 4 days later. The results are plotted relative to the plaque-forming response of control (nonirradiated) mice. (After Kennedy *et al.*, 1965.)

of IgG responses may involve T cell and B_γ precursor cell radiosensitivities. Furthermore, the question of possible precursor–progeny relationships between B_μ and B_γ cells is clearly relevant and has yet to be fully elucidated (see Warner, 1974), particularly in view of the potential importance of IgD-bearing lymphocytes in this regard.

In studying the relative radiosensitivity of IgM and IgG responses, various patterns have been observed. Several groups have reported that the IgM response is more radioresistant than the IgG response (Svehag and Mandel, 1964; Robbins and Smith, 1964; Nettesheim *et al.*, 1969). These studies involved estimations of serum antibody levels in irradiated animals. However, given the problem of differential Ig class loss through the gut of irradiated animals (Bazin *et al.*, 1970), direct assessment of antibody-forming cells represents a more critical approach to the issue. In a detailed study of this question, Zaalberg *et al.* (1973) observed almost identical D_{37} values for the radiosensitivity of IgM and IgG plaque-forming cells (PFC) to SRBC from both primary and secondary immunization regimes (primary IgM, 88 r; IgG 99 r; secondary IgG, 93–102 r).

Several basic radiobiological problems concerning these studies remain unresolved. First, it is frequently observed that n values greater than 1 occur in such studies (e.g., Fig. 11) (Zaalberg *et al.*, 1973), i.e., that a shoulder is observed in the radiosensitivity curve at low doses. Several explanations have been proposed but require further elucidation; these include: (i) "multihit" effect in that several hits on a cell are required for inactivation; (ii) antigen can induce blast transformation and thereby activate repair mechanisms before interphase death occurs; and (iii) the expected inhibitory effect of irradiation at low doses is masked by the preferential abrogation of a regulatory mechanism—possibly either humoral antibody itself, or a cellular suppressor mechanism (see Section III,C).

A second problem that has received some attention is whether resistance to irradiation is increased in animals repeatedly exposed to radiation. Although initial studies inferred that this might occur within the antibody-forming cell lineage (Petrov and Cheredeev, 1968), subsequent studies (Price and Makinodan, 1970; Petrov and Cheredeev, 1972) suggested that this was not the case and that the data might be explained in terms of altered proportions of various cell types.

b. Timing of Irradiation and Antigenic Challenge. Whole-body irradiation may either depress, augment, or not alter the primary humoral antibody response. In most of the studies discussed above in which radiation has inhibited immune responses, antigenic challenge was given at the time of or closely after irradiation.

It has been frequently observed, however, that irradiation given *after* antigenic challenge can result in an enhancement of antibody production. After the initial studies and reports of this phenomenon (Taliaferro and Taliaferro, 1954; Dixon and McConahey, 1963), many situations have been described in which the particular conditions of antigen timing, dosage, form of antigen, etc., have resulted in enhancement rather than depression of immunity. Before considering some of the possible mechanisms involved, several of the variables that can lead to this observation will be discussed.

In the original studies of Taliaferro *et al.* (1964), it was proposed that two types of radiation-induced enhancement exist. One type (type A, Table VI) is found with relatively low doses of irradiation (of the order of 25–200 r) and is associated with a heightened peak titer and a shortened latent period (see below). The second type (type B, Table VI), which has been more frequently described, involves exposures to higher doses (e.g., over 400 r) and is associated with an increased peak titer but a lengthened latent period prior to the peak titer. This situation has been observed in several studies with various antigens in rabbits (Talia-

TABLE VI
POSSIBLE MECHANISMS OF RADIATION ENHANCEMENT OF ANTIBODY PRODUCTION

Type A: Low-dose irradiation (25–200 r) before antigen
Type B: High-dose irradiation (>400 r) after antigen
1. Disproportionate repopulation of depleted lymphoid tissues by rapidly dividing antigen-stimulated cells
2. Endotoxin release from radiation damaged gut
3. Local cellular destruction creating a *milieu* for proliferation of more immunocompetent cells than would normally respond
4. Selective inhibition of IgG-mediated feedback control
5. Preferential inhibition of regulatory cells (? suppressor T cells)

ferro *et al.*, 1964; Lebedev, 1965; Keuning *et al.*, 1963), mice (Gengozian and Makinodan, 1958; Morgan *et al.*, 1960; Hoffstein and Dixon, 1974; Schmidtke and Dixon, 1973b), and guinea pigs (Vlahovic and Stankovic, 1961), and was most exhaustively analyzed by Dixon and McConahey (1963). They observed that (i) the degree of optimum stimulation varied from one antigen to another; (ii) the time interval between antigen challenge and irradiation differed in terms of optimal stimulation for different antigens; (iii) the dose of radiation for optimal stimulation also varied for different antigens. In general it appeared that the more rapid the antibody response, the earlier must irradiation be given to enhance the response. Irradiation after antigen injection has not always been observed to result in higher titers than in controls, but in such situations, the degree of immunodepression observed with a given dose may not be as great as when irradiation precedes antigen (Fitch *et al.*, 1956).

The first type of radiation enhancement described by Taliaferro *et al.* (1964) (type A) involves antigen injection *following* relatively low doses of irradiation. Using doses of 25–100 r given within a week *prior* to antigen, Taliaferro and Taliaferro (1969, 1970) observed prolonged production and transient high peak titers of hemolysins in rabbits. Similarly, Hoffstein and Dixon (1974) observed that primary antihemocyanin antibody responses in mice could be markedly enhanced by single weekly doses of irradiation. Strain differences were observed in that 175 R per week (given for 4 weeks *prior* to antigen injection) was the optimal stimulatory exposure for SWR/J mice whereas 125 R/week was the optimal dose for C3H mice. Weekly exposure of these strains to 300 R or 150 R, respectively, was immunosuppressive in comparison to unirradiated controls. These studies clearly demonstrate stimulatory effects of low dose irradiation given *prior* to antigen challenge.

Observations on potential radiation enhancement of secondary responses have been more variable. Type A enhancement (Table VI) was

not observed by Taliaferro and Taliaferro (1970), and Dixon and McConahey (1963) observed only limited enhancement (type B) of secondary responses in rabbits to hemocyanin and no enhancement to BSA or BGG. Under very specific conditions of radiation dose and timing, enhancement of secondary responses of both type A (Hoffstein and Dixon, 1974) and type B (Schmidtke and Dixon, 1973b) was observed in mice. In the former instance, strain differences with respect to optimal radiation dose were again observed, and the optimal doses for both strains were slightly higher than the optimal weekly dose for primary enhancement. In the second instance (type B), when 440 R was given at a critical time (4 days) after a primary injection, the number of antibody-forming cells in the spleen after a secondary antigen injection was increased significantly compared to those in unirradiated controls. Irradiation at times other than 4 days after primary antigen injection (i.e., 3, 5, or 6 days) did not result in enhancement, but in fact suppressed the response. Increased responsiveness induced by irradiation was also dependent on the priming dose of antigen (Schmidtke and Dixon, 1973b). However, not only does antigen dosage play a role in determining degree of radiation enhancement, but the actual form of antigen presentation can be a factor. Stoner *et al.* (1974) observed that radiation enhanced secondary antibody responses to tetanus toxoid when the priming antigen was presented as an antigen–antibody complex at equivalence, but not when the priming antigen was free tetanus toxoid.

Augmentation of the immune response has also been shown after local irradiation. Again, the timing is critical, in that local irradiation (920 R) of lymph nodes draining the sites of antigen injection 1 or 2 days after antigen challenge resulted in augmented levels of serum antibody, whereas irradiation 3–4 days after challenge gave immunosuppression, and irradiation at 6–9 days had no effect (Eltringham and Weissman, 1971).

In certain situations, radiation-induced enhancement of antibody production has been specific for only some classes of response. Homocytotropic antibody production was markedly enhanced by 400 R whole-body irradiation several days after antigen injection, whereas IgG antibody responses in the same animals were profoundly suppressed (Tada *et al.*, 1971). Since similar observations were made in animals partially depleted of lymphocytes in other ways, and in all cases the enhanced response was abrogated by injection of thymus cells (Okumura and Tada, 1971), this situation seems most likely to involve selective depletion of a homeostatic regulator (Table VI, mechanism 5). The possible importance of cell interactions is also inferred from *in vitro* studies, in that radiation enhancement of antibody formation to bacteriophage was not seen with

an *in vitro* primary or secondary response using radiation and timing conditions that *in vivo* did show enhancement (Tao and Leary, 1969).

These various studies therefore clearly demonstrate that whole-body irradiation can under specific circumstances either augment or inhibit immune responsiveness. Inhibition is clearly to be anticipated in view of the known capacity of radiation to induce lymphocyte destruction. What, however, are the mechanisms of radiation enhancement? In considering several possibilities (Table VI), it is essential to recognize that there are perhaps two distinct types of radiation enhancement—differing both with respect to radiation dose and relative timing of antigen injection—and that different mechanisms may be involved in these situations. The possible mechanisms may not be mutually exclusive and may interact in various ways.

1. On the basis of early results (Dixon and McConahey, 1963), it was proposed that lymphoid tissues are depleted of cells by irradiation and are then disproportionately repopulated by rapidly dividing antigen-stimulated cells. Histological studies are consistent with this view (Dixon and McConahey, 1963; Lebedev, 1965), which might be expected to be more relevant to type B enhancement. Direct analysis of antibody-producing cells has shown significantly greater numbers of such cells in the spleens of irradiated mice (Schmidtke and Dixon, 1973b). Although irradiation would be expected to inhibit the proliferative potential of all cell types in the irradiated lymphoid tissues, it may be that antigenically stimulated cells have a significantly increased relative or absolute resistance over nonstimulated cells. Increased radioresistance has been observed in lymphocytes after activation with: mitogens (Conard, 1969; Cirkovic, 1969, 1970; Renner and Renner, 1971; Brannon and Dewey, 1969; Field *et al.*, 1972), allogeneic lymphocytes (Schrek, 1968), and tuberculin (Stefani, 1966). One study failed to show a similar increase after *in vivo* antigen injection (Berenbaum, 1966).

2. An additional mechanism, perhaps partially operative in some but not all studies, might be antibody enhancement in response to endotoxin released from the gut after irradiation. Transient bacteremia by gut organisms released after irradiation has been shown to have stimulatory activity on the reticuloendothelial system (Sljivic, 1970a,b).

3. An alternative or perhaps modified view of mechanism 1 above has been proposed by Makinodan and Price (1972). Basically, this view does not require disproportional proliferation, but simply an enhanced involvement of *potentially* reactive cells. It is based on the concept that the maximum immune expression observed in any response situation need *not* necessarily reflect the full immunological potential to the antigenic challenge. Several experiments clearly indicate that the immune system

is capable of enhanced responses that are much greater than are achieved in conventional immunization schemes. Cell impermeable diffusion chambers containing antigen and spleen cells from preimmunized mice, when implanted in irradiated recipients, can generate ten times more antibody-producing cells per unit number of spleen cells than *in situ* immunization (Makinodan and Albright, 1967). Therefore, Makinodan and Price (1972) suggested that radiation augmentation is mediated by a sufficient amount of cell destruction to create a suitable *milieu* for proliferation and differentiation of more immunocompetent cells than normally would participate in a response. Nucleic acids and other degradation products resulting from irradiation damage might aid in this stimulation or proliferation (Taliaferro and Taliaferro, 1970).

4. Feedback regulation of the antibody response is particularly controlled by IgG antibody (Uhr and Möller, 1968; Diener and Feldmann, 1972). If, as discussed above, IgG serum antibody levels might on occasion be preferentially inhibited by radiation, then feedback control might be reduced and result in an overall increased antibody response (of predominantly IgM type). No direct evidence is available for this possibility.

5. Of perhaps more relevance in relation to homeostatic control mechanisms is the suppressor T-cell system (Gershon, 1975; Herzenberg *et al.*, 1975; Tada *et al.*, 1971). As discussed later (Section III,C), suppressor T cells are particularly radiosensitive and may be preferentially abrogated by type A radiation treatments. This would result in increased immune responsiveness, as observed in the study of Tada *et al.* (1971). Since current studies are suggesting that suppressor T cell control is primarily directed at helper T cells (Herzenberg *et al.*, 1975), this would infer that this mechanism of radiation enhancement is operative only with respect to T cell-dependent immune responses.

2. *The Productive Phase of Antibody Formation*

The productive phase of antibody formation is a heterogeneous phenomenon involving both memory cell differentiation and antibody secretion by lymphocyte-like cells (Bosman *et al.*, 1969; Cunningham *et al.*, 1965), by DNA-synthesizing immature plasma cells (Nossal and Mäkelä, 1962) and by mature plasma cells (Fagraeus, 1948; Leduc *et al.*, 1955; Nossal, 1959a). Many investigations on the comparative radiosensitivity of primary and secondary immune responses have inferred a greater radioresistance of secondary responses (Taliaferro *et al.*, 1964; Makinodan *et al.*, 1959; Silverman and Chin, 1954) and have suggested after a given antigen challenge, the immune response progresses from a radiosensitive to a relatively radioresistant phase (Dixon *et al.*, 1952; Taliaferro *et al.*, 1952). Although it is not always possible to distinguish these two aspects,

we will separately consider the radiosensitivity of antibody-producing cells and of the secondary response.

a. Plasma Cells and the Active Immune Response. Various early studies on whole-body irradiation after antigen injection have clearly indicated that immunodepression does not necessarily ensue (see above). Thus, Dixon *et al.* (1952) showed that 800 R given 3 days after antigen was not immunosuppressive in rabbits; Hale and Stoner (1956) found no antibody depression in mice given 650–775 r at the time of peak serum antibody formation; and Taliaferro *et al.* (1952) also concluded that radiosensitivity was limited to the early stages of the antibody response. In other studies, however, a balanced situation may apply. Claman (1963) showed that irradiation during the steady state of the primary response produced a continuous fall in antibody levels but was without effect when given during the declining phase of the secondary response. Thus, the steady state of persisting serum antibody, particularly after a primary response, is maintained by a balance between proliferation of differentiating cells and the decay (half-life) of mature plasma cells and of their secreted antibodies. This balance may well vary for different antigens, and will change with time in a given response, progressing toward increased radioresistance.

Before considering the possible effect of irradiation on antibody-producing cells, it is relevant to note that antibody molecules themselves do not lose biological activity (antigen-binding capacity) after irradiation unless doses in the megarad range are used (Rosse *et al.*, 1967).

The majority of plasma cells are relatively short-lived and survive for only a few days, although a minority, long-lived population also exists that can survive for many months (Miller, 1964). The latter type thus contributes increasingly to the latter stages of the productive phase. Both types of plasma cells are extremely radioresistant. Miller and Cole (1967) could find no change in numbers of long-lived plasma cells following 800 r. Exposure of spleen cells in diffusion chambers to 10,000 R during the plateau phase of secondary antibody formation results in a relative increase in the proportion of plasma cells (Makinodan *et al.*, 1967), indicating their radioresistance. Based on the incorporation of amino acids into specific antibody, Vann and Makinodan (1969) also showed that the rate of antibody synthesis is not affected by 10,000 R, and morphological confirmation of the integrity of the cytoplasmic structures of plasma cells given 10,000 R was shown by Sado (1969).

Direct assessment of radiobiological parameters of plasma cell radiosensitivity has been made using the plaque-forming assay with either: (i) cells irradiated *in vitro* and then immediately assayed (Kennedy *et al.*, 1965); or (ii) irradiated *in vivo* in chambers and then transferred to a

new host for 3 days before assay (Sado *et al.*, 1971). Survival curves show D_{37} values of the order of 6000–9000 R for IgM PFC and comparable values for IgG cells, with a clear threshold for inactivation as reflected by the extrapolation number (see Fig. 13). Similarly, the numbers of background or natural PFC found in spleens of unimmunized mice are not significantly reduced following whole-body irradiation, indicating that their maintenance is not markedly dependent on continued cell proliferation (Hege and Cole, 1967).

b. The Secondary Antibody Response. In general, the secondary response, when considered in its entirety, appears to be more radioresistant *in vivo* than the primary response. Thus, at comparable times of antigenic challenge after a relatively low dose of irradiation (200 r), a greater depression of primary than secondary antibody responses is observed (Fig. 14) (Stoner and Hale, 1962). Numerous similar studies have shown

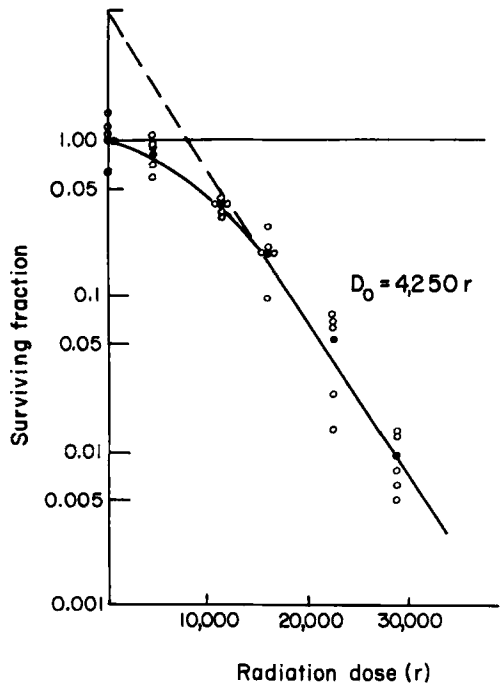


FIG. 13. Relation between magnitude of irradiation and numbers of indirect plaque-forming cells (PFC). Mice bearing diffusion chambers containing immunized spleen cells were irradiated with indicated doses, and the chambers then transferred to new hosts. PFC in the chambers were assayed 3 days later. ○——○, values obtained from individual chambers; ●——●, the mean for each radiation dose. (After Sado *et al.*, 1971.)

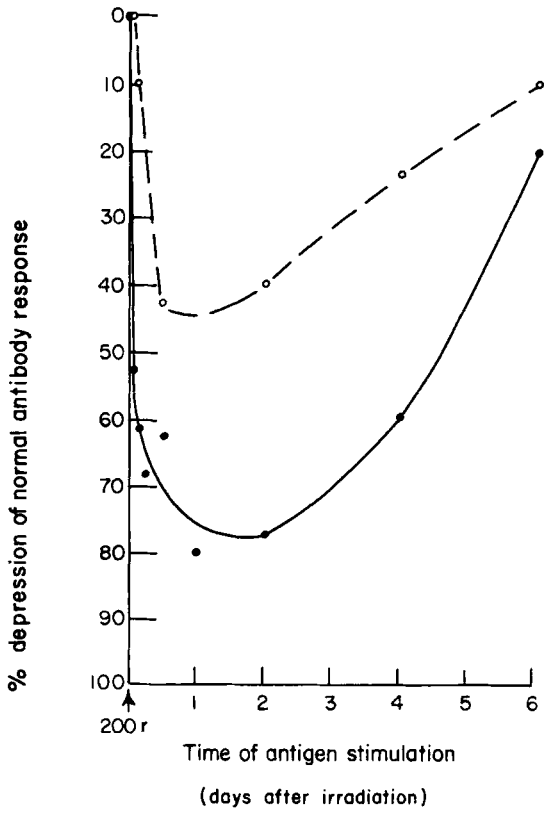


FIG. 14. Relative radiosensitivity of the primary (●——●) versus secondary (○— —○) antibody response. In these studies, the effect is estimated in the whole animal by measuring overall antibody response for both the primary and secondary components, and the results are plotted relative to control nonirradiated animals given primary or secondary immunization, respectively. (After Stoner and Hale, 1962.)

that secondary responses can be considerably depressed by preirradiation, but to varying degrees relative to the primary response, the magnitude of the discrepancy between the primary and secondary responses apparently being dependent on the system under study (Taliaferro *et al.*, 1952; Crosland-Taylor, 1955; Makinodan *et al.*, 1959; Porter, 1960, Thorbecke *et al.*, 1964). Subsequent studies, however, have led to the conclusion that the actual radiosensitivities of the cells mediating primary and secondary responses are identical. Detailed measurements of the radiosensitivity of the secondary response were made by Makinodan *et al.* (1962) using the cell transfer system, under conditions in which linear relationships between viable cell numbers and antibody titers occur. The magnitudes of

the decreases in the primary and secondary antibody-forming activities of spleen cells after a given dose of X-rays were approximately the same. Taliaferro and Taliaferro (1969) also observed that the IgM response to Forssman antigen was of equal radiosensitivity in the primary and secondary response. *In vitro* responses to bacteriophage antigen also showed similar levels of depression by radiation for primary and secondary responses (Tao and Leary, 1969). In these latter two studies, it was in fact inferred that the memory cells might be *more* sensitive to irradiation than the initial immunocompetent cells. Cells responding in the secondary response are thought to represent specific differentiation products of the memory cell pool induced by the primary antigenic stimulus (Sercarz and Coons, 1962). As the antigenic stimulus subsides, expansion of the pool decreases and may not regenerate after injury if the appropriate immunological stimulus is lacking. Thus, irradiation may permanently reduce or abolish the memory cell pool (Nettesheim *et al.*, 1969). Recovery of the memory cell pool after irradiation depends upon available antigen and accordingly could be influenced by many factors including the type and amount of primary antigen, the primary irradiation interval, and the radiation dose (Nettesheim *et al.*, 1967; Nettesheim and Williams, 1968).

These various studies therefore infer that if the radiosensitivity of the cells involved in primary and secondary responses are similar, other factors must be operative *in vivo* to affect the actual expression of immunity. Perhaps the major considerations in this regard concern cell kinetics and the role of germinal centers.

The basis of the apparently conflicting data relative to the radiosensitivity of the secondary response has been discussed by Makinodan and Price (1972) in terms of the full potential response. Previous studies had shown that although the difference in magnitude of serum antibody levels in primary and secondary responses might only be 2-fold in certain situations, the primed animal actually possessed up to 100 times more potentially responsive immunocompetent units than the unprimed host (Makinodan and Albright, 1967). In other words, the ratio of actual immunological expression to potential expression is greater in a primary than in a secondary response. Thus, for a given radiation dose and with identical radiosensitivities of primed and unprimed cells, a much greater number of potentially reactive cells would remain in the primed animal and a change in the ratio of actual to potential expression might occur. Such a shift would mute the effect of radiation.

Various studies on radiosensitivities of germinal center structures have suggested that a close correlation exists between reappearance of these structures and recovery of specific immune potential (memory) following

irradiation (Thorbecke *et al.*, 1964). As noted previously, the radiosensitivity of the antigen-trapping network of lymphoid follicles may particularly play a role in the radiation-induced suppression of memory development (Nettesheim and Hanna, 1969). A detailed analysis of this problem was recently made comparing tertiary response potential to germinal center damage (Grobler *et al.*, 1974). It had previously been observed that, whereas 800 r had no effect on secondary antibody responses when given 4 days after secondary challenge, a marked suppression of anamnestic responses occurred with irradiation on days 1–3 (Hale and Stoner, 1963). Cytokinetic analysis (Grobler *et al.*, 1974) revealed that plasma cellular proliferation in lymph node medullary cords reached a marked and narrow peak on day 3 and had subsided almost to control levels between days 4 and 5—hence, the marked suppressive action of irradiation given up to day 3 on the ongoing antibody response. In contrast, however, germinal center formation did not attain full development until day 5 to 6 after the booster. Radiation on day 4, although not suppressing the ongoing antibody response, almost totally abrogated priming for subsequent teritiary responsiveness, indicating that germinal center formation was particularly relevant to subsequent memory generation but was not involved in the concurrent antibody-producing plasma cell response. This study particularly demonstrates how radiation given at precise stages of the immune response can be extremely useful to the immunologist in dissecting different cellular components of an active immune response.

C. Radiosensitivity of Lymphocyte Subpopulations Involved in Antibody Formation

From an analysis of the various studies considered in the preceding two sections, it would seem clear that lymphocytes are the prime target of radiation-induced inhibition of humoral antibody formation. In general, those studies seem to show a radiation sensitivity similar to that affecting the proliferative ability of a wide variety of other mammalian cell types (Puck and Marcus, 1956; Elkind and Whitmore, 1967). However, since many of the *in vivo* studies can be influenced by an abscopal, or remote, effect, and since it is now firmly established that many humoral antibody responses require interaction between T and B lymphocytes, it is accordingly relevant to attempt to assess directly the radiosensitivity of the various lymphoid cell populations, with particular reference to their possible stage of activation. Since we have considered other approaches to direct T- and B-cell radiosensitivity elsewhere (Sections II and IV), this section concerns only direct studies involving antibody formation.

In most cell-interaction systems studied, it has been proposed that the T cell is the limiting cell type (Groves *et al.*, 1970; Price and Makinodan, 1970; Vann and Dotson, 1974; Addison, 1974a; Janeway, 1975a), and accordingly the radiosensitivity of the overall response (e.g., to SRBC) is primarily a measure of T-cell radiosensitivity. The results of several such studies are listed in Table VII, and in general, most of the D_{37} values fall in the range defined for other proliferating cells. In relation to subsequent comments on the possible radioresistance of helper T-cell activity, it is to be noted that all the listed studies involve unprimed T-cell help situations. For comparison, Table VII also lists several other studies on T-cell radiosensitivity, which again show values predominantly in the range of 70–100 r.

Relatively few reports have dealt directly with B-cell radiosensitivity. Several studies have described considerable radiosensitivity of the avian B-cell lineage (derived from the avian bursa of Fabricius) (Warner, 1967). Although embryonic bursectomy by hormones (Warner *et al.*, 1969) or surgery (Cooper *et al.*, 1969) will totally prevent antibody and Ig synthesis, surgical bursectomy at hatch is less effective. This is presumed to be due to the movement of bursa-derived cells into the peripheral lymphoid tissues prior to hatching. If sublethal whole-body irradia-

TABLE VII
RADIOSENSITIVITY OF LYMPHOCYTE SUBPOPULATIONS AS ASSESSED IN VARIOUS SYSTEMS

System	D_{37} (r)	Reference
T-dependent humoral response	70	Makinodan *et al.*, 1962
T-dependent humoral response	47–58	Celada and Carter, 1962
T-dependent humoral response	81	Simic *et al.*, 1965
T-dependent humoral response	80	Kennedy *et al.*, 1965
T-dependent humoral response	214	Haskill *et al.*, 1970
T-dependent humoral response	188	Petrov and Cheredeev, 1972
T-dependent humoral response	50	Addison, 1974a
B-cell responses (antibody production)	96	Addison, 1974a
B-cell responses (antibody production)	70	Janeway, 1975b
B-cell responses (Ig production)	70–145	Anderson and Warner, 1975
B-cell responses (cell viability)	40	Anderson *et al.*, 1975
T-cell responses (GVH)	74	Smith and Vos, 1963
T-cell responses (GVH)	100	Sprent *et al.*, 1974
T-cell responses (GVH)	75	Blackett, 1965
T-cell responses (proliferation)	64	Anderson *et al.*, 1972
T-cell responses (cell viability)	100	Anderson *et al.*, 1974
T-cell responses (allograft)	75	Celada and Carter, 1962
T-cell responses (helper)	230	Janeway, 1975b

tion is given to newly hatched bursectomized chickens, much greater immunodepression is observed even with doses of 250 r (Cooper *et al.*, 1966). Direct *in vivo* irradiation studies have also shown massive destruction of bursal lymphoid follicles without normal regeneration (Weber and Weidanz, 1969). In the relatively few reports on mammalian B cells (Table VII), D_{37} values similar to those for T cells were observed. These studies have included *in vivo* immune responses to T-independent antigens (Addison, 1974b), direct survival estimates of B cells irradiated *in vitro* (Anderson *et al.*, 1975), and cell-transfer studies of B-cell populations irradiated *in vitro* and then assessed for *in vivo* antibody (Janeway, 1975b) or Ig-producing capacity (Fig. 15) (Anderson and Warner, 1975). In most of these systems continued cell proliferation is required, and accordingly it is not possible to conclude whether radiation damage is due to interphase death or to inhibition of cell proliferation. These reports are comparable, however, with the conclusions (see Section II) that: (i) when cell division is required, the D_{37} of T and B cells are approximately similar; and (ii) interphase death occurs very rapidly with B cells, somewhat less rapidly with T cells (Anderson *et al.*, 1974), and comparatively slowly (if at all) with activated T cells. The functional expression of irradiated activated T cells may, however, be dependent

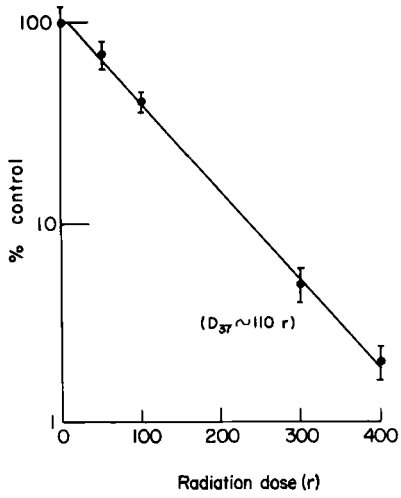


FIG. 15. Immunoglobulin (Ig) synthesis as a function of radiation dose. BABB/14 spleen cells, 1×10^6, were irradiated *in vitro* and transferred BALB/c recipients which had received 400 r 24 hours prior to cell transfer. The recipient serum was assayed for donor allotype Ig by radioimmunoassay, and the results are expressed as a percentage of the amount of Ig produced with control (nonirradiated) cells. (After Anderson and Warner, 1975.)

upon additional factors, particularly on whether the cells are located in the appropriate microenvironment.

Considerable controversy has arisen concerning the radiosensitivity of helper T cells in different experimental situations. Although initial studies indicated that the collaborative activity of helper T cells in primary antibody formation was quite radiosensitive (Miller and Mitchell, 1968; Claman and Chaperon, 1969; Ito and Cudkowicz, 1971), subsequent studies on carrier-primed helper cells in guinea pigs indicated a very high degree of radioresistance (5000 r) (Katz *et al.*, 1970), and *in vitro* analysis of the radiosensitivity of primed mouse T cells also showed resistance up to 4000 r (Kettman and Dutton, 1971). It was accordingly proposed by Katz *et al.* (1970) that the apparent discrepancy might relate to the stage of activation of the T cell at the time of irradiation. In other words, antigen-activated helper T cells might be protected from interphase death by virtue of their heightened metabolic activity, which serves to activate repair mechanisms, or otherwise stabilize radiosensitive lymphocyte structures and/or metabolic processes. Many precedents exist for this, in that other T-cell subpopulations activated in various ways are more resistant to irradiation than their corresponding nonactivated controls (Stefani, 1966; Schrek, 1968; Conrad, 1969; Cirkovic, 1969; Brannon and Dewey, 1969; Rickinson and Ilbery, 1971; Renner and Renner, 1971; Field *et al.*, 1972; Nichols *et al.*, 1975). Activation in these studies does not protect cells from radiation-induced mitotic death, but rather only permits their survival (Conard, 1969; Rickinson and Ilbery, 1971). Thus, if helper effects do not require continued cell division, various functions of these cells might continue, and although the *generation* of helper T cells induced by specific antigen would be radiosensitive since it involves cell division (e.g., Kappler *et al.*, 1974), the effectors of helper activity would be radioresistant.

Subsequent studies by Anderson *et al.* (1972) showed that, in a different experimental design, *in vitro* irradiated primed helper T cells *were* radiosensitive as shown by their inability to help in an *in vivo* adoptive transfer (Table VIII). Therefore, it appears that other factors, besides the activation stage, can affect the radiosensitivity of helper T cells, foremost among which is the ability of the irradiated cells to repopulate the lymphoid organs of the adoptive host.

Analysis of the radiosensitivity of helper T cells must involve specific consideration of the particular experimental design. Various studies in this field have been listed in Table IX on this basis and will now be briefly considered.

1. Unprimed T cells. Regardless of whether irradiation is given *in vivo* or *in vitro*, or whether the assay system is adoptive transfer (classical

TABLE VIII

EFFECT OF *in Vitro* IRRADIATION OF CARRIER-SPECIFIC THORACIC DUCT LYMPHOCYTES (TDL) ON ANTIHAPTEN ANTIBODY RESPONSE[a,b]

Group	Number and source	Radiation dose (r)	Number of irradiated recipients	7 S PFC/spleen		*P* values (cf. group 2)	
				FγG	NNP	FγG	NNP
1	—	—	4	40 (150–10)	230 (1820–30)	<0.005	<0.005
2	10^6 FγG-primed TDL	—	7	18,030 (23,410–13,890)	88,870 (116,120–68,020)	—	—
3	10^6 FγG-primed TDL	1000	6	2 (3–1)	70 (200–30)	<0.005	<0.005
4	10^7 FγG-primed TDL	1000	7	10 (20–30)	50 (100–20)	<0.005	<0.005
5	5×10^7 FγG-primed TDL	1000	5	3 (9–1)	5 (16–2)	<0.005	<0.005

[a] After Anderson *et al.* (1972).

[b] TDL from CBA donors primed to FγG 5 months previously were exposed to 1000 r *in vitro* and various cell numbers were injected intravenously into lethally irradiated recipients together with 6×10^6 anti-θ serum-treated spleen cells from CBA mice primed to NNP-OVA 6 weeks before cell transfer. The irradiated recipients were challenged with 50 μg of fluid NNP-FγG at the time of transfer, and PFC responses to both FγG and NNP were determined 7 days later. Results expressed as geometric mean ± SE. FγG, fowl immunoglobin; NNP, 4-hydroxy-3,5-dinitrophenylacetic acid; 7 S PFC, indirect plaque-forming cells.

TABLE IX
RADIOSENSITIVITY OF T-CELL HELPER FUNCTION

T cells	Irradiation	Assay	Radio-sensitivity[a]	Reference
Unprimed T cells				
Normal	*In vitro*	Adoptive transfer	Sensitive	Miller and Mitchell, 1968
Normal	*In vitro*	Adoptive transfer	Sensitive	Claman and Chaperon, 1969
Normal	*In vitro*	Adoptive transfer	Sensitive	Ito and Cudkowicz, 1971
Normal	*In vivo*	*In vitro*	Sensitive	Kappler *et al.*, 1974
Primed T, *in vitro* assay				
Primed	*In vitro*	*In vitro*	Resistant	Kettman and Dutton, 1971
Primed	*In vivo*	*In vitro*	Resistant	Katz *et al.*, 1973
Primed	*In vivo*	*In vitro*	Resistant	Katz and Unanue, 1973
Primed T, adoptive transfer assay				
Primed	*In vivo*	Adoptive transfer	Sensitive	Hamaoka *et al.*, 1972
Primed	*In vitro*	Adoptive transfer	Sensitive	Hamaoka *et al.*, 1972
Primed	*In vitro*	Adoptive transfer	Sensitive	Anderson *et al.*, 1972
Primed	*In vitro*	Adoptive transfer	Sensitive	Anderson *et al.*, 1974
Primed	*In vitro*	Adoptive transfer	Sensitive	Janeway, 1975b
Primed	*In vitro*	Adoptive transfer	Resistant	Katz *et al.*, 1970
In situ irradiation				
Primed	*In situ*	*In vivo*	Resistant	Hamaoka *et al.*, 1972
Primed	*In situ*	(No transfer)	Resistant	Klinman, 1972
Primed	*In situ*	(No transfer)	Resistant	Playfair, 1972
Primed	*In situ*	(No transfer)	Resistant	Janeway, 1975b
Nonspecific help				
Normal	*In vitro*	*In vitro*	Resistant	Hirst and Dutton, 1970
Normal	*In vitro*	*In vitro*	Resistant	Munro and Hunter, 1970
Normal	*In vitro*	*In vitro*	Resistant	Farrar, 1974
Normal	*In vitro*	Adoptive transfer	Resistant	Warner and Anderson, 1975
Normal	*In vitro*	Adoptive transfer	Resistant	Gershon *et al.* 1974

[a] In most of these studies, dose-response relationships were not determined. "Resistant" usually refers to no change in activity following high-dose exposure (800 r or greater); and "sensitive," to significant inhibition with doses in the range of 300–1000 r.

cell interaction) or *in vitro*, unprimed T cells are quite radiosensitive. These data are consistent with other approaches that clearly indicate that helper T cell activity is generated only after T-cell proliferation (Davies, 1969; Miller and Mitchell, 1969b).

2. Primed T cells, *in vitro* assay. Effector helper T cells are clearly radioresistant when their activity is assayed *in vitro*. This assay system does not put other constraints on the helper T cells, such as homing requirements, and would therefore imply: (i) that antigenically activated helper T cells are not sensitive to radiation-induced interphase death; and (ii) that further cell proliferation is not required on behalf of the T cell

for its helper expression. The only caution that might be raised in this context concerns the need to demonstrate antigenic specificity of the helper cell (see nonspecific help below).

3. Primed T cells, adoptive transfer assay. In distinction to the clear demonstration of radioresistance of primed helper T cells when assayed *in vitro,* most studies with adoptive transfer systems have shown that helper expression is radiosensitive with D_{37} values of the order of 230 r (Janeway, 1975b). If radiation has not caused interphase death in these cells, and proliferation is not required, other factors must influence the ability of the helper cells to mediate their effects after transfer. Recent studies by Anderson *et al.* (1974) have demonstrated marked alteration in homing patterns of irradiated lymphocytes, thereby suggesting that a failure of irradiated helper cells to home properly to lymphoid organs is the reason for their failure to satisfactorily interact with B cells.

4. *In situ* irradiation. Confirmation of this latter concept is found during studies in which cell homing is not required after irradiation, and helper effects have been found to be radioresistant. Recipient animals were first primed with carrier, heavily irradiated, and then provided with a source of B cells. High titers of carrier determined antibody result. A variation on this system has been to transfer adoptively primed helper cells, to irradiate the recipient at various times after transfer, and then to provide a B-cell source. Cell migration during the initial 24 hours after cell transfer appears to be quite critical. The capacity of adoptively transferred carrier-primed lymphocytes to exhibit helper activity is abolished by irradiation of the adoptive host immediately, but not 24 hours, after cell transfer (Hamaoka *et al.,* 1972). Factors that might influence specific cell homing might also affect the apparent radiosensitivity of the helper effect. In this connection, significant helper activity has been shown to persist when carrier antigen was given to the recipients immediately after 500 R, irrespective of whether the recipients were irradiated immediately or 24 hours after helper cell transfer (Janeway, 1975b). Although a mechanism of altered homing has not been well defined in this situation, injection of antigens has been shown to affect the regulation of lymphocyte trapping in various lymphoid tissues (Zatz and Gershon, 1975).

5. Nonspecific help. Many mechanisms of nonantigen-specific, but T cell-mediated, help have recently been described (e.g., Katz and Benacerraf, 1972). When examined with *in vitro* or *in vivo* assay systems, these effects have all been found to be radioresistant. These studies include: (i) allogeneic (Hirst and Dutton, 1970; Munro and Hunter, 1970) and xenogeneic (Farrar, 1974, 1975) helper effects on *in vitro* SRBC responses; (ii) syngeneic thymic helper effects on the *in vivo* transfer of Ig expression (Warner and Anderson, 1975); (iii) and *in vivo* prolifera-

tive responses of T cells against allogeneic antigens (Gershon *et al.*, 1974).

From these various studies, it therefore appears that T-cell helper function in antibody production, like that of T-cell function in cellular immunity (see Section IV), does not require proliferation once the initial antigen-induced process of differentiation and clonal expansion has occurred (Hamaoka *et al.*, 1972), and that activated T cells are resistant to radiation-induced interphase death.

As discussed above in Section III,B, many situations have been observed in which radiation results in augmentation of a given immune response. One possible mechanism (see Table VI) involves the elimination of a homeostatic regulatory cell-mediated mechanism. Such a cell type has been described and extensively studied in the last few years. Although many current studies are now attempting to characterize this cell in terms of known lymphocyte cell surface receptors and antigens, it is already reasonably certain that suppressor T cells are a distinct, functional T-cell subpopulation. Many aspects of their role in the regulation of the immune response and in tolerance have been recently reviewed (*Transplantation Reviews,* 1975, Vol. 26). In considering the possible effects of irradiation on this cell type, the assay systems available at present are far from simple in that most studies involve assessment of an alteration in the balance of suppressor and helper functions. The available systems might be considered from two points of view.

a. Systems in which whole-body irradiation results in augmentation of the immune response: a similar augmentation can be shown by other means of lymphocyte depletion (adult thymectomy or antilymphocyte serum), *and* thymus cell injection results in depression of the immune response. This combination has been seen in humoral antibody responses, namely, homocytotropic antibody production (Tada *et al.*, 1971) and in the response to polyvinyl pyrrolidone (Rotter and Trainin, 1974). In both cases, thymus cells are capable of reducing the enhanced response engendered by whole body irradiation. Similarly, Nichols *et al.* (1975) observed enhanced cytotoxic lymphocyte generation following low-dose irradiation (50–150 r), and Sabbadini (1974) reported increased cell-mediated cytotoxicity following whole-body irradiation. Again, thymus-cell injections suppressed the response.

Various groups have previously reported (e.g., Dixon and Weigle, 1957; Nossal, 1959b) that neonatal animals are poor recipients for the adoptive transfer of primary antibody formation by adult cells. Recent studies by McCullagh (1975b) have shown that this inability to respond may be due to the presence of a thymus-derived suppressor cell. In this model, he also observed that relatively low doses of whole-body irradiation

(350 r) would overcome this neonatal suppressive activity (McCullagh, 1975a).

From the doses of irradiation used in some of these studies, it would appear that suppressor T-cell activity is quite radiosensitive. The particular model systems do not reveal whether suppressor cell proliferation is necessary for the effect, but from the short time involved in some of the experiments, the possibility clearly exists that suppressor cells are undergoing radiation-induced interphase death. McCullagh (1975a) also observed that the exposure of neonates to antigen (SRBC) before irradiation increased their resistance to adoptive immune responses, providing irradiation was given no later than 24 hours after antigen. This suggests that the antigenically activated suppressor cell becomes more radioresistant, at least for a short period. This parallels observations on another suppressor system, which is not antigen specific, namely the ability of Con A-activated T cells to suppress *in vitro* antibody formation to SRBC. In these studies (Dutton, 1972; Rich and Pierce, 1973) irradiation of the T cells before incubation with mitogen abrogates the generation of suppressor cells, whereas the mitogen-activated cells are still capable of suppression even after 2000 R.

b. The second type of model involves situations in which the addition of a T-cell population has an enhancing effect on the particular immune response and after irradiation of this T cell source an even greater enhancement is observed. Such a result might be interpreted as follows: The T-cell population contained a radioresistant helper component and a regulating (suppressor) radiosensitive component. The latter tended to minimize the effect of the former, and thus, irradiation would result in greater enhancement. Systems in which this has been observed have not shown antigen specificity of the suppressor component, so the nature of the suppressor cell's specificity is not clear. The system would best be explained perhaps by an idiotypic type of recognition of the suppressor cell for the corresponding helper cell. Results of this type have been observed in the nonspecific helper effect on *in vitro* antibody formation (Fig. 16) (Hirst and Dutton, 1970), in thymus cell augmentation of the adoptive transfer of Ig production between allotype congenic mice (Warner and Anderson, 1975), and in the ability of thymocytes to enhance proliferative responses to allogeneic antigens (Gershon *et al.*, 1974). In this latter system, it was concluded that feedback signals between different populations of T lymphocytes are particularly important in immunological homeostasis, and irradiation can clearly alter the balance by affecting one specific subpopulation in a preferential manner.

Although these data are all fairly preliminary and the systems employed are quite complex, at present it might be proposed that the suppressor

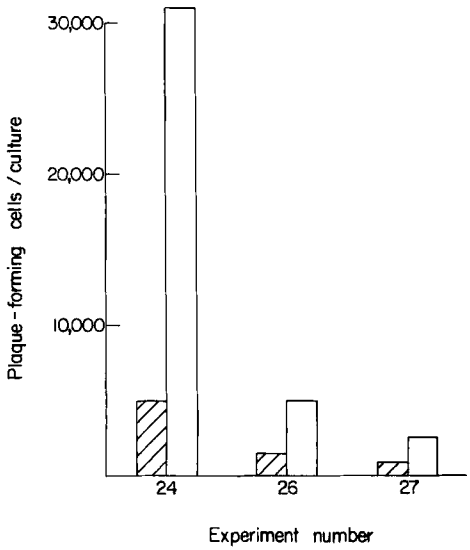


FIG. 16. Capacity of nonirradiated (□) and irradiated (▨, 1100 r) nonattached BALB/c spleen cells to restore the plaque-forming response to neonatal thymectomy (nTx) BDF_1 spleen cells Irradiated cells received 1100 r *in vitro;* results are expressed as arithmetic mean with 3–4 nTx mice/group. (After Hirst and Dutton, 1970.)

lineage of T cells is a distinct subpopulation with a suppressor precursor and a suppressor effector cell, and that the former is quite radiosensitive, perhaps susceptible to interphase death, and the activated cell is more resistant. However, both stages may have suppressive properties, perhaps to varying degrees. This subpopulation would thus parallel the helper T and cytotoxic T cell subpopulations in the conversion from radiosensitivity to resistance on activation. The balance and interaction between these cell types is clearly of major importance in any future considerations of how radiation may affect any particular immune response.

IV. Effects of Radiation on Cellular Immune Reactions

A. Delayed Hypersensitivity

Until recently, evaluation of the relationship between radiation and DTH reactions was complicated by the fact that *in vivo* these reactions could only be assessed semiquantitatively and that little information was available on the relation between the development of a skin lesion and the number of sensitized lymphocytes. Accordingly, the possibility of

detecting small radiation-induced changes has been more limited than with antibody production, particularly when the latter included measurement of actual numbers of antibody-forming cells. In the absence of a sensitive assay, much of the work to date has involved the measurement of indurated skin lesions in guinea pigs or footpad or ear swelling in mice. Considerable radiation-induced impairment of this component of the immune response probably must take place before it is detectable by this approach.

With these reservations in mind, however, there is general agreement that the induction of DTH is more radioresistant than that of the antibody response. Thus, whole-body exposure of guinea pigs to 300 r prior to sensitization with diphtheria toxoid markedly suppressed the development of circulating antitoxin but failed to inhibit the induction of DTH. As a consequence, exposure to 300 r 18–24 hours before sensitization extended the period of "pure" DTH (e.g., no demonstrable antibody) from about 7 days in the control group to approximately 12 days in the irradiated animals. Similar results were noted with 200 r. Doses of 50 and 100 r caused a decrease in antitoxin titers although DTH lasted for a normal period of time. On the other hand, when 300 r was given 18–72 hours after sensitization, DTH persisted for the usual period and circulating antibody first appeared at the usual time after sensitization (Salvin and Smith, 1959). Similarly, Schipior and Maguire (1966) showed that a single exposure to 200–250 R failed to suppress the acquisition of allergic contact dermatitis to dinitrochlorobenzene in guinea pigs. These findings were confirmed by Uhr and Scharff (1960), who also investigated a second species, the rabbit, and other antigen–antibody systems. Moderate doses of radiation (400 r) given to rabbits before sensitization suppressed antibody formation to a greater degree than DTH responses. However, large doses (800 r) completely suppressed both types of responses. The febrile reaction often associated with the development of DTH may be a more sensitive end point than evaluation of skin reactions. In guinea pigs given 200 r before sensitization, a febrile response occurred on systemic challenge with antigen in both irradiated and control groups (Uhr and Scharff, 1960). This response occurred in animals showing suppression of their antibody responses, again suggesting that DTH reactions are more radioresistant than antibody responses.

The systems reported above are clearly not capable of providing accurate estimates of the radiosensitivity of cells involved in DTH reactions. Accordingly, adoptive transfer studies will be required to give more reliable estimates of the radiosensitivity of both the precursor and effector cells involved in delayed-type reactions. Recent studies have, however, indicated that certain histocompatibility restrictions operate in control-

ling effective transfer of DTH reactions (Miller *et al.*, 1975), and this factor may have to be considered in assessing radiosensitivity when nonsyngeneic transfer systems are used. Using syngeneic combinations, M. A. Vadas, R. E. Anderson, N. L. Warner, and J. F. A. P. Miller (unpublished results, 1975) have recently observed moderate radiosensitivity of the cells capable of transferring DTH reactions (complete suppression observed with 500 r of donor primed cells).

Apparent inconsistencies also exist regarding the radiosensitivity of DTH reactions in cell transfer systems that probably relate to impaired homing by irradiated cells. Thus, as shown in groups C and D of Table X, Kettman and Mathews (1975) noted radioresistance of DTH reactivity in a cell transfer system only when the irradiated cells were injected into the site of antigen challenge. Intravenous transfer of sensitivity could be affected by nonirradiated spleen cells from primed mice, but not by irradiated cells (group A and B, Table X). Similar results have been reported (Asherson and Loewi, 1967) utilizing lymph node and peritoneal exudate cells; DTH activity was reduced by 1500–3000 r administered *in vitro* prior to intravenous transfer.

TABLE X

EFFECT OF IRRADIATION ON CAPACITY OF PRIMED SPLEEN CELLS TO TRANSFER DELAYED-TYPE HYPERSENSITIVITY[a,b]

	Conditions of cell transfer			
Group	Number and source of spleen cells	Radiation dose (R)	Route of cell transfer	Anti-SRBC response, footpad swelling at 24 hr Δ mm (±SEM)
A	10×10^7 SRBC primed	—	I.v.	0.60 ± 0.06
	10×10^7 SRBC primed	1500	I.v.	−0.03 ± 0.03
B	3×10^7 SRBC primed	—	I.v.	0.60 ± 0.15
	3×10^7 SRBC primed	1500	I.v.	0.03 ± 0.09
C	4×10^6 SRBC primed	—	Footpad	0.50 ± 0.20
	4×10^6 SRBC primed	1500	Footpad	0.60 ± 0.10
D	8×10^6 SRBC primed	—	Footpad	0.93 ± 0.10
	8×10^6 SRBC primed	1500	Footpad	0.80 ± 0.08

[a] After Kettman and Mathews (1975).

[b] In groups A and B, spleen cells used for transfer were taken from mice 4 days after priming with 0.2 ml of a 0.01% suspension of SRBC. Half of the sample was exposed to 1500 R. Four days after the footpad challenge, the recipients were sacrificed. In groups C and D, spleen cells used for transfer were taken from mice 6 days after priming with 0.2 ml of a 0.01% suspension of SRBC. Half of the sample was exposed to 1500 R. The recipients were primed with 0.2 ml of a 0.01% suspension of SRBC 4 days prior to footpad challenge. Half of these primed mice were exposed to 1500 R immediately before challenge.

Recent observations by Elliott *et al.* (1975) have inferred that two populations of lymphocytes are competent in the transfer of DTH to normal recipients. It is possible that putative differences in the radiosensitivity of these two populations may contribute to the apparent contradictions among experiments designed to evaluate the radiosensitivity of DTH reactions.

Irradiation of the recipients prior to cell transfer generally results in a diminution of the DTH response in the recipient animals. Transfer of primed spleen cells into the footpads of normal mice exposed to 1500 R did not result in a delayed-type response (Kettman and Mathews, 1975). Smaller doses are also effective. Thus, Coe *et al.* (1966) reported that irradiation of recipient rats (550 R) before transfer of sensitized cells totally prevented the expected delayed reaction, provided the skin-test challenge was given soon after the cell transfer and irradiation. Whole-body exposure of recipient guinea pigs to 130 r at 4 days before cell transfer from sensitized donors produced a diminution in the tuberculin reactivity of the recipient animals (Cummings *et al.*, 1955). The passive transfer by sensitized cells of experimental allergic encephalitis (EAE) into recipients was also inhibited by irradiation of the recipients 24 hours before cell transfer (Levine *et al.*, 1969). Complete inhibition occurred with 700 or 1000 R, partial inhibition with 400 R, and no inhibition with 100 R. These experiments suggest that two components are necessary for the successful transfer of DTH. One component is a radioresistant T cell contributed by the donor, and the second is a more radiosensitive component derived from the host. This hypothesis is consistent with various studies which indicate that the majority of infiltrating cells in DTH lesions are of host origin (Bloom and Chase, 1967) and involve the proliferation of bone marrow-derived cells (Lubaroff and Waksman, 1968).

In summary, DTH reactions appear at present to be relatively radioresistant, especially when the sensitized cells are irradiated *in situ* or are transferred in concert with the involved antigen to the specific site where the evaluation will transpire. In such a setting, the apparent radioresistance of the involved lymphocytes probably relates to one or more of the following observations: (1) DTH reactions involve T cells that appear to be more radioresistant than B cells; (2) many of the experimental systems evaluated to date require limited traffic on the part of the involved T cell; (3) many of the experimental systems involve antigen-activated T cells, which are more radioresistant than nonactivated cells; (4) some of the experimental models employed to evaluate the relationship between radiation and DTH reactions involve numbers of sensitized cells greatly in excess of that required to evoke a

maximum response; therefore, radiation-induced death of a significant number of the involved cells does not result in a detectable diminution of the response in question. Further studies on the radiosensitivity of the cells mediating this response are required, preferably using cell transfer systems and more quantitative assays such as have been recently described (Vadas *et al.*, 1975).

B. Graft-versus-Host Reactions

Closely related to the above is the GVH response. With respect to radiation, the magnitude of this response may be studied by (1) irradiation of the parental-strain lymphocytes prior to their injection into F_1 hybrids; (2) irradiation of the hybrid recipients prior to the injection of parental-strain lymphocytes.

In the latter situation, irradiation of the F_1 recipient 2 days prior to injection influences the subsequent GVH reaction in several ways depending upon the involved dose. With adult rats as recipients, small doses of radiation (up to 300 r) increase the severity of the GVH reaction. Thus, such doses increase peripheral vasodilatation (a manifestation of GVH disease in the rat) and permit maximum splenomegaly with small numbers of parental cells. Larger doses (above 300 r) are associated with decreased peripheral vasodilatation and suppression of the associated splenomegaly (Blackett, 1965). The latter findings presumably relate to the observation that, except for the first 2 days, splenomegaly is predominantly due to proliferation of host cells (Davies and Doak, 1960; Fox, 1962; Nowell and Defendi, 1964; Owen *et al.*, 1965; Nisbet and Simonsen, 1967; Zeiss and Fox, 1963). The decrease in peripheral vasodilatation noted with large doses may relate to the poor condition of the heavily irradiated host (Blackett, 1965), since it was not noted in a second study when a variety of additional signs and symptoms were coupled to form an index of the severity of the GVH response (Field and Gibbs, 1965).

The effect of radiation upon the capacity of parental lymphocytes to elicit a GVH response is more complex and has been used effectively to investigate T–T cell cooperation. As shown in Fig. 17, irradiation inhibits the capacity of parental cells to elicit a GVH response in F_1 hybrids as measured by the Simonsen assay (Sprent *et al.*, 1974). Similar results have been reported by Biggar *et al.* (1971) in mice and by Blackett (1965) in rats. Radiation also inhibits the ability of parental cells to incorporate [^{3}H]TdR when introduced into lethally irradiated F_1 hybrids as shown in Table XI (Anderson *et al.*, 1972). At very large dose levels (3000 r), parental spleen cells, although themselves inactive,

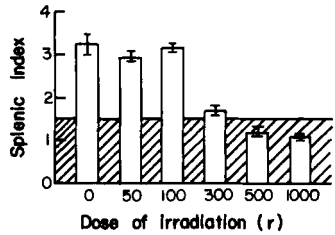


FIG. 17. Spleen indices in (CBA × C57BL)F_1 mice injected at birth with 2.5×10^6 CBA thoracic duct lymphocytes irradiated *in vitro* with indicated doses. Results are expressed as aritmetic mean (±SE) of 6–8 mice per group. Indices exceeding a value of 1.5 (hatched area) are considered significant. (After Sprent *et al.*, 1974.)

augmented the GVH reactivity of nonirradiated cells from the same inoculum (Blackett, 1965). Although no correlation with the number of exposed cells could be demonstrated, these results suggest that highly irradiated parental cells can release factors which activate other cells. In an investigation of T–T cell interactions, Gershon *et al.* (1974) have provided considerable insight into this unexpected observation.

In a series of experiments, Gershon and others have shown that the addition of F_1 spleen cells or thymocytes regulates the magnitude of splenomegaly and DNA synthesis produced by parental thymocytes in irradiated F_1 hybrid mice (Hilgard, 1970; Barchilon *et al.*, 1972; Lieb-

TABLE XI

PROLIFERATIVE CAPACITY OF IRRADIATED CBA THORACIC DUCT LYMPHOCYTES (TDL) TRANSFERRED TO LETHALLY IRRADIATED (CBA × C57BL) F_1 MICE[a,b]

Radiation dose (r)	Number of TDL administered			
	1×10^6	5×10^6	25×10^6	62.5×10^6
0	408,386 ± 63,089	—	—	—
50	389,159 ± 33,682	439,370 ± 10,540	—	—
100	303,571 ± 27,699	525,774 ± 5,018	—	—
200	120,541 ± 8,040	371,227 ± 29,622	—	—
300	36,563 ± 985	83,946 ± 16,794	—	—
500	22,169 ± 1,751	20,028 ± 1,503	25,682 ± 3731	–
1000	47,711 ± 17,749	34,134 ± 2,293	27,167 ± 385	31,882 ± 5566
5000	22,289 ± 1,928	23,406 ± 2,923	19,680 ± 1442	32,617 ± 4877

[a] After Anderson *et al.* (1972).

[b] Results are expressed as total disintegrations per minute per F_1 spleen 4 days after TDL injection and 30 minutes after the mice received an intravenous pulse of 25 μCi T^3H. Each figure represents the arithmetic mean (±SE) of the activity obtained from four such spleens. Background disintegrations when no TDL were injected = 21,543 ± 140.

haber *et al.*, 1972; Gershon *et al.*, 1972, 1974). F_1 thymocytes suppress the response of a highly responding inoculum of parental thymocytes and enhance the response of an inoculum that responds less well. Exposure of the F_1 thymocytes to 900 R tends to reverse the character of the regulatory effect: irradiation of F_1 cells with suppressor activity converts their regulatory effect to an augmentative one and vice versa. Irradiated (900 R) parental thymocytes, themselves incapable of DNA synthesis, affect the response of nonirradiated parental thymocytes in much the same way. When the response is high, the addition of 900 R parental thymocytes is suppressive; and when it is low, the addition of the irradiated cells is augmentative (Gershon *et al.*, 1974). Although a complete understanding of the various involved phenomena will require additional work, it is clear that F_1 and parental thymocytes exert a regulatory effect in the allogeneic transfer system, and that exposure of these populations of cells to 900 R does not abolish such regulatory activities but generally changes the character of the effect (from suppressive to augmentative and vice versa).

C. Transplantation Immunity

1. Experimental Allograft Rejection

The feasibility of pretreating prospective recipients with ionizing radiation to promote survival of foreign grafts was clearly demonstrated by Murphy and Taylor in 1918. This work appears to have been forgotten until the early 1950s when, after the pioneer studies of Medawar (1946) on the immunological basis of transplantation rejection, Dempster *et al.* (1950) showed suppression of skin homograft rejection by pretreatment of the recipients with radiation. An exposure of 250 R given to rabbits before the application of allogeneic skin grafts unmistakably prolonged the survival of the grafts. The second-set response, however, was unaffected by this dose of radiation.

Prolonged survival of skin grafts with only minor genetic differences can be induced by pretreating the recipients with ionizing radiation in nonlethal doses. A moderate delay in primary homograft rejection was observed (Micklem and Brown, 1961) in mice given 400 r. As shown in Fig. 18, the magnitude of this delay is proportional to dose (Brent and Medawar, 1966). Prolonged rejection of male skin grafts on female syngeneic mice has also been induced by exposing recipients to 300 r (Kelly, *et al.*, 1966).

The effect on graft survival of ECI of the circulating blood of calves before and after skin homografting has been described in 13 recipients (Chanana *et al.*, 1966). In all the ECI-treated calves, the normal acute

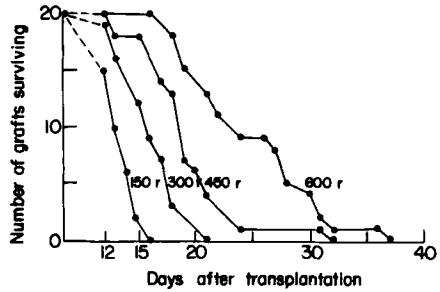


FIG. 18. Survival of A-strain skin grafts transplanted onto adult male CBA recipients 3 days after whole-body exposure to indicated doses. (After Brent and Medawar, 1966.)

rejection phenomenon, which characteristically occurs 9–10 days after grafting, was modified to a less violent process with a 1–11-day increase in rejection time.

These results clearly indicate that the homograft-rejecting capacity could be depressed by prior irradiation, although the relative radiosensitivity of primary versus secondary graft rejection was not defined. Tyan and Cole (1963a,b) analyzed this problem in a series of studies in which some variables, such as radiation dose and method of presensitization, were considered and xenogeneic grafts were compared with allogeneic grafts. Second-set responses of mice presensitized by means of allogeneic or xenogeneic skin grafts were more resistant to a lethal (850 r) or sublethal (670 r) dose of irradiation than first-set responses. Differential radiosensitivity of the xenogeneic and allogeneic reactions was also observed, but in opposite directions in primary versus second-set rejections (Tyan and Cole, 1963b).

Accurate measurements of the radiosensitivity of the immune mechanisms involved in homograft rejection are again difficult in the absence of a quantitative cell assay system. Two approaches to this problem have been reported. In one case (Celada and Makinodan, 1961), for estimating second-set rejection, recipient mice were primed with donor transplantation antigens, irradiated, and then given an injection of spleen cells of donor type, which have been previously sensitized to SRBC. The ability of the transferred cells to form anti-SRBC antibody in the recipient was then dependent upon radiosensitivity of the cellular immune response of the recipient. When recipients were given 500 r, only a few animals responded to the SRBC challenge, indicating almost complete rejection of the transferred cells. With 700–850 r the antibody responses by the donor cells were intermediate, and, with 900 r, titers comparable to isologous controls were observed indicating complete suppression of the homograft response.

Further quantitative evidence of the radiosensitivity of homograft immunity comes from a second assay method (Celada and Carter, 1962) in which the killing effect of parental (P_1 or P_2) cells was studied in irradiated, immunologically inert ($P_1 \times P_2$) F_1 recipient mice by determining the decrease of anti-rat agglutinins synthesized by P_2 cells. The data showed that the homograft-rejecting capacity was more radioresistant than the agglutinin-forming capacity. Slight strain differences were also observed. The D_{37} values for agglutinin formation by C3H and C57 cells were 58 and 47 r, respectively. The corresponding value for homograft-rejecting capacity (C3H cells) was calculated to be 78 r, which is in the range of radiosensitivity calculated for cells in the inductive phase of the humoral antibody response. This suggests that cell proliferation is the major radiosensitive step in the development of a homograft response.

2. *Cytotoxic Lymphocytes*

One problem with the interpretation given in this latter section is that the particular assay system used has not been proved to represent graft rejection by a direct T-cell process, and that cytotoxic or protective antibody formation may also be involved. In fact, in a further extension of this assay method (Capalbo, 1968), evidence was presented that the reaction could proceed through a porous membrane.

Further critical studies of the radiosensitivity of the actual effector cells that mediate cellular immunity are still needed, and several suitable methods for this have recently become available. These involve *in vitro* assays directly measuring cytotoxic effects of sensitized lymphocytes on target cells (Brunner *et al.*, 1968, 1970; Ginsburg, 1968; Perlmann and Holm, 1969). Studies of this type have suggested that there are two categories of specific cytotoxic lymphocytes: one retains cytotoxicity after doses of 2000 r, whereas the other is much more radiosensitive, being markedly inhibited after doses of around 500 r (Denham *et al.*, 1970; Song and Levitt, 1974; R. Burton, N. L. Warner, and R. E. Anderson, unpublished results, 1975). Some evidence also suggests that stimulation by antigen renders the cytotoxic lymphocytes more radioresistant (Grant *et al.*, 1972). Thus, educated human lymphocytes maintain the majority of their lympholytic activity in the MLC-CML system despite exposures up to 15,000 r (Nichols *et al.*, 1975). Similarly, Denham *et al.* (1970) showed that murine cytotoxic lymphocytes activated 7 days previously, still showed cytotoxic activity after exposure to 20,000 R. When sensitization was accomplished 21 days previous to irradiation, doses less than 500 r were inhibitory. Song and Levitt (1974) also observed a multi-

component type of dose effect curve for murine cytotoxic lymphocytes activated *in vivo* 9–11 days previously, with about 83% of the cells being very radioresistant with a D_0 of the order of 5.5 kilorads, and the remaining cells being sensitive to doses of about 200–500 r. In our own studies (R. Burton, N. L. Warner, and R. E. Anderson, unpublished results, 1975), we used *in vitro* educated murine cytotoxic lymphocytes (Burton *et al.*, 1975), and also found a biphasic dose-effect curve (Fig. 19). The cells were irradiated after 5 days of activation and then held for 18 hours in medium prior to the cytotoxic assay.

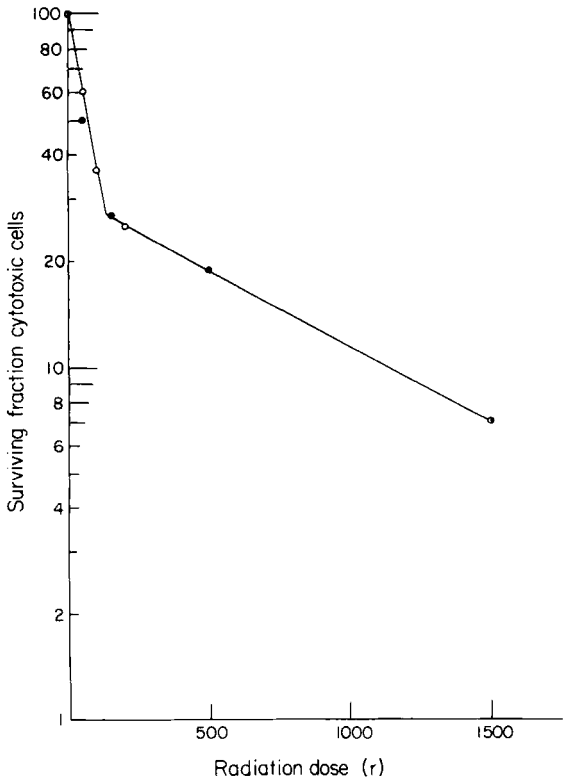


FIG. 19. Effect of irradiation on cytotoxic lymphocytes. In both experiments *in vitro*-activated murine T cells were harvested, irradiated, cultured for 18 hours and then assayed for cytotoxicity as described by Burton *et al.* (1975). The surviving cytotoxic fraction is expressed relative to controls as a function of the specific lysis value and the number of viable lymphocytes at the time of assay. The two experiments shown involve: (1) CBA spleen cells activated against BALB/c spleen with ^{51}Cr P815 cells as target cells (○——○); (2) BALB/c spleen cells activated against plasma cell tumor MPC-11 with ^{51}Cr MPC-11 cells as target cells (●——●). (After R. Burton, N. L. Warner, and R. E. Anderson, unpublished results, 1975.)

From these various studies, it would seem clear that a majority of activated cytotoxic lymphocytes are extremely radioresistant, implying that they are not sensitive to interphase death, and that they can still mediate their function (like activated helper cells) even after high doses of radiation. Depending on the conditions of activation, the T-cell population also appears to contain a subpopulation of cells that are likewise contributing to the cytotoxic effect in the control (nonirradiated) situation, but which are inactivated by even moderate doses of radiation. Since the assay conditions or preculture period would in general be long enough for a wave of cell division, it is not known whether this subpopulation has undergone mitotic or interphase death.

3. *Hematopoietic Grafts*

Bone marrow transplants into syngeneic recipients lethally irradiated in the hematopoietic dose range results in the restoration of the bone marrow of the host by the progeny of the transferred cells and prevents death (Lorenz *et al.*, 1951, 1952; Ford *et al.*, 1956; Lindsley *et al.*, 1955; van Bekkum, 1966). However, when allogeneic marrow is transplanted, two problems are encountered. First, the immune competence of the host must be sufficiently depressed to permit the survival of the injected cells. The magnitude of exposure required for such immunosuppression depends upon the histocompatibility discrepancies between the donor and recipient (Davis and Cole, 1963; Trentin, 1958) and the rate of exposure (Courtenay, 1963; Gengozian *et al.*, 1969). In mice, inadequate suppression results in graft rejection and early (within 5–21 days) mortality (Trentin, 1958). This occurs even in the high sublethal range, presumably because either: (i) T cells are more radioresistant than stem cells or (ii) fewer cells are required to reject hematopoietic grafts than are required to repopulate the bone marrow. The second problem with allogeneic grafts occurs when the host possesses a major transplantation antigen not expressed by the donor. This results in late mortality (21–60 days) due to a GVH reaction (Cole *et al.*, 1955; Congdon and Urso, 1957; de Vries and Vos, 1959). The immunological nature of both of these problems has been well documented (Simonsen, 1962; Elkins, 1971) and will not be reviewed herein. Instead, a brief consideration of hematopoietic transplantation in larger animals and man will be undertaken.

In man, bone marrow grafts were first introduced in patients with leukemia (Mathé, 1959) and in the victims of the radiation accident in Vinca, Yugoslavia (Mathé *et al.*, 1959). Although it has been clearly indicated that such marrow grafts can take initially, secondary disease

in man is very severe with an early onset. In mice and rats, although takes of bone marrow require suppression of the host by reasonably high radiation doses, permanent chimeras are often established. In primates, on the other hand, secondary disease is a far more common problem (de Vries *et al.*, 1961). This difference may be partly due to (i) the number of cells required to protect the lethally irradiated recipient and especially the number of immunocompetent cells in the donor bone marrow inoculum and (ii) the number of immunocompetent cells required to initiate the GVH reaction. For example, Vos (1966) has shown that mouse bone marrow contains fewer immunologically active cells than monkey bone marrow. Dicke *et al.* (1969) has also shown that mouse bone marrow contains far fewer PHA-sensitive cells than monkey bone marrow. It is not known how many immunocompetent cells are necessary to initiate a GVH reaction in a lethally irradiated (mid-hematopoietic syndrome dose) human.

Several groups have studied the effect of marrow infusion in patients with leukemia. Using dose rates of up to 2 R min^{-1}, total exposures of 1200–2000 R do not appear to induce early gastrointestinal complications. However, even in this exposure range, it was found (Thomas *et al.*, 1961) that, although initial takes of allogeneic marrow (usually from related donors) occurred, survival of the patient was only of 2–4 weeks' duration. Death was either from infection or, less commonly, from recurrent leukemia. It appears that extremely high doses of radiation would be needed to completely eradicate the leukemic cells.

Although occasional remission of leukemia has been observed (Thomas *et al.*, 1959, 1961) with whole-body irradiation in a sublethal exposure range of 325 or 880 R, most studies have involved higher exposures combined with marrow transfusion. In several studies with identical twins, the leukemic individual was given 800–1600 R and marrow from the normal twin. In each instance (Thomas *et al.*, 1959, 1961), a remission was achieved, but the improvement was followed by an early return of the leukemia (Thomas and Epstein, 1965). These studies confirm, however, that the lethal effect of high doses of radiation in man can be counteracted by marrow transfusion, although very large doses may be required to eliminate all the neoplastic cells.

With respect to allogeneic marrow grafting in irradiated leukemic patients, Mathé *et al.* (1969) reviewed 24 patients and showed that in 17 the marrow grafts had taken. However, of the 17, 10 died with acute secondary disease, 3 with subacute or chronic secondary disease, and 4 with recurrent leukemia. This experience is important, despite the fact that, today, radiotherapy in leukemia is limited to the CNS, because it suggests that the eradication of secondary disease is the major problem in

human bone marrow transplantation. Thus, although low dose rates were generally employed, the majority of the grafts took initially, and it appears that a sufficient depression of host immunity was achieved. Approaches to the prevention of secondary disease consist mainly of (i) pre- or posttreatment of the host with immunosuppressive agents and (ii) efforts to reduce or remove immunocompetent cells from the donor inoculum. Immunosuppression of the irradiated host has had limited success. Current efforts with respect to alternative (ii) are directed toward the complete histocompatibility typing of man. However, the problems of procurement and storage are enormous. Future efforts will probably be directed toward the use of allogeneic hematopoietic tissues that lack immunocompetent cells.

Fetal liver is a major site of hematopoiesis and, if taken at an early stage, does not contain any immunocompetent cells. Studies in mice (Urso *et al.*, 1959; Tyan *et al.*, 1966; Tyan and Cole, 1966) clearly show that fetal liver cell suspensions do not induce secondary disease in primary irradiated hosts. However, provided the host has an intact thymus, the cells become differentiated to immunocompetent cells and at the same time become tolerant to the host's histocompatibility antigens. The use of fetal liver cell suspensions for the treatment of irradiated rhesus monkeys has been studied by van Bekkum *et al.* (1969), who found that more than one complete fetal liver was required per recipient. However, it is possible that the optimal gestational age was not used in these experiments.

In man, van Putten *et al.* (1968) concluded that the pooled frozen liver cells of roughly 50 human fetuses of 20–26 weeks' gestation would be required for one adult. Since a large amount of material cannot be transfused safely, the use of fetal liver cells was thought to be clinically unrealistic. On the other hand, it is possible that a more judicious choice of fetal age for the liver source, and possible fractionation of the cell populations to enrich for stem cells, might permit the use of this approach. Alternatively, if allogeneic bone marrow is to be the source of donor cells, the approach must be either to purify the stem cells or to kill selectively, or otherwise remove, the immunocompetent cells. Both methods offer considerable promise but neither has been sufficiently refined to be employed clinically on a routine basis.

At the present time, none of the above approaches to the prevention of secondary disease has yet been shown to work completely satisfactorily, which probably indicates that several variables are involved. This is substantiated by the studies of Congdon *et al.* (1965, 1967), who undertook a comprehensive "4-factorial" study in mice, assessing the effects of variation in: the interval between whole-body irradiation and injection of

allogeneic bone marrow; the number of bone marrow cells; the age of bone marrow donor; and the sex of the donor and recipient. The 90-day mortality could be reduced 10-fold by controlling these factors. These results, combined with other approaches mentioned above, indicate that the complete elimination of the mortality due to secondary disease is a realistic objective.

As noted previously, T–T cell collaboration is involved in cellular immune responses and in particular the GVH reaction. How such cell collaboration influences GVH disease in irradiated recipients of allogeneic bone marrow is presently unclear. More specifically, a key question is whether the bone marrow population contains immunocompetent T lymphocytes which can initiate a GVH reaction immediately upon injection into an irradiated recipient, or whether maturation of the potentially immunocompetent cells (stem cells) in the bone marrow is required. If the latter is true, or if a cell collaboration step is involved, the process may take place in the host, and the radiation dose rate used in man and other primates may be insufficient to prevent a rapid expression of secondary disease by the donor cells. This possibility is based on the following experiments: Adult thymectomized irradiated mice given syngeneic bone marrow are immunologically unreactive. When an allogeneic thymus graft is also placed in the recipient, the mice recover their immune capabilities and reject the thymus graft itself (Miller *et al.*, 1964). At no time is there any evidence of repopulation of the allogeneic graft. This indicates that the injected syngeneic bone marrow cells already carry the potential to react against the histocompatibility antigens of the allogeneic graft but first require something from the graft, probably humoral in nature (Osoba and Miller, 1964), to express this activity. Thymus grafts irradiated *in vitro* (2000 R) fail to restore neonatally thymectomized mice to full immunological capacity (Miller *et al.*, 1966), thus suggesting the existence of a radiosensitive stage in the synthesis or release of the thymic factor. These experiments therefore support the concept that bone marrow contains an immunocompetent cell capable of reacting against histocompatibility antigens provided a thymic factor is available.

If these observations are also applicable to the injection of bone marrow cells into allogeneic recipients, they imply that the host must provide a thymic factor for the injected cells to be able to induce the GVH reaction. Since this effect of the host thymus may be radiosensitive, it is quite possible that the relatively late onset of secondary disease in lethally irradiated mice is from radiation damage to the host's thymus, and that this must first recover before the injected cells can attack. Since the dose rates used in man and primates are of a low order (<5 R min^{-1}), it is

quite possible that this radiosensitive phase of the thymic effect has not been sufficiently destroyed, thus permitting the immediate maturation of injected stem cells to immunocompetence. This concept would suggest that higher dose rates might also be advantageous in delaying thymic restoration and therefore development of immune competence. It is possible that additional local thymic irradiation might even produce a sufficient delay to permit the injected cell population to become tolerant to host antigens.

Experimental verification of this concept could come from a direct demonstration that removal of the host thymus prevented the induction of secondary disease in irradiated mice given allogeneic cells. Such experiments have been reported by three groups, but the interpretation of the results is difficult, because ATxXBM mice also develop a wasting phenomenon associated with lymphoid atrophy. Thus, even if secondary disease were prevented in thymectomized allogeneic recipients, the mice might still die of the wasting disease associated with lymphoid aplasia. In one study (van Putten, 1964) with heterologous combinations, a marked reduction in the incidence of secondary disease was observed in thymectomized mice, and in two other studies a marginal prolongation of life was observed (Goedbloed and Vos, 1965; Simmons *et al.*, 1965). A critical test of this hypothesis, however, would require the use of germfree ATxXBM recipients, to avoid wasting disease from lymphoid atrophy.

4. Organ Grafts

Human renal allograft transplantation has become a major accepted form of clinical therapy for several forms of end-stage kidney disease. Inherent in any successful organ transplantation is the prevention of graft rejection. This can basically be approached in two ways: (i) by avoiding presenting the recipient with an effective foreign antigen; and (ii) by suppressing the host's immune response. The majority of current approaches to immunosuppression do not involve irradiation, and accordingly the field of organ transplantation is currently of less direct relevance to the topic of radiation and immunity. However, since radiation has been used frequently in the past 10–15 years, selected aspects will be discussed here although they do not represent an ideal approach. Suppression of homograft immunity by using radiation has been aimed at either: (i) the graft itself; or (ii) the immune system of the host. Since it has been shown that the entire sequence of cellular events connected to allograft rejection can take place in the grafted kidney (Pedersen and Morris, 1970), local irradiation might be expected to lower the intensity of the rejection process.

An impressive body of data clearly indicates that local irradiation of the kidney soon after transplantation is of definite value in delaying acute rejection (Hume *et al.*, 1966; Hume and Wolf, 1967; Wolf *et al.*, 1967; Kauffman *et al.*, 1965; Ono *et al.*, 1967). Local graft irradiation is usually performed by a fractionated total dose of about 1000 r. These results and related data suggest that, in general, local irradiation of the kidney *soon* after transplantation may be beneficial and that several factors are possibly involved, including both the destruction of any donor lymphoid cells, which may act as a strong immunogen and the destruction of early infiltrating host cells, many of which may be acting in a nonspecific but destructive manner. The latter might be acting in a fashion analogous to the recruitment of normal host lymphoid cells in DTH reactions (Bloom and Chase, 1967).

Whole-body irradiation for suppression of organ-graft rejection poses many problems. If radiation were to be the sole agent for immunosuppression, the accompanying problems of bone marrow transplantation would also have to be solved since the dose required to create sufficient immunosuppression would lead to marrow aplasia. Accordingly, only sublethal exposures are practical which, although aiding in immunodepression, may still be expected to provide significant radiation damage. Before the advent of the currently popular methods of immunodepression, whole-body irradiation at doses of the order of 400 r was used with some possible success (Merrill *et al.*, 1960; Hamburger *et al.*, 1962). Additional localized irradiation of the spleen and the right lower abdomen has also been given to depress immunity and to obliterate the lymphatic field draining the transplant (Woodruff *et al.*, 1962).

Under certain experimental circumstances, whole-body irradiation has actually accelerated the destruction of renal grafts. Studies in inbred rats (Feldman *et al.*, 1968) with renal grafts placed into immunologically tolerant hosts have afforded a means of examining the rejection process under controlled conditions. Graft rejection could be induced by the injection of large numbers of competent syngeneic lymphoid cells. If whole-body irradiation (550 R) was also given, graft rejection was greatly facilitated, in that fewer injected cells were needed to induce rejection, and graft destruction was hastened. Total body irradiation per se was occasionally followed by the destruction of skin homografts. This effect may have occurred through a variety of mechanisms, such as (i) depletion of lymphoid cells in host organs allowing better seeding of the injected cells; (ii) enhanced cell growth and preferential mitosis in the presence of antigen; (iii) alterations in the target cells rendering them more susceptible to rejection; or (iv) reduction or suppression of the state of tolerance in the host, particularly if T suppressor mechanisms

were involved. The latter hypothesis, which is currently thought to be of primary importance in this regard, will be discussed in more detail in the next section.

V. Radiation and Immunological Tolerance

A. Induction and Breakdown of Tolerance by Radiation

In the preceding sections we have considered the effects of radiation on the variety of cell types that together are involved in the production of a "positive" immune response. However, it has also become evident that the induction of a "negative" immune response, i.e., immunological tolerance, may sometimes involve the interaction of several different cell types. Although a discussion of the many forms of immunological tolerance is beyond the scope of this review, several aspects are particularly relevant to a consideration of tolerance and radiation.

1. The state of immunological tolerance can be produced by several different and distinct mechanisms. The original concept of Burnet and Fenner focused on clonal deletion of the immunocompetent cell (see review by Burnet, 1959). In several experimental situations, this concept may be applicable, in that immunocompetent cells of the particular specificity under study appear to be totally absent (Nossal, 1974). In this context, recent studies have suggested that a particular cell stage early in differentiation may be acutely sensitive to tolerance induction (Nossal and Pike, 1975) possibly owing to the expression of only the IgM surface receptor rather than the dual expression of IgM and IgD (Vitetta and Uhr, 1975).

Recent attention has also focused on two other possible tolerance mechanisms, involving either blockade of effector cells by antigen or antigen–antibody complexes (Stocker and Nossal, 1975) or the generation of a T-dependent suppressor system that acts to specifically inhibit the immune response to a given antigen (Basten *et al.*, 1975; Nachtigal *et al.*, 1975; Gershon, 1975).

2. In immune responses to antigens requiring interaction between cells of the T- and B-cell series, the state of tolerance can result from T- and/or the B-cell series being rendered specifically unresponsive to the particular antigen (Mitchison, 1972; Weigle, 1973). The possible role of radiation in augmenting or terminating such a tolerant state might thus depend on which cell series was primarily involved in the tolerant state.

3. The persistence of the state of tolerance will thus depend on the particular mechanism involved, the need for antigen persistence, and the

possible influence of the input of newly derived cells into the immune system.

4. Given these variations, radiation might act in alternative ways on either the ability to induce a tolerant state or on its persistence. Tolerant states involving suppressor activation would be most liable to termination by irradiation if the particular suppressor cell type were especially radiosensitive. Alternatively, if, after irradiation, an increased proportion of immature cells (e.g., pre-B cells) existed in the population, tolerance induction might occur more readily.

Irradiation has, in fact, been shown to be particularly useful in aiding tolerance induction in adult animals when moderate to highly immunogenic materials are used. In the absence of irradiation, the antibody formation that results from antigen-reactive cells being driven toward immunity masks any simultaneous tolerance induction among other susceptible cells. On the other hand, when sublethal irradiation precedes antigen injection, antigen-reactive cells are killed in proportion to the dose of radiation used. Since the subsequent recovery of the immune system occurs primarily by differentiation of hematopoietic stem cells, a considerably higher proportion of immature cells are present than under normal circumstances, and these cells are particularly susceptible to tolerance induction (Nossal and Pike, 1975). Thus, the postirradiation recovery phase closely resembles, in this respect, the relative immunological immaturity of the newborn, a stage that is also especially susceptible to tolerance induction (Nossal *et al.*, 1965; Dresser and Mitchison, 1968).

Examples of tolerance induction in irradiated adult animals include: (i) the use of 150 r to facilitate skin graft tolerance to weak histocompatibility antigens (Kelly *et al.*, 1966); (ii) induction of tolerance to a single dose of BSA given after irradiation (Linscott and Weigle, 1965); and (iii) the injection of multiple doses of antigen given over a prolonged period immediately or within a few weeks after a single dose (550–600 r) of irradiation in rabbits (Nachtigal *et al.*, 1968) or mice (Mitchison, 1968). In these studies, it appeared that multiple small doses of antigen given in the postirradiation period were more effective than a single large dose. This observation suggests that tolerance induction is not simply related to antigen overloading, but rather that cells at certain early periods of differentiation are especially susceptible to tolerance induction.

In most situations, the state of immunological tolerance persists for only a finite period. Loss of tolerance can result in several ways, depending upon the particular mechanism of tolerance involved. Critical factors in this regard include a balance between the persistence of antigen, the

emergence of new immunocompetent cells, and if relevant, the persistence of suppressor cells. Thus, measures, such as thymectomy, that reduce the rate of appearance of new competent cells can prolong the state of tolerance (Claman and Talmage, 1963; Claman and McDonald, 1964).

Denhardt and Owen (1960) proposed that radiation of tolerant animals could result in termination of the tolerant state either through radiation-induced destruction of the cells storing antigen or through the enhanced stem cell proliferation which follows whole-body irradiation. Although this was not observed in two studies of BSA-tolerant rabbits irradiated with either 300 r (Denhardt and Owen, 1960) or 450 or 1000 r (Weigle, 1964), studies in erythrocyte-tolerant rats have shown that tolerance breakdown can follow irradiation (Nossal and Larkin, 1959; Mäkelä and Nossal, 1962; Stone and Owen, 1963). Similarly, partial transplantation tolerance induced across H-2 barriers at birth was completely abrogated by exposure to 350–450 r (Fefer and Nossal, 1962). Low zone tolerance to the bacterial antigen flagellin has also been broken by the combined injection of TDL and host irradiation—even when only the recipient's spleen was irradiated (Shellam, 1971).

Why can tolerance be broken by radiation in some situations but not others? In view of the current evidence suggesting that immunological tolerance can involve one of several different mechanisms, it is most likely that radiation will have a differential effect in each of these situations. Various studies have clearly demonstrated that T cell-dependent suppressor activity is associated with, if not causative of, certain tolerant states (Basten *et al.*, 1975; Nachtigal *et al.*, 1975). In such a circumstance, irradiation, by removing suppressor cells, could terminate the state of tolerance. In other situations in which true clonal detection may exist, postirradiation recovery could result in the exaggerated emergence of potentially new immunocompetent cells. If tolerogenic concentrations of antigen were not present, immune recovery would be expected.

Further definition of the exact mechanisms of tolerance in given situations will therefore be required to determine whether radiation would be expected to enhance or inhibit the induction or maintenance of the tolerant state.

B. Implications for Autoimmunity

The pathogenesis of autoimmune disease is exceedingly complex and involves the interaction of a variety of distinct components that together results in the activation and expression of autoreactive T and B lymphocytes. Since autoimmunity has frequently been considered to be analo-

gous to a breakdown in tolerance, i.e., self-tolerance, consideration of the possible effects of radiation in the generation or loss of self-tolerance is clearly relevant. Three general hypotheses might be considered for possible radiation induction or acceleration of autoimmune processes, and these are listed in Table XII. Although few definitive statements can be made at this time concerning these possibilities, several studies have been reported that tend to stress the third alternative. However, any or all of these three mechanisms may be operative under particular circumstances and each warrants further investigation.

1. Antigenic Changes

Irradiation has been shown to produce changes in the antigenic structure of tissues, or to result in the release of several components which are normally inaccessible. After irradiation to the head and neck region in man, necrotizing vasculitis and damage to thyroid epithelial cells have been observed (Lindsay *et al.*, 1954). Under these conditions, leakage of thyroglobulin into the circulation can be detected (Robbins *et al.*, 1954) and autoantibodies to thyroglobulin may be found in the serum (O'Gorman *et al.*, 1964). However, in other situations not involving radiation, such as after cardiac surgery, when thyroglobulin can also be detected in the circulation (Torrigiani *et al.*, 1969), autoantibody formation does not necessarily ensue; these observations suggest that at least after head and neck radiation, the release of thyroglobulin is not in itself sufficient to result in autoantibody formation.

Several examples of actual changes in the antigenic structure of tissues after either external or internal radiation have been noted and these may be followed by autoantibody production (Zilber *et al.*, 1956; Kiselev and Semina, 1959; Shubik *et al.*, 1969; Alekeoff, 1970). Further investigation in this area is required to characterize these apparent antigenic changes at the molecular level.

2. Mutagenic Potential

A second hypothesis that relates to the immunological consequence of radiation is that mutagenic effects may result in a relative increase in numbers or frequency of cells expressing autoimmune potential, and that such cells, in sufficient numbers, initiate an autoimmune response. In one study, the spleens of mice taken 7 days after lethal whole-body irradiation were injected subcutaneously into the skin of normal syngeneic mice resulting in a marked reaction that was not observed with cells taken only 1 day after irradiation (Allegretti and Dekaris, 1969). These observations were interpreted as suggesting the acquisition of self-

reactivity induced by radiation. Further analysis of this model would, however, be required to demonstrate immunological specificity, in distinction to possible pharmacological effects.

The effects of chronic low-dose radiation on autoimmune expression have also been studied (Croft *et al.*, 1975) in several mouse strains, using a dose of 20 R per year (approximately a 200-fold increase over background exposure). Although no changes were observed in the survival of a strain [(NZB $\times$ NZW)F_1] with a high predisposition to a lethal autoimmune glomerulonephritis, randomly bred mice and NZB inbred mice (having a lower incidence of glomerulonephritis than the BW hybrid) died significantly earlier. No evidence of increased autoimmune disease was, however, observed in these animals. It was proposed that either impairment of immunity to infection or increased somatic mutations might be involved. The former possibility has not received any significant experimental support in low- to moderate-dose radiation studies. For example, the mortality curves of irradiated mice (1 dose of 300 r at 6 weeks) show similar patterns of radiation-induced life shortening in both germfree and conventional mouse strains (Walburg and Cosgrove, 1970). No role of bacterial flora was observed in this study.

The general concept of mutagenesis in relation to radiation-induced effects on autoimmunity is analogous to several general concepts of radiation and aging. Various studies have demonstrated that although aging is associated with a general decline in immune responsiveness to extrinsic antigenic challenges (Waldorf *et al.*, 1968; Roberts-Thomson *et al.*, 1974), an increased incidence of serum autoantibodies is observed in old age (Hooper *et al.*, 1972). This raises the possibility that the aging process itself involves autoimmune expression. Walford (1969) has proposed that aging is due to somatic cell variation, particularly of those factors that determine self-recognition patterns among cells. In higher animals, the cells of the reticuloendothelial system are especially involved. Aging among these species is proposed to be related to the unleashing of self-destructive processes analogous or identical to autoimmune phenomena or transplantation disease. The initial cause of the somatic cell variation, whatever it may be, is extrinsic to this pathogenetic mechanism, although cell variation may be further stimulated by autocatalytic immune processes. If irradiation increases the rate of somatic cell variation, and therefore the potential development of an autoimmune state, and if at the same time is immunosuppressive, it will tend to inhibit the autoimmune tendencies of the somatically variant cells. Thus, irradiation may have two opposing effects on the onset of autoimmune disease, one accelerating and one retarding. The actual result might therefore depend on the balance of these two factors and in turn depend

upon the type of radiation, total dosage, dose rates, age of animals at time of radiation, species, nutrition, and many other factors. In particular, if age is a factor, it may well relate to the greater radiosensitivity of the young animal. If aging is an autoimmune process, then in adults the process may well be sufficiently underway to be autocatalytic, and irradiation at this time would not lead to any greater observable rate of change. This conclusion is indeed similar to that reached by Anderson (1971) in considering the preliminary data available on the immunological effects of radiation on atomic bomb survivors.

Although there is still much to be learned about the fundamental relationship of aging, radiation and autoimmunity, one major objection might be made with respect to the concept of radiation-induced (by mutation) autoreactive cells. Namely, that in several species it has been shown that potentially autoreactive cells, i.e., cells capable of binding autoantigens, already exist in the normal animal (Unanue, 1971; Bankhurst *et al.*, 1973; Coates and Lennon, 1973) in a frequency similar to that of cells capable of binding extrinsic types of antigens. These experiments were performed under conditions that primarily detect B cells, and thus suggest that there is no restriction in the normal animal on the emergence of autoreactive B cells. These studies do not, however, exclude the possibility that if helper or other immunocompetent T cells are required for the expression or activation of an autoimmune response, then these cells may show radiation-induced mutagenesis leading to their emergence. This possibility would need to be restricted to a type of variable region gene expression shown only by the T cells involved in DTH or helper function, since several studies have shown that autoreactive cytotoxic T cells can be activated *in vitro* (Cohen *et al.*, 1971; Ilfeld *et al.*, 1975).

3. *Loss of Regulatory Mechanism*

In the past several years, considerable interest and experimentation has focused on the concept that autoimmunity represents an imbalance in a natural regulatory control process. According to this hypothesis, potential autoreactivity is normally controlled or suppressed by a T-cell population (Fudenberg, 1971; Allison, 1974). Experimental support for this concept has been reviewed (Allison, 1974; Talal, 1975) and basically demonstrates that the NZB mouse strain, which expresses autoimmunity, shows a deficiency in a regulatory T-cell population that can be restored by either (i) populations of intact T cells or (ii) factors (thymopoitin) capable of inducing appropriate T-cell differentiation. If autoimmune expression is thus associated with a decline in function or numbers of the T-dependent suppressor mechanism, then radiation in-

duction, or acceleration, of autoimmunity might be expected to occur since this regulatory system is known to be acutely radiosensitive (see Section III,C). Several observations on the effects of sublethal irradiation on the incidence of thyroiditis in experimental animals are consistent with this interpretation. In a strain of chickens showing spontaneous autoimmune thyroiditis, whereas bursectomy at hatch decreased the incidence of disease, neonatal thymectomy increased the disease incidence (Wick *et al.*, 1970a), indicating that depletion of a "regulatory" T-cell function more readily permitted disease expression. Neonatal sublethal whole-body irradiation had a similar effect in increasing the incidence of thyroiditis (Wick *et al.*, 1970b), although it did not result in any detectable increase in nonthyroid autoantibody production (Wick *et al.*, 1970a).

Similarly, whole-body irradiation of Wistar rats (5×200 r every 2 weeks) resulted in a significant incidence of thyroiditis (Penhale *et al.*, 1973). Irradiation and thymectomy (at 5 weeks) resulted in an even larger increase. Of particular significance in relation to mechanisms 1 versus 3 (Table XII), was the observation that irradiation of the head and neck (including the thyroid) region alone did not induce disease, whereas radiation of the trunk and limbs (i.e., including lymphoid tissues) did so.

The relationship between radiation and experimental autoimmune diseases, and in particular EAE, is confused by a number of apparently conflicting reports. Since these responses appear to involve predominantly a cellular immune reaction (Paterson, 1966), radiation in sufficient amounts might be expected to reduce the magnitude of the response. However, administration of 150 r 18 hours prior to antigen was reported to result in an *increased* severity of EAE in guinea pigs, although there was no diminution of DTH to the material used for sensitization (Field, 1961). Allegranza (1959) reported a similar relationship also in guinea pigs. On the other hand, Paterson and Beisaw (1963) showed that 400 r whole-body exposure of rats prior to sensitization with spinal cord and adjuvant suppressed the development of EAE. This suppression was dose dependent and was observed in two strains of rats sensitized by either

TABLE XII
POTENTIAL MECHANISMS OF RADIATION-INDUCED AUTOIMMUNITY

1. Alteration of tissue constituents to create new autoantigens or release previously inaccessible components
2. Mutation leading to the emergence of autoreactive clones
3. Alteration of the balance of natural regulator mechanisms that control potential autoimmune expression

of two routes. A reduced production of complement-fixing antibodies also occurred, but there was little, if any, suppression of DTH reactivity as evaluated by tuberculin skin testing. Irradiation also suppressed the development of EAE in rabbits (Condie and Nicholas, 1962). These apparent contradictions in the effect of radiation on the induction of EAE may be due to differences in the preparation of antigen, the immunization schedule, the experimental animal employed, and the radiation dose. In this connection, the studies of Paterson (1966) appear to indicate a relationship between cytotoxic antibody formation and the magnitude of clinical disease. It might also be speculated that a radiation-induced reduction in antibody formation could lead to the more severe disease observed in irradiated guinea pigs. Since enhancing antibodies may protect animals from EAE (Paterson, 1966), it is possible that these, rather than cytotoxic antibody, are normally produced in the guinea pig with the immunization scheme used. Accordingly, radiation-induced depression of this type of antibody formation would lead to a more aggressive response that would further the disease process.

An alternative explanation of these reports would be that enhancement of disease was due to radiation-induced elimination of suppressor cells. Prevention of disease might in turn be due to irradiation with an appropriate dose or at an appropriate time when the inductive stage of the eventual effector arm was particularly radiosensitive. As in several other situations (Gershon *et al.*, 1974), this area is complex in that T–T regulatory interactions with cell types of differential radiosensitivity are probably involved.

Further analysis of the potential of radiation to induce or accelerate autoimmune disease will require more specific evaluations of particular lymphoid subpopulations in order to delineate the possible significance of this mechanism. Other factors may be involved, since it has been observed, for example, that the NZB mice have a high resistance to radiation—owing to an unusually large pool of hematopoietic stem cells (Morton and Siegel, 1971). Genetic analysis of this stem cell abnormality does not, however, provide any support for the concept that the enlarged stem cell pool is associated with the autoimmune disorders of these mice (Warner and Moore, 1971).

VI. Radiation, Immunity, and Neoplasia

The general subject of radiation in relation to immunity and neoplasia is itself a major area that is too broad to be fully covered in this review. However, in view of the importance of several aspects of the immune response to radiation-related neoplasms, brief mention of select areas

will be made. For more extensive coverage, the reader is referred to a recent conference report on this subject (Bond *et al.*, 1974).

A. Radiation and Carcinogenesis

Ionizing radiation in sufficient quantities is both immunosuppressive and tumorigenic. These two effects may be related, in that radiation-induced suppression of the immune response may permit the uninhibited proliferation of neoplastic cells. In addition, the latter may have been directly activated by one of several postulated radiation-related events—direct injury of DNA, activation and/or release of latent virus, virus-related derepression of host genes, etc. Central to this putative relationship between radiation-induced immunosuppression and carcinogenesis is the concept of immune surveillance (Smith and Landy, 1971).

The hypothesis of immune surveillance, proposed by Thomas (1959) and developed by Burnet (1970), suggests that clones of malignant cells arise periodically, but seldom survive because they are rejected as allografts unless a defect in the immune response permits these abnormal cells to be tolerated. The key to this thesis is the observation that some tumors are antigenic to the autochthonous host and thus would be expected to elicit an immune response (see Keast, 1973). Of particular relevance is the observation that immune deficiencies, congenital as well as acquired, are associated with an increased prevalence of neoplasia. However, the distribution of the various types of tumors in these immunodeficient individuals is different from that found in the general population, with a marked preponderance of lymphomas and reticuloendotheliioses (Penn and Starzl, 1972; Kersey *et al.*, 1973). This unexplained association suggests that the regulation of some tumors may be more susceptible to immune surveillance than others. Alternatively, Gershwin and Steinberg (1973) have proposed that a loss of suppressor activity, occasioned by prolonged administration of ALS or congenital absence of the thymus, may lead to unregulated proliferation of the lymphoreticular system and malignancy.

The relevance of considering neoplasia and immunity in relation to radiation and immunity in general is, therefore, based upon the following set of premises: (i) many tumors are antigenic and may initiate an immune response in the host against the tumor cells (cellular and/or humoral response); (ii) many carcinogens, both chemical and viral, are immunosuppressive; (iii) upon the development of a clone of malignant cells, an interaction occurs in which the growth rate of the tumor is pitted against the developing immune response. The immune response may either retard or enhance the growth rate of tumors (Baldwin, 1973; Hellström and Hellström, 1974; Klein, 1973–74).

Accordingly, the relationship of the immune response to tumor growth is pictured in an analogous fashion to the balance between immunity and infection, and just as radiation depresses immunity and permits a greater spread of infection, so it is proposed that radiation-induced depression of immunity may permit more rapid growth and spread of neoplastic cells. Subtraction of select components of the immune response at an appropriate age may also retard or promote tumorigenesis. The essential questions relevant to this presentation are therefore as follows: (i) What role does radiation-induced depression or augmentation of the immune response play in the development of radiation-related neoplasms? (ii) Does radiation augment or interfere with immunotherapeutic approaches to cancer management?

Before considering whether radiation-induced immune depression is a critical factor in radiation induction of cancer, it is relevant to evaluate whether radiation can depress immune responses to established tumors and especially neoplasms with tumor-specific transplantation antigens.

Many observations indicate that mouse tumors are antigenic to their syngeneic strain and that irradiation, like many other forms of immunodepression, permits more rapid tumor growth or shortens the latent period (e.g., Klein and Klein, 1962; Glynn *et al.*, 1964; Fefer *et al.*, 1967; Warner and Rouse, 1972). For example, in a study (Rosenau and Moon, 1967) on methylcholanthrene-induced sarcomas in mice, whole-body irradiation prior to transplantation resulted in a marked increase in tumor growth. A dose of 400 r gave a maximum effect, and enhanced growth could even be detected among mice in which the tumor was transplanted 4 months after exposure, although the maximum effect was observed with transplantation 24 hours after irradiation. In a similar vein, Grant and Miller (1965) noted an accelerated appearance of chemically induced tumors in mice immunodepressed by neonatal thymectomy.

Some caution might be introduced here concerning general statements on immune deficiency and tumor growth. Several recent studies have suggested that *inhibition* of tumor growth can be observed in T cell-deficient animals (Stutman, 1975; Bonmassar *et al.*, 1975; Umiel and Trainin, 1974; N. L. Warner and M. F. A. Woodruff, unpublished results, 1975). Most of these studies have involved athymic (nude) mice, which also possess a greater than normal number of natural cytotoxic cells that are capable of killing many tumor cells *in vitro* (Kiessling *et al.*, 1975; Herberman *et al.*, 1975; R. Burton, D. Barr, and N. L. Warner, unpublished results, 1976). These cytotoxic cells are not T cells, B cells, or "classical" adherent macrophages, and potentially they represent another mechanism of immune surveillance, one that may be particularly relevant in explaining why athymic nude mice do not show a high incidence of

spontaneous tumors (Custer *et al.*, 1973; Rygaard and Povlsen, 1974). In this context, it is important to note that these effector cytotoxic cells appear to be quite radioresistant (at least to 1000 R) *in vitro* (R. Burton, D. Barr, and N. L. Warner, unpublished results, 1976).

Unfortunately, there are as yet only a few studies dealing with primary or spontaneous tumors and radiation-induced immune depression. In one such study (Anderson *et al.*, 1972), the following groups of mice were compared: (1) germfree, sublethally irradiated; (2) barrier-maintained, sublethally irradiated; (3) germfree, nonirradiated; (4) barrier-maintained, nonirradiated. Both gemfree groups developed fewer neoplasms than their barrier-maintained counterparts, but such tumors as did develop appeared at an earlier average age. These observations suggest that germfree mice, with decreased numbers of recirculating small lymphocytes and probably a decreased exposure to environmental carcinogens, are particularly at risk with respect to neoplasia, especially after irradiation, but only when the appropriate cocarcinogenic agents are present. In an extension of this study, preliminary evidence suggests that irradiated nTx germfree mice develop a somewhat different spectrum of neoplasms at an earlier age than do their sham-operated contemporaries, but the incidence of such tumors is not nearly so great as might be anticipated (R. E. Anderson and W. E. Doughty, unpublished results, 1975). Although in relation to the low incidence of tumors in nude mice (Rygaard and Povlsen, 1974; Custer *et al.*, 1973) and the above discussion, this might be expected.

Osteosarcomas which arose in mice following administration of ^{90}Sr have been shown to carry tumor-specific transplantation antigens, in that immunization of recipients with 15,000 irradiated tumor cells resulted in a lower incidence of takes of transplanted tumor providing the recipients were also exposed to 400 R one day before transplantation (Nilsson *et al.*, 1972). These experiments confirm a previous suggestion (Nilsson *et al.*, 1965) that radiation-induced sarcomas may be antigenic, and that this antigenicity may be a factor in the development of the primary tumor. Infection of ^{90}Sr-treated mice by bacillus Calmette Guérin (BCG) at a time close to the expected appearance of the first bone tumors resulted in a delay of the development and a significant decrease of the total incidence of such tumors, which may have been due to stimulation of the immune system by the BCG.

One of the strongest arguments relating radiation-induced immunosuppression to tumor induction comes from the study of radiation-induced mouse leukemias. Before considering this argument in detail, however, it must be emphasized that the model system used is not ideal, since it involves neoplasia of a component cell of the immune system. The

changes that have been attributed to radiation-induced alterations in the host immune system might alternatively be explained by a direct interference with the potentially neoplastic line of lymphoid cells.

In any discussion of radiation-induced lymphosarcomas and lymphatic leukemias in mice, two experimental observations are of primary importance (Kaplan, 1954, 1964): (1) there is a far greater incidence of tumors when the dose is fractionated with successive increments spaced a few days apart; and (2) the entire body must be irradiated since shielding of the spleen or bone marrow, or injection of normal bone marrow after whole-body irradiation, drastically reduces tumor incidence (Kaplan, *et al.*, 1953; Lorenz *et al.*, 1953). Three separate factors appear to relate to these observations: injury to the extrathymic sites of storage of the latent virus with its concomitant release; injury to the thymus followed by a variable period of regeneration; and injury to the bone marrow, which in turn interferes with the thymic regeneration. A long period of regeneration permits prolonged exposure of immature thymus cells to circulating virus. Such cells appear to be particularly susceptible to this oncogenic agent. Lymphoma can also be induced by the direct injection of the leukemogenic filterable agent from irradiated C57 mice into a thymus graft carried by a thymectomized irradiated host (Haran-Ghera *et al.*, 1966). If the host is not irradiated, however, leukemia does not develop, suggesting that something more is required than the presence of active virus in association with thymocytes. Haran-Ghera (1967) and Haran-Ghera and Peled (1967) have presented evidence to suggest that the other essential factor in leukemogenesis may be radiation-induced immunological depression. Tests on the immunological reactivity of irradiated mice were performed by evaluating the production of antibodies to *Shigella* antigen. The four weekly whole-body exposures of 170 R used for leukemia induction resulted in marked immunological depression, with the minimal antibody production in these mice persisting for about 1 week after irradiation, and coinciding with the release of the filterable agent into bone marrow. Therefore, it was postulated that the inoculation of normal bone marrow immediately after irradiation reequips the immune system and accordingly reduces tumor incidence. An alternative explanation is that the inoculated stem cells promote repopulation of the host thymus, thus interfering with the prolonged maturation arrest of thymic cells.

It therefore appears that in the induction of murine leukemia: (1) a transient radiation-induced depression in host immunity [possibly mainly homograft immunity, Haran-Ghera (1967)] is an important factor when combined with (2) the activation or release of a latent virus which can (3) attack a susceptible (immature) thymus cell. Depression of the im-

mune response of the host may also be of importance in other experimental tumor models that involve radiation.

Another effect of radiation on the incidence of murine leukemia has been observed (Ludwig *et al.*, 1969). The same radiation dose that enhances leukemogenesis in a nonirradiated mouse counteracts leukemia development if given to a mouse that was previously irradiated but has not yet developed leukemia. This suggests that the preleukemic interval, the period between recovery from the first dose of radiation and the development of the disease, includes a vulnerable radiosensitive stage. However, it might also be proposed that the first dose of radiation has broken a state of tolerance to a vertically transmitted leukemia virus (see Wahren and Metcalf, 1970). Loss of tolerance would be expected to be followed by a period of time during which an immune response to the viral antigen occurs, which may be critical for neoplastic development. A second dose of radiation in this period would largely suppress this newly emerging state of immunity against cell surface-expressed viral antigen and therefore suppress tumor development.

B. Radiation and Immunotherapy

One of the interesting problems in relation to the general study of immunotherapy as an adjunct to the treatment of cancer is its possible interrelationship with irradiation. Three general areas might be considered: (1) the use of irradiated cells as immunogens to elicit antitumor responses; (2) the effect of host irradiation on tumor immunity; (3) the use of radiolabeled antitumor antibodies.

1. When tumors carry unique tumor specific antigens, the autologous tumor may have to be used as the immunizing agent. Since the inoculation of viable autologous tumor cells may lead to its regrowth, or to the release of soluble antigen that might result in complex-induced blockage, the tumor cells must first be inactivated by some treatment that does not destroy immunogenicity. Radiation appears to meet these requirements in most cases. Lymphoid cells appear to retain their normal immunogenicity in MLC reactions after irradiation with 1200 R (Elves, 1969), although some reduction in activity has been reported (McKhann, 1964). While several investigations have failed to show any effect of irradiated autologous cells alone in promoting tumor rejection (Matsuyama *et al.*, 1963), other investigators have observed a significant increase in immunogenicity of irradiated isologous tumor cells (Maruyama, 1967, 1968). No difference was observed between cells exposed to 100 R and controls, but with 1000 R an increased reaction against the tumor was evident. It appears that irradiation may have caused greater exposure of the antigenic

sites of the tumor cells or may have enriched the tumor population for the more antigenic cells by selectively removing the less antigenic ones.

In view of the marked changes that irradiation can cause to the plasma membrane of the cell (see Section VII), these latter results might suggest that irradiation has resulted in release of immunogenic cell membrane fragments. Several other *in vitro* studies have also shown that high doses of irradiation (2000–4000 r) given to tumor cells will release an antigenic component into the tissue culture medium that is capable of activating a cellular immune response against the tumor (Sato *et al.*, 1968; Nio, 1970).

In general, these and other studies show that irradiation does not destroy tumor immunogenicity, and in fact may well enhance the release of active material from the tumor cells.

2. The effect of irradiating the host on the antitumor immune response is basically the same as for any other specific immune response. When radiotherapy is used to primarily reduce the tumor mass, the significant question is whether the immune response can then assist in the destruction of the remaining viable tumor cells. Accordingly, in each particular situation one must consider whether the active local immune response is in the field of radiation, whether it is of a type that is radiosentitive, and whether radiation, through release of free tumor antigen, may block rather than induce immune responses. An example of the combined use of radiation and immunotherapy is a study of Haddow and Alexander (1964) in which a sample of fibrosarcoma was removed from a rat host and exposed to 10,000 r *in vitro*. The irradiated cells were then given back to the autologous animal, and the remaining large mass of the primary tumor was locally exposed (*in vivo*) to 2000 r. A striking regression in size of the primary tumor occurred in many cases. Injection of irradiated autografts alone had no effect without local irradiation. It is known that local irradiation does not kill all cells in such large masses of tumor tissue. A significant fraction remains. However, the growth of the surviving cells may be considerably inhibited by the immune response initiated by the irradiated autologous graft. As noted previously in this review, certain cell types, including nonspecifically activated macrophages, can be quite radioresistant, and, if these cells are playing a role in the antitumor response, their activities would not be expected to be inhibited by irradiation. On the other hand, in some situations in which heterologous antibodies have been shown to be capable of mediating destruction of a carcinoma, whole-body irradiation with 400 r completely inhibited the therapeutic effects of the serum (Order *et al.*, 1974). As noted at the start of this section, several studies have shown that irradiation depresses potential antitumor responses, and further considera-

tions in this area with tumors that are less immunogenic than those usually employed in experimental studies would be warranted, particularly in relation to radiotherapeutic studies (see Bond *et al.*, 1975).

3. Specific antitumor antibodies, which can be radioactively labeled, may provide a powerful tool for the assessment of the number and distribution of tumor cells. Such an approach may be of eventual diagnostic, and perhaps even therapeutic, importance in man (Day *et al.*, 1956; Weissman *et al.*, 1972; Primus *et al.*, 1973).

Immune sera prepared against tumor-specific antigens can occasionally be shown *in vivo* to reduce the growth rate of tumors (Gorer and Amos, 1956), and cytotoxic complement-fixing antibodies are most likely involved. However, in many cases, such inhibition is not found, and antibody-mediated enhancement of tumor growth is more likely. It is clear, from a variety of experiments, that antitumor antibodies can localize on the surfaces of tumors *in vivo*. If the antibody carried with it a source of radiation with high specific activity, then selective radiation killing of the tumor cells might occur. This finds a good precedent in the work of Ada and Byrt (1969), who showed that ^{125}I-labeled antigen bound to the surfaces of antigen-reactive cells specifically killed those cells without affecting normal cells. In a report on the production of a specific precipitin to a renal cancer in man, Nairn *et al.* (1963) suggested that specific antibody to tumors might localize on the surfaces of tumor cells and act as a homing carrier for radiotherapeutic or chemotherapeutic agents. This was then demonstrated in mice by Ghose *et al.* (1967, 1972), who treated Ehrlich-ascites cells *in vitro* with a ^{131}I-labeled antibody to the tumor. After inoculation of these cells into mice, the tumor did not grow. In another series of investigations (Day *et al.*, 1965a,b; Mahaley *et al.*, 1965), it was shown by means of radiolabeled antibodies that antibody molecules to human brain tumors could be localized *in vivo*, and radiolabeled antifibrin antibodies have been shown (McCardle *et al.*, 1966) to localize preferentially in association with certain tumors, since the deposition of fibrin often occurs in these areas.

A detailed investigation has been made on the many factors that influence the binding of radiolabeled antibodies to tumors, by using a model lymphoma system in mice (reviewed by Weissman *et al.*, 1974). It was concluded that considerable investigation is still required to optimize the system in terms of specific binding, which involves problems of purifying specific antibody and suitably radiolabeling the antibody without causing inactivation. This general system may have only limited use with tumors that readily exfoliate tumor-specific cell-surface antigens.

Another recent approach has been the demonstration (Ghose and Cerini, 1969) that antibody-treated Ehrlich-ascites cells are rendered

more radiosensitive than control tumor cells. This effect may be mediated through antibody fixation on the membrane, thereby interfering with the cell membrane permeability and making some of the radiation effects more damaging (Vermund and Gollin, 1968). Doses of radiation which did not greatly influence the growth rate of normal rabbit serum-treated tumor cells severely inhibited the antibody-treated cells. It was suggested that this phenomenon may be related to the correlation of "observed durability of the response to chemotherapy in a Burkitt lymphoma" with the observed frequency of preferential binding of a globulin fraction on the tumor cell's surface (Klein *et al.*, 1967).

VII. Effect of Variation of Irradiation Conditions on Immunological Responses

Much of the experimental work described above has involved single exposures to relatively large doses of radiation, the latter often in the form of X-rays or γ particles. Data derived from such experiments are of particular importance to immunologists, but are of less relevance to radiobiologists. In particular, very little information is currently available on low-dose effects, the consequences of exposure to heavily ionizing versus lightly ionizing radiation, and the influences of fractionation, molecular oxygen, etc., especially as such variables relate to the individual components of the immune response. In large measure, this paucity of data relates to the fact that much of the experimental work on the radiobiology of the immune response was completed prior to the formulation of many of the current concepts in cellular immunology, especially the definition of subpopulations of lymphocytes and of the cooperative relationships that characterize the interactions of these cells. Therefore, many of the relevant radiobiological data need to be reexamined in the light of current immunological thought, and a significant number of the experiments will have to be repeated with defined populations of lymphocytes and more sensitive end points than have been employed in the past.

A. Low-Dose Effects

Studies of radiation-induced inactivation of antibody-forming capacity have usually yielded D_{37} values in the 60–100 r range (see Table VII). Such doses, when administered in whole-body fashion, have usually resulted in significant suppression of antibody formation.

The effect of very low doses (<50 r) upon the immune response has not been extensively investigated to date, primarily because the test systems employed have not been sufficiently sensitive to detect small variations from normal. Recently, the development of extremely sensitive test systems has engendered considerable interest in low-dose effects with

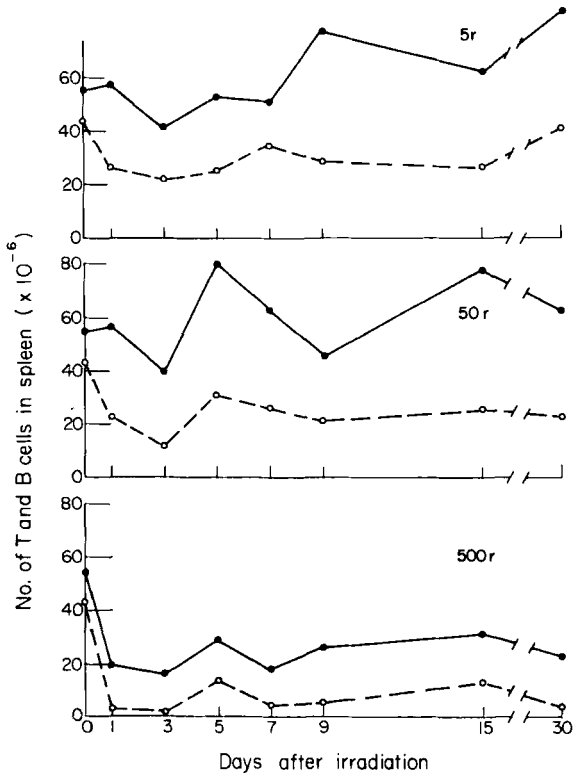


FIG. 20. Absolute numbers of splenic T (●——●) and B (○— —○) cells as a function of whole-body radiation dose and time after exposure. Results represent arithmetic means from 9–10 female, 10–12-week-old CBA/J mice per datum point with cell counts and immunofluorescence testing performed in triplicate. (After R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976.)

some unexpected results. For example, Fig. 20 shows the effect of 5, 50, and 500 r whole-body radiation upon the absolute numbers of T and B cells in spleens of 10–12-week-old female CBA/J mice sacrificed in serial fashion after such exposure. The remarkable increase in T and, to a lesser degree, B cells on day 5 after exposure to 50 r, accompanied by a significant increase in spleen size, was unexpected and remains unexplained, as does the reduced numbers of B cells on day 30 (R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976). Human B and T cells of peripheral blood origin exposed to the same doses of radiation also show significant changes in morphology and responsiveness to LPS, Con A, and PHA (G. B. Olson, R. E.

Anderson, and P. H. Bartels, unpublished results, 1976). Related to these observations, Fig. 21 (R. E. Anderson, J. V. Scaletti, and J. C. Standefer, unpublished results, 1975) shows some of the morphological abnormalities occasioned by exposure of B (nu/nu splenic lymphocytes) and T (BALB/c thymocytes) cells to 50 r *in vitro*. Data for identical cell populations exposed to 0 and 500 r are included for comparison. Table XIII shows the frequency of occurrence of these and related radiation-induced morphological abnormalities as a function of time after exposure. Exposure of T and B cells results in marked abnormalities of the plasma membrane that become more pronounced with time.

The above radiation-induced morphological abnormalities are reflected in dose-dependent abnormalities in specific glycoproteins obtained from aqueous extraction of the plasma membranes of irradiated T and B cells (R. E. Anderson, J. V. Scaletti, and J. C. Standefer, unpublished results, 1975). Loss or alteration of these specific components, kown to be constituents of defined receptor sites, may account for the reduced reactivity of irradiated lymphocytes with specific ligands (Standefer *et al.*, 1975). Conversely, stimulation of T, and possibly B, cells with such ligands prior to or immediately after irradiation may stabilize the involved receptor sites and thereby account in part for the radioprotective effect of PHA and related substances as discussed previously. Alternatively, stimulation of lymphocytes with specific ligands may serve to activate membrane-

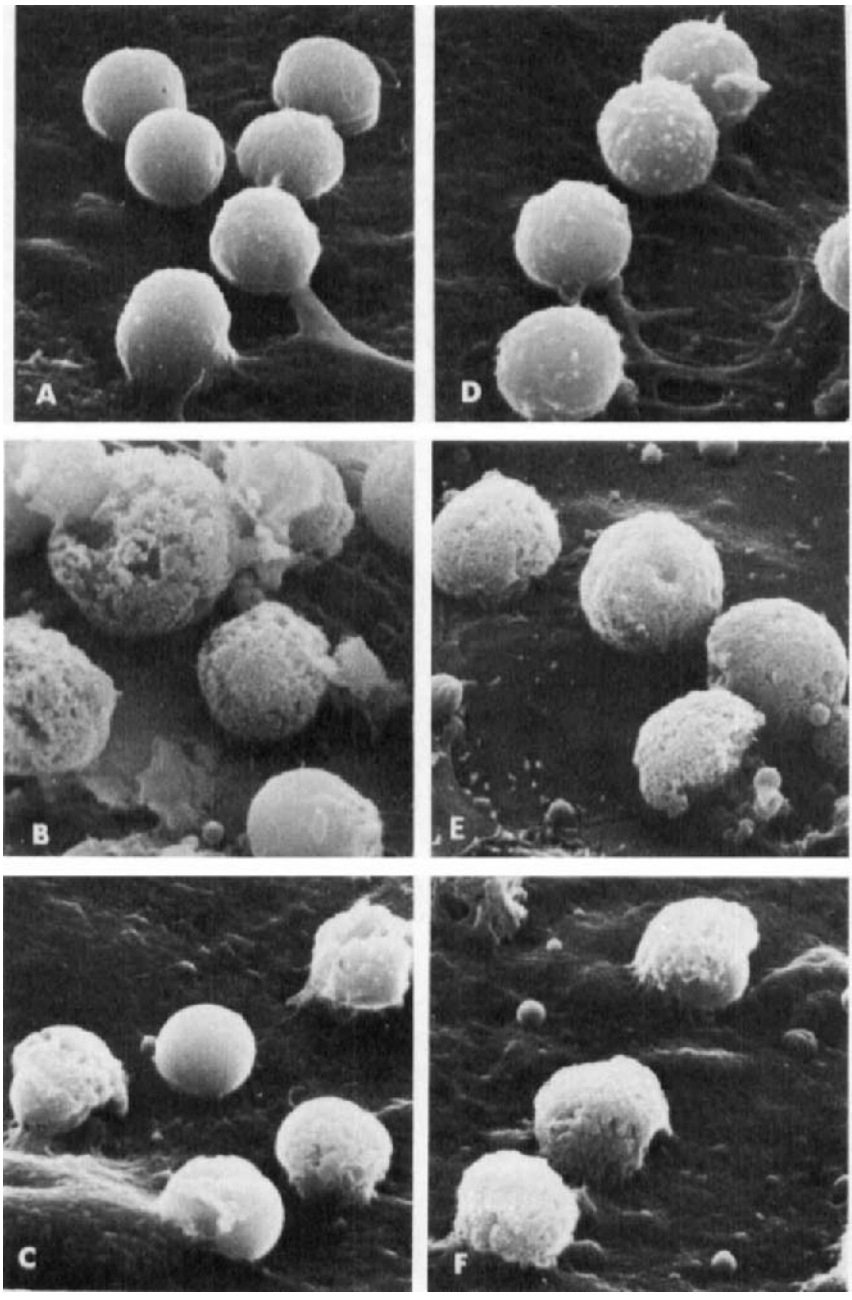
TABLE XIII
MORPHOLOGICALLY ABNORMAL T AND B CELLS AS A FUNCTION OF TIME AFTER EXPOSURE AND RADIATION DOSE[a,b]

Cells	Radiation dose (r)	Percent of cells with abnormalities[c] at times after irradiation		
		2 Hr	4 Hr	24 Hr
T	0	19.8	20.5	15.7
	50	39.0	ND	45.8
	500	47.0	57.2	72.8
B	0	20.6	32.7	34.8
	50	50.2	ND	75.8
	500	58.4	51.5	75.2

[a] After R. E. Anderson, J. V. Scaletti, and J. C. Standefer, unpublished results, 1975.

[b] Experimental protocol is similar to that described in Fig. 21.

[c] Results are expressed as percent of cells with morphological abnormalities evident on SEM.


A
D
B
E
C
F

associated enzymes, which are also active in postirradiation repair processes. Loss or alteration of receptor sites prior to activation of these enzymes would preclude their participation in the reparative processes.

Radiation-induced enhancement of the immune response has also been observed with relatively low doses (see Section III). An enhanced rate of antibody synthesis (heightened peak titer and shortened latent period) was observed in rabbits given 25 r 2 days to 2 hours after antigen injection (Taliaferro and Taliaferro, 1954), and production of hemolysins was prolonged when rabbits were given 25 r as long as 1 month before the injection of antigen (Taliaferro and Taliaferro, 1969). These results show that single doses of radiation in the range of 25–50 r may either depress or enhance the antibody response, the direction of the effect apparently being determined mainly by the temporal relationship between the injection of antigen and exposure to radiation. Preliminary studies on the relative numbers of PHA-responsive versus Con A-responsive cells in mouse spleen, lymph node, and thymus support this concept. The differential response to these mitogens appears to reflect the relative numbers of at least two T-cell subpopulations, perhaps with enhancer and suppressor cell activity (Greaves *et al.*, 1974). Acutely PHA-responsive (? suppressor) cells in CBA/J mice exposed to 5, 50, and 500 r decrease in relative number more markedly than do Con A-responsive (? enhancer) cells in all three tissues. However, with respect to recovery, the converse situation appears to exist, although the data are somewhat less conclusive —PHA-responsive cells appear to regenerate more quickly than Con A-responsive cells (R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976).

With respect to the immune response, a key problem in attempting to extrapolate from high-dose to low-dose effects is that most responses are composed of a variety of interrelated but separable interactions that differ significantly in their response to radiation injury. Some of the known differences in this regard include: susceptibility to interphase cell death, capacity to traffic in a normal fashion, kinetics of cell death and recovery, release of immunologically active subcellular factors at the time of cell

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FIG. 21. Representative SEM photomicrographs of T and B cells exposed to 0, 50, and 500 r *in vitro*. Thymocytes from 20-week-old female BALB/c mice and splenic lymphocytes from 30-week-old female nu/nu mice (BALB/c background) were irradiated or sham irradiated, layered over monolayers of African green monkey fibroblasts grown on cover slips and held at 37°C for 24 hours. The cell suspensions were then processed in standard fashion for SEM. (A) T cells, 0 r; (B) T cells, 50 r; (C) T cells, 500 r; (D) B cells, 0 r; (E) B cells, 50 r; (F) B cells, 500 r. All cells were photographed at the same magnification (×5000). (After R. E. Anderson, J. V. Scaletti, and J. C. Standefer, unpublished results, 1975.)

death, efficiency of repair mechanisms, and response to radioprotective agents, such as mitogens and antigens. The situation is further confused by the possible presence of competing effects. Thus, one day after exposure to 500 r *in vivo*, a more profound decrease in PHA-responsive than Con A-responsive spleen cells is noted although both cell types are decreased in absolute numbers (R. E. Anderson, J. Autry, G. B. Olson, G. M. Troup, and P. H. Bartels, unpublished results, 1976). As a consequence, in some systems, such as the cellular transfer of Ig among congenic strains augmented by irradiation of the thymus (Warner and Anderson, 1975), the net effect of irradiation in this dose range may be an enhanced immune response since reduced numbers of effector cells function more effectively in the absence of an even larger fraction of the more radiosensitive suppressor cells. Clearly, further studies are urgently needed to provide additional information on the relative number of residual suppressor and enhancer cells at various whole-body exposure levels and the kinetics of recovery especially in view of the potential therapeutic implications of the apparent differences described above.

Related to these discussions is the effect of multiple or continuous low doses of radiation on the immune response. A key question in this regard is whether the immune response eventually declines or adapts to continuous low-level exposures. This will be considered in the next section.

B. Fractionated and Prolonged Doses

The amount of data available on the effects of continuous or repeated low-dose exposures is quite limited, but at least provides an order of magnitude of exposure associated with immune suppression. In a study on pathogen-free mice exposed to 1–4 r per mouse, Stoner and Hale (1963) showed that the ability of irradiated animals to produce antibody to some, but not all, antigens was inhibited by cumulative doses in the sublethal range. In several studies in the Russian literature, mice, rats, guinea pigs, rabbits, and monkeys were given daily exposures of 1–21 r for varying periods and examined for their responses to various bacterial antigens or for serum bactericidal activity. In general, no inhibition was observed in immune responses except at the higher daily dose (UNSCEAR Report, 1972).

In considering data on the effects of fractionated low doses, it is important to remember that dose rate as well as absolute dose are important in evaluating the degree of radiation-induced inhibition of immune function(s). Since many fractionated or continuous radiation studies have been performed at very low dose rates, it is difficult to compare these results with similar experiments in which an identical single dose was

employed and administered over a short period of time. Varying degrees of repair, occurring between or even during low-dose exposures, undoubtedly confound the results. In this connection, it would be of great value to select a sharply quantifiable end point and then compare (i) the single dose versus (ii) the fractionated dose versus (iii) the continuous-exposure dose required to achieve this end point.

Several recent studies have extensively explored the question of the effect of dose rate on immunodepression, with both neutron and X-irradiation (Carlson and Gengozian, 1971; Gottlieb and Gengozian, 1972a,b). With neutron irradiation, antibody formation was markedly inhibited with antigen given 12 hours after exposure, and the degree of depression was dependent only on the total dose, not on the dose rate. In contrast, with X-irradiation, the dose rate as well as the total dose affected the degree of immunodepression (Fig. 22). This discrepancy probably relates to the fact that dose-rate effects are reduced or absent with high linear energy transfer (LET) particles, such as neutrons. Cells exposed to high

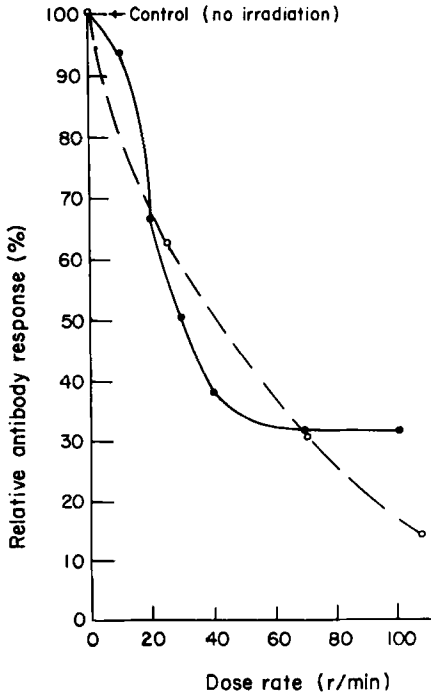

FIG. 22. Effect of exposure rate on antibody responses of mice (●——●) and rats (○— —○). Mice were given a total exposure of 700 r and rats 500 r. Results are expressed as percent of control (no irradiation) values. (After Gengozian *et al.*, 1968; Simic *et al.*, 1965.)

LET radiations generally either die or escape unscathed—in either case, no sublethal damage is present to be repaired.

A question that is especially relevant to this discussion is whether repeated small doses or low-level continuous irradiation give rise to an accumulation of damage, or whether restoration (and/or repair) after small doses is complete and thus adaptation of the immune system to repeated or protracted exposures occurs. Although a large amount of data is available on the susceptibility of the immune response to large doses of radiation, very little information exists that is relevant to the issue of restoration and/or repair.

It is important to distinguish the concept of adaptation to repeated low-level irradiation from the suggestion that the antibody–cell series may develop acquired radioresistance. The latter view was first proposed in studies with mice (Petrov and Cheredeev, 1968), but, as has been discussed previously, the data can be explained by radiation-induced alterations in the proportions of interacting cells. Instead, the concept of adaptation implies that (i) the precursor cells involved in the immune response are all equally radiosensitive; (ii) they are being continually replaced throughout life; and (iii) at any given time, there are many more potentially immunocompetent cells with respect to a given antigen than are required to produce the usual level of response. Kennedy *et al.* (1965) in fact suggested, on the basis of their dose-response data, that the immune system could suffer at least a thousandfold depletion of the proliferative capacity of its cells without completely losing the ability to respond to an antigen by the production of PFC. This actual number varies for different antigens, and further studies of this type are clearly indicated.

C. Radioisotopes

Radioisotopes have been used experimentally to deliver radiation at localized sites in the lymphatic system. This includes such methods as the application of ^{32}P-impregnated polythene strips to the surface of the spleen (Ford, 1968), intraatrial implantation of a β-emitting source (Barnes *et al.*, 1964), and intralymphatic infusions of radioisotope-labeled agents (Tilak and Howard, 1964; Wheeler *et al.*, 1965; Edwards *et al.*, 1967). Unfortunately, accurate dosimetry has been a persistent problem in the use of these approaches experimentally or therapeutically. This problem is especially acute with radioactive substances administered parenterally. In this situation, dosimetry depends not only on the tissue distribution of the substance under consideration, but also, on its biological and physical half-life, the energies of the particles emitted, and the radiosensitivity of the adjacent tissues. For example, ^{32}P-labeled col-

loidal chromic phosphate in a dose of 780 μCi administered intravenously to rabbits (Yohn and Saslow, 1964) was calculated to yield 14,000 r during the 14 days between isotope injection and introduction of antigen. As might be expected, this approach resulted in a marked depression of antibody formation. The resultant effect could be counteracted by multiple antigen injections, which might indicate that the major effect of the intravenously injected isotope was on the spleen, and that, as a consequence of multiple injections, nonsplenic sites participated in the response. However, in a related study (Chiba *et al.*, 1967), the injection of ^{32}P chromic phosphate showed some effect on all organ systems when administered intravenously, whereas selective injury to lymphoid tissues occurred only in association with intralymphatic injection.

The literature (see UNSCEAR Report, 1972) also contains a large amount of data concerning the effects of various internally deposited radioisotopes (^{210}Po, ^{90}Sr, ^{45}Ca, ^{32}P, ^{131}I, ^{198}Au, ^{65}Zn and an unseparated mixture of nuclear-fission products) on immunogenesis when experimental animals are immunized with a wide variety of bacterial antigens. In most cases immunodepresssion occurs with suitable doses.

VIII. Conclusions

As has been emphasized throughout this review, much of the experimental work involving radiation and the immune response was performed prior to the development of many of the precepts that guide current immunological thought, in particular the definition of the cooperative relationships among the various subpopulations of immunocompetent cells. Since it is now possible to dissect many of the individual components of the immune response and to isolate functionally uniform subpopulations of lymphocytes, many classical experiments that relate radiation to immune dysfunction should be repeated with defined populations of cells.

One of the best illustrations of the injurious effects of ionizing radiation on immunity is the decreased resistance of irradiated animals (usually in the 200–600 R exposure range) to specific pathogens. This has been demonstrated countless times with many different pathogens of bacterial, viral, rickettsial, and fungal types. Decreased resistance to infection varies considerably for different infectious agents, species of host, types of infections (acute or chronic), and radiation parameters. Part of this variation may depend on the type of assay system employed. For example, it appears that radiation-induced decreased resistance to infection primarily occurs not immediately but several days after exposure. Challenge with an acutely infectious agent at the time of radiation does not produce

any decrease in resistance. Challenge with an agent that induces a more chronic infection is more likely to produce decreased resistance when introduced at the time of or immediately after irradiation. Radiation-induced decreased resistance to infection is primarily mediated by a decrease in the host's specific immune response, although other nonspecific factors may also be of importance, particularly macrophage handling of antigen and granulocyte functions.

Phagocytosis of antigens and antigen degradation are relatively radioresistant with doses of the order of 1000 r. Some changes in granulocyte activities have been reported even with relatively low doses (100 r), but the significance of this for the eventual immune response is probably minor. In several studies, although irradiated macrophages successfully phagocytosed antigen, they did not appear to be capable of processing it in a manner that is obligatory for the initiation of the immune response. Alternatively, with other antigenic systems, irradiated macrophages were more efficient than controls in presenting immunogenic material. Antigen handling in lymphoid follicles appears to be particularly important for the development of the secondary response, and radiation inhibition of this function may be a factor in antibody depression.

Depending upon dose, dose rate, and time of irradiation relative to antigen injection, the immune response may show either a shortened lag phase and higher antibody levels, or a lengthened lag phase and a reduced antibody response. Increased antibody responses may occur either (1) when the radiation dose is low (e.g., observed with 25 r) or (2) when the antigenic stimulation is administered at very specific critical times after irradiation.

In primary responses, if the involved T cells need to proliferate for collaboration with B cells, then the response will appear radiosensitive, whereas in carrier functions of secondary responses proliferation may not be as important, and radiation in doses of up to 2000 r does not seem to interfere with this function, *providing* the activated T cells are in their correct microenvironment.

The logarithmic phase of antibody production is only moderately radiosensitive because of the mixture of proliferating immature plasmablasts and of highly radioresistant mature nondividing antibody-synthesizing cells. No significant depression of antibody secretion is observed in the populations of cells irradiated in late logarithmic, plateau, and decline phases. During these periods, most of the involved cells are mature plasma cells.

The secondary antibody response has often been described as radioresistant in studies of overall antibody production in whole animals. However, many of the differences between primary and secondary responses

can be accounted for by the numbers of potentially available cells that can be called upon for the particular immune response.

In considering the radiosensitivities of various subpopulations of lymphocytes, several preliminary conclusions might be drawn: (1) B lymphocytes are quite radiosensitive and probably undergo interphase as well as mitotic death following irradiation. (2) Within all functional categories of T-lymphocyte subpopulations (i.e., helper, suppressor, and cytotoxic), there is a clear change from extreme radiosensitivity of the precursor cell to radioresistance of the effector cell. Suppressor cells are particularly radiosensitive, and may be the only T-cell subpopulation to undergo interphase death. (3) In evaluating the effect of radiation on any type of lymphocyte, specific attention must be paid to the assay system, since several plasma membrane-related properties of the cell may be affected by irradiation (e.g., homing potential to an appropriate microenvironment) even though the main differentiated function of the cell type in question may not be affected.

The basis of the remarkable radiosensitivity of the small lymphocyte remains to be defined especially with respect to interphase cell death. Definition of apparent differences in the radiosensitivity of subpopulations of lymphocytes may provide a key with which to attack this problem. Two possible approaches are readily apparent:

1. An investigation of radiation-induced lesions in the plasma membrane of uniform subpopulations of lymphocytes. As currently defined, each subpopulation of lymphocytes possesses a uniquely characteristic surface. These membrane-associated differences may relate to the apparent discrepancies in radiosensitivity referred to above. Currently available SEM and biochemical data would tend to support this concept. A major problem in this area, however, is the determination of whether specific lesions are primary (for example, caused directly by ionizing radiation) or secondary. For example, radiation-induced inactivation of membrane-associated Na^+–K^+-dependent ATPase could result in a variety of profound alterations, which would all be secondary phenomena.

2. An investigation of repair of radiation-induced cell injury in uniform populations of lymphocytes. Techniques are now available to evaluate repair of radiation-induced DNA lesions. Comparisons among uniform subpopulations of lymphocytes of the activity of such enzymes would be of particular interest. A similar comparison utilizing single versus split-dose exposures would provide possible insight into other repair mechanisms. Careful low-dose experiments addressed to interpreting interphase death and defining the character of the dose-response curve at low levels of exposure would also be relevant to this point.

In a broader context, much remains to be learned about the mecha-

nisms involved in the suppression of the immune response that often follows whole-body irradiation. It is clear that irradiation interferes with the normal traffic of injured but still viable cells. To date, such distortions of cell traffic have been evaluated on an organ-by-organ basis. Future experiments will need to address the issue of what proportion of irradiated cells home to the appropriate organ(s) but fail to navigate to the proper microenvironment where cell–cell cooperation can take place. In addition, *in vivo* observations should be correlated with comparable data obtained *in vitro* and putative differences reconciled. Accurate measurements of the radiosensitivity of the various human lymphoid cells involved in the development of an immune response should be made *in vitro*. There is virtually no well controlled careful assessment of the radiosensitivity of the antibody response in man. Since this may be extremely relevant to certain aspects of radiation and neoplasia, some effort should be made to ascertain the radiosensitivity *in vitro* of the individual human lymphoid populations involved in different stages of immune responses, with due consideration to effects of different forms of radiation, dosage, and dose rate. With the various cell-separation systems available and the methods at hand for inducing primary antibody formation *in vitro* and for quantitating numbers of antibody-producing cells, it would be possible to carry out the following determinations: (a) *macrophages:* with certain antigens macrophages are required for the primary induction of antibody formation. Through the use of a macrophage-free test system, assays could be performed on the effect of adding macrophage preparations that were previously subjected to a series of radiation exposures. (b) *Antigen-reactive cells and antibody-forming precursor cells:* in similar fashion, T cells and B cells could be irradiated separately at various doses, recombined with the appropriate nonirradiated cell type, and studied for the capacity to collaborate toward antibody production *in vitro*. In man, bone marrow cells and thymus cells (obtained from tissue fragments at surgery or at autopsy in forensic cases) could be assayed, together with T cells and B cells derived from normal human blood, and fractionated by some of the immunological techniques now available. (c) *Plasma cells:* antibody-PFC from any primary induction system with human lymphoid cells could be assayed for radiosensitivity *in vitro*.

In studies with *Shigella* antigen and mice, the radiosensitivity of the macrophages has been stressed. This type of study in experimental animals should be extended to other antigens, since, if this is a major factor with bacterial antigens, then susceptibility to infection following sublethal irradiation with doses above 100 r might primarily involve interference with macrophage function.

Additional low-dose experiments need to be performed with individual

subpopulations of lymphocytes. Enhancement of some immune phenomena appears to occur after low-dose exposures. Such augmentation may relate to alterations in the proportions of interacting subpopulations, or radiation injury may augment the release of immunologically active molecules. Another intriguing possibility is that repair processes that are activated after nonlethal exposures may also serve nonspecifically to improve the efficiency of the immune response. For example, electron spin resonance studies suggest that irradiation may alter the fluidity of the plasma membrane of lymphocytes (R. E. Anderson, J. V. Scaletti, and J. C. Standefer, unpublished results, 1975). Alterations in membrane fluidity could enhance a variety of immune processes.

With respect to the radiosensitivity of suppressor and enhancer cells, much additional work needs to be done. Most of the currently available evidence suggesting that suppressor cells are more sensitive than enhancer cells is derived indirectly by utilizing complex systems in which a balance between the two cell types determines the actual net effect. In addition, it will be of considerable interest to determine the kinetics of regeneration of suppressor and enhancer cells postirradiation. Preliminary data suggest that such regeneration occurs asynchronously. Computer-assisted morphometric analysis may be of assistance in this regard since analysis of apparently uniform populations of murine T cells shows 3 and possibly 4 subpopulations.

In the field of transplantation, there are at least two areas in which radiation can be of considerable value. In organ transplantation, it is clear that immunosuppression from whole-body irradiation with sublethal doses is not feasible. However, ECI of the blood and local graft irradiation soon after grafting both have been shown to be of value, particularly in acute rejection reactions. Further experimental studies with these techniques, preferably in large animals, should be continued, and in particular should address alternative schedules of irradiation. The feasibility of other approaches, such as intralymphatic injection of radioactive colloids in the suppression of allograft rejection, also needs further evaluation, with much emphasis on a more accurate determination of the dose received by various cells and tissues.

In marrow transplantation, several promising approaches to the elimination of the secondary disease syndrome must be actively pursued if elimination of leukemic cells by radiation, followed by marrow transplantation, is to be a practical form of therapy. Such approaches include comprehensive multifactorial studies, fractionation of immunocompetent cells from hematopoietic stem cells, and elimination of immunocompetent cells by appropriate pretreatment. Host thymic factors (? epithelial in origin) are probably involved in inducing differentiation of donor mar-

row stem cells into immunocompetence. This host component might be vulnerable to radiation suppression and thereby result in a depression of the initiation of secondary disease. Further studies of this phenomenon are needed to determine whether this could lead to a practical approach for the elimination of secondary disease.

If the concept of immunological surveillance is applicable to most forms of cancer, it might be expected that irradiated individuals would show an increased susceptibility to all types of neoplasms, approximately in proportion to their normal incidence. Alternatively, since serum-blocking factors involve antibody, then depression of these factors could effectively result in an increased efficiency of cell-mediated tumor regression.

Since radiation does not destroy the immunogenicity of tumors, but rather may actually enhance it, further studies are fully warranted on the possible cumulative effects of combined immunotherapy and radiotherapy of certain tumors. Further technological studies are also required to optimize the potential of using radiolabeled antibodies in either immunodiagnosis or immunotherapy.

Experimentally, it will be of interest to see whether the development of radiation-induced neoplasms in mice can be aborted by the periodic administration post-exposure of defined subpopulations of uneducated syngeneic T cells. If radiation-induced neoplasia is related to impaired immune suvreillance, then transfusion of the deficient or defective subpopulations should reduce tumor incidence. Suppressor and enhancer T cells are of particular interest in this regard.

The concept of loss of the tolerant state after irradiation leads naturally to a consideration of the possible autoimmune consequences. Although several potential mechanisms exist, current immunological interest is concerned with the balance of suppressor control over potential and preexisting autoimmune aggressor cells. Radiation may precipitate the activity of the latter cells by selectively inhibiting the suppressor cell type. Further studies on this aspect are clearly required, with due consideration to radiobiological parameters such as dose, dose rate, and quality of irradiation as well as persistence of the effect.

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