ADVANCES IN IMMUNOLOGY

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F. J. Dixon &
Henry G. Kunkel

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PREFACE

Immunologic research covers a broad field ranging from the molecular structures of Ig molecules and their genetic implications to the nature of immunologic diseases of man and animals. These varied studies are interdependent in that what is being learned about Ig molecules at the molecular level is being translated into mechanisms of immunologic responses and reactions in experimental situations, and the results of these studies serve as a basis for our developing understanding of the pathogenesis of clinical immunologic diseases. The reviews included in Volume 16 cover this broad spectrum of investigation and illustrate well the cohesiveness of immunologic research.

In the first contribution, Drs. Natvig and Kunkel present in detail the antigenic and biochemical basis for the classification of Ig molecules. Antigenic analyses of Ig molecules from different individuals have provided a long list of genetic markers of the Ig genes, the behavior of which appears to be controlled by regular Mendelian laws. The distribution of Ig classes and subclasses in normal serum and the selective nature of Ig classes in certain antibodies are discussed, along with the characterization of Ig molecules on the surfaces of normal lymphocytes, leukemic lymphocytes, and myeloma plasma cells. Myeloma proteins and related homogeneous Igs are compared, and their biological activities and structurally abnormal forms are described. Finally, the idiotypic antigens of the variable portions of light and heavy chains of myeloma proteins and specific antibodies are discussed and their use in the study of the variable regions in different Ig molecules is described.

The second article, by Dr. Weigle, is an authoritative discussion of immunologic unresponsiveness. Certainly, therapeutic manipulation of the immune response to achieve specific unresponsiveness in a variety of immunologic diseases is a prime goal, and recent advances in our understanding of this phenomenon bring us closer to its realization. As Dr. Weigle explains, the induction and termination of unresponsiveness can now be described in terms of B and T lymphocytes, their antigen receptors, serum antibody, and antigenic structure and dose. Finally, the implications of these mechanisms of induction and termination of unresponsiveness for autoimmune and neoplastic diseases are placed in good perspective by the author.

In the third contribution, Drs. Wheelock and Toy describe the complex interrelationship between lymphocytes and infecting viruses. Lym-

phocytes may play a variety of roles in viral infections—from acting as primary targets of infection to providing an immunologic barrier against infection and, finally, to mediating the destructive changes in a virus-induced immunopathologic disease. Infection of lymphocytes in both acute and chronic viral infections is common and may be evidenced by both morphologic and functional abnormalities of the lymphocytes. The basis of the inadequacy of the lymphoid system in immunodeficiency states and after immunosuppression which predisposes to viral infections and to activation of latent viruses is discussed, along with the ways in which lymphocytes may normally act to terminate viral infections. Finally, the means by which lymphocytes contribute to viral disease—by harboring virus and allowing replication, by themselves undergoing malignant transformation, and by inducing allergic inflammation—are considered in detail.

Drs. Cochrane and Koffler provide, in the fourth article, a complete and critical statement of our knowledge of immune complex diseases. There can be little doubt of the frequency of immune complex diseases caused by circulating antigen–antibody complexes. Many of the mechanisms by which these complexes cause disease are recognized, and the authors define them clearly in molecular and cellular terms. The missing element in most clinical immune complex diseases is identification of the antigen or etiologic agent. However, the description of the presently known etiologic agents and their roles in pathogenesis which is presented here will certainly contribute to an intensified search for other agents and an improved understanding of this important pathogenic mechanism.

In the final contribution, Dr. Zvaifler describes immunopathologic events contributing to joint disease in rheumatoid arthritis. The peculiar anatomy of the joint which permits or encourages the production of chronic inflammation is considered. The phlogogenic events, including the accumulation and precipitation of immunoglobulins presumably as antibody–antigen complexes, the activation of complement, kinins, and clotting factors, and the participation of polymorphonuclear and mononuclear leukocytes in the inflammation, are critically discussed and integrated. As the author points out, our understanding of the pathogenesis of rheumatoid arthritis is reasonably clear but we still await identification of the etiologic agent and/or antigens involved.

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FRANK J. DIXON
HENRY G. KUNKEL

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Human Immunoglobulins: Classes, Subclasses, Genetic Variants, and Idiotypes

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I. Immunoglobulin Classes and Subclasses

A. Introduction

The immunoglobulin classes and subclasses represent a group of structurally related proteins, and in all instances consist of two pairs of polypeptide chains held together by disulfide bridges and noncovalent forces. Each chain has an N-terminal part of about 107 to 115 amino acids, which contains the variable areas and is primarily involved in the specific interaction with the antigen, and a constant portion of about

107 to 110 amino acids for the light chain and about 310 to 330 amino acids for the heavy chain. The constant part, particularly of the heavy chains, carries the sites for many of the other important biological activities, such as complement fixation, interaction with rheumatoid factors, interaction with receptors on cell membranes, transplacental transfer, and passive cutaneous anaphylaxis (PCA). The heavy chains (about 450 amino acids) are distinctly different in biochemical and antigenic structure for each of the immunoglobulin classes and subclasses (see Killander, 1967; Cairns, 1967; Edelman *et al.*, 1969; Bearn, and Kunkel, 1968). These differences form the basis for the classification of the immunoglobulins into five main classes and into four IgG and two IgA subclasses (Grey and Kunkel, 1964; Terry and Fahey, 1964; Kunkel and Prendergast, 1966; Feinstein and Franklin, 1966; Vaerman and Heremans, 1966). At present it is not known whether there are subclasses within the three other immunoglobulin classes, IgM, IgD, and IgE. The previously reported subclasses of IgM were probably related to variable region subgroups of IgM proteins. Each chain can interact with any type of light chain (κ or λ) and any light-chain subgroup to make an immunoglobulin molecule.

The light chains (about 214 amino acids) are common to all immunoglobulin classes although the ratio of κ to λ is not always the same. Table I shows the distribution of light-chain types within the various IgG subclasses.

The accumulated evidence indicates that the synthesis of the variable and the constant region (Hilschmann and Craig, 1965) of the chains is governed by different genes, one variable (V) and one constant (C) (Hood and Prahl, 1971). There appear to be three main groups of the V-region genes: those for κ light chains, those for λ light chains, and those for all the heavy chains. Each of the variable groups consists of a number of subgroups (Milstein, 1967; Solomon *et al.*, 1965; Tischendorf *et al.*,

TABLE I
RATIO OF κ TO λ PROTEINS IN IMMUNOGLOBULIN G SUBCLASSES

	IgG1		IgG2		IgG3		IgG4	
Protein	1[a]	2[b]	1[a]	2[b]	1[a]	2[b]	1[a]	2[b]
κ	104	155	11	30	9	15	5	14
λ	43	109	10	31	8	12	1	2
Ratio κ/λ	2.41	1.42	1.10	0.96	1.12	1.25	5.0	7.0

[a] (1) Data of Terry *et al.* (1965).
[b] (2) Data of Schur *et al.* (see Schur, 1972).

1970; Köhler *et al.*, 1970). Thus, there exists probably a pool of heavy-chain V genes which can be combined with any heavy-chain C gene to make heavy polypeptide chains. Similarly, the pool of κ V genes combines with κ C genes to make κ light chains, and the pool of λ V genes behaves similarly.

The immunoglobulins can, therefore, be classified according to three different parameters related to different antigenic and biochemical structures:

1. Heavy-chain C regions constitute the basis for the different classes and subclasses.
2. Light-chain C regions specify the types or subtypes.
3. Variable regions of both heavy and light chains can be divided into different groups and subgroups.

It appears that the respective genes specifying these classes and subclasses, types and subtypes, and groups and subgroups are present in the normal genome of every single individual. Furthermore, there are also genetic variants imposed on these structures. So far, genetic markers in the human have only been detected for the constant region of heavy and light chains. However, markers in the variable regions are likely to be found and have been described in other species (Todd, 1966; Edelman and Gottlieb, 1970).

Immunoglobulin G is the major immunoglobulin class making up about 80% of serum immunoglobulin. There are four subclasses which were first distinguished by antigenic differences. These also show distinct peptide and amino acid differences and differences in biological activities which will be considered in detail later.

Immunoglobulin A is the dominant immunoglobulin class in secretions and may be present in serum as a monomer or polymer. Immunoglobulin contains two subclasses, IgA1 and IgA2. The latter shows a genetic variant, Am+, which is of special interest because it lacks disulfide linkage between the heavy and light chain (Kunkel *et al.*, 1969b; Grey *et al.*, 1968a; Jerry *et al.*, 1970). In the secretions the proteins contain an additional protein termed *secretory piece* (see review Tomasi and Grey, 1972). The IgA polymer together with IgM also appear to contain an extra chain called *J-chain* (Halpern and Koshland, 1970).

Immunoglobulin M is usually found as a pentamer structure, but 7 S monomers have been observed (see review Metzger, 1970); IgD and IgE (see review Bennich and Johansson, 1971) are present as monomers.

B. Antigenic Relationships

The antigenic properties of the immunoglobulins can be divided into three main categories: (*1*) antigens present in all normal sera are called

isotypes; (2) antigens present in some but not in all normal sera and which segregate as if controlled by allelic genes are called *allotypes;* (3) antigenic properties related to one particular antibody population are called individual specificities or *idiotypes.* First consideration will be given to the isotypes and allotypes, which are related to the C heavy-chain region. We shall return to idiotypes and other V-region markers in Section V.

1. Antigenic Markers Present in All Normal Sera (Isotypes)

Class-specific antigens represent one group of isotypes. No cross-reactions have been demonstrated for antigens of the C regions of the different immunoglobulin classes, although homologies are clearly apparent from sequence studies. The subclasses, however, show many shared antigens. In addition, they show a variety of subclass-specific antigens which are present in all normal sera. They were originally defined by precipitation techniques (Grey and Kunkel, 1964; Terry and Fahey, 1964), but recently many more antigens have been delineated by employing sensitive hemagglutination inhibition methods (Natvig and Kunkel, 1968, 1969). Table II shows isotypes restricted to single subclasses as well as isotypes shared by all proteins in more than one particular subclass but omits the nonmarkers described below.

a. Immunoglobulin G Subclass Antigens and Methods to Obtain Subclass Antisera. The subclass-specific isotypes are usually brought out by relatively strong antisera made against one subclass protein and

TABLE II
IMMUNOGLOBULIN SUBCLASS-SPECIFIC ISOTYPES AND ISOTYPES SHARED BY ALL PROTEINS OF MORE THAN ONE SUBCLASS

Subclass-specific isotypes	Shared isotypes
IgG1 k Fab	IgG1-2-3 Fc
IgG1 $F(ab')_2$	IgG2-3 Fc
IgG1 Fc[a]	IgG2-4 Fc
IgG2 k Fab	IgG1-3 Fc
IgG2 Fc	
IgG3 Fab	
IgG3 $F(ab')_2$	
IgG3 Fc	
IgG4 k Fab	
IgG4 Fc	

[a] For each of the 4 IgG subclasses, there are two Fc antigens, one for each Fc homology region.

absorbed extensively with other subclass proteins (Grey and Kunkel, 1964; Terry and Fahey, 1964). A similar approach was used for establishing isotype antigens in hemagglutination inhibition. Here, the same antisera were used together with red cells coated by IgG carrying the respective antigens tested for (Natvig and Kunkel, 1969). It has also been shown that human anti-IgG antibodies may detect common subclass antigens (Allen and Kunkel, 1966) and also antigens confined to one subclass (Natvig, 1970; Natvig and Turner, 1971; van Loghem and deLange, 1970).

Antisera specific for each subclass that can be used by simple agar diffusion methods are obtained with considerable difficulty and have not become generally available. In particular, anti-IgG1 antisera have proven very hard to obtain. Antisera to IgG3 can be obtained readily in rabbits by ordinary immunization procedures; this is also the case with IgG4, although this is somewhat more difficult. Immunoglobulin G2 antisera are obtained readily in monkeys. In the authors' laboratories, antisera specific for IgG2 and IgG1 have been obtained in a few rabbits by immunization with isolated heavy chains. At present, the method of choice appears to be that of Spiegelberg and Weigle (1968), where tolerogenic injections are included in the rabbit regimen. Good antisera to IgG1 have been obtained by this method in some but certainly not all animals. There are many pitfalls in the use of subclass antisera by agar precipitation techniques, the chief of which relates to the influence of the light-chain types used in the immunization. Many antisera made against κ proteins show specificities that relate to the light chain but are not absorbed out by or inhibited by κ proteins of other classes (Tables II and IX). This situation is well described for IgA proteins (Prendergast *et al.*, 1966) and has also been described for IgM (Terry *et al.*, 1969). Recently it has also been encountered with antisera to the IgG subclasses in the authors' laboratories and caused considerable difficulty. The problem is less for λ proteins which, therefore, offer advantages for most immunizations.

Another problem relates to common antigens between certain subclasses, particularly those between IgG3 and IgG2. Many monkey and goat antisera against IgG2 proteins cross-react very strongly with IgG3 and lose precipitation capacity after IgG3 absorption. However, they are good IgG2–IgG3 antisera. With nonprecipitating systems, such as hemagglutination inhibition, the antisera are a much smaller problem. Ordinary rabbit immunization in many instances have provided antisera that are specific for each subclass. However, the problems mentioned above also are evident depending on the standard used on the red cells. Subclass-specific determinants can be detected by immunofluorescence technique and also here the antisera are a smaller problem than with precipitating systems (Munthe and Natvig, 1971).

TABLE III
HEMAGGLUTINATION INHIBITION ASSAY SYSTEM FOR DETECTING THE $\gamma_{1\text{-}2\text{-}3}$ FC ISOTYPE ANTIGEN[a]

Immunoglobulin subclass	Concentration of inhibitor				
	0.5	0.12	0.03	0.08	0.002
IgG1[b]	0	0	0	±	2
IgG2	0	0	0	0	2
IgG3	0	0	0	1	2
IgG4	2	2	2	2	2
Protein La[c]	0	0	0	1	2
IgG1 Fab	2	2	2	2	2
IgG1 Fc	0	0	0	0	2

[a] Test system: rabbit anti-IgG2 absorbed with IgG4 and red cells coated with IgG1 Gm(a) anti-Rh.

[b] No differences were observed between various allelic types of the subclass proteins.

[c] The hybrid IgG4–IgG2 protein mentioned on p. 27.

When studying the antigens present in more than one subclass, one should generally coat the cells with protein from a different subclass than that used for immunization to prevent idiotypic dominance and the detection of the most narrow subclass-specific antigens. Table III shows as an example the results obtained for the IgG1-2-3 antigen. This antigen is clearly related to heavy-chain subclasses irrespective of light-chain types and variable region subgroups and resides in the Fc part of the heavy polypeptide chain.

b. Quantitation of Subclass Levels. Table IV shows the mean IgG subclass concentration in normal adults as obtained in independent studies from several different laboratories. The series of Leddy was determined by a radioimmunoassay procedure. The agreement is quite

TABLE IV
PERCENTAGE DISTRIBUTION OF IMMUNOGLOBULIN G SUBCLASSES IN NORMAL SERA AND INCIDENCE AMONG MYELOMA PROTEINS

IgG1	IgG2	IgG3	IgG4	Reference
66 ± 8[a]	23 ± 8	7.3 ± 3.8	4.2 ± 2.6	Yount *et al.* (1970)
64[b]	28	5	3	Leddy *et al.* (1970)
69[c]	18	8	5	(Authors' material)
72[d]	17	7	4	Schur (1972)

[a] Normal values ± 1SD for 145 adult Caucasian sera.

[b] Mean values for 10 normal adults.

[c] Myeloma incidence (144 cases).

[d] Myeloma incidence (368 cases).

striking. These levels are very close to the incidence of myeloma proteins of the different subclasses in one series studied (Natvig *et al.*, 1967b). Several other series have shown similar results except for a slightly lower incidence of IgG2 myeloma proteins (Terry *et al.*, 1965; Virella and Hobbs, 1971; Schur, 1972).

The IgG subclass levels are of course determined by both synthetic rate and catabolism. The catabolic rate ($T/2$) appears to be in the range of 11-12 to 20-21 days for IgG1, IgG2, and IgG4 (Spiegelberg and Weigle, 1968; Morell *et al.*, 1970), whereas the fractional catabolic rate for IgG3 is much lower. This may be due to the aggregation of IgG3 proteins recently described (Capra and Kunkel, 1970).

It is of special interest that the subclass levels are clearly related to allotype. This was first described for the IgG3 system (Yount *et al.*, 1967) where the product of the Gm^b allele is present at two times the level of the product of the Gm^g allele. These results have been confirmed recently by Litwin who also found some effects in the IgG1 system although they were not as marked as for the IgG3 (Litwin and Balaban, 1972). Preliminary findings for IgG4 also indicate a very marked effect (van Loghem, 1971) of allotype on the serum IgG4 levels. Recent work in other species has shown similar relationships between allotype and Ig levels as well as the immune response to certain antigens.

2. *The Principle of the Nonmarkers (Markers Shared by at Least Two Subclasses Which Show Genetic Variability within One Subclass)*

The wide use of myeloma proteins in studies on the genetic antigens in the human has brought forth an important new principle not observed in animal studies. This is the concept of the nonmarker which represents an antigen shared by different subclasses but is changed in one subclass by a genetic event in that subclass only. This expression has been utilized because in most instances the regular genetic markers were first characterized. It was convenient to employ an expression to show that the new marker was antithetic to an already known genetic marker. Those antigens that are present on all proteins of more than one subclass reflect the many structural homologies preserved in the recently duplicated subclass genes. In contrast, the subclass-specific antigens reflect unique structures that have developed by mutations or other genetic events, occurring in one cistron after the dissociation of the subclasses (see later). In accord with this concept, the immunoglobulin system shows two different types of genetic markers, depending on whether a genetic event is related to a subclass-specific region or a region shared by other subclasses: in the first instance, two regular allelic genetic

markers appear, such as Gm^r and Gm^z; in the other instance, one might get one regular Gm marker, which behaves as an allele in one subclass, such as Gm^a whereas the antithetic marker is simultaneously shared by other subclasses. This latter type of genetic marker is called a *nonmarker*. These nonmarkers are not detected in the usual isoimmunization procedure used in experimental animals and were only detected in the human through the study of myeloma proteins and their antisera made by heteroimmunization. Table V summarizes the distribution of nonmarkers among IgG subclasses and allelic variants.

The first such genetic marker shared by other subclasses was "non-a." The peptide (Frangione *et al.*, 1966) and the corresponding antigenic marker (Natvig *et al.*, 1969) were present in all Gm(a)-negative IgG1 proteins and in all IgG2 and IgG3 proteins irrespective of genetic type and were absent in the IgG4 subclass and in other immunoglobulin classes. There was striking agreement between the chemical and antigenic analyses.

Another similar antigen "non-g" was present in Gm(g)-negative IgG3 proteins, but also in all IgG2 proteins (Natvig *et al.*, 1969). These nonmarkers are always present in normal human serum but show dose variations in different individuals. This is particularly clear for non-a since it is a genetic marker of the major subclass IgG1. Although it is difficult to utilize non-a and non-g as genetic markers in whole serum, they have proven to be valuable in characterizing isolated myeloma proteins and other immunoglobulin molecules. Recently, a technique was developed to isolate IgG1 and IgG3 Fc fragments from normal serum whereby non-a and non-g could be used as genetic markers in these sub-

TABLE V

DISTRIBUTION OF THE VARIOUS NONMARKERS AMONG IMMUNOGLOBULIN G SUBCLASSES AND ALLELIC VARIANTS

Immunoglobulin subclass and Gm variant	Non-a	Non-g	Non-b^0	Non-b^1	4a	4b
IgG1 Gm(a)	−	−	+	+	+	−
Gm(f)	+	−	+	+	+	−
IgG2 n+	+	+	+	+	−	+
n−	+	+	+	+	−	+
IgG3 b^0+ b^1+	+	+	−	−	+	−
b^0+ b^1−	+	+	−	+	+	−
g	+	−	+	+	+	−
IgG4 4a	−	−	−	−	+	−
4b	−	−	−	−	−	+

classes, respectively (Michaelsen and Natvig, 1971). This has had valuable implications in characterizing some rare IgG gene complexes (Natvig *et al.*, 1971) which will be discussed later (p. 27).

The IgG4 subclass shows a unique relationship between two genetic antigens shared by other subclasses (Kunkel *et al.*, 1970). No regular genetic marker has been found in this subclass. One antigen, 4a, is present in some IgG4 proteins, whereas another antigen, 4b, is present in all 4a-negative IgG4 proteins. No protein containing both antigens was found. However, 4a is also found in all IgG1 and IgG3 proteins, but 4b is shared only with IgG2 proteins. Both markers thus behave as nonmarkers similar to non-a and non-g but with the unusual characteristics of being mutually exclusive in their subclass distribution. Since all normal human sera are, therefore, positive for both markers, they can only serve as genetic markers of IgG4 after isolation of IgG4 Fc fragments from normal human serum. This can be performed by papain digestion and zone electrophoresis. Thus by removing all contaminating IgG1, IgG2, and IgG3, the markers 4a and 4b were shown to behave as if controlled by allelic IgG4 genes. Furthermore, there was a close genetic linkage to the Gm markers of the other subclasses of IgG (Kunkel *et al.*, 1970). Abel (1972) has demonstrated the chemical basis for these two antigens.

Test systems have also been developed for two non-b markers (Gaarder and Natvig, 1972; van Loghem and deLange, 1972). They are both distributed within all Gm(b)-negative IgG3 proteins, but in addition are found in IgG2 and IgG1 proteins irrespective of genetic type. The two markers differ in their specificity and molecular localization. One appears to be antithetic to Gm(b^0) and is called "non-b^0." The other is antithetic to Gm(b^1) and is called "non-b^1" (Gaarder and Natvig, 1972). All these different nonmarkers are present on Fc; they have also been localized to the various subclass pFc′ fragments (see pp. 19–21).

Rivat *et al.* (1971) have described a genetic marker, Gm(e), which shows some parallelism to non-a but with certain differences. It is present in Gm(f), IgG1 proteins and in IgG2 proteins, as in the case of non-a; but, it is absent in IgG3 proteins which contain non-a. Furthermore, in population studies it behaves like an ordinary genetic marker rather than like non-a. This marker holds some special significance because it would be the only regular genetic marker present in more than one subclass. Observations in the authors' laboratories indicate that Gm(e) may not be a single antigenic system but a combination of two specificities (Gaarder and Natvig, 1970)—one component of the system

being non-a and the other the Ga specificity which is absent in IgG3 proteins (Allen and Kunkel, 1966) (see p. 21).

3. *Regular Genetic Gm and Am Markers*

The regular genetic markers of heavy polypeptide chains are found in the following subclasses: IgG1, IgG2, IgG3, and IgA2. In accordance with the concept that one gene codes for the constant part of one polypeptide heavy chain, the Gm and Am groups may be classified in the following five categories:

1. A number of identical antigens separately named because of independent discovery and paucity of reagents for comparison. There is very good evidence that Gm(f), (b^w), and (b^2) are identical and specified by Leu in position 214 (Eu numbering); furthermore, (b^a) and (b^3), (b^β) and (b^0), and (b^γ) and (b^1) appear to be identical. Also Gm(p), (e), and (y) may not be regular Gm markers but related to non-a which appears to determine the only amino acid differences from Gm(a+) chains in the Fc region.

2. Different Gm antigens but still located on the same polypeptide chain. These have been shown to differ in distribution in population studies and show different molecular localization as in the case with Gm(z) vs. Gm(a) and Gm(x) and also for the several Gm(b) subfactors.

3. The Gm markers belonging to different chains of the same subclass and behaving as if under the control of allelic genes Such alleles may either be homoalleles and, thus, reflect base interchanges in the same position of the structural genes; this is so, for example, for Gm(f) and Gm(z) which probably depend on an Arg-Lys interchange in position 214. However, often a Gm marker has a nonmarker in the antithetic position, and these two markers behave as under the control of homoalleles, as in the case with Gm(a) and non-a, with Gm(g) and non-g, with Gm(b^1) and non-b^1, and with (b^0) and non-b^0. In contrast, markers in different positions within allelic genes of the same cistron are heteroalleles. Because of the close linkage between such markers, they may still be used as allelic markers in population studies. Homoalleles are by definition exclusive of each other with respect to the same gene or chromosome but, in rare cases, which are discussed on p. 27, homoalleles are found on the same chromosome. This probably reflects the results of unequal homologous crossing-over giving rise to gene duplication.

4. The Gm antigens of different subclasses which are inherited as closely linked combinations and very rarely segregate in family studies. However, they may vary greatly in different populations. In a given population, these Gm markers are nearly always in coupling giving rise to gene complexes involving the four IgG cistrons.

5. Genetic markers of different classes. Gm antigens of the four IgG subclasses are also closely linked to the Am_2+ marker of IgA2 (Kunkel *et al.*, 1969b).

A summary of the immunoglobulin heavy-chain allotypes, listed according to the two nomenclatures commonly utilized, is shown in Table VI. This table also includes the subclass to which each antigen is restricted. It was unfortunate that this subclass distribution was entirely ignored in the preparation of the "new" numbering nomenclature system.

TABLE VI
IMMUNOGLOBULIN HEAVY-CHAIN ALLOTYPES AND RELATION TO IMMUNOGLOBULIN CLASSES AND SUBCLASSES

Nomenclature		Subclass of heavy chain
New	Original	
Gm markers		
1	a	IgG1
2	x	IgG1
3	b^w or b^2	IgG1
4	f	IgG1
5	b and b^1	IgG3
6	c	IgG3
7	r	IgG1
8	e	[a]
9	p	[a]
10	b^a	IgG3
11	b^β	IgG3
12	b^γ	IgG3
13	b^3	IgG3
14	b^4	IgG3
15	s	IgG3
16	t	IgG3
17	z	IgG1
18	Rouen 2	IgG1
19	Rouen 3	?
20	San Francisco 2	IgG1
21	g	IgG3
22	y	[a]
23	n	IgG2
	b^0	IgG3
	b^5	IgG3
	c^3	IgG3
	c^5	IgG3
Am markers		
1	1 or +	IgA2

[a] May partly measure non-a (see pp. 9, 10, 21).

IgA2	IgG4	IgG2	IgG3	IgG1		
Am+	4b	n+	b	f	non-a	C
Am+	4a	n−	b	f	non-a	C
Am+	4a	n−	g	z	a	C,M
Am+	4a	n−	g	z	ax	C
Am−	4a	n−	b	z	a	N
Am+	4a	n+	b	f	a	M
Am−	4a	n−	b	z	a	M

FIG. 1. Major gene complexes detected in different population groups. Some of these gene complexes differ only by a shift of several genetic markers indicating that they may have arisen by rare recombinational events. C = Caucasian; M = Mongoloid; N = Negro.

As a result many workers have continued to use the lettering system which appeared in the original descriptions of the markers.

The major gene complexes that are found in the different population groups are shown in Fig. 1; rarer gene complexes are shown in Fig. 2. These Gm marker systems have been extensively reviewed previously as to the distribution in various population groups (Natvig and Kunkel, 1968). Further detailed description of the various genetic antigens have been presented elsewhere (Grubb, 1970). Recently, a linkage between the Gm and α_1 antitrypsin (Pi) systems has been shown (Gedde-Dahl *et al.*, 1972).

IgG2	IgG3	IgG1		
(n+)	g	z	a	C
n−	(g)	f	non-a	C
(n−)	b	f	a	M
n+	b	f	non-a(x)	C
(n+)	b	z	a	M

FIG. 2. Rare gene complexes detected in Caucasian populations and which can be followed in families. They have probably evolved through rare inter- or intra-cistronic recombinations. M = Mongoloid; C = Caucasian. Parentheses indicate unusual marker.

C. Biological Activities

The main biological activity of immunoglobulins is their antigen binding which resides in the Fab fragment and is specified by the V regions. However, increasing attention has been focused on other biological activities which mainly involve the Fc part of the heavy chains, such as C1q fixation, transplacental transfer, PCA reactivity, binding to receptors on macrophages and lymphocytes, binding with rheumatoid factors, and interaction with staphylococcal A protein. There are pronounced differences between the classes and subclasses in these biological activities (Table VII).

Complement activation through binding of C1q is most efficient with IgG1 and IgG3 proteins, although the IgG2 is also active; the IgG4 proteins are completely inactive. This pattern has been demonstrated both by the interaction with aggregated proteins of each subclass (Ishizaka *et al.*, 1967) as well as by complex formation demonstrated in the ultracentrifuge (Müller-Eberhard, 1968). However, it has recently been shown that complement can also be activated to some extent through the Fab fragments (Sandberg *et al.*, 1970; Götze and Müller-Eberhard, 1971).

Another biological activity with selective subclass distribution is the fixation of IgG to heterologous skin, the PCA reaction. This reaction can easily be obtained with IgG1, IgG3, and IgG4, but not with IgG2 (Terry, 1966). The latter distinction has been confirmed in the authors' laboratories.

Immunoglobulin G is the only Ig class that clearly passes through

TABLE VII
Biological Properties of Immunoglobulin G Subclass Proteins

Biological parameters	IgG1	IgG2	IgG3	IgG4
Complement fixation	++	+	+++	0
PCA[a] reactivity	+++	0	+++	+++
Placental transfer	+++	+++[b]	+++	+++
Macrophage receptor	+++	0	+++	0
Reaction with protein A[c]	+++	+++	0	+++
Antibody activities[d]	Anti-Rh	Antidextran Antilevan	Anti-Rh	Anti-Factor VIII

[a] Passive cutaneous anaphylaxis.

[b] One study indicated a lower concentration of IgG2 in the cord sera (Wang *et al.*, 1970b).

[c] Protein A of staphylococci.

[d] These activities are dominant in the subclasses listed.

the placenta. The degree of placental transfer of the four IgG subclasses is, however, still a matter of discussion. Several workers have shown that IgG1, IgG3, and IgG4 are present in the cord blood in quantities corresponding to those of the mother; IgG2 also clearly passes the placenta. However, some workers have reported considerably lower levels of IgG2 in cord blood than in the mothers' sera (Wang *et al.*, 1970b). In contrast, other investigations have not confirmed this difference and have shown similar levels of IgG2 in cord blood and sera of the mothers (Mellbye *et al.*, 1971; Morell *et al.*, 1971). In the authors' laboratories the hemagglutination inhibition technique with genetic markers for IgG2 as well as for other subclasses were utilized to show that the IgG subclass proteins detected in the cord blood were of maternal origin.

The binding to macrophage receptors is most active with IgG1 and IgG3 proteins (Abramson *et al.*, 1970; Huber *et al.*, 1971). Similar receptors have been detected on human neutrophile granulocytes (Messner and Jelinek, 1970). It also appears that the same structural site on the IgG1 and IgG3 is involved and these proteins compete for the same receptor on the macrophages.

Another selective biological activity is shown by the interaction between staphyloccocal A protein and certain IgG subclasses. Strong reactivity is seen with IgG1, IgG2, and IgG4, whereas IgG3 does not react (Kronwall and Williams, 1969). This distribution has been confirmed by the authors using aggregated proteins of each subclass.

Anti-immunoglobulin antibodies or rheumatoid factors in human sera show a striking relationship to certain subclass proteins and their genetic variants. One frequent pattern is seen with antibodies that react with IgG1, IgG2, and IgG4 proteins and involves a site called Ga which is present on all these subclass proteins except IgG3 (Allen and Kunkel, 1966; Gaarder and Natvig, 1970). This subclass distribution is the same as that for protein A mentioned above, but it is not known whether the same site is involved in these reactions. In addition, other anti-Ig antibodies react with specific Gm sites and nonmarkers and various other isoantigens shared by subclass proteins. Many of these anti-Ig antibodies can be used for the detection of these antigens. Some of these subclass-specific anti-Ig antibodies also involve reaction with hidden sites on the Ig subclass molecules (Natvig, 1966, 1970; Williams and Lawrence, 1966). An unusual number of patients with cryoglobulins of the IgG type have anti-IgG antibody activity of the IgG3 subclass (Grey *et al.*, 1968b). Antibodies directed against IgA subclass proteins are more rare, but are found particularly in patients lacking IgA (Vyas *et al.*, 1968b; Kunkel *et al.*, 1970; Natvig *et al.*, 1970). Severe reactions have been reported

in such patients if blood containing IgA is given to them (Vyas *et al.*, 1968b).

The IgG3 proteins have a number of special properties that appear significant in disease. They show a striking concentration-dependent aggregation (Capra and Kunkel, 1970) which probably is involved in their affinity for C1q, the first complement component (Müller-Eberhard, 1968). In addition, hyperviscosity of the serum results from relatively low level of this subclass because of this aggregation. This has been implicated in a significant number of clinically important hyperviscosity states (Capra and Kunkel, 1970). Also, the IgG3 proteins are selectively retained in the sera of a number of patients with generalized hypogammaglobulinemia, particularly the Gm(b) genetic type (Yount *et al.*, 1970).

D. Chemistry of Immunoglobulins and Their Subfragments

1. Structural Subfragments of IgG

Several new subfragments of IgG have been delineated since the first successful splitting of rabbit (Porter, 1959) and human fragments by papain digestion (Edelman *et al.*, 1960). In the Fab region it is primarily the $F(ab')_2$ and Fab' fragments obtained by pepsin digestion which have been utilized. In addition, various fragments of light and heavy chains have been obtained by cyanogen bromide cleavage at methionine residues. Since the variable part of the chain contains different numbers of methionine residues at several positions this splitting gives rise to a variety of fragments. In the Fc region, particularly, fragments of the C-terminal half of the Fc have been studied. By pepsin digestion at pH 4.5, it is possible to release pFc' fragments of molecular weight 26,000 starting from amino acid 336 to the C-terminus. The fragments contain one intrachain disulfide bond and are held firmly together as a dimeric unit by noncovalent forces. All the four Ig subclasses release these fragments, but their ease of production varies being least for IgG1 and then progressively increasing through IgG4 (Turner *et al.*, 1970a,b) (Fig. 3).

There are also subfragments of pFc'. By further digestion with trypsin about 7 N-terminal residues are lost, but the tryptic fragment still extends to the C-terminal end. This fragment has a slightly faster electrophoretic mobility than pFc', although it contains all the same genetic antigens as found in pFc'. Finally, by papain digestion, an Fc' fragment that lacks 8–9 N-terminal residues but also 13 or 14 C-terminal residues as compared to pFc' (Figs. 4 and 5) can be obtained. Recently also chymotryptic subfragments have been characterized

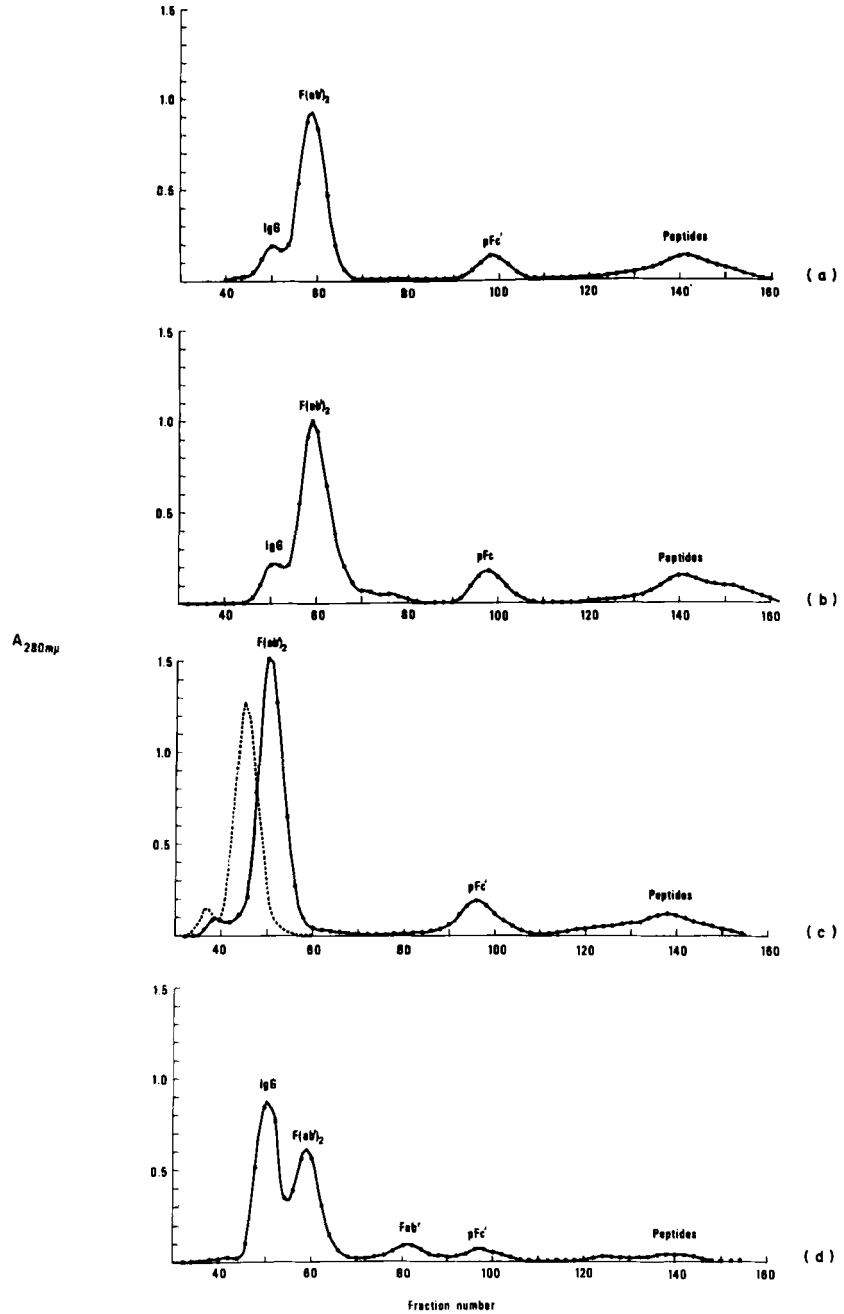

FIG. 3. Profiles of the fractionation on Sephadex G-150 of various IgG subclass proteins after mild pepsin digestion which gives rise to pFc' fragments. (a) 24 hours peptic digest of IgG1; (b) 6 hours peptic digest of IgG2; (c) 1 hour peptic digest of IgG3; (d) 2 hours peptic digest of IgG4. The pFc' fragment is eluted in the same position from all the four subclasses. (From Natvig and Turner, 1971.)

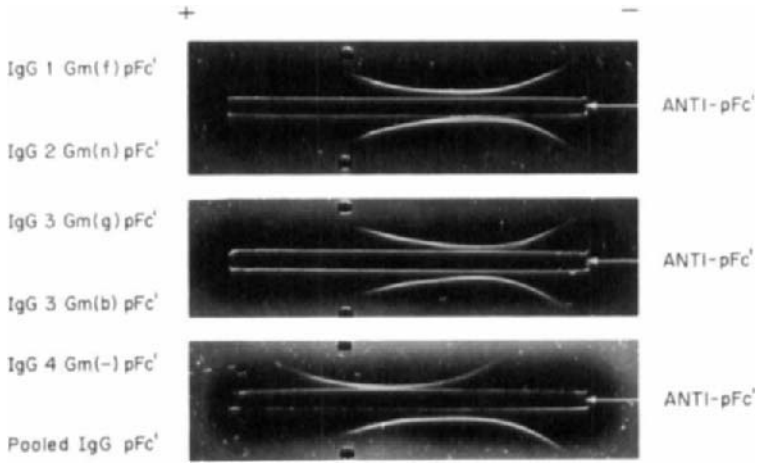

FIG. 4. Immunoelectrophoresis of pFc' fragments from each of the four IgG subclasses. The mobility is very similar for IgG1, IgG2, and IgG3 pFc' fragments, but IgG4 pFc' has a faster mobility. (From Turner *et al.*, 1970b.)

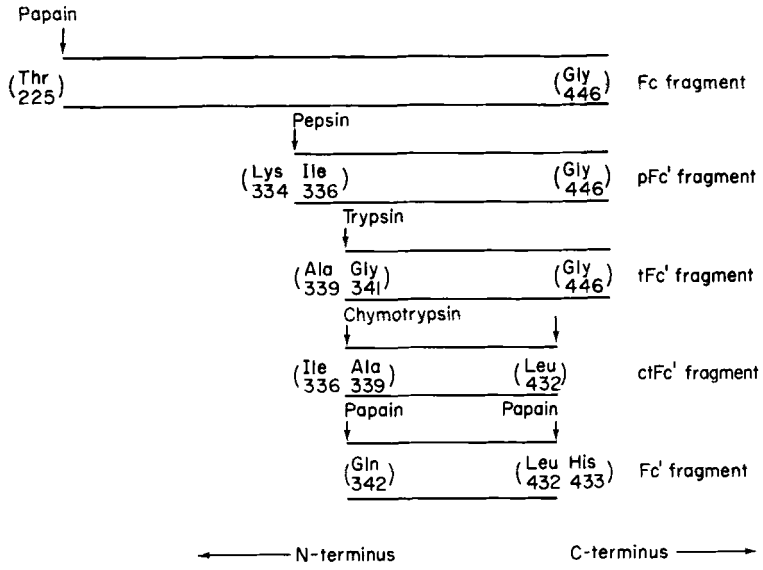

FIG. 5. Schematic illustration of different subfragments of Fc and their probable molecular localization. The numbering corresponds to that of the Eu protein. Residue 446 is the C-terminal residue. (See Natvig and Turner, 1971; Turner *et al.*, 1972a.)

(Turner *et al.*, 1972a). In addition, new subfragments have been characterized by papain digestion. Connell and Porter (1971) have obtained an Facb fragment from rabbit IgG. Michaelsen and Natvig (1972) have characterized various other subfragments by papain digestion of human native IgG: Fabc, $F(c)_2$, and $F(ab)_2$. The yield of these fragments from various IgG subclasses is very different.

Recently, Givol (1972) has described an Fv fragment consisting of the variable part of heavy and light chains of a mouse IgA myeloma protein. This fragment was obtained by pepsin digestion at low pH and had a molecular weight of 29,000. The antibody activity of the whole protein was recovered from the fragment. Also, another group claims to have some evidence for a fragment corresponding to the homology region CH2 (Painter *et al.*, 1972).

2. *Homology Regions*

On the basis of structural similarities, the polypeptide chains can be subdivided into homology regions, each centered around one intrachain disulfide bridge and comprising about 115 amino acids for the heavy chain and 107–110 amino acids for the light chain. The N-terminal homology region, V_H or V_L, is the variable part of the chain. The constant homology region of the light chain, C_L, has the same size as V_L. For the heavy chain the first homology region of the constant part, CH1, corresponds to the constant part of the Fd fragment and can be studied by isolating the Fab fragment which does not contain the homology regions CH2 and CH3. The second region, CH2, corresponds to the N-terminal half of the Fc fragment up to a position from 330 to 340, whereas the third homology region extends to the C-terminus. The CH3 region corresponds closely to the pFc′ fragment obtained by mild pepsin digestion and can be isolated as a separate fragment from all the four IgG subclasses (Turner *et al.*, 1970a,b). However, as for the different enzyme susceptibility of the IgG subclass molecules to give Fc fragments, there are also striking differences in the conditions for release of pFc′ fragment from intact molecules. This property can be utilized for subclass determination of IgG proteins (Turner *et al.*, 1970a).

The CH2 region has not generally been isolated readily as a separate fragment and is digested to smaller peptides under the conditions used to release pFc′. However, Connell and Porter (1971) have recently described a technique for isolating a so-called Facb fragment which appears to contain the Fab fragment plus the CH2 region of Fc, but lacks CH3 (pFc′). Thus, by comparing the activities of Fab, pFc′, Fc, and Facb, it is possible to localize various biological activities and antigenic specificities to each of the three homology regions. As mentioned above, a fragment has recently been isolated which is claimed to correspond to the CH2

TABLE VIII
ASSIGNMENT OF VARIOUS ANTIGENS TO DIFFERENT FRAGMENTS OF IMMUNOGLOBULIN G

Immunoglobulin fragments	Antigen[a]		
	γ2-3	γ1-2-3	γ 2k Fab
IgG2 unsplit	0.008	0.008	0.015
$F(ab')_2$	>0.5	>0.5	0.008
Fc	0.004	0.004	>0.5
pFc′	0.12	0.008	0.12

[a] The γ2-3 is located in Fc outside pFc′ and thus resides in CH2; γ1-2-3 is in the pFc′ region and thus in CH3; and γ 2k Fab is in the constant portion of Fd and thus in CH1.

homology region and which has a retained complement fixation activity (Painter *et al.*, 1972).

3. *Relationship of Genetic Markers to Subfragments and Homology Regions*

Hemagglutination inhibition studies with the various fragments and subfragments of IgG have been used to locate the various antigens to different homology regions. Constant heavy-chain markers which are detected in the F(ab) fragment are assigned to CH1; test systems that are inhibited by Fc and pFc′ detect antigens in the CH3 region; other systems which are inhibited by Fc, but not by pFc′, are probably located in the CH2 region. Examples of certain antigens that are distributed within different homology regions, as indicated by their inhibition patterns, are shown in Table VIII. With this localization technique the subclass antigens can be assigned to each of the constant homology regions as indicated in Table IX. It is significant that each of the three homology regions can be characterized by antigenic markers, either specific for one

TABLE IX
ASSIGNMENT OF VARIOUS ISOTYPES TO CONSTANT HOMOLOGY (CH) REGIONS

Immunoglobulin subclass	CH1	CH2	CH3
IgG1	γ1 Fab k, γ1 $F(ab')_2$	γ1 Fc	γ1-2-3 Fc
IgG2	γ2 Fab k	γ2 Fc, γ2-3 Fc, γ2-4 Fc	γ1-2-3 Fc
IgG3	γ3 Fab, γ3 $F(ab')_2$	γ3 Fc, γ2-3 Fc	γ1-2-3 Fc
IgG4	γ4 Fab k	$\gamma 4\ Fc_I$, γ2-4 Fc	$\gamma 4\ Fc_{II}$[a]

[a] Also for each of the other subclasses the authors have characterized a subclass specific antigen in the CH3 region.

TABLE X

LOCALIZATION OF GM MARKERS AND NONMARKERS ON THE CH REGIONS OF THE FC FRAGMENT OF DIFFERENT IG'S

Immunoglobulin	CH2			CH3	
IgG1 a	4a		non-b^1	a	non-b^0
IgG1 f	4a		non-b^1	non-a	non-b^0
IgG2 n	4b	non-g	non-b^1	non-a	non-b^0
IgG2 n−	4b	non-g	non-b^1	non-a	non-b^0
IgG3 b	4a	non-g	b^1	non-a	b^0
IgG3 g	4a	g	non-b^1	non-a	non-b^0
IgG4 a	4a			γ4 non-a	
IgG4 b	4b			γ4 non-a	

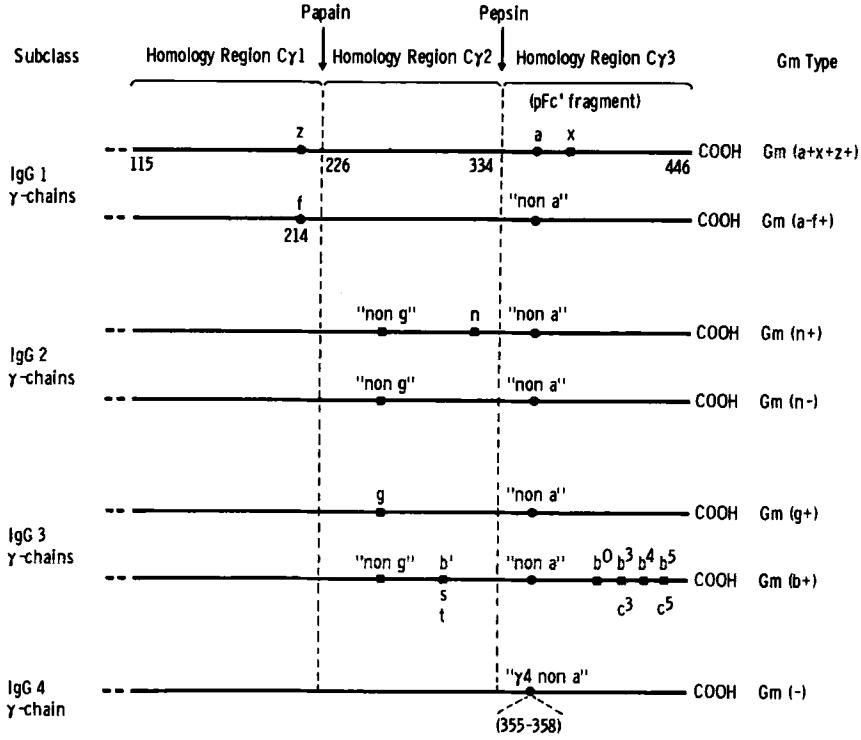

FIG. 6. Distribution of the Gm markers and certain nonmarkers within each of the three constant homology regions of the four IgG subclasses. (●) Antigens of which the exact sequence localization is known; (■) genetic markers where no precise sequence localization is available. (From Natvig and Turner, 1971.)

particular subclass or, in some cases, shared by more than one subclass. When the genetic Gm markers and the nonmarkers are similarly localized (Table X), a whole series of antigens that can be used for mapping of immunoglobulin molecules is obtained. For some of the homology regions, particularly CH2 and CH3, there are several antigenic specificities which distinguish the different subclasses (Fig. 6).

4. Amino Acid Sequences and Their Relationship to Subclasses and Genetic Markers

The whole amino acid sequence of one IgG1 Gm(f) molecule is now known, and several stretches of other IgG subclasses are characterized. Recently amino acid differences between various IgG subclasses and genetic variants in the pFc′ region have been characterized (Turner *et al.*, 1972b). There are also striking differences in the interchain disulfide bridges (Milstein and Pink, 1970). However, as yet no complete study has been made of all the IgG subclasses and their genetic variants. From amino acid interchanges in certain positions, from peptide mapping analysis, and the relationship of certain antigens to structural subfragments, good evidence is available for some amino acids being directly related to specific antigenic determinants. Some known amino acid interchanges appear rather certainly to be related to specific antigens, e.g., Gm (a) and non-a (see below), whereas others are not so clearly related because of alternative possibilities. The amino acid interchanges that appear to be related to the various antigens are listed in Table XI.

Recently, studies by Ponstingl and Hilschmann (1972) indicate that there are only two amino differences in the Fc fragments between Gm (z + a + x −) and Gm(f + non-a +) IgG1 proteins. This gives further structural evidence that the Gm(e) and some other Gm markers of IgG1 are indeed as genetic marker systems measuring non-a.

5. Relationship between Tertiary or Quaternary Configuration and Primary Structure

As previously discussed, the Gm markers have successfully been related to immunoglobulin fragments and subfragments. Also the genetic markers of the light chains, the InV markers, are definitely located on the constant region of the κ light chains. One particular property of the Gm(f) and Gm(z) markers of the Fd fragment and InV (1) and (2) markers of the light chains is their requirement for the integrity of the quaternary structure between heavy and light chains for the expression of the antigenic activity. However, the specificity always resides in the

TABLE XI
SPECIFIC AMINO ACIDS RELATED TO GENETIC ANTIGENS

Antigens	Chain	Subfragment and homology region	Sequence No.	Amino acids
Gm a	γ1	pFc′ CH3	355–358	Arg[a] Asp[a] Glu Leu[a]
Non-a	γ1-2-3	pFc′ CH3	355–358	Arg[a] Glu[a] Glu Met[a]
γ4 non-a	γ4	pFc′ CH3	355–358	Gln[a] Glu[a] Glu Met[a]
Gm f	γ1	Fd CH1	214	Arg[a]
Gm z	γ1	Fd CH1	214	Lys[a]
Gm 4a	γ4	Fc CH2	309	Val Leu His
Gm 4b	γ4	Fc CH2	309	Val — His
Gm g	γ3	Fc CH2	296	Tyr[a]
Non-g	γ2-3	Fc CH2	296	Phe[a]
Gm b^0	γ3	pFc′ CH3	436[b]	Phe[a]
Non-b^0	γ1-2-3	pFc′ CH3	436	Tyr[a]
γ1-2-3	γ1-2-3	pFc′ CH3	445	Pro[a]
γ4 Fc	γ4	pFc′CH3	445	Leu[a]
InV (1)	κ	Ck	191	Leu[a]
InV (2)	κ	Ck	191	Val[a]
Oz+	λ	$C\lambda_1$	190	Lys[a]
Oz−	λ	$C\lambda_2$	190	Arg[a]

[a] Can be explained by point mutation.
[b] This position may be related to another Gm(b) marker.

heavy chain for the Gm markers and in the light chain for the InV markers, so that heavy chains from the Gm-positive molecules can hybridize with light chains from Gm-negative molecules and still express the antigenicity and vice versa for the InV system (Polmar and Steinberg, 1964; Litwin and Kunkel, 1967).

The need for a well-preserved tertiary configuration is also evident with regard to the Gm(a), non-a, and γ4 non-a markers. Here the primary Gm(a) and non-a amino acid sequences, which appear to be related to a peptide between residue 355 and 360, are present both in the pFc′, tFc′, ctFc′, and Fc′ fragments. However, the antigenic property is lost upon degradation of pFc′ or tFc′ to ctFc′ or Fc′ (Natvig and Turner, 1971; Turner *et al.*, 1972a).

6. *Localization of Biological Activities*

Attempts have also been made to localize the various biological activities of the IgG molecules to different subfragments. Complement fixation activity has been tentatively related to the CH2 region (Kehoe *et al.*, 1971). Rheumatoid factor may either interact with the pFc′ frag-

ment or with antigenic structures in the N-terminal part of Fc Thus, several antigens in both main homology regions of the Fc fragment are involved in rheumatoid factor reactions (Natvig and Turner, 1970, 1971; Natvig *et al.*, 1972).

E. Genetic Events during the Evolution of the Immunoglobulin Classes and Subclasses

1. Mutations

There is ample evidence for continued genetic events occurring among the Ig genes. Basically, mutations might give rise to polymorphisms within a single subclass cistron. There are examples of such polymorphisms that can be explained by a single point mutation, e.g., Gm^f–Gm^z, InV^1–InV^3. In addition, several subclass- or subtype-specific amino acids can also be explained by single point mutations, e.g., Oz^+ and Oz^- (Ein, 1968). Thus, not only the development of allelic markers, but also the first series of genetic events which led to the divergencies among the subclasses appears to be due to point mutations. Examples are shown in Table X.

2. Regular Recombinations

There are also examples of genetic events which can most readily be explained on the basis of recombinations either intercistronic or intracistronic. However, it should be stressed that it is difficult to tell the direction of the genetic event. Thus, it is very difficult to tell which genes were the primary ones and which resulted from recombination among ancestral genes.

One strong line of evidence for recombination stems from the many different stable gene complexes that are detected in different population groups. Within these gene complexes all the regular genetic markers that are known from other population groups are found. With the exception of some rare Gm(b) subtype markers, Gm(c^3) and (c^5), all known genetic markers are distributed widely in differing populations. It would be much more difficult to conceive of these genetic markers developing independently in all these populations than to explain their different relationships by a single or a small series of recombinational events.

As previously shown there are some gene complexes that are more prevalent in certain populations than others, for example:

$Gm^{za}Gm^{b}Gm^{n}$–$Gm^{4a}Am^{2}$+	Negro
$Gm^{za}Gm^{g}Gm^{n}$–$Gm^{4a}Am^{2}$+	Caucasian
Gm^{f} $Gm^{b}Gm^{n}$+$Gm^{4b}Am^{2}$+	Caucasian
$Gm^{fa}Gm^{b}Gm^{n}$+$Gm^{4a}Am^{2}$+	Mongoloid
$Gm^{za}Gm^{b}Gm^{n}$–$Gm^{4a}Am^{2}$–	Mongoloid

Most likely these gene complexes evolved through rare, equal, homologous cross-over events. However, other genetic mechanisms such as inversions, deletions, duplications, hybridizations, and gene conversions might be considered. In addition there is also evidence for intragenic recombination in case of the Mongoloid gene Gm^{fa} (see later).

Another example of a possible intragenic recombination is the unusual Gm^{fx} gene. Nearly always Gm(x) is connected to Gm(a) and has been shown to reside in the pFc′ fragment together with Gm(a) (Natvig and Turner, 1971). Studies in the authors' laboratories have shown that Gm(x) is associated with non-a in this unusual situation as well as with f, giving the gene $Gm^{fx\ non\text{-}a}$, which, in turn, is closely linked to Gm^{b} and Gm^{n}. Such a relationship for Gm(x) can be readily explained by an intragenic crossover resulting in a shift of the C-terminal portion of the pFc′ of the usual Gm(azx) chain to the Gm(f non-a) chain. This would require a positioning of Gm(x) to the C-terminal side for which there is some chemical evidence. Such a crossover also fits well with the order of the IgG cistrons presented later (see p. 29).

3. *Evidence for Unequal Homologous Crossover*

Three different types of genetic events can result from unequal homologous crossover. First, there may be deletions which can affect a whole cistron or only parts of a cistron and give rise to deleted proteins; second, there may be hybridizations whereby new genes arise which contain parts from different cistrons; and third, there may be duplications whereby partially larger chains or new chains may originate. Table XII summarizes the different types of genetic events described.

a. Deletions. Evidence for deletions of a whole γ-cistron was first described for IgG3 where certain gene complexes appeared to lack all common alleles of the IgG3 subclass cistron. The gene complex (n+)(−)(f) was inherited by regular Mendelian principles in several families (Fig. 7) and, in addition, the affected persons had close to half the normal amount of IgG3 protein in their sera (Natvig *et al.*, 1968). This was strong evidence for deletions rather than for a third silent allele. The most plausible explanation was a structural gene defect affecting the IgG3 gene (Natvig *et al.*, 1968). Further evidence for this explanation has been obtained in other similar families.

TABLE XII
KNOWN CASES OF DELETIONS, HYBRIDIZATIONS, AND DUPLICATIONS AMONG HUMAN IMMUNOGLOBULINS

Immunoglobulin gene complex	Family case	Reference
IgG1 IgG3 IgG2		
	Deletions	
f, −, n+	B 968	Natvig *et al.* (1968)
	GJ	Natvig *et al.* (1968)
	La	Natvig *et al.* (1968)
	I	Natvig *et al.* (1968)
	SO	Natvig *et al.* (1968)
	Dnl	van Loghem and Natvig (1970)
	Vis	van Loghem and Natvig (1970)
	Goe	van Loghem and Natvig (1970)
	Hen	van Loghem and Natvig (1970)
−, b, n−	Kr	Natvig *et al.* (1968); Litwin *et al.* (1973)
−, b, n+	E.K.	Litwin *et al.* (1973)
	K. N.	Litwin *et al.* (1973)
	Vri	van Loghem and Natvig (1970)
	Pie	van Loghem and Natvig (1970)
	Duplication	
zaf non-a, g,n+	H. W.	Natvig and Kunkel (1969); Natvig *et al.* (1971)
	Hye	van Loghem and Natvig (1970)
zaf non-a, g,n−	I	Natvig and Kunkel (1969); Natvig *et al.* (1971)
	Hybridization	
γ3 Fd-γ1 Fc	2902	Kunkel *et al.* (1969a)
γ4CH1,2-γ2CH3	La	Natvig and Kunkel (1972)

There is also similar evidence for a structural deletion of IgG1 genes, and four families have been studied where these defects are found in different members (Yount *et al.*, 1970; Litwin *et al.*, 1973). The homozygous state has as yet not been encountered. However, this gene is implicated in some cases of immune deficiencies, where it appears to interact with other abnormal genes to produce disease. The best example is a patient with one parent showing the IgG1 defect, and the other, the IgG3 defect (Yount *et al.*, 1970).

There are also a number of genes in the population where one genetic type of immunoglobulin is produced at a lower level. These genes have also been encountered in the families of cases of immunodeficiencies (Rivat *et al.*, 1970a; Yount *et al.*, 1970). In the authors' laboratory we

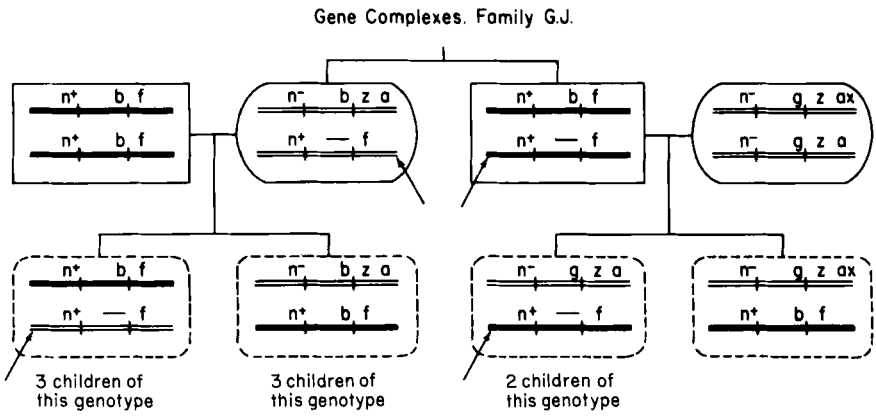

FIG. 7. Family which illustrates inheritance of a rare gene complex with deletion of the IgG3 cistron on one chromosome. This chromosome is indicated by an arrow and the gene complexes are drawn on the basis of the known phenotypes of the respective individuals. Heavy lines—paternal chromosomes; double lines—maternal chromosomes. The pedigree represents a family with a sister and brother with a rare Gm^{n+}, −, Gm^{f} gene complex. Two of the brother's children and three of the sister's children appeared to have the same rare gene complex. Also in the sister and three of her children, a rare Gm^{n-}, $Gm^{b^0 * b^3 b^5}$, Gm^{za} gene complex was present. (From Natvig *et al.*, 1968.)

have encountered a $Gm^{a}Gm^{g}$ weak gene inherited through several members of the same family (Gedde-Dahl *et al.*, 1971).

b. Hybridization. The second type of result from unequal homologous crossover is hybridization. This can be observed as a duplication but is most readily seen as a result of a deletion. One well-documented instance of hybridization is due to a deletion of the Fd part of IgG1 and of the Fc part of IgG3 with subsequent repair and occurrence of one gene coding for IgG3 Fd and IgG1 Fc (Kunkel *et al.*, 1969a). This case was noted because of an apparent lack of all normal genetic markers in the serum. This individual was homozygous for the extremely rare gene complex that could be followed in his family (Steinberg *et al.*, 1968); he also lacked Fd IgG1 and all Fc IgG3 antigens, but IgG1 Fc antigens including non-a and IgG3 Fab antigens were present. Furthermore, IgG1 Fc antigens precipitated with antisera specific for Fab IgG3 showing that the respective antigens were on the same molecules (see Fig. 8). On the antigenic site of the $F(ab')_2$ fragment revealed by pepsin digestion, the hybrid molecule shows an IgG3 antigen. Thus the molecule appears to be of IgG3 type up to the site of pepsin digestion which is not known. The hybrid molecules contained IgG1 Fc antigens present in the CH2 region and non-a present in CH3. There was also evidence for antibody

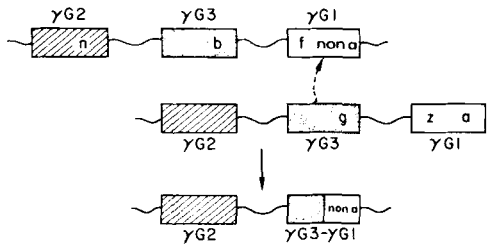

FIG. 8. Schematic representation of the two IgG gene complexes with unequal homologous crossover giving rise to a hybrid gene which could code for the hybrid IgG3 Fab–IgG1 Fc molecule described. The hybrid gene is devoid of all regular Gm markers but contains the non-a marker. (From Kunkel *et al.*, 1969a.)

activity in the hybrid molecule. Thus there appear to be complete heavy chains with a V region followed by a first CH region which is of the IgG3 type, whereas regions CH2 and CH3 are both of the IgG1 type. This was termed a Lepore type of γ-globulin (Kunkel *et al.*, 1969a; Natvig and Kunkel, 1969).

Another probable hybrid molecule was represented by a myeloma protein which showed many of the unique properties of the IgG4 subclass and contained certain IgG4 Fc antigens. However, it lacked certain other IgG4 Fc antigens. When this protein was studied with several subclass markers and all the different Gm markers and nonmarkers available, it was found to contain certain antigens of other IgG subclasses that had not been found in any IgG4 proteins previously studied. It appeared that the CH1 and CH2 homology regions were all IgG4 type, whereas the CH3 region was not IgG4 but had antigenic properties of the IgG2 subclass. Also the normal counterpart of this protein has been detected in certain rare Negro sera which appear to result from this rare IgG4–IgG2 hybrid gene in a homozygous state.

c. Duplications. There is also evidence for the occurrence of recent duplications among the γ heavy-chain genes, giving rise to two IgG1 genes on one chromosome. This was obtained for a rare gene complex Gm^{fza}, which was inherited as a stable complex in two different families. Since Gm^{z} and Gm^{f} are homoalleles in the region of the IgG1 genes coding for the Fd part of γ1 chains, these findings suggested some type of duplication. This was further verified by testing for Gm(a) and the antithetic marker, non-a, in the IgG1 Fc fraction. These studies showed that Gm(a) and non-a originated from the rare gene complex. Since Gm(z) and Gm(f) are located in the CH1 region, and Gm(a) and non-a in the CH3 region this strongly indicates that there was a complete or almost complete duplication of the whole C-region genes in IgG1. The

duplication was, however, not similar to the subtotal duplication giving rise to an enlarged polypeptide chain as known for haptoglobulin. In the case of the IgG1 genes described above, gel filtration, ultracentrifugation, and specific absorption studies indicated a complete duplication with the production of two different IgG1 polypeptide chains under separate genetic control (Natvig *et al.*, 1971) (Fig. 9).

Two other families have recently been described with Gm^{za} Gm^{f} inherited as one gene complex. In one of these families studied by the authors, evidence from non-a typing of the IgG1 fraction supports the same interpretation of a total IgG1 gene duplication. The other family described from another laboratory (Steinberg *et al.*, 1968) was interpreted quite differently by these authors. A series of mutational events was postulated; but, the gene complex in this family can also be most readily explained on the basis of a single unequal homologous cross-over, very similar to the ones proposed.

The fact that the gene for the Lepore type of immunoglobulin and the presumed duplicated gene were found in a rare rather "inbred" isolate of Caucasian Hutterites is of some interest because the presence of these rare duplicated and deleted genes will necessarily give a situation where there is always some misalignment of IgG genes at meiosis which might promote further recombination.

Recently, evidence has also been obtained for recent duplication of the C region of the light chain. This was first made evident by studies on the Oz marker which demonstrated a subtype of λ light chains (Ein and Fahey, 1967; Ein, 1968). Evidence has been presented for three subtypes of λ light-chain C regions (Hess *et al.*, 1971).

4. Number and Order of IgG Cistrons

a. Number of Cistrons. At present, there is the following strong evidence from normal immunoglobulins and particularly from studying myeloma proteins that there are at least four different IgG heavy-chain cistrons, one for each of the four IgG subclasses. (Initially this was a subject of considerable controversy.)

1. The IgG myeloma proteins can be classified into four well-defined subclasses representing different proteins with distinct antigenic, physical chemical, and basic structural amino acid and peptide differences.

2. Similar normal IgG fractions representing each of these four IgG subclasses can be found in all normal individuals.

3. In each of these subclasses genetic markers have been detected by which the subclass protein can be classified into different types, behaving as if controlled by allelic genes. In single heterozygous individuals eight different allelic genes can occur. Since a single cistron

can only have two alleles in a single given individual, the eight alleles can only be accounted for by at least four different cistrons.

4. The evidence for hybridization of two different subclass genes to make one hybrid IgG3 Fab–IgG1 Fc gene is further strong genetic evidence for different cistrons controlling the synthesis of the subclass genes.

A related question concerns the point as to whether there is more than one cistron for each subclass. There is evidence that definitely restricts the number of cistrons to not more than one for each subclass in most normal individuals.

1. There is a very striking "homozygocity" of the Gm gene complexes even in population groups where multiple allelic gene complexes are found, e.g., Caucasian and Mongoloid populations. An alternative argument would be a mechanism that selected against the presence of allelic genes on one chromosome, but such a selection appears very unlikely (Ohno, 1970).

2. Further evidence along the same line stems from the studies on IgG in the individual homozygous for the rare hybrid IgG3 Fab–IgG1 Fc molecule. In this situation no normal IgG1 or IgG3 molecules were found. Since it is unlikely that the deletion and hybridization would delete exactly any surplus of IgG1 and IgG3 cistrons totally, the most likely explanation is again that only one cistron exists for each of the subclasses IgG1 and IgG3.

Therefore, the accumulated evidence favors only one cistron for each subclass normally, and the number of heavy-chain subclass cistrons can then be roughly estimated. Although there may be as yet unknown subclasses and classes, they are unlikely to play a quantitative major role in the immunoglobulin system; the possibility of silent IgG cistrons remains. However, most likely there are four IgG and two IgA cistrons. For IgM, IgD, and IgE, subclasses have so far not been defined. There has been considerable work performed in several laboratories to delineate IgM

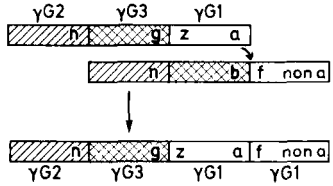

FIG. 9. Diagram for postulated mechanism of gene duplication with mispairing of the IgG heavy-chain genes followed by an intercistronic crossover at meiosis giving rise to two IgG1 genes, one with Gm^{a} and another with $Gm^{f\,non\text{-}a}$. (From Natvig *et al.*, 1971.)

subclasses, but so far without success. Too few IgD and IgE myeloma proteins are known to exclude subclasses. The total number of immunoglobulin heavy-chain C-region cistrons can probably be estimated to be not less than nine and probably not more than twelve to fifteen.

For the light-chain C regions, there is only one κ cistron known, and, for the λ C regions, three cistrons are known.

b. Order of Heavy-Chain Cistrons. The most likely interpretation of the Lepore type of IgG is a deletion type of hybridization. Since the N-terminal part of the molecule is γ3 and the C-terminal is γ1, this can only be explained by having the γ3 cistron to the "N-terminal" side of γ1. Also, since there is no deletion of γ2 and γ4 and other classes in this instance, γ1 and γ3 are likely to be adjacent. This is also compatible with gene complexes in the population groups which are easily explained by this order. The order of the two other subclasses is not definitely established, but data from another hybrid IgG heavy-chain molecule, probably IgG4–IgG2, indicate that IgG4 for the same reasons is "N-terminal" and adjacent to IgG2. This order is used in Figs. 8 and 9.

There is no definite genetic proof for placing the IgG4–IgG2 and the IgG1–IgG3 complexes to one or the other side of each other. However, since there are so many antigenic similarities between IgG2 and IgG3, they are highly likely to be adjacent. Thus the order is probably IgG4, IgG2, IgG3, and IgG1. This is also compatible with previous suggestions for the order of the γ-cistrons (Natvig *et al.*, 1967a,b).

The IgA2 is closely linked to IgG (Kunkel *et al.*, 1969b), and it is highly likely that IgA1 together with IgM, IgD, and IgE heavy chains will make one large linkage group of C-region heavy-chain genes. However, the positions of IgA1 and IgA2, IgM, IgD, and IgE are not known. They might be placed on the "N-terminal" side of the IgG cistrons because of more similarities with IgG4 than with IgG1.

5. *Genetic Markers and Regulatory Genes*

There has been some discussion whether immunoglobulins are under the control of regulatory genes that can turn on or off the whole IgG gene complex for production of IgG circulating antibodies. There are two observations supporting the contention that lymphocytes may contain Ig genetic markers that are not present in the respective serums.

1. Ropartz and associates have recently presented data which were interpreted as proof for the production by the lymphocytes, in short-term cultures after phytohemagglutinin stimulation, of immunoglobulin allotypes not present in the respective serums (Rivat *et al.*, 1970b).

2. The fluorescent antibody technique was used in another study where also evidence was obtained for genetic markers of lymphocytes that were not present in the respective serum (Curtain and Baumgarten,

1965; Lobb, 1968). However, this was performed with heteroantisera which require very strong absorptions, particularly for genetic markers belonging to minor subclasses such as Gm(b) and Gm(g) of IgG3. There are thus some technical questions that have not yet been fully answered.

To the contrary, all the other results described in this review regarding genetic markers of regular immunoglobulin genes including deletions, duplications, and hybridizations can readily be explained in terms of classical genetics. The bulk of present evidence thus strongly indicates that the genetic markers are inherent properties of the structural immunoglobulin genes and controlled by regular Mendelian laws.

6. Genetic Markers in Subhuman Primates

Certain Gm genetic markers, nonmarkers, and other subclass antigens have been detected in some subhuman primates, particularly in the higher species, e.g., gorilla and chimpanzee (for review, see Goldsmith and Moor-Jankowski, 1969). However, the biological implications of these antigens which are restricted to specific IgG subclasses in humans are not clear until a subclass differentiation has been carried out in the primates. The detection of such antigens alone does not prove that similar subclasses are present in the primate. However, there is some additional evidence from the authors' laboratories for similar subclasses in the higher primates. This stems from the appearance of two different, nonfusing lines in immunoelectrophoresis of primate serum employing anti-IgG3 antisera. One of these was a major line, whereas the other was fainter and resembled the human IgG3 line. So far these different proteins have not been isolated, partly because the primates have not shown myeloma proteins. It is, therefore, not known whether genetic antigens which appear on different subclass molecules in human IgG are similarly separate in these primates. One striking finding is that several genetic markers which behave as allotypes in the humans, are present in all sera of certain primate species. In other instances, differences are found within a species, but these human allotype markers have not been shown to be inherited as Mendelian traits in the primates. The IgG3 antigens appear particularly interesting and are frequently detected in these primate sera. Several Gm(b) markers have been found. Primate sera frequently have the human IgG3 Fc antigen which was used above. In contrast, the very strong human IgG3 $F(ab')_2$ antigen is not detected in primate sera by precipitation techniques.

The accumulated evidence suggests that the IgG subclasses as well as some of the genetic variants developed prior to the dissociation of the human and subhuman primate species. However, further work is needed to prove these relationships.

From an evolutionary point of view, it is also interesting to note that in Old World monkeys there is a peptide which is intermediate in amino acid differences between the Gm(a) and non-a sequences (Wang *et al.*, 1969a):

Gm(a) peptide:	Asp-Glu-Leu-Thr-Lys
Old World monkey peptide:	Glu-Glu-Leu-Thr-Lys
Non-a peptide:	Glu-Glu-Met-Thr-Lys

II. Antibodies: Subclass Distribution

From the early work with anti-Rh antibodies it became apparent that isolated antibodies did not reflect all the genetic markers and subclasses that were present in the whole serum of the patient. In many instances they represented a striking selectivity (Grubb, 1956; Harboe, 1959; Natvig and Kunkel, 1968). Similarly, other antibodies were also shown to represent a subclass selectivity (Allen *et al.*, 1964). This work has been extended to a wide variety of antibodies particularly those to various carbohydrates where the most extreme selections have been seen (Yount *et al.*, 1968).

Anti-Rh antibodies appear to be distributed mainly within the IgG1 and IgG3 subclasses, and in a large panel the proportion of IgG1 to IgG3 is rather similar to the proportion of IgG1 to IgG3 in normal serum, approximately 10:1 (Natvig and Kunkel, 1968). However, there is a striking disproportion of IgG2 which represents about 20% of normal IgG; on several hundred anti-Rh antibodies tested, no IgG2 types have been clearly detected. Recently there have been reports of IgG4 anti-Rh antibodies (van Loghem and deLange, 1970; Frame *et al.*, 1970).

A striking selective subclass distribution has been shown particularly for antidextran and antilevan and other precipitating antibodies to carbohydrates which very frequently appear to be pure IgG2 (Yount *et al.*, 1968). However, other antibodies with defined activities to specific antigens were nearly 100% of the IgG1 subclass. The selective subclass distribution was paralleled by similar restriction of genetic markers. Isolated antibodies thus in many instances appear to represent "monoclonal" responses in humans. Other human antibodies shown to represent isolated subclasses are antibodies against Factor VIII, which usually appear to be IgG4 (Anderson and Terry, 1968; Robboy *et al.*, 1970).

III. Surface-Bound Immunoglobulins on Lymphocytes

A. Immunoglobulin Classes, Subclasses, and Allotypic Markers

Recently, antisera specific for classes, subclasses, and genetic markers have been used to detect and characterize immunoglobulins on human

lymphocytes. In human peripheral blood, about 10 to 20% of lymphocytes carry membrane-bound immunoglobulin as demonstrated by staining with anti-F(ab')$_2$ or anti-κ and anti-λ antisera. About two-thirds of the cells with membrane-bound immunoglobulin stain for IgM while the majority of other cells stain for IgG and only a few cells, about 1%, stain for IgA (Grey *et al.*, 1971; Fröland and Natvig, 1971, 1972a; Siegal *et al.*, 1971; Pernis *et al.*, 1971). Even IgG subclasses can be detected on lymphocytes but are not distributed among the lymphocytes in the same proportions as in serum (Fröland and Natvig, 1971, 1972a). IgG2 appears to be the dominant IgG subclass on lymphocyte membranes, making up about 60 to 90% of IgG, while IgG1 and IgG3 make up 10 to 30% each of the IgG positive lymphocytes. IgG4 is only rarely detectable and usually represents less than 5% of the positively staining IgG lymphocytes.

Gm markers have also been detected (Fröland and Natvig, 1971, 1972a,c). The Gm(f) and Gm(z) markers have been looked for primarily since they are present on the Fd part of the molecules. They are only detected in persons who at the same time are positive for the respective genetic marker in their serum. The cells staining for Gm(z) and those staining for Gm(f) added up to the approximate number of cells staining for IgG1. Also genetic markers in the Fc region have been detected by the immunofluorescence technique, such as Gm(n) and Gm(g). These markers are located in the N-terminal half of Fc (homology region CH2). In contrast, antigens in the C-terminal half of Fc, e.g., Gm(a) and non-a, are not exposed on the surface of the cells. Also anti-pFc' gives staining of none or only a very small percentage of cells ($<1\%$), compared to the mean of about 5% of cells staining with anti-IgG Fc (Fröland and Natvig, 1972c). These findings suggest strongly that the IgG receptor molecule is oriented with the C-terminal part towards the lymphocyte surface while the N-terminal part is free to react with antigen.

B. Surface-Bound Immunoglobulin as a Marker of B-Lymphocytes in Man

The cells staining for immunoglobulin in humans as in the mouse and chicken appear to be bone marrow-derived (B-lymphocytes) and thus belong to the plasma cell line. Particular interest has centered on these lymphocytes in patients with various types of immune deficiencies showing extremely low levels of circulating Ig and few or no plasma cells. In some of these cases, particularly those with Bruton type of agammaglobulinemia, no lymphocytes staining for surface-bound immunoglobulin are detected (Grey *et al.*, 1971; Siegal *et al.*, 1971; Fröland and Natvig, 1971, 1972a). In contrast, patients with cellular immunodeficiencies have normal or even elevated numbers of cells staining for surface-bound

immunoglobulin. It has become clear that not all patients with agammaglobulinemia lack circulating B-lymphocytes, and this may be evidence of a maturation defect where the differentiation of B-lymphocytes to plasma cells is blocked. In some of these cases percentages of B-lymphocytes above the normal level have been observed (Siegal *et al.*, 1971; Fröland, 1972b; Preud'homme and Seligmann, 1972).

The question of whether immunoglobulins are present on the membranes of T type lymphocytes remains controversial. Most workers in the field have failed to find any immunoglobulins by fluorescent antibody studies and this is the authors' experience. Immunoglobulins, if present, would have to be there in very low concentrations or in a buried state. The report (Marchalonis *et al.*, 1972) that T cells have IgM in amounts comparable to B cells is a major exception. The detailed studies of Uhr and associates (Vitetta *et al.*, 1972), using the same methods as Marchalonis, have failed to find evidence of immunoglobulins. One group (Warner *et al.*, 1970) has reported light chains on the surface of T cells in the mouse.

C. B-Lymphocytes in the Newborn

Immunoglobulins on lymphocytes in the neonatal period have also been studied (Fröland and Natvig, 1972b). In humans, umbilical cord serum samples show extremely low levels of immunoglobulins produced by the fetus itself, and most IgG, including all the four IgG subclasses, is transferred from the mother (Mellbye *et al.*, 1971; Wang *et al.*, 1970b; Morell *et al.*, 1971). However, newborn children have normal levels of circulating B-lymphocytes in their umbilical cord blood with a mean of immunoglobulin-positive lymphocytes somewhat above that for adults including the mothers. Distribution of classes and subclasses of immunoglobulins on the lymphocytes was similar to that described in adults. The present data therefore indicate that the neonatal period represents a stage where B-lymphocytes probably have not as yet differentiated to any large extent into mature plasma cells (Fröland and Natvig, 1972b).

D. Restriction of Membrane-Bound Immunoglobulins on Single Lymphocytes

The accumulated evidence indicates that each normal lymphocyte stains only for one immunoglobulin class. This was first indicated by the findings that the sum of percentages of the various classes and subclasses in normal adults added as the total sum for immunoglobulin-positive lymphocytes (Grey *et al.*, 1971; Fröland and Natvig, 1971, 1972a). However, also in double staining experiments, the main immunoglobulin classes and also the four IgG subclasses were represented

on different normal lymphocytes (Fröland and Natvig, 1972c). In the latter experiments it was also shown that one lymphocyte only carried the allele of one subclass gene and it appeared that the lymphocytes showed allelic exclusion as to their surface-bound immunoglobulins. Similar data on allelic exclusion have been obtained by Litwin (1972).

Further evidence for the restriction of immunoglobulin on the lymphocyte surface has been obtained by studying chronic lymphatic leukemia. Most patients with chronic lymphatic leukemia have lymphocytes with surface-bound immunoglobulin which is primarily IgM (Grey *et al.*, 1971; Pernis *et al.*, 1971; Fröland *et al.*, 1972; Seligmann and Preud'homme, 1972). These cases are of particular interest in this respect because of the homogeneity of the cell population and their surface immunoglobulin: it is either κ IgM or λ IgM but not both in the majority of cases. Some individuals have shown only light chains on their surfaces of either the κ or λ type. In rare cases IgG is observed, which in one case was shown to be IgG2, Gm(n−).

The accumulated evidence from studying both normal lymphocytes and lymphocytes in chronic lymphatic leukemia indicates that a large proportion of immunoglobulin-positive lymphocytes shows restriction of membrane-bound immunoglobulin similar to that of mature plasma cells and their immunoglobulin product. However, one report (Papamichail *et al.*, 1971) presents very different findings in chronic lymphocytic leukemia with multiple immunoglobulins on the lymphocyte surface.

In marked contrast to chronic lymphatic leukemia, lymphocytes in acute lymphoblastic leukemia are negative in the immunofluorescent test for surface-bound immunoglobulin and may thus represent a malignant development of T-lymphocytes (Fröland *et al.*, 1972; Fröland, 1972a).

IV. Myeloma Proteins and Related Homogeneous Immunoglobulins

A. Relationship to Normal Immunoglobulins

The close relationship of the myeloma proteins and Waldenström-type macroglobulins to the normal immunoglobulins is now abundantly clear. It took more than 20 years to establish this point following early evidence of their close antigenic relationship (Kunkel *et al.*, 1951). In fact little evidence is available that these proteins are in any way abnormal. The only clear exception to this rule is in the case of the heavy-chain disease syndromes; this point will be discussed in detail in a later section. In all other instances, analogous proteins have been found in the normal pool of the immunoglobulins. In addition, it has been established that the incidence of a given type of myeloma protein matches very

closely the percentage level of this protein in the immunoglobulin pool. This was first established for κ and λ proteins and has subsequently been found to be true for the heavy-chain classes and the genetic markers (Kunkel, 1968). In the human situation, no clear exception has been encountered which suggests that the origin of a specific type of myeloma is primarily a random event relating directly to the normal frequency. In the mouse myeloma situation the high frequency of the IgA type myeloma appears to represent an exception (Potter and Lieberman, 1970). However, this probably can be explained on the basis of the site of origin of these proteins which may involve areas where IgA-producing cells and their precursors predominate. Another example involving the V regions has been found recently (Grant and Hood, 1971).

There may be abnormalities in the differential production of light chains as opposed to whole molecules in myeloma clones, but the chains themselves appear normal in antigenic and sequence studies. A number of light-chain fragments reflecting both the variable and the constant part of the molecule have been found in the urine of a few patients (Solomon *et al.*, 1966; Cioli and Baglioni, 1968). Some of these appear at constant levels over long periods of time as in the case of one patient studied in the authors' laboratory. However, their significance has not been determined and evidence for synthesis in this form has not come forward. An IgM Fab-like fragment has also been described appearing constantly in large amounts (Winchester *et al.*, 1967), but the same questions arise as for the light-chain fragments.

The unusual IgG4 myeloma protein described above initially was thought possibly to represent an abnormal product of the myeloma clone because it lacked certain IgG4 antigens in the Fc region (Natvig and Kunkel, 1972). However, further investigation indicated that this protein represents a hybrid molecule with the N-terminal portion of the heavy-chain C-region being IgG4 and the C-terminal portion being of the IgG2 type. This protein was found in a Negro, and detailed examination of normal sera from this population indicated that a similar protein existed as a genetic variant. Here again the normal counterpart was found.

B. Biological Activity

One of the important properties of the myeloma group of proteins is their specific binding properties for antigens. This property, where it has been identified, is proving of great utility in studying the combining site of antibodies. This property was first identified in the case of the cold agglutinins, and these were termed Waldenström macroglobulins

with specific binding properties for certain red cells many years ago (Fudenberg and Kunkel, 1957). They were selectively recognized because this property produced hemolytic anemia in these individuals. Subsequently monoclonal anti-γ-globulins (Kritzman *et al.*, 1961; Metzger, 1967) and anti-lipoproteins (Beaumont, 1967) were described, and now a long list of activities has become clear (Ozer and Chaplin, 1963; Waldenström *et al.*, 1964; Wernet *et al.*, 1972a) (Table XIII). The description of proteins that bind dinitrophenyl (DNP) and other haptens has been of special importance and utility (Eisen *et al.*, 1967). There have been some pitfalls in the screening of these proteins for certain activities with reactive molecules particularly the acidic type. It is now agreed that the many proteins that precipitate with DNP proteins do not represent antibodies in many instances (Terry, 1970). The same is probably true for certain deoxyribonucleic acid (DNA)-precipitating proteins (Hannestad, 1969). It is not enough to show that the specific property resides in the Fab portion of the molecule; other criteria, such as binding affinity and characteristics of the binding, must be applied.

In addition to the cold agglutinin syndrome, a number of other disease pictures have been described which relate to the specific activity of monoclonal proteins. The IgM anti-γ-globulins usually have been termed mixed cryoglobulins because they react with IgG very visibly in the cold in most instances. They also appear to react with IgG or, perhaps, complexes of IgG under *in vivo* conditions to produce an immune complex type of nephritis (Meltzer *et al.*, 1966). Hyperglobulinemic purpura which was originally thought to represent a typical polyclonal immunoglobulin elevation has been shown to represent an extreme elevation of monoclonal IgG anti-γ-globulins in many instances (Capra *et al.*, 1971). These form complexes with themselves and other IgG molecules which appear to be involved in the purpuric skin lesions characteristic of these patients. Recently, a monoclonal IgG protein that reacts with transferrin *in vitro* and *in vivo* has been described (Wernet *et al.*, 1972a). The transferrin reactivity has resulted in an interference in iron transport in this patient with resulting liver disease

TABLE XIII
ACTIVITIES CLEARLY DELINEATED FOR HUMAN MYELOMA PROTEINS AND WALDENSTRÖM MACROGLOBULINS

1. Cold agglutinins	5. Old cell agglutinin
2. Anti-γ-globulins	6. Anti-DNP[a]
3. Anti-streptolysin O	7. Anti-transferrin
4. Anti-lipoprotein	8. Anti-galactomannan

[a] 2,4-Dinitrophenyl.

from an accumulation of the iron. In a somewhat analogous fashion the anti-lipoproteins interfere with lipid transport and cause lipid accumulations in various organs resulting in malfunction. Several of these have been studied in the authors' laboratories, and lipid deposits in the skin are a frequent finding. In one case the deposits were extremely widespread including organs such as the liver and spleen. In this instance the deposits were surrounded by plasma cells.

A number of additional examples are known where an activity of a monoclonal protein is strongly suspected but remains unproven. The disease, papular mucinosis, is associated with an extremely basic IgG band that has been studied by several investigators (McCarthy *et al.*, 1964; James *et al.*, 1967; Lawrence *et al.*, 1971). In each case the protein has had λ light chains and is IgG1, where the latter has been studied. It seems highly likely that a special activity is involved, perhaps a reactivity to some normal or altered skin constituent, in view of the extensive skin lesions in these patients. Also in Gaucher's disease, monoclonal bands are common (4 of 16 cases in one series) (Pratt *et al.*, 1968). A unique type of protein has not been noted as in the case of the papular mucinosis patients. It is possible that the lipid deposits in this disease contain an antigen that would react with these proteins.

A different aspect of monoclonal immunoglobulins is afforded by the electrophoretically homogeneous populations of IgG in the cerebrospinal fluid (CSF) in patients with multiple sclerosis and certain encephalitides, such as subacute sclerosing panencephalitis (SSPE). Recent investigations of the latter condition (Vandvik, 1972) have shown that protracted infection in the central nervous system with measles virus may result in synthesis in this organ of homogeneous IgG antibodies to antigenic subcomponents of the virus. These antibodies appear to be "monoclonal" both with respect to their light chain determinants and to their monospecific activity to a given virus antigen component.

C. "Benign" Monoclonal Bands

Through the widespread use of zone electrophoresis analysis of serum samples in hospitals, it became apparent that many individuals showed γ-globulin bands in the serum without accompanying signs of multiple myeloma. In the vast majority of cases these bands have not been related to any disease process although their occurrence in various diseases such as pernicious anemia, rheumatoid arthritis, and malignancy, has caused speculation about such a possibility. The extensive survey of Axelsson and associates in normal individuals aided greatly in clarifying these questions. These workers found a definite incidence in normal individuals

over the age of 30 which increased to a level of 5.7% in people over the age of 80 (Axelsson *et al.*, 1966).

One of the major problems that has arisen concerns the question of whether such bands indicate malignant disease and whether vigorous therapy should be instituted in such individuals as for multiple myeloma. This has been done in many areas and probably erroneously. Several groups have now followed such individuals for extensive periods of time, and there is universal agreement that in the vast majority myeloma with invasive bone lesions does not ensue, at least not over a 5–10 year period. Thirty-five such cases have been followed at the Rockefeller Hospital for more than 5 years, and in no instance has serious disease ensued. One individual has been followed for 13 years and more than fifty serum samples have been analyzed. The monoclonal band has remained at exactly the same level over this entire period. Figure 10 shows the earliest and the latest electrophoretic run in the cellulose acetate system on the serum of this individual. In this case the band was of the IgG λ type and was associated with considerable suppression of the other immunoglobulins. It illustrates a number of characteristic findings. The band is usually relatively low in concentration and rarely exceeds the albumin level as is usually the case in overt myeloma; there is almost invariably an extraordinary constancy of the level over long periods of time; but the other immunoglobulins are usually not suppressed to the degree observed in the patient illustrated. Bence Jones proteins are extremely rare and were not observed in any of the authors' 35 cases. Similar findings have been made by others (Waldenström, 1964; Danon

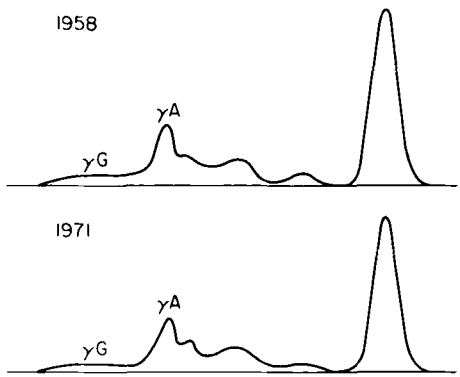

FIG. 10. Comparison of the electrophoretic pattern by cellulose acetate electrophoresis of sera from patient Co taken 13 years apart. The γA band and low γG remained essentially unchanged. The two sera were separated in the same experiment on frozen and stored samples.

et al., 1967). Most investigators in the field now believe that the vast majority of the monoclonal bands do not necessarily signify malignant disease.

There are, however, reports in the literature which raise the possibility that invasive multiple myeloma may develop in individuals who carried the "benign" monoclonal bands for many years. The most significant of these is the report (Norgaard, 1964) where 5 cases of myeloma were recorded which previously had been studied up to 13 years earlier because of unusual anticomplementary activity in the serum. The latter activity is often seen in myeloma with large γ-globulin peaks. Another similar instance was observed by Witebsky (see Bloom *et al.*, 1958) in a patient who developed multiple myeloma after it had been known for many years that he showed unique anticomplementary activity.

A number of instances have been recorded where monoclonal bands have disappeared spontaneously or more commonly through surgical manipulation. Removal of tumors has caused such disappearance in several cases (Bohard, 1957; Clubb *et al.*, 1964). The case studied by the latter authors, the disappearance of a band after removal of a parathyroid tumor, is particularly striking and well documented. Another case studied at Rockefeller University by Winchester and the authors showed an IgA monoclonal band along with general elevation of immunoglobulins for several years in a patient with chronic gall bladder disease. Removal of the gall bladder by surgery led to a disappearance of the band along with a decrease in the general immunoglobulin level. Osserman and co-workers have emphasized a relationship between chronic biliary disease and myeloma (Osserman and Takatsuki, 1965). In this study, they reported a band that developed in a patient after a reaction to Gantrisin and which subsequently regressed. Ritzmann and associates have also described an interesting case where the liver appeared in some way involved in a monoclonal IgM protein (Ritzmann *et al.*, 1970). Here the band disappeared completely after an episode of infectious hepatitis. Cases such as these raise the possibility that a number of the bands result from antigenic stimulation in these individuals, and certainly monoclonal antibodies have been described in the human (Yount *et al.*, 1968) as well as high levels in rabbits and other species (Eichmann *et al.*, 1970). However, the remarkable regulatory mechanism that controls such a constant level of protein in these individuals does not relate well to the antigen stimulation concept. In the authors' view some cases may result from constant antigenic stimulation, but the majority are the result of what might be considered as benign tumors of the lymphocyte–plasma cell series. As with other benign tumors, most of these remain benign but some undergo malignant trans-

formation to invasive myeloma. Also, as with other tumors, malignant myeloma may arise directly in some instances and from the benign plasma cell tumors in other instances.

D. "Abnormal" Proteins

A very different situation from that emphasized above for most myeloma proteins has been observed with respect to another group of proteins which includes the heavy-chain disease types. Two cases have been studied in detail, and strong evidence obtained that these are, indeed, abnormal proteins. The first case involves a protein described by Deutsch which was demonstrated from peptide analysis to have a deletion in the heavy chain (Deutsch and Susuki, 1971). Our studies (Kunkel and Deutsch, 1971) indicated that this was an IgG1 protein antigenically and contained the non-a antigen in the Fc portion. However, it lacked the Gm(f) marker in the Fab region which would be expected in such a protein. No other genetic markers were found. Studies on the normal γ-globulin of the serum of this individual showed that it contained the IgG1 markers, Gm(az) and Gm(f), in the ratio characteristic of heterozygous Caucasians. Thus the two IgG1 genes of this individual were producing the expected normal products. Family studies confirmed these interpretations. Thus the special protein lacking Gm(f) was not a genetic variant but an abnormal product of a specific clone of cells.

A similar situation was observed for the heavy-chain disease protein, case Ma (Osserman and Takatsuki, 1963). This protein was found to be an IgG1 type containing the Gm(a) genetic marker. However, the expected associated Gm(z) marker was not detected. The normal γ-globulin was readily separable from the heavy-chain disease protein by simple zone electrophoresis (Fig. 11). The inhibition titers of the fractions are shown for Gm(a), (z), and (f). The heavy chains migrating in the α_2 region (tubes 2–6) contain all three markers. Thus in this heterozygous individual, Gm(z) is present in the expected ratio to Gm(a) in the normal γ-globulin along with Gm(f). The two IgG1 genes produced the expected normal products; the heavy-chain proteins were clearly not the products of an altered gene but abnormal proteins arising in one clone of cells. It appears probable that all of the heavy-chain disease proteins are of this type. Attempts to prove this in the others were not possible because the individuals were not heterozygous as in the cases described. These cases stand in sharp contrast to those described in an earlier section where altered proteins are found which clearly result from genetic events and are carried through families.

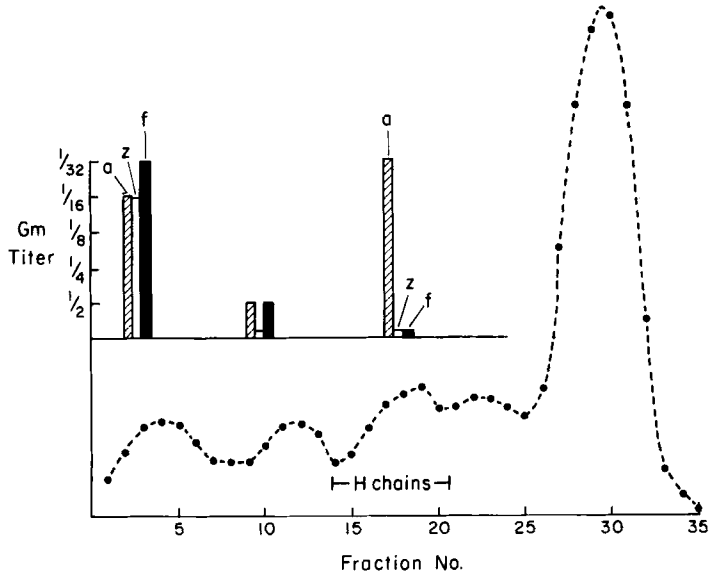

FIG. 11. Block electrophoresis experiment on the serum of the heavy (H)-chain disease case Ma. The position of the rapidly migrating heavy chains is indicated. The normal γ-globulin of this patient contains the Gm(a, z) and Gm(f) markers in approximately the usual proportions indicating normal production of the two IgG1 allelic products. The heavy chains are Gm(a) positive but lack the usual associated Gm(z) marker.

E. DELETIONS IN HEAVY-CHAIN DISEASE PROTEINS

Recent structural studies (Frangione and Milstein, 1969; Franklin and Frangione, 1971a) have demonstrated large deletions in the heavy chains of several of the heavy-chain disease proteins. Characteristically they show an N-terminal sequence that is not abnormal and then a large deletion extending through part of the V region through most of the CH1 area. The size of the deletion appears to be different in each case, but it is of special interest that the deletion ends at position 216 in the two proteins where it has been thoroughly studied. Figure 12 illustrates the deletion schematically. These proteins show no binding to light chains which would not be expected in view of the deletion and, therefore, appear only as small heavy chains.

The Deutsch protein, mentioned above, shows a much smaller deletion and the heavy chains still have affinity for light chains even though no disulfide bonds exist between the heavy and light chains (see Fig. 12). Another protein that appears very similar to this has been

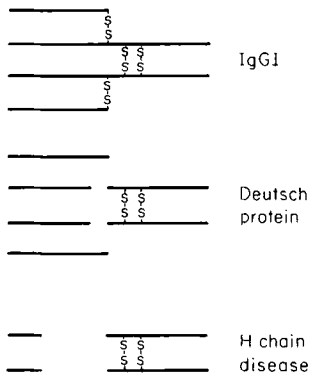

FIG. 12. Diagram showing the deleted portions of the heavy (H) chains in certain abnormal proteins including those of Franklin's heavy-chain disease cases. The Deutsch protein has a much smaller deletion and the light chains are still attached by noncovalent bands.

described (Parr *et al.*, 1971). These may be much more common and just not recognized by the usual screening methods.

V. Idiotypic Specificities

One of the most characteristic features of immunoglobulins is their capacity to induce individual specific or idiotypic antibodies on immunization. These have long been known for myeloma proteins and were probably involved in immunization experiments as early as 1945 where they formed the basis for the general impression that these were abnormal proteins with no counterparts in normal serum. Antisera to crude myeloma fractions after absorption with normal serum still reacted with the myeloma serum. For many years this abnormal or foreign protein character of myeloma proteins was universally recognized on the basis of these experiments until it was demonstrated that antisera to Fraction II γ-globulin prepared from normal plasma specifically reacted with myeloma proteins. The recognition of both cross-reactivity with normal γ-globulins and individual antigenic specificity for each myeloma protein came from the studies of Slater and associates (1955). However, it was not until considerably later that this principle was also demonstrated for antibodies. Many earlier studies on antibodies had failed to show any specificities that were not found in normal γ-globulin. Studies from the authors' laboratories (Kunkel *et al.*, 1963) clearly demonstrated that a variety of isolated human antibodies could elicit immune antisera which reacted specifically with the antibody used for immunization after absorption with normal immunoglobulins. Similar observations were made

at approximately the same time for isolated rabbit antibodies (Oudin and Michael, 1963). It was thus clear that in this respect antibodies behaved in exactly the same fashion as myeloma proteins. Many isolated antibodies, particularly those to protein antigens, were too heterogeneous to show such individual specificity. More homogeneous human antibodies such as those to polysaccharide antigens readily produced specific antisera.

A. Individual Antigenic Specificity vs. Idiotypy

"Individual antigenic specificity" was the first name applied to these specificities and was observed initially on immunization of a foreign species, either rabbit or monkey, in the case of human immunoglobulins. With the observation that similar specificities could be detected by immunization of animals in the same species the term *idiotypy* was applied. Initially this was only used for such intraspecies immunization, and individual antigenic specificity was restricted to the antigens observed with the heterologous antisera. More recently the use of the term idiotypy has been broadened to include both types since it is clear that related antigens are involved. It will be used in this way in the present review. However, it should be emphasized that there are striking differences in the antisera produced by the two procedures. Further differences also must exist for systems where the antisera are made in the same inbred strains of animals where the antigen is formed. Such a system has been demonstrated recently in BALB/c mice (Sirisinha and Eisen, 1971); previously it had been thought that heterologous strains were necessary for the production of idiotypic antibodies. It would appear that the largest number of idiotypic antigens are revealed by interspecies immunization, considerably less by intraspecies immunization, and the least by immunization within the same inbred strain of animals.

B. Methods of Detection

Most of the work with the determination of idiotypic specificities has been carried out by precipitation methods primarily in agar gels. In the intraspecies immunizations, absorptions are not necessary except for the allotype antigenic differences where they occur. In the systems involving heterologous immunization, however, absorption of the antisera is required to remove class, subclass, and genetic-type antibodies. A very important point here relates to the degree of absorption of such heterologous antisera and the results obtained are in large part determined by this absorption, a factor not always considered by investigators in this field. Very extensive absorption with immunoglobulin of the same species as the antigen, using up to 100 mg. of pooled immunoglobulin per cubic

centimeter of heterologous antiserum, is usually required to provide an antiserum that resembles that obtained by intraspecies immunization. Such antisera are maximally specific and are most useful in defining differences between monoclonal immunoglobulins. However, it is important to recognize that heterologous antisera that are minimally absorbed are also extremely useful, particularly in defining relationships between proteins and between a given protein and the normal γ-globulin of different individuals. This point will be discussed again later in this section.

Figure 13 illustrates some of these points. Here antisera to three different myeloma proteins were used after absorption with different amounts of Fr II γ-globulin, and these no longer gave precipitin lines with heterologous myeloma proteins nor with Fr II in agar gel plates. However, Fr II when put in adjoining wells inhibited the lines obtained with the homologous proteins. This was a characteristic finding with all these idiotypic antisera; Fr II γ-globulin or γ-globulin from single normal individuals, although failing to precipitate, inhibited the homologous reaction. This inhibition reflected the fall in the precipitin curves shown in Fig. 10 after further absorption with γ globulin. These results can probably be explained on the basis of multiple antigens in the pool of normal γ-globulins which relate partially to the homologous antigens;

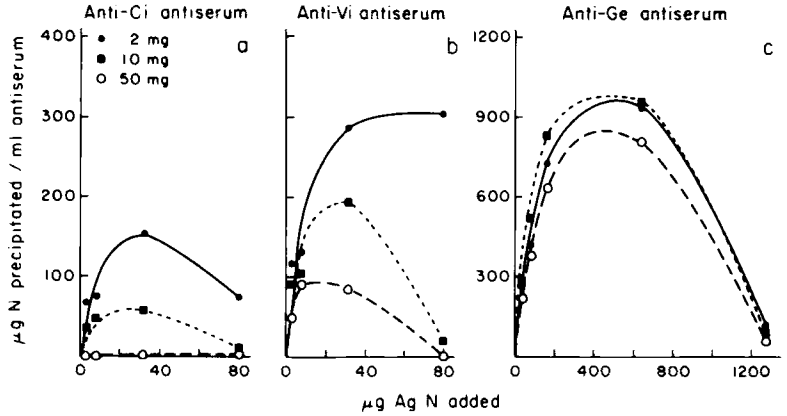

FIG. 13. Precipitin curves of idiotypic rabbit antisera to human myeloma proteins and their homologous antigens after different degrees of absorption with high concentrations of Fraction II γ-globulin. The usual picture is shown with the anti-Vi serum (b)—extreme absorption lowers the precipitation but residual precipitation remains. The anti-Ci antiserum (a) becomes negative after absorption with 50 mg. Fraction II per cubic centimeter of antiserum. The anti-Ge serum (c) is unusual in that it is little affected by extreme absorption (Grey *et al.*, 1965).

however, there are not enough of these on single molecules to give a precipitate in the agar gel assay. Wide differences are encountered with different antisera even against the same proteins. Figure 13 illustrates these wide differences. Another pattern that is sometimes seen but not shown clearly is the situation where absorption with Fr II reduces the precipitin curve after initial absorption but then additional amounts have no further effect, and agar gel analysis shows no inhibition with Fr II. Here the antiserum appears completely specific for the homologous protein and probably has reached the state seen with idiotypic antisera obtained by intraspecies immunization.

Seligmann and associates have carried out detailed precipitin studies of the idiotypic system. They obtained considerable evidence of myeloma heterogeneity at the idiotypic level in these studies and were able to demonstrate the appearance of fractions of the myeloma antigen in the region of antibody excess. They emphasized the utility of the idiotypic system for bringing out heterogeneity in these proteins but were unable to relate this heterogeneity to that observed in starch gel electrophoresis where multiple bands for the light chains are not uncommon (Seligmann *et al.*, 1965).

Harboe and associates have studied the idiotypic specificities of monoclonal IgM proteins in various precipitin systems. They observed a number of different types of idiotypic antigens in this work. Particularly interesting was one type which was destroyed by reduction and alkylation (Harboe *et al.*, 1969). Recent studies demonstrate that the latter involved the splitting of the disulfide band linking μ and κ chains (Solheim *et al.*, 1971).

Very different results are obtained when the antisera described above are tested in inhibition systems involving the myeloma protein used for immunization (Kunkel, 1970). In agglutination systems the homologous

TABLE XIV

IDIOTYPIC SPECIFICITY AS SHOWN BY SPECIFIC INHIBITION BY MYELOMA PROTEIN GR OF ANTI-GR AGGLUTINATION SYSTEM[a]

	Inhibitor protein concentration (mg./ml.):						
	50	1	0.1	0.01	0.001	0.0001	0.00001
Gr		0	0	0	0	0	2
Na		2	2	2	2	2	2
Fr II	2	2	2	2	2	2	2
Gr in Fr II		0	0	0	0	1	2

[a] Fraction II (Fr II) fails to inhibit.

myeloma protein inhibits the agglutination to very low concentration, whereas Fr II γ-globulin or normal individual γ-globulin fails entirely to show inhibition even at very high concentrations and irrespective of the degree of absorption of the antiserum. It would appear that the residual homologous specificity shown in the precipitin curves after great absorption dominates the reaction in this nonprecipitating system. Table XIV illustrates typical findings with this system. If the homologous myeloma protein is added to high concentrations of Fr II, it can be detected at very low concentrations. As little as 1 part in 20 million of Fr II could be recognized. This method failed to detect molecules in pooled γ-globulin that were identical by this idiotypic criterion to the myeloma protein and offered some minimum estimates of the degree of antibody diversity.

Nisonoff and associates have employed primarily a radioimmunoassay system in which inhibition by homologous unlabeled protein as well as other proteins can be measured (Hopper and Nisonoff, 1971). This system avoids the problem of nonprecipitating idiotypic antibodies through the use of a goat antiserum to rabbit Fc in the case where the idiotypic antiserum is from an immunized rabbit. In this system marked specificity for the homologous protein was obtained and pooled γ-globulin showed little or no inhibition as in the case of the hemagglutination experiments described above.

C. Localization of Idiotypic Antigens

As would be expected from the relative invariability of the Fc fragment of immunoglobulins, initial experiments demonstrated that the idiotypic antigens were localized on the Fab fragment (Kunkel *et al.*, 1963; Grey *et al.*, 1965; Harboe *et al.*, 1969). Antisera made to whole myeloma proteins and antibodies usually showed idiotypic specificities that related to the combination of heavy and light chains (Grey *et al.*, 1965). However, in addition, clear evidence for chain localization of antigens was obtained with many antisera to whole proteins and both the light and heavy chains could be shown to be involved. Antisera made to light chains or Bence Jones proteins readily produced idiotypic antibodies (Mannik and Kunkel, 1962). This was particularly true of the λ-type light chains, and strong antisera in the authors' experience usually showed idiotypic specificity. Many κ antisera also showed this but these antibodies were frequently weak and were dominated by the C-area specificities. Antisera made to isolated heavy chains produce idiotypic antibodies with considerable difficulty, and when they are produced it is hard to rule out contaminant whole molecules, at least in the IgG system, as acting as the stimulus.

It was apparent from the above studies that the V areas of the heavy and light chains were involved in these specificities. At the moment it is not clear whether certain antibodies, such as those absorbed out by large amounts of Fr II, relate to one part of the V area while other antibodies that show the unique specificity, demonstrated by hemagglutination, relate to another area. Studies on this point are extremely difficult. However, the work of Nisonoff and his associates has demonstrated clearly that at least some of these specificities relate to the antibody-combining site. These workers were able to show that the presence of haptene on the antibody Fab fragments blocked the reaction with idiotypic antibodies. Strong blocking was also obtained in experiments where the mouse anti-DNP myeloma protein, MOPC 315, was affinity labeled with DNP haptene (Brient and Nisonoff, 1970). Some such blocking effects were also observed in the studies on the cold agglutinins and their cross-idiotypic specificity (Williams *et al.*, 1968). The cold agglutinin antibodies when bound to the red cell failed to absorb out the cross-idiotypic antibodies. This system, however, does not have the advantages of the haptene antigens used by Nisonoff and associates. Recent work with a haptene system in inbred mouse strains has also demonstrated a specific blocking effect of DNP ligands on the idiotypic antigens of the myeloma protein MOPC-315 which had been extensively studied earlier in regard to its DNP binding (Sirisinha and Eisen, 1971). All of these studies strongly implicate the region of the antibody-combining site in the idiotypic specificities. It would appear that the three hypervariable regions that have been demonstrated for the light chains (Wu and Kabat, 1970) and the three hypervariable regions on the heavy chains (Kehoe and Capra, 1971) might be involved. It, of course, remains unclear whether all of these or just some play a dominant role.

D. Diclonal Immunoglobulins

One of the most significant usages to which idiotypic antisera have been applied has been in the demonstration of similar V regions for different classes of heavy chains. Situations where more than one monoclonal immunoglobulin appears in the same serum have proven of particular value for such studies. These were first studied a number of years ago in the authors' laboratory, and one serum was demonstrated to have an IgG and an IgA peak which weer shown to have identical light chains by both starch gel electrophoresis and idiotypic antiserum analysis (Prendergast *et al.*, 1966). Later studies of this case demonstrated identical idiotypic reactions with the whole protein. An additional case (Wang *et al.*, 1969b) was subsequently described which was also

shown to have similar light chains by sequence analysis. Later independent studies from the authors' laboratory (Penn *et al.*, 1970) as well as by Wang and associates (Wang *et al.*, 1970a) demonstrated idiotypic similarity between the IgG and IgM peaks of diclonal sera that related to similar V regions of the heavy chains. In the study by Penn and associates, both the light and heavy chains were demonstrated to have similar idiotypic specificity by means of multiple antisera. More recently, Penn has studied a number of additional diclonal and triclonal sera which show similar idiotypic relationships (Penn, 1972). Diclonals consisting of IgA and IgG proteins have shown the same similarity of light and heavy chain idiotypy that was found for the IgM–IgG combinations. Table XV lists the various examples that have been studied thus far. In all instances where the light chains have shown similar idiotypy, the heavy chains have shown it as well despite the C-region class differences.

It should be emphasized that not all diclonal cases show these relationships and a number have been studied in the authors' laboratory where no idiotypic relationship is demonstrable. There are also many examples where one band has κ light chains and the other band has λ light chains. The possibility that the heavy chains in these cases have similar V regions despite the difference in light-chain class has not been ruled out, although some studies by Penn failed to offer evidence for this

TABLE XV
KNOWN CASES OF DICLONAL AND TRICLONAL SYSTEMS SHOWING SHARED IDIOTYPIC SPECIFICITY

Case	γG	γA	γM	Reference
Ba	γG1λ	γA1λ		Prendergast *et al.* (1966); Penn (1972)
Mi	γG3λ		γMλ	Penn *et al.* (1970)
Til	γG2κ		γMκ	Wang *et al.* (1970a)
Di	γG1κ		γMκ	Penn (1972)
We	(γG1κ)[a]	γA2λ	γMλ	Penn (1972)
To			γMκ	Hannestad (1969)
			γMκ	
Na			γMκ	Hannestad (1969)
			γMκ	
—			γMκ[b]	Freedman *et al.* (1971)
			γMκ	

[a] This protein in a triclonal serum did not share idiotypic specificity with the other two.

[b] Heavy chains were identical by chemical criteria. Idiotypic specificity was not studied.

possibility. Table XV shows one triclonal situation where the two λ proteins had idiotypic similarities, whereas the κ protein failed to react. Because the light chains play a major role in eliciting idiotypic antisera in most instances, even though the heavy chains are also involved, it is not clear that heavy-chain similarity would be detectable in such a situation.

In the one case studied by the authors (Mi in Table XV) the light chains showed idiotypic identity with a light-chain antiserum despite the fact that mobility differences were present by gel electrophoresis. The latter was shown to be due to the presence of histidine in one protein and not in the other. This small difference in the light chains of the two proteins is of particular interest. Recently (Hannestad and Sletten, 1971) diclonal IgM systems have been demonstrated where differences in the IgM proteins exist despite idiotypic relationships. In all instances the diclonal proteins have been found in separate cells by fluorescent antibody staining. The idiotypic similarities strongly suggest that separate cell lines developed from the same original stem cell. In those instances, mentioned above, where slight V-region differences have been found, it is suggested that mutations occurred after the cell lines diverged. Further studies of the exact sequence basis for these slight differences will be of special interest. The possibility exists that such differences occur with considerable frequency and are present in many situations where only a single monoclonal protein is apparent. It has long been known that idiotypic antisera frequently provide evidence of heterogeneity in such proteins in precipitin curve analysis as well as through agar diffusion studies.

E. Cross-Idiotypic Specificity

The first example of cross-reactions in the idiotypic specificities of monoclonal immunoglobulins from different individuals was observed in the case of the human IgM cold agglutinins. Here antisera made to a single isolated monoclonal cold agglutinin, after absorption with different IgM proteins without such activity, continued to react with cold agglutinins other than the one used in the immunization (Williams *et al.*, 1968). Some antisera show little or no specificity for the immunizing antigen and the cross-specificity dominates; other antisera show the reverse picture with primary specificity for the original antigen. The cross-specificity is a property of the Fab portion of the molecule and the main antigens appear to be localized to the V region of the heavy chains. Those antisera that show light-chain dominance in the idiotypic specificity are in general less useful for demonstration of the cross-idiotypic specificity.

It has become apparent that a number of antigens are involved in cross-specificity, and a wide assortment of spurs are seen between different cold agglutinins in agar plate analysis with many of the antisera (Williams, 1971). Figure 14 illustrates the differences between different cold agglutinins brought out by two different antisera. Both antisera in the main recognize the same proteins. However, the spurs obtained are quite different and the assortment of antigens involved are quite apparent. Recently considerable information has been obtained on the I antigen with which the cold agglutinins react (Feizi *et al.*, 1971). It is clear that blood group-related antigens are involved and that different monoclonal cold agglutinins show a number of different specificities. Studies are currently underway in the authors' laboratory to attempt to relate these differences to the cross-idiotypic differences. In general it has been found that the cold agglutinins with small-i specificity react weakly or not at all with antisera made to proteins possessing big-I specificity (two of the negative proteins in Fig. 14 are small-i proteins). The accumulated evidence obtained thus far in this system indicates that cross-idiotypic specificity bears a relationship to the antigen-combining property of these proteins.

Recently preliminary findings have been reported by Franklin and Frangione (1971b) suggesting that cross-reactions similar to those in the cold agglutinin system are present for monoclonal anti-γ-globulins.

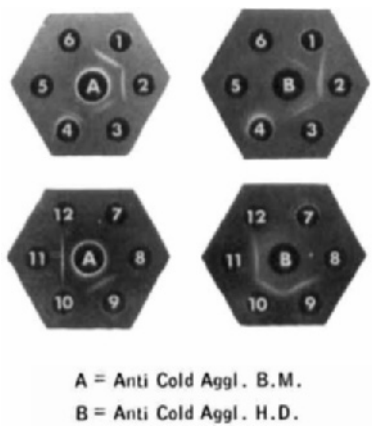

FIG. 14. Precipitin reactions between isolated IgM cold agglutinins and two cold agglutinin antisera which have been absorbed with IgM proteins lacking this activity. Only cold agglutinins precipitate with these antisera. The two antisera show related but somewhat different specificities when tested with the same twelve, different cold agglutinins, and spurs between the proteins are evident. The proteins used to produce the antisera are omitted. Proteins 7 and 8 are IgM cold agglutinins with i specificity; the others are I or non-I cold agglutinins.

These proteins are much more difficult to study in precipitating systems because many of them react with rabbit γ-globulin particularly if it is present in the form of soluble or insoluble complexes. In the latter situation they augment precipitin lines giving the impression of specificity. It remains unclear as to whether these problems were overcome in the above study; they have proved difficult to avoid in the authors' studies with antisera to these proteins even when absorptions were made with solid adsorbents. A better system has been obtained by coating red cells with these proteins and employing hemagglutination and hemagglutinating-inhibition reactions. This has brought out clear evidence of cross-idiotypic specificity for certain of the monoclonal anti-γ-globulins (Kunkel *et al.*, 1973). Six of eight isolated IgM proteins with this activity show cross-specificity with two different antisera. Table XVI illustrates the inhibition by four positive anti-γ-globulins, one negative anti-γ-globulin, and three IgM proteins without known activity. Fraction II as well as the Fab fraction of Fr II at higher concentrations also show inhibition in this system, but no monoclonal IgG proteins nor IgM proteins of the approximately fifty proteins tested show similar inhibition. Thus it appears clear that the principles laid down for the cold agglutinins also apply to a second monoclonal protein.

Cross-idiotypic specificity has also been demonstrated between mouse myeloma proteins possessing similar combining activity for the antigen, phosphorylcholine (Potter and Lieberman, 1970). Here, however, the

TABLE XVI

CROSS-IDIOTYPIC SPECIFICITY OF ISOLATED IMMUNOGLOBULIN M ANTI-γ-GLOBULINS[a]

Macroglobulin	Inhibitor protein concentration (mg./ml.):						
	1.0	0.25	0.06	0.015	0.004	0.001	0.0002
Wa[b]	0	0	0	0	0	1	2
BI[b]	0	0	0	0	0	2	2
Si[b]	0	0	0	0	0	1	2
Ea[b]	0	0	0	0	0	1	2
La[b]	1	2	2	2	2	2	2
Fr II[c]	0	0	1	2	2	2	2
Ga[c]	2	2	2	2	2	2	2
St[c]	1	1	2	2	2	2	2
Sz[c]	2	2	2	2	2	2	2

[a] Four of the five anti-γ-globulins inhibit the reaction but other macroglobulins do not.

[b] Immunoglobulin M anti-γ-globulins.

[c] Immunoglobulin M proteins without activity. Antiserum made against anti-γ-globulin Ma; red cell coat = Wa.

proteins are all obtained in the highly inbred BALB/c strain which makes the situation quite different from that with the human proteins. Similar cross-idiotypic specificity for certain homogeneous streptococcal antibodies has been reported (Eichmann and Kindt, 1971). This was only found within single families of immunized rabbits, and antistreptococcal antibodies from outbred rabbits failed to react. More recently, it has been demonstrated that pooled γ-globulin from outbred rabbits inhibits their system which may relate to the inhibition described above for the anti-γ-globulin cross-idiotypy. It is of special interest that the normal γ-globulin of rabbits from the same family showed considerably greater inhibition.

F. Other Applications

Since idiotypic antisera are so readily obtained against myeloma proteins and many isolated antibodies, their value as markers for the V regions of these proteins in many diverse types of studies is obvious. They have become an important supplement to allotypic markers in genetic studies particularly since the mechanisms involved in V-region diversity remain so poorly understood. Their application, however, extends well beyond such usage as genetic markers of V regions and is currently extensive in such diverse areas as lymphocyte receptors and clinical glomerulonephritis. In the latter situation it is possible to identify the deposition of specific serum antibodies in the glomerular lesion. Here idiotypic antibodies have been made to anti-γ-globulins which appear as cryoglobulins in the sera of patients with systemic lupus erythematosus (Agnello *et al.*, 1971). These same antibodies have been identified in the glomerular deposits by means of fluorescent antibody staining using fluorescent idiotypic antisera, and the significance of the serum antibodies was thus demonstrated. Studies are under way on amyloid where idiotypic antisera to associated Bence Jones proteins are being used to demonstrate their significance in the amyloid deposits (Glenner *et al.*, 1972). In addition, usage has been made of idiotypic antisera in the authors' laboratory as a tag to follow the disappearance of infused proteins, to discover the site of synthesis of a specific serum immunoglobulin, to detect residual monoclonal proteins which, by less sensitive techniques, appeared to disappear following therapy, etc. The technique also offers a method to relate specific serum immunoglobulins to those on lymphocyte surfaces; idiotypic markers have been identified on the lymphocyte surface (Wernet *et al.*, 1972b) in a number of cases where monoclonal proteins have been found in the serum to which idiotypic antisera could be produced. This undoubtedly will be a rapidly expanding usage particularly in fluorescent antibody studies.

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Immunological Unresponsiveness[1]

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I. Introduction

Following appropriate contact with an antigen, an animal may respond in a number of different ways. The host may produce circulating antibody, become primed without producing antibody, develop delayed hypersensitivity, or become immunologically unresponsive. Which of the above events occurs depends on the conditions under which the antigen

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is injected. If appropriate conditions are selected, an unresponsive state can result, in that the animal does not respond to subsequent injection of the same antigen given under conditions which would otherwise favor an immune response. Since Burnet and Fenner (1949) first postulated that animals could be made specifically unresponsive to a foreign substance by injecting that substance during neonatal life, antigen-directed unresponsiveness has been induced with numerous antigens in both neonates and adults of a variety of species. Of particular importance is that the induced unresponsive state often mimics the unresponsiveness that animals enjoy to their own body constituents. Recent observations demonstrating the cellular complexity of the immune response and the presence and nature of antigen receptors on lymphocytes warrant a reevaluation of vast amounts of data accumulated over the years on immunological unresponsiveness. This chapter is primarily devoted to discussing the cellular events involved in the induction, maintenance, and termination of antigen-directed unresponsive states.

II. Conditions Affecting Induction of Immunological Unresponsiveness

The induction, degree, and duration of immunological unresponsiveness are dependent on the species and strain of animals, the immunocompetence of the host, nature of the antigen, dose of antigen, and route of injection. In most situations, a combination of several of the above conditions is involved.

A. Species and Strain of Animal

The ease with which immunological unresponsiveness is induced is markedly dependent on the species. In addition to the usual laboratory animals, i.e., rabbits, mice, and guinea pigs (cited in Weigle, 1967a), immunological unresponsiveness has been induced in chickens (Tempelis *et al.*, 1958), goats (Carter and Cinader, 1960), opossums (Major and Burrell, 1971), toads (Marchalonis and Germain, 1971), primates (Cotes *et al.*, 1966; R. J. Howard *et al.*, 1969), and man (Rossen *et al.*, 1971). Since different antigens and different aged animals have been used, results are difficult to compare among these species. However, sufficient data are available employing serum protein antigens injected during neonatal life to permit a comparison among rabbits, mice, guinea pigs, and chickens. With these antigens, a complete unresponsive state of long duration can be induced neonatally in rabbits (Dixon and Maurer, 1955; Smith and Bridges, 1958). A complete unresponsive state can also be induced neonatally in mice (Terres and Hughes, 1959; Thorbecke *et al.*, 1961; Dresser, 1961; Dietrich and Weigle, 1963) and guinea pigs

(Humphrey and Turk, 1961), but of shorter duration than that observed in rabbits. Induction in chickens during either embryonic or neonatal life results in only a hyporesponsive state (Tempelis *et al.*, 1958).

A marked variation in the ease with which unresponsiveness is induced can be seen among various strains of animals within a species. The dose of deaggregated (ultracentrifuged) human γ-globulin (HGG) required to induce unresponsiveness to HGG varies among the strains of mice used—C57BL/6J mice become unresponsive following a single injection of as little as 0.1 mg., whereas BALB/cJ mice do not become unresponsive to as much as 10 mg. injected under identical conditions (Golub and Weigle, 1969). Injection of 1 mg., but not 0.5 mg., is sufficient to induce an unresponsive state in A/J mice. Data obtained by cross-mating and backcrossing of these strains strongly suggest that the ease with which unresponsiveness is induced is under genetic control. These findings are not surprising, since such genetic control of the immune response is well documented (McDevitt and Benacerraf, 1969).

As with BALB/cJ mice, Staples and Talal (1969) reported that the induction of unresponsiveness in NZB and NZB/NZW hybrid mice to deaggregated HGG was difficult. Subsequently, Cerottini *et al.* (1969) reported that there was no difference in the ease of induction of unresponsiveness to deaggregated HGG in NZB/NZW hybrid mice and mice of several other strains. Russell and Denman (1969) were able to induce an unresponsive state to rabbit γ-globulin in NZB/NZW hybrid mice; however, no comparison was made with other strains. Although these differences observed in the induction of unresponsiveness in NZB/NZW hybrid mice may be the result of differences in the protocols used by the investigators, they may also be the result of genetic drift in the different colonies of the NZB and/or NZW strains. In any event, studies on the induction of both immunological unresponsiveness and immunity with these mice are of particular interest since they acquire with age spontaneous autoimmune-like phenomena (Howie and Helyer, 1968).

B. Immunocompetence

As revealed by previous studies, the immunocompetence of the host is the major factor in the induction of immunological unresponsiveness. Usually, the higher the degree of competence the more difficult it is to induce an unresponsive state and, with most antigens, the induction of unresponsiveness in immature animals is more easily achieved than in mature animals. However, in special situations, immunological unresponsiveness can be induced in both normal adults and adults previously sensitized to the antigen.

1. Neonatal and Embryonic Animals

During neonatal or embryonic life, animals are not immunologically competent, thus an unresponsive state to a particular antigen is most readily induced during neonatal life in species that are least immunologically mature at birth. Rabbits develop the ability to make a humoral antibody response to bovine serum albumin (BSA) between days 8 and 21 after birth (Eitzman and Smith, 1959), and the unresponsive state to BSA can be induced in neonatal rabbits if the BSA is injected before the ninth day after birth (Smith and Bridges, 1958). A similar correlation between immunocompetence and the induction of unresponsiveness has been made in other species (Smith, 1961). However, the ability of the fetus to produce antibody to certain antigens upon appropriate stimulation is not uncommon (Silverstein and Prendergast, 1970). The human fetus responds to injection of bacteriophage with both plasma cell formation (Uhr *et al.*, 1960) and development of cellular hypersensitivity (Uhr *et al.*, 1962). Silverstein *et al.* (1963) observed that immunocompetence to injections of bacteriophage, horse ferritin, and ovalbumin incorporated into adjuvant developed on approximately days 53, 65, and 125, respectively, of a 150-day gestation period in sheep, whereas immunocompetence to diphtheria toxoid, *Salmonella typhosa* or Bacillus Calmette-Guérin (BCG) did not develop during fetal life. It would be of interest to know if immunocompetence during fetal life correlates with the ability to induce unresponsiveness in neonatal life. Unfortunately, the latter antigens do not lend themselves well to studies on the induction of immunological unresponsiveness.

2. Adult Animals

Immunological unresponsiveness can also be induced in adult animals. However, the animal's immunocompetence must be inhibited temporarily or the antigen given in either a nonimmunological form or by a nonimmunizing route. In some cases, the degree and duration of the unresponsive state is the same as that of early life where natural immunoincompetence exists.

a. Normal Adults. If the immunocompetence of the host is abolished or interfered with by irradiation, immunosuppressive drugs, thoracic duct drainage, or treatment with antilymphocyte globulin (ALG) accompanied by injection of antigen, the animal most often fails to respond to that antigen for a period of time thereafter. Whole-body irradiation given in sublethal doses almost completely eliminates lymphocytes from both primary and peripheral lymphoid tissue temporarily. Since the animal cannot respond to antigens during this period, injection of anti-

gen after irradiation can lead to a specific unresponsive state (Dixon and Maurer, 1955; Nachtigal and Feldman, 1963; Rittenberg and Nelson, 1963; Linscott and Weigle, 1965; Kawaguchi, 1970a) which remains for a significant period of time after recovery from irradiation and reconstitution of the lymphoid organs. All rabbits given 500 R of whole-body irradiation and then injected 2 and 7 days later with a total of 300 mg. of an aqueous preparation of BSA failed to respond to a subsequent injection of BSA in Freund's adjuvant given 2 months later (Linscott and Weigle, 1965). The unresponsive state was of a long duration since at 4 months the rabbits were still 80% unresponsive. Doses of 12 and 1 mg. of BSA given at similar times after irradiation resulted in a 95 and 70% inhibition, respectively, of antibody production at 2 months. The amount of BSA per kilogram body weight required to induce an unresponsive state appears to be approximately the same for irradiated adults as for neonates.

Antigenic competition and depletion of lymphocytes by either ALG or thoracic duct drainage also favor the induction of immunological unresponsiveness. It is of interest that in all three situations the thymus-derived lymphocytes appear to be preferentially affected. As the result of antigenic competition, a large percentage of rabbits injected with a mixture of antigens in complete Freund's adjuvant does not make an antibody response to aqueous BSA given 2 days later. Most of the non-responders become unresponsive in that they fail to respond to a subsequent injection of BSA (Weigle and High, 1967). Although it is not known for certain whether antigenic competition is at the level of the thymus-derived cell, it is thymus cell-dependent (Gershon and Kondo, 1970). Similarly, the depletion of their recirculating lymphocytes by thoracic duct cannulation of adult rats permits the induction of an unresponsive state. Unresponsiveness was induced to flagellin following injection of rats, previously drained of lymph and thymocytes from the thoracic duct for 5 days, with 100 μg. of flagellin, 3 times weekly for 6 weeks (Shellam, 1969b). It is well known that thoracic duct drainage preferentially affects thymus-derived cells. As with thoracic duct drainage, treatment with ALG preferentially depletes lymphoid organs of thymus-derived cells and favors the induction of unresponsiveness in adult animals. Heterologous antibody to lymphocytes has been shown to be a potent immunosuppressive agent capable of prolonging the survival time of allografts in both experimental animals (Waksman *et al.*, 1961; Woodruff and Anderson, 1963; Monaco *et al.*, 1966; Levey and Medawar, 1966; Jeejeebhoy, 1967) and man (Monaco *et al.*, 1967; Starzl *et al.*, 1967). However, it is questionable whether "tolerance" induced to allografts is a true unresponsive state or the result of

favorable competition between humoral and cellular hypersensitivities (Hellström *et al.*, 1971). The humoral antibody response is also affected by ALG, although larger amounts are required to cause inhibition (Monaco *et al.*, 1966; James and Anderson, 1967; Barth *et al.*, 1968). More recently, the injection of ALG with flagellin was shown to inhibit antibody formation and to induce an unresponsive state to a subsequent injection of flagellin (Shellam, 1969b). In the above situations where there is a depletion of the thymus-derived cell, it would be interesting to know the fate of the unresponsive state following the natural reconstitution of these cells.

There are many chemical agents that prolong allograft survival and inhibit the production of circulating antibody (cited in Schwartz, 1965). Since a different mechanism may be involved in "tolerance" to allografts, this portion of the review will only be concerned with antibody production. By using production of circulating antibody as the end point, only 6-mercaptopurine (Schwartz and Dameshek, 1959), amethopterin (Barlow and Hotchin, 1962), cyclophosphamide (Maguire and Maibach, 1961; Aisenberg, 1967; Salvin and Liauw, 1967; Dietrich and Dukor, 1968; Nakamura and Weigle, 1970; Kawaguchi, 1970b), cytosine arabinoside (Gordon *et al.*, 1969), and acriflavine (Farr *et al.*, 1965) have been used to induce specific immunological unresponsiveness to defined antigens. To achieve a completely unresponsive state, these drugs are injected over a prolonged period of time beginning several days before injecting the antigens and ending several days later. The immunosuppressive agents probably circumvent an immune response which then allows an unresponsive state to develop.

Doses of antigens also can be selected that favor the induction of unresponsiveness in adults, in neonatal animals, in sensitized animals, or in *in vitro* experiments and are discussed below (Section II,D) as one of the general conditions for induction of immunological unresponsiveness.

In situations where two forms of the antigen are available, one which induces immunity (immunogen)[2] and one which induces unresponsiveness (tolerogen), prior injection of the tolerogenic form induces a specific unresponsive state to subsequent injections of the immunogenic form. Classic examples of immunogens and tolerogens are aggregated and deaggregated forms of mammalian γ-globulins, respectively. Commercial preparations of γ-globulins owe their antigenicity to the presence of small amounts of aggregated material. When these preparations are further aggregated by heating, their immunogenicity is markedly enhanced. Commercial preparations can be deaggregated by

[2] *Immunogen* refers to a substance that induces immunity, whereas *tolerogen* refers to a form of antigen that induces unresponsiveness (tolerance).

ultracentrifugation to yield tolerogenic monomers. Injection of heterologous, deaggregated γ-globulin not only does not give rise to antibody production but also leads to an unresponsive state to subsequent injection of aggregated γ-globulin. Dresser (1962), by using bovine γ-globulin (BGG), was first to demonstrate that the injection of deaggregated preparations (centrifuged for 30 minutes at 20,000 *g*) into adult mice led to a specific immunological unresponsive state. Other mammalian γ-globulins have since produced equivalent results in other species. A single injection of deaggregated (centrifuged at 100,000 *g* for 90 minutes) HGG into adult A/J mice results in complete unresponsiveness that lasts for approximately 4 months (Chiller *et al.*, 1971). Protocols have been described for producing effective preparations of immunogenic and tolerogenic HGG by heat aggregation and ultracentrifugation, respectively (Chiller and Weigle, 1971). Effective preparations of deaggregated HGG have also been prepared by "biological filtration" where the HGG is injected into mice and the mouse serum, removed later, used as the source of HGG (Golub and Weigle, 1969). In this situation, the aggregated material is apparently removed by the reticular endothelial system (Frei *et al.*, 1968).

Immunological unresponsiveness is also induced in adult rabbits after injection of deaggregated BGG (Biro and Garcia, 1965). However, usually only a proportion of the rabbits becomes unresponsive unless a deaggregated myeloma IgG is used (Spiegelberg and Weigle, 1967). The efficiency of myeloma IgG in inducing an unresponsive state may be due to the homology of the variable regions. Unresponsiveness has also been induced in adult rabbits to H-chain protein obtained from patients with H-chain disease and with deaggregated (centrifuged at 105,000 *g* for 90 minutes) Fc fragments isolated from human IgG. Morse (1965) demonstrated that certain myeloma IgG preparations could be aggregated by heat, but others could not. Apparently, a portion of the IgG proteins obtained from the serum of any individual has the ability to aggregate, but another portion has not. The site responsible for aggregation has been shown to reside mainly on the Fab fragments (Augener and Grey, 1970). Rabbits made unresponsive to the Fc fragment when immunized with intact γ-globulin made antibody to only the Fab fragment. In a similar manner, specific antibody for IgG subclasses was obtained (Spiegelberg and Weigle, 1968)—rabbits were made unresponsive to a myeloma protein of one subclass and then immunized with another subclass incorporated into complete adjuvant. An unresponsive state to heterologous ALG has been induced in mice (Golub and Weigle, 1969) and humans (Rossen *et al.*, 1971) by prior injection of deaggregated preparations of the normal γ-globulins.

Some evidence suggests that aggregated and deaggregated proteins

other than γ-globulins may also play a role in immunity and unresponsiveness. Frei *et al.* (1965) reported that some adult rabbits injected with BSA deaggregated by biological filtration became unresponsive to BSA. These studies were extended (Frei *et al.*, 1968) and in addition the authors reported that monomeric, but not polymeric BSA induced an unresponsive state in rabbits. Another group of workers (Pinckard *et al.*, 1968) reported that a large percentage of adult rabbits injected with ultracentrifuged BSA became unresponsive. In contrast, injection of adult guinea pigs with aqueous preparations of BSA and human serum albumin (HSA) containing both monomers and polymers induced an unresponsive state, and the isolated polymers were as effective in inducing unresponsiveness as the monomers (Dvorak *et al.*, 1969). Although these differences could be explained if the guinea pig does not possess an effective mechanism for handling the polymers, there may be an alternative explanation. Dvorak and Bast (1970) reported that the induction of unresponsiveness in rabbits to HSA correlated with the absence of endotoxin and not the absence of polymers. Endotoxins have been clearly shown to interfere with the induction of unresponsiveness in the mouse (Claman, 1963; Golub and Weigle, 1967a). One would predict then that the immune mechanisms in rabbits are more sensitive to endotoxin than those in guinea pigs. In any event, the answer to whether molecular aggregation of antigen plays a crucial role in immunity and unresponsiveness will have to await further experimentation.

Specific unresponsiveness to an antigen can also be induced in adults by injecting a molecule that is not antigenic but has similar determinants as the antigen. Poly-γ-D-glutamic acid (PGA) capsular polypeptide of *Bacillus anthracis* is nonimmunogenic in adult rabbits, but its injection does induce an immunologically unresponsive state (Roelants and Goodman, 1970). Although immunization with the polypeptide alone does not induce an immune response, injection of the polypeptide conjugated to methylated BSA results in the production of antibody specific for PGA. However, if PGA alone is injected prior to injection of the conjugated preparation, no antibody to PGA is produced. Similarly, Sela *et al.* (1971) have shown that a synthetic multichain polypeptide is not immunogenic if injected into adult rabbits in a soluble form, but it is soluble if incorporated into adjuvant. If an injection of the soluble form precedes the adjuvant injection, no antibody is produced. Nonimmunogenic synthetic polypeptides have also been used to induce unresponsiveness in neonatal rabbits (Maurer *et al.*, 1963; Schechter *et al.*, 1964).

Certain antigens induce an unresponsive state if they are given by an unusual route. Certain haptens, when injected intradermally into guinea pigs couple, through the free ε-amino group of lysine, to the tissue pro-

teins and give rise to both circulating antibody and delayed hypersensitivity. However, if the antigen is first given orally, the guinea pigs cannot be sensitized by subsequent intradermal injections (Chase, 1946; Chase and Battisto, 1959; Coe and Salvin, 1963). Intravenous injections of large doses of haptens alone into adult guinea pigs also inhibit sensitization to subsequent intradermal injections (DeWeck and Frey, 1966). Battisto and Miller (1962) were able to induce an unresponsive state in adult guinea pigs by injecting small amounts of picryl chloride or BGG into the mesenteric veins, whereas injection into the jugular veins resulted in immunity. Both circulating antibody and delayed hypersensitivity were effected. In the case of the hapten, reactions to different routes may be either the result of the manner in which the hapten is coupled to the tissue or the distribution of the hapten–protein conjugate after the *in vivo* conjugation. Possibly the hapten, when given orally or injected intravenously, conjugates to circulating proteins. It has been shown (Havas, 1969; Golan and Borel, 1971) that an unresponsive state to haptens can be induced in adult mice if the haptens are conjugated to isologous serum proteins before injection. The unresponsive state to BGG obtained by injection into the mesenteric vein was probably the result of deaggregation in the liver before the antigen reached the lymphatic tissue. Injection of BGG directly into the thymus of adult rats enhanced the induction of unresponsiveness to BGG (Horiuchi and Waksman, 1968). The interpretation of this observation was that the thymus is involved in the induction of immunological unresponsiveness.

b. Sensitized Adults. Although an unresponsive state can be induced in sensitized adults, the events leading to unresponsiveness are more complex than those involved in the induction in normal adults. With sensitized adults, both virgin and memory cells must be affected. It is unlikely that virgin cells in sensitized adults are affected differently from those in similar cells of normal adults. The available data indicate that the memory cells are eliminated by exhaustive differentiation to antibody-producing cells as first suggested by Sterzl and Trnka (1957) who observed a transient production of antibody preceding the induction of an unresponsive state to *Salmonella paratyphosa* in rabbits. Similarly to Sterzl and Trnka (1957), Matangkasombut and Seastone (1968) and J. G. Howard *et al.* (1969) demonstrated that an immune response to pneumococcal polysaccharide occurred during the induction of immunological paralysis in mice. Paul *et al.* (1969a) found that sequential injection of pneumococcal polysaccharide (tolerogen) into adult rabbits previously sensitized with intact pneumococci (immunogen) led to a progressive decrease in the magnitude of the immune response. It has also been shown that the injection of a paralytic dose of bacterial

lipopolysaccharide in previously sensitized mice results in a decline in the number of antibody-producing cells (Britton, 1969a). It is probable that induction of unresponsiveness to bacterial antigens involves memory cells which have arisen as the result of prior contact with that antigen or related antigens. Induction of unresponsiveness in animals previously sensitized with serum protein antigens has been reported by several groups of investigators. Unresponsiveness to BSA was readily reinduced in rabbits in which the unresponsive state had spontaneously terminated and a response to BSA had occurred (Humphrey, 1964). Dorner and Uhr (1964) were able to induce an unresponsive state in rabbits minimally sensitized to BSA by injecting large amounts of soluble BSA. Byers and Sercarz (1968) observed that rabbits previously sensitized with BSA made a secondary response upon restimulation with a large dose of BSA, but were exhausted in that lymph node cultures made after these injections did not respond to subsequent *in vitro* stimulation with BSA, possibly due to exhaustive differentiation of memory cells *in vivo*. An unresponsive state also has been induced *in vivo* with C57BL/6J mice previously immunized with aggregated HGG (von Felten and Weigle, 1972). Induction was difficult in that it required several repeated injections of deaggregated HGG and probably involved exhaustive differentiation of memory cells. Difficulties in obtaining an unresponsive state seem to stem from aggregation of the injected deaggregated HGG by persisting antibody. Frey *et al.* (1964b), studying delayed hypersensitivity to a hapten, were able to induce an unresponsive state by injecting sensitized guinea pigs intravenously with aqueous preparations of the hapten.

Induction of unresponsiveness in sensitized animals has been achieved with the aid of immunosuppressive agents. The secondary response to BSA is suppressed in mice treated with 6-mercaptopurine (La Plante *et al.*, 1962) and in rats treated with cyclophosphamide or cytosine arabinoside (Gordon *et al.*, 1971). Cyclophosphamide has also been used to reintroduce specific immunological unresponsiveness to self-antigens. Experimental autoimmune thyroiditis induced in rabbits can be reversed by injecting rabbit thyroglobulin and cyclophosphamide. These rabbits become unresponsive in that they can no longer respond to subsequent injections of rabbit thyroglobulin (Nakamura and Weigle, 1970). Paterson (1971) observed that Lewis rats with paralytic experimental allergic encephalomyelitis (EAE) when treated with cyclophosphamide alone showed clinical remission and reversal of histological evidence of disease, however, this phenomenon probably arose from inhibition of proliferation of inflammatory cells and release of injurious factors within the sites of the lesions, rather than a reinduction of unresponsiveness. However,

the clinical symptoms of EAE could be reversed by injecting human encephalitogenic proteins or an active peptide incorporated into *incomplete* adjuvant into monkeys in which the disease had been induced by prior immunization with the encephalitogenic protein in complete adjuvant (Eylar *et al.*, 1972). Since injection of the protein in incomplete adjuvant does not result in disease or an immune response, it appears that an unresponsive state may have been induced or reinduced to the encephalitogenic protein.

C. Nature of Antigen

Unresponsive and hyporesponsive states have been induced to a variety of antigens with diversified properties. The nature of the antigen plays a major role in the degree and duration of the unresponsive state which is induced. Among other things, the nature of the antigen determines its own *in vivo* fate and tissue distribution. Specific immunological unresponsiveness has been studied with purified serum proteins, viruses, bacteria and their products, heterologous erythrocytes, haptens, and synthetic polypeptides.

1. Serum Proteins

Heterologous serum proteins readily lend themselves to investigation of a number of aspects of immunological unresponsiveness. They are relatively simple and of low antigenicity as compared to bacterial and viral antigens. These proteins can be obtained in large quantities and in a highly purified form. The immune response to them can be readily quantitated either at the serum or cellular level. They are efficiently labeled with ^{131}I or ^{125}I and are readily followed in the circulation and in tissue. After injection, these proteins equilibrate between the intra- and extravascular spaces and come in contact with all of the antigen-reactive cells. If injected in adults under usual procedures, an antibody response ensues and the protein is eliminated from the body fluid by an immune mechanism. If given under selective conditions in which an immune response does not occur, an unresponsive state to subsequent immunogenic injection develops. This latter property is exemplified by the immunogenic (aggregate) and tolerogenic (monomeric) forms of γ-globulins discussed above (Section II,B,2) and by neonatal animals injected with heterologous serum proteins.

When equilibration is complete, after intravenous injection, the concentration of heterologous or homologous proteins present in the lymph and the plasma (C_L/C_P ratio) correlates exponentially with the effective hydrodynamic diffusion radius of the protein (Fig. 1). With globular proteins, the higher the molecular weight, the greater is the concentra-

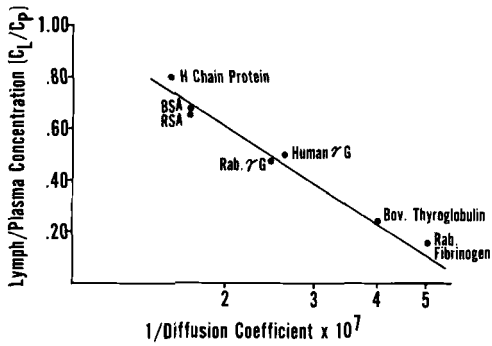

FIG. 1. Relationship between lymph to plasma concentration (C_L/C_P) and logarithm of 1/diffusion coefficient of various plasma proteins. The effective hydrodynamic diffusion radius of a particular protein is directly related to 1/diffusion coefficient. Rab—rabbit; Bov—bovine; BSA—bovine serum albumin; RSA—rabbit serum albumin; γG—γ-globulin. [Reprinted from *J. Immunol.* **100,** 380 (1970).]

tion in the plasma (Nakamura *et al.,* 1968). The extravascular space itself is heterogeneous in that some compartments preferentially accept small proteins. These events, along with the marked variation in the half-life of the rate of catabolism of various heterologous proteins in mice (Dietrich and Weigle, 1963) and rabbits (Nakamura *et al.,* 1968), strongly indicate that the concentration of serum proteins in the various compartments of the extravascular fluid space varies considerably with different proteins. This distribution has important immunological implications since immunological phenomena are probably dependent on the concentration of these antigens at the surface of antigen-reactive cells in the extravascular spaces. It may be that the concentration of antigen at these surfaces that is necessary to induce an unresponsive state is the same for all antigens. However, to provide a given concentration of antigen at crucial extravascular compartments containing these cells, the antigen concentration of the blood undoubtedly varies markedly with different serum protein antigens. It is not surprising that immunological unresponsiveness is so readily induced in animals to heterologous serum proteins since self-serum proteins are so well tolerated.

2. *Bacterial and Viral Antigens*

In contrast to the ease with which immunological unresponsiveness is induced to soluble serum proteins, it is difficult to induce a completely unresponsive state to many bacterial and viral antigens.[3] In most cases,

[3] The induction of unresponsiveness to bacterial and viral antigens has been extensively dealt with in *Immunological Tolerance to Microbial Antigens* (Friedman, 1971).

the antigens are particulate, complex, and of high antigenicity, especially when intact bacteria or viruses are used. These antigens are usually rapidly removed from the circulation, do not equilibrate effectively between intra- and extravascular spaces, and thus do not come into contact in effective concentrations with all of the antigen-reactive cells. Thus, only a hyporesponsive state is obtained often and its maintenance requires repeated injections of the antigens. However, there are certain exceptions among bacterial antigens. Immunological unresponsiveness can be induced in adult mice after injection of moderate amounts of purified pneumococcal polysaccharides, as was first observed by Felton (1949) and has been more actively pursued in the past few years by Howard and co-workers (Howard, 1972). Pneumococcal polysaccharides, which persist for many months *in vivo*, probably owe their tolerogenic properties to the lack of specific depolymerases for these polysaccharides in mice (Kaplan *et al.*, 1950; Stark, 1955). Injections of minute amounts (250 μg.) of the polysaccharide result in an apparent unresponsive state characterized by the absence of circulating antibody, but antibody-producing cells are present (J. G. Howard *et al.*, 1969). The apparent unresponsive state probably results from a "treadmill" neutralization of the antibody by the persisting antigen, an explanation offered previously by Dixon *et al.* (1955) to explain the mechanism of "paralysis" to pneumococcal polysaccharide. Higher doses, however, result in a central inhibition lacking the appearance of antibody-producing cells. Injection of extremely small amounts (0.5–5.0 μg.) does result in immunity with the appearance of only IgM antibody in the circulation. An unresponsive state has also been induced in mice by injection of a detoxified endotoxin of *Escherichia coli* into adult mice (Britton, 1969b). This endotoxin is a lipopolysaccharide that persists in tissues much as does pneumococcal polysaccharide.

A completely unresponsive state has been induced in neonatal rats to the monomeric subunit (flagellin) of the flagella of *Salmonella adelaide* (Nossal *et al.*, 1965). Appropriate acetoacetylation of the hydroxyl groups of flagellin markedly inhibits its ability to elicit production of antibody and drastically reduces its affinities for anti-flagellin; its injection results in an unresponsive state to a subsequent injection of flagellin in adult rats (Parish, 1971a). Although intact flagellin is a good antigen in adult rats, certain subfractions are not. Flagellin, which has a molecular weight of approximately 40,000, can be cleaved into four fragments with cyanogen bromide (Parish and Ada, 1969). The largest fragment (Fragment A) has a molecular weight of 18,000 and reduced antigenicity compared to whole flagellin. Repeated injections of Fragment A into adult rats resulted in a hyporesponsive

state to subject injection of flagellin. Flagellin, its chemical derivative, and Fragment A have been used extensively to study the mechanisms of immunological unresponsiveness and have obvious advantages (Nossal and Ada, 1971) over flagella or the intact bacteria. One disadvantage is that the unresponsiveness to flagellin is apparently not very stable, since it terminates after immunization with flagella incorporated into complete Freund's adjuvant (Lind, 1968).

From limited data, it appears that an unresponsive state is induced in neonatal rabbits injected with small amounts (2 mg.) of glucose-6-phosphate dehydrogenase from yeast. This enzyme is, however, a unique antigen in that it apparently equilibrates between intra- and extravascular spaces and persists in the body fluids in the same manner as do serum protein antigens.

The induction of immunological unresponsiveness to viral antigens has not been well studied. Injection of inactivated viruses during neonatal life does result in a temporarily hyporesponsive state, e.g., multiple injections of influenza virus beginning at birth depress the antibody response to a subsequent injection during adult life (Slotnick, 1971). Neonatal infections of mice with lymphocytic choriomeningitis virus (LCM) (Hotchin *et al.*, 1962) and chickens with avian leukosis virus (Rubin *et al.*, 1962) have been reported to result in an unresponsive state to these viruses, in that viremia persists during adult life without detectable antibody. In the case of LCM virus, if an unresponsive state does exist, it is only partial, since Oldstone and Dixon (1970) have detected antibody deposited as antibody–virus complexes along the glomerular basement membrane of the kidney in animals with viremia. A recent examination of the immunological state of these mice suggests that antibody production does occur, but it is depressed (Volkert and Lundstedt, 1971). The immunological state of animals infected with tumor viruses has been investigated to a limited extent. Although there is no evidence for an unresponsive state to deoxyribonucleic acid (DNA) tumor viruses (Defendi, 1971), antibody has not been detected to the group-specific antigen of ribonucleic acid (RNA) tumor viruses in a number of species so infected (see Huebner *et al.*, 1971), although antibody to other antigens of the viruses was present. In view of the recent findings with LCM virus, it is not unlikely that a responsive or hyporesponsive state exists but is masked by the antigen.

3. *Heterologous Erythrocytes*

Because of the advantages offered by heterologous red blood cells in their availability, high antigenicity and sensitivity of detection of both circulating antibody and antibody-producing cells, this antigen

is one of the most popular used to investigate the mechanisms of the immune response. For the same reasons, heterologous erythrocytes have been used to investigate unresponsiveness in neonatal animals (Hasek, 1954; Billingham *et al.*, 1953; Simonsen, 1956; Nossal, 1958; Calabresi and Edwards, 1958; Mitchison, 1962a) and in adults treated with immunosuppressive agents (Dietrich and Dukor, 1968; Gershon and Kondo, 1970; Miller and Mitchell, 1970). Unfortunately, heterologous red blood cells do not lend themselves readily to the induction of unresponsiveness. Like intact bacteria, they are complex, particulate antigens and neither persist in the circulation nor equilibrate into the extravascular spaces. As a result, it is difficult to induce a completely unresponsive state even with multiple injections, and unresponsiveness is rapidly lost when the injections are discontinued.

4. Haptens and Synthetic Polypeptides

In addition to the induction of unresponsiveness in guinea pigs by feeding or injection of hapten–protein conjugates into the mesenteric vein, unresponsiveness to contact sensitivity has been induced by intravenous injection of adult guinea pigs with large amounts of free hapten (Sulzberger, 1929; DeWeck *et al.*, 1964; Frey *et al.*, 1964a) before sensitization. It seems likely that the effective tolerogens in these studies were conjugates formed *in vivo* between circulating proteins and the haptens, since other workers have shown that injection of conjugates between 3,4-dinitrophenyl (DNP) sulfonic acid and isogeneic serum proteins into adult mice results in an unresponsive state specific for the DNP group (Havas, 1969; Golan and Borel, 1971). In these latter studies, circulating antibody was assayed, and the DNP–isogeneic protein conjugates were shown to be nonimmunogenic. Similarly, DNP–polylysine, containing 5–31 residues, was nonimmunogenic, but capable of inducing unresponsiveness in adult mice (Golan and Borel, 1971). In contrast, when delayed hypersensitivity was assayed, unresponsiveness was induced in neonatal guinea pigs with only hapten conjugates which were immunogenic. Jones and Leskowitz (1965) observed an unresponsive state which was specific for the hapten in guinea pigs injected at birth with polytyrosine–azobenzenearsonate (ABA-polytyr) conjugates in that immunization with either ABA–polytyr or a conjugate of polyhistidine–azobenzenearsonate (ABA-polyhist) failed to produce delayed hypersensitivity to azobenzenearsonate coupled to guinea pig serum albumin. An unresponsive state was also induced in some guinea pigs following neonatal injection of mono- and divalent haptens which were prepared by coupling azobenzenearsonate to tyrosine and dityrosine in which the amino groups were blocked by acetylation. Such compounds when incorporated into complete adjuvant and injected

into normal guinea pigs induced delayed hypersensitivity. Since no data were given concerning the production of circulating antibody, these small chemicals may have induced delayed hypersensitivity by their ability to stimulate thymus-derived cells but not precursor cells. It has been more recently shown by Collotti and Leskowitz (1970), with delayed hypersensitivity as the test system, that only those conjugates that were immunogenic in adult guinea pigs could induce unresponsiveness when injected during the neonatal period. These results contrast to those from adult mice where unresponsiveness for circulating antibody was induced by nonimmunogenic conjugates between hapten and isogeneic serum proteins. The difference observed between these two systems may be the result of differences in the ages of the hosts, species of animals, or assay systems used. Inhibition of antibody production by immunogenic, synthetic polypeptides in adult rabbits injected with synthetic polypeptides that are nonimmunogenic further emphasizes that an unresponsive state can be induced to nonimmunogenic substances (Maurer *et al.*, 1963; Schecter *et al.*, 1964; Roelants and Goodman, 1970).

Unresponsive states to haptens have also been induced by injecting neonatal rabbits with the haptens conjugated to heterologous serum proteins. Cinader and Pearce (1958) did so in a fraction of the rabbits injected during neonatal life with HSA conjugated with the diazonium derivative of sulfanilic acid. However, whether or not the unresponsive state was directed to the sulfanil groups per se was not reported. An unresponsive state was also induced to BSA conjugated to 2,4,6-trinitro-1-chlorobenzene (TNP–BSA) when injected into rabbits during neonatal life (Weigle, 1965a). In this situation there was, at least, a degree of unresponsiveness directed to the hapten. A hyporesponsive state has been induced to polyalanyl haptenic groups in rabbits injected at birth with poly-DL-alanyl ribonuclease (RNase) and poly-DL-alanyl-BSA (Bauminger *et al.*, 1967). In both of the above situations, circulating antibody was assayed. A hyporesponsive state has also been induced in adult mice to the 4-hydroxy-3,5-dinitrophenacetyl (NNP) hapten after either repeated injections of NNP–BSA conjugates with (Seppälä and Mäkelä, 1971) or without cyclophosphamide treatment (E. Möller *et al.*, 1971). It seems most likely that the specificity of the unresponsive state with heterologous carriers may be broader than that with isologous carriers and may include a portion of the protein.

D. Dose of Antigen

The dose of antigen injected is one of the major factors that determines both the degree of immunological unresponsiveness that is

established and its duration. Usually the larger the dose of antigen that is injected, the more complete is the unresponsive state and the longer the duration (Smith and Bridges, 1958; Eitzman and Smith, 1959); yet with serum protein antigens, multiple injections of small doses into neonatal rabbits are more effective than one single large injection (Weigle, 1972). A single injection of 100 mg. of BSA on the day of birth results in unresponsiveness in 95% of the rabbits when challenged 3 months later, whereas at this time all of the rabbits that had been injected twice a week for 10 weeks with 0.01 mg. were unresponsive. In the latter case, a total dose of only 0.2 mg. was given. Once the unresponsive state is established by neonatal injections of a large dose of antigen, it can be perpetuated by subsequent injections of small doses given in adult life (Smith and Bridges, 1958). As discussed previously, the minimal dose of antigen required to induce unresponsiveness can vary from one strain to another within a species (Golub and Weigle, 1969) and probably varies from species to species. Large doses of antigens have been used to induce an unresponsive state in adult animals. The classic example is where extremely small doses of pneumococcal polysaccharides result in an immune response and larger doses induce an unresponsive state (Felton, 1949; Howard, 1972). Exceptionally high doses of serum protein antigens given as multiple injections in rabbits (Dixon and Maurer, 1955; Johnson *et al.*, 1955) and in mice (Sercarz and Coons, 1963) produce an unresponsive state, and a hyporesponsive state has been produced in adult chickens after a single injection of 2.61 gm. of BSA (Mueller and Wolfe, 1961).

With certain antigen and species combinations, two doses have been reported, one high and one low, which produce an unresponsive or hyporesponsive state, with doses in between which result in immunity (cited in Dresser and Mitchison, 1968). This phenomenon was first reported by Mitchison (1964) in adult mice receiving injections of various doses of BSA 3 times a week and was termed "high-low zone tolerance." When similar studies were carried out with lysozyme, ovalbumin, diphtheria toxoid, and RNase, only a "high zone tolerance" was observed (Mitchison, 1968). A "high-ultra low zone tolerance" was observed in rats injected periodically with flagellin for 2 weeks starting at birth (Shellam and Nossal, 1968). A dose of 10^{-4} pg./gm. body weight produced the "low-zone tolerance." However, a similar high-low zone tolerance could not be induced in adult rats (Shellam, 1969a). Similarly, a high-low zone tolerance to HSA was not observed in adult rats when a similar protocol was used as that used in mice (Bell, 1971). Low zone tolerance has been reported for BSA in 50% of rabbits given periodic injections of 1 mg. of BSA, and the ability to paralyze the immune

response in adult rabbits with extremely large injections given daily has been mentioned above. In any event, the occurrence of high-low zone tolerance is an unusual phenomenon in the induction of immunological unresponsiveness. When it does occur, there are probably two requisites: first, the animal must be able to respond to an immunogenic form of the antigen; and, second, the antigen preparations must contain both an immunogenic (even if only in trace amounts) and a tolerogenic form of the antigen. The high-low zone phenomenon could then be explained by a competition between the two forms which would depend on the absolute amount of each form and not on a ratio of the two. The two zone phenomenon is not seen when either the animal is unable to respond or when the antigen preparation contains only tolerogen. Only one zone is observed in rabbits given biweekly injections of BSA or heterologous thyroglobulin starting at birth (Weigle, 1971) or in adult mice given a single injection of deaggregated HGG (Golub and Weigle, 1969).

E. Route of Injection

As discussed previously, there are situations in adults where certain routes of injection fail to elicit an immune response and as a result the animal becomes unresponsive to injections of the antigen by more appropriate routes. Although the route of injection has not been seriously investigated in neonatal animals, it is probably less important than among adults, since there is no need to bypass immunocompetence. One would not expect the route of injection to be important for unresponsiveness to heterologous serum proteins in either neonates or adults since after injection into any site these antigens equilibrate throughout the intra- and extravascular fluid spaces. With antigens that do not readily equilibrate and are rapidly eliminated, the route of choice probably would be intravenous.

III. Transfer of Cells

A. Transfer from Unresponsive Animals to Normal Animals

It is well established that, with certain limitations, cells from peripheral lymph node tissues and blood of unresponsive animals remain unresponsive when transferred to normal recipients. The response to pneumococcal polysaccharides in lethally irradiated mice was reconstituted with lymph node and spleen cells from normal mice, but not from mice made unresponsive to the polysaccharide, 9 days previously (Brooke and Karnovsky, 1961). Similar observations with unresponsiveness to pneumococcal polysaccharides were made by Neeper and Sea-

stone (1963a). White blood cells of guinea pig unresponsive to the hapten 2,4 dinitro-1-chlorobenzene were unable to transfer delayed hypersensitivity for this hapten, yet readily transferred tuberculin sensitivity (Battisto and Chase, 1963). The unresponsive state of lymph node and spleen cells taken from selected inbred rats injected periodically for 3 weeks with sheep red blood cells (SRBC) remained after these cells were transferred to lethally irradiated recipients. Similar results were obtained with mice made unresponsive as adults by injections of deaggregated HGG. Spleen cells taken from such animals were unable to reconstitute the response to aggregated HGG of irradiated recipients of the same inbred strain (Dietrich and Weigle, 1964; Golub and Weigle, 1967b; Chiller *et al.*, 1970). In all of the above studies, the cells were transferred shortly after the unresponsive state was induced. More recent studies with long-term unresponsiveness gave different, but interesting results (Chiller and Weigle, 1973). Strain A/J mice injected with 2.5 mg. of deaggregated HGG (centrifuged 100,000 *g* for 150 minutes) remained unresponsive to subsequent injections of aggregated HGG for 120 to 130 days. Spleen cells taken at 28 days failed to reconstitute irradiated recipients; however, spleen cells taken at day 80 or later did respond to injections of aggregated HGG in irradiated recipients, although the donors themselves did not. Cells taken on day 60 behaved as did cells taken from normal animals, whereas cells taken later were hyperresponsive. It may be that small amounts of antigen persisting in the mice maintained unresponsiveness, and the removal of these cells from the antigen released them from their unresponsive state. If this is the case, the persisting antigen must either be supertolerogenic or concentrated at a critical site, since the amount of antigen present at 120 to 130 days is not enough to induce unresponsiveness in normal animals. Ultimately, other factors in the unresponsive donor may regulate the unresponsive and the responsive states, as will be discussed later.

B. Transfer from Normal Animals to Unresponsive Animals

In contrast to the ease with which unresponsive cells remain so when transferred to normal animals, it usually has been difficult to transfer a responsive state to unresponsive animals. There have been several reports that the immune response can be transferred to unresponsive recipients with normal lymphocytes (Weigle and Dixon, 1957; Mitchison, 1962b; Denman *et al.*, 1967). However, one of these reported minimal antibody titers in allogeneic recipients (Weigle and Dixon, 1957), and in another (Denman *et al.*, 1967) no comparison was made with normal recipients. In any event, with a number of systems, it has been difficult to reconstitute unresponsive recipients with normal cells. In contrast

to the above studies, the unresponsive state to pneumococcal polysaccharide in mice could not be terminated with normal spleen cells (Neeper and Seastone, 1963b) nor could normal spleen cells transfer a responsive state to BGG to syngeneic mice unresponsive to deaggregated BGG (Tong and Boose, 1970). Similarly, McCullagh (1970a) was not able to reconstitute the immune response of rats made unresponsive by neonatal injections of SRBC by injecting normal syngeneic lymphocytes, and Miller and Mitchell (1970) experienced difficulty in getting syngeneic lymphocytes to respond in mice made unresponsive as adults with injection of SRBC and cyclophosphamide. Similar to the observations with BGG, syngeneic spleen cells failed to reconstitute mice made unresponsive to HGG, whether the unresponsive mice were or were not irradiated (Chiller and Weigle, 1972). The failure of the transfer of normal lymphocytes to reconstitute unresponsive animals may be the result of the induction of unresponsiveness in these transferred lymphocytes. Precursor cells can be found in the spleens of unresponsive rats for 3 days after injection of normal lymphocytes, but they then disappear (McCullagh, 1970b). It appears that either there is some central control of the unresponsive state or that persisting antigen is inducing unresponsiveness in the normal lymphocytes. Either case would be compatible with the observations that, in long-term unresponsiveness, spleen cells removed from unresponsive mice respond well to the antigen in syngeneic recipients (Chiller and Weigle, 1973). Persisting antigen could certainly be involved in the case of pneumococcal polysaccharides (Neeper and Seastone, 1963b), since this antigen is known to persist in the tissue for long periods of time (Kaplan *et al.*, 1950; Stark, 1955). Circulating antibody has been offered as a possible mechanism of central control. In support of this possibility was the observation of Tong and Boose (1970) that a factor is present in the serum of unresponsive mice which inhibits the immune response of normal murine lymphocytes. However, since in their experiments the normal cells were injected only 12 days after deaggregated BGG and since BGG has a half-life of approximately 5 days (Dietrich and Weigle, 1963), a sufficient level of antigen would be present in the body fluids of the recipient to induce unresponsiveness in the transferred cells. In further evidence that the factor is not antibody, antibody-producing cells cannot be detected for at least 22 days after the injection of deaggregated HGG into adult mice. If the controlling factor originates from one of the cell types involved in the immune response, this is not made evident by injecting a mixture of normal cells and unresponsive cells into an irradiated normal recipient (Chiller and Weigle, 1973). Unresponsive cells in such a mixture do not interfere with the responses of normal cells. Whatever

the mechanism that controls inhibition of the expression of competent cells in unresponsive animals, its identification will have to await further experimentation.

IV. Cellular Events

A. Induction of Antibody Production

In the past few years, it has become obvious that stimulation of the immune mechanism to produce antibody is not a simple process but involves a series of events and at least several different cell types (see Miller *et al.*, 1971). It is well accepted that responses to most antigens require synergism between thymus-derived and bone marrow-derived (bursa equivalent) cells (Claman *et al.*, 1966a; Miller and Mitchell, 1967). After these two cell types interact, it is the bone marrow (precursor) cell that differentiates into the antibody-producing cell (Mitchell and Miller, 1968; Nossal *et al.*, 1968; Jacobson *et al.*, 1970). It has been suggested that the synergism between the thymus- and bone marrow-derived cells is via two different determinants on the same antigen (Mitchison, 1969a; Rajewsky *et al.*, 1969; Katz *et al.*, 1970). In the immune response to the hapten portion of hapten–protein conjugates, the transfer of syngeneic cells sensitized with the carrier to normal animals endows these animals with the ability to make a secondary response to the hapten present on that carrier but not on a different carrier. That the hapten and protein carriers react with two cells was suggested by the ability to inhibit the secondary response with excess protein carrier (Mitchison, 1971a). In the above situations, it is assumed that the protein carrier reacts with thymus-derived cells and the hapten reacts with the precursor cells of the bone marrow-derived cells.

Synergism between thymus- and bone marrow-derived cells can also take place with two unrelated antigens, one reacting with thymus-derived cells and the other reacting with bone marrow-derived cells. The population of specific thymus-derived cells can be expanded ("educated") by injecting lethally irradiated mice with normal syngeneic thymus cells and SRBC. Spleen cells of the recipient can then be used as the expanded population of SRBC helper cells. When such educated thymus cells are mixed *in vitro* with a pure population of bone marrow cells plus SRBC and an unrelated antigen, there is an enhanced antibody response to the unrelated antigen (Hartmann, 1970). These results have recently been confirmed by Vann and Kettman (1972). Similarly, Rubin and Coons (1971) cultured mouse spleen cells and added small amounts of a heterologous antigen to which the donor mice had been primed, thereby increasing the *in vitro* response to SRBC. Nonspecific synergism

has also been reported *in vivo* during graft-versus-host (GVH) reactions. When thymus-derived cells are grafted, a large proportion of the cells are specific for the histocompatibility antigens of allogeneic cells (Wilson, 1969) and are capable of being stimulated by these antigens. During GVH reactions, an unrelated enhanced antibody response has been demonstrated for SRBC in rats (McCullagh, 1970c) and DNP–protein conjugates in guinea pigs (Katz *et al.*, 1971a). In the latter experiments, viable spleen and lymph node cells were transferred. The inability of irradiated cells to enhance the unrelated response supports the suggestion that the effect was the result of GVH. A similar nonspecific enhancement has been reported by Green *et al.* (1969) who demonstrated that otherwise nonresponding strain 13 guinea pigs respond to DNP–poly-L-lysine if a large amount of mycobacteria is incorporated into the adjuvant. In all of the above situations, nonspecific enhancement probably is the result of antigen-stimulated, thymus-derived cells nonspecifically stimulating adjacent bone marrow-derived cells at the same time that bone marrow-derived cells are reacting with an unrelated antigen. Stimulated thymus cells have been postulated to release a factor that, over a short range, can stimulate bone marrow cells that have reacted with an antigen (Rubin and Coons, 1971; Dutton *et al.*, 1971). Dutton *et al.* (1971) have recently isolated material from educated thymus cells which appears to have some stimulating effects on bone marrow-derived cells. Thus, although the synergism between thymus and bone marrow cells may involve the reaction of these cell types with different determinants on the same molecule, such an interaction is not mandatory.

In addition to the thymus and bone marrow-derived cells, macrophages have been implicated in the immune response. Although macrophages have been reported to transfer specific information to lymphocytes (Fishman, 1961; Adler *et al.*, 1966), their major role appears to be that of antigen handling. Unanue and Askonas (1968) have shown that macrophages of mice treated with antigen and washed extensively contain a trace amount of the antigen on their surfaces. Such macrophages, when injected into normal syngeneic mice initiate an antibody response, whereas injection of a similar amount of free antigen does not. The strongest support for the role of macrophages in the immune response is Mosier's (1967) observation that a response to SRBC would not proceed in the absence of macrophages *in vitro*. Cultures in which the cells that adhere to surfaces (macrophages) were removed did not respond to the antigen. However, if the culture was reconstituted with adherent cells, the ability to respond was restored. Additional support for a role for the macrophages in the induction of immunity is the

ability of macrophages to establish immunocompetence in newborn animals. Martin (1966) observed an immune response to BSA in newborn rabbits injected with adult macrophages, whereas Argyris (1968) established immunological competence in newborn mice with adult macrophages. It has been recently observed that the injection of serum proteins in physical forms which increase uptake by macrophages results in an immune response in neonatal rabbits. Antibody-producing cells appear in the spleens of rabbits on day 9 and peak on day 15 after subcutaneous injection on the first day of life with BSA incorporated into incomplete Freund's adjuvant (Chiller *et al.*, 1973). Rabbits also develop a normal complement of antibody-producing cells if injected with heat-aggregated HGG on the day of birth. Neonatal rabbits fail to respond to injections of aqueous and nonaggregated preparations of either BSA or HGG. A deficiency in the antigen-handling mechanism may be responsible for the immunoincompetence of newborn animals. An alternative explanation for the ability of the neonatal rabbits to produce antibody in these situations is that there is a deficiency in the thymus-derived cell which is compensated for by the particulate nature of the antigen. The latter suggestion is compatible with the failure of thymus cells of 1-day-old mice to collaborate with adult murine bone marrow cells in the reconstitution of the immune response of irradiated mice to SRBC (Claman *et al.*, 1966b).

B. Induction and Maintenance of Immunological Unresponsiveness

A complexity of cellular events similar to that involved in the immune response is also involved in the induction of immunological unresponsiveness. Again, several cell types can be affected in the induction, maintenance, and termination of the unresponsive state. In addition, the spontaneous termination and the induction in at least some cell types are time-dependent events which can be studied kinetically.

1. Cellular Interactions

Both the thymus- and bone marrow-derived cells have been implicated as cellular sites for unresponsiveness. That the thymus is involved in immunological unresponsiveness was first suggested by the prolongation of the unresponsive state in mice after thymectomy (Claman and Talmage, 1963; Taylor, 1964). More recently, bursectomy of chickens has been reported to prolong the unresponsive state to BSA (Peterson *et al.*, 1971). The first direct evidence that thymus-derived cells were affected during the induction of immunological unresponsiveness was published by Isakovic *et al.* (1965), who studied the unresponsive state to BGG

in adult rats. In these experiments, it was shown that the immune response to BGG in thymectomized-irradiated rats given normal bone marrow could be reconstituted with thymus cells from normal but not from unresponsive rats. Taylor (1968), by using similar reconstitution experiments with mice observed unresponsiveness to BSA in thymus cells, but not in bone marrow cells. However, his observations were made only at 24 hours after injection of the BSA. Similarly, Many and Schwartz (1970) reported the thymus but not the bone marrow cells to be unresponsive 48 hours after injection of SRBC and cyclophosphamide. However, Playfair (1969) reported unresponsiveness to SRBC in bone marrow cells but not in thymus cells of adult mice injected with SRBC and treated with cyclophosphamide. The responsiveness in the thymus cells in Playfair's work may have been caused by his use of NZB × BALB/c F_1 hybrid mice. The spontaneous autoimmune disease in the NZB mice is thought to be the result of abnormality in cellular hypersensitivity (thymus-dependent). In accord, observations of Talal *et al.* (1971) showed a hyporesponsive state in bone marrow cells of (NZB × NZW)F_1 hybrid mice made unresponsive by multiple injections of SRBC and treated with cyclophosphamide, yet the thymus cells were completely responsive. By using a different regimen to induce an unresponsive state to SRBC in mice treated with cyclophosphamide, Miller and Mitchell (1970) obtained unresponsiveness in the spleen and thoracic duct cells, but not in thymus or bone marrow cells. As suggested by the authors, their failure to induce unresponsiveness may have been lack of penetration into thymus or bone marrow by the antigen in an effective concentration. One would not expect a sufficient concentration of SRBC antigens from a single injection to equilibrate into the extravascular fluids of thymus and bone marrow.

Lasting and complete unresponsiveness was investigated in both thymus and bone marrow cells (Chiller *et al.*, 1970). Experiments were designed to test the ability of thymus and bone marrow cells from adult A/J mice injected with deaggregated HGG (tolerogen) to collaborate with normal cells in the immune response to aggregated HGG (immunogen). The mice were injected intraperitoneally with 2.5 mg. of tolerogen and on day 21 the bone marrow and the thymus were removed. Single cell suspensions were prepared, and either thymus (100×10^6) or bone marrow cells (30×10^6) were injected into lethally irradiated syngeneic recipients along with normal bone marrow or thymus cells, respectively. The mice were injected with aggregated preparations of both HGG and turkey γ-globulin (TGG); at appropriate times, they were sacrificed, and their spleens were analyzed for plaque-forming cells (PFC) to both HGG and TGG by a modification (Golub

TABLE I

SPECIFICITY OF UNRESPONSIVENESS IN THYMUS AND BONE MARROW CELLS OBTAINED FROM MICE INJECTED WITH DEAGGREGATED HUMAN γ-GLOBULIN

Cell combinations[a]	Reconstitution of irradiated recipients[b]	
	HGG	TGG
nT + nBM	+	+
tT + nBM	−	+
nT + tBM	−	+
tT + tBM	−	+

[a] n—Cells from normal donors; t—cells from tolerogen-treated donors; T—thymus cells; BM—bone marrow cells.

[b] HGG—human γ-globulin; TGG—turkey γ-globulin.

et al., 1968) of the hemolytic plaque assay (Jerne and Nordin, 1963). All combinations of cells responded immunologically to TGG, whereas the only combination that responded to HGG was that of normal thymus and normal bone marrow cells (Table I). If either the thymus or bone marrow cells came from an unresponsive donor, the recipient was unresponsive. It has been shown that the failure of thymus and bone marrow cells from unresponsive mice to collaborate in the immune response is not the result of the exodus of competent cells from these organs (Weigle *et al.*, 1971a). The above results demonstrate that an unresponsive state can be induced in both cell types and is compatible with the presence of antigen receptors on both thymus and bone marrow cells. Furthermore, they suggest that only the thymus- or bone marrow-derived cells, but not both, need to be unresponsive for an unresponsive state to exist in the intact animal.

2. *Cellular Kinetics*

The *in vivo* induction of unresponsiveness does not occur immediately after the injection of a tolerogen, but develops gradually. Spleen cells taken from mice at various times after injection of the tolerogen were transferred to irradiated mice. In order to determine the immune state of the transferred cells, the recipients were challenged with the immunogen. By using this experimental design, induction of a hyporesponsive state to BSA in adult mice required 24 hours (Mitchison, 1968), and a significant hyporesponsiveness in mice was present 4 hours after injection with deaggregated BGG (Das and Leskowitz, 1970). Four days were required *in vivo* before a complete unresponsive state to deaggregated HGG was present in mice (Golub and Weigle, 1967b), a

period similar to that observed for the induction of unresponsiveness to pneumococcal polysaccharides in mice (Matangkasombut and Seastone, 1968). By using a quantitative assay and a similar reconstitution experiment in A/J mice, it was observed that although a 4-day period was required for the induction of unresponsiveness in all cells *in vivo*, 75% of the cells were unresponsive 6 hours after injection of the deaggregated HGG (Fig. 2) (Chiller and Weigle, 1971). Only several hours elapsed before there was an *in vitro* induction of unresponsiveness to bacterial antigens (Britton, 1969c; Diener and Armstrong, 1969).

The kinetics of induction and spontaneous termination of unresponsiveness in thymus and bone marrow cells are quite different. Since only one of the two cell types has to be unresponsive in order that the intact animal is unresponsive, the kinetics of the induction and spontaneous termination of the unresponsive state can be investigated separately in both cell types (Chiller *et al.*, 1971). Strain A/J mice were injected with 2.5 mg. of deaggregated HGG, at various times their bone marrow and thymuses were removed, and single cell preparations were injected into lethally irradiated recipients (syngeneic) along with normal thymus and bone marrow cells, respectively. The recipients were injected with aggregated HGG and their spleens later assayed for PFC to HGG. At each time interval, the percentage of unresponsiveness obtained in the recipients receiving one cell type from normal donors and the other cell type from donors injected with deaggregated HGG compared to the response obtained in animals receiving both normal cell types was calculated and plotted as shown in Fig. 3. An unresponsive state was rapidly induced in the thymus cells, being virtually complete by the second day

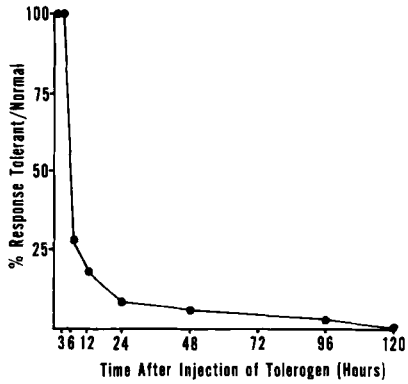

FIG. 2. Cellular kinetics of induction of unresponsiveness to deaggregated human γ-globulin (tolerogen).

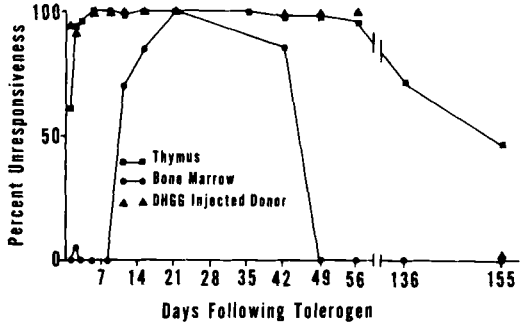

FIG. 3. Kinetics of the induction of immunological unresponsiveness to human γ-globulin (HGG) in thymus and bone marrow cells of adult A/J mice. DHGG—deaggregated HGG.

and totally complete by the fifth day. Unresponsiveness remained complete for 120 to 130 days, and at 155 days responsiveness was only 50% of that of normal thymus cells. This kinetic pattern for induction and termination of unresponsiveness in thymus cells is reflected in the kinetic pattern observed in the intact animal. In comparison, a longer latent period characterizes the induction of unresponsiveness in bone marrow cells. Unresponsiveness is not observed until after day 8 and is not complete until day 21. Furthermore, it is rapidly lost in bone marrow cells, and they spontaneously return to a normally responsive state by day 49. Thus, unresponsiveness in the thymus cells and the intact animal is of relatively long duration and is rapidly induced, whereas the unresponsive state in bone marrow cells is of relatively short duration and is slowly induced. There are several possible explanations for the delay in the latter.

1. Although unlikely, the difference in the time of onset of the induction of unresponsiveness between thymus and bone marrow cells may be quantitative, in that the thymus cells require less antigen; however, a tenfold increase in the dose of deaggregated HGG did not change the kinetic pattern of the induction in bone marrow cells (Table II) (Chiller and Weigle, 1972).

2. Induction of unresponsiveness in the bone marrow cells may be under thymic control and depend on either direct interaction between bone marrow cells and a small number of responsive thymus cells migrating to the bone marrow or on a humoral factor secreted from thymus-derived cells or the intact thymus gland. Gershon and Kondo (1970) reported that induction of unresponsiveness in bone marrow cells was thymus cell-dependent. They were able to induce unresponsiveness in bone marrow cells injected into thymectomized and irradiated

TABLE II

EFFECT OF DEAGGREGATED HUMAN γ-GLOBULIN (TOLEROGEN) ON THE INDUCTION OF UNRESPONSIVENESS IN THYMUS AND BONE MARROW CELLS

Day after administration 25 mg. of tolerogen	No. of mice	% Unresponsiveness (day 11)[a]		
		T	BM	Animal
3	16	100	11	>99
8	16	100	0	>99

[a] T—thymus cells; BM—bone marrow cells; animal—donor of tolerant T and BM cells.

mice by also injecting these mice with large doses of SRBC over a period of 30 days, but only if thymus cells (15×10^6) were also injected. There is also strong evidence of thymus control in thymus-independent immune responses. Although with thymus-independent antigens, thymus-derived cells are not needed for bone marrow cells to differentiate into antibody-producing cells, thymus or thymus-derived cells appear to play a role in the regulation of antibody production by the bone marrow-derived cells. Treatment of mice with antilymphocyte serum (ALS), which preferentially depletes the recirculating pool of long-lived thymus-derived cells, results in a tenfold increase in the response to pneumococcal polysaccharides (Baker *et al.*, 1970a). The immune response to pneumococcal polysaccharide is thymus-independent. Similar enhancement of the thymus-independent immune response to polyvinylpyrolidone (PVP) also occurs after treatment of mice with ALS (Kerbel and Eidinger, 1971). Furthermore, there is evidence that two types of thymus-derived cells act in opposing ways to regulate the antibody response. The ability of ALS to increase the magnitude of the response to pneumococcal polysaccharide is thought to be the result of inactivation of the cell type that normally suppresses that response (Baker *et al.*, 1970b). The loss of long-term unresponsiveness to HGG in mouse spleens which have been removed and subsequently transferred to irradiated hosts may be the result of disruption of thymic control (Chiller and Weigle, 1973). If the thymus-derived cell or the thymus itself is playing a role in the induction of unresponsiveness in bone marrow cells, unresponsive thymus cells are effective, since it has been possible to reinduce unresponsiveness in bone marrow cells after it is spontaneously lost (60 days after initial injection of deaggregated HGG) at a time when the thymus cells are still unresponsive (Chiller and Weigle, 1973). In fact, the kinetic pattern of primary induction and reinduction appears to be the same.

3. The unresponsive state may occur in thymus cells immediately or shortly after interaction with antigen as a direct result of covering of receptors or killing of the cell, whereas the induction of unresponsiveness in bone marrow cells may be an active process. The active process may require antigen-directed maturation of bone marrow, since it has recently been reported (Weigle *et al.*, 1971a) that bone marrow-derived cells in the spleen require less of a latent period than those cells remaining in the bone marrow. In these experiments, A/J mice were injected with deaggregated HGG, and 3 days later spleen cells were used as a source of bone marrow cells. Injection of these cells along with normal thymus cells into irradiated syngeneic recipients failed to reconstitute immunocompetence to a subsequent injection of aggregated HGG. It appears that both bone marrow-derived cells in the peripheral lymphoid tissue and bone marrow cells (in the bone marrow) have predetermined specificity but that the specificity is more developed in the former and that the time required for the induction of unresponsiveness in bone marrow cells is the result of an antigen-directed maturation of these cells. Whatever the events, they do not involve antibody, since antibody-producing cells could not be detected in mice during the induction of the unresponsive state (Fig. 4) (Habicht *et al.*, 1970; Chiller and Weigle, 1971).

It is also difficult to explain the kinetic difference between the thymus and bone marrow cells in the termination of unresponsiveness. In both bone marrow cells and bone marrow-derived cells in the spleen, this termination is rapid (Chiller and Weigle, 1973). In all probability, termination of unresponsiveness in both thymus- and bone marrow-

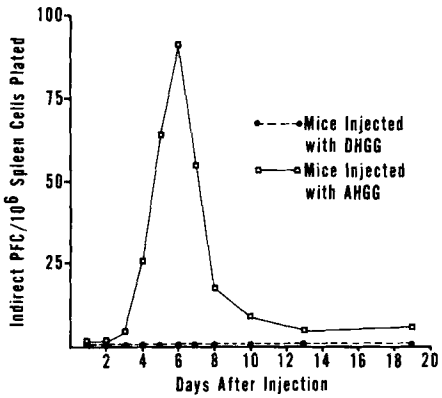

FIG. 4. Cellular response [plaque-forming cells (PFC)] of mice injected with either tolerogen, i.e., deaggregated human γ-globulin (DHGG) or immunogen, i.e., aggregated human γ-globulin (AHGG). Each point represents average responses of 10 individual mice. [Reprinted from *J. Immunol.* **106**, 1651 (1971).]

derived cells is related to the decrease in the deaggregated HGG *in situ* by normal catabolism. Differences in the nature of receptors on bone marrow and thymus cells may influence maintenance of unresponsiveness at low concentrations of tolerogen. Rapid termination of unresponsiveness in bone marrow cells is compatible with observations that turnover of antigen receptors on bone marrow-derived cells is surprisingly more rapid than turnover of receptor sites on thymus cells (Wilson *et al.*, 1972). Still, it is difficult to explain the long duration of unresponsiveness in the thymus gland in view of the high turnover rate of cells in the thymus, unless only extremely small concentrations of tolerogen are needed to maintain the unresponsive state or that tolerogen is concentrated in critical areas of this organ.

That bone marrow cells have preformed specificity has recently been confirmed by Unanue (1972), who demonstrates specific killing of bone marrow cells incubated with antigen heavily labeled with ^{131}I. Similar irradiation suicide under less favorable conditions was observed by Basten *et al.* (1971) with bone marrow-derived cells of the spleen but not with cells from bone marrow. The difficulty in obtaining such irradiation suicide of bone marrow cells with ^{125}I-labeled antigen strengthens the view that the predetermined specificity in the bone marrow is not fully developed.

Since thymus-derived cells are responsible for the development of cellular or delayed hypersensitivity, it is not surprising that they have been shown to be a site of unresponsiveness for delayed hypersensitivity in the rat (Isakovic *et al.*, 1965). Although bone marrow cells are required for expression of delayed hypersensitivity (Lubaroff and Waksman, 1968), they act nonspecifically, since in reconstitution experiments with normal thymus cells, bone marrow cells are effective when their source is an unresponsive donor (Tubergen and Feldman, 1971).

The importance of the dose of antigen on the induction of unresponsiveness in intact animals has been extensively discussed above (Section II,D). Of additional interest is the effect of antigen doses on the induction of unresponsiveness in thymus and bone marrow cells. In this respect, it has been shown that the unresponsive state can be induced in thymus cells with much less antigen than in bone marrow cells (Chiller *et al.*, 1971). In these experiments, adult A/J mice were injected with various amounts of deaggregated HGG; 11 days later preparations of their thymus and bone marrow cells along with normal bone marrow and thymus cells, respectively, were injected into lethally irradiated recipients which were subsequently injected with aggregated HGG, and their spleens were analyzed for PFC. In these experiments (Table III), considerably larger doses of deaggregated HGG were required to induce

TABLE III
EFFECT OF DOSE OF TOLEROGEN (DEAGGREGATED HUMAN γ-GLOBULIN) ON INDUCTION OF UNRESPONSIVENESS IN THYMUS AND BONE MARROW CELLS OF ADULT A/J MICE

Dose of tolerogen injected (mg)	% Unresponsiveness (day 11)	
	Thymus	Bone marrow
2.5	99	70
0.5	99	56
0.1	94	9
0.01	54	0
10^{-11}	0	0
10^{-13}	0	0

an unresponsive state in bone marrow cells than in thymus cells. Apparently, when unresponsiveness is induced with low doses of antigen, thymus-derived cells and not bone marrow cells are affected. In agreement with the latter, Mitchison (1971b) reconstituted the unresponsive state in mice made unresponsive with a low dose of BSA, but not with high doses of BSA, by injecting previously stimulated (educated) thymus cells. It should be pointed out that with the deaggregated HGG system, although it takes only small amounts of tolerogen to induce an unresponsive state in thymus cells, a high-low dose phenomenon was not observed (Table III). Induction of unresponsiveness in mice to the 4-hydroxy-3,5-dinitrophenacetyl hapten affects the thymus-derived cell more efficiently than the bone marrow-derived cell (E. Möller *et al.*, 1971). These observations probably result from the difference in the dose required for induction of unresponsiveness in the two cell types.

Although macrophages apparently play an important role in the immune response, this role is nonspecific, since the macrophages are just as effective if they come from an unresponsive animal as from a normal animal (Mitchison, 1969b). Very little is known concerning the possible role of macrophages in the induction and maintenance of immunological unresponsiveness. Circumvention of the induction of unresponsiveness in neonatal rabbits by either injection of antigen in a form more readily taken up by macrophages (Chiller *et al.*, 1973) or by injection of adult macrophages (Martin, 1966; Argyris, 1968) suggests that the manner in which antigen reacts with macrophages may play an important role. As shown previously with other antigens (Unanue and Askonas, 1968), macrophages treated with soluble aggregated HGG fix antigen to their surfaces and, when injected into normal mice, initiate an immune response (Hyde and Weigle, 1969). With identical concentrations of deaggregated

HGG (monomeric), only one-twentieth as much HGG fixes to macrophage surfaces, and the injection of such macrophages does not initiate an immune response. Whether these observations have anything to do with the immunogenic nature of aggregated HGG and the tolerogenic properties of monomeric HGG is not known.

V. *In Vitro* Induction of Unresponsiveness

Specific immunological unresponsiveness has been induced *in vitro* with certain antigens and under selective conditions. Usually the effective antigens are thymus-independent antigens, and the immune response is limited to IgM production. The rate of induction is rapid, and a significant level of unresponsiveness can be detected in several hours. An unresponsive state has been induced in cell cultures to the lipopolysaccharide antigen of *Escherichia coli* (Britton, 1969c), high doses of polymerized flagellin (POL) (Diener and Armstrong, 1969), and highly substituted conjugates of DNP–POL (Feldmann, 1971). In all three of these studies, the antigens which were added to mouse spleen cells were polymers of repeating antigenic determinants. This suggests that the induction of unresponsiveness in an antigen-sensitive cell *in vitro* involves the simultaneous reaction of many antigenic determinants with the surface of the cell (Feldmann, 1971). A similar situation can be created by adding low concentrations of specific antibody to a culture with antigens that do not induce unresponsiveness *in vitro*. A small amount of antibody added to an otherwise immunogenic dose of POL induces a hyporesponsive state in mouse spleen cells *in vitro* (Diener and Feldmann, 1970). Fragment A of flagellin fails to induce immunological unresponsiveness *in vitro*. However if a small amount of antibody is added, a hyporesponsive state results. The $F(ab)_2$ but not the Fab fragment of antibody is effective in inducing this state (Feldmann and Diener, 1972). Apparently, antibody links the molecules together to form a multideterminant aggregate. Antigens that lend themselves to the induction of unresponsiveness *in vitro* are polymers of repeating units in contrast to those which are required for induction of unresponsiveness *in vivo*. Human γ-globulin is an example of the latter case; monomeric preparations of HGG readily induce an unresponsive state, whereas injection of aggregates of multiunits of HGG results in an immune response. It seems likely that mechanisms of *in vivo* induction of unresponsiveness to polymers and to monomeric proteins must differ.

In contrast to the IgM unresponsiveness produced by polymeric thymus-independent antigens, the unresponsiveness to serum protein antigens is difficult to induce *in vitro*. Mitchison (1968) had difficulty

in inducing unresponsiveness to BGG in lymphocytes of mice *in vitro*. Scott and Waksman (1969) were unable to induce an unresponsive state *in vitro* to BGG in dissociated rat spleen cells, but could do so when intact spleens were injected *in vitro* and, 2 hours later, these cells were transferred to syngeneic recipients. Byers and Sercarz (1970) were able to induce immunological "paralysis" to BSA *in vitro* by adding relatively large amounts of BSA to primed lymph node fragments. However, the unresponsive state was reversed, and an immune response occurred upon removal of the BSA. The difficulty in inducing an unresponsive state *in vitro* to serum protein antigens may be the failure to use polymeric forms, the small amount of IgM antibody produced after injections of aqueous serum protein antigens, or interference in the induction by the thymus-derived cells. If a system that mimics the induction of the unresponsive state *in vivo* can be developed *in vitro*, an important tool would be available to study the kinetics of cellular interaction, the changes occurring on membranes, and the subcellular events involved in the induction of immunological unresponsiveness. Such studies would especially be approachable if sophisticated techniques can be devised for isolation of purified populations of thymus and bone marrow cells of a single specificity.

VI. Role of Antigen Receptors

A. Induction of Immunity

Numerous reports in the past 2–5 years have demonstrated the presence of receptor sites on lymphocytes. Most of the evidence has been indirect in that immunoglobulin-like substances on lymphocytes have been detected, or addition of antigen to the cells has caused a specific change in their function. The presence of receptor sites on lymphocytes was implied when the ability of these cells to reconstitute irradiated recipients was inhibited by affinity labeling the cells with the specific antigen (Plotz, 1969; Segal *et al.*, 1969) and when potential antibody-producing cells were removed by passage through column of antigen-coated beads (cited by Wigzell, 1971). Sell and Gell (1965) were the first of a number of workers to demonstrate immunoglobulins on lymphocytes. In their studies, the addition of anti-immunoglobulin to lymphocytes resulted in transformation of the cells. Others were able to localize fluorescent-labeled antibody to immunoglobulins on the surface of bone marrow-derived cells (Pernis *et al.*, 1970; Rabellino *et al.*, 1971; Greaves, 1970; Raff, 1970). It has been more difficult to localize labeled antibody to immunoglobulin on thymus or thymus-derived cells. By using ^{125}I-

labeled rabbit antibody, Jones *et al.* (1971) were able to detect L chains on only 4% of thymus cells. Other workers (Pernis *et al.*, 1970; Unanue *et al.*, 1971; Raff *et al.*, 1970) have been able to localize labeled anti-immunoglobulin on neither thymus nor thymus-derived cells. In addition, thymus cells could not be transformed to blast cells by either anti-allotype sera (Sell *et al.*, 1970) or anti-immunoglobulin sera (Sell, 1967). Failure of blast transformation after treatment with anti-immunoglobulin sera has also been observed with chicken thymocytes (Ivanyi *et al.*, 1969). However, by using radioautographic techniques (with prolonged exposures of 30 to 60 days), Bankhurst *et al.* (1971) were able to demonstrate the localization of ^{125}I-labeled immunoglobulin on lymphoid cells from the thymus and thoracic duct lymph from mice and in lymphoid cells from the thoracic duct lymph of irradiated mice reconstituted with allogeneic cells. Approximately 14 and 37% of thymus and thoracic duct cells, respectively, were labeled with ^{125}I antibody to light-chain protein; no labeling was observed with ^{125}I antibody to μ, α, or γ_2 chains. Additional evidence for the presence of receptors on thymus-derived cells is the ability to inactivate the function of these cells by treatment with anti-light-chain sera (Greaves *et al.*, 1969; Greaves and Hogg, 1971; Mason and Warner, 1970; Warner *et al.*, 1970). Suicide experiments showing specific irradiation killing of immunocompetent lymphocytes incubated with antigen heavily labeled with ^{125}I (Ada and Byrt, 1969; Humphrey and Keller, 1970) are also evidence for the presence of antigen receptors on lymphocytes. Specific killing with heavily labeled antigens has been shown for thymus-derived cells (Basten *et al.*, 1971; Roelants and Askonas, 1971), bone marrow-derived cells (Basten *et al.*, 1971), and bone marrow cells (Unanue, 1972). The induction of immunological unresponsivenes in thymus, bone marrow, and bone marrow-derived cells (Weigle *et al.*, 1971a) is further indirect evidence that these cells contain specific receptors for antigen.

More direct evidence for the presence of receptors for antigen on the surface of lymphocytes are the experiments with radioactive-labeled antigens and radioautographic techniques (Naor and Sulitzeanu, 1967; Ada *et al.*, 1970; Humphrey and Keller, 1970). Identification of these receptors with immunoglobulins was suggested by the inhibition of ^{125}I antigen uptake by antibody to L chains. Although the uptake of antigen on the surface of bone marrow or bone marrow-derived cells was clearly demonstrated, no significant uptake could be shown for thymus cells (Ada *et al.*, 1970; Humphrey and Keller, 1970), possibly because of the paucity of receptors on these cells. By using an extremely sensitive system, Modabber *et al.* (1970) were able to show antigen binding to thymus cells of normal mice.

The available data strongly indicate that the receptor on precursor cells of bone marrow-derived cells is an immunoglobulin. Still, the evidence is inconclusive as to whether the effective receptor site on the thymus-derived cell is an intact immunoglobulin, the Fab piece, or the variable portions of the heavy and light chains. Although the receptor sites on bone marrow-derived cells are apparently immunoglobulins, there is some question as to what class or classes are present. Vitetta *et al.* (1971) were able to isolate ^{125}I-labeled protein which was a 7 S molecule from the surfaces of BALB/c mouse lymphocytes labeled enzymatically with ^{125}I, and it specifically reacted with antisera prepared against the μ heavy chain. Thus, they postulated that the receptor on these cells was a 7 S monomer of IgM. However, Davie and Paul (1971) reported that the great majority of guinea pig lymphocytes that bind specific antigens contain IgG receptor sites. By inhibiting rosette formation to SRBC with antibody specific to IgG and IgM, Greaves (1971) obtained data suggesting that during the 4–5 day period after primary injection (at a time when the lymphocytes were either not secreting or minimally secreting antibody), both IgM and IgG receptors were present.

Whatever the immunoglobulin class, or portion thereof, that acts as receptor, it is the events that occur after their reaction with antigen that lead to antibody production. With fluorescent-labeled anti-immunoglobulin, Taylor *et al.* (1971) demonstrated that if lymphocytes of mice were suspended in the Fab fragments (monovalent) of the fluorescent antibody, the receptors were uniformly dispersed over the surface; but, if the cells were suspended in preparations containing intact fluorescent anti-immunoglobulin (bivalent), patchy aggregates appeared on the surface and rapidly formed polar caps which were pinocytized by the lymphocytes. These observations are compatible with those of Vitetta *et al.* (1971) who reported that the immunoglobulin on the surface of lymphocytes is free rather than trapped in the matrix of the membrane. It is tempting to relate the events that follow the interaction of the receptors with intact anti-immunoglobulins to possible events that may occur after the interaction of multivalent antigens with specific immunoglobulin receptors. In preliminary results, it was shown that similar patchy aggregates and cap formation do occur with multivalent antigens but not with monovalent antigens (Taylor *et al.*, 1971). The role of the thymus cell in the events that occur on the precursor cells is not known; nor is it known whether similar events occur on thymus-derived cells but to a lesser degree. It may be that despite their complexity, events that occur on the precursor cells cannot be expressed without either a direct or indirect interaction with sensitized thymus cells in thymus-dependent responses.

B. Induction of Immunological Unresponsiveness

In all probability, the manner in which antigen reacts with the receptor on the lymphocytes designates whether an immune or unresponsive state will result. If antibody production is initiated by the reaction between thymus and bone marrow-derived cells with antigen and subsequent events in the bone marrow-derived cell, the presentation of the antigen in an unfavorable form may interrupt these events. In the latter case, an unresponsive rather than a responsive state could result. This possibility may suggest why the injection of monomer HGG leads to immunological unresponsiveness in adult mice, whereas injection of aggregated HGG results in antibody production. Revelation of the exact cellular phenomena that take place during the induction of unresponsiveness will have to await the isolation of purified lymphocytes with a given specificity and emergence of sophisticated tissue culture systems.

One of the major issues that can be studied is the fate of the receptor sites during the induction of immunological unresponsiveness. There is not complete agreement on whether lymphocytes containing specific receptor sites for the tolerated antigen are or are not present in unresponsive animals. Ada *et al.* (1970) found a normal complement of antigen-binding cells in the spleens of rats made unresponsive to flagellin by injecting the cyanogen bromide fragment (tolerogen) of flagellin. They also observed approximately the same number of antigen-binding cells in the spleens of either normal rats or rats made unresponsive by periodic injections of hemocyanin starting at birth. In contrast to the results of Ada *et al.* (1970), Naor and Sulitzeanu (1969) observed only a few antigen-binding cells in the lymph nodes of mice made unresponsive in comparison to the numbers found in the lymph nodes of normal mice. Humphrey and Keller (1970), studying unresponsiveness in mice to both the synthetic polypeptide [^{125}I](T,G)-A- -L(TIGAL) and hemocyanin found approximately 80% as many antigen-binding cells in the spleen and lymph nodes of treated mice as in normal mice. Similarly to Naor and Sulitzeanu, Katz *et al.* (1971b) also found significantly more antigen-binding cells in the lymph nodes and spleens of normal guinea pigs than in the lymph nodes and spleens of guinea pigs made unresponsive to DNP coupled to a copolymer of glutamic acid and lysine. Sjöberg (1971) and G. Möller *et al.* (1971) were able to detect rosette-forming cells (antigen-binding cells) in mice made unresponsive to the lipopolysaccharide antigen of *Escherichia coli*. With the exception of BSA, the antigens employed in the above studies do not readily lend themselves to the induction of immunological unresponsiveness, although they are effective in detecting antigen-binding cells. In less than ideal

conditions, unresponsiveness may only be present in the thymus-derived cells. In this situation, the intact animal would be unresponsive, but would contain competent bone marrow-derived cells capable of binding the antigen. In the case of unresponsiveness induced in mice to HGG, there were no significant antigen-binding cells detected in the spleen at a time (days 5–20) when it was shown that both thymus- and bone marrow-derived cells were unresponsive [Louis *et al.* (1972)]. When the bone marrow cells began to recover from the unresponsive state (day 42), antigen-binding cells were detected in both the spleen and bone marrow although the thymus and intact animal were still unresponsive. Thus, antigen-binding cells may exist in unresponsive animals; however, unresponsive thymus cells are the only requirement in order that the intact animal remain unresponsive. A normal or almost normal complement of antigen-binding cells is found in unresponsive animals probably because antigen-binding cells are detectable only in bone marrow-derived cells by the procedures used. It is safe to assume that detectable receptor sites for an antigen are absent in cell populations that are unresponsive to that antigen. Whether these cells have been eliminated or stripped of their receptors or the receptors have been covered by antigen cannot be answered at the present time.

VII. Role of Antibody

Although some theories of antibody production implicate circulating antibody in the induction of unresponsiveness (Eisen and Karush, 1964; Bretscher and Cohn, 1970) and, in certain cases, a transient production of antibody precedes the induction of immunological unresponsiveness, it is unlikely that antibody production is a requirement for the induction of a complete and stable unresponsive state *in vivo*. Transient production of antibody followed by an unresponsive state was observed in newborn rabbits injected with large amounts of *Salmonella paratyphi B* (Sterzl and Trnka, 1957) and during the induction of unresponsiveness in newborn rabbits and piglets injected with large doses of SRBC (Sterzl, 1966). These observations were interpreted as being the result of a terminal exhaustive differentiation, where in the presence of a large excess of antigen, almost all immunocompetent cells and their activated descendants differentiated into short-lived antibody-producing cells. Rowley and Fitch (1965) also observed transient production of antibody-producing cells (PFC) in rats during the induction of an unresponsive state with periodic injections of SRBC starting at birth. They suggested that subsequent induction of unresponsiveness was the result of "antibody feedback." However, it is more likely that the appearance and disappearance of these cells have little to do with unresponsiveness

but result from exhaustive differentiation of immunocompetent cells. A similar situation occurs in adult rabbits receiving repeated injections of BSA. Rabbits injected daily with 1 gm. BSA for approximately 2 months develop unresponsiveness to BSA (Dixon and Maurer, 1955), and their spleens contain PFC to BSA (Chiller *et al.*, 1973). These cells appear between days 9 and 11 and disappear by day 21. Although the injections were given for a total of 36 days, reappearance of PFC did not occur during this period of time nor during a 47-day period after the injections were terminated. One of the features in the induction of unresponsiveness to pneumococcal polysaccharides in mice is the transient production of antibody preceding establishment of an unresponsive state (Siskind and Howard, 1966; Matangkasombut and Seastone, 1968; J. G. Howard *et al.*, 1969; Kerman *et al.*, 1970). Transient production of antibody in these animals and the subsequent induction of unresponsiveness may involve an exhaustive differentiation of primed cells resulting from natural injection with related bacteria. A similar exhaustive differentiation and loss of a responsive state was observed in rabbits immunized to pneumococcal polysaccharide by injecting the intact bacteria (Paul *et al.*, 1969a). Mice so immunized responded to a single injection of the polysaccharide given alone but failed to respond to subsequent injections.

An active role is played by antibody in certain cases where unresponsiveness is induced *in vitro*. Immunological unresponsiveness to flagellin can be induced *in vitro* by the exposure of mouse spleen cells to an immunogenic dose of polymerized flagellin in the presence of low concentrations of specific antibody (Diener and Feldmann, 1970). Similarly, Fragment A of flagellin by itself fails to induce unresponsiveness to flagellin *in vitro*, yet does so if a small amount of antibody is added. This situation is probably not one of antibody feedback but rather of presenting antigen in a favorable form to the precursor cells.

Contrary to the above observations, antibody-producing cells cannot be detected during the induction of unresponsiveness in adult mice to deaggregated HGG (Chiller and Weigle, 1971) or to BSA in neonatal rabbits (Chiller *et al.*, 1973). In the latter studies, rabbits were injected subcutaneously with 150 and 250 mg. of BSA on the first and third days of life, respectively. Neither direct nor indirect PFC to BSA could be detected in their spleens during the 22 days following the first injection. However, as mentioned previously, rabbits did develop a large number of specific PFC after injection with BSA incorporated in incomplete Freund's adjuvant on the day of birth. Neither direct nor indirect PFC were detected in the spleens of adult mice during a 19-day period after injection of deaggregated HGG (Fig. 4), and these mice developed an unresponsive state of long duration. Although the above data dealing

with soluble serum proteins do not rule out a possible role for antibody in the induction of unresponsiveness, they do not support the mandatory requirement of the formation of antibody by either exhaustive differentiation or effective presentation of the antigen to competent cells. In the cases where antibody production precedes induction of unresponsiveness, induction may result despite antibody production and not because of it. It has been suggested that transient production of antibody observed with the thymus-independent antigens (pneumococcal polysaccharides) compared to lack of production related to serum protein antigens may arise from rapid induction of unresponsiveness in thymus-derived cells in the latter case which prevents activation of bone marrow-derived cells to antibody-producing cells (Howard, 1972).

VIII. Split Unresponsive States

Split unresponsiveness refers to situations where immunological unresponsiveness is established for one of the parameters of the immune response but not for another. When an unresponsive state is induced, usually production of both circulating antibody and delayed hypersensitivity are affected; however, in some cases, delayed hypersensitivity is preferentially affected. Administration of haptens through non-immunogenic routes affects both delayed hypersensitivity and circulating antibody (Chase, 1946; Battisto and Miller, 1962; Battisto and Chase, 1965). Turk and Humphrey (1961) and Gregg and Salvin (1963) observed that the induction of both delayed hypersensitivity and humoral immunity was inhibited by injecting protein antigens into newborn guinea pigs. Similar results have been obtained in adult guinea pigs (Dvorak *et al.*, 1965; Weigle, 1966). On the one hand, delayed hypersensitivity and production of circulating antibody were inhibited in rabbits made unresponsive to HSA (Chutna and Hraba, 1962), but, on the other hand, several studies revealed an unresponsive state which affected delayed hypersensitivity but not the production of antibody. Delayed hypersensitivity to tuberculin but not the production of circulating antibody was inhibited in guinea pigs receiving injections of tuberculoprotein prior to immunization with BCG (Boyden, 1957). Also working with guinea pigs, Asherson and Stone (1965) were able to inhibit preferentially the development of delayed hypersensitivity by prior treatment with a number of specific antigens. Borel *et al.* (1966) reported that injection of DNP–BGG into newborn guinea pigs resulted in the suppression of delayed hypersensitivity to DNP in 50% of the animals, without affecting production of antibody. In all these cases, guinea pigs were the experimental animals and delayed hypersensitivity was preferentially affected. The preferential suppression of delayed

hypersensitivity could be readily explained by unresponsiveness of only thymus-derived cells since, in the mouse, these cells are more susceptible to the induction of unresponsiveness than are bone marrow-derived cells. However, it is difficult to understand the ability of unresponsive thymus cells to collaborate in the production of circulating antibody. If the antigens used in the guinea pig are thymus-dependent, then either the immunization procedures must have bypassed the thymus-derived cells or two types of thymus cells are required—one specific for delayed hypersensitivity and another specific for participation in the production of circulating antibody. If two types of thymus-derived cells are present, the experiment showing split unresponsiveness suggests that activation of the cells involved in delayed hypersensitivity may require a lower concentration of antigen than stimulation of those that collaborate with the precursors of antibody production. However, an entirely different situation has been reported in rats immunized with appropriately acetoacetylated flagellin (Parish, 1971b). After immunization with highly conjugated acetoacetyl flagellin, rats developed delayed hypersensitivity but could not be stimulated to produce antibody to flagellin. These results could be explained either by a preferential induction of unresponsiveness in bone marrow-derived cells or the existence of two types of thymus-derived cells. These conflicting results might be reconciled by determining if the antibody response in the spleen cells of these rats could be reconstituted with normal thymus-derived cells.

A split unresponsive state has also been reported between different antibody subclasses. Dvorak *et al.* (1965) preferentially induced unresponsiveness to serum protein antigens for the production of γ_2 rather than γ_1 antibodies in guinea pigs. Coe (1971) observed that hamsters injected with hen egg albumin (HEA) incorporated into complete Freund's adjuvant produced both γ_1 and γ_2 antibody. However, injections of soluble HEA (over a large dose range) resulted in specific unresponsiveness for the γ_2 antibody, while γ_1 antibody was being produced. Subsequent injections of HEA in complete adjuvant resulted in the production of only γ_1 antibody. Cells committed to production of antibody of various subclasses may respond differently to contact with antigen; they may contain receptors of different avidity or the receptors may be present on the cell surfaces in different amounts.

IX. Termination of Immunological Unresponsiveness

A. Spontaneous Termination

Although immunological unresponsive states that are induced after injection of tolerogen may, at times, be of long duration they are finite,

and in the absence of further injection of tolerogen, the responsive state returns (Smith and Bridges, 1958). The time at which the unresponsive state is spontaneously terminated is related to the persistence of the antigen. As mentioned previously, serum protein antigens persist in the circulation for a long period as does the duration of unresponsiveness to these proteins. It may be that a critical concentration of antigen which maintains unresponsiveness over a long period of time also inhibits the expression of newly arising thymus cells that have specificity for that antigen. Thereby, the early spontaneous loss of unresponsiveness in bone marrow-derived cells is probably related to the necessity for higher concentrations of antigen for induction of unresponsiveness in bone marrow cells than in thymus cells.

Several investigators have observed that during the spontaneous loss of unresponsiveness there is a concomitant antibody response in the absence of further injection of antigen. Terres and Hughes (1959) induced unresponsiveness to BSA in neonatal mice and observed immunity coinciding with the spontaneous loss of the unresponsive state. Thorbecke *et al.* (1961) showed that neonatal mice receiving a single injection of 1 mg. of BGG were unresponsive 2 weeks later, but similarly treated mice when challenged at 2 months of age gave a secondary immune response, indicating that they had been primed. In addition, unresponsiveness induced by neonatal injection of pneumococcal polysaccharide disappears slowly with time and is followed, without further injection of antigen, by immunity (Siskind *et al.*, 1963). These observations are compatible with the existence of long-term unresponsiveness where there is an expanded population of competent bone marrow-derived cells and unresponsive thymus-derived cells (Chiller and Weigle, 1973). When the antigen level falls below that needed to maintain unresponsiveness in thymus-derived cells, it may still be present in amounts effective in stimulation of some of these cells. In the case of thymus-dependent antigens (Terres and Hughes, 1959; Thorbecke *et al.*, 1961), collaboration of these cells with the expanded population of sensitized bone marrow cells would lead to antibody formation. In any event, these experiments agree with others where only small amounts of antigen were needed to stimulate thymus-derived cells and suggest that the sensitization of these cells requires less antigen than does the induction of unresponsiveness, contradictory to the observations that low doses of antigen are particularly effective in inducing unresponsiveness.

Once the spontaneous termination of the unresponsive state begins, it can be augmented by whole-body irradiation of the animal. Irradiation might be expected to terminate an unresponsive state by producing mutant lymphocytes specific for the antigen, or, if antigen is needed, for the maintenance of unresponsiveness by diluting out the antigen by

either cell death after irradiation or cell proliferation during recovery. However, irradiation has not been observed to alter a complete and stable unresponsive state. Whole-body irradiation of adult rabbits made unresponsive by neonatal injection of 500 mg. BSA failed to terminate the unresponsive state (Weigle, 1964a). Conversely, whole-body irradiation of adult rabbits made hyporesponsive by injecting only 10 mg. of BSA at birth did result in an increased antibody response over that of nonirradiated controls (Weigle, 1967a). Termination of unresponsiveness following whole-body irradiation has been reported by several groups of investigators (Fefer and Nossal, 1962; Mäkelä and Nossal, 1962; Stone and Owen, 1963). The experimental design of these studies also involved a hyporesponsive state rather than an unresponsive state and the animals used undoubtedly contained both competent thymus-derived and competent bone marrow-derived cells. It is likely that irradiation enhances the spontaneous termination of unresponsiveness, as it augments the immune response of normal rabbits previously injected with antigen (Taliaferro, 1957; Dixon and McConahey, 1963). In this situation, lymphocytes that have both survived irradiation and have been stimulated by antigen multiply more rapidly than those that survive but are not stimulated, and the former preferentially repopulate the lymphatic tissue (Dixon and McConahey, 1963). This sequence appears to be similar in irradiated hyporesponsive animals. A significant increase in the number of stimulated cells would result in a responsive rather than an unresponsive state.

The scanty data available suggest that once a stable unresponsive state is induced, it is not readily terminated by other agents which either directly or indirectly affect lymphocytes. Phytohemagglutinin, which induces an intense proliferation of thymus-derived cells *in vitro*, has no effect on the unresponsive state induced in adult mice to HGG (Golub and Weigle, 1967b). Similarly, endotoxin (Claman, 1963; Golub and Weigle, 1967a) and actinomycin D do not interfere with the unresponsive state to HGG in mice once it is induced. However, it has been shown that endotoxin (*Escherichia coli*) (Claman, 1963; Golub and Weigle, 1967a) and actinomycin D (Claman and Bronsky, 1965) inhibit the induction of unresponsiveness in adult mice to deaggregated γ-globulin. Injection of *Corynebacterium parvum* into rabbits simultaneously with deaggregated BSA also inhibits the induction of an unresponsive state (Pinckard *et al.*, 1968). Like endotoxin, injection of *C. parvum* both augments the immune response and enhances the phagocytic activity of the reticuloendothelial system. With endotoxin, it appears that the inhibition is independent of the granulopectic activity of the reticuloendothelial system and does not occur in endotoxin-tolerant

mice. That actinomycin D also inhibits RNA synthesis may further emphasize the possibility that the induction of immunological unresponsiveness is an active process. Inhibitors of DNA synthesis employed under similar circumstance not only do not interfere with the induction of unresponsiveness (Claman and Bronsky, 1965), but also in many situations, promote it.

B. Specific Termination

A complete and stable unresponsive state can be specifically terminated by means which involve immunological phenomena. It is well established that the unresponsiveness to a number of antigens can be terminated by immunization with certain chemically altered preparations of the tolerated antigen (Cited in Weigle, 1967a), in that the animal responds to subsequent injection of the unaltered antigen (Weigle, 1962). Although the termination of immunological unresponsiveness[4] with altered preparations of the tolerated antigen gives some important insights into immunological unresponsiveness, of far more interest is the termination of unresponsiveness with cross-reacting antigens which allows some appraisal of the cellular mechanisms involved. As with altered antigens, immunization of unresponsive animals with certain antigens that cross-react with the tolerated antigen results in a termination of the unresponsive state (Weigle, 1961a; Benjamin and Weigle, 1970a). A stable, unresponsive state to BSA is present in adult (3 months of age) rabbits injected with 500 mg. of BSA at birth. Such rabbits immunized with aqueous preparations of cross-reacting albumins synthesize circulating antibody that reacts with both the immunizing albumin and BSA (Weigle, 1961a). Rabbits made unresponsive to BSA at birth and given no additional injections of BSA as adults make normal levels of binding (Table IV) and precipitating antibody to BSA when immunized with various cross-reacting albumins (Benjamin and Weigle, 1970a). Furthermore, the anti-BSA produced in the unresponsive rabbits is qualitatively the same as that produced by injecting cross-reacting albumins into normal rabbits. One or two months after the termination of the unresponsive state, antibody production can be restimulated by injecting BSA (Benjamin and Weigle, 1970b). However, if additional periodic injections of BSA are given, the rabbits return to the unresponsive state (Weigle, 1964b, Benjamin and Weigle, 1970b). The antibody that is made after injection of BSA is directed to only

[4] The phrase "termination of immunological unresponsiveness" as used in this review denotes an immune response to the injection of an antigen to which the animal was previously unresponsive and has no implication as to the mechanism involved.

TABLE IV

MEAN ANTIGEN-BINDING CAPACITIES OF SERA FROM NORMAL AND BOVINE SERUM ALBUMIN-UNRESPONSIVE RABBITS AFTER TWO COURSES OF VARIOUS SOLUBLE ALBUMINS[a]

Terminating antigen[b]	Status	No. of animals	Antigen tested[b,c]				
			BSA	PSA	HSA	ESA	GPSA
PSA	Unres.	19	11.6	188.8	3.3	7.0	9.4
	Norm.	12	11.7	249.1	3.7	9.2	8.0
HSA	Unres.	26	9.1	8.3	200.8	8.9	11.5
	Norm.	26	7.8	6.9	142.7	7.6	10.0
GPSA	Unres.	9	7.1	32.6	15.7	10.8	324.4
	Norm.	14	8.7	33.7	14.2	12.7	289.3
ESA	Unres.	15	6.6	18.7	15.1	170.2	11.9
	Norm.	26	5.6	14.4	10.9	139.4	8.3

[a] Reprinted from *J. Exp. Med.* **132,** 69 (1970).

[b] BSA—bovine serum albumin; PSA—pig serum albumin; HSA—human serum albumin; GPSA—guinea pig serum albumin; ESA—equine serum albumin.

[c] Antigen-binding capacity measured as micrograms antigen N bound to the globulin [precipitated with 50% saturated $(NH_4)_2SO_4$] present in 1.0 ml. of serum.

determinants on BSA that are related to those on the albumin used to terminate the unresponsive state and can be completely absorbed by the terminating albumin.

The normal response to BSA-related determinants in rabbits unresponsive to BSA indicates that these animals contain immunocompetent precursor cells. The presence of a normal complement of precursor cells to BSA in a rabbit unresponsive to BSA suggests that the site of unresponsiveness is at some cell type other than the precursor. All of the events involved in the termination of immunological unresponsiveness to BSA, after immunization with cross-reacting albumins, could be explained if thymus-derived but not bone marrow-derived cells were unresponsive (Weigle *et al.*, 1971b; 1972). The presence of normal precursor cells and unresponsive thymus-derived cells in such rabbits 90 days after injection of tolerogen would be consistent with the observations that adult mice made unresponsive to deaggregated HGG spontaneously lose unresponsiveness in their bone marrow cells by day 49 after injection of tolerogen (Chiller *et al.*, 1971). It thus appears that although unresponsiveness may be induced in both thymus- and bone marrow-derived cells after rabbits are injected at birth with BSA, it is lost in the bone marrow-derived cells by the time the adult rabbits are injected with the cross-reacting albumins. Interaction between bone marrow- and thymus-derived cells in unresponsive rabbits injected with a cross-reacting antigen could take place if determinants not related to BSA react with specific thymus-derived cells, while the determinants

related to BSA react with the normal complement of precursor cells present in the bone marrow-derived cells. A normal antibody response to the BSA-related determinants would then be expected. That thymus- and bone marrow-derived cells react with different antigen determinants is suggested by the observations of Mitchison (1969a) and Rajewsky *et al.* (1969), although, as discussed above, direct interaction between these cells is not mandatory. The failure of subsequent injections of BSA to produce antibody production specific for BSA would be expected, since the thymus-derived cells are still unresponsive to BSA and additional precursor cells with new specificities cannot be recruited. However, bone marrow-derived cells carrying memory specific for determinants related to BSA may be restimulated by BSA. Return to the unresponsive state after additional injections of BSA may be the result of an exhaustive differentiation of these memory cells in the absence of further recruitment of precursor cells. Bone marrow-derived cells of mice have memory (Miller and Cudkowicz, 1971; Miller and Sprent, 1971; Takahashi *et al.*, 1971) that is expressed only in the presence of thymus cells; however, thymus cells from specifically unresponsive mice are as effective as those from normal mice (Miller *et al.*, 1971). Simultaneous injections of BSA with the injections of cross-reacting albumins inhibit the termination of the unresponsive state (Weigle, 1964b) which could be explained by reinduction of unresponsiveness in the precursor cells.

When the unresponsiveness is terminated by injection of chemically altered preparations of the tolerated antigen, it is probably because competent thymus cells to altered determinants are present. Injection of antigens complexed to heterologous antibody most likely results in the termination of an unresponsive state in the same manner. Rabbits made unresponsive to BSA at birth make an antibody response to BSA when injected as adults with BSA complexed with anti-HSA (Habicht *et al.*, 1972). In this situation the heterologous anti-HSA adds determinants capable of reacting with thymus-derived cells, and since it cross-reacts only 15% with BSA (Weigle, 1961b), it leaves determinants free to react with bone marrow-derived cells. Antibody production has also been reported for spleen cells from unresponsive mice stimulated *in vitro* with antigen–antibody (heterologous) complexes (Intini *et al.*, 1971). Spontaneous termination of unresponsiveness in mice to HGG has been enhanced by immunization with human rheumatoid factor (Azar, 1966). Similarly, antibody to HGG was produced in rabbits after immunization with rheumatoid factor (Biro and Arroyave, 1970). Since new antigen determinants are presented via the μ chains, it may be that in these latter two studies antibody produced was reactive with only the L chains of HGG. In any event, the above observations are compatible with the finding of unresponsive thymus-derived and responsive bone marrow-derived cells.

The presence of unresponsive thymus-derived cells and competent precursor cells can also explain several other immunological phenomena. Guinea pigs which are genetically nonresponders to DNP conjugated to poly-L-lysine (Kantor *et al.*, 1963) respond to DNP if the DNP–poly-L-lysine is conjugated to BSA, but not if the guinea pigs are first made unresponsive to BSA (Green *et al.*, 1968). These nonresponders apparently contain competent precursor cells to DNP but not competent thymus-derived cells with specificity for poly-L-lysine. Conjugation of DNP–poly-L-lysine to BSA most likely permits interaction between thymus-derived cells (via BSA) and precursor cells (via DNP). This suggestion is compatible with the observations that, although nonresponders make circulating antibody to the DNP–poly-L-lysine portion of the conjugate, they do not demonstrate thymus-dependent functions such as delayed hypersensitivity and blast cell transformation to this portion. Paul *et al.* (1969b) were able to terminate the unresponsive state induced in neonatal rabbits to BSA by injecting DNP–BSA if the unresponsive state was induced with small but not with large doses of BSA. With large doses, unresponsiveness probably occurs in both the thymus- and bone marrow-derived cells, whereas with small doses, unresponsiveness most likely exists in only thymus-derived cells. Unresponsiveness in the thymus- but not in the bone marrow-derived cells would also explain why injections of 2,4,6-trinitrophenyl–horse γ-globulin (TNP–HoGG) are able to induce a normal response to TNP in rabbits that were made unresponsive to TNP–BSA at birth (Weigle, 1965a). The TNP–BSA is a poor tolerogen since unresponsiveness was induced in only a proportion of the rabbits, and it is unlikely that an unresponsive state is present in the bone marrow-derived cells in adult life. The ability of a GVH reaction to terminate unresponsiveness to SRBC in rats (McCullagh, 1970c) when both allogeneic lymphocytes and SRBC are injected also arises from the presence of competent precursor cells. Stimulation of thymus-derived cells among the transferred cells adjacent to a competent precursor that has reacted with the tolerated antigen could induce the precursor cell to differentiate into an antibody-producing cell. A similar phenomenon may explain why the unresponsiveness to flagellin can so readily be terminated in rats by injecting the flagellin incorporated into complete Freund's adjuvant (Lind, 1968). The mycobacteria in the adjuvant may stimulate the host's own thymus-derived cells near competent bone marrow cells that have reacted with flagellin.

Direct termination of immunological unresponsiveness by injecting altered or cross-reacting antigens has most often been accomplished in rabbits, but two studies utilized mice. Dietrich (1966) was able to terminate unresponsiveness induced to HGG in newborn mice by immunization with the HGG coupled to the diazonium derivatives of benzoic,

arsanilic, or sulfanilic acid. As is the case with the termination of unresponsiveness to BSA in rabbits (Weigle, 1962), injection of the antigen coupled to two derivatives was more effective than if it was coupled to only one derivative. The difficulties that Scales and Cruse (1970) encountered in terminating the unresponsive state to BGG by immunization with a cross-reacting γ-globulin most likely resulted from the time between injection of the tolerogen and immunogen. The HGG was injected 20 days after deaggregated BGG had been given; a time when both bone marrow and thymus cells would be expected to be unresponsive (Chiller *et al.*, 1971).

X. Autoimmunity

The best example of antigen-directed immunological unresponsiveness is the unresponsive state enjoyed by animals to their own body constituents. During early life before maturation of the immune mechanisms, animals appear to develop a specific immunological unresponsiveness to most of their own body constituents. The available evidence (Triplett, 1962; Rosenberg and Tachibana, 1962; Cinader *et al.*, 1964) suggests that unresponsiveness to self-antigens results from direct contact between self-components and receptors on antigen-reactive cells. In all likelihood, the natural unresponsive state to self-antigen is identical to the unresponsive state induced after injection of foreign proteins during neonatal life. If these two types of unresponsiveness are the same, the mechanisms involved in the termination of acquired unresponsive states may be similar to those of some autoimmune phenomena. Certain experimental autoimmune states can be induced by similar procedures to those that terminate unresponsiveness to BSA in the rabbit. In at least some of these situations, unresponsiveness appears to be at the level of thymus cells and not precursor cells. Although thyroiditis can readily be produced in rabbits following injection of homologous thyroglobulin incorporated into complete Freund's adjuvant (Rose and Witebsky, 1956), it can also be produced by injections of aqueous preparations of altered homologous thyroglobulin (Weigle, 1965b). Injection of aqueous preparations of homologous thyroglobulin coupled to the diazonium derivatives of arsanilic and sulfanilic acids into rabbits causes termination of their unresponsive states, in that thyroid lesions develop and antibody is produced which is reactive with rabbit thyroglobulin. Similar injections of native homologous thyroglobulin fail to produce either antibody or lesions, and convincing data demonstrate that rabbits do possess an unresponsive state to their own thyroglobulin (Nakamura and Weigle, 1967b). Injection of aqueous preparations of certain heterologous thyroglobulins also results in thyroiditis and formation of circulating antibody to rabbit thyroglobulin (Weigle and Nakamura, 1967).

This latter phenomenon has the same characteristics as does the termination of unresponsiveness in rabbits to BSA after the injection of cross-reacting albumins. Once the unresponsive state is terminated by immunizing with heterologous thyroglobulin, the rabbits respond to a subsequent injection of rabbit thyroglobulin. However, with additional injections the rabbits regain their unresponsive state. None of the antibody produced after subsequent injections of rabbit thyroglobulin is specific, since it can be completely absorbed with the heterologous thyroglobulin. Simultaneous injection of homologous and heterologous thyroglobulin inhibits the production of both thyroiditis and circulating antibody to rabbit thyroglobulin (Weigle, 1967b). Once again, termination of unresponsiveness to autologous thyroglobulin can be explained by the presence of unresponsive thymus-derived cells and immunocompetent precursor cells (Weigle, 1971). Although thyroglobulin can be detected in the circulation of animals (Daniels *et al.*, 1967) and after equilibration between intra- and extravascular spaces persists (in rabbits) in the circulation with a half-life of 2.5 days (Nakamura and Weigle, 1967a), it is present in concentrations too low to expect unresponsiveness to be maintained in bone marrow-derived cells. The absence of thymus-derived cells specific for homologous thyroglobulin in rabbits immunized with bovine thyroglobulin and producing circulating antibody to the thyroglobulin is suggested by the ability of these cells to release migration inhibition factor (MIF) in the presence of bovine thyroglobulin but not rabbit thyroglobulin (Clinton, 1972). Evidence implicates altered thyroglobulin in the production of thyroiditis in rabbits injected with homologous thyroglobulin incorporated into complete adjuvant. It appears that alteration of the thyroglobulin in the granuloma by local pH changes and later by cathepsins (Weigle *et al.*, 1969) permits the thyroglobulin to react with thymus-derived cells specific for these alterations. Both thyroiditis and circulating antibody to rabbit thyroglobulin are induced in rabbits injected with aqueous preparations of rabbit thyroglobulin partially degraded with pepsin (Weigle *et al.*, 1969) or papain (Anderson and Rose, 1971). Autoimmune thyroiditis also has been induced in mice following immunization with aqueous preparations of heterologous thyroglobulin (Nakamura and Weigle, 1968). Here, both the thyroiditis and production of circulating antibody are thymus-dependent, and the spleens of normal mice contain antigen-binding cells to mouse thyroglobulin (Clagett, 1972).

In contrast to thyroiditis in the rabbit, subjects with other experimental diseases which are accompanied by delayed hypersensitivity may not have unresponsiveness in either the thymus- or bone marrow-derived cells to the antigen involved, e.g., EAE and thyroiditis in the guinea pig. Both examples may result from concentrations of antigens in the body fluid insufficient to maintain an unresponsive state in either cell type.

The half-life of guinea pig thyroglobulin in the guinea pig is only several hours (Nakamura, 1967), whereas the half-life of homologous and several heterologous thyroglobulins in rabbits is 2.5 days. Thus, the concentration of thyroglobulin present in the body fluids of guinea pigs may be insufficient to maintain a completely unresponsive state of even thymus-derived cells.

Numerous investigators have been able to induce autoimmune phenomena by immunizing with either cross-reacting tissue components (cited in Weigle, 1972) or altered organ-specific antigen (Porkorna and Vojtiskova, 1964; Swanborg and Radzialowski, 1970), but these studies were not concerned with the cellular mechanisms involved.

Several human autoimmune diseases appear to have similar mechanisms to those described above for experimental thyroiditis in the rabbit. Addition of IgG protein as antigen to peripheral lymphocytes of patients with rheumatoid arthritis failed to induce any increase in blast transformation of uptake of thymidine-^{3}H, although some of these patients' sera contained antibody that reacted with IgG (Kacaki *et al.*, 1969). Thus, the immune response to IgG may not have involved an interaction of the IgG with thymus-derived cells, since the blastogenic activity is associated with thymus-derived cells. Similar observations have been made in patients with rheumatic fever. These patients contain circulating antibody to heart antigen which cross-reacts with streptococcal antigens, and this disease is associated with streptococci infections. Peripheral blood lymphocytes of these patients can be stimulated to transform into blast cells by the streptococcal antigen but not by the heart antigen (McLaughlin *et al.*, 1972), suggesting that, despite the presence of cells making antiheart antibody, the thymus-derived cells are not responsive to these antigens. It may be that determinants on the streptococcal antigens, which are unrelated to the determinants present on the heart antigens, react with thymus-derived cells specific for them and permit competent precursor cells to react with determinants related to those present on the heart antigen.

Because of the low level of human growth hormones in the body fluids, one would not expect an unresponsive state to these hormones to exist in the precursor cells of bone marrow-derived cells. Therapeutic injection of aggregated (altered) preparations of growth hormones into children with hypopituitary dwarfism resulted in production of antibody to growth hormones in a large percentage of the children (Illig, 1970). Yet, similar injections of clear (unaltered) preparations did not cause antibody production, and children who made antibody after long-term treatment with the aggregated preparations lost that capacity with substituted injections of clear hormone preparations. This situation resembles the events in termination of immunological tolerance to BSA after injection of altered preparations of BSA.

Autoimmune phenomena may also involve nonspecific stimulation of thymus-derived cells which subsequently stimulate competent bone marrow-derived cells (Allison *et al.*, 1971). Virus infection could lead to stimulation of thymus-derived cells in close proximity to competent precursor cells. Either by direct interaction of these cells, possibly via macrophages, or through a humoral factor released from the stimulated thymus-derived cells, the precursor cells could be stimulated to differentiate into cells producing antibody to self. If cellular immunity does not result in rejection of the virus, such a phenomenon could culminate in a progressive autoimmune disease because body components were altered by chronic viral infection. Examples of nonspecific stimulation of thymus-derived cells are observed in the enhanced response to unrelated antigens during GVH reactions (McCullagh, 1970a; Katz *et al.*, 1971a) and the conversion of guinea pigs nonresponsive to DNP–poly-L-lysine to responders by incorporating large amounts of mycobacteria in the adjuvant containing the DNP–poly-L-lysine (Green *et al.*, 1968).

XI. Specificity

In general, the unresponsive state to a given antigen is specific in that the unresponsive animal usually makes a normal response to an unrelated antigen. Nevertheless, there are several situations where the specificity of the unresponsive state is broader than the specificity of the antibody resulting from immunization. Rats rendered unresponsive to *Salmonella* flagellin serotype fg respond suboptimally to two other *Salmonella* flagellin antigen serotypes, i and d, even though the three types appear to be serologically unrelated (Austin and Nossal, 1966). Similarly, rabbits made unresponsive by neonatal injections of BSA, as adults, fail to respond to sheep serum albumin (SSA) (Weigle, 1961a) which cross-reacts serologically only 75% with BSA (Weigle, 1961b). In addition, A/J mice made unresponsive to deaggregated HGG are 97 and 91% unresponsive to pig γ-globulin (PGG) and horse γ-globulin (HoGG), respectively (Ruben *et al.*, 1971), although the anti-HGG antibody produced in similarly immunized mice cross-reacts serologically only 1% with PGG and HoGG. Bauminger and Sela (1969) failed to find a straightforward correlation between the specificity of serological reactions and the cross-reaction involved in immunological unresponsiveness. Since antibody specificity is directed by the precursor cell which produces it, it would appear that the thymus cells are responsible for the broad specificity of the unresponsive state and that their receptor sites probably recognize a larger number of antigen determinants present on an antigen. The specificity of delayed hypersensitivity is also broad (Benacerraf and Gell, 1959; Gell and Benacerraf, 1959) and entirely dependent on thymus-derived cells.

XII. Tumor and Transplantation "Tolerance"

Although antibody is not involved in inducing or maintaining a complete and stable unresponsive state, circulating antibody apparently plays an active role in acceptance of allografts. Failure to reject allografts and tumors may result from a state of immunological "tolerance" in which there is antibody production rather than immunological unresponsiveness. Specific serum antibody enhances the survival of both allografts (Voisin *et al.*, 1966) and tumors (Kaliss, 1958). Lymphocytes sensitized to tumor antigens are capable of killing specific tumor cells *in vitro*, and serum antibody to tumor antigens prevents *in vitro* killing (Möller, 1965; Brunner *et al.*, 1967; Hellström and Hellström, 1969). Recently, Hellström and Hellström (1969) demonstrated that tumors are accompanied by cellular immunity to the tumor and that the ability of the tumor to survive and grow *in vivo* may be attributed to a specific ability of the serum of the tumor-bearing individual to prevent tumor cell destruction by the sensitized lymphocytes. Similarly, mice retaining skin allografts have both cellular immunity to the donor cells and circulating antibody that inhibit destruction of these cells by sensitized lymphocytes (Hellström *et al.*, 1971). Furthermore, tetraparental mice have lymphocytes capable of reacting against the cells of each parent and a serum factor ("antibody") capable of inhibiting the lymphocytes (Wegmann *et al.*, 1971; Phillips *et al.*, 1971). Since these mice possess a mixture of cells containing different H-2 antigens, the cells react to each other *in vitro* by undergoing blastogenesis four- to fivefold greater than do lymphocytes from either parental strain alone (Wegmann, 1971). However, a mixture of lymphocytes of the two parental strains undergoes blastogenesis *in vitro* fourteen-fold greater than do lymphocytes from either parent alone, suggesting that considerable central unresponsiveness may be present in these animals. It is difficult to evaluate what role blocking antibody and central unresponsiveness play in successful renal allografts in the human. A large percentage of patients who receive renal grafts from relatives have lymphocytes capable of reacting *in vitro* with the donor's fibroblasts, and in many cases blocking antibody has been detected in the serum of the host (Pierce *et al.*, 1971). There was a good correlation between the patients' high titer of blocking antibody and a favorable clinical course. One year after transplantation, the number of patients possessing sensitized lymphocytes capable of reacting to the donor markedly decreased, suggesting that in the more successful grafts central unresponsiveness had been established. At the present time, it is not known how central unresponsiveness participates in allograft persistence. In any event, serum from F_1 hybrid mice did not inhibit the blastogenesis resulting from mixing lymphocytes from the two parents, implying the absence of autoreactivity against H-2 antigens in normal biparental animals (Phillips *et al.*, 1971).

XIII. Possible Mechanisms

Although the mechanisms involved in tolerance to tumors and allografts may become more obvious, those concerned with the induction and maintenance of central immunological unresponsiveness where there is no evidence of enhancing antibody are not understood. It is important to keep in mind that only one cell type is required in order for the intact animal to be unresponsive, and in thymus-dependent responses, when only one cell type is involved, it is usually the thymus-derived cell. Obviously, both the immune response and the induction of immunological unresponsiveness require that the immunogen or tolerogen reacts with receptor sites, and it is also likely that the manner in which this interaction occurs directs which of the two phenomena takes place. Bretscher and Cohn (1970) postulated that induction of humoral antibody formation requires the recognition of two identical determinants on an antigen by a bivalent receptor on antigen-sensitive cells and that the two determinants are presented to the antigen-sensitive cell by bivalent carrier antibody. Immunological unresponsiveness was thought to involve the absence of carrier antibody and thus the recognition of only one determinant by the antigen-sensitive cell. This theory permits carrier antibodies to be receptors on thymus-derived cells and suggests that unresponsiveness may involve the absence of thymus-derived cells. It also predicts that unresponsiveness to self is at both the precursor and thymus-derived cellular levels. However, neither situation is absolute since experimental unresponsiveness can occur in both cell types, and there is good evidence that unresponsiveness to some self-antigens is at the level of thymus-derived cells only. In any event the change in the configuration of the receptors on the precursor cell caused by the reaction of two identical determinants with two receptor sites on the precursors supposedly leads to immunity, and failure to present antigen in this manner may lead to unresponsiveness. The Bretscher–Cohn theory does not consider the fate of receptors during the induction of unresponsiveness. Does the failure to detect antigen-reactive cells in animals unresponsive to that antigen mean that these cells have been eliminated? Jerne's (1971) recent theory of antibody production visualizes unresponsiveness to self as elimination of lymphocytes containing receptors to self-histocompatibility antigens. Unresponsiveness may also result from death of competent cells after interaction between immunoglobulin receptors on the cell with antigen and the subsequent fixation of complement (Azar and Good, 1971). In any event, direct evidence for or against the presence or absence of unresponsive cells is not available, and the best proof for the absence of such cells is the failure of workers to detect receptor sites in completely unresponsive animals. Even this evidence

is not conclusive since lymphocytes containing receptors in unresponsive animals may be hidden because they are covered with tolerogen or receptors may not be replaced once they are stripped from the cell. Reactions between receptors on precursor cells and multivalent antigen may reseult in migration of antigen–receptor complexes to form polar caps which enter the cell by pinocytosis where digestion of the complexes takes place. If these events fail to cause antibody production, unresponsive cells may form in that receptor sites are not regenerated. Conversely, if antigen reacts with the receptor sites so that cap formation and subsequent pinocytosis do not occur, an unresponsive state may result. Tolerogen may remain fixed to the receptor sites, thus blocking further stimulation, and the unresponsive state is only lost after the effective concentration of tolerogen is removed from these sites. Such may be the case with monomeric and aggregated preparations of HGG where the monomers may not be able to aggregate the receptor sites on the surface of competent cells resulting in the failure of subsequent cap formation and pinocytosis. Nevertheless, aggregated HGG may successfully induce an immune response because its multivalence permits it to aggregate receptor sites, thus initiating the subsequent events. In addition to clonal elimination or clonal suppression, another possibility for the mechanism of the induction of unresponsiveness in both thymus- and bone marrow-derived cells is that of clonal conversion. In such conversion, cells containing receptors of one specificity would be converted to cells containing receptors of a different specificity. Thus, the genetic information coding for the production of a phenotype specific for the tolerogen would be suppressed, while genetic information coding for the production of a phenotype specific for an unrelated antigenic determinant would be expressed. There exist biological precedents for such a mechanism in other systems. Immunization of tadpoles with adult hemoglobin results in both the suppression of appearance of adult hemoglobin during metamorphosis of tadpoles to adult frogs and the expression of another hemoglobin which is electrophoretically and serologically distinct (Maniatis *et al.*, 1969). Similarly, a switch in a gene product resulting in suppression of IgG allotypes in rabbits can be induced by antibody specific for an allotype. It has recently been shown that although the allotypic marker specific for the antibody is absent in such allotypically suppressed rabbits, there appear IgG molecules which carry a new set of allotypic determinants (Tosi *et al.*, 1972). The manner (qualitative or quantitative) in which the antigen is presented to the antigen-reactive cells in order to induce immunological unresponsiveness is of utmost importance, as each of the above possibilities stresses.

If macrophages are nonspecific handlers of antigen and can, indeed,

present antigen to specific antigen-reactive cells, then how they present the antigen may be critical in the subsequent events. It has been clearly shown that antigen reacts directly and in large amounts with receptor sites present on precursor cells. Conversely, receptor sites have been shown only indirectly to be present on thymus-derived cells. It may be that effective stimulation of thymus-derived cells, at least with certain antigens, requires the antigen to be presented to these cells on the surfaces of macrophages. Failure of macrophages to fix deaggregated HGG to their surfaces effectively could explain why injection of such preparations leads to immunological unresponsiveness rather than to immunity. Whatever the mechanisms involved in the induction of immunological unresponsiveness, they may differ for thymus- and bone marrow-derived cells.

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Participation of Lymphocytes in Viral Infections[1]

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I. Introduction

Despite years of interest and research, the role of lymphocytes in viral infections today remains poorly defined. Delayed hypersensitivity reactions have been reported in many viral diseases (Allison, 1967), and it has been tempting to believe that lymphocytes play an important role in the defense of the host against these viruses. However, the evidence supporting this belief is largely indirect. Lymphocytes have been found to produce factors that inhibit virus production in host cells. Furthermore, the depletion of lymphocytes or the impairment of their function *in vivo* leads to increased susceptibility of the host to many viruses. In addition, the presence of lymphocytes in large numbers at foci of virus infection suggests that they have responded to a chemotactic factor and are fulfilling some role in containing the virus at the site of infection. Finally, many viruses are able to subvert the function of lymphocytes suggesting that viral mutants with increased pathogenicity have been selected through an acquired ability to impair an important mediator of host defense, i.e., lymphocytes. In contrast, there is increasing evidence that lymphocytes may participate in the production of certain viral diseases, serving either as host cells for viral replication or as mediators in an immune response which itself can result in overt disease.

There are many aspects of virus–lymphocyte interactions which occur both *in vitro* and *in vivo*. We have attempted to present an overall view of the possible roles lymphocytes play, both in production of and defense against viral disease. We also discuss the effects of viral infections on cell-mediated immunity (CMI). The reader is referred to Notkins *et al.* (1970) for a review of the effects of viral infections on humoral immunity.

II. Problems Associated with *in Vitro* Studies on Lymphocytes

The acquisition of evidence to define the role of lymphocytes in viral infections has been hampered by two factors. First, the lymphocyte which is actively involved either as a host cell for viral replication or in an attack on the virus and virus-infected cell has usually infiltrated into the site of infection and is relatively inaccessible to the investigator. A study of lymphocytes present in the peripheral blood could, therefore, yield negative and misleading data, since this population may consist of resting, non-virus-involved cells. Second, in attempting to study the lymphocyte *in vitro,* the investigator must employ culture conditions which are far from ideal and which produce alterations in lymphocyte structure and function, thus possibly negating the relevance of results obtained.

Below are listed some of the problems encountered when one attempts to study lymphocytes *in vitro*. These difficulties should be considered both in the design of experiments and in the interpretation of their results.

A. Purification

Many techniques have been developed for the preparation of pure populations of lymphocytes for *in vitro* experiments (Ling, 1968, p. 55). Separation of lymphocytes from other types of leukocytes has been achieved by adsorption of nonlymphoid cells onto materials such as glass beads (Rabinowitz, 1964) or cotton wool (Lamvik, 1966), by removal of phagocytic cells with a magnet following exposure of the leukocyte culture to iron particles (Kuper *et al.*, 1961), and by differential centrifugation (Ling *et al.*, 1965). All these techniques can provide lymphocyte cultures of better than 99% purity. However, a defect common to all is that lymphocytes, which at the time of collection are actively involved in a cellular or humoral immune response, may be separated from resting lymphocytes on the basis of differences in size (Crowther *et al.*, 1969) or glass-attaching properties (Fishman, 1961) associated with lymphocyte activation. The former lymphocytes would tend to remain with the phagocytic cell [monocyte–macrophage and polymorphonuclear leukocyte (PMN)] population during the separation procedures. Since many experiments with lymphocytes are designed to study their role in the immune response, it is important to avoid using purified cultures of lymphocytes which may be composed almost entirely of resting cells.

B. Lymphocyte–Macrophage Interactions

Lymphocytes have been shown to require an association with macrophages in order to produce an optimal response to certain stimuli. Therefore, the use of purified lymphocytes *in vitro* to study these responses may yield data not truly reflective of the *in vivo* state. An intimate association of lymphocytes with macrophages has been shown to be essential for primary antibody production *in vitro* (Mosier, 1969). In contrast, purified lymphocyte cultures are capable of producing interferon in response to various stimuli; however, interferon production is augmented when a small number of macrophages is added to the culture (Epstein *et al.*, 1971). Highly purified lymphocyte preparations show a moderate blastogenic response to either nonspecific mitogens or antigens to which they are sensitive. However, the addition of small numbers of macrophages to lymphocytes greatly enhances their blastic transformation (Levis and Robbins, 1970a,b). Since any agent that stimulates a lymphocyte induces interferon, and since the amount of

interferon produced is related to the degree of stimulation (Wheelock and Toy, unpublished results), it is probable that macrophages enhance interferon production of lymphocytes by augmenting their biosynthetic response to stimulating agents. Alternatively, macrophages may produce interferon-inducing agents and present them to lymphocytes in a more active form.

C. Alterations in Metabolism

Uninfected lymphocytes placed in culture undergo morphologic and metabolic changes which lead to cell death (Ling, 1968, p. 69). Continuous cultures can be achieved readily when lymphocytes contain the Epstein-Barr virus (EBV) genome prior to their removal from the peripheral circulation or when the virus is added to normal lymphocytes at initiation of the culture (Henle *et al.*, 1967; Grace, 1967; Pope *et al.*, 1968b; Gerber *et al.*, 1969a). The finite life-span of uninfected lymphocytes in culture imposes severe restrictions on the types of experiments which can be performed and prevents extrapolation of results obtained *in vitro* to the *in vivo* state.

Resting lymphocytes may undergo spontaneous transformation to fibroblast-like (Prempree and Merz, 1966) or macrophage-like cells (Berman and Stulberg, 1962). An additional and more frequent problem arising in lymphocyte cultures is spontaneous lymphoblastic transformation (Johnson and Russell, 1965). Little is known about the initiating causes of this phenomenon. However, lymphocytes which are either primed *in vivo* by antigens or involved in transformation to functionally active cells at the time of their removal from the body may quickly convert to a blastic metabolism and morphology when placed in culture. Most, if not all, cultures of adult lymphocytes contain a small percentage of lymphocytes which are undergoing this spontaneous transformation. These cells markedly affect experiments on virus–lymphocyte interaction, for they support viral replication and interfere with investigations on the resting lymphocyte which cannot support viral replication unless first stimulated with a mitogen (Wheelock and Edelman, 1969).

D. Variability of Response

It is our experience that lymphocytes removed from the same individual on different days and placed in culture with a mitogen vary greatly in their capacity to support vesicular stomatitis virus (VSV) and 17D yellow fever virus replication and in their ability to produce interferon. Incorporation of thymidine-^{3}H into phytohemagglutinin (PHA)-stimulated lymphocytes derived from the same volunteers on numerous occasions also varied greatly (Stjerholm, 1971 personal communication).

Nothing is known of the *in vivo* factors that affect lymphocytes and result in this variation. It is conceivable that the variability may in part be due to viral and bacterial infections in process at the time the lymphocytes are collected. These infections together with their associated inflammatory and immune responses may elaborate humoral factors, such as interferon or lymphotoxin, *in vivo,* which can subsequently alter the *in vitro* characteristics of lymphocytes. When such infections involve the lymphocytes themselves, either a direct or an interferon-mediated indirect interference with viral replication *in vitro* can be expected.

The use of more efficient techniques for culturing lymphocytes combined with judicious selection of appropriate combinations of leukocyte types will permit experiments to be conducted *in vitro* which are truly analogous to the *in vivo* process under investigation.

III. Morphological Alterations in Virus-Infected Lymphocytes

During the course of most acute viral infections *in vivo* and *in vitro,* infected cells undergo morphological alterations which may lead to cell death. These cellular alterations can consist of (*1*) production of chromosomal aberrations, (*2*) formation of syncytia, (*3*) appearance of inclusion bodies, (*4*) extensive vacuolization of the cytoplasm, and (*5*) disruption of the cell. In addition, less drastic changes may occur. For example, the cytoplasmic–nuclear volume ratios may change, resulting in the appearance of morphologically transformed "atypical" cells.

With lymphocytes, only chromosomal aberrations and the appearance of morphologically transformed, atypical cells have been reported to occur during the course of viral infections. The presence of viruses or viruslike particles in lymphocytes taken from patients with viral or suspected viral infections is not considered a morphological alteration and will be covered separately in Section V.

A. Chromosomal Aberrations

Nichols *et al.* (1962) made the original observation that lymphocytes from patients with clinical measles virus infections had a higher frequency of chromosomal aberrations than did lymphocytes from healthy donors. Other investigators confirmed Nichols' findings and extended the observation to several other viral infections (see Table I).

Included in Table I are also several negative reports. These discrepancies as well as the variations in the percentage of cells containing chromosomal aberrations can be explained in several ways. The presence of chromosomal aberrations in lymphocytes is of short duration. Nichols *et al.* (1962) reported that chromosomal aberrations which were present in measles virus-infected lymphocytes removed on one day were

TABLE I
STUDIES ON VIRUS-INDUCED CHROMOSOMAL DAMAGE IN LYMPHOCYTES[a]

Virus	Host cell[b]	References
1. Measles	Human lymphocytes	Nichols *et al.* (1962, 1968); Tanzer *et al.* (1963); Harnden (1964); Gripenberg (1965); Aula (1965)
2. Measles	Human leukocytes	Aula (1965); Nichols *et al.* (1965)
3. Rubella	Human leukocytes	Makino and Aya (1968)
4. Mumps	Human lymphocytes	Aula (1965); Gripenberg (1965); Chun *et al.* (1966)
5. Yellow fever virus	Human lymphocytes	Harnden (1964)
6. Rous sarcoma virus (Schmidt-Ruppin and Bryan strains)	Human leukocytes	Nichols *et al.* (1964)
7. Poliovirus	Human lymphocytes	Aula and Nichols (1968)
8. Infectious and serum hepatitis	(a) Human lymphocytes	Aya and Makino (1966)
	(b) Human normal leukocytes plus sera from hepatitis patients	El-Alfi *et al.* (1965)
	(c) Leukocytes + PHA	Kerkis *et al.* (1967); Barinskii *et al.* (1968)
	(d) Normal human leukocytes + sera from hepatitis patients + PHA	Baroyan *et al.* (1970); Shubladze *et al.* (1969)
9. Adenovirus-12	Human leukocytes	Nichols (1966); Nichols *et al.* (1968)
10. Chickenpox	Human lymphocytes	Aula (1965); Gripenberg (1965); Chun *et al.* (1966)
11. Infectious mononucleosis	Human lymphocytes	Gripenberg (1965)
12. Herpes simplex virus	Normal leukocytes	Makino and Aya (1968)
13. Herpeslike virus	Burkitt lymphoma	Gripenberg *et al.* (1969)
	Cultured lymphoblasts from patients with Burkitt lymphoma	Kohn *et al.* (1967); Miles and O'Neill (1967); Tomkins (1968)
14. Virus induced murine leukemias	Mouse leukocytes	Koller *et al.* (1963) (review)

[a] See Stich and Yohn (1970), text and Tables I and III, for description of types of chromosomal breakage, number of patients studied, and sampling times for some of the above viruses.

[b] PHA—phytohemagglutinin.

not present prior to or after that day. Likewise, Harnden (1964) observed chromosomal damage in lymphocytes removed 8 or 9 days after 17D yellow fever virus vaccination but not in cells removed from the peripheral circulation before or after these days. Thus, the demonstration of chromosomal aberrations in lymphocytes is dependent upon the time after infection when the cells are removed and examined.

Two additional factors, the immune status of the patient and the severity of the illness, could also account for the variability in chromosomal aberrations seen in patients. It is a reasonable assumption that chromosomal damage which occurs in lymphocytes during the course of viral disease is the result of virus–cell interaction. This interaction might occur at the level of the lymphocyte precursor or take place between lymphocytes and virus particles in the circulation during viremia. Thus, viruses would appear in the bloodstream earlier and in greater quantity in a severe infection than in a mild one. The immune response would also vary accordingly and affect the viremia and the magnitude of virus–lymphocyte interaction. For further explanation of factors possibly influencing virus-induced chromosomal damage, see Stich and Yohn (1970).

Most of the evidence on the mechanisms by which viruses produce chromosomal damage has been derived from experiments that use cells other than lymphocytes (review by Nichols, 1970). These studies suggest several possible mechanisms: (*1*) inhibition of deoxyribonucleic acid (DNA) or protein synthesis by viruses; (*2*) virus-induced alterations of lysosomal membranes; and (*3*) integration of the viral genome into cellular DNA.

The use of selective inhibitors of DNA synthesis causes chromosomal damage indistinguishable from that seen in lymphocytes from patients with certain viral infections (Nichols and Heneen, 1964; Brewen, 1965; Nichols, 1966; Brewen and Christie, 1967). Similar chromosomal aberrations are seen in leukocytes cultured in the absence of the essential amino acid, arginine (Aula and Nichols, 1967). In addition, several viruses that interfere with PHA stimulation of lymphocytes have been shown to cause chromosomal damage, e.g., measles and rubella (Smithwick and Berkovich, 1966; Montgomery *et al.*, 1967; Olson *et al.*, 1969). Thus, viruses that alter host DNA or protein synthesis may be capable of inducing chromosomal aberrations (Nichols, 1970); most viruses of this type, however, produce rapid gross cytopathology which may explain the absence of reported chromosomal aberrations in cells infected with these viruses.

Allison and Paton (1965) proposed that viruses may affect lysosomes causing them to release enzymes which would then produce chromo-

somal damage. Aula and Nichols (1968), however, failed to demonstrate lysosomal damage in cultures of measles or adenovirus-infected leukocytes displaying chromosomal aberrations.

Attempts to localize viral nucleic acid on damaged chromosomes have been far from satisfactory. Although viral nucleic acid has been found diffusely within nuclei (Rapp and Hsu, 1965) or in association with host cell chromosomes (zur Hausen, 1967, 1968; Sambrook *et al.*, 1968), it has not been localized at the site of chromosomal damage nor necessarily limited to those cells displaying chromosomal aberrations (Nichols *et al.*, 1968).

Numerous lymphoblastic cell lines derived from patients with Burkitt lymphoma (Stewart *et al.*, 1965; O'Conor and Rabson, 1965; Epstein *et al.*, 1964, 1967; Pope *et al.*, 1968a), infectious mononucleosis (IM) (Henle *et al.*, 1968), and leukemia (Iwakata and Grace, 1964; Zeve *et al.*, 1966) contain herpeslike virus particles and chromosomal aberrations. Karyograms of numerous lymphoblastic cell lines from different sources have been made and compared in the hope of finding a "marker" or specific chromosomal aberration common to all these cell lines. Kohn *et al.* (1967) found a specific abnormal constriction or achromatic gap on either one or both long arms of the No. 10 chromosome in four of the five Burkitt lymphoma cell lines studied. In addition, an extra Group C chromosome was also detected in some Burkitt cell lines (Kohn *et al.*, 1967; Miles and O'Neill, 1967; Tomkins, 1968), in one cell line derived from a patient with IM (Tomkins, 1968), and in another from a leukemic patient (Tomkins, 1968). Other chromosomal aberrations have been reported by these investigators; however, a specific abnormality could not be confidently designated as a marker chromosome for all of the lymphoblastic cell lines studied. Since chromosomes of many cells undergo numerous aberrations in continuous culture, it is not certain whether the anomalies found in lymphoblastic cell lines represent herpes virus-induced damage or simply instability of specific chromosomes.

B. Atypical Lymphocytes

Atypical lymphocytes appear in the circulation of infected individuals in many illnesses (Wood and Frenkel, 1967; Finch, 1969). The best studied atypical lymphocytes, however, are those found in patients suffering from IM (see Carter and Penman, 1969). Atypical lymphocytes are large cells with abundant cytoplasm and irregular cell boundaries. The cytoplasm is moderately to intensely basophilic and contains intracytoplasmic vacuoles. The endoplasmic reticulum, Golgi apparatus, and mitochondria are more developed than those found in small lymphocytes

and closely resemble those structures present in PHA-transformed lymphocytes. The ribosomal content of the cells is greatly increased. Nuclei are large, pleomorphic, and eccentrically located. The chromatin is loosely packed and similar to that seen in cells about to divide (Cooper, 1969; Finch, 1969).

Atypical lymphocytes are pleomorphic and, therefore, difficult to classify on a morphological basis alone (Ghaemi and Seaman, 1963; Carter, 1966). However, one can differentiate atypical lymphocytes from other mononuclear cells on the basis of cytochemical reactions coupled with morphological and ultrastructural observations. In culture, these cells resemble PHA-transformed lymphocytes in that DNA and ribonucleic acid (RNA) synthesis are increased when compared with normal unstimulated lymphocytes. The number of cells which have increased metabolism, however, is not as great as in PHA-stimulated normal lymphocyte cultures. Furthermore, when atypical lymphocytes are exposed to PHA, RNA synthesis is markedly depressed unless the cells are first incubated in culture for 48 to 72 hours, after which time they respond normally (Rubin, 1966).

The metabolism of atypical lymphocytes as well as their initial unresponsiveness to PHA suggests that they may contain a latent virus. In at least three infections in which atypical lymphocytes occur in the circulation, namely, IM (Henle *et al.*, 1968; Chessin *et al.*, 1968; Moses *et al.*, 1968), Burkitt lymphoma (Carbone *et al.*, 1969; Weinberg and Becker, 1969), and cytomegalovirus (Paloheimo *et al.*, 1968), a herpeslike virus has been associated with lymphocytes. Further discussion on the association of herpeslike viruses with lymphocytes can be found in Section V.

IV. Lymphocytopenia in Viral Infections

Viral infections in man and experimental animals have been reported to alter both the relative and absolute numbers of leukocytes in the circulation (Gresser and Lang, 1966). Of interest to this review are only the effects of viral infections on lymphocytes; therefore, only papers reporting alterations in the number of circulating lymphocytes will be considered.

Transient lymphocytopenia in man has been observed during infections caused by poliovirus, coxsackie A, rhinovirus, yellow fever virus, chickenpox, adenovirus, measles, and both influenza A and B viruses (see Table II). Table II also lists viruses causing lymphocytopenia in lower animals. Although the majority of the viruses listed possess membranes, there is no correlation between the presence of this membrane and the ability of a virus to cause lymphocytopenia. Only two DNA

TABLE II
VIRUSES CAUSING LYMPHOCYTOPENIA IN THEIR HOST

Virus	Host	References
1. Poliovirus	Monkey	Taylor (1919)
	Man	Peabody *et al.* (1912)
2. Coxsackie A	Man	R. G. Douglas *et al.* (1966)
3. Rhinovirus	Man	R. G. Douglas *et al.* (1966)
4. Colorado tick fever	Man	Johnson *et al.* (1960)
5. Venezuelan equine encephalitis	Guinea pig	Airhart *et al.* (1969)
6. Yellow fever virus	Monkey	Daberkow and Knüttgen (1966)
	Man	Wheelock and Toy (unpublished)
7. Tick-borne encephalitis	Mouse	Malkova *et al.* (1961)
8. Measles	Man	Benjamin and Ward (1932)
9. Rubella	Man	Hillenbrand (1956); Hynes (1940); Carroll (1934)
10. Mumps	Rabbit	Harris and Henle (1948)
11. Newcastle disease virus	Mouse	Woodruff and Woodruff (1970)
	Rat	Woodruff and Woodruff (1970)
12. Parainfluenza I, Sendai	Mouse	Degré (1971)
13. Influenza A	Man	Henle *et al.* (1946); Salk *et al.* (1945); Henle *et al.* (1943); Burden (1933); Keegan (1918); Bloomfield and Harrop (1919); Smorodintsev *et al.* (1937)
	Rabbit	Harris and Henle (1948)
14. Influenza B	Man	Henle *et al.* (1946)
	Rabbit	Harris and Henle (1948)
15. Chickenpox	Man	Holbrook (1941)
16. Adenovirus	Man	Parrott *et al.* (1954); R. G. Douglas *et al.* (1966); Dascomb and Hilleman (1956)

viruses have been reported to produce a lymphocytopenia, adenovirus, and chicken pox, and the data for the latter suggest a reduction in other leukocyte types as well.

In experimental animal systems, the degree of lymphocytopenia has been found to be related to the quantity of inoculum (Harris and Henle, 1948; Woodruff and Woodruff, 1970) and in man, to the severity of the viral illness (Douglas *et al.*, 1966). In most reports, the lymphocytopenia is transient, lasting no more than a few days with lymphocyte counts rapidly returning to normal levels thereafter. In human infections with influenza A or B, the reduction in the number of circulating lymphocytes usually occurs simultaneously with the onset of symptoms (Douglas *et al.*, 1966). In 56 patients with chickenpox and lymphocytopenia, Holbrook (1941) reported that lymphocyte counts reached their lowest level on the second or third day after the appearance of the

rash. A slight reduction in circulating lymphocytes occurred during the incubation period (i.e., 11–1 days prior to the onset of the rash). However, since the data were pooled and individual cell counts were not reported, it is impossible to determine how many of the decreased cell counts were significant.

The transient nature of the lymphocytopenia may explain why several authors have failed to observe its occurrence in patients infected with influenza or adenoviruses (cf. Parrott *et al.*, 1954; Dascomb and Hilleman, 1956; Smorodintsev *et al.*, 1937; Henle *et al.*, 1946; Douglas *et al.*, 1966). Thus, demonstration of lymphocytopenia depends on how soon a patient is first seen by the investigator and the frequency with which cell counts are performed. Therefore, any attempts to correlate lymphocytopenia with a viral infection should be done under the strictest laboratory conditions in which the time of infection and dose of virus administered as well as the immunological status of the volunteer to the virus are precisely controlled.

The mechanism(s) by which a viral infection results in a lymphocytopenia are not known. Few investigators have attempted to study this problem. Evidence suggests that the virus particle itself is responsible. Harris and Henle (1948) reported that inoculation of as little as 0.03 ml. of undiluted allantoic fluid containing either influenza A or B virus into rabbits markedly decreased the peripheral lymphocyte count, whereas 0.003 ml. failed to alter the count significantly. Increase in the injected volume of influenza virus-containing allantoic fluid resulted in a more severe lymphocytopenia. Injection of 20 ml. of either infective allantoic fluid or influenza virus concentrated by centrifugation resulted in widespread necrosis of the liver and spleen and death of the animals within 48 hours. They concluded that the degree of lymphocytopenia was directly related to the number of infective virus particles injected. Woodruff and Woodruff (1970) reported similar results by using Newcastle disease virus (NDV) in mice and rats. Neither Harris and Henle (1948) nor Woodruff and Woodruff (1970) were able to separate the lymphocytopenic effect of the inoculum from the virus particle by centrifugation. Both laboratories also reported that prior incubation of the virus inoculum with virus-specific antisera completely abolished the lymphocytopenic effect of the inoculum.

In one report, the lymphocytopenia was related to increased titers of virus in the blood and spleen (Malkova *et al.*, 1961). Viral replication was not a prerequisite for induction of lymphocytopenia (Woodruff and Woodruff, 1970); NDV exposed to ultraviolet (uv) irradiation for as long as 15 minutes was rendered completely noninfective, but still able to induce marked lymphocytopenia in mice similar to that caused by the

nonirradiated virus. Incubation of the virus inoculum at 56°C for 30 minutes reduced infectivity and both the hemagglutinating and neuraminidase activity of the preparation. In contrast to uv irradiation, this treatment impaired the ability of the inoculum to produce lymphocytopenia (Woodruff and Woodruff, 1970).

Harris and Henle (1948) suggested that virus-induced lymphocytopenia was a result of stimulation of the adrenal glands. This suggestion was prompted by the observations of Dougherty and White (1945) who reported that injection of adrenocorticotropic hormone (ACTH) and adrenal cortical steroids resulted in lymphocytopenia. In order to test this hypothesis, Woodruff and Woodruff (1970) compared the effects of NDV and ACTH in bilaterally adrenalectomized mice. They reported that although the lymphocytopenia associated with injection of ACTH was abolished in these animals, NDV was still effective in lowering the peripheral lymphocyte count.

Lymphocytopenia may result from a direct cytotoxic action of viruses on lymphocytes or lymphoid organs leading to cell destruction. Harris and Henle (1948) noted necrosis in rabbit spleens after injection of large quantities of influenza virus, and Hanaoka *et al.* (1969) reported selective destruction of small lymphocytes in lymphoid and splenic tissues of mice infected with lymphocytic choriomeningitis virus (LCM). Woodruff and Woodruff (1970) showed that injection of mice with NDV resulted in rapid depletion of small lymphocytes from the cortex of lymph nodes and from the periarteriolar lymphoid sheaths of the spleen. As with lymphocytopenia, the depletion was transient with repopulation of the cortex occurring in 72 hours. The same authors also measured the output of small lymphocytes from the thoracic duct of rats similarly infected. Within 12 hours after infection the lymphocyte population had decreased by 60%. By 72 hours, the count had returned to slightly more than normal. These studies suggest that destruction of small lymphocytes is responsible for lymphocytopenia.

The significance of lymphocytopenia in viral disease is not known. An explanation alternative to the direct cytotoxic action of viruses on lymphocytes is the transient sequestration of circulating lymphocytes into various organs of the infected animal. Recent evidence suggests that NDV interferes with the normal pattern of lymphocyte migration (Woodruff and Woodruff, personal communication).

V. Virus–Lymphocyte Associations *in Vivo*

Lymphocytes from diseased and apparently healthy human beings and experimental animals have been found to be associated with viral particles. Virus–lymphocyte associations have been demonstrated by

direct electron-microscopic examination, or isolation of virus either from the leukocyte fraction of peripheral blood, or from lymphocytes cocultivated on feeder cell layers with subsequent cytopathology. In addition, lymphoid cells from individuals having diseases of suspected viral etiology have been placed in long-term cultivation and found by electron microscopy and immunofluorescence to contain herpeslike viruses.

A. Burkitt Lymphoma

A herpeslike virus, first described by Epstein *et al.* (1964) and referred to thereafter as the *Epstein-Barr virus* has frequently been seen by electron microscopy in different cell lines of Burkitt lymphoblasts maintained in culture (Epstein *et al.*, 1965; Stewart *et al.*, 1965; O'Conor and Rabson, 1965; Epstein *et al.*, 1967; Epstein and Achong, 1968a,b). Attempts to isolate EBV have been uniformly unsuccessful. Early isolations of a herpeslike virus from a small proportion of biopsy specimens (Woodall and Haddow, 1962; Simons and Ross, 1964) of reovirus (Bell *et al.*, 1964) and an as yet unidentified agent (Dalldorf and Bergamini, 1964) from cases of Burkitt lymphoma have not been confirmed.

Epstein-Barr virus is found in both the nucleus and cytoplasm of cultured Burkitt lymphoblasts (Epstein *et al.*, 1965). Approximately 1–2% of the cells contain the virus which exists in two forms: (*1*) an immature particle with a diameter of 75 mμ found in both nucleus and cytoplasm and (*2*) a larger particle with a diameter of 110 to 115 mμ found on the cell surfaces and within cytoplasmic membrane-bound vesicles.

Immunofluorescent and complement fixation (CF) tests have demonstrated the presence of EBV antigens in freshly biopsied material and in cultured lymphoblasts from patients with Burkitt lymphoma. The immunofluorescent test for EBV capsid antigen (Henle and Henle, 1966b) has proved useful in serological surveys and also has allowed investigators to monitor the levels of EBV in continuous lymphoblastic cell lines. Three types of antigens have been demonstrated in EBV-carrying cell lines: (*1*) a viral capsid antigen found inside cells containing virus-like particles (Henle and Henle, 1966a,b); (*2*) a membrane-associated antigen (Klein *et al.*, 1966, 1967); and (*3*) an early antigen found in both the nucleus and cytoplasm of experimentally infected blastoid cell lines (Henle *et al.*, 1970). Pope *et al.* (1969) and Walters and Pope (1971), by using the CF technique, also were able to identify three virus-related components in EBV-infected continuous cell lines: (*1*) an unenveloped, heat-stable EBV particle, (*2*) a heat-labile, subviral particulate antigen, and (*3*) a heat-resistant soluble antigen. All cell lines

studied which contained virus, as demonstrated by electron microscopy or immunofluorescence, harbored these antigens. Another cell line, Raji, derived originally from Burkitt's lymphoma and later shown to be virus-free, contained only the heat-resistant soluble antigen. This latter cell line may, therefore, contain a defective EBV incapable of forming complete virus particles (zur Hausen and Schulte-Holthausen, 1970). Further studies are required in order to relate the antigens detected by immunofluorescence to those demonstrated by CF and to associate these antigens with the two viruslike particles within cells described by Epstein *et al.* (1965).

B. Infectious Mononucleosis

Lymphocytes removed from patients with IM can be maintained in long-term cultures with comparative ease (Henle *et al.*, 1968; Diehl *et al.*, 1968; Pope, 1967; Chessin *et al.*, 1968; Glade *et al.*, 1969). A small proportion of the cells in each culture (0.1–6%) harbors EBV as demonstrated by immunofluorescence and electron microscopy (Chessin *et al.*, 1968; Diehl *et al.*, 1968; Henle *et al.*, 1968; Klein *et al.*, 1968; Moses *et al.*, 1968). Unlike the reports concerning EBV and Burkitt lymphoma, the demonstration of EBV antigen in freshly isolated or cultured lymphocytes has been more difficult. Epstein-Barr virus has not been isolated nor has it been seen in leukocytes taken directly from patients with IM either during acute disease or after recovery, when all individuals have virus-specific antibodies (Henle *et al.*, 1968; Evans *et al.*, 1968; Marker, 1970). When the leukocytes are placed in culture, however, three different kinds of particles eventually emerge (Moses *et al.*, 1968): (*1*) a herpeslike virus particle with a diameter of 90 to 100 mμ and found in the cell nucleus. Larger, enveloped particles (160 mμ in diameter) are often found outside the cells in the medium, (2) small (25–50 mμ) extracellular particles, and (3) small particles (22 mμ) budding from the inner surface of membranes of the rough endoplasmic reticulum. The relationship of these particles to the disease is unknown. Moses *et al.* (1968) and Moore *et al.* (1967) have observed similar virus particles in leukocyte cultures derived from apparently normal individuals or patients with disorders other than IM.

C. Other Viral Infections

1. Man

Herpeslike viruses have been seen in lymphocytes from certain blood dyscrasias such as chronic myelogenous leukemia (Chandra *et al.*, 1971),

idiopathic thrombocytopenia purpura (Sincovics, 1969), and various types of leukemia (Jensen *et al.*, 1967; Rauscher, 1968). In addition, lymphoma and leukemia buffy coats have been reported to contain viruslike particles (Moore and Ito, 1966; Moore *et al.*, 1966; Zeve *et al.*, 1966), but, as mentioned previously, Moore *et al.* (1967) and Moses *et al.* (1968) have also seen these particles in cultured lymphocytes and leukocytes derived from normal healthy individuals.

Continuous lymphoblastoid cell lines derived from patients with serum (Glade *et al.*, 1968; S. D. Douglas *et al.*, 1969b) and infectious hepatitis have been shown to contain EBV (Stevens *et al.*, 1969, 1970). In the latter studies, all individuals from whom lymphoblastoid cell lines were established had had prior exposure to EBV as indicated by the presence of EBV antibodies in their serum at the time blood was obtained. The EBV was detected in six of eight cell lines by electron microscopy. The authors felt a role for EBV as an etiologic agent of infectious hepatitis was unlikely because (*a*) no significant rises in anti-EBV antibodies were evident during infection and (*b*) EBV is structurally different from and more labile than the purported agent of viral hepatitis.

Rubella virus has been isolated from leukocyte cultures obtained from infants with rubella syndrome (Jack and Grutzner, 1969). Cytomegalovirus was isolated from cultured cells of infants suffering from congenital cytomegalovirus (Lang and Noren, 1968) or from hepatitis (Stulberg *et al.*, 1966). Fresh or relatively fresh, transfused blood was implicated in cytomegalovirus infection of open heart surgery patients (Paloheimo *et al.*, 1968). Because free virus has not been demonstrated in the circulation of humans infected with cytomegalovirus but has been found associated with the buffy coat layer (Stulberg *et al.*, 1966; Lang and Noren, 1968), it is probable that in Paloheimo's study the virus was cell-bound. Papp (1937) was able to transmit measles virus infections to healthy human volunteers by using washed leukocytes from the buffy coats of infected individuals.

Measles virus was isolated in Hela cells cocultivated with lymph node cells taken from 2 of 5 patients with subacute sclerosing panencephalitis. The virus was identified by hemagglutination-inhibition, immunofluorescence, and neutralization tests (Horta-Barbosa *et al.*, 1971).

2. *Animals*

Viruses have been found in lymphocytes or leukocytes taken from chickens, cows, guinea pigs, chimpanzees, and horses (no attempt will be made here to review the literature concerning avian, murine, and feline leukemia viruses).

A herpeslike virus was found in circulating lymphocytes of chickens suffering from Marek's disease (Wight *et al.*, 1967)—an infectious lymphoproliferative disorder characterized by neural infiltration with lymphocytes and plasma cells. As many as twelve particles (diameter approx. 90 mμ) could be seen within cytoplasmic vesicles of infected lymphocytes. Only a small proportion of the cells contained these particles, however. The disease could be transmitted by injecting infected whole blood into normal healthy chickens which also developed virus particles within their lymphocytes. Lymphocytes placed in culture with PHA became transformed, and most of these transformed cells contained herpeslike viruses in their nuclei (Campbell and Woode, 1970). The majority of these particles were immature, having a single membrane surrounding a nucleoid measuring approximately 94 × 74 mμ in diameter. Empty and double-membraned particles were also seen but less frequently. They measured 120 mμ and were seen within vesicles in the nucleus. Campbell and Woode were not able to locate any of the particles in the cytoplasm. The addition of fresh or cultured lymphocytes from chickens infected with Marek's disease to healthy chick kidney monolayers induced a cytopathic change in the monolayers typical of that seen when monolayers were established from Marek diseased chickens. Indirect immunofluorescence indicated the presence of viral antigen in a high proportion of the transformed lymphocytes or cocultivated kidney monolayers.

Four groups of investigators have reported type C particles in leukocytes of cows with bovine lymphosarcoma (Dutta and Sorensen, 1969; Theilen and Kawakami, 1969; Miller *et al.*, 1969; Lee *et al.*, 1970). These particles, measuring 90–120 mμ, were seen in both mixed leukocyte and in lymphocyte cultures stimulated with PHA, were found primarily extracellularly, and were rarely seen budding from cell surfaces. Particles of this description were observed in lymphocytes from 15 of 24 cattle in a multiple-case herd, in 15 of 26 cattle injected with lymphosarcoma material, and in 2 of 16 aged cows from a herd with no history of lymphosarcoma (Miller *et al.*, 1969).

Herpeslike virus particles were found in leukocytes taken from guinea pigs infected for varying periods of time with a guinea pig herpeslike virus (Hsiung *et al.*, 1971). These authors were unable to see the virus in the leukocytes unless the cells were cultured for 3 to 22 days. Even so, the number of cells harboring viruslike particles was low (0.3–8%). Chimpanzees were inoculated with human lymphoblastoid cell lines and concentrated herpeslike virus particles isolated from these cells (Landon *et al.*, 1968). Lymphoblastoid cell cultures were successfully established from 8 of the 11 inoculated chimpanzees. One of the cell

lines appeared to be of human origin and may have come from the initial inoculum. From 1 to 60% of the cells from the various cultures contained herpeslike viruses, similar in appearance to the virus particles seen originally in the inoculated cells. Attempts to isolate the virus from the chimpanzee lymphoblastoid cell lines by using herpes virus-susceptible, monkey, kidney cell cultures were unsuccessful.

By immunofluorescence, Benda *et al.* (1970) were able to demonstrate viral antigens within lymphocytes, monocytes, and atypical mononuclear cells from guinea pigs infected with LCM. The number of leukocytes showing immunofluorescence paralleled increasing titers of LCM in the plasma. Large fluorescent granules were seen in the cytoplasm of the leukocytes at later stages in the infection, demonstrating clearly that the virus was not only attaching to the surface of the circulating leukocytes but was also penetrating into the cells and probably replicating. (See review by Sommerfield, 1968, for a discussion on detecting viral antigens in PMNs and macrophages by immunofluorescence.)

The precise role of lymphocyte-associated virus in some pathological conditions is unclear, even in the more intensively studied models, namely IM and Burkitt's lymphoma. Since EBV is ubiquitous, one must exercise caution in assigning this virus the role of an etiologic agent. As has been mentioned, herpeslike viruses have been found in normal lymphocytes from man (Moore *et al.*, 1967; Moses *et al.*, 1968) and other animals, e.g., horses (Kemeny and Pearson, 1970). Therefore, EBV may have only a passenger relationship in Burkitt's lymphoma and IM.

In most of the studies mentioned, virus was not discernible in freshly isolated cells, but was seen only after cultivation of these cells for several days or even weeks. The addition of PHA to some cultures improved the chances of demonstrating virus. That the virus was infectious in some of the studies was shown by the fact that primary or secondary cells cocultivated with leukocytes from animals with various disorders underwent cytopathic changes; however, not all attempts have been successful.

Cells which eventually develop EBV or EBV antigens can be established in continuous culture with relative ease. Stevens *et al.* (1970) hypothesized that EBV was necessary for the establishment of these cultures. Prior exposure of patients to EBV could result in the virus becoming associated with leukocytes, and upon removal from the peripheral circulation, those cells containing the virus would begin to proliferate and at the same time produce virus or viral antigens. Isolation of EBV and other viruses, such as rubella or measles, from leukocytes of infected individuals may merely reflect an attraction of the leukocyte

cell surface for these viruses. Therefore, it remains to be seen whether virus–leukocyte interaction is an integral part of the infectious process or merely a fortuitous association.

VI. Effect of Viruses on Lymphocytes

A. *In Vitro* Studies

1. The Stimulated Lymphocyte

Lymphocytes removed from the peripheral circulation and placed in cell culture remain viable for several weeks. For the most part these lymphocytes are metabolically dormant. Only 0.06% of freshly isolated lymphocytes incorporate thymidine-^{3}H (Bond *et al.*, 1958). After approximately 5 days, however, some of the cells undergo spontaneous transformation, and as many as 40–70% can become blastoidlike with increased DNA, RNA, and protein synthesis (Johnson and Russell, 1965).

Lymphocytes can be transformed earlier if certain nonspecific mitogenic substances, such as PHA (Hungerford *et al.*, 1959; Nowell, 1960), concanavalin A (Olson and Liener, 1967), pokeweed (Farnes *et al.*, 1964), and certain bacterial products including staphylococcal exotoxin (Ling and Husband, 1964) or streptolysin S (Hirschhorn and Schreibman, 1964), are added to the culture medium. During transformation, the stimulated lymphocytes synthesize DNA, RNA, and proteins at rates greatly increased over those present in unstimulated lymphocytes. This activity is usually of short duration, however, and metabolic rates eventually fall as the cells in the culture die (Ling, 1968, pp. 175–192). In addition, the number of lysosomes per cell increases as does the activity of the enzymes usually associated with these structures (e.g., acid phosphatase and dehydrogenase activities). Increases in metabolism and, likewise, enzymatic activities usually reach their peak between 2 and 3 days after stimulation, and the peaks are dependent upon the concentration of mitogen used. Deoxyribonucleic acid synthesis in small lymphocytes isolated from normal human peripheral blood begins to increase at about 30 hours after the addition of PHA to the cultures, reaches its peak at 44 to 60 hours, and remains there for 12 to 24 hours more (Michalowski, 1963; Cooper *et al.*, 1961; McIntyre and Ebaugh, 1962). Ribonucleic acid synthesis in PHA-stimulated cells begins as early as 0.5 hour after addition of the mitogen (McIntyre and Ebaugh, 1962; Torelli *et al.*, 1963; Epstein and Stohlman, 1964) and continues to rise until 48 to 60 hours at which time the rate of RNA synthesis remains constant. Protein synthesis parallels RNA synthesis. Some of

the products produced by stimulated lymphocytes will be discussed in Section VI,C.

In addition to being stimulated nonspecifically, lymphocytes can be transformed by specific substances to which the lymphocytes are sensitized. Some commonly used substances for sensitization are tuberculin purified protein derivative (PPD) (Marshall and Roberts, 1963; Cowling *et al.*, 1963; Witten *et al.*, 1963), tetanus toxoid (Elves *et al.*, 1963), penicillin (Hirschhorn *et al.*, 1963), and smallpox, poliovirus (Elves *et al.*, 1963), measles (Bach and Hirschhorn, 1965), and pertussis (Hirschhorn *et al.*, 1963) vaccines. Specific stimulation of lymphocytes differs from nonspecific stimulation in several ways. Fewer cells enter blastogenesis during specific stimulation; the number of cells responding to the stimulus may vary from less than 5 to approximately 50%; however, usually no more than 5% of the cells become stimulated (Ling and Husband, 1964; Cowling *et al.*, 1963). Cowling *et al.* (1963) also reported that stimulation proceeded more slowly in response to specific antigens. The type of RNA made in specifically stimulated lymphocytes is of a different size than the RNA synthesized in nonspecifically stimulated cells (Cooper and Rubin, 1965a,b; Rubin and Cooper, 1965).

In summary, lymphocytes transformed by either specific or nonspecific stimuli undergo blastogenesis with increased rates of protein, RNA, and DNA synthesis. Concomitantly with increased metabolic rates, there is an increase in the total volume of the cell and development of the endoplasmic reticulum. All of these changes in stimulated lymphocytes might therefore be expected to favor virus replication.

2. *Viral Replication in Stimulated Lymphocytes*

Virus–lymphocyte interactions have been studied *in vitro* primarily in terms of viral replication and interferon production. Viruses that have been shown to replicate in human lymphocytes are listed in Table III; for comparison, replication of viruses in other leukocytes or in mixed leukocyte cultures have been included. As was indicated earlier, only stimulated lymphocytes can support viral replication. In the case of EBV, the virus particle itself acts as a mitogen and stimulates blastoid transformation of lymphocytes thereby permitting viruses to replicate (Gerber *et al.*, 1969a). The PMNs and unstimulated lymphocytes are not capable of supporting viral replication. The monocyte–macrophage fraction of peripheral blood was found to support replication of all viruses which were tested in these cells.

a. Measles Virus. Measles virus has been shown to be closely associated with peripheral leukocytes during viremia. Hektoen (1905),

TABLE III

VIRAL REPLICATION AND INTERFERON PRODUCTION IN HUMAN LEUKOCYTES *in Vitro*[a,b]

Virus	Mixed leukocytes				Lymphocytes				Monocytes–macrophages		References
	No PHA		With PHA		No PHA		With PHA				
	VR	IF	VR	IF	VR	IF	VR	IF	VR	IF	
Measles	+	+	ND	ND	ND	ND	ND	ND	ND	+	Berg and Rosenthal (1961); Gresser (1961)
Echovirus	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	Berg (1961)
Poliovirus	+	ND	+	ND	0	ND	+	ND	+	ND	Gresser and Chany (1964); Willems *et al.* (1969a,b)
Mumps	0	+	+	+	ND	ND	ND	ND	ND	ND	Duc-Nguyen and Henle (1966); Willems *et al.* (1969b)
Newcastle disease	+	+	ND	+	0	+	ND	ND	ND	ND	Duc-Nguyen and Henle (1966); Wheelock (1965, 1966)
Vaccinia	0	ND	+	ND	0	ND	+	ND	ND	ND	Miller and Enders (1968); Willems *et al.* (1969b)
Herpes simplex[c]	0	ND	+	ND	ND	ND	ND	ND	ND	ND	Nahmias *et al.* (1964)
Vesicular stomatitis	+	0	+	+	0	0	+	+	+	0	Duc-Nguyen and Henle (1966); Edelman and Wheelock (1966, 1967, 1968a); Willems *et al.* (1969b)
17D Yellow fever	+	+	+	+	0	+	+	+	+	+	Wheelock and Edelman (1969)
Sendai	ND	+	ND	ND	ND	+	ND	ND	ND	+	Duc-Nguyen and Henle (1966); Gresser (1961); Lee and Ozere (1965)
Rubella	ND	ND	ND	ND	ND	ND	ND	+	ND	ND	Mellman *et al.* (1965); Olson *et al.* (1968)

[a] Polymorphonuclear leukocytes have not been shown to support virus replication or interferon production.

[b] +—Virus replication or interferon production; 0—no virus replication nor interferon production; ND—not done; VR—viral replication; IF—interferon; PHA—phytohemagglutinin.

[c] For a discussion of Epstein-Barr virus replication, see Section VI,B,*5*.

using whole blood, and Papp (1937), using washed leukocytes, were successful in transmitting measles infections from patients to healthy individuals.

Leukocytes derived from healthy human subjects and from 2 leukemic patients supported viral replication when infected with an attenuated strain of measles virus passed in human kidney cell cultures (Berg and Rosenthal, 1961). Virus titers peaked 4–7 days after infection with an increase of 2.1 $\log_{10}$ $TCID_{50}$ in titer over the input virus. In one experiment, no viral replication occurred. Attempts to grow measles virus in leukocytes from chickens, dogs, rabbits, mice, and guinea pigs were unsuccessful. The only animal leukocytes other than human which supported viral replication were monkey. Leukocytes from a single monkey that had been immunized with attenuated measles viruses supported viral replication in only one of three experiments. Although the monkey leukocytes had been washed before infection with virus, the possibility remained that antibody against measles had entered the culture.

b. Enteric Viruses. Berg (1961) tested, in leukocytes, the replication of two other viruses shown to cause viremias in infected individuals, namely poliovirus and enteric cytopathic human orphan virus-9 (ECHO-9). The latter virus replicated slowly in human leukocyte cultures and not at all in suspensions of red blood cells or rabbit leukocytes. Poliovirus failed to replicate in any of the cultures.

Gresser and Chany (1964) reported the propagation of poliovirus Type I in human leukocytes. Titers up to 10^7 $TCID_{50}$/ml. were readily obtained. Viruses were added to leukocytes at inception of the culture, yet no replication took place until the third or fifth day after which viral titers rose rapidly. If leukocytes were maintained in culture for 3 or 4 days before infection with poliovirus, no lag was seen. These experiments were significant because a cell type other than PMNs was implicated in the replication of the virus, since most PMNs are gone from leukocyte preparations after 3 days in culture. In addition, since small lymphocytes were reported to transform spontaneously into blastoidlike cells after several days in culture, it was likely, in view of Gresser and Chany's data that lymphocytes were the target cells for poliovirus replication. The authors also reported that human interferon added to the leukocyte cultures inhibited replication of poliovirus.

Further evidence implicating lymphocytes as one of the cell types supporting poliovirus replication was reported by Willems *et al.* (1969a,b). The addition of PHA to leukocyte cultures enhanced viral replication and increased the rate at which virus was taken up by cells. These studies also showed that the PHA was increasing the number of

cells able to replicate virus and not just increasing the capacity of cells in unstimulated cultures to produce more virus, since the yield of approximately 10 P. F. U. virus/cell was alike for both stimulated and unstimulated cultures. Based on their data, Willems *et al.* (1969b) suggested that monocytes were the cell type initially infected and producing viruses which then spread to PHA-stimulated lymphocytes undergoing blastogenesis.

Specific stimulation of lymphocytes from human donors immunized with poliovirus vaccine and tetanus toxoid rendered these cells capable of supporting poliovirus replication (Willems and Vondrovicova, 1970). Viral titers were 1–1.5 log greater in antigen-stimulated lymphocytes than in unstimulated cultures. Tuberculin-stimulated lymphocytes derived from Mantoux-positive donors also supported the replication of either measles or poliovirus. Viral replication in specifically stimulated lymphocytes has also been reported when heterologous antilymphocyte serum (ALS) (Edelman and Wheelock, 1968b) and PPD were used (Wheelock and Toy, unpublished data).

c. Myxoviruses. Only two members of the myxovirus group, namely mumps and NDV, have been shown to replicate in leukocyte cultures (Duc-Nguyen and Henle, 1966; Wheelock, 1966). Unlike the viruses mentioned so far, mumps viruses would only replicate in cultures exposed to PHA. When leukocytes were infected on the same day that the cells were placed in culture, there was a delay of approximately 3–4 days before viruses could be detected. If, however, as Gresser and Chany (1964) had done, the cells were maintained in culture in the presence of PHA for 4 to 5 days, viral replication could be detected within 48 hours after infection. In the latter case, more than 50% of the cells contained viral antigen, whereas cultures infected on the same day that the cells were prepared showed only rare cells containing viral antigen within the first 48 hours. Despite the number of cells presumably producing virus, reported titers did not exceed 1.7×10^4 P.F.U./ml.

With the immunofluorescent antibody technique, Duc-Nguyen and Henle (1966) were also able to demonstrate viral replication in NDV- and VSV-infected leukocytes. After incubation of uv-irradiated NDV with leukocyte cultures, no cells demonstrated immunofluorescence, thus confirming that the immunofluorescence seen in unirradiated NDV infections was due to replication and not just to attachment of NDV to cell surfaces.

Further evidence for the replication of NDV in human leukocyte cultures was provided by Wheelock (1966). Newly made viral antigen in leukocyte cultures was first detected 4 hours after NDV infection and accumulated until approximately 14 hours at which time the cells began

to show marked cytopathology. New infective virus was detected 7 hours after infection and persisted in the cultures for approximately an additional 65 hours. Interferon production also occurred simultaneously with viral replication.

Wheelock (1966) compared the production of interferon and NDV in leukocyte and pure lymphocyte cultures maintained for varying periods of time at 37°C before infection. Interferon production decreased steadily with *in vitro* aging of the leukocytes or lymphocytes. This effect of aging could be detected as early as 2 hours after incubation; after 48 hours in culture, no interferon was produced upon the addition of NDV. In contrast, no differences in interferon production were seen when PHA was added to leukocyte cultures of different ages.

Leukocyte cultures aged for 48 hours before NDV infection produced more infective virus than did fresh cultures. Enhanced viral replication probably was a result of the inability of leukocytes to produce interferon. Wheelock suggests that this alteration of the interferon response might explain the reported shortened eclipse period for poliovirus in aged leukocyte cultures (Gresser and Chany, 1964).

d. Vaccinia. The only DNA viruses reported to replicate in human leukocyte cultures are vaccinia virus (Miller and Enders, 1968) and herpes simplex (Nahmias *et al.*, 1964; Gönczöl *et al.*, 1969; Bouroncle *et al.*, 1969, 1970). Attempts to demonstrate cytomegalovirus and varicella zoster viral replication in human leukocyte cultures have failed (Gönczöl *et al.*, 1969).

Vaccinia viral replication began within 8 hours after infection of PHA-treated leukocytes and reached its peak at approximately 72 hours (Miller and Enders, 1968). The yield of virus was calculated to be 30–40 P.F.U./cell with approximately 60% of the cells producing virus. Infected cells slowly degenerated in culture; the most striking alteration in morphology consisting of fragmentation of the nuclei or condensation of chromatin material within the nuclei of infected cells. In their studies, Miller and Enders showed that vaccinia virus either entered or at least attached to nonstimulated leukocytes, that PHA was required for viral replication, and that replication required the continued presence of PHA. Leukocytes which were washed after exposure to PHA for 3 days before infection did not support viral replication to any great extent unless PHA was added back to the culture medium.

e. Herpesvirus. Herpes simplex virus was first reported to replicate in human leukocytes by Nahmias *et al.* (1964). No virus replication was demonstrated unless the cultures were exposed to PHA. Herpes simplex virus titers began to increase between 4 and 6 days after infection and continued to rise until the eighteenth day. Virus titers dropped slowly

thereafter, and virus was still present in the culture medium 26 days after infection. No differences were noted in the degree of viral replication in leukocytes obtained from donors with different levels of antibodies against herpes simplex in their serum. Gönczöl *et al.* (1969) also demonstrated the need for the continued presence of PHA in order for virus to replicate, confirming the earlier observations of Miller and Enders (1968). The addition of PHA to cultures 1 day before viral infection did not accelerate the appearance of virus after infection (Gönczöl *et al.*, 1969; Nahmias *et al.*, 1964). In addition to using leukocytes from healthy donors, Gönczöl *et al.* (1969) infected cells from 10 patients with chronic myeloid leukemia in both the presence and absence of PHA. Neither herpes simplex nor cytomegalovirus would replicate in these cultures.

Contrary to previous reports (Nahmias *et al.*, 1964; Gönczöl *et al.*, 1969), Bouroncle *et al.* (1970) observed maximum titers of herpes simplex virus in leukocyte cultures at 48 hours after infection. By using electron microscopy, these authors were able to find viruslike particles within the cytoplasm of some cells and in the intracellular spaces at 6 hours, but not at 2 hours after infection. At 48 hours, nearly every cell examined contained herpeslike virus particles.

The EBV, a member of the herpesvirus group, was first described in association with lymphoblastoid continuous cell lines derived from patients with Burkitt's lymphoma (Epstein *et al.*, 1964). Since then, this virus has been detected in several other continuous cell lines derived from patients with leukemia, IM, hepatitis, and from apparently normal individuals (see Sections III and V). Viral replication has been measured by the continued presence of EBV in dividing cell cultures and by the presence of viral antigens detected by immunofluorescence and CF (Gerber and Deal, 1970). Unlike herpes simplex virus, EBV can replicate in leukocyte cultures without the addition of PHA or other mitogens (Gerber *et al.*, 1969a). *In vitro* infection of normal leukocyte suspensions with EBV stimulates DNA synthesis in lymphocytes and causes them to undergo blastogenesis (Gerber and Hoyer, 1971). In this respect, the virus acts as its own mitogen. The resulting lymphoblastoid cells can then be kept in continuous culture indefinitely. These cells harbor EBV and show morphological characteristics similar to those seen in other lymphoblastoid cell lines containing EBV, namely chromosomal aberrations and the presence of an extra Group C, No. 10 chromosome, diffuse staining of the nucleus, and changes in the endoplasmic reticulum (Kohn *et al.*, 1967; Miles and O'Neill, 1967; Tomkins, 1968; Cooper, 1969; Finch, 1969). Uninfected control cultures or leukocytes exposed

to material from an EBV-free lymphoblastoid cell line did not undergo blastogenesis and rarely survived beyond 45–50 days.

f. Vesicular Stomatitis Virus. In order to determine the effect of PHA on viral replication in human leukocytes *in vitro*, Edelman and Wheelock (1966, 1967, 1968a) studied the interaction of VSV with various cell types present in mixed leukocytes, both in the presence and absence of PHA. They showed that VSV would replicate in mixed leukocyte cultures in the absence of PHA (Edelman and Wheelock, 1966). Unlike the reports for poliovirus and mumps (Gresser and Chany, 1964; Duc-Nguyen and Henle, 1966), prior addition of PHA to leukocyte cultures delayed viral replication 2–4 hours. However, when viral replication did occur, titers were 2 logs higher than in nontreated cultures.

Analysis of this paradoxical observation required preparation of relatively pure cultures of each leukocyte type. This was accomplished by separating the monocyte–macrophage fraction from the lymphocytes by passage of leukocyte-rich plasma through columns of glass beads according to the method of Rabinowitz (1964). The PMNs and monocytes that had adhered to the column of glass beads were selectively eluted using ethylenediaminetetraacetic acid (EDTA) buffered in saline. Thus cultures rich in each cell type were obtained. Lymphocyte cultures could be established which contained less than 1% of other type cells; PMN cultures and cultures of monocytes greater than 95% pure likewise could be prepared.

Vesicular stomatitis virus was shown to replicate in monocyte and PHA-stimulated lymphocyte cultures but not in PMN or unstimulated lymphocyte cultures. Addition of PHA to lymphocytes induced interferon production but stimulated these cells to produce virus eventually after they underwent blastogenesis. If exogenous interferon (i.e., interferon prepared in cells other than those being treated) was added to unstimulated mixed leukocyte cultures and retained in the culture medium after viral infection, no viral replication occurred. Thus, it was evident, that the delay in viral replication seen in cultures exposed to PHA before infection was caused by interferon. The addition of PHA to cultures of leukocytes induced interferon synthesis in lymphocytes. This interferon was taken up by monocytes and inhibited VSV replication in these cells. The PHA-stimulated lymphocytes overcame the interferon effect and were able to support VSV replication to higher titer (Edelman and Wheelock, 1966, 1967, 1968a).

The effect of varying doses of PHA on lymphocytes was studied in relationship to the degree of stimulation of the cells' metabolism (as measured by the incorporation of uridine-^{3}H into RNA) and the amount

of virus produced (Wheelock *et al.*, 1972a). A critical level of stimulation was necessary in order for virus synthesis to occur. In these studies, 0.05 μg. of PHA induced a twofold increase in incorporation of uridine but had no effect on virus replication. Addition of 0.5 μg. PHA brought about a tenfold increase in viral replication with a fourfold increase in uridine incorporation. Addition of 5 μg. of PHA to the cultures brought about a peak titer of virus 3 $\log_{10}$ greater than the input virus and a ten- to twenty-fold increase in incorporation of uridine-^{3}H. By using lymphocytes from tuberculin-positive individuals, it was also shown that the degree of stimulation caused by the specific sensitizing antigen, PPD, was related to the amount of interferon released and the titer of virus produced.

g. 17D Yellow Fever Virus. In an attempt to relate the VSV experiments to human viral infections, another virus was selected, the 17D strain of yellow fever (17D virus). This virus produces an asymptomatic infection in man associated with transient low levels of virus and interferon in the blood (Wheelock and Sibley, 1965), followed by the development of long-lasting immunity to the wild strain of yellow fever virus. This virus was chosen because experiments could be conducted under highly controlled conditions; specifically the dose of virus and the time and site of inoculation could be precisely controlled. In addition, the exact immune status of human volunteers could be determined prior to vaccination.

As had been reported with VSV, 17D virus replicated in unstimulated mixed leukocyte cultures with peak titers reached on the third or fourth day (Wheelock and Edelman, 1969). Addition of PHA to the cultures delayed the peak viral titers by 6 to 8 days, and virus titers were generally 1 $\log_{10}$ lower than those in untreated cultures. Inclusion of interferon into the culture medium of mixed leukocytes prevented 17D virus from replicating.

Replication of 17D virus in homogeneous cultures of monocytes, PMNs, and lymphocytes was studied. Virus replicated to high titers in monocyte cultures, both in the presence and absence of PHA; however, viral titers were somewhat lower in PHA-treated monocytes and peaked usually one day later than in untreated preparations. Low levels of interferon were measured in the medium from PHA-treated monocytes. No viral replication was noted in relatively pure cultures of PMNs or unstimulated lymphocytes. Unlike VSV, however, exposure of lymphocytes to 17D virus resulted in the production of low levels of interferon in the culture medium. The addition of PHA to infected lymphocyte cultures resulted in 17D viral replication with the production of interferon.

In order to study the role of lymphocytes in the defense against 17D virus infection, pure lymphocyte cultures were prepared from the peripheral blood of human donors on various days after vaccination and studied *in vitro* for their ability to support 17D viral replication (Wheelock *et al.*, 1970).

Several attempts to detect 17D viral replication in leukocytes and lymphocytes removed on various days after vaccination and placed in culture in the presence of PHA proved fruitless. This was disappointing since 17D virus produces a transient viremia in vaccinated individuals (Wheelock and Sibley, 1965), and viruses have been shown to be associated with leukocytes isolated from the peripheral blood (Papp, 1937; Berg and Rosenthal, 1961).

Viral replication occurred, however, if PHA-stimulated cultures prepared from donors during the first 5 days postvaccination were infected *in vitro* with 17D virus. Under the same conditions, lymphocytes removed on the seventh through fourteenth day failed to produce infective virus. By the twenty-fifth day, however, peripheral lymphocytes had regained their ability to support 17D viral replication.

The mechanism involved in the transient loss of ability of postvaccination lymphocytes to support 17D viral replication was studied in a variety of ways (Wheelock *et al.*, 1972a). When the PHA response of lymphocytes removed from the peripheral circulation on various days after vaccination was studied by measuring the incorporation of thymidine-^{3}H, no significant differences were seen. In addition, the 17D virus refractory lymphocytes produced no greater amounts of interferon when stimulated with PHA than normal lymphocytes with PHA. No 17D virus-specific neutralizing antibody could be detected in the culture medium of virus refractory lymphocytes, and all attempts failed to detect virus-specific immunoglobulins on the surface of PHA-stimulated or unstimulated lymphocytes.

Finally, the specificity of the 17D virus refractory state was tested by studying the replication of VSV in these lymphocytes. It was found that VSV replicated equally well in normal lymphocytes and in lymphocytes removed from the circulations of donors on various days following 17D virus vaccination. Thus, the refractory state was immunologically specific.

Further studies on 17D virus refractory lymphocytes revealed that these cells could inhibit viral production in a 17D virus, persistently infected human cell line (Toy and Wheelock, 1970; Wheelock *et al.*, 1972a), whereas normal lymphocytes showed no effect. Cytotoxic factors similar to those released by LCM-immune lymphocytes on exposure to the virus (Oldstone and Dixon, 1970) were sought but not found. In

addition, no direct cytotoxic action of virus refractory lymphocytes on virus-producing normal lymphocytes was found.

h. Tick-Borne Encephalitis (TE) Virus. The TE virus replicates in suspension cultures of human leukocytes. Employing two strains of TE virus, Mayer and Dobrocká (1969) demonstrated replication only of the more virulent strain. Approximately 40% of the cells produced viral antigen as shown by specific immunofluorescence. No fluorescence was seen in cells infected with the attenuated TE strain.

B. Products of Virus–Lymphocyte Interactions

When lymphocytes are stimulated by either nonspecific agents, such as PHA, or specific antigens to which they have been previously sensitized, they release a number of factors that affect neighboring cells. Not all of these factors have, as yet, been demonstrated during the course of viral infections. Factors that are clearly involved in the host defense include interferon, an inhibitor of viral replication, and lymphotoxin, a cytotoxic factor which may be involved in destruction of virus-infected cells.

1. Interferon

a. Unstimulated Lymphocytes. As mentioned previously, virus–lymphocyte interactions have been studied primarily in terms of viral replication and interferon production. Interferon, a protein with a molecular weight of approximately 30,000 daltons, is produced by cells in response to a wide variety of substances—most notably viruses, viral nucleic acid, synthetic polynucleotides, and extracts from assorted microorganisms. It can inhibit most viruses and has been found to be effective both *in vitro* and *in vivo* (Vilcek, 1969). Table III lists viruses that can induce interferon production in human leukocytes and in cultures of unstimulated lymphocytes without PHA. The one virus that failed to produce interferon in these latter cultures was VSV; also, of all the viruses tested, VSV alone did not induce interferon in monocyte–macrophages. Perhaps the rapidity with which this virus replicates accounts for its inability to induce interferon (Edelman and Wheelock, 1966, 1967, 1968a,b). Production of interferon in human PMNs has not been demonstrated.

Factors that affect virus-induced interferon production in human leukocyte suspensions have been described by numerous investigators (e.g., Gresser, 1961; Wheelock, 1966; Lee, 1969; Strander and Cantell, 1966; Gresser and Chany, 1964; Strander and Cantell, 1967; Falcoff *et al.*, 1966; Strander, 1969; Horowitz *et al.*, 1970). Since most of these investigators used leukocytes mainly as a source of interferon and not for the purpose of studying virus–lymphocyte interactions directly, their

work is not described in detail. Some conditions that affect interferon production in leukocyte cultures include: (*1*) the type of virus used, (*2*) the state of the virus, e.g., active or inactive, (*3*) the multiplicity of infection, (*4*) the method used for collection of blood, (*5*) the age of the leukocyte cultures, (*6*) the temperature of incubation, and (*7*) the concentration of serum present in the culture medium.

Freshly isolated leukocytes from patients with chronic lymphocytic leukemia produced markedly less interferon in response to virus inoculation than did normal leukocytes (Gresser, 1961; Lee *et al.*, 1966; Hadhazy *et al.*, 1967; Strander *et al.*, 1970). In contrast, leukocytes from 3 patients with congenital agammaglobulinemia produced normal amounts of interferon *in vitro* (Gresser, 1961).

Bone marrow cell cultures from patients with leukemia and other diseases were studied for the presence of interferon (Northrop and Deinhardt, 1967). Of the 356 bone marrow cultures tested, only 17% contained interferon. No correlation was evident between the interferon-producing capacity of these cells and the disease afflicting the patient from whom they were derived.

Lymphoblastoid cell lines established from patients with Burkitt's lymphoma (Henle and Henle, 1965; Rabson *et al.*, 1966; Swart and Young, 1969; Zajac and Kohn, 1970; Zajac *et al.*, 1969), IM (Kasel *et al.*, 1968; Haase *et al.*, 1970; McCombs and Benyesh-Melnick, 1967), and from patients with lymphocytic and myelogenous leukemia (Deinhardt and Burnside, 1967; Blume *et al.*, 1969; Haase *et al.*, 1970; Minnefor *et al.*, 1970; Kasel *et al.*, 1968; Strander *et al.*, 1970) have been reported to produce interferon. Interferon production also occurs in lymphoblastoid cell lines derived from normal individuals (Haase *et al.*, 1970).

Interferon production appears to be related to the presence of the EBV genome (Henle and Henle, 1966b; Zajac *et al.*, 1969; Zajac and Kohn, 1970; Swart and Young, 1969); however, some lymphoblastoid cell lines with virus did not produce interferon (Haase *et al.*, 1970). Swart and Young (1969) found an inverse relationship between the percentage of cells in culture containing EBV and the amount of interferon produced. Zajac and Kohn (1970) derived twenty-three subcultures from single cells of the EB-2 lymphoblastoid cell line in which 0.2–0.6% of the cells showed EBV-specific immunofluorescence. All clones produced interferon and showed immunofluorescence similar to that found in the parent cell line. This study suggested that every cell in the parent line contained the EBV genome and, thereby, possessed the ability to produce interferon.

b. Stimulated Lymphocytes. Wheelock (1965) reported the induction of interferon synthesis in human leukocyte cultures incubated in the

presence of PHA. Interferon was demonstrated in culture fluids 2 hours after addition of PHA to the leukocytes and peaked by the twentieth hour. Interferon from these cultures was compared with interferon from NDV-infected leukocytes, and several minor differences were noted: (*1*) only NDV-induced interferon was stable at pH 2 or pH 10 for 24 hours; both interferons were stable at pH 3–9, (*2*) both interferons were stable at 46°C for 1 hour, but only the NDV-induced interferon retained its biological activity at 56°C. It was suggested by this author that PHA might be inducing interferon synthesis in lymphocytes, since PHA had been reported to affect these cells (Robbins, 1964). Subsequent studies with purified lymphocytes (Edelman and Wheelock, 1966, 1967, 1968a) proved this assumption to be correct. Later studies (Wheelock *et al.*, 1972a, and unpublished observations), revealed a positive correlation between the degree of stimulation of these cells and the titer of interferon produced. In addition to PHA, other nonspecific stimulators of lymphocytes, such as pokeweed mitogen and streptolysin (Friedman and Cooper, 1967), have been reported to induce interferon production.

In 1966, Glasgow reported that leukocytes removed from the peritoneal cavity of mice previously immunized against chikungunya virus produced more interferon than unsensitized leukocytes when these cells were exposed to the virus. This phenomenon suggested to Green *et al.* (1969) that interferon may be produced by sensitized lymphocytes exposed to the specific sensitizing antigen. Human lymphocyte cultures were prepared from individuals previously immunized with tetanus or diphtheria toxoids and from donors proven to be tuberculin PPD-positive. The addition of each specific antigen to sensitized lymphocytes resulted in the production of interferon. Other investigators have confirmed these findings (Lackovic and Borecky, 1970; Milstone and Waksman, 1970). The degree of lymphocyte stimulation produced by either PHA or PPD as measured by the incorporation of uridine-^{3}H into RNA, correlated with the amount of interferon produced (Wheelock and Toy, 1972). The role which interferon from sensitized lymphocytes may play in controlling viral infections will be discussed in Section IX.

2. Proliferative Inhibitory Factor

Normal murine lymphocytes have been found to inhibit tumor cell growth *in vitro*. Direct contact between lymphocytes and tumor cells was essential for this inhibition and no elaboration of a cytotoxic factor could be detected (Jamieson and Wallace, 1970). A similar inhibition of tumor cell growth, with no loss of viability, could be produced by PHA-stimulated lymphocytes (Green *et al.*, 1970). This inhibition was

found to be mediated by a soluble factor called *proliferative inhibitory factor* (PIF) produced by PHA-stimulated lymphocytes. The PIF is a macromolecule produced by stimulated human lymphocytes; it cannot be extracted from unstimulated fresh cells. Its inhibitory effect is reversible, although the treated cells require a few days to recommence growth. Proliferative inhibitory factor has not as yet been detected during the course of viral infections (Cooperband and Green, 1971).

3. *Lymphotoxin*

Lymphocytes play a key role in tissue destruction by participating in delayed hypersensitivity, homograft, and graft-vs.-host reactions. This cytotoxic activity of lymphocytes results either from their direct attack on target cells (Möller and Lundgren, 1969; Perlmann and Holm, 1969) or through elaboration of soluble factors that can destroy nonlymphoid cells (David, 1971).

Investigators have found that sensitized lymphocytes can produce a marked toxic effect on target cells containing antigens to which the lymphocytes are sensitive (Govaerts, 1960; Rosenau and Moon, 1961; Wilson, 1965). The toxic effect can also be elicited by stimulation of lymphocytes with mitogens such as PHA (Holm *et al.*, 1964; Holm and Perlmann, 1969; Möller and Lundgren, 1969). Histoincompatibility between lymphocytes and target cells is not required for lymphocytes from sensitized donors to destroy syngeneic target cells provided that the target cells have sensitizing antigens on their surfaces (Möller, 1965; Hellström and Möller, 1965).

This cytotoxic effect has been found to be mediated by a soluble factor called *lymphotoxin.* Released from lymphocytes after stimulation by either specific antigens (Ruddle and Waksman, 1968a) or mitogens such as PHA (Granger and Kolb, 1968), lymphotoxin acts on cell membranes and can destroy cells derived from several mammalian species including man. The capacity of lymphocytes to elaborate lymphotoxin has been correlated with the appearance of delayed hypersensitivity reactions in the animals from which the lymphocytes were derived (Ruddle and Waksman, 1968b). Coyne *et al.* (1971) found that guinea pig lymphotoxins were more heat-labile than migration inhibitory factor (MIF) and could be separated from the latter by electrophoresis but not by elution from Sephadex. In addition, they found lymphotoxins to be resistant to treatment with neuraminidase.

Lymphotoxin has been related to at least one viral infection. A soluble cytotoxic factor with the properties of a lymphotoxin has been found to be released from immune mouse lymphocytes after exposure to LCM. Although the release of this factor was immunologically spe-

cific, in that it was produced only on exposure to LCM, its cytotoxic action was nonspecific, since it destroyed both the viral-infected and noninfected target cells (Oldstone and Dixon, 1970). In another system where lymphotoxin might be involved, spleen cells from mice immunized with mumps virus exerted a cytotoxic effect on epithelial cells persistently infected with mumps virus (Speel *et al.*, 1968). This cytotoxic reaction was immunologically specific and appeared to be induced by direct contact between immune spleen cells and target cells. Lymphotoxin was not sought in this system.

The mechanism whereby lymphotoxin acts remains conjectural, but it may facilitate the movement of immunoblasts through tissues by transiently damaging the endothelium of local capillaries. Electron-microscopic studies of the sequence of events leading to destruction of target cells by PHA-stimulated lymphocytes have been described by Biberfeld (1971).

4. Migration Inhibitory Factor

Lymphocytes from guinea pigs which exhibit delayed hypersensitivity reactions produce a factor which inhibits macrophage migration when exposed *in vitro* to the specific sensitizing antigen (Bloom and Bennett, 1966; David, 1966; Granger *et al.*, 1970). This factor, MIF, is heat-stable at 60°C for 30 minutes, nondialyzable and has a molecular weight reported to be 25,000 (Bloom and Jimenez, 1970)–50,000 daltons (Remold *et al.*, 1970). The production and action of MIF are dependent on protein synthesis and are destroyed by chymotrypsin (Remold and David, 1970) and neuraminidase (David, 1971). It is likely, therefore, that MIF is a glycoprotein. Potentially at least, MIF can have profound effects on the immune response, specifically on the delayed hypersensitivity reaction. Migration inhibitory activity has been demonstrated in a number of animal virus systems. Feinstone *et al.* (1969) found that migration of peritoneal cells from mice immunized with mumps or influenza viral antigen was inhibited when the immunizing antigen was present in the incubation mixture. Tompkins *et al.* (1970) also reported inhibition of migration of peritoneal cells from rabbits infected with fibroma virus. They plated peritoneal cells on fibroma virus-infected monolayers and were able to correlate the time at which migration inhibition first appeared with the onset of the cutaneous delayed hypersensitivity response and resistance to infection in the intact animal from which the cells were derived. Finally, release of MIF from LCM, lactic dehydrogenase, or mumps virus-immune spleen cells exposed *in vitro* to the specific viral antigens has been demonstrated by Tubergen and Oldstone (1971). They found that in some

instances the virus-infected lymphoid cells released MIF even without additional exposure to the virus.

5. *Chemotactic Factor*

Lymphocytes from guinea pigs exhibiting delayed hypersensitivity, on exposure to a specific sensitizing antigen *in vitro,* can produce a soluble factor which is chemotactic for mononuclear macrophages (Ward *et al.,* 1969). Like MIF, the macrophage chemotactic factor is relatively heat-stable and nondialyzable. It can be separated from MIF by disc electrophoresis on acrylamide gels (Ward *et al.,* 1970); in contrast to MIF, it has no species specificity and appears to be resistant to neuraminidase. Injection of supernatants of antigen-stimulated lymphocytes into the skin of guinea pigs elicits mononuclear cell infiltration into the area. The chemotactic activity of the supernatants may draw monocytes to the site of injection, and MIF may then prevent migration of these cells away from the site. A factor chemotactic for neutrophils is also produced by antigen-stimulated guinea pig lymphocytes. This factor is different from the macrophage chemotactic factor, being slightly smaller in size (Ward *et al.,* 1971). Recently, Ward *et al.* (1971) found that a factor was released from antigen-stimulated lymphoid cells which was chemotactic for lymphocytes. There is, however, as yet no evidence that this lymphocyte chemotactic factor is an entity distinct from the chemotactic factors for neutrophils and mononuclear cells mentioned above. Of interest is the fact that antigen-stimulated cells can produce MIF, chemotactic factor, and lymphotoxin in the same culture. However, the biological activity of lymphocyte medium containing more than one of the factors is dependent upon the type of target cell employed (David, 1971). Chemotactic factors have not, as yet, been demonstrated in virus–lymphocyte interaction neither *in vivo* nor *in vitro.*

6. *Blastogenic Factor*

Sensitized lymphocytes stimulated with specific antigens release a factor that can induce mitosis in fresh lymphocytes from nonsensitized individuals (Kasakura and Lowenstein, 1965; Gordon and Maclean, 1965). The authors suggested that the mitogenic factor might, in fact, be HL-A antigens that stimulated lymphocyes lacking these antigens. More recently, a factor released from lymphocytes stimulated by specific antigens (Maini *et al.,* 1969; Wolstencroft and Dumonde, 1970) has been shown to be mitogenic on autologous and allogeneic lymphocytes. A related factor released by concanavalin A-stimulated lymphocytes has been found to be mitogenic only on lymphocytes from the same or

genetically similar donors (Powles *et al.*, 1971). These mitogenic factors have not as yet been associated with viral disease.

7. *Transfer Factor*

Transfer factor is a small nonantigenic nonimmunoglobulin, dialyzable moiety of less than 10,000 mol. wt. which is extracted from sensitized lymphocytes and can convert the normal host as well as his circulating lymphocytes to a specific antigen-responsive state (Lawrence, 1949, 1969). Lymphocyte-transforming factor, similar to transfer factor is produced by tuberculin-sensitive lymphocytes *in vitro* after exposure to PPD (Valentine and Lawrence, 1969). Transfer factor has not as yet been demonstrated as a product of any virus–lymphocyte interaction.

8. *Implications*

Lymphocytes release many factors within a few hours after either specific or nonspecific stimulation. These factors can act in a variety of ways to suppress viral replication and eradicate the infection. Suppression of viral replication results from the intracellular action of interferon and from destruction of virus-producing cells by lymphotoxins. Several of the factors also act as mediators of cellular immunity. A major effort has been made in recent years to develop *in vitro* techniques to assay each of these factors and relate their activities to the *in vivo* state. The application of knowledge gained from a study of lymphocyte products has already yielded promising results, e.g., transfer factor has been employed clinically to treat children with immune deficiency syndromes and has been found to induce a state of increased resistance to viral infections (Levin *et al.*, 1970).

C. Inhibition of Blastogenesis

The ability of viruses to mutate has led to the selection of strains with selective advantages over the host. Perhaps one such advantage is the ability of some viruses to subvert the immune response. Both the humoral and CMI responses have been shown to be affected by viruses (Notkins *et al.*, 1970). Observations on the inhibitory effects of viruses on delayed hypersensitivity reactions *in vivo* have been made with measles (Von Pirquet, 1908, 1911), influenza (Bloomfield and Mateer, 1919), and varicella and polioviruses (Starr and Berkovich, 1964; Berkovich and Starr, 1966). Only recently, however, techniques were developed to study this phenomenon *in vitro*.

Von Pirquet's original observations with measles virus were tested in an *in vitro* system by Smithwick and Berkovich (1966). They found that the addition of live measles virus to PPD-sensitive lymphocytes

significantly reduced the response of the cells to PPD stimulation. Later reports (Montgomery *et al.*, 1967; Olson *et al.*, 1969; Willems *et al.*, 1969b; Willems and Rawls, 1969; Häyry *et al.*, 1970) described the inhibitory effects of a number of viruses, including a murine leukemia virus, on the lymphocyte response to PHA. Olson *et al.* (1967) earlier reported that lymphocytes taken from patients with congenital rubella syndrome did not respond optimally to PHA. Others (Simons and Fitzgerald, 1968; Marshall *et al.*, 1970), however, have not been able to confirm this lack of responsiveness. Olson *et al.* (1969) suggested that viruses take over critical sites of cellular biosynthesis and in this way inhibit the PHA response.

In general the PHA response *in vitro* seems to be related to CMI, since neonatal thymectomy and not bursectomy drastically diminished the PHA responsiveness of chicken peripheral lymphocytes (Greaves *et al.*, 1968). However, infants with rubella who contain lymphocytes with decreased *in vitro* reactivity to PHA have been shown to develop normal hypersensitivity responses to 2,4-dinitrofluorobenzene and *Candida* (Olson *et al.*, 1967). It is necessary, in specific situations, therefore, to use caution in equating *in vitro* lymphocyte transformation data with *in vivo* delayed hypersensitivity reactions.

In addition to the reported decreases in *in vitro* reactivity of lymphocytes from patients with congenital rubella, lymphocytes from patients with IM and infectious hepatitis also are depressed in their ability to undergo blast cell transformation (Rubin, 1966; Mella and Lang, 1967; Willems *et al.*, 1969b; Yam *et al.*, 1967). The presence of EBV in lymphocytes from IM suggests a viral causation for the observed decreased lymphocyte reactivity. (See Section VII,A.)

It is tempting to attribute the decreased reactivity of lymphocytes in diseases of unknown etiology to the presence of a virus. Many of these diseases, Hodgkin's disease, chronic lymphocytic leukemia, Down's syndrome, leprosy, multiple myeloma, Waldenström's macroglobulinemia, thymic dysplasia, ataxia telangiectasia, and rheumatoid arthritis, are manifested by immunological disorders involving suppressed, delayed hypersensitivity reactions and antibody production. It is entirely possible that decreased lymphocyte reactivity is an end product of altered cellular metabolism which can be produced by many factors of which viruses are but one. If, indeed, viruses can enhance their pathogenicity by subverting the host's immune response, then it should be possible to increase host resistence to infection by administering agents that restore immunological competence. This approach to therapy might apply not only to viral infections but to any disease in which lymphocyte function is impaired.

VII. Immunological Defects and Their Effects on Viral Infections

The importance of lymphocytes in the defense against viral infections can be understood by studying the severity of infections produced in hosts with impaired lymphocyte function. Such impairment can occur either naturally, associated with congenital, immune deficiency syndromes involving thymic dysplasia, or experimentally, after administration of immunosuppressive agents. In this section, whenever possible the discussion is confined to hosts in which CMI is selectively impaired. In many situations, however, broad immunosuppressive effects have been produced and no attempts made to differentiate between impairment of CMI and antibody production. In analyzing these systems, it is important to remember that immunosuppressive agents may have multiple effects on the host in addition to inhibiting the immune response. For example, ALS can (*a*) blockade the reticuloendothelial system (Sheagren *et al.*, 1970), (*b*) produce transformation of lymphocytes to blast cells (Grasbeck *et al.*, 1964) which can support viral replication (Edelman and Wheelock, 1968b), (*c*) inhibit hepatic phagocytosis (Pisano *et al.*, 1969), and (*d*) affect interferon production (reviewed by Glasgow, 1971).

A. Induced Immunosuppression in Animals

It is possible, by correlating immunosuppression with altered pathogenicity, to identify some viral diseases in which CMI is unimportant in the recovery process. Table IV presents a list of viruses that do not have increased pathogenicity in hosts given sufficient immunosuppressive therapy to inhibit CMI.

Table V lists viruses with increased pathogenicity in mice given immunosuppressive therapy, but having unaltered production of antibody to the virus.

In addition to viruses listed in Table V, a large number of cytocidal and oncogenic viruses have increased pathogenicity in animal hosts which have been immunosuppressed by various methods with undetermined effects on CMI and antibody production. For a review and discussion on the effect of immunosuppression on acute viral infections, the reader is referred to Nathanson and Cole (1971; Glasgow, 1971;

TABLE IV

Viruses without Increased Pathogenicity after Immunosuppression

Influenza (Hirsch and Murphy, 1968)
17D Strain of yellow fever (Zisman *et al.*, 1971)
Lymphocytic choriomeningitis (Gledhill, 1967; Hirsch *et al.*, 1967, 1968a; Lundstedt and Volkert, 1967; Volkert and Lundstedt, 1968)
Rabies (Allison, 1970b)

TABLE V
VIRUSES WITH INCREASED PATHOGENICITY IN IMMUNOSUPPRESSED HOSTS PRODUCING NORMAL AMOUNTS OF ANTIBODY

Vaccinia (Hirsch *et al.*, 1968b)
Ectromelia (Blanden, 1970)
Herpes simplex (Mori *et al.*, 1967)
Simian virus (Tevethia *et al.*, 1968)
Polyoma (Vandeputte, 1968)

Merigan and Stevens, 1971; Hirsch, 1970). Those viruses that have increased oncogenic effects in immunosuppressed hosts include adenovirus Type 12, murine sarcoma virus, leukemia viruses of the Friend, Moloney, and Rauscher groups, and Gross virus-induced lymphomas (reviewed by Allison, 1969; Hirsch *et al.*, 1971). In contrast, immunosuppression, by thymectomy at least, had no effect on Gross-type virus (Gross, 1959) and the Bittner agent (Martinez, 1964). Law (1969) also found that the frequency and latent period of chemically induced fibrosarcomas containing tumor-specific antigens and mammary tumor virus-induced tumors was not increased by ALS or neonatal thymectomy.

B. Immune Deficiency Syndromes in Man

Discovery of new congenital immunodeficiency syndromes with different types of immunological incompetence, together with acquired immunological deficiency states resulting from both surgical procedures and administration of immunosuppressive agents, has permitted correlations between specific types of immunological deficiencies and susceptibility to specific viral infections. Agammaglobulinema (of the Bruton type) has not been associated with severe or disseminated viral disease. In contrast, immunological deficiency syndromes involving thymic dysfunction and lymphopenia (including DiGeorge syndrome, Wiscott-Aldrich syndrome, Swiss-type agammaglobulinemia, ataxia telangiectasia and cartilage hair syndrome) predispose the individual to severe infections by a number of viruses listed below. These viruses in general have in common the ability to spread from cell to cell directly, thereby avoiding neutralization by antibody in the extracellular fluids. Consequently the cell-mediated mechanism is believed to be the important immunological defense against such viruses (Gatti and Good, 1970).

1. Vaccinia

Fulginiti studied a number of patients with progressive vaccinia and concluded on the basis of their specific immunological deficiencies that failure to heal after dermal smallpox vaccination was the result of

defective CMI (Fulginiti *et al.*, 1966, 1968; Fulginiti, 1971). A number of patients developed progressive vaccinia despite their production of circulating virus-neutralizing antibody but displayed reduced CMI responses to several antigens. In these patients, the infection was progressive from the local site of vaccination, and the absence of secondary lesions was believed due to interruption of viremia by neutralizing antibody in the serum.

Interruption of progressive vaccinia by transfusions of immune leukocytes, but not serum, has been reported by O'Connell *et al.* (1964). In contrast, individuals with congenital hypogammaglobulinemia and intact CMI produced a typical local pock which resolved in 2 to 3 weeks followed by development of the capacity to produce a general reaction of the delayed hypersensitivity type when challenged with vaccinia virus (Good and Varco, 1955; Kemp, 1960). Only in one instance did an infant with pure hypogammaglobulinemia develop progressive vaccinia and that was associated with a concomitant infection with mumps virus. It is possible in this case that the associated mumps viral infection led to suppression of CMI resulting in activation and increased susceptibility to vaccinia virus. Another syndrome, generalized nonprogressive vaccinia, consists of localized vaccinial lesions appearing at many sites throughout the body (Chandra *et al.*, 1969). These lesions occur in the presence of apparently normal CMI and are believed to be due to an IgM deficiency.

Fulginiti (1969) studied intradermal vaccinia virus inoculation in monkeys immunosuppressed by either X-irradiation or ALS. He concluded that the CMI plays a primary role in defense against vaccinia virus and that antibody and interferon play secondary roles. Bone marrow reconstitution of irradiated monkeys with depressed CMI restored their resistance to vaccinia viral infection. Other workers made neonatal rabbits tolerant to vaccinia virus by injection of inactivated virus early in life (Flick and Pincus, 1963). These animals had a higher mortality rate and lacked CMI compared with control rabbits. Hirsch *et al.* (1968b) with vaccinia and Blanden (1970) with ectromelia found significantly increased viral replication and mortality in mice treated with antithymocyte serum. Such mice had depressed CMI but normal levels of virus-neutralizing antibody and interferon. These animal studies confirm the conclusion that CMI is the principal host defense against pox viral infection.

2. Measles

Measles virus can be lethal in children who have defects in CMI associated with congenital deficiency syndromes and malignancies or

acquired by immunosuppressive therapy. Nahmias *et al.* (1967) reported the occurrence of fatal, giant cell, measles virus pneumonia in a lymphopenic infant who had no identifiable thymus. The almost complete absence of small lymphocytes in the lymph nodes, spleen, and intestines in this infant suggested that these cells play an important role in host resistance to measles virus. A similar disease occurred in a sibling who had the same type of immunological defect.

Lipsey *et al.* (1967) studied 3 patients with defects in CMI, who had developed fatal measles viral infections. Identification of the exact immunological deficiency syndrome in these patients was not made, but all had thymic dysplasia and one had normal immunoglobulin and antibody-producing capacities. Fatal measles has been reported in patients with leukemia (Mitus *et al.*, 1965) and in children receiving immunosuppressive therapy (Meadow *et al.*, 1969). Furthermore, children with leukemia can develop severe and often fatal infections after exposure to live measles virus vaccine (Mitus *et al.*, 1962).

3. Varicella-Zoster

Varicella and herpes zoster are viral diseases in which mortality is rare enough to warrant publication. Hook *et al.* (1968) investigated the immune system in surviving members of a family in which 2 of 5 children who contracted varicella died. They found normal delayed hypersensitivity to a number of antigens and normal antibody production to various virus antigens including varicella. At autopsy the thymus in 1 patient was small but appeared histologically normal.

Lux *et al.* (1970) reported unusually severe varicella infections in 2 children with cartilage hair hypoplasia. These children had persistent lymphocytopenia, diminished delayed skin hypersensitivity to a number of antigens, and diminished *in vitro* transformation of lymphocytes. In 1 child, rejection of a skin allograft was delayed. Serum immunoglobulin levels were normal, and the antibody response to a variety of viral and bacterial antigens was normal. The authors concluded that a defect in CMI was responsible for the unusual susceptibility to varicella infection.

Fulginiti *et al.* (1966) reported a case of progressive varicella in a patient with impaired CMI and normal production of antiviral antibodies. Children with Wiscott-Aldrich syndrome have lymphocytopenia, deficient delayed hypersensitivity reactions, defective lymphocytoblastogenesis *in vitro*, and defective humoral antibody responses to carbohydrate but not to protein antigens. Such patients often sustain severe viral infections including varicella (Cooper *et al.*, 1968; Gatti and Good, 1970).

In contrast, Eisen *et al.* (1965) studied 5 children with ataxia

telangiectasia, an immunological deficiency syndrome involving an absent or underdeveloped thymus and reduced IgA levels. He found that the children with grossly impaired CMI had recovered normally from varicella, measles, and mumps infections. The author suggested that these viral infections might have occurred at a time when the immunological mechanisms were only partially impaired. One of the patients, at an advanced stage of ataxia telangiectasia, contracted varicella at the age of 12. Serious illness followed with slow healing and considerable scarring.

Huntley *et al.* (1963) reported a case of dysgammaglobinemia in which recovery from varicella was normal. Gitlin *et al.* (1962) and Eysserig *et al.* (1960) reported fatal varicella in patients with agammaglobulinemia. Nakano and Kojima (1965) reported a fatal case of varicella in a 10-month-old female who had dysgammaglobulinemia and hypoplasia of the thymus. They noted a complete absence of lymph nodes. Others reporting small or absent lymph nodes associated with fatal varicella are deVaal and Seynhaeve (1959), Ackerman (1964), and Hitzig and Willi (1961).

Varicella and herpes zoster infections have been reported to occur in 9% of patients after renal transplantation and immunosuppressive therapy (Rifkind, 1966; Spencer and Anderson, 1970), suggesting a relationship of this infection to the induced immune deficiency state. Further evidence for this comes from the high incidence of herpes zoster infections in patients with Hodkin's disease where deficiencies in CMI may activate or increase susceptibility to the virus (Sokal and Firat, 1965).

4. Herpes Simplex and Cytomegaloviruses

Cooper *et al.* (1968) studied 18 patients with Wiscott-Aldrich syndrome and frequently found severe infections produced by herpes simplex and cytomegaloviruses. The marked lymphocytopenia and impaired CMI in these young males was associated with completely competent antibody production against protein antigens, suggesting the importance of lymphocytes in resistance to these viruses.

Cytomegaloviral infections have been reported to occur often after transplantation surgery. For example, 91% of patients receiving renal transplants developed evidence of infection with cytomegalovirus within 2 months of surgery (Anderson and Spencer, 1969). Interestingly, infection occurred more frequently in patients containing virus-specific antibody before transplantation (Craighead *et al.*, 1967) suggesting viral activation after impairment of CMI by immunosuppressive therapy. Fatal disseminated cutaneous and visceral herpes simplex virus infections have

developed in patients after renal transplantation (Montgomerie *et al.*, 1969). Also, herpes simplex virus pneumonia developed after immunosuppression associated with lung surgery (R. G. Douglas *et al.*, 1969; Schwartz, 1969).

5. Adenoviruses

Wigger and Blanc (1966) reported a fatal infection with adenovirus Type 2 in a 10-month-old infant with hypogammaglobulinemia. Thymic aplasia, massive hepatic necrosis, and bronchopneumonia were found on autopsy. Extensive necrosis occurred in lymph nodes, pancreas, and biliary ducts. A combined immunodeficiency syndrome involving low levels of immunoglobulins and alymphoplasia was present in the patient and prevented identification of the precise immunological defect responsible for increased susceptibility to adenovirus infection.

6. Poliovirus

Hypogammaglobulinemic individuals have been reported to develop severe infections to naturally acquired poliovirus (Good *et al.*, 1962). However, in only 1 recorded case has a hypogammaglobulinemic infant developed a fatal infection to poliovirus vaccine (Cheng *et al.*, 1966); on autopsy, a normal thymus was found but lymphocytes in the medulla of lymph nodes were depleted. There is no report of children with impaired CMI developing severe infections to poliovirus vaccine.

C. Activation of Latent Viral Infections

Activation of viruses in immunosuppressed animals was first noted by Woodruff (1967) who reported that dogs previously vaccinated with canine distemper virus developed overt disease after treatment with ALS. In these cases, it is clear that immune lymphocytes participating in the CMI response to the virus did not always eradicate the infection but could permit dormant viruses to persist within the host, to emerge, and to produce disease when CMI became impaired.

Activation of viruses after immunosuppression has been shown in mice which have latent LCM infections. Adult mice infected with sublethal doses of LCM usually develop immunity to the virus with high titers of complement-fixing antibody in their blood together with prolonged latent infection. Treatment of these mice with ALS has no effect on antibody titer or on pathogenic effects of the virus, but it does lead to the development of viremia. The viremia disappears on the cessation of ALS treatment (Volkert and Lundstedt, 1968).

VIII. Role of Lymphocytes in the Production of Viral Disease

In recent years, attention has focused on EBV as a possible etiologic agent for at least two diseases of man: IM and Burkitt lymphoma. Evidence to support this role is discussed below; however, certain aspects of infections produced in lymphocytes by EBV have been considered in other sections of this review and are not included here. The reader is referred to Section III, A and B for a discussion of chromosomal aberrations and atypical lymphocytes, Section V, A and B for EBV–lymphocyte associations *in vivo* and *in vitro,* Section VI,B,5 for EBV replication in lymphocytes, and Section VI,C,*1* for interferon production.

A. Host Cells for Acute Infections of Man

It is perhaps remarkable that lymphocytes have been implicated as host cells for viral replication in but one acute disease of man, IM. A viral etiology for this disease had long been suspected, but no significant progress toward confirmation was made until 1968 when Henle *et al.* reported the presence of EBV antigen in leukocyte cultures from a patient with IM (Henle *et al.,* 1968). A herpeslike virus, EBV, had been originally observed by Epstein and Barr with the electron microscope in cultures of Burkitt lymphoma cells (Epstein *et al.,* 1964). Studies of college students, representing prospective high risk groups, revealed that cases of IM were confined to individuals lacking antibody to EBV prior to illness (Evans *et al.,* 1968). Antibody production to EBV consistently followed IM and persisted for life. Finally, EBV has been shown to have proliferative effects on human lymphocytes in that it stimulates DNA synthesis (Gerber and Hoyer, 1971) and enables cells to grow in continuous culture.

Despite the strong seroepidemiologic and virologic relationships which have been established between EBV and IM, there has been some reluctance to accept EBV as the etiologic agent for IM. This reluctance is based in part on the fact that EBV has also been implicated as the etiologic agent of another disease, Burkitt lymphoma (see Section VIII,B). It is possible that EBV is an ubiquitous virus and finds suitable host cells for its replication in any lymphoproliferative disease. The inability to isolate the virus by conventional means and to perform experiments necessary to fulfill Koch's postulates prevents universal acceptance of the virus as the cause of IM. As with Burkitt's lymphoma, EBV may require an additional factor for production of acute disease. Of special interest here is the persistence of EBV for years after infection in the leukocytes of individuals who contain EBV antibody (Diehl *et al.,* 1968). Such persistence may lead to lifelong production

of virus-specific antibody and be the basis for transmission of the virus in blood transfusions during surgery, producing an IM-like post-transfusion syndrome (Gerber *et al.*, 1969b).

B. Host Cells for Oncogenic Infections of Man

Burkitt lymphoma, a neoplastic disease composed of poorly differentiated lymphocytic cells, is the one neoplastic disease of man in which a virus has been implicated as the etiologic agent. This lymphoma occurs in Africa in a geographic distribution suggesting that causation might depend on the arthropod vector spread of an etiologic agent (Burkitt, 1962). Although this initial hypothesis has not been confirmed, additional epidemiologic evidence supports the infectious nature of the disease (Pike *et al.*, 1967; Burkitt and Wright, 1966).

Early attempts with conventional methods to demonstrate a virus in the lymphoma cells were uniformly negative, and it was not until the cells were placed in culture and examined with the electron microscope that virus was detected. The virus is similar in structure to herpes virus, but all attempts to isolate it from the lymphoma *in situ* or from cultured lymphoma cells have been unsuccessful. The virus, however, can infect several types of hematopoetic cells *in vitro* and enable these cells to divide and grow in long-term culture (Henle *et al.*, 1967; Grace, 1967; Pope *et al.*, 1968b; Gerber *et al.*, 1969a). Antigen within EBV-infected cells does not react with sera made against other herpes viruses (Henle and Henle, 1966a,b). However, 100% of sera from patients with Burkitt's lymphoma contained antibody to the EBV antigen (Levy and Henle, 1966), and specific antibody was also detected in patients after recovery from IM (Henle *et al.*, 1968). The demonstration of EBV in the peripheral leukocytes of patients with and patients recovered from IM combined with seroepidemiologic studies have strongly implicated EBV as the etiologic agent of this disease (see Section VIII,A). The question has been raised, then, as to why EBV may produce two different types of disease.

Burkitt (1969) suggested that a co-factor may be required for EBV to produce a lymphoma. Present in the geographic area of high lymphoma incidence is malaria. The stimulatory effects of malaria on the reticuloendothelial system are well known and may, when combined with EBV, result in malignancy. Conversely, infection with EBV in the absence of lymphoreticular stimulation could produce an acute infection of the IM type. This hypothesis is difficult to prove, since the oncogenic potential of EBV precludes experimental studies on man. However, the demonstration that an EBV vaccine could lower the incidence of Burkitt's lymphoma in a high incidence disease area would provide strong evidence of the etiologic role of EBV.

C. Mediators of Immunopathological Disease

Intracerebral inoculation of adult mice with LCM regularly produces a lethal disease within 6 to 8 days (Traub, 1936; Cole *et al.*, 1971). Histological examination of the brain reveals marked mononuclear cell infiltration in all membranes but no discrete neuronal pathology (Lundstedt and Volkert, 1967), suggesting an immunopathological basis for acute LCM disease. Experiments with various immunosuppressive agents support this view; adult mice after X-irradiation, neonatal thymectomy, and methotrexate, chlorambucil, cortisone, ALS, and cyclophosphamide treatment are, to varying degrees, protected against the acute manifestations of disease and survive the infection (reviewed by Cole *et al.*, 1971).

A series of elegant experiments have implicated lymphocytes in the pathogenesis of LCM. Cole *et al.* (1971) observed that growth of virus in the brain of adult mice treated with cytophosphamide 3 days after infection was identical to that in untreated mice but that 85% of the treated mice survived and became permanent virus carriers. Histological examination of immunosuppressed mice infected with LCM revealed minimal or no lymphoid cell infiltration of the choriod plexus ependyma and meninges. Corroborative evidence for the pathological action of immune lymphocytes came from adoptive cell transfer experiments. Immune lymphocytes were obtained from mice in which a virus-carrier state had been produced by intraperitoneal inoculation of LCM virus. Inoculation of these cells into mice in which LCM disease had been prevented by cyclophosphamide produced death in 100% of recipients. Histological examination of these mice revealed marked infiltration of mononuclear cells and severe inflammation of all cerebral membranes.

Other members of the arenovirus group have also been found to produce immunorelated cerebellar necrosis. Borden *et al.* (1971) reported that antithymocyte serum prevented development of cerebellar necrosis caused by Tacaribe virus but had no effect on replication of the virus in the central nervous system. Similar protective effects of immunosuppression on the lethal effects of Tamiami virus (Calisher *et al.*, 1970), Junin virus (Weissenbacher *et al.*, 1969), and 17-D yellow fever virus (Hirsch and Murphy, 1967) have been reported.

IX. Role of Lymphocytes in the Defense against Viral Disease

A. Acute Infections

There exist in scientific literature few papers dealing with mechanisms by which lymphocytes act in the host defense against viral infections.

In most of these reports, investigators compared the interactions of viruses with normal and immune lymphocytes and studied various ways by which immune cells resist viral infection, either directly by attack on virus and virus-infected cells or, indirectly, through the action of antiviral factors elaborated during the course of virus–immune cell interactions.

1. Direct Cytotoxic Action

Speel *et al.* (1968) demonstrated that splenic cells of mice immunized with mumps virus produced cytotoxic effects on epithelial cells persistently infected with mumps virus *in vitro*. Under normal culture conditions, the persistently infected cells supported replication of mumps virus and produced viral antigen on their surface membranes with no associated cytopathic effect. Mumps virus-sensitized splenocytes reacted specifically with mumps virus-infected target cells thereby producing destruction of the monolayer. A similar cytotoxic action of immune splenocytes on LCM virus-containing target cells was studied by Oldstone *et al.* (1969). Splenic cells from LCM-immunized mice produced cytotoxic effects *in vitro* on mouse embryo or monkey kidney cells infected with LCM virus. The mechanisms involved in these two systems are not known, but Oldstone did find that a cytotoxic effect similar to that produced by immune cells could be produced by anti-LCM viral antibody and complement.

2. Release of Antiviral Factors

Normal and immune lymphocytes can, when exposed to nonspecific mitogens or to antigens to which they are sensitive, produce a number of factors which act in various ways to suppress virus production and confine the virus to the primary site of infection. These factors, as they relate to viral diseases, are discussed in Section VI,C.

3. Viral Resistance of Immune Cells

Douglas and Smith (1930) found that inoculation of leukocytes from vaccinia virus-immune animals increased the resistance of susceptible rabbits to infection with vaccinia virus. They concluded that a component of cellular immunity was acting to prevent initiation of the infection. Steinberger and Rights (1963) found that cultures of spleen cells of vaccinia-immune rabbits were less susceptible to infection by vaccinia virus than were nonimmune cells. No virus-specific antibody could be detected in the immune cultures, suggesting that the mechanism for increased virus resistance was at the cellular level.

Maral (1957) studied the replication of rabbit myxoma virus in

peritoneal cells *in vitro*. Viruses replicated to high titers with formation of giant cells but without cell destruction. Reduced yield of virus were obtained when peritoneal cells from immune rabbits were used, and the author believes this reduction was due to cellular immunity.

4. Increased Virus Uptake

There is no published report of enhanced virus uptake per cell when normal and immune human leukocytes are compared. Willems *et al.* (1969a), however, found an enhanced uptake and penetration of poliovirus by lymphocytes in mixed leukocyte cultures after stimulation with PHA. However, the greater yields of virus in stimulated cultures did not result from monocytes, which support replication in unstimulated cultures, but rather from lymphocytes taking up and sustaining the replication of virus.

B. Oncogenic Infections

1. Polyoma

Cellular immunity is of prime importance in delayed hypersensitivity reactions and is believed to be the principal mechanism involved in allograft and tumor rejection in man and experimental animals. The demonstration that animal tumors possess tumor-specific transplantation antigens (TSTA) was first made by Gross (1943). It was subsequently found that tumors of viral etiology are different from spontaneous or chemically induced tumors in that the former have TSTA which is common to all tumors induced by one virus but different from tumors induced by other viruses. Thus, immunization of an animal with a particular virus will produce resistance against all tumors induced by that virus but not against tumors from other viruses (Hellström and Möller, 1965). Here, only tumors induced by viruses are considered. A more extensive review of CMI against both viral and nonviral tumor antigens can be found in K. E. Hellström and Hellström (1969).

Polyoma virus-induced tumors were the first viral neoplasms in which TSTA was detected. The existence of TSTA was suspected when Sjögren *et al.* (1961a,b) and Habel (1961, 1962), working independently, found that polyoma virus tumors grew better in normal mice than in syngeneic adult mice which had been immunized with polyoma virus as adults. Resistance also occurred in adult mice immunized with a non-virus releasing allogeneic polyoma tumor; no polyoma virus-specific antibodies could be demonstrated in these mice (Sjögren, 1961, 1964). The existence of a TSTA unrelated to polyoma structural proteins was, therefore, suspected and resistance believed due to cellular immunity

mediated through immune lymphocytes. Hellström (1967) demonstrated that immune lymphocytes exist in polyoma virus-immunized mice and exert a cytotoxic action against polyoma tumor cells. By using the colony inhibition (CI) test, she found that lymph node cells from polyoma virus-immune mice reduced polyoma cell colony formation *in vitro*. The inhibitory effects noted were complement-independent.

Polyoma-immune lymph node cells were also studied by adoptive transfer experiments. Strain C57 mice, normally resistant to polyoma virus oncogenesis, can be rendered susceptible by neonatal thymectomy, probably as a result of suppression of CMI (Ting and Law, 1967). Thymectomized C57 mice can be protected against formation of polyoma tumors by adoptive transfer of lymph node cells from C57 mice which have been immunized against polyoma virus TSTA. Immunized lymph node cells offer protection when they are inoculated as late as 30 days after the animal has been virally infected (Law *et al.*, 1967; Allison, (1970a).

2. *Adenovirus*

The CI test has been applied to a number of other virus-induced tumors as a means of demonstrating CMI. Hellström and Sjögren (1967) found that lymph node cells from mice immunized with allogeneic adenovirus-12 tumor cells were cytotoxic against colony formation of adenovirus-12 sarcoma cells *in vitro*. The immunity was specific in that control lymph node cells were not inhibitory regardless of whether they were derived from normal mice or from mice immunized with other TSTA. The adenovirus-12-immune mice from which the immune lymphocytes had been derived contained antibody which produced a complement-dependent inhibition of colony formation against the target cells.

3. *Moloney Sarcoma*

Hellström *et al.* (1968) and I. Hellström and Hellström (1969) studied mice in which Moloney sarcoma virus had produced tumors that eventually regressed spontaneously. Mice with both actively growing and regressing tumors contained lymph node cells which were cytotoxic against Moloney sarcoma cells in the CI test. Sera from mice with progessively growing Moloney sarcomas but not from mice with spontaneous or methycholanthrene-induced sarcomas abrogated the inhibitory effect of cytotoxic lymph node cells against Moloney sarcoma cells. When the serum was absorbed with Moloney sarcoma cells, and then retested in the CI test, the protective effect against Moloney sarcoma virus had been removed. It was suggested that sera from mice with progessively growing tumors contained antibodies that blocked the cyto-

toxic action of immune lymphocytes and, thus, mediated an efferent form of immunological enhancement.

4. Mammary Tumor Virus

A variety of other virus-induced tumors have been studied for their ability to elicit CMI. Heppner and Pierce (1969) studied mammary carcinoma in BALB/C-F C3H mice which contained mammary tumor virus as a result of foster nursing of C3H mice. They removed primary carcinomas from the mice and used them as targets for lymph node cells derived from mice from which the tumors had been removed. They found that these lymphoid cells were cytotoxic against the mammary tumor cells *in vitro*. Lymph node cells from mice which did not bear mammary carcinomas or from normal BALB/C mice were not inhibitory. The authors thus showed that CMI could be demonstrated against the TSTA of virus-induced mammary tumors.

5. Shope Papilloma

In rabbits, Shope papilloma virus produces papillomas which usually regress after a few weeks. The probability of regression varies among different strains of rabbits. Some papillomas, however, develop into carcinomas. Colony inhibition of papilloma cells was demonstrated using autochthonous lymph node cells from rabbits in which papillomas had either regressed or developed into carcinomas (I. Hellström and Hellström, 1969). Such lymph node cells also inhibited papilloma cells from other rabbits indicating a cross-reacting TSTA. Serum from rabbits with persistent Shope papillomas or Shope carcinomas abrogated the inhibitory effect of cytotoxic lymph node cells on Shope tumor cells, whereas serum from rabbits in which the tumors had regressed had no such effect. It was postulated that sera from rabbits with persistent papillomas or carcinomas contain antibodies that mediate an efferent form of immunological enhancement (I. Hellström *et al.*, 1969).

6. Shope Fibroma

The relationship between delayed hypersensitivity and resistance to infection was studied by Allison and Friedman (1966). They found that resistance of rabbits to superinfection with fibroma virus appeared 5 days after the primary viral inoculation, at the time when delayed hypersensitivity to the virus developed. From a series of experiments involving various immunosuppressive agents, they concluded that antibody prevents the spread of virus but that CMI brings about regression of the primary tumor. To support this conclusion, Allison (1966) found

that in newborn rabbits fibromas progress to enormous size and kill the animals; antibody is formed but CMI cannot be demonstrated.

C. Roles of Interferon, Antibody, and Lymphocytes

Three major components in the host defense against viral infection are believed to be interferon, antibody, and lymphocytes. Silverstein (1970) reviews macrophages and viral immunity. Each of these components has been shown, under experimental conditions *in vitro* and *in vivo,* to be capable of inhibiting specific viral infections. In naturally occurring infection, however, a sequence of these host responses may be necessary for containment of the virus and complete recovery. Studies on the kinetics of appearance of each response reveal that interferon is produced within hours after infection (Vilcek, 1969), followed by antibody and CMI responses (Oren *et al.*, 1971).

Relatively few investigators have attempted to dissect these three defense mechanisms rigorously and to establish the relative roles of each in the recovery process. The research which has been done, however, reveals that viruses vary in their susceptibility to inhibition by interferon, in their exposure during spread from cell to cell to extracellular fluids containing antibody, and in their ability to stimulate and, in turn, to be destroyed by a lymphocyte-mediated CMI response.

Analysis of the individual host-protective mechanisms is made difficult by the overlapping effects of experimental manipulations. For example, ALS inhibits both CMI and thymic-dependent antibody production (James, 1970; Levey, 1970). Furthermore, ALS has a marked effect on interferon production related to the interferon inducer employed (reviewed by Glasgow, 1971). Interferon inducers also have multiple effects; for example, soon after its discovery (Field *et al.*, 1967), poly I:poly C was found to have potent antitumor activity in mice (Levy *et al.*, 1969) which was attributed to the direct action of interferon. This belief was in part based on the *in vivo* antileukemic effects of interferon preparations (Gresser *et al.*, 1967). More recently, however, it was found that no correlation exists between interferon production and the antitumor effects of poly I:poly C (Weinstein *et al.*, 1970). The antitumor properties of poly I:poly C may result from its inhibitory effects on cellular RNA and protein synthesis (Weinstein *et al.*, 1970; Larson *et al.*, 1970) but are more likely produced by stimulation of an immune response against tumor-specific surface antigens. Cantor *et al.* (1970) have shown that poly I:poly C enhances graft-versus-host activity in BALB/C mice, and Turner *et al.* (1970) have reported that poly I:poly C increases both humoral and CMI responses in mice. The hypothesis that poly I:poly C acts as an adjuvant is supported by the

ability of statolon, a double-stranded RNA-containing mycophage, to suppress completely an established Friend leukemia virus infection in DBA/2 mice. Statolon induces interferon and reverses the immunosuppression caused by Friend virus in that it stimulates the production of a cytotoxic antibody against Friend leukemic cells which converts a lethal infection into a dormant one (Wheelock *et al.*, 1969, 1971, 1972b; Wheelock and Caroline, 1970; Weislow *et al.*, 1972).

Analysis of the defensive roles played by interferon, antibody, and lymphocytes is further complicated by the temporal and spatial overlapping of their activation and production. Interferon is induced in lymphocytes after either nonspecific (PHA) (Wheelock, 1965) or antigen-specific stimulation (Green *et al.*, 1969). Also PHA stimulation transforms lymphocytes into cells morphologically resembling those involved in CMI responses (Wilson *et al.*, 1968; Bach *et al.*, 1969). Finally, increased amounts of immunoglobulin have been observed in PHA-stimulated as well as antigen-stimulated lymphocytes (Ripps and Hirschhorn, 1967).

Perhaps the best evidence for determining the relative roles of interferon, antibody, and CMI responses comes from observations on immune deficiency syndromes in man where specific defects in immunocompetence have been identified (see Section VII,B). Here, situations exist in which patients have intact antibody- and interferon-producing systems but lack CMI. These patients are highly susceptible to viruses which have in common their ability to spread from cell to cell without exposure to extracellular factors such as antibody.

The demonstration that the interferon system itself is incapable of halting the spread of these viruses in immunologically deficient patients rules against the importance of interferon in the host defense against such viruses. Support for this conclusion comes from Fulginiti's (1969) experiments with immunosuppressed monkeys, Flick and Pincus' (1963) studies on tolerant rabbits, and Hirsch's *et al.* (1967) studies on ALS-treated mice.

Blanden (1970), studying ectromelia in mice, found that antithymocyte serum suppressed CMI and increased pathogenicity and mortality from the virus; normal amounts of interferon and neutralizing antibody to the virus were produced. The conclusions of these workers is that normal interferon and neutralizing antibody responses were insufficient to contain poxviruses; their experiments pointed to the primary role of CMI in the defense against poxviral infections.

In contrast, certain viruses (see Table IV) have no increased virulence for hosts in which CMI has been severely impaired by ALS. Normal amounts of virus-specific antibody are produced in such hosts,

and it may be concluded that viruses which require a viremic phase for production of disease can be contained by the presence of antibody in the circulation.

It is likely that the interferon, antibody, and CMI responses to viral infections are intimately related. Although in special situations one or more of these responses may be selectively suppressed, in general, the three act together in defense of the host. There is, however, great variation among viruses in their susceptibility to each response. Therefore, no general rule can be made as to the relative importance of each response in the host defense. Rather the defense against each virus must be considered separately and different approaches employed therapeutically to stimulate the most effective combination of host responses against that virus.

Recent studies on a number of synthetic double-stranded ribonucleotides reveal that preparations vary in their interferon and adjuvant activities (Hilleman *et al.*, 1971). Further, the adjuvant activity of poly I: poly C, a good interferon inducer, can be enhanced by combining it with emulsified peanut oil. Such experimental approaches aimed at the stimulation of host responses individually and in combination with each other may lead to effective treatment of viral diseases.

X. Summary

In the foregoing review we have seen that lymphocytes may act to man's detriment as well as to his benefit. Lymphocytes may participate in the production of viral disease in a number of ways. They may serve as host cells for viral replication by being stimulated by foreign antigens. In the case of EBV, the virus itself can act as a mitogen and induce neoplastic transformation with associated EBV replication. Immune lymphocytes may also produce disease by infiltrating into infected foci causing an acute inflammatory reaction. Such viral infections are immunopathogenic.

In contrast, lymphocytes can participate actively in the host defense against viral infections, either directly as aggressor immunocytes exerting cytotoxic effects on virus-containing host cells or indirectly by releasing a number of factors that suppress virus production. Important among these factors is interferon which increases the viral resistance of contiguous uninfected cells. Another factor, lymphotoxin, can destroy infected cells which are in proximity to the sensitized lymphocyte.

Theoretically, lymphocytes can act in the host defense in two additional ways. Immune lymphocytes have been shown to carry immunoglobulins on their surface membranes, but a cytophilic virus-specific antibody has not yet been demonstrated. Such an antibody on the sur-

face of lymphocytes which infiltrate infected foci could neutralize the virus and eradicate the infection. In addition, lymphocytes could take up and inactivate infective viruses.

Analysis of the roles of interferon, CMI, and antibody in the defense against viral diseases reveals that these three responses often occur as a sequence following infection. Viruses vary in their sensitivity to each response. Any attempt to utilize these host defenses in the treatment of viral infections must take the specific sensitivities of viruses into account.

Future studies should be directed toward elucidation of the precise ways by which lymphocytes act in the host defense. Identification of these mechanisms could lead to the development of therapeutic approaches which would effect rapid recovery from viral infections.

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Immune Complex Disease in Experimental Animals and Man[1]

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I. Experimental Immune Complex Disease with Nonreplicating Antigens

No experimental model has provided us with greater insight into the mechanism of immune complex disease than experimental serum sickness. The morphological, immunohistological, and serological features of the laboratory models have provided a basis for understanding the pathogenic mechanisms responsible for human glomerulonephritis, vasculitis, and a variety of systemic connective tissue diseases.

The subject of experimental acute and chronic immune complex disease produced by nonliving antigens has received extensive review in this series (Weigle, 1961; Unanue and Dixon, 1967). In this chapter, we summarize the data presented in these two reviews and then treat in detail more recent studies. References prior to 1967 will not be dealt with comprehensively.

A. Acute Immune Complex Disease

1. Description

Experiments to study acute immune complex disease (serum sickness) have been performed in rabbits almost exclusively. Previously serum was injected to induce the disease, but, it is now preferable to employ a purified heterologous plasma protein, such as bovine serum albumen (BSA). Injection intravenously of a single foreign protein yields a predictable nonimmune half-disappearance time ($T_{½}$) in the recipient rabbit allowing one to determine when the disease commences. After injection, BSA-^{131}I is eliminated from the circulation in three phases (Fig. 1): (*1*) in which the BSA equilibrates between intra- and extravascular spaces; (*2*) a slower phase in which BSA is catabolized at about the rate of the rabbit's own albumin; and (*3*) a more rapid phase which is now recognized as being caused by an immune response to the antigen. After antigen has been completely removed from the circulation, free antibody appears. However, of greatest importance in understanding the mechanism of this disease is the observation that during immune elimination of the antigen, complexes of antibody and antigen can be detected in the circulation (Fig. 1). It is when immune complexes circulate that lesions of acute immune complex disease develop in arteries, glomeruli, joints, and heart. The incidence of arteritis is greatest in the coronary outflow area at the points of branching of the aorta and

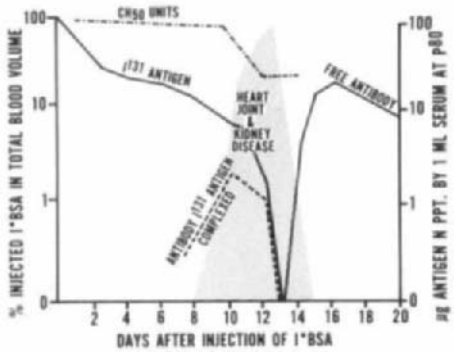

FIG. 1. Elimination of bovine serum albumin (BSA)-^{131}I antigen from the circulation of a typical rabbit, showing appearance of immune complexes in the circulation and development of lesions. Free antibody was measured by precipitation (p80) with antigen.

in the pulmonary arteries. Glomerulonephritis (Fig. 2) is marked by endothelial swelling with, perhaps, some mild lifting of endothelial cells from the underlying basement membrane. There is a paucity of leukocytic emigration. Proteinuria is marked with little or no hematuria. With

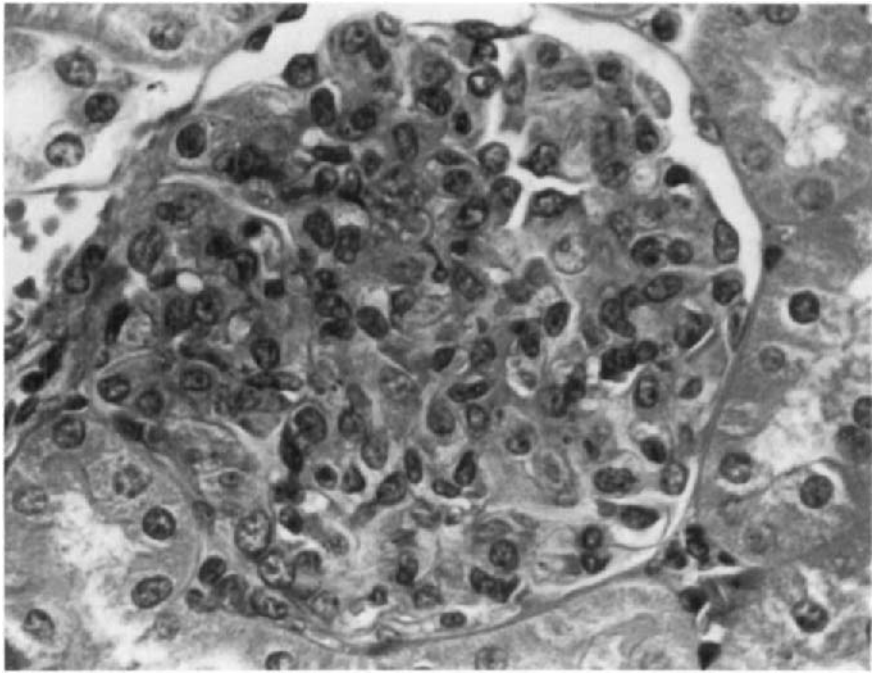

FIG. 2. Glomerulitis in acute complex disease of a rabbit. Endothelial cells have enlarged to fill the capillary spaces. Note the absence of neutrophiles. ×250.

fluorescent antibodies, BSA antigen, host γ-globulin, and the third component of complement (C3) are detectable in a granular pattern along the glomerular basement membrane (GBM) (Fig. 3). This granular appearance has become the hallmark of immune complex disease in all species. It allows one to determine that a given glomerular disease has an immune complex pathogenesis. With the electron microscope, little is seen in the acute disease beyond the swelling of endothelial cells. Occasionally, in rabbits sacrificed several days to weeks after elimination of the antigen, electron-dense deposits are seen along the outer aspect of the GBM. At this time, the immune complexes are not identifiable with fluorescent antibody techniques, although C3 is occasionally observed.

Arteritis begins with mild proliferation of intimal endothelial cells. This may be observed at the onset of immune elimination of the antigen and becomes more striking in the subsequent days. Neutrophiles enter

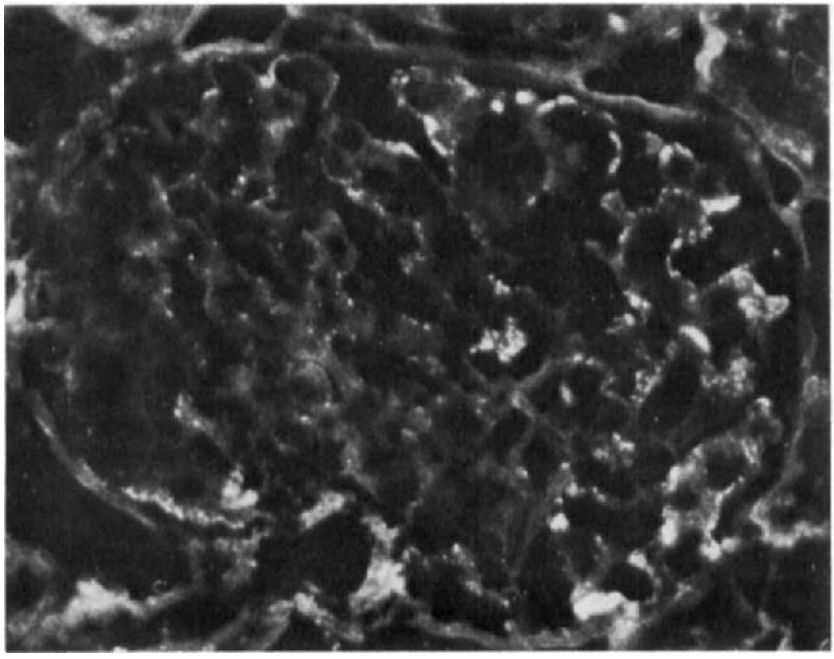

FIG. 3. Fluorescent photomicrograph of a glomerulus, similar to that in Fig. 2, stained for the presence of host immunoglobulin. The fine granular fluorescence along the basement membrane is the characteristic feature of immune complex disease. Antigen and host C3 are similarly deposited. ×250. (Courtesy of Dr. Curtis Wilson.)

the reaction site and rapidly degrade underlying membranes, notably the internal elastic lamina, to gain entrance to the media and adventitia. Fibrinoid necrosis then develops in the arterial wall (Fig. 4). By fluorescent microscopy, antigen, host γ-globulin, and C3 are detected at the onset of injury lying in the vicinity of the internal elastic lamina. They form globular and some finely granular deposits. However, they are rapidly removed so that 1–2 days after the onset of arteritis, that is, even before complete immune elimination of the antigen, the immune reactants are no longer detectable. Phagocytic cells, in particular neutrophiles, rapidly engulf the offending complexes and destroy them. This is useful knowledge for investigators studying vasculitis in humans by means of fluorescent techniques. The pathogenic agents are removed extremely rapidly, leaving little to observe.

Several features of this disease should be stressed to understand its

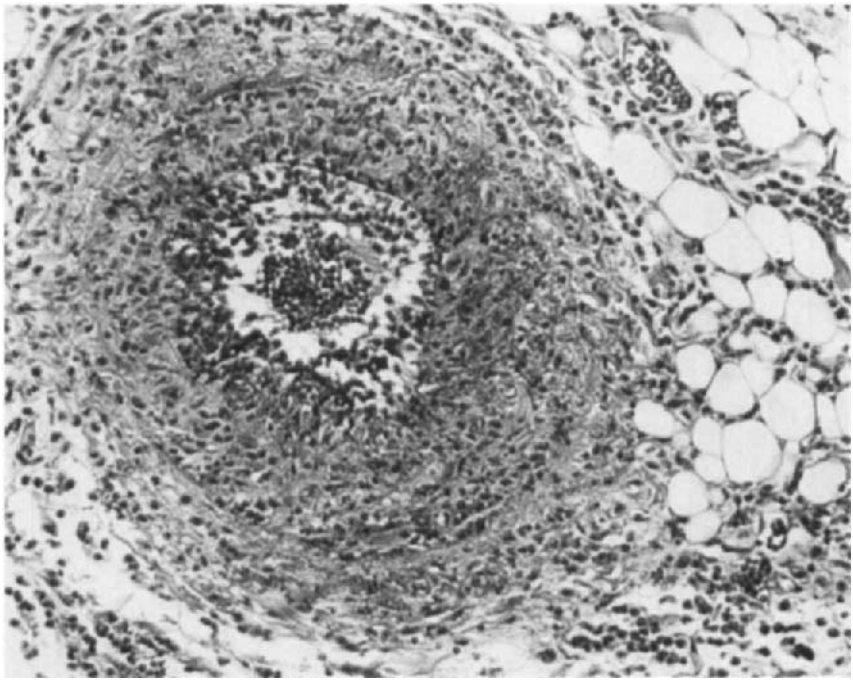

FIG. 4. Arteritis observed in acute immune complex disease of rabbits. The lesion is marked by early neutrophile accumulation followed by appearance of mononuclear cells. Destruction of the internal elastic lamina by neutrophiles precedes progression of inflammation into the media and adventitia. Fibrinoid necrosis of the arterial wall is frequent. ×100.

pathogenesis. The immune reactants without doubt circulate and deposit as immune complexes. Antigen does not first deposit in the blood vessel wall so that antibody can then react with it, but, instead, when antigen is in excess, immune complexes form and circulate prior to deposition in sites of injury. Complement levels are depressed during the development of this disease, but platelet levels remain normal. Removal of neutrophiles abolishes arteritis but does not affect glomerulonephritis. This might be expected, since neutrophiles accumulate in the former but not the latter site of injury. The mediation of lesions will be dealt with in greater detail below.

The quantity of antigen, bound in glomeruli in acute immune complex disease has been measured (Wilson and Dixon, 1970). At the height of the disease, 1–35 μg. BSA per rabbit was bound in the kidneys, with a mean of about 20 μg. The antigen was located primarily in the renal cortex. With each kidney containing 200,000 glomeruli (Smith, 1951), this amounted to 4.4×10^8 molecules of BSA per glomerulus. The amount of antibody present was estimated (with assumptions) to be about 1.3×10^9 molecules/glomerulus, which falls in the range of antibody molecules per glomerulus required to induce acute nephrotoxic nephritis by using heterologous antibody to GBM (Unanue and Dixon, 1967). The $T_{½}$ disappearance of antigen from the kidneys was found to be 10 days, i.e., well beyond the time that antigen could be detected by immunofluorescence (Wilson and Dixon, 1970).

Although not appreciated in the early studies of immune complex disease in rabbits, Fish *et al.* (1966) observed electron-dense granules developing in association with the GBM 2–3 days after elimination of circulating antigen. As seen by immunofluorescence the granules contained C3 and γ-globulin, but antigen was not detected (although, as noted above, when isotope detection was used antigen was probably present in small amounts). The antigen was presumably coated by the γ-globulin and complement. Lambert (1971) found recently that radioisotope-labeled anti-BSA, injected after complete immune elimination of the antigen, bound to the deposits in the glomeruli. The presence of these granules in other experiments has been confirmed (Wilson and Dixon, 1970). The deposits developing after elimination of circulating antigen are not associated with injury and proteinuria. Indeed, they deposit during the healing phase. This suggests that the immunoglobulin and complement binding along the GBM at this time are not strongly phlogogenic.

2. *Mediation of Lesions in Acute Immune Complex Disease*

Two different mechanisms appear to mediate the development of lesions in acute immune complex disease of rabbits. Arteritis exhibits

large numbers of neutrophiles in the early phase of its development, and it is completely inhibited if neutrophiles are eliminated when immune complexes enter the circulation (Kniker and Cochrane, 1965). As in other immunological reactions where neutrophiles play an important role in mediation (Cochrane, 1968), if complement is depleted with cobra venom factor, neutrophiles do not enter the reaction (Henson and Cochrane, 1971a). Necrotizing injury of the arteries is thus averted. By contrast, glomerulitis most frequently develops in the absence of significant numbers of neutrophiles, and elimination of neutrophiles (Kniker and Cochrane, 1965) or depletion of C3 and terminal components of complement (Henson and Cochrane, 1971a) have no effect on the development of injury. This directs attention to a second, as yet unknown, mechanism of mediation of glomerular injury. Studies of nephrotoxic nephritis have also demonstrated a complement–neutrophile mechanism of immunological injury of the glomerulus (Henson and Cochrane, 1971b).

B. Chronic Immune Complex Disease

1. Description

Experimental chronic immune complex disease has proved to be a most useful model in understanding human glomerulonephritis. When injected daily with heterologous serum protein antigens, rabbits with strong antibody responses develop chronic membranous glomerulonephritis in about 5 weeks (see review, Unanue and Dixon, 1967). The amount of antigen injected intravenously is critical, for the dose must be varied daily to obtain a temporary state of excess antigen after each injection to allow the formation of immune complexes which circulate in an excess of antigen. Glomerulonephritis, but not arteritis, develops. If the dose of antigen is insufficient to clear the circulating antibody, the antigen is removed in seconds and complexes do not circulate. Animals that fail to respond to the antigen do not develop disease, whereas those responding poorly require longer periods (>12 weeks) than good responders to develop chronic glomerulonephritis.

Chronic glomerulonephritis is detectable clinically by proteinuria, hypoproteinemia, and elevated cholesterol and urea levels in the blood. If antigen injections continue, the clinical course is progressive, leading to fatality. In rabbits giving a moderate antibody response, the most common and earliest form of the disease is thickening of the glomerular capillary membranes without cellular proliferation or swelling and without accumulation of leukocytes. In rabbits producing a larger immune response, neutrophiles accumulate, and proliferation and swelling of endothelial cells occur (Fig. 5). Crescents of epithelial cells form in

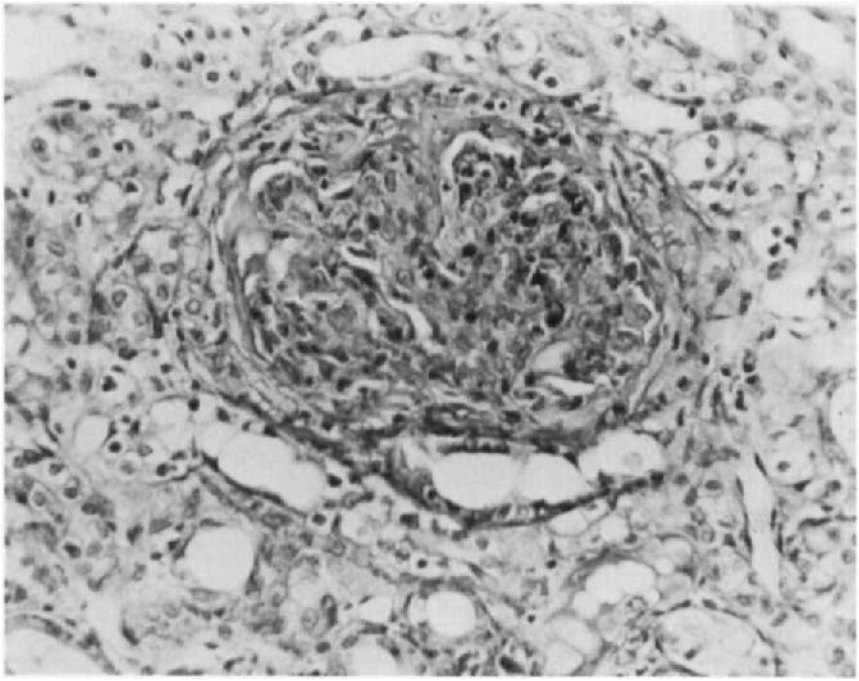

FIG. 5. Glomerulonephritis seen in chronic immune complex disease of a rabbit. Note the thickening of capillary walls, cellular accumulation, and proliferation of epithelial cells in a "crescent." ×150. (Courtesy of Dr. Curtis Wilson.)

Bowman's space, and the animals frequently run a rapid terminal course. Immunofluorescent studies reveal large granular deposits of antigen, host immunoglobulin and C3, lying along the GBM, principally on the external surface (Fig. 6). By electron microscopy, the deposits appear as electron-dense masses formed within and along the external surface of the GBM (Fig. 7). Epithelial cells show broadening of their foot processes forming a continuum along the basement membrane. Of great interest in the pathogenesis of chronic immune complex disease in rabbits is that arteritis and endocarditis fail to occur. The reason for this remains enigmatic.

The quantity of antigen, radiolabeled BSA, deposited in the kidneys (presumably glomeruli) depends upon the amount of antigen injected each day and the stage of the disease. And as mentioned before, the amount of antigen injected is determined by the amount of antibody produced by the rabbit, enough antigen being required to clear the free antibody and place the rabbit temporarily in a state of excess antigen. Before the onset of proteinuria, 47 ± 18 μg. of antigen resides in the

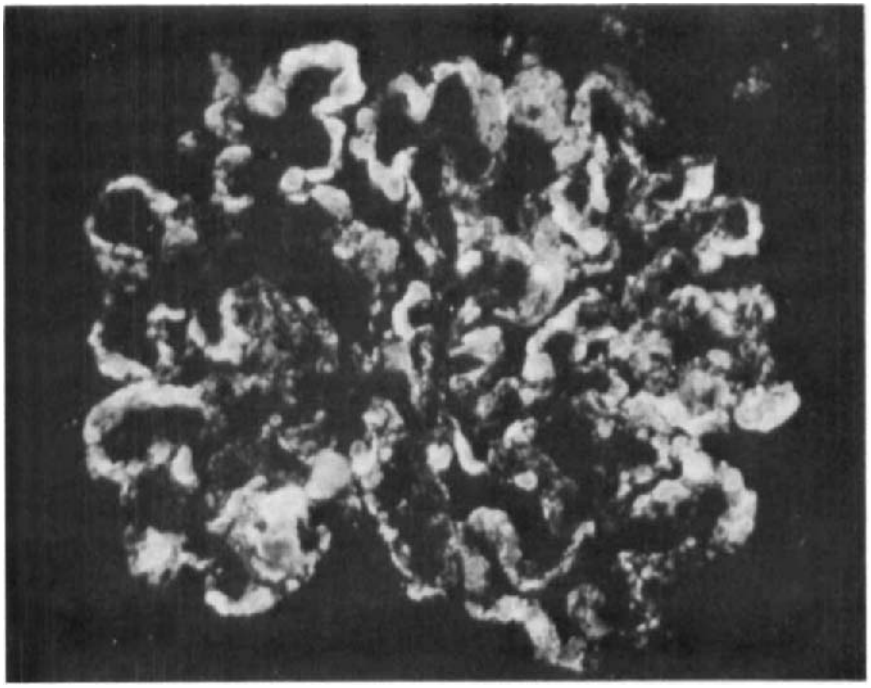

FIG. 6. Fluorescent photomicrograph of a glomerulus similar to that in Fig. 5. The granular deposition of immune complexes collecting along the outer edge of the basement membrane is the characteristic feature of the lesion. Complexes may also deposit along the inner aspect of the basement membrane immediately after injection of antigen. ×250.

kidney when 50–200 mg. are injected each day. After proteinuria has commenced, 601 ± 73 μg. are found in the kidneys with the same daily antigen dose (Wilson and Dixon, 1971). Whereas 0.04% of the injected antigen is deposited in the kidneys before onset of proteinuria, 0.5% is deposited when proteinuria appears. The heavier deposits seen by immunofluorescence reflect the increased rate of deposition. Apparently, the glomeruli are altered during the time when injury develops so that increasing numbers of circulating complexes become entrapped. It is quite possible that the increasing permeability of the GBM is responsible for the increased rate of deposition; and, conversely, the increased rate of deposition may adversely affect the GBM. Fatigue of the reticuloendothelial system, especially the mesangial cells, may also occur so that systemic and local clearance of the immune complexes may be impaired. This would allow greater numbers of complexes to become entrapped in and along the GBM (Wilson and Dixon, 1971).

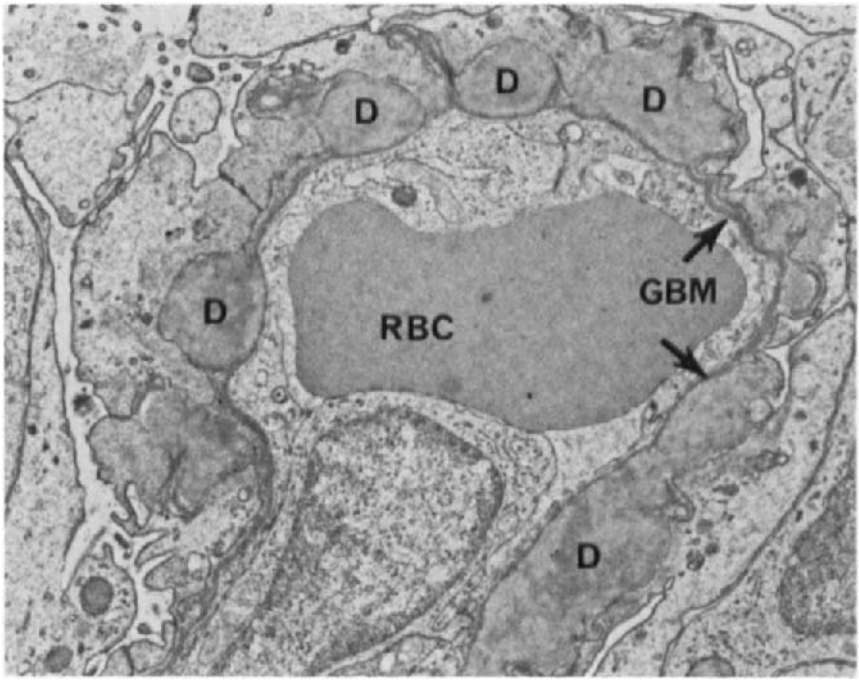

FIG. 7. Electron photomicrograph of a glomerulus in chronic immune complex disease. The feature most noteworthy is the presence of electron-dense deposits (D) lying in and along the external aspect of the glomerular basement membrane (GBM). These deposits are rich in antigen and antibody. (RBC), red blood cells. (Courtesy of Dr. Curtis Wilson.)

The importance of the pathogenic capacity of antibody in forming immune complexes has been stressed (Pincus *et al.*, 1968; Christian, 1969, 1970; Christian and Lightfoot, 1970). When fixed amounts of antigen (BSA) were injected daily, about half the rabbits produced sufficiently large amounts of antibody to remove the antigen immediately, about one-third failed to respond immunologically, and 10–20% responded with poorly precipitating antibody. The poorly precipitating antibody formed complexes with the injected antigen that persisted, at least for a short time, in the circulation. This group of rabbits developed chronic nephritis after 14 to 18 weeks. Evidence suggested that the poor precipitability of the antibody resulted from limited recognition of the antigenic determinants (Christian, 1970; Lightfoot *et al.*, 1970). The complexes sedimented at 19 S or more, indicating they were sufficiently large to be entrapped in the vessels. These studies of Christian and his colleagues draw attention to the quality of antibody in determining whether complexes circulate and become deposited. Nevertheless, Dixon

et al. (1961) and Wilson and Dixon (1971) have stressed that any immunologically responsive rabbit develops chronic glomerulonephritis provided the quantity of antigen injected each day removes the antibody from the circulation and remains temporarily in excess. In fact, rabbits responding with large amounts of antibody develop more severe nephritis sooner than poor producers provided the doses of antigen are increased (Wilson and Dixon, 1971).

Presumably in naturally occurring immune complex disease, both quality and quantity of the antibody formed may be important. On the one hand, if the antigen responsible for immunization is present in sufficient quantities, even large amounts of precipitating antibody bring about injury; on the other hand, if the antigenic stimulus is limited, then production of either small quantities of antibody or antibody with limited reactivity with the antigen induces a state of circulating complexes that predisposes to disease.

2. *Mediation of Lesions in Chronic Immune Complex Disease*

There are no firm data on the mediation of chronic glomerulonephritis in chronic immune complex disease. As was noted above, in the course of this disease, neutrophiles can accumulate in the glomeruli, and fibrinoid necrosis may be associated. This suggests a role of neutrophiles under these conditions, but supporting evidence is lacking.

3. *Beneficial Effect of Overloading Purified Antigen in Chronic Immune Complex Glomerulonephritis*

When rabbits with chronic glomerulonephritis were injected with a ten- to forty-fold excess of purified antigen the existing process of injury was reversed dramatically (Valdes *et al.*, 1969). Proteinuria disappeared over a 4-week period once the high level of antigen was started. The heavy deposits of γ-globulin in glomeruli, present at the time high levels of antigen commenced, decreased in intensity in the first 7–10 days and disappeared after treatment for several weeks. Wilson and Dixon (1971) found that the $T_{\frac{1}{2}}$ disappearance of antigen in glomerular-bound complexes fell from 5.0 to 0.9 days upon increasing the daily dose of antigen to more than 1 gm./day. Deposits of complexes in the glomeruli regressed. Proteinuria was not affected, however, possibly owing to irreversible structural injury that had occurred prior to administration of large doses.

4. *Immune Complex Nephritis Induced with Antigens of Renal Tubular Cells*

When rats are repeatedly injected intraperitoneally with homologous or heterologous kidney extract in complete Freund's adjuvant, chronic

glomerulonephritis appears in 3 to 5 months. Besides proteinuria, the rats demonstrate hyperlipemia, hypercholesterolemia, and elevated levels of blood urea nitrogen (Heymann *et al.*, 1959). When purified antigen is used (see below) the immunizing injection may be given in the rear foot pads (Edgington *et al.*, 1968). Although originally immune complex nephritis was thought to exemplify disease produced by antibody to glomerular constituents, strong evidence favors the supposition that this disease results from the formation of immune complexes (reviewed by Unanue and Dixon, 1967).

1. Host γ-globulin and C3 are present in association with the GBM forming granular and not linear deposits, typical of immune complex glomerulonephritis. The immunofluorescent granular deposits correspond to electron-dense deposits seen lying along the external aspect of the GBM.

2. Renal tubular antigen is apparently responsible for the immune response. This antigen is not found in normal glomeruli indicating that an external antigen is involved.

3. The injected antigen has to be present in rats given crude renal extracts in order for nephritis to develop that is typical of the disease. The continued presence of antigen was of special significance when lymphoid cells were transferred from immunized rats to induce nephritis in normal ones. Lymphoid cells obtained from rats injected in the foot pads and subcutaneous space produced a mild disease upon transfer to normal recipients (Glassock *et al.*, 1969). However, the reaction period required after transfer was sufficiently long to suggest that active immunization of the recipient had occurred from small quantities of antigen transferred with the lymph node cells.

4. When diseased kidneys are homogenized and eluted, the γ-globulins so obtained react with the tubular cells but not with glomeruli (Grupe and Kaplan, 1967).

The antigen responsible for development of this autologous immune glomerulonephritis has been extracted from renal tubular epithelium in relatively pure form (Edgington *et al.*, 1968); it is called RTE-α_5 and is large, $S^{\circ}_{w,\,20} = 28.6$. The antigen is a lipoprotein, probably derived from membranes of subcellular particles distributed in the brush border of the renal proximal tubular epithelium. Injection of as little as 3 μg. provokes nephritis with a morphological pattern typical of the disease evoked when crude renal extract is employed. The RTE-α_5 does not appear in normal glomeruli; but after disease is induced, this antigen is present in the granular deposits along the GBM.

The amount of purified RTE-α_5 required to induce this disease is insufficient to account for the amount of antigen present in the glomeru-

lar deposits along the GBM. It seems that the injection of RTE-α_5 antigen in complete Freund's adjuvant brings about deposition of the autologous antigen present in the circulation of normal rats, since rapid elimination of a significant amount of antibody directed aganst RTE-α_5 occurs after intravenous injection in normal rats (Glassock *et al.*, 1968a), and since autologous renal tubular antigen is found in the glomerular deposits after immunization with heterologous antigen (Edgington *et al.*, 1967). The possibility must be considered that the injected antigen terminates a tolerant state to the tubular antigen that is normally secreted into the bloodstream. When $HgCl_2$ is injected into rats, glomerulonephritis sometimes results (Bariety *et al.*, 1971). The data suggest that an autoimmune, soluble, complex nephritis developed as a result of the release of autoimmunogenic tubular antigens following $HgCl_2$-induced renal tubular injury.

II. Deposition of Circulating Immune Complexes in Tissues

A. Evidence of an Active Process

Circulating complexes encounter one of two fates—either they are phagocytized and removed by the reticuloendothelial cells and circulating phagocytic cells, or they become deposited in blood vessels and tissues setting the stage for subsequent injury. Deposition in vessels involves many factors and is not merely a process of simple diffusion into vessel walls from the bloodstream. This concept is important in view of its therapeutic implications. By preventing circulating immune complexes from gaining entrance to vessel walls, one would avert disease and allow the reticuloendothelial system to clear the complexes completely.

In order to study the mechanism of deposition of circulating immune complexes, passive induction of immune complex disease has been attempted. Although it has proved most difficult and hard to reproduce, suggestive evidence that at least mild immune complex disease can be induced passively has been obtained. Germuth and Pollack (1958) infused antibody into rabbits having antigen in their circulations. The antigen was eliminated at much the same rate as in serum sickness, and the rabbits developed mild swelling of glomerular endothelial cells and occasional foci of arterial inflammation in vessels of the stomach, duodenum, or peripancreatic tissues. The coronary arteries, most frequent targets in serum sickness, were not involved. About half the rabbits showed one or a combination of these lesions. McCluskey and Benacerraf (1959) injected immune complexes into mice, some of which developed glomerular, endocardial, and arterial lesions within 36 hours

after the first injection. The glomerular lesions contained large numbers of neutrophiles and thereby differed from the acute lesions in serum sickness of rabbits. Others (Cochrane and Weigle, 1958; Fish *et al.*, 1966) have not been able to repeat these results by using preformed immune complexes and studying over 80 rabbits and 200 mice. Hyperimmune rabbit antibody was used which may explain the negative results in rabbits as will be discussed below. The difficulty in obtaining reproducible morphological injury with passively transferred preformed immune complexes suggested that some essential factor was missing and promoted a series of investigations dealing with the mechanisms responsible for deposition.

Evidence of an active process required for deposition of circulating colloidal particles was obtained by Benacerraf *et al.* (1959). Mice were first injected intravenously with colloidal carbon and then given histamine, 5-hydroxytryptamine (serotonin), epinephrine, or preformed immune complexes. In each case, carbon deposited in the intimal layer of large arteries, endocardium of the heart, and walls of venules in various sites. The immune complexes were thought to liberate vasoactive amines *in vivo,* leading to deposition of the carbon. Circulating immune complexes themselves could be made to deposit in venules in guinea pigs by simultaneous infusion of agents that induced liberation of vasoactive amines from mast cells (Cochrane, 1963a,b). Anaphylatoxin, passive anaphylaxis, and the known histamine liberator, octylamine, all were effective in releasing the vasoactive amines to bring about deposition of the circulating immune complexes. Pretreatment with antihistamines prevented the released histamine from increasing vascular permeability, and circulating complexes failed to deposit in vessel walls.

Morphological studies of the deposition of particles from the bloodstream into vessel walls after local injection of vasoactive amines have been performed in several laboratories (Alksne, 1959; Majno and Palade, 1961; Peterson and Good, 1962; Cochrane, 1963a). In each case, the particles passed between endothelial cells to become entrapped along a limiting basement membrane of the venule. Immune complexes could also be seen entrapped at this location (Cochrane, 1963a). This suggested a process of filtration by the limiting membrane.

If immune complexes deposit by filtration, then one might expect large, but not small complexes to become entrapped. This proved to be the case. Complexes of various sizes were prepared and assayed for their ability to deposit in vessel walls; other molecules of different sizes were also used. It was found that only complexes or protein molecules larger than 19 S in size became entrapped (Figs. 8 and 9). In Fig. 8 are shown the sedimentation patterns of small and large complexes,

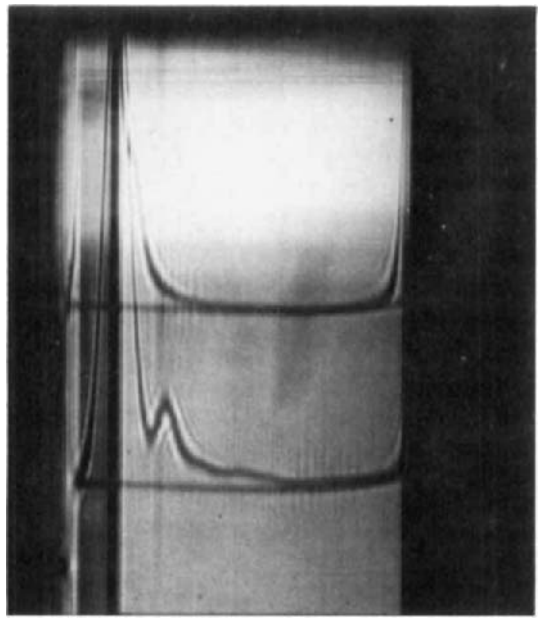

FIG. 8. Analytical ultracentrifugation patterns of the same two soluble bovine serum albumin (BSA)–anti-BSA complexes shown in Fig. 9. Upper frame—complexes prepared at 100 × equivalence; lower frame—complexes prepared at 20 × equivalence. Frames, reading from left to right, taken at 16, 32, and 40 minutes after centrifuge reached two-thirds maximum speed. A rapidly sedimenting boundary is apparent in the complexes prepared at 20 × equivalence.

and Fig. 9 demonstrates that only the large immune complexes were able to deposit in vessel walls. Regarding other molecules, hemocyanin from the keyhole limpet in its associated form (7×10^6 mol. wt.) was capable of deposition, whereas in its dissociated form (0.8×10^6 mol. wt.) it was not; aggregated human γ-globulin would deposit, but non-aggregated γ-globulin would not. These experiments emphasize the importance of molecular size.

In acute immune complex disease of rabbits, striking parallels were observed to that in guinea pigs. In both models, evidence indicated that a state of increased vascular permeability existed during the time immune complexes were depositing from the circulation. Colloidal carbon, injected intravenously became localized along with the complexes in the developing intimal lesions (Kniker and Cochrane, 1968). The carbon filtered between endothelial cells and lined up along the internal elastic lamina of the coronary arteries. This elastic lamina apparently acted as the filtration barrier and lost its integrity only

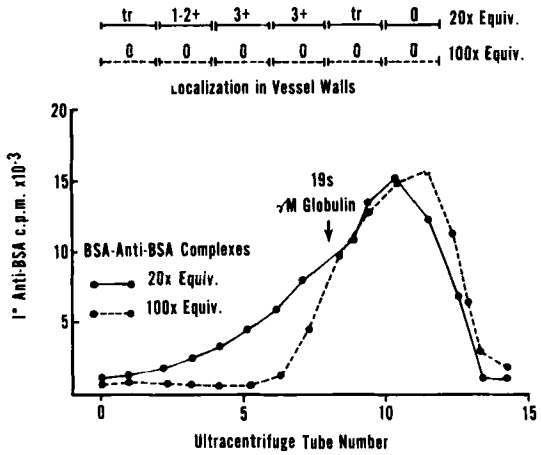

FIG. 9. Patterns of sedimentation of anti-bovine serum albumin (BSA)-*I complexes with BSA using two quantities of BSA. Sedimentation carried out in a linear sucrose gradient, 10–37%, 4 hours at 50,000 rpm. Purified human γM-globulin served as marker. Those made by using a large excess of BSA (100 × equivalence) yielded slowly sedimenting complexes, whereas those prepared in moderate antigen excess (20 × equivalence) demonstrated both slowly sedimenting and more rapidly sedimenting components. Fractions collected after centrifugation and injected into guinea pigs showed localizing complexes only in tubes taken from complexes prepared at 20 × equivalence and greater than 19 S in size.

when neutrophiles entered the reaction (Kniker and Cochrane, 1965). Evidence of active induction of vascular permeability was also observed. Antagonists of vasoactive amines, given at the time immune complexes appeared in the circulation, prevented in great part the deposition of immune complexes in arteries and glomeruli (Table I). In addition, when the most important reservoir of vasoactive amines in the circulation of rabbits, i.e., platelets (Humphrey and Jaques, 1954; Waalkes and Coburn, 1959), was removed in rabbits subjected to serum sickness, deposition of immune complexes and development of arterial and glomerular lesions were markedly suppressed (Table I). Additional studies provided evidence that treatment with antagonists of vasoactive amines inhibited deposition of immune complexes in glomeruli and development of lesions in chronic glomerulonephritis (Kniker, 1968). Rabbits receiving constant treatment with cyproheptadine or chlorpheniramine and methysergide maleate showed distinctly reduced proteinuria and deposition of immune complexes in glomeruli. Inhibition in the latter treatment group was greater than in the former. Blood urea nitrogen levels were also reduced. Similar treatment of rabbits that had already developed chronic immune complex glomerulonephritis ame-

TABLE I
INCIDENCE AND SEVERITY OF SERUM SICKNESS LESIONS IN TREATED AND CONTROL RABBITS[a,b]

Lesions	Treated rabbits (% positive), antihistamine and antiserotonin, 11 rabbits	Control rabbits (% positive)	
		Platelet depletion, 16 rabbits	10 rabbits
Coronary artery			
Endothelial proliferation	9	44	90
Medial necrosis	9	19	80
Glomeruli			
Immunofluorescent deposits[c]	0 to + (Fine)	0 to+ (Fine)	+ to ++ (Coarse)
Endothelial swelling and proliferation[d]	1.0+	1.7+	2.2+

[a] Data from Kniker and Cochrane (1968).
[b] Values tabulated are averages for each group.
[c] Grading of immunofluorescent deposits of IgG, antigen (bovine serum albumin): 0 to +++.
[d] Grading of glomerular endothelial swelling and proliferation: 0 to 3+.

liorated the disease (Kniker, 1970). The incidence of serum sickness in adult humans receiving antidiphtheria toxin globulin was lessened four-fold by treatment with antagonists of vasoactive amines (Kniker *et al.*, 1971). Fever and rash, the most striking signs of a serum sickness in humans, do not necessarily result from circulating complexes.

In rabbits, the size of the immune complex is also important in determining whether deposition of complexes and development of lesions occur. In a correlative study, deposition of circulating complexes and development of glomerular and arterial lesions in acute immune complex disease of rabbits were found almost exclusively in animals forming large complexes, i.e., greater than 19 S in size (Cochrane and Hawkins, 1968) (Table II). Rabbit serum sedimentation patterns of complexes that were sufficiently large to deposit in vessels as opposed to those that were too small are shown in Fig. 10. In chronic immune complex disease in rabbits, a similar correlation has been observed (Wilson and Dixon, 1971).

Evidence is, therefore, at hand indicating that a complicated series of events takes place leading to deposition of circulating complexes. Increased vascular permeability occurs, brought about most probably by the release of vasoactive agents from their reservoirs in the circulation

TABLE II

RELATIONSHIP OF THE SEDIMENTATION CHARACTERISTICS OF CIRCULATING IMMUNE COMPLEXES TO THE DEVELOPMENT OF LESIONS IN SERUM SICKNESS[a]

Rabbits	Average amount BSA-^{131}I bound to globulin at maximum[b] (%)	Pattern of BSA-^{131}I sedimentation[c]	Glomerular lesions	Total amount proteinuria (mg)	Maximal complement depletion (%)
9	43.3	Heavy	2.3+	567	78
5	41.6	Light	0 to ±	0	67

[a] Data taken from Cochrane and Hawkins (1968).

[b] Percent (average) of bovine serum albumin (BSA)-^{131}I bound to globulins determined by precipitation of the globulins with ammonium sulfate at 50% saturation.

[c] Pattern of sedimentation of BSA-^{131}I complexed to globulin as determined in sucrose gradient. Heavy = BSA-^{131}I sedimentation pattern extends below 19 S marker; light = BSA-^{131}I sedimentation pattern does not reach 19 S marker (see Fig. 2).

or the tissues. Then in the presence of increased permeability the large macromolecular immune complexes deposit along a filtering membrane. Inflammation ensues.

In humans, there is abundant evidence that the earliest deposition of immune complexes occurs along vascular basement membranes. In glomeruli, for example, electron microscopy has revealed electron-dense deposits falling between endothelial cells and the basement membranes (Michael *et al.*, 1966).

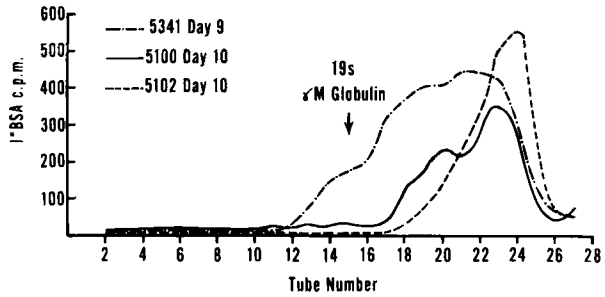

FIG. 10. Sedimentation patterns of bovine serum albumin (BSA)-*I in sera obtained from 3 rabbits at the times shown after injection of BSA-*I. Centrifugation was carried out in linear sucrose density gradient 10–37% at 50,000 r.p.m. for 4 hours. No. 5100 demonstrates a typical "light" pattern, and No. 5341 a "heavy" pattern. No. 5102 failed to develop antibodies and showed a sedimentation pattern of free BSA in normal serum.

Hydrodynamic forces also play roles in the deposition of circulating complexes. Arterial lesions in acute immune complex disease of rabbits occur most commonly at the entrance of the coronary arteries and at branches and bifurcations of the aorta (Germuth, 1953; Kniker and Cochrane, 1968). The heart valves are often involved. In addition, if a coarctation is induced artificially in the lower aorta, lesions develop on the inferior surface of the constricted zone (Kniker and Cochrane, 1968). This is of great interest, since it has been shown that platelets clump and adhere to endothelia in these areas of severe turbulence (Geissinger *et al.*, 1962).

The intensity of lesions in serum sickness increases in the presence of hypertension (Fisher and Bark, 1961), again drawing attention to the importance of hydrodynamic factors. Hüttner *et al.* (1970) found that in hypertensive rats, the passage of both small and large molecules into the subendothelial spaces of the aorta was increased over normal. Evidence was offered showing that small molecules, i.e., horseradish peroxidase, passed through intercellular junctions, whereas a large molecule, ferritin, passed through the endothelium via cytoplasmic transport vesicles. It is well known in addition that hypertension is associated with the development of arteritis (Wilson and Byron, 1941; Smith *et al.*, 1944; Smith and Zeek, 1947; Zeek, 1952).

B. Mechanisms of Release of Vasoactive Amines from Platelets

1. Immune Complexes and Platelets in the Presence of Plasma

The rabbit has served as a most useful model in studies of immune mechanisms by which vasoactive amines are released from blood cells. Not only are the platelets well endowed with histamine which allows easy quantitation of released products, but the rabbit is particularly prone to acute and chronic immune complex disease. These observations made *in vitro* can be tested for relevance in a disease situation *in vivo*. Clearly, a subject of pressing interest has been the immunological mechanisms responsible for the release of histamine from platelets.

Immune complexes have been found by many investigators to bring about the release of amines from rabbit platelets in the presence of plasma (Humphrey and Jaques, 1955; Barbaro, 1961a; Gocke and Osler, 1965; Gocke, 1965; Bryant and Des Prez, 1968; Henson and Cochrane, 1969a; Henson, 1969b). The reaction is augmented greatly when antibody is in excess (Barbaro, 1961a; Gocke and Osler, 1965). The requirement of complement has been shown in several laboratories (Humphrey and Jaques, 1955; Gocke, 1965; Henson and Cochrane,

1969b), although others have questioned the need for complement based on incomplete inhibition by conditions affecting complement (Barbaro, 1961b) and a requirement of Mg^{++} but not Ca^{++} together with plasma (Siraganian *et al.*, 1968a). Regarding the latter data, complement activity could still exist through the activation of C3 proactivator by preformed complexes.

Strong evidence favoring the participation of complement components in the immunological release of vasoactive constituents of platelets emerged from the use of plasma from rabbits genetically deficient in C6. Immune complexes failed to induce release of histamine and serotonin from rabbit platelets in the presence of C6-deficient plasma, but this ability was restored by addition of semipurified rabbit C6 (Henson and Cochrane, 1969b). Three complement-dependent reactions were described resulting in the release of histamine and serotonin from platelets in these studies. The first, noted above, resulted from the interaction of immune complexes in an excess of antibody with platelets in the presence of normal rabbit plasma. Through the action of C3 immune adherence, platelets clumped to complexes, and through the action of terminal components, lysis of the platelets followed. Lysis was evident with the electron microscope and by measuring release of cytoplasmic and granular constituents of the platelets. Clumping of platelets by strong immune adherence was not observed by Gocke and Osler (1965), although the lower concentration of platelets in their study could account for the difference.

2. *Immune Complexes and Platelets in the Presence of C6-Deficient Plasma and Neutrophiles*

In a second mechanism, when C6-deficient plasma was employed, release of histamine and serotonin could be induced by adding a few neutrophiles (Henson and Cochrane, 1969a,b; Henson, 1970a). Lysis of platelets did not result.

3. *Immunological Activation of Platelets Using a Particulate Antigen*

Third, if a particulate antigen such as zymosan was used, together with antibody to its surface determinants, complement components through C3 were required to induce immune adherence of platelets and particles, and release of histamine and serotonin occurred without participation of the terminal complement components (Henson and Cochrane, 1969a,b). These two latter release mechanisms required the active participation of the platelets, since inhibitors of glycolysis and serine esterases diminished the release of histamine (Henson, 1969a).

4. Interaction of Sensitized Leukocytes and Platelets

A fourth mechanism of immunological release of constituents from rabbit platelets which has received recent attention involves synergy between sensitized leukocytes and platelets in the presence of antigen. Plasma is not required. Schoenbechler and Sadun (1968) and Siraganian *et al.* (1968b) who used washed leukocytes and platelets from immunized rabbits, described this mechanism independently. Protein antigens have also been used to elicit this reaction (Schoenbechler and Barbaro, 1968; Barbaro and Schoenbechler, 1970; Henson, 1969a; Henson and Cochrane, 1969b; Siraganian and Osler, 1969; Henson, 1970b). The leukocyte involved was thought at first to be mononuclear in type, although direct evidence of its participation was uncertain (Schoenbechler and Barbaro, 1968; Henson, 1970b). More recently, the basophile has been recognized as the leukocyte responsible for the synergistic action. Siraganian and Osler (1971b) correlated the histamine content of leukocytes (and thereby the basophiles) and induction of the histamine-releasing reaction with platelets. Further, a correlation was found between basophiles that were counted and the leukocyte–platelet mechanism (Henson and Benveniste, 1971), and basophiles have been identified at the center of clumped platelets immediately after initiating the reaction (Benveniste *et al.*, 1972). When the serum of rabbits having strong reactivity was transferred to normal recipient rabbits, the reaction could also be transferred (Colwell *et al.*, 1970; Benveniste and Henson, 1971). The serum also was rich in homocytotropic antibody, as determined by its capacity to endow passive cutaneous anaphylaxis in normal recipients. A correlation between the presence of homocytotropic antibody and the mechanism of histamine release from platelets was described earlier by Barbaro and Zvaifler (1966) and has been confirmed by Siraganian and Osler (1970). Fractionation of antiserum by anion exchange and gel filtration chromatography indicated that the antibody responsible migrated as a fast γ and was of about 200,000 daltons in size. Passive transfer was prevented by pretreatment with anti-IgE (Benveniste and Henson, 1971). The leukocyte–platelet reaction could be elicited with purified anti-IgE.

A Soluble Factor Released from Basophiles Causing Aggregation of Platelets and Release of Their Vasoactive Amines. After reaction with antigen, the sensitized leukocytes release a soluble factor (Henson, 1969a, 1970b; Henson and Benveniste, 1971; Siraganian and Osler, 1971a), which has been termed the *platelet-activating factor* (PAF). This soluble factor binds rapidly to albumin and causes platelets to clump and release their vasoactive constituents through an energy-

TABLE III
EFFECT OF C3 DEPLETION IN ACUTE IMMUNE COMPLEX DISEASE (SERUM SICKNESS) OF RABBITS[a]

Rabbit	Glomerulitis (No. positive/total)	Proteinuria (mg./day), average	Necrotizing arteritis (No. positive/total)
C3-depleted[b]	13/13	664	0/6[c]
Control	43/45	456	20/34

[a] Data from Henson and Cochrane (1971).

[b] C3 depleted by injections of cobra venom factor just before appearance of circulating immune complexes.

[c] Arterial intimal proliferation and edema present in all six rabbits, but neutrophil accumulation did not occur in C3-depleted rabbits.

requiring process. It is of small molecular weight, as it causes no apparent change in the size of albumin to which it binds in active form. Platelet-activating factor is a distinct product of basophiles, to be added to the list of pharmacologically active constituents of these cells. Preliminary studies of leukocytes from allergic humans together with rabbit platelets have demonstrated the presence of a similar if not identical mechanism.

C. Lack of Participation of Complement Components in Release of Vasoactive Amines from Platelets in Acute Immune Complex Disease in Rabbits

Experiments have been conducted to determine if the complement-dependent mechanisms of histamine release play a role in the deposition of circulating immune complexes in rabbits. Rabbits were depleted of C3 and terminal components of complement by the injection of cobra venom factor (Cochrane *et al.*, 1970). Immune complexes appeared in the circulation and, despite depletion of complement, they deposited in glomeruli to induce injury (Table III) (Henson and Cochrane, 1971). Arteries were also affected, but in the absence of sufficient C3, neutrophiles did not accumulate to produce necrotizing arteritis. The fact that the immune complexes are in antigen excess in acute immune complex disease may be important in this observation. The complement-dependent mechanisms of release of vasoactive amines from platelets are significantly augmented in antibody excess as noted above.

D. Correlation of the Basophile-Dependent Mechanism of Histamine Release from Platelets and Deposition of Immune Complexes with Development of Injury

A correlative study of the basophile-dependent mechanism of histamine release from platelets and deposition of immune complexes and

TABLE IV
CORRELATION OF LEUKOCYTE-DEPENDENT RELEASE OF HISTAMINE FROM PLATELETS AND GLOMERULAR INJURY IN ACUTE IMMUNE COMPLEX DISEASE (SERUM SICKNESS) OF RABBITS

No. of rabbits[a]	Glomerulonephritis	Rabbits with leukocyte-dependent release
17	Present	16
8	Absent	1[b]

[a] All rabbits exhibited circulating immune complexes. Amount of complexes was comparable between the two groups.

[b] Complexes of "light" type in sedimentation pattern.

injury is shown in Table IV. Only animals with circulating immune complexes are included. As noted, when circulating complexes deposited and injury resulted, the mechanism was detected in all but one instance (Henson and Cochrane, 1971). By contrast, rabbits with immune complexes but without deposition of complexes and development of injury failed in all but one case to demonstrate the basophile–platelet mechanism. Most interestingly, the single rabbit with circulating immune complexes and the basophile–platelet mechanism was found to have complexes of the "light" type that were too small to be entrapped in vessel walls. This correlation implicated the basophile–platelet mechanism as being the important immunological method of releasing vasoactive amines and thereby increasing vascular permeability and bringing about deposition of the circulating complexes. Furthermore, the basophile–platelet mechanism may also be detected in rabbits with chronic immune complex disease.

The application of these findings in human immune complex disease is uncertain, although data are becoming available. For example, Kniker, in a study of patients receiving foreign serum (antitoxin) employed antagonists of vasoactive amines. The drugs were started immediately prior to the time lesions and symptoms were expected. The disease was markedly inhibited in patients receiving drugs, less than 5% showed signs of immune complex disease. This contrasted with 25% of controls who developed serum sickness (Kniker, personal communication). The results are reminiscent of those wherein immune complex disease was inhibited by antagonists of vasoactive amines in rabbits.

E. SUMMARY OF THE PROCESS OF IMMUNE COMPLEX DEPOSITION

Mechanisms by which deposition of immune complexes may occur in acute immune complex disease of rabbits are shown in Fig. 11 and may be summarized as follows. (*1*) Immune complexes and protein

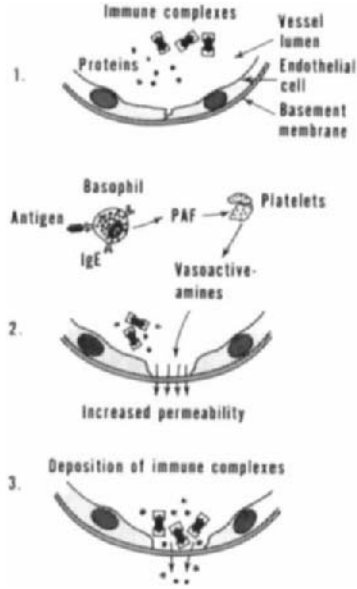

FIG. 11. Summary of mechanisms of immune complex deposition in rabbits.

molecules circulate in blood vessels. (2) In the presence of basophilic leukocytes with adherent IgE antibody, antigen induces release of the soluble intermediary, PAF. This intermediary activates platelets to clump and to release vasoactive amines. The amines then cause an increase in permeability of blood vessels, especially in areas where the platelets clump and impinge upon blood vessels. (3) With increased permeability, macromolecular (>19 S) immune complexes enter and become entrapped along filtering membranes in the vessel wall to induce injury.

F. Relationship to Development of Arteriosclerosis

As emphasized by Mustard and his colleagues (Geissinger *et al.*, 1962; Mustard *et al.*, 1962), atheroma formation occurs in the same areas where aggregates of platelets and white cells are found along arterial intima. Formation of atherosclerotic plaques from platelet aggregates in the intimal layers of blood vessels in pigs (Geissinger *et al.*, 1962) and rabbits (Chandler and Hand, 1961; Hand and Chandler, 1962) has been demonstrated.

A marked potentiation of atherogenesis has been noted in rabbits receiving diets high in cholesterol when foreign plasma proteins are injected (Roberts, 1960; Minick, 1966; Minick *et al.*, 1966; Levy, 1967; Van Winkle and Levy, 1968, 1970). By examining arterial lesions after a

single injection of bovine albumin to induce serum sickness followed by 2 weeks of high cholesterol diet, Levy (1967; Van Winkle and Levy, 1968) noted a marked increase in the incidence of atheromatous lesions over rabbits receiving a high cholesterol diet alone. Prolongation of the cholesterol diet with continued injections of foreign protein led to severe atherosclerotic lesions (Minick *et al.*, 1966). These resembled human atherosclerotic lesions in several ways: rabbit lesions contained lipid-filled "foam" cells throughout the thickened intima; changes including reduplication of the intimal elastic lamina were apparent, the media contained lipid; degenerative changes were involved; and cellular infiltration was noted. A parallel was drawn to the heightened atheromatous lesions observed in certain cases of rheumatic diseases (Minick *et al.*, 1966). The lesions were observed up to 135 days after beginning the experiments and continued even when the cholesterol diet was terminated. Van Winkle and Levy (1970) noted no decrease in incidence of atheromatous lesions up to 8 weeks after termination of the cholesterol diet in rabbits receiving both foreign protein and cholesterol diet, whereas those on the cholesterol diet alone showed a markedly decreased incidence. As noted previously (Germuth, 1953), the arteritic lesions of acute immune complex disease healed and disappeared within 1 to 2 weeks of the time antigen was eliminated from the circulation. This suggests that the combination of inflammatory injury and lipid deposition in arterial walls leads to alterations in the vascular structure that become self-perpetuating. This may occur even when the level of inciting agents is greatly reduced.

Injury to arteries from a variety of sources leads to accumulation of lipid in hypercholesterolemic rabbits (Prior and Hartmann, 1956; Prior and Hutter, 1955; Kelly *et al.*, 1952; Friedman and Byers, 1961). Lipids were not observed in the lesions unless the rabbits were given a prolonged hypercholesterol diet starting shortly after the trauma. The experimental lesions resembled closely the naturally occurring arteriosclerotic plaques in human arteries (e.g., see Friedman and Byers, 1961). The mechanism by which lipid enters the plaque has been the subject of much consideration but is not central to the present review.

III. Fate and Disappearance of Immune Complexes

A. Circulating Immune Complexes

The subject of the disappearance and fate of circulating immune complexes was the subject of extensive review in the first volume of this series (Weigle, 1961). This review will briefly summarize the subject and discuss advances that have occurred subsequently.

When a circulating antigen induces formation of antibody, measurable complexes are formed. The complexes are generally eliminated at a rapid rate from the circulation. In a situation where antigen is infused into sensitized animals with circulating antibody, the complexes formed are deposited in the lung, liver, and spleen where they are rapidly catabolized (Dixon and Maurer, 1953; Francis *et al.*, 1957). A great deal has been learned from studies of immune complexes formed *in vitro* that are infused intravenously *in vivo*. Immune precipitates prepared at equivalence and washed have been dissolved in an excess of antigen with increasing amounts of the antigen (Weigle, 1958). The size of the soluble complexes thus formed varies inversely with the amount of antigen added. When injected intravenously into rabbits, the bulk of complexes was rapidly removed from the circulation in the first 24 hours. A slower rate of elimination followed over the next several days. The level at which the complexes equilibrated in the circulation after 24 hours depended on the amount of antigen employed to dissolve the precipitate. The more antigen used, the smaller the complexes formed and the greater the resulting levels in the circulation. In other studies, immune complexes were prepared by using an amount of antigen (20 times the amount needed at the point of equivalence) that produced soluble complexes varying greatly in size. When injected intravenously, the large complexes (>19 S) disappeared rapidly (in a few seconds), whereas the smaller complexes (<19 S) remained in the circulation (Cochrane and Hawkins, 1968). The animal was, therefore, able to remove large complexes preferentially. Similar findings were obtained by Lightfoot *et al.* (1970). With oligovalent hapten–protein conjugates as antigens, immune complexes were prepared, and by limiting the amount of hapten, the valency of antigen could be reduced leading to the formation of soluble immune complexes with the antibody. Again, complexes >19 S in size (Ag_2Ab_5) were rapidly removed from the circulation of mice after infusion, while many smaller complexes (Ag_1 Ab_2 or Ag_1 Ab_1) persisted in the circulation. Mannik *et al.* (1971) and Arend and Mannik (1971) examined the capability of numerous immune complexes to circulate. Again, the large complexes were rapidly removed from the circulation, a significant percentage being taken up by the liver of rabbits. Since the large complexes are of greater pathogenic significance than the small, the capacity to remove the former rapidly must be considered as an important defense mechanism.

A slower initial rate of elimination of passively injected avian and canine antibody–antigen complexes than of similar rabbit antibody–antigen complexes was noted in rabbits by Patterson and his colleagues (Patterson and Suszko, 1966, 1967; Patterson *et al.*, 1968). The rate of

elimination of antibodies was dependent upon the species of recipient: avian antibody was compared with rabbit antibody in its ability to remove antigen (BSA) from the circulation. Avian antibody produced rapid elimination of antigen in birds but not in rabbits or guinea pigs; rabbit antibody produced rapid elimination of BSA in rabbits, guinea pigs, and birds (Patterson and Suszko, 1966). This suggested that receptors on the reticuloendothelial cells recognize different immunoglobulins with varied affinities.

The role of plasma factors has been analyzed in the elimination of immune complexes from the circulation. Depletion of complement by aggregated IgG or the anticomplementary factor in cobra venom had no effect on the rate of disappearance of the complexes from the circulation (Mannik and Arend, 1971; Arend and Mannik, 1971). Complement depletion had no effect on the capacity of circulating complexes to deposit in blood vessel walls as well (Cochrane and Hawkins, 1968; Henson and Cochrane, 1971). Of interest, reduction and alkylation of antibody before preparation of the soluble complex permitted formation of large complexes but resulted in a markedly decreased rate of removal of the complexes from the circulation. Complexes so formed consisted of large as well as small aggregates. Although 40% of the untreated immune complexes initially injected were found in the livers of rabbits after 40 minutes, only 1.3% of the complexes made with reduced alkylated antibody were deposited in that organ.

These observations lead to the important question of recognition of immune complexes by cells responsible for the elimination of the complexes. The cells undoubtedly are mostly members of the reticuloendothelial system and apparently are capable of recognizing the surface conformations of large but not small immune complexes. The presence of complement components is not necessary for recognition when the complexes are sufficiently large. But size is not the only criterion for removal, for when the complexes were prepared with antibody having its interchain disulfide bond reduced and alkylated, a marked reduction in the initial rate of elimination of the complexes in rabbits and rhesus monkeys was noted (Mannik and Arend, 1971).

Receptors for immunoglobulins are present on the surfaces of macrophages (or monocytes) and neutrophiles. In general, this has been demonstrated by the binding of immune complexes or immunoglobulins to the surface of leukocytic membranes. Antigens either on, or bound to, red cell surfaces have been employed to study conveniently the interaction between immune complexes and white cells. When immunoglobulins have complexed with antigens, binding to leukocytes and presumably to reticuloendothelial cells is greatly enhanced (Uhr, 1965;

Uhr and Phillips, 1966; Berken and Benacerraf, 1966; Phillips-Quagliata *et al.*, 1971; LoBuglio *et al.*, 1967; Huber *et al.*, 1968; Henson, 1969b; Lay and Nussenzweig, 1969). Binding of complexes to macrophages is mediated by the Fc portion of the antibody molecule (Uhr and Phillips, 1966) and might result from changes in the immunoglobulin structure upon complexing with antigen or from the sum of multiple binding sites of immunoglobulin molecules held together by antigen. Structural changes develop in antibody molecules upon complexing with antigen as indicated by direct observation (Feinstein and Rowe, 1965; Valentine and Green, 1967) or by physical data (Ishizaka and Campbell, 1959; Grossberg *et al.*, 1965; Heeney and Stanworth, 1966; Warner *et al.*, 1970). However, Phillips-Quagliata *et al.* (1971) have presented evidence with hapten–protein antigens of varied valencies indicating that the more important factor in the aggregation of immune complexes to macrophages lies in the summation of individual sites on immunoglobulin molecules rather than in structural changes of the immunoglobulin upon complexing with antigen. The requirement of complement, especially C3, for binding of the IgM–antigen complex to macrophages (monocytes) and neutrophiles has been observed by Henson (1969b), and inhibition studies with nonaggregated IgG allowed the distinction between receptors for IgG and C3 on the surfaces of human monocytes (Huber *et al.*, 1968).

The data present a plausible explanation for the rapid elimination of large complexes. In addition, they predict that elimination of complexes containing IgM antibodies may well be augmented by complement.

B. Immune Complexes Deposited in Sites of Injury

Studies have been conducted of the mechanisms responsible for the removal of injurious complexes after their deposition in sites where injury takes place.

Immune complexes formed in blood vessel walls in Arthus reactions are short-lived. Disappearance rates of antigen, injected locally to form the complexes, is rapid with >90% disappearance in 24 to 48 hours (Korngold *et al.*, 1953; Cochrane *et al.*, 1959). The presence of antibody retards the initial loss of injected antigen from the site. However, with fluorescent antibody techniques, the majority of injurious complexes in vessel walls were eliminated by 24 hours (Cochrane *et al.*, 1959). After neutrophiles were depleted, complexes remain in the vessel walls without apparent change for periods up to 72 to 96 hours, when observations were terminated. Similarly, in experimental immune complex arteritis, deposits of immune complexes and complement were rapidly removed by incoming neutrophiles (Kniker and Cochrane, 1965).

Only in the earliest lesions were complexes demonstrated by fluorescent techniques.

Neutrophiles and occasional monocytes removed from immune complex lesions contain granules of immune complexes (Cochrane *et al.*, 1959). These neutrophiles when placed in culture degrade the complexes, secreting fragments of the cleaved proteins into the surrounding medium. This may well account for the observed degradation and disappearance of complexes in the lesions. In studies of phagocytosis, Sorkin and Boyden (1959, 1961, 1962) demonstrated that peritoneal exudate cells of rabbits and guinea pigs phagocytize immune complexes and degrade them. Patterson *et al.* (1962) and Hawkins and Peeters (1971) have shown that only large complexes are taken up by leukocytes, in keeping, perhaps, with the rapid elimination of large but not small complexes.

In lesions not readily attacked by phagocytic cells, the deposited complexes are able to survive for longer periods. In acute immune complex disease, the $T_{½}$ disappearance of radiolabeled BSA in the renal glomeruli was about 10 days (Wilson and Dixon, 1971). These lesions in rabbits, as opposed to those of acute glomerulonephritis in humans, rarely contained neutrophiles. In chronic glomerulonephritis, where the deposits of complexes formed along the epithelial (external) aspect of the GBM, the disappearance rate of the antigen was dependent upon the dose of antigen employed to maintain complexes in the circulation. As mentioned previously, when injections of antigen were discontinued, the antigen persisted with a $T_{½}$ of 5.6 ± 1.1 days (Wilson and Dixon, 1971). When the usual doses of antigen were given to maintain chronic immune complex disease the $T_{½}$ of antigen was 5.0 ± 0.7 days, or if large injections of antigen (1.2–3.0 gm.) were given, 0.9 ± 0.1 day. These values confirm and extend in a quantitative manner the observations of Valdes *et al.* (1969), who showed that administration of large doses of antigen to rabbits with chronic immune complex disease cleared the complexes and dramatically improved the state of the injured glomeruli.

IV. Immune Complex Disease and Living Antigens

A. Immunopathological Mechanisms in Disease of the New Zealand Mouse

1. Background Immunopathology

A disease in the New Zealand (NZ) strain of mice has been observed and well studied since its original description by Bielschowsky *et al.*

(1959). The NZ mice with black coat color (NZB) developed autoimmune hemolytic disease and progressive chronic membranous glomerulonephritis. Hybridization with other strains, especially the white-coated (NZW) strain, was found to induce a far more rapid onset of disease with increased severity of glomerulonephritis and early death. Females were affected more rapidly and severely than males. The disease in hybrid mice resembled closely the nephritis of systemic lupus erythematosus and established the NZ mice as prime animals in which to study naturally occurring autoimmune disease. The description of lesions and immunological background in their development has formed the basis of a comprehensive analysis by Howie and Helyer (1968) in this series to which the reader is referred for studies performed before 1969. In the present review, we focus on developments occurring since that time, referring back only for points of clarification.

Evidence of Immune Complex Disease. The morphological features of nephritic lesions in both NZB and (NZB × NZW)F_1 hybrid mice are characteristic of the lesions in known immune complex nephritides. Thickening of the GBM seen by light microscopy and ultrastructural deposits of material along the external and, to a lesser extent, the internal aspect of the GBM are characteristic. Deposits are also prominent along the GBM in the mesangial region. By fluorescent microscopy, mouse γ-globulin and C3 are detectable forming a granular pattern along the GBM and in the mesangium (Aarons, 1964; Mellors, 1965; Nairn *et al.*, 1966; Dubois *et al.*, 1966; McGiven and Hicks, 1967; Lambert and Dixon, 1968; Seegal *et al.*, 1969). γG-Globulin and C3 appear in glomeruli prior to the appearance of several other plasma proteins that accumulate in severely diseased glomeruli (Nairn *et al.*, 1966).

With fluorescein-conjugated human antideoxyribonucleic acid (DNA) antibodies, Lambert and Dixon (1968) demonstrated the presence of DNA in a pattern identical to that of immunoglobulin in glomeruli of diseased NZB/W hybrid mice. Antibodies specific for the pyrimidines, thymine and cytosine, were found by Seegal *et al.* (1969) to bind to antigens in the glomerular capillary walls in 40 of 51 NZB/W hybrid mice over 5 months of age. These pyrimidine antigens occurred in the same locations as deposits of mouse immunoglobulin. Strikingly lower amounts of immunoglobulin and pyrmidine antigens were found in NZB or NZW mice, in keeping with a lower incidence of renal disease. The authors pointed out that the antibodies to thymine and cytosine demonstrated denatured and not native DNA.

Elution of immunological reactants from washed renal homogenates of NZB/W hybrid mice has been performed by Lambert and Dixon (1968). Eluates contained only the γG_{2a} class of immunoglobulin. When

eluates were examined for antinuclear constituents by immunofluorescence, a "rim and fibrillar" pattern of fluorescence on target nuclei was observed, suggesting that anti-DNA antibody was present (Tan, 1967b). This hypothesis was born out experimentally by suppressed fluorescence after absorption of the eluted antibody with DNA or nucleoprotein and by treatment of target nuclei with deoxyribonuclease (DNase) (Lambert and Dixon, 1968). The anti-DNA antibody of the eluates was eighty-fold greater in concentration than that in serum at a comparable concentration of γG-globulin. Eluates failed to bind to the GBM. Thus strong evidence supports the thesis that the glomerular disease in NZB and NZB/NZW F_1 hybrid mice is caused by immune complexes.

2. Possible Viral Etiology

Major purposes in studying these mice have been to elucidate the initiating factors of the immune response and to characterize the source of DNA to which they are responding. Many of the theories concerning pathogenesis about these mice in the 1960s were reviewed by Howie and Helyer (1968), and the present remarks will be limited to subsequent observations.

Attention has been directed to the wild-type Gross (G) leukemia virus by Mellors and co-workers (Mellors, 1965; Mellors *et al.*, 1969, 1971; Mellors and Huang, 1966). Like many mouse strains, NZB and NZB/NZW F_1 hybrid mice harbor G virus. The virus is detected by the presence of antigens of the murine leukemia virus (MLV) in tissues by using immunofluorescence and by antibodies to cellular G antigen(s) and G antigen(s) of the viral envelope (Aoki *et al.*, 1970). Virus G-soluble antigen (GSA) may be found in the circulation of infected mice (Aoki *et al.*, 1968).

In most mouse strains, wild-type G leukemia virus and GSA remain in the tissues and plasma for the duration of life. By contrast, in NZB and NZB/NZW F_1 hybrids, GSA is eliminated from the circulation of adults. Tests for GSA elimination in NZB and NZB/NZW F_1 hybrids indicated that 50% of mice had eliminated the antigen by 13.3 and 7.6 months, respectively. Antibody to G antigen thereafter increased in incidence (Fig. 12) (Mellors *et al.*, 1969, 1971). By immunofluorescence, MLV antigens were located in the mesangium and in capillary loops in glomeruli of proteinuric NZB/NZW F_1 hybrid mice (Mellors *et al.*, 1971). Immunoglobulins were associated with the MLV antigen. By contrast, in the more slowly developing membranous nephritis of NZB mice, MLV antigen and immunoglobulin were detected only in the mesangium (Mellors *et al.*, 1969). Kidneys from NZB and NZB/NZW

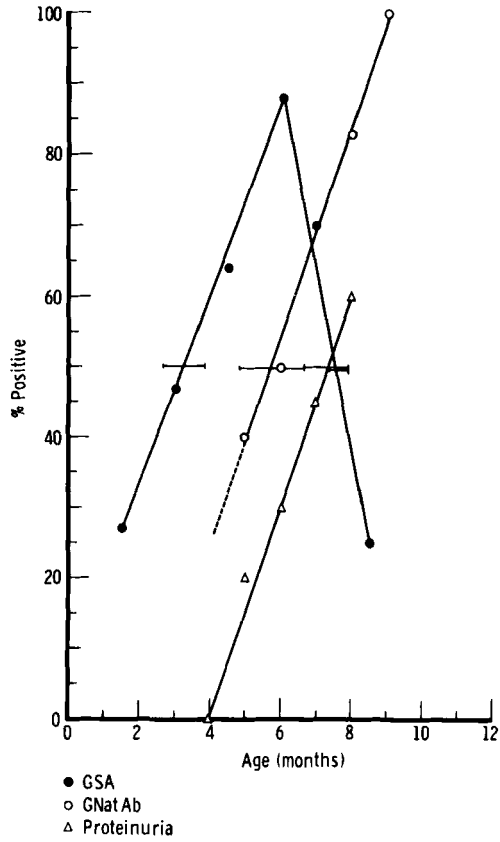

FIG. 12. Tests (% positive) for Gross virus-soluble antigen (GSA), Gross natural antibody (G Nat Ab), and significant proteinuria performed on (NZB × NZW)F_1 hybrid mice at various ages. Horizontal bar at 50% positive; age, mean ± S.E.

F_1 hybrid mice were homogenized and eluted under acid conditions. Eluates of the NZB kidneys contained GSA that had appeared when the animals were about 6 months of age. Antibody to G antigens was measurable in the sera of mice by 14 months of age and in the renal eluates as early as 2–4 months of age (Mellors *et al.*, 1971). Thus, G antigens and antibodies appeared in the kidneys prior to development of proteinuria and well in advance of measurable free antibodies to G antigens in the circulation. In NZB/NZW F_1 hybrid mice, GSA was found in the circulation earlier than in NZB mice and appeared before 2 months of age. It reached peak incidence in the hybrid mice by 6 months and diminished thereafter. Antibody to G antigen was first

found at 4 months, far earlier than in NZB mice (14 months). Renal disease (proteinuria) occurred in some hybrid mice as early as 5 months, shortly after the earliest appearance of free antibody (Mellors *et al.*, 1971).

Cellular G antigens were present in the acid eluates of diseased NZB kidneys, since antibody from immunized C57B1 mice reacted with the antigen (this antibody is known to react only with cellular G antigen and not with the viral envelope). When tested with acid eluates of the diseased kidneys, antibody from an NZB mouse gave a far stronger immunofluorescent reaction than did antibody from C57B1 mice, suggesting that antigens of the viral envelope may also be present (Mellors *et al.*, 1971). Further work will be required to substantiate this notion.

Although this discussion dwells on findings in NZB mice, glomerulonephritis has also been observed in AKR mice similarly infected with wild-type G leukemia virus (Richer *et al.*, 1966) and in mice infected experimentally with Rauscher and Friend viruses (Richer *et al.*, 1966; Dunn and Green, 1966). Similarly, BALB/c mice bearing persistent infections with Moloney sarcoma or Moloney leukemia virus develop glomerulonephritis (Hirsch *et al.*, 1969).

Several immune complexes having various antigens may circulate simultaneously and induce glomerulonephritis. In order to identify and quantitate antibodies to various antigens in affected glomeruli of NZ mice, Dixon *et al.* (1971) tested acid eluates from washed renal homogenates. The quantity of IgG in the eluates was then determined by quantitative immune diffusion before and after absorption with insoluble nucleoprotein, GSA, and G virus (Table V). The results indicated that,

TABLE V

SPECIFICITY OF IMMUNOGLOBULIN G ELUTED FROM KIDNEYS OF NZ MICE

Strain	Age (months)	% eluted IgG absorbed with			
		NP[a]	Gross[b]	NP + Gross	LCM[c]
NZW	8–14	35	5	59	—
NZB	4–12	48	17	62	—
NZB × W					
Male	9–10	44	7	64	—
Female	9–10	45	21	72	—
SWR/J					
LCM carriers	4–9	3	0	—	45

[a] NP, nucleoprotein.

[b] Eluates were absorbed with both Gross virus (G + E G2 cells) and Gross-soluble antigen (EL4 cells coated with Gross-soluble antigen) (from Dixon *et al.*, 1971).

[c] LCM, lymphocytic choriomeningitis.

whereas 35–48% of the eluted IgG was removed by absorption with insoluble nucleoprotein (depending upon the sex and strain of NZ mouse), 5–21% was absorbable by G virus and GSA. The results indicated that only a portion of the antibody in the glomerular deposits was directed toward G antigen. This brings attention to the possibility that the G virus may be but one agent contributing to the pathogenesis of the disease.

To underscore the possibility that several viruses may contribute to immune complex glomerulonephritis in NZ mice, Tonietti *et al.* (1970) induced superinfection of NZ mice with two viruses, lymphocytic choriomeningitis (LCM), a ribonucleic acid (RNA) virus, and polyoma, a DNA virus. The glomerulonephritis that developed was of greater severity than that in untreated NZ mice. When the glomeruli of such mice were eluted and the eluates tested for the amount of IgG reacting with nucleoprotein and the injected virus (polyoma and LCM), it was found that a significant amount (20–45%) of IgG was contributed by antibody to the polyoma or to the LCM (Dixon *et al.*, 1971). Recently, Oldstone *et al.* (1971) have shown that infection with LCM virus of NZW, NZB, and hybrid mice within 15 hours of birth induced production of detectable GSA in 84 to 90% of all mice by 3 months of age. Noninfected controls showed development of G antigens in only 3 to 5% of mice tested. Similar infection with polyoma virus was without effect. It might be expected (although it has not yet been demonstrated) that the amount of G virus and GSA in immunological complexes in the glomeruli would be increased by coinfection with LCM virus.

Of further interest in the pathogenic picture in NZB/NZW mice is the recent observation that superinfection of these mice with lactic dehydrogenase virus reduces the formation of antinuclear antibody and the development of glomerulonephritis (Dixon *et al.*, 1971). At 9 months of age, twice the number of female mice survived over noninfected animals when infected with this virus. The mechanism of this protection is not known.

3. *Immunological Responsiveness of NZ Mice*

The abnormal immunological reactivity of NZ mice has been implicated in the development of autoimmune disease. As noted previously, NZB and NZB/NZW mice are unique in their response to MLV. In addition, when challenged with DNA bound to methylated bovine albumin, NZW and NZB/NZW hybrids, but not NZB mice, gave a heightened response to DNA (Lambert and Dixon, 1968, 1970). The response was manyfold higher than responses in several other inbred strains. Strain NZ mice produced anti-RNA and anti-DNA in a greater

than normal response to immunization with synthetic polyribonucleotides and polyinosinic–polycytidylic acid (rI–rC) (Steinberg *et al.*, 1969; Talal, 1970; Talal *et al.*, 1971a). Associated with immunization were accelerated immune complex nephritis and death (Carpenter *et al.*, 1970). Antibodies to RNA in NZB/NZW F_1 hybrids and in patients with systemic lupus erythematosus have been examined for specificity (Talal *et al.*, 1971b). The murine and human antibodies showed reactivity with double-stranded RNA. The source of stimulation from antibodies to double-stranded RNA is unknown but could come from either virus or host.

Many antigens, such as sheep erythrocytes (sheep RBC's) (Playfair, 1968; Morton and Siegel, 1969), nucleic acids (Steinberg *et al.*, 1969; Lambert and Dixon, 1968, 1970), rI-rC (Talal, 1970), and foreign proteins (Weir *et al.*, 1968; Staples and Talal, 1969), evoke a greater immune response in NZ mice than in other strains. This is not true of all antigens, e.g., keyhole limpet hemocyanin (Cerottini *et al.*, 1969). In addition, evidence indicates that in older NZB/NZW hybrid mice that have developed disease, the immune response is hampered (Solomon and Benveniste, 1969; Morton and Siegel, 1969). Strain NZ mice reach immunological maturity earlier than other strains (Evans *et al.*, 1968). Perhaps, as a result of their unusual capacity to respond to antigens with humoral antibody, it is difficult to produce lasting immunological unresponsiveness to certain antigens in these mice (Staples and Talal, 1969; Weir *et al.*, 1968; Staples *et al.*, 1970; Steinberg *et al.*, 1970), although to other antigens (human and rabbit IgG), lasting tolerance can be induced (Russell and Denman, 1969; Cerottini *et al.*, 1969). Talal (1970) has also reported that thymic cells obtained from NZB/NZW mice tolerant to sheep RBC's were not able to convey tolerance in the presence of normal bone marrow cells to an irradiated, syngeneic recipient. Similar thymic and bone marrow cells from C57B1/6 mice did convey tolerance.

The importance of these observations lies in the finding that NZ mice respond to DNA in a heightened fashion. Presumably viruses that are tolerated by most strains of mice are immunogenic to NZ mice. Although NZB mice gradually develop immunity to DNA in the course of their lives, they do not respond particularly well to immunization with DNA bound to methylated bovine albumin. By contrast, NZW along with hybrid mice do give a heightened response to such immunization (Lambert and Dixon, 1970). This suggests that the great augmentation of disease in hybrids may result from the natural presence of stimulating viral infection governed by the genetic character of the NZB mouse, coupled with the hyperresponsiveness to stimulation with nuclear antigens conveyed by the genotype of the NZW mouse.

Aside from an unusually augmented immunoglobulin response to nuclear antigens, NZ mice show certain irregularities in cellular immunity. The graft-versus-host reaction in newborn, histoincompatible recipients was deficient when adult NZ mouse spleen cells were employed (Cantor *et al.*, 1970). The response of NZ mouse spleen cells to phytohemagglutin (Leventhal and Talal, 1970; Teague *et al.*, 1970), the ability of older NZB and hybrid mice to reject tumors induced by Moloney sarcoma virus (Gazdar *et al.*, 1971), and the rejection of skin allografts (Teague *et al.*, 1970) were poorer than in normal controls. With these data taken together, the theory has been espoused that disease in NZ mice results from two factors—the poor cellular response which allows freedom of growth to the virus(es) native to these mice and the excessive immunoglobulin response which they mount to infection. The results are large amounts of antibody–antigen complexes in the circulation and glomerulonephritis. Factors associated with these pathogenic mechanisms must be included in the early immunological maturity of these mice (Evans *et al.*, 1968) and the immunological effect of the decrease of recirculating lymphocytes (Zatz *et al.*, 1971; Denman and Denman, 1970).

4. Genetic Factors in Development of Immune Complex Disease in NZ Mice

As previously reviewed (Howie and Helyer, 1968), the incidence of antierythrocyte (Coombs) antibodies, antinuclear antibodies (ANA), and glomerulonephritis varies markedly not only between NZB, NZW, and (NZB × NZW)F_1 mice, but also in other mating combinations with other strains of mice. In general, antierythrocyte antibodies are noted in all NZB, and variably but to a lesser degree in (NZB × NZW)F_1, but not at all in NZW mice. The incidence of ANA is about 25% in NZB mice at 9 months of age, 60% in (NZB × NZW)F_1, and less than 10% in NZW mice. Glomerulonephritis develops slowly in the great majority of NZB mice and is membranous in character; it occurs rarely in NZW, but is a fulminating, lethal disease in the (NZB × NZW)F_1 hybrid. As noted previously, NZW mice respond to injections of DNA coupled to methylated BSA much better than do NZB mice even though NZB mice gradually develop ANA spontaneously over the course of their lives. These observations have led to several genctic studies to determine the background of these variations. To date, however, a clear genetic answer is not at hand. Glomerulonephritis with immunoglobulins bound along the GBM develops in virtually all NZB and (NZB × NZW)F_1 mice as noted. The (NZB × F_1) backcross mice are all positive, and backcross of F_1 with NZW yields about 50% positives (Ghaffer and Playfair, 1971).

This would speak in favor of a single dominant gene. However, crossing NZB and BALB/c mice should yield similar results, but only about 20% of the F_1 hybrids and 50% of the ($F_1 \times$ NZB) backcross mice have glomerular deposits as seen by fluorescence. Braverman (1968) performed genetic studies in NZ mice and obtained data suggesting that production of antierythrocyte antibody and ANA is under the control of a single gene. The studies by Ghaffer and Playfair (1971) of antierythrocyte antibody, however, suggest recessive gene control of antierythrocyte antibody in mice tested at 9 months of age. From available data, most students of the genetic aspects of NZ disease conclude that multiple factors control the development of antibodies and disease: environmental factors, degree and types of viral infection as well as genetic predisposition.

McDevitt and his colleagues (Grumet *et al.*, 1971) have observed an association between patients with systemic lupus erythematosus and the histocompatibility (HL-A) type of the individuals. With the previous relationships of histocompatibility and the immune response (McDevitt and Chinitz, 1969) noted in mice, McDevitt has predicted a linkage between H-2 histocompatibility type and development of autoimmunity in NZ mice.

B. Aleutian Disease in Mink

In 1941, a mutation occurred in a population of ranch mink associated with a change in coat color. The color represented the phenotypic expression of a homozygous recessive gene, and mink so affected possessed the "Aleutian" trait. The genetic types were written aa, with aA and AA—aa represented mink with the Aleutian trait. The Aleutian mink developed illness readily which pathologically affected many organs, especially livers and kidneys (Hartsough and Gorham, 1956). Plasma cells were present in lesions in great abundance along with widespread arteritis (Helmboldt and Jungherr, 1958). The presence of such large numbers of plasma cells in diseased mink brought Obel (1959) to the conclusion that the disease was a plasma cell myeloma.

Experimental transmission of the disease has been performed with extracts of diseased tissues (Karstad and Pridham, 1962; Henson *et al.*, 1962; Trautwein and Helmboldt, 1962; Russell, 1962; Henson *et al.*, 1963). The agent was filtered through Krueger "E" and Berkefeld N filters and sedimented in 1 hour at 95,000 *g*. Development of disease paralleled the rise in γ-globulin levels. Transmission of disease to aa mink was more readily achieved than to Aa or AA pastel mink, although transmission could be effected in the latter.

With the development of disease, the characteristic histopathological

lesions were those of glomerulonephritis, widespread arteritis, and diffuse plasmacytosis involving many organs. The glomerulonephritis bore all the features of immune complex disease histologically. A proliferation of cells occurred in glomeruli, and white blood cells accumulated (Henson *et al.*, 1966, 1967, 1968; Kindig *et al.*, 1967; Thompson and Aliferis, 1964).

Examination of the glomerular lesions with fluorescent antibodies revealed a granular pattern of IgG and complement along vascular membranes (Henson *et al.*, 1968; Porter *et al.*, 1969). Weak staining for virus was also observed in a few glomeruli with the same pattern of fluorescence as that of IgG. Viral antigen was found only early in the disease (Porter *et al.*, 1969a); lesions in arteries also contained IgG (Porter *et al.*, 1965a). The pattern of fluorescence in the diseased glomeruli was associated with the presence of lumpy deposits along the GBM as seen with the electron microscope (Henson *et al.*, 1968, 1969).

Aleutian disease virus (ADV) has been recovered from serum, urine, and diverse organs of infected mink (Henson *et al.*, 1962; Kenyon *et al.*, 1963a; Gorham *et al.*, 1964; Ecklund *et al.*, 1968), and the ADV antigen is detectable in phagocytic cells in spleen and liver and other tissues (Porter *et al.*, 1969a). Although originally the suggestion was made that Aleutian disease represented an example of a "slow" virus disease (Sigurdsson, 1954), studies of transmission have indicated that the virus multiplies rapidly after transfer into a normal, uninfected animal (Ecklund *et al.*, 1968; Porter *et al.*, 1969a). Rapid viral growth occurs in both violet (aa) and pastel (aA or AA) mink. Ecklund *et al.* (1968) reported aa mink to be more susceptible to the virus than other genotypes with death used as a measure of susceptibility. Porter *et al.* (1969b), however, presented evidence that the virus replicated rapidly in each genetic type. Thus, whereas the genetic type of the mink is important in the severity of disease, it does not apparently influence the rate of viral replication after transfer.

The antibodies in Aleutian disease are of particular interest. After infection commences and plasma cells proliferate, γ-globulin production ensues leading in time to marked hypergammaglobulinemia (Henson *et al.*, 1961; Kenyon *et al.*, 1963b; Porter *et al.*, 1965a,b). The spectrum of immune globulins becomes quite limited, often leading to a monoclonal gammopathy. This is evidence that the responding clones are greatly restricted in type (Porter *et al.*, 1965b). As would be anticipated from the vast numbers of plasma cells, the hypergammaglobulinemia results from increased production rather than deficient catabolism (Porter *et al.*, 1965a). Specificity for ADV has been observed in the

γ-globulin produced (Porter *et al.*, 1965a; Kenyon, 1966; Porter and Larsen, 1968); antibody to virus is detectable by 10 days after transmission and reaches considerable proportions as the disease progresses (Porter *et al.*, 1969a). The finding of free antibody in the presence of infectious virus sets this disease apart from other chronic viral diseases such as lymphocytic choriomeningitis. Aleutian disease viral antibody does not neutralize the virus and, hence, does not offer protection (Porter *et al.*, 1969a). In fact, immunization or transfer of antibody can heighten the disease (Porter *et al.*, 1971).

The presence of immune complexes in infected mink was demonstrated by Porter and Larsen (1967). By using antibody to mink IgG, precipitation of the IgG from infective mink serum considerably reduced the infective capacity of the serum; antibody to mink albumin did not share this property. Deoxyribonucleic acid was detected in sera before and after disease developed in mink that were experimentally infected, but not in sera from normal mink. Anti-DNA antibodies appeared after infection developed (Barnett *et al.*, 1968). In keeping with the presence of circulating virus–antivirus complexes were arteritis and glomerulonephritis, the hallmarks of immune complex disease (Hartsough and Gorham, 1956; Helmboldt and Jungherr, 1958; Obel, 1959; Leader *et al.*, 1963; Henson *et al.*, 1966, 1967; Kindig *et al.*, 1967; Porter *et al.*, 1965a).

The majority of IgG in diseased mink is a 6.4 S protein (Kenyon *et al.*, 1963b; Porter *et al.*, 1965a,b); however, large complexes, 19 S and far greater in size, were also demonstrated (Porter *et al.*, 1965a). The data indicate that diseased mink are in a state of antibody excess. Since it has been shown that complexes greater than 19 S in size were associated with deposition in glomeruli and arteries in immune complex disease of rabbits (Cochrane and Hawkins, 1968), it is of interest that complexes greater than 19 S in size are present in mink with Aleutian disease. Antibody to single- and double-stranded DNA were observed in these mink (Barnett *et al.*, 1969). In addition, DNA was detected by using rabbit antibody to DNA, although the DNA detected in mink serum was not destroyed by prior treatment with DNase. Protein-free extracts of mink serum reacted to deoxypentose with dephenylamine reagent. Both anti-DNA and DNA were present prior to infection, but levels rose after inoculation of mink with infectious, cell-free filtrates or after spontaneous infection. The relationship of DNA and anti-DNA to virus–antivirus complexes remains to be clarified.

Elution of diseased kidneys yielded IgG that bound to particles in cells of diseased mink. These particles were identical in appearance to those demonstrable with known antiviral antibody (Porter *et al.*, 1969b).

C. Immune Complex Disease Associated with Infection of Mice with Lymphocytic Choriomeningitis Virus

Manifestations of Lymphocytic Choriomeningitis

Lymphocytic choriomeningitis virus was found originally to be highly virulent for certain strains of mice (Traub, 1935, 1939). Five to 10 days after infection, diseased mice exhibited somnolence, photophobia, tremor, and spasms and frequently died. Mononuclear inflammatory cells were found in meninges, ependyma, choroid plexus, and around vessels generally in affected mice. Surviving mice were at times noted to contain neutralizing antibody, fitting the pattern of recovery from several other well-studied viral agents. By contrast, in occasional surviving adult mice, and in many mice infected either *in utero,* or shortly after birth, persistence of the virus was observed despite apparent recovery (Traub, 1936b, 1938, 1939; Whitney, 1951; Hotchin and Cinits, 1958; Traub, 1960; Hotchin and Weigand, 1961a; Weigand and Hotchin, 1961; Hotchin *et al.,* 1962; Volkert and Hannover, 1964; Oldstone and Dixon, 1970a). Such mice have been classed as "carriers." Although they resisted the effects of intracerebral injection of virus (Traub, 1938), free neutralizing antibody was not detected in the blood of these mice (Traub, 1936a).

Considerable strain specificity is exhibited in determining whether mice develop carrier disease after neonatal exposure to the LCM virus (Table VI). Traub noted in his early description that certain mouse strains were not subject to infection with LCM (Traub, 1936b)—the strains of mice developing disease (and immune complexes) were those susceptible to the virus (Oldstone and Dixon, 1968b, 1969). A strong correlation was found between mice in which carrier disease developed and from which infective virus could be obtained. It was originally postulated that the chronic disease of carrier mice was caused by an autoimmune phenomenon (Hotchin *et al.,* 1962; Hotchin and Cinits, 1958; Hotchin, 1965). This was based on the investigators inability to detect free antiviral antibody in the circulation and to produce further injury by injection of anti-LCM virus antibody. However, the data given in the preceding two paragraphs indicate that the most important antigen involved in the immune complex disease of LCM carrier mice is the viral antigen, i.e., an antigen exogenous to the host.

Aside from viremia, the tissues of the infected carrier mice contain infectious virus or viral antigen (Traub, 1935; Oldstone and Dixon, 1969, 1970a; Wilsnack and Rowe, 1964). Based on (*1*) the presence of virus in the carrier, (*2*) the ease with which the carrier state is initiated *in utero,* and (*3*) the absence of detectable antibodies to virus in the circulation, investigators have postulated that a state of immunological

TABLE VI
INFECTIVITY TITERS AND IMMUNOFLUORESCENT STAINING IN 2- TO 3-MONTH-OLD LYMPHOCYTIC CHORIOMENINGITIS CARRIER MICE[a]

Strains	Kidney			
	Titer[b]	LCM Ag[c]	γG[d]	C3
SWR/J	5.4	+	++	++
B10D2 new	3.8	+	++	++
B10D2 old	3.6	+	++	++
NZB	3.4	+	++	++
NZB/W	—	+	++	++
AJAX	—	+	+	+
AKR	2.0	+	±	±
C3H	1.6	+	±	±

[a] From Oldstone and Dixon (1968b).

[b] Reciprocal of log dilution giving a 50% lethal end point per 0.03 ml. intracerebral inoculum. Number is the mean value of the LD_{50} end points obtained with organs from 4 individual mice.

[c] Degree of involvement as determined by direct immunofluorescence: (+) 2–10% cells involved.

[d] Degree of glomerular involvement as determined by direct immunofluorescence: (±) minimal globulin deposit in general limited to mesangial area; (+) moderate globulin deposit in mesangial and peripheral loop area; (++) marked globulin deposits in mesangial and peripheral loop areas. Neither γ-globulin nor C3 was found deposited in any other tissues studied except in areas of focal necrosis in the liver.

tolerance exists in these mice (Burnet and Fenner, 1949; Hotchin and Cinits, 1958; Volkert and Hannover, 1964; Volkert, 1964; Hotchin, 1965). Evidence fails to support this hypothesis, however, since carrier mice were found to resist the severe effects of virus injected intracerebrally (Traub, 1938). This observation has been repeated (Volkert and Larsen, 1965; Volkert, 1964). Cells *in vitro,* exposed to LCM virus, developed a noncytopathic infection (Bender and Cinatl, 1962; Wilsnack and Rowe, 1964; Peterson and Volkert, 1966; Oldstone and Dixon, 1968b). In addition, LCM virally infected mice in the absence of an immune response, i.e., in mice irradiated, thymectomized, or treated with immune suppressants prior to infection, did not develop the inflammatory lesions of the carrier state (Rowe, 1956; Haas and Steward, 1956; Hotchin and Weigand, 1961b; East *et al.,* 1964; Hirsch *et al.,* 1967).

Strong evidence for the participation of an immune response in the carrier has more recently been obtained. Both humoral and cellular immune mechanisms are detectable, and lesions of immune complex disease have been recognized that clearly point to LCM antigen–anti-

LCM pathogenesis. Circulating infective virus is in large part bound to γ-globulin and C3 (Oldstone and Dixon, 1969). Examination of the glomeruli in carrier mice revealed the characteristic appearance of immune complex glomerulonephritis (Oldstone and Dixon, 1968a, 1969). Fluorescent antibodies allowed detection of granular deposits rich in mouse immunoglobulin and C3 along the GBM (Oldstone and Dixon, 1968a, 1969). Acid eluates of glomeruli in kidneys of carrier mice revealed complement-fixing antibody to LCM (Oldstone and Dixon, 1968a, 1969). When guinea pig C3 was injected into LCM carrier mice, elimination of the heterologous C3 as well as uptake of the C3 in the glomerular deposits became more rapid than before (Oldstone and Dixon, 1969). Carrier mice exhibited changes not only typical of chronic glomerulonephritis but also with foci of acute and chronic inflammation in the liver, lungs, kidney, and, to a lesser extent, pancreas, omental fat, spleen, thymus, and, infrequently, the brain (Oldstone and Dixon, 1969). Arteritis histologically similar to that in acute immune complex disease appeared shortly after birth in neonatally infected mice (Oldstone and Dixon, 1970c). These data establish the LCM carrier mouse as one of the best examples of chronic immune complex disease resulting from a living antigen.

Delayed cellular sensitivity is readily detected in carrier mice. Their spleen cells are cytotoxic to cells containing LCM virus but not normal target cells, and a cytotoxic soluble factor was released from the carrier spleen cells upon contact with antigen (Oldstone and Dixon, 1970b, 1971a).

The reason for development of a carrier state, allowing progressive immune complex disease to occur, rather than a state of complete immunity is unclear. When mice are infected in adulthood, the latter condition prevails, and immune complex disease does not develop. Evidence exists that carrier mice are somehow deficient in their ability to make an immune response (Oldstone and Dixon, 1971a), but not as a result of thymic deficiency (Oldstone and Dixon, 1971a,b). Nor is there good indication why certain strains are less susceptible than others to LCM virus and, therefore, to immune complex disease.

Association between H-2 type of mice and susceptibility to immunological leptomeningitis induced by LCM virus has recently been observed (Oldstone and McDevitt, 1972). Adult SWR/J mice (H_2QQ genotype) are susceptible to LCM leptomeningitis when infected with LCM virus, whereas C3H/J (H_2KK) are not. All F_1 hybrids (H_2QK) of these two strains were found to be susceptible. Experiments in which F_1 hybrids were back-crossed with SWR/J homozygotes produced uniformly susceptible mice (H_2QQ or H_2QK), which with C3H/J mice

produced half-susceptible and half-resistant strains. The susceptible mice were H_2QK genotype, and the resistant were H_2KK, indicating a close association between H_2-type and production of the appropriate antibodies required for development of leptomeningitis.

D. Immune Complex Disease Associated with Lactic Dehydrogenase Virus in Mice

Lactic dehydrogenase virus (LDV) is typical of agents that produce prolonged, most often life-long, levels of circulating immune complexes. This virus is a small RNA virus with a calculated spherical diameter of 40 mμ. (Notkins, 1965), and is named for the enzyme, the levels of which are elevated in the circulation of mice bearing the virus. This high enzymatic level apparently results from decreased rates of clearance (Notkins, 1965), possibly owing to diminished function of the reticuloendothelial system. The virus invades and persists in macrophages and cells of the reticuloendothelial system (Notkins, 1965; DuBuy and Johnson, 1966; Evans and Salaman, 1965; Porter *et al.*, 1969a); infectious virus persists also in the circulation.

The infected mice were originally thought to be tolerant of the virus, since neutralizing antibody was not readily detected. However, when the virus in the blood was killed *in vitro* by ultraviolet irradiation or other mild means, neutralizing antibody of modest potency against LDV was detected (Notkins *et al.*, 1966). The virus was also found to circulate as a virus–antibody complex (Notkins, 1965; Notkins *et al.*, 1968), since heterologous antibody to mouse immunoglobulin neutralized the virus, whereas such antibody to virus not exposed to mouse immune serum had no neutralizing effect. This has led to a useful assay for infectious virus–antivirus complexes, i.e., the use of a heterologous antibody capable of neutralizing an infectious viral complex (Ashe and Notkins, 1966).

Not only has heterologous antibody to immunoglobulin proved to neutralize virus, but complement components also neutralize (Notkins, 1971). Components 1 and 4 effected considerable neutralizing capacity to the herpes simplex virus–mouse antivirus complex, and this was augmented by the addition of C2 and C3. Rheumatoid factor added to the complex did not result in neutralization, but when anti-IgM or complement components were added, neutralization occurred (Daniels *et al.*, 1969, 1970; Ashe *et al.*, 1971; Notkins, 1971). The authors implied that neutralization resulted from a sequential addition of proteins to the virion.

Injury from prolonged infection with LDV was minimal. Mice developed splenomegaly and lymphoid hyperplasia (Riley, 1964), but only after months of infection was there definite glomerular alteration. Mild

cellular proliferation was then observed in glomeruli (Porter and Porter, 1971; Oldstone and Dixon, 1971c). In independent studies, Porter and Porter (1971) and Oldstone and Dixon (1971c) found immune complexes deposited in the glomeruli of LDV-infected mice. The deposits contained IgG, C3, and small amounts of viral antigen. Elution of glomeruli revealed the presence of anti-LDV antibody—37 μg. immunoglobulin per gram of kidney was eluted from a pool of the kidneys of 10 mice infected 28 days before (Porter and Porter, 1971). Despite these findings, evidence of cellular proliferation and mild thickening of the basement membrane was reported, but proteinuria was not observed.

The cause of the persistence of LDV infection in mice is not certain. Since the antibody formed is not strongly neutralizing, the animal may be unable to purge the virus. The rate of production of virus or viral antigen must also be taken into account. Interferon does not apparently play a major role in counteracting LDV virus (Notkins and Shochat, 1963; Porter *et al.*, 1969b; Evans and Riley, 1968).

V. Spontaneous Immune Complex Disease of Unknown Cause in Animals

Sheep (Lerner and Dixon, 1966; Lerner and Dixon, 1968), rats (Foley *et al.*, 1964), hamsters (Guttman and Kohn, 1960), mice (Gude and Upton, 1962), steer, and goats (Lerner and Dixon, 1968) develop spontaneous glomerulonephritis. Over 95% of sheep develop a slowly progressive nephritis marked by proteinuria and mild azotemia. Immunofluorescent analyses revealed granular depositions of IgG and C3 along the GBM. Eluates of diseased kidneys failed to bind to normal glomeruli. The pathogenic agents in these diseases are not known and present the same enigma as many of the spontaneously occurring immune complex glomerulonephritides in humans.

VI. Glomerulonephritis of Possible Immune Complex Origin in Humans

A. Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) closely resembles experimental chronic serum sickness mediated by antigen–antibody complexes. Although multiple systems are affected in SLE, the most significant lesions that determine the course of the disease are found in renal glomeruli and blood vessels. Early studies called attention to the fibrinoid necrosis prominent in glomeruli and connective tissue (Klemperer *et al.*, 1941), extracellular hematoxylin bodies, and bone marrow cells containing similar bodies (Hargraves *et al.*, 1948). Considerable interest was aroused

when the serum from patients with SLE was found capable of reacting with cell nuclei (Holman and Kunkel, 1957; Robbins *et al.*, 1957; Seligmann, 1957; Miescher and Strassle, 1957). Understanding of the genesis of lupus erythematosus (LE) cells was enhanced by the finding of γ-globulin within the phagocytized nuclear material (Vazquez and Dixon, 1957) and the demonstration that antinucleoprotein antibodies were responsible for inducing the LE cell reaction (Holman and Deicher, 1959). In addition, a variety of antibodies were found in sera directed against multiple nuclear and cytoplasmic constituents. Concurrent studies with tissues obtained from patients with SLE indicated that γ-globulin and complement were found in areas of fibrinoid necrosis in the vascular and glomerular lesions (Mellors *et al.*, 1957). The relationship between the humoral "autoantibodies" and tissue lesions, other than LE cells, was not defined.

The broad spectrum of antibodies present in the sera of patients with SLE are directed against a variety of host cellular antigens, although many of the antibodies are reactive with antigens common to bacteria and viruses. A heterogeneous population of antibodies directed against DNA, for both native and single-stranded determinants, has been characterized (Deicher *et al.*, 1959; Barbu *et al.*, 1960; Stollar and Levine, 1961; Stollar *et al.*, 1962; Stollar and Levine, 1963; Arana and Seligmann, 1967; Tan *et al.*, 1966; Wold *et al.*, 1968; Carr *et al.*, 1969; Pincus *et al.*, 1969; Koffler *et al.*, 1969b, 1971a; Cohen *et al.*, 1971). Antibodies unique for native DNA, single-stranded DNA, and antibodies common to both native and single-stranded DNA have been demonstrated. Most antibodies reactive with native DNA react more avidly with the "backbone determinants" of single-stranded DNA, which may indicate that this polynucleotide is an important immunogen for DNA antibodies found in human sera (Koffler *et al.*, 1971a). Antibodies reactive with native DNA determinants are restricted primarily to patients with SLE and are useful from the diagnostic standpoint, whereas antibodies reactive only with single-stranded DNA occur in patients who have a variety of diseases associated with tissue destruction (Koffler *et al.*, 1969b, 1971a). Antibodies reactive with particulate and soluble nucleoprotein differ from antinative DNA antibodies with respect to the requirement of a protein–DNA complex (Holman and Deicher, 1959; Tan, 1967a). A high incidence of antibodies to a periodate-sensitive nuclear antigen, the Sm antigen, obtained from saline extracts of nuclei, has been demonstrated in SLE sera by agar gel diffusion (Tan and Kunkel, 1966a). Hemagglutinating antibodies reactive with an antigen derived from calf thymus nuclei (ENA) have also been described (Holman, 1965; Sharp *et al.*, 1971). Although the antigens involved in

this reaction have not been completely characterized, ribonucleoprotein has been demonstrated to be one of the reactive antigens in this extract (Koffler *et al.*, 1971a; Mattioli and Reichlin, 1971). Antiribosomal antibodies have been observed in association with renal disease (Schur *et al.*, 1967); anticytoplasmic antibodies have also been found in lower incidence in SLE sera (Asherson, 1959; Deicher *et al.*, 1960) and antibodies reactive with double-stranded RNA have been demonstrated (Koffler *et al.*, 1969b; Schur and Monroe, 1969; Schur *et al.*, 1971). The latter antibodies are a heterogeneous population that cross-react with single-stranded polyribonucleotide and single-stranded DNA (Koffler *et al.*, 1971a).

Several lines of evidence suggest that the antinuclear and anticytoplasmic antibodies are not cytotoxic for tissues but rather that the lesions are induced by local deposition of antigen–antibody complexes. Therefore, the presence of an appropriate circulating antigen would be required to render the antibody of pathogenic significance. Although antinative DNA antibodies were found to be unique for SLE, other antinuclear antibodies were found in a variety of diseases without evidence of tissue lesions resembling those in SLE. No evidence for *in vivo* localization of antinuclear or anticytoplasmic antibodies in parenchymal organs was obtained from immunofluorescent studies except for occasional *in vivo* binding of antinuclear antibodies in tubular cells of kidney (Paronetto and Koffler, 1965) and in nuclei of skin (Tan and Kunkel, 1966b). Antinuclear antibodies were not found to be cytotoxic on transplacental passage (Bridge and Foley, 1954) or when passively administered to animals (Bencze and Lakatos, 1962).

The basis for implicating antigen–antibody complexes as the mediators of the phlogistic response in SLE tissue rests on a variety of investigations: serological studies of the serum complement system, immunofluorescent and electron-microscopic studies of renal biopsies and kidneys obtained at autopsy, characterization of glomerular eluates from nephritic kidneys, and serial studies of antibodies and antigens during the course of the disease. The investigations have helped to delineate one system, the DNA–anti-DNA system, as a likely pathogen in SLE and have implicated several other systems in the pathogenesis of the renal and vascular lesions.

Decrease in serum complement and the presence of fluctuating levels of DNA antibody and DNA antigen (Fig. 13) were found to be related to clinical exacerbations of disease (Tan *et al.*, 1966; Schur and Sandson, 1968). Over 90% of patients with significant renal disease have marked depression of serum complement (Schur and Sandson, 1968). Although decreased levels of serum complement do not unequivocally indicate

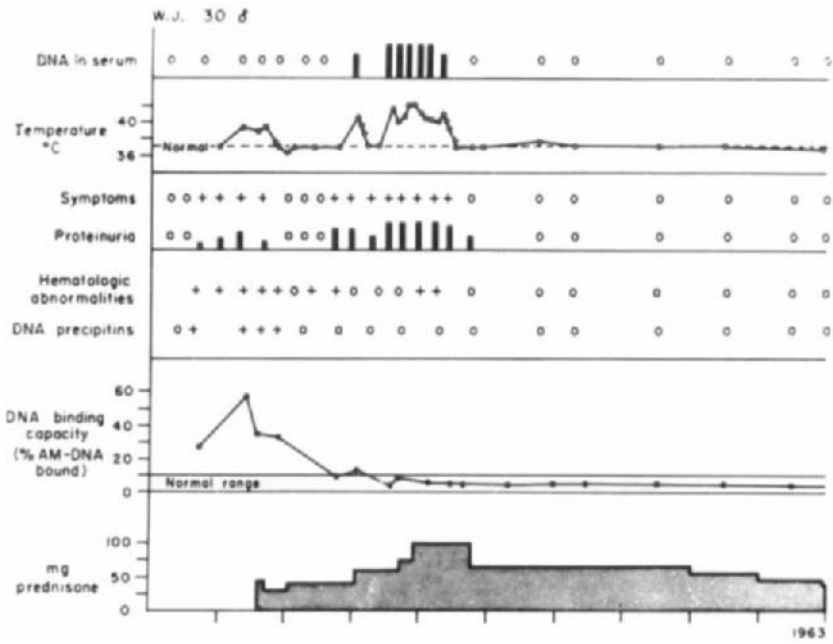

FIG. 13. Serial study of a patient with systemic lupus erythematosus showing two episodes of proteinuria. One is associated with the presence of anti-deoxyribonucleic acid (DNA) antibodies, measured by agar gel precipitin and actinomycin-labeled DNA (AM-DNA) binding tests; the second is associated with free DNA antigen in serum.

fixation to antigen–antibody complexes, several factors may be cited which support this hypothesis. There is a significant depression of early complement components in hypocomplementemic SLE sera (Kohler and Bensel, 1969), suggesting an activation mechanism proposed for antigen–antibody–complement interaction. Several early components, C1q, C3, and C4, have been identified in the renal lesions (Lewis *et al.*, 1970). A high incidence of complement-fixing antinuclear antibodies is present in SLE patients with active nephritis in contrast to patients with other forms of SLE or other diseases associated with antinuclear antibodies (Tojo and Friou, 1968). Recent studies have suggested that hypocomplementemic sera from patients with SLE contain γ-globulin complexes. These sera are frequently anticomplementary and precipitate with Clq globulin (Agnello *et al.*, 1970).

The γ-globulin and complement deposits demonstrable by immunofluorescence in the glomerular lesions assume a granular pattern characteristic for antigen–antibody complexes in nephritic kidneys, closely resembling the pattern found in chronic serum sickness disease of rab-

bits (Dixon *et al.*, 1961; Cochrane and Dixon, 1968). Complement-fixing γG and γM globulins predominate in the deposits (Paronetto and Koffler, 1965). Very fine granular deposits have been found in patients with early renal disease, whereas in patients with more advanced lesions coarser granules of protein are observed (Fig. 14) (Koffler *et al.*, 1969a). In certain kidneys with morphological features of membranous nephropathy, the granular lesions may persist without accumulation of the irregular lumpy deposits found in kidneys in the advanced stages of glomerular disease (Fig. 15). Patients without renal symptoms but with evidence of active disease also appear to have protein deposits in their renal glomeruli, but these are confined to the mesangial areas (Fig. 16). The mesangium of the glomerulus has an active phagocytic capability and may clear circulating complexes, preventing their deposition along the GBM. It is not clear if a specific type of complex has a preferential affinity for the mesangium or GBM or if the mesangium in certain cases

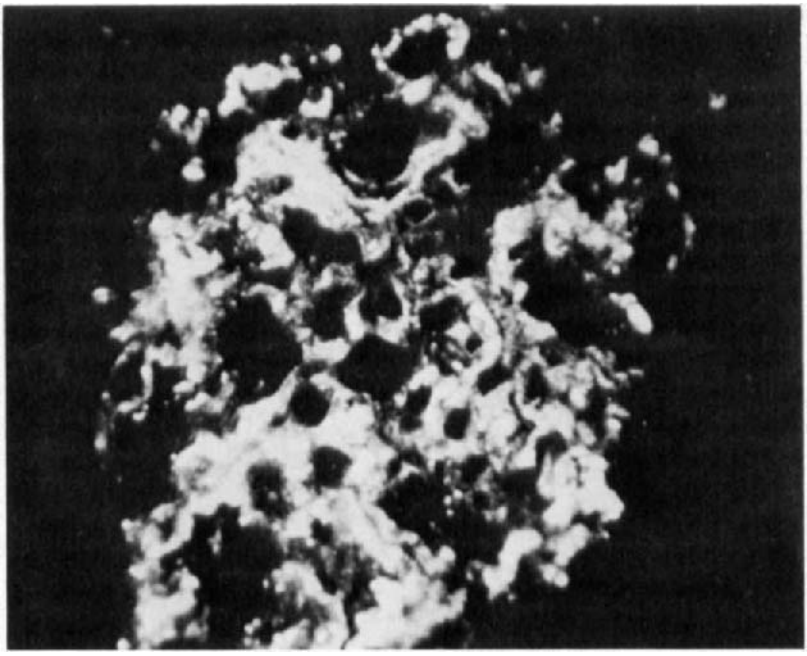

FIG. 14. Patient V.B. with systemic lupus erythematosus and moderate proteinuria with normal blood urea nitrogen. Characteristic granular deposition of γG-globulin along glomerular basement membrane. Note granules are of varying size and are distributed irregularly throughout glomerulus. Magnification: ×250. (From Koffler *et al.*, 1969a.)

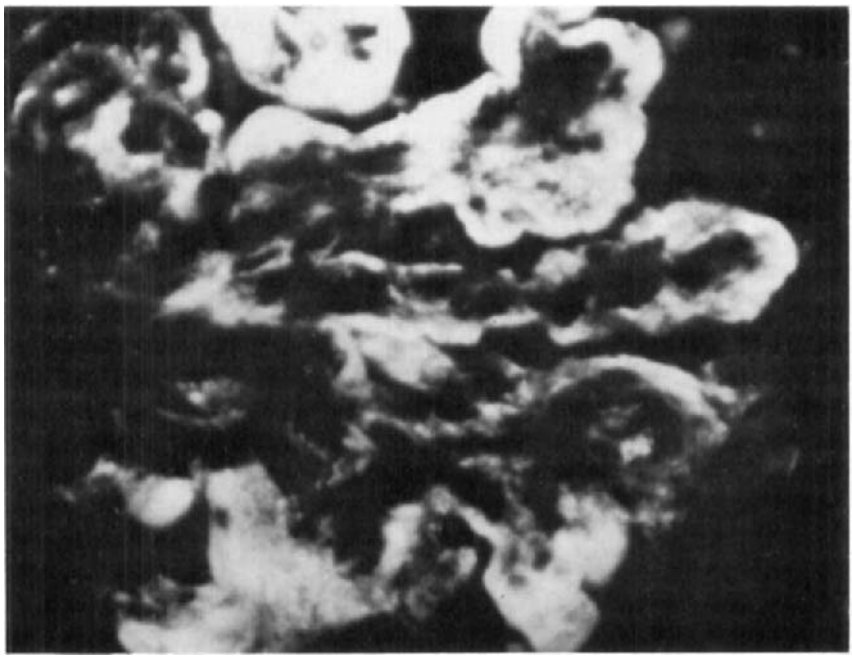

FIG. 15. Lumpy deposits of γG-globulin outlining tufts of a glomerulus from a kidney with severe systemic lupus erythematosus nephritis. The section was incubated with a fluorescein-labeled anti-γG-globulin serum. Magnification: ×500. (From Koffler *et al.*, 1967a.)

becomes saturated and incapable of further clearing action, allowing the deposition of complexes along the GBM. The variety of patterns of immunoglobulin and complement deposition, the persistence of granular deposits in some patients and the progression to lumpy deposits in other patients may reflect the heterogeneous nature of the immune complexes found in different patients.

Electron-microscopic studies of SLE kidneys indicate that proteinaceous deposits are present in several locations in the glomerulus (Comerford and Cohen, 1967). Dense deposits within the mesangium are especially prominent and are associated with cellular proliferation and organization. Large subendothelial deposits are most common, in addition to intramembranous and subepithelial localization. The ultrastructural pattern of deposition indicates that proteinaceous deposits are prominent with electron-dense, finely granular masses, which may show a crystalline or fingerprint pattern (Churg and Grishman, 1972). Although the precise nature of the dense deposits is not discernible by

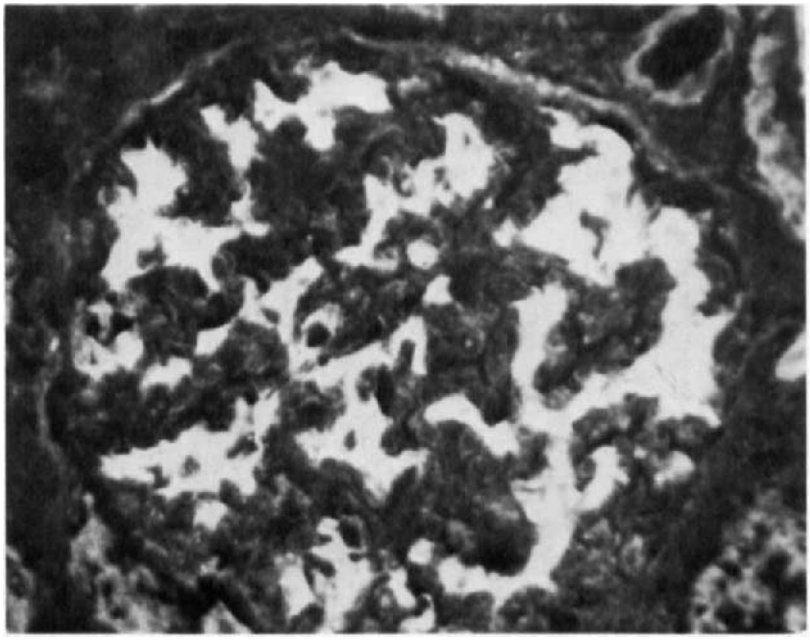

FIG. 16. Patient P.W. with systemic lupus erythematosus and no proteinuria. Mesangial deposits of protein shown as irregular strandlike areas of fluorescence lying between capillary loops. Localization of β1C-globulin. Magnification: $\times$250. (From Koffler *et al.*, 1969a.)

electron microscopy, the typical fibrillar ultrastructural appearance of fibrin is minimal in glomeruli. Immunofluorescent studies also indicate that the deposition of fibrin in renal lesions is variable and does not constitute a major portion of the protein deposits. The electron-microscopic findings are, therefore, consistent with the deposition of aggregates of protein along the GBM.

Immunochemical analysis of the specificity of the γ-globulin deposited in glomeruli has been performed by elution of isolated glomeruli separated from nephritic kidneys (Koffler *et al.*, 1967a; Krishman and Kaplan, 1967). Glomeruli obtained from renal cortical homogenates have been subjected to acid buffer and enzyme treatments in order to elute antibodies bound in the form of antigen–antibody complexes in glomeruli. Treatment of glomeruli with acid buffer eluted antibodies reactive with both native and single-stranded DNA, nucleoprotein, and phosphate buffer-extractable nuclear antigens, and occasionally with a ribonucleoprotein. Antibodies reactive with double-stranded RNA were not

TABLE VII
CONCENTRATION OF ANTIBODIES IN GLOMERULAR ELUATES

Antibodies[a]	Acid buffer eluates	Deoxyribonuclease eluates
Total No. studied	9	9
Anti-NDNA	5[b]	6
Anti-SDNA	6	8
Anti-RNA Pr	3	1
Anti-DSRNA	0	0

[a] (NDNA), native deoxyribonucleic acid; (SDNA), single-stranded deoxyribonucleic acid; (RNA Pr), ribonucleoprotein; (DSRNA); poly A · poly U.

[b] Number of eluates with increased antibody activity per milligram of γ-globulin in comparison with serum, assayed by hemagglutination.

demonstrable in acid buffer eluates. Deoxyribonuclease treatment was a useful technique for separating both antinative DNA and anti-single-stranded DNA antibodies from immune complexes containing DNA (Table VII) (Koffler *et al.*, 1971b). Quantitative studies performed with eluted antinuclear antibodies indicated that those antibodies were present in significantly higher concentrations per milligram of γ-globulin than were antinuclear antibodies found in serum. The high specific activity of antinuclear antibodies in glomerular eluates is most likely accounted for by the concentration of antigen–antibody complexes along the GBM. Detection of other antibodies present in lower titer may be obscured by the parallel elution of antigen, which is not susceptible to elimination by specific enzyme treatment, such as deoxyribonuclease.

The presence of native DNA antigen has been observed in serum during periods when antinative DNA antibodies were not demonstrable (Tan *et al.*, 1966). Recent studies indicate that single-stranded DNA antigen is present in SLE sera as well (Koffler *et al.*, 1972). Other nuclear antigens, such as Sm antigen, have been found in urine and joint fluids but have not been identified in serum. Multiple factors may be responsible for the appearance of DNA antigens in the sera of patients with SLE. Tissue necrosis and steroid treatment have been implicated in the release of DNA antigen into serum (Hughes *et al.*, 1971).

Deoxyribonucleic acid (DNA) antigen has been found in glomeruli of kidneys from which anti-DNA antibodies have been eluted (Koffler *et al.*, 1967a). The DNA was demonstrable after elution of *in vivo* bound antibodies, which blocked the *in vitro* reaction of fluorescein-labeled anti-DNA antibodies. The antigen was distributed in glomeruli in a pattern similar to that of γ-globulin and complement (Fig. 17). The demonstration of antigen in glomeruli adds further support to the

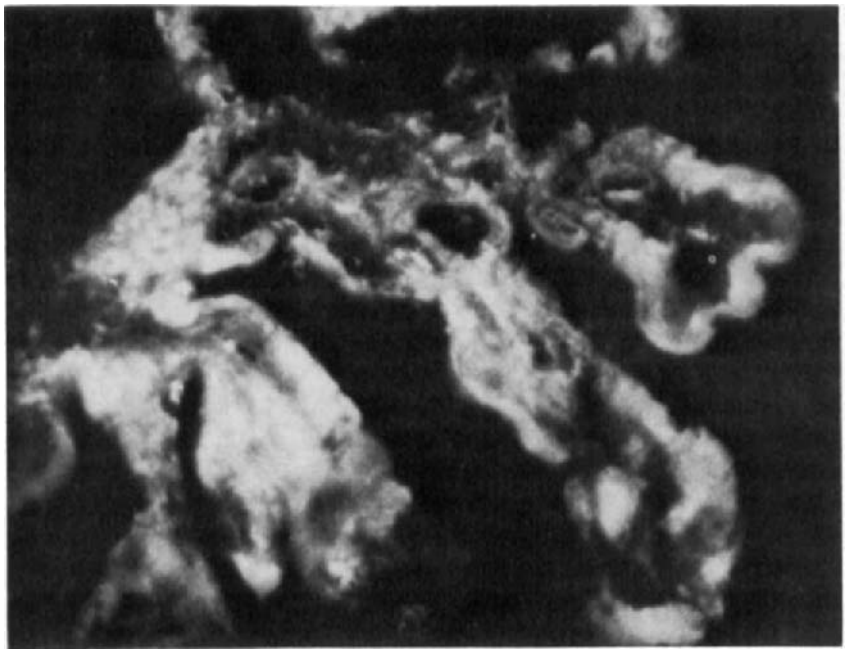

FIG. 17. Localization of deoxyribonucleic acid (DNA) antigen in a renal glomerulus of a kidney with severe systemic lupus erythematosus nephritis. Deposits of DNA along the glomerular basement membrane demonstrated after treatment of a cryostat section with 2 *M* NaCl for 120 minutes, followed by incubation with fluorescein-labeled anti-DNA antibody. Magnification: ×540. (From Koffler *et al.*, 1967a.)

concept that DNA–anti-DNA complexes are present in glomeruli. Determinants of denatured DNA have also been observed in glomerular deposits (Andres *et al.*, 1970a), although it is not clear if this represents *in situ* denaturation of segments of native DNA or deposition of circulating single-stranded DNA.

Serial studies of selected antipolynucleotide antibodies during the course of the disease indicate that certain antibodies are associated with disease activity (Koffler *et al.*, 1971a). The relationship of antinative DNA antibodies to clinical exacerbations is well documented. In addition, the titers of antibodies reactive with single-stranded DNA and with double-stranded RNA increase during periods of clinical activity but may also be elevated during clinically quiescent periods. Anti-ribonucleoprotein antibodies, in contrast, do not show any clear relationship to clinically active stages of the disease. Glomerular elution, antigen localization, and serial studies implicate both native and single-stranded

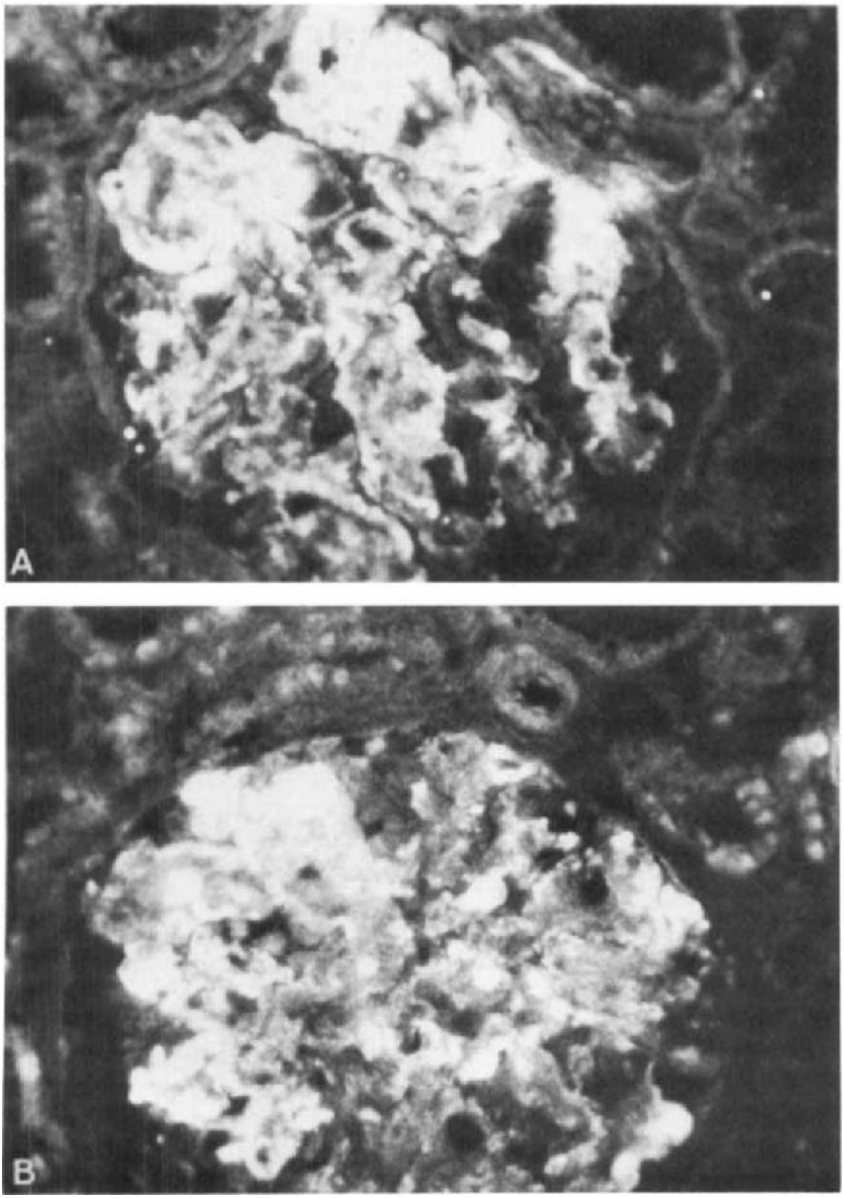

FIG. 18. Renal biopsy obtained from patient with serum γM–γG cryoglobulin, a C1q precipitin reaction, and renal disease. (A) Granular and lumpy deposits of γM-globulin are demonstrable by immunofluorescence. Magnification: ×250. (B) Deposits reactive with fluorescein-labeled aggregated γ-globulin demonstrating rheumatoid factor activity are in a glomerulus from the same biopsy. Magnification: ×250. (From Koffler *et al.*, 1971b.)

DNA immune complexes as agents involved in the glomerular lesions (Koffler *et al.*, 1971b). The participation of other antigen–antibody systems, such as Sm antigen, ribonucleoprotein, nucleoprotein, and ribosomes, remains to be demonstrated.

In addition to the evidence implicating antipolynucleotide antigen–antibody complexes, serological and immunofluorescent studies indicate that other types of γ-globulin complexes occur in glomerular deposits and sera of patients with SLE (Agnello *et al.*, 1970). Sera from patients with active SLE may contain mixed 19 S–7 S cryoglobulins (Christian *et al.*, 1963; Koffler *et al.*, 1970; Agnello *et al.*, 1971). Renal biopsies obtained from patients with circulating 19 S–7 S cryoglobulins with rheumatoid factor actively manifest moderate-to-heavy granular deposits of γM-globulin in glomeruli (Koffler *et al.*, 1970, 1971b) (Fig. 18a). Rheumatoid factor activity is demonstrable in these deposits in several cases when cryostat sections of renal biopsies are incubated with fluorescein-labeled aggregated γ-globulin (Fig. 18B; Table VIII). Recent studies based on a precipitin reaction between isolated C1q globulin and aggregated γ-globulin in agar gel have permitted the detection of γ-globulin complexes in the sera of these patients (Agnello *et al.*, 1970, 1971). These unknown high molecular weight complexes (i.e., greater than 19 S) which consist mainly of γG-globulin may react *in vivo* with rheumatoid factors and along with complement enhance the nephritic process. Cryoglobulinemia appears to be a manifestation of this interaction. Both cryoglobulins and C1q precipitable complexes have been associated with low complement levels and clinical activity (Fig. 19). More direct evidence is provided by studies that demonstrate the same rheumatoid factor in the patient's cryoprecipitate and glomerular deposits by use of fluorescein-labeled antibody with idiotypic specificity for the rheumatoid factor (Agnello *et al.*, 1971).

Low molecular weight C1q precipitins also occur in SLE, and this type of C1q reactant may account for hypocomplementemia in a significant number of patients (Agnello *et al.*, 1971). The nature of these reactants is also unknown; however, C1q is known to react with anionic substances in addition to γ-globulin complexes (Agnello *et al.*, 1970), and it is possible that low molecular weight anionic substances, possibly of foreign origin, may be involved directly or complexed with γ-globulin. These low molecular weight complexes are not reactive with rheumatoid factors and, although they are associated with hypocomplementemia and clinical activity, they have not thus far been implicated in the nephritic process (Agnello, personal communication). The high and low molecular weight complexes have not as yet been demonstrated to be related to antipolynucleotide antigen–antibody systems and are

TABLE VIII

SEROLOGICAL AND IMMUNOFLUORESCENT FINDINGS IN SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS WITH CRYOPRECIPITINS AND C1q PRECIPITIN REACTIONS[a]

	Cryoprecipitins				Renal biopsy[c]				
Subject	mg./100 ml.	Rheumatoid factor (RF)	CH_{50}	C1q precipitation	Pattern of deposit	γG	γM	RF	Proteinuria (gm./24 hr.)
M. R.	36.8	+	<30	++	Granular	3+	3+	2+	4.8
J. M. H.	14.4	+	33	+++	Granular	3+	2+	Tr	5.7
J. H.	10.0	+	<30	++	Granular	3+	3+	2+	1.4
A. B.[b]	12.0	+	<30	++	Granular	1+	3+	0	1.6
G. G.	2.8	0	35	++	Mesangial	2+	Tr	NS	0
S. V.	3.6	0	38	++	Mesangial	3+	0	NS	0.1
D. S.	2.7	0	66	+	Mesangial	3+	0	NS	0

[a] From Agnello *et al.* (1971).

[b] With Sjögren's syndrome.

[c] (NS) not studied; (Tr), trace.

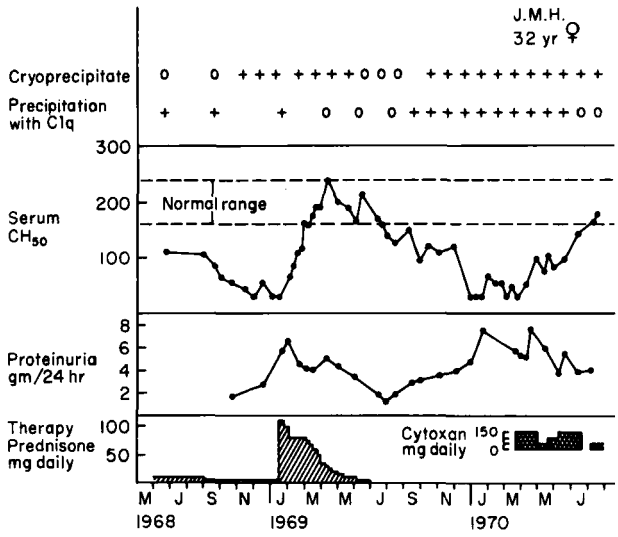

FIG. 19. Serial study of a patient with systemic lupus erythematosus, showing several episodes of clinical activity with moderate renal disease. Anti native deoxyribonucleic acid, anti-double-stranded ribonucleic acid, and anti-single-stranded DNA antibodies are absent throughout the course. High titers of antiribonucleoprotein antibodies are demonstrable in all sera tested. Note the correlation of the following parameters with disease activity: amount of γM-γG cryoglobulin, a C1q precipitin reaction, and serum complement depression. (From Koffler *et al.*, 1971b).

found both in association with and in the absence of anti-DNA antibodies.

Cryoglobulins with rheumatoid factor activity have also been associated with glomerulonephritis and vasculitis in a syndrome which bears certain analogies to SLE but appears to be an independent entity (Meltzer *et al.*, 1966). In these patients studied at autopsy, diffuse proliferative glomerulonephritis, necrotizing arteritis, and endocarditis were found; γG and γM globulins were observed in glomerular lesions, but staining for rheumatoid factor was not performed.

Localization of immune complexes in SLE is not limited to renal glomeruli. Immunofluorescent and electron-microscopic studies have demonstrated proteinaceous deposits in medium-sized blood vessels, small blood vessels of the skin (Grishman and Churg, 1970), spleen, joints, and other organs (Grishman and Churg, 1971) and in subepidermal locations at the site of cutaneous lesions. Deoxyribonucleic acid antigen has been demonstrated in larger blood vessels in which heavy deposits of γ-globulin and complement were found (Fig. 20). (Koffler *et al.*, 1967a). Nodular aggregates of γ-globulin and complement

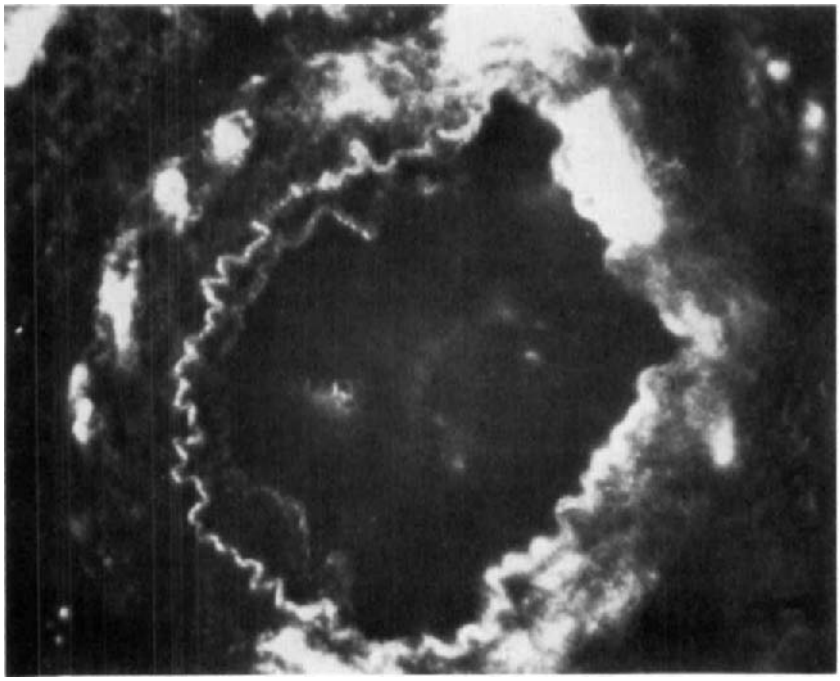

FIG. 20. Deposits of deoxyribonucleic acid (DNA) antigen in the wall of a medium-sized artery showing fibrinoid necrosis. Section was treated with fluorescein-labeled anti-DNA antibody. (From Koffler *et al.*, 1967b.)

at the dermal–epidermal junction have been associated with DNA (Tan and Kunkel, 1966b). Whereas deposits in vascular locations most probably derive from circulating complexes, the origin of the cutaneous deposits is less certain. *In vivo* deposition of antinuclear antibodies in superficial nuclei of skin cells has been found upon biopsy of skin from patients with rashes, and this material may be secondarily released into the dermis.

The stimulus for the formation of the various antibodies and the release of antigen has not been clarified. Recent studies have suggested that a viral infection may be present in patients with SLE. Clusters of microtubular structures resembling myxo- and paramyxoviruses have been found in the cytoplasm of glomerular and cutaneous endothelial cells (Gyorkey *et al.*, 1969; Kawano *et al.*, 1969; Grausz *et al.*, 1970; Pincus *et al.*, 1970; Garancis *et al.*, 1971; Hurd *et al.*, 1971). Similar structures have been found in patients with other forms of glomerulonephritis, but the tubular structures found in tissues from patients with SLE are more

widespread and comprised of larger aggregates. Serological studies have revealed elevated antibody titers to several viruses including myxoviruses (Phillips and Christian, 1970; Hollinger *et al.*, 1971). The presence of antibodies reactive with double-stranded RNA has implicated double-stranded viral RNA antigen. Although strong reactivity with reovirus RNA has been demonstrated (Talal *et al.*, 1971a), certain of these antibodies have been shown to cross-react with single-stranded DNA and RNA, indicating that polynucleotides other than viral RNA are potential immunogens (Koffler *et al.*, 1971a). The present evidence for a pathogenic role for viral infection is incomplete, although it is an area of active investigation. The reader is referred to experimental studies of viral pathogenicity discussed in Section IV.

Chemical and physical agents have also been implicated as potential pathogens which may induce tissue damage and in some instances initiate a syndrome resembling SLE. Diphenylhydantoin, isoniazid hydralazine, and procainamide have been associated with the appearance of several types of antinuclear antibody, and sometimes with the appearance of a lupoidlike syndrome (Alarcon-Segovia *et al.*, 1967; Dubois, 1969; Siegel *et al.*, 1967). Contact with physical agents, such as ultraviolet irradiation, may induce cutaneous lesions and exacerbation of systemic symptoms. Ultraviolet irradiation has been shown to alter native DNA both *in vitro* (Tan, 1968) and *in vivo* (Tan and Stoughton, 1969) and may enhance the antigenicity of DNA in addition to facilitating its release into the circulation.

Recent investigations have contributed to understanding the mechanism of tissue damage in SLE. Several different antigen–antibody complexes appear to be deposited in the renal and vascular tissues of patients with SLE. The variegated clinical symptoms in patients with SLE may reflect the heterogeneous nature of the immune complexes localized in tissues. Multiple factors appear to be involved in the formation of the various antibodies observed in SLE sera and the release of circulating antigens. In addition to the potential pathogens previously cited, there is evidence supporting the role of a genetic predisposition for the development of antinuclear antibodies (Brunjes *et al.*, 1961). Future investigations will attempt to define further the nature of the various immune complexes, the relative cytotoxicity of these systems, and the factors responsible for their appearance in the sera and tissues of patients with SLE.

B. Acute Glomerulonephritis

Acute glomerulonephritis characteristically follows acute streptococcal infection, after a latent period of 1 to 2 weeks. During this period, the

infection resolves, titers of antibodies to a variety of streptococcal antigens increase (Rammelkamp, 1954), and, in the small percentage of patients destined to develop glomerulonephritis, serum complement depression becomes manifest (Fischel, 1957; Lange *et al.*, 1960). Although many strains of streptococci elicit infection, only a few, most notably β-hemolytic *Streptococcus* of Type 12 (Rammelkamp, 1957), have been consistently associated with the development of glomerulonephritis.

Renal biopsies obtained from patients early in the course of acute glomerulonephritis showed granular deposits of γ-globulin and complement scattered along the glomerular capillary walls (McCluskey *et al.*, 1966; Michael *et al.*, 1966; Feldman *et al.*, 1966; Seegal *et al.*, 1965). In addition, variable amounts of complement and fibrinogen were observed within the mesangial areas of glomeruli. By using fluorescein-labeled antisera to Type 12 nephritogenic streptococci, deposits of streptococcal antigen were observed in areas not directly related to the granular deposits of γ-globulin and complement (Seegal *et al.*, 1965; Koffler and Paronetto, 1965); i.e., within mesangial cells and among proliferating endothelial cells. Subsequently, several investigators reported a similar distribution for streptococcal antigen (Michael *et al.*, 1966; Treser *et al.*, 1969). Since there have been failures to demonstrate streptococcal antigen by several investigators (McCluskey *et al.*, 1966; Feldman *et al.*, 1966), it appears that certain factors are critical for the demonstration of antigen. The antigenic sites available for detection of antigen are most prominent early during the course of the disease and appear to be saturated with antibody at later stages of disease. It has also been observed recently that fluorescein-labeled serum IgG fractions obtained from patients convalescing from early stages of the disease may be successfully employed for demonstrating streptococcal antigen (Treser *et al.*, 1969).

Electron-microscopic examinations of renal biopsies have also demonstrated the presence of γ-globulin and streptococcal antigen by using ferritin-labeled antisera (Andres *et al.*, 1966). Here the ability to demonstrate streptococcal antigen in the subepithelial deposits was lost, although antigen clearly remained in subendothelial locations. This indicates either that the complexes which migrated across the basement membrane were saturated with excess antibody or that antigen was denatured.

Although the precise localization of streptococcal antigen in glomeruli does not parallel the granular deposition, it is plausible that many of the complexes initially deposited in the mesangium retain the ability to complex *in vitro* with labeled antibody. Considering the difficulties

encountered in demonstrating antigen in other systems, it would be necessary to perform extensive elution-type studies prior to attempts at antigen localization. Another factor which may be involved in the attraction of streptococcal antigen to the mesangium is the affinity of certain streptococcal proteins for the fibrinogen complexes (Kantor, 1965a) which appear to have a propensity for lodging among the proliferating mesangial cells of kidneys with acute glomerulonephritis.

The roles of streptococcal membrane and cell wall antigens have not been clarified. Antibodies induced to streptococcal membrane may result in glomerular damage either by cross-reaction with GBM or by localization as part of an immune complex. At present, there is no evidence that cross-reactive basement membrane antibody deposition results in a granular pattern, although this possibility has been raised (Markowitz and Lange, 1969). The streptococcal membrane has been implicated as an antigenic component of an immune complex in glomeruli (Kantor, 1965b), although other studies have indicated that material derived from streptococcal cell walls is present in glomerular deposits (Zabriskie, 1971). The self-limited nature of acute glomerulonephritis and the difficulty in obtaining kidneys from autopsy have not permitted evaluation and quantitation of glomerular-bound antibody in order to clarify further the nature of the immune complexes deposited in the kidney.

Acute glomerulonephritis has been associated with other bacterial infections, including pneumococcal otitis, staphylococcal otitis, and staphylococcus aureus infection of a subarachnoid-jugular shunt (Michael *et al.*, 1971). Glomerular γ-globulin and complement deposits, similar to those observed in acute poststreptococcal glomerulonephritis, were found, but bacterial antigen was not demonstrated. These patients also had depressed serum complement levels. Viral disease has been associated with glomerulonephritis (Minkowitz *et al.*, 1968), but little information is available concerning deposition of immune complexes in glomeruli. Glomerulonephritis occurring in patients with infectious mononucleosis is associated with focal deposits of γ-globulin, complement (Randell *et al.*, 1969) and heterophile antigen (Peters, 1967), although viral antigen has not been demonstrated in the deposits.

Cryoglobulins of the 19 S–7 S type have been associated with focal glomerulitis and vasculitis, as previously discussed (see Section VI,A). An unusual episode of acute glomerulonephritis was reported in a young man with chronic hepatitis after exposure to the cold (Feizi and Gitlin, 1969). The patient's serum contained a γG–γM cryoglobulin, and glomeruli showed granular deposits of both γG and γM globulins, which raised the possibility that the cryoglobulin induced the glomerular lesions.

C. Subacute and Chronic Glomerulonephritis

Kidneys showing progressive subacute and chronic glomerulonephritis may be classified on the basis of immunofluorescence and elution studies as either anti-GBM or antigen–antibody complex mediated. The latter group comprises the vast majority of cases. In the subsequent section, certain forms of glomerulonephritis are cited which offer information concerning a specific immune complex system or which have distinctive morphological, clinical, or serological features that delineate a group of related cases.

There is a paucity of data concerning the localization of specific antigen and characterization of eluted antibody from "idiopathic" immune complex-type glomerulonephritis. Several infectious agents have been identified as components of immune complexes. The nephrotic syndrome associated with malarial infection appears to be related to glomerular localization of complexes containing malarial antigen (Ward and Conran, 1969). Australia antigen has been demonstrated in glomerulonephritis after post-transfusion hepatitis (Combes *et al.*, 1971). Elution studies have not been performed in either case to add further substantiation of the nature of the immune complexes. Serological studies, however, in both instances have provided evidence for circulating immune complexes. By Sephadex G-200 gel filtration of sera obtained from patients with malarial nephrosis, β1C was detected in a complexed macromolecular form (Soothill and Hendrickse, 1967). Evidence has been presented that circulating Australia antigen–antibody complexes are present in the sera of patients with chronic active hepatitis (Schulman and Barker, 1969). Syphilis has been associated with nephrotic syndrome (Braunstein *et al.*, 1970): kidneys manifest lumpy and granular deposits of immunoglobulins and complement, but no antigen or specific antibody has been demonstrated. Glomerulonephritic patients who have associated infected ventriculoatrial shunts exhibit γ-globulin and complement along the GBM (Black *et al.*, 1965; Stickler *et al.*, 1968), and in one instance staphylococcal antigen was demonstrated in the glomerular deposits (Kaufman and McIntosh, 1971).

Host tissue antigens have occasionally been implicated as a component of immune complexes. Antithyroglobulin antibodies have been eluted in several cases of glomerulonephritis occurring in patients with chronic thyroiditis, and thyroglobulin antigen was found in one of these cases (Koffler *et al.*, 1968; R. Schwartz, personal communication). A kidney with nephrotic syndrome associated with bronchial carcinoma contained antibodies in glomerular eluates reactive with tumor cell membranes (Lewis *et al.*, 1971), raising the possibility that soluble antigens from the

tumor formed complexes with antibody induced by foreign tumor antigens.

In patients with chronic sclerosing glomerulonephritis, serological findings have not contributed to understanding the pathogenesis of the disease. Granular or lumpy deposits of γ-globulin may be identified in the few remaining nonhyalinized glomeruli in many cases, although in some instances immune complex-type deposition is not apparent. Elution studies have not revealed specific antibody, although often only small amounts of γ-globulin may be eluted from such kidneys.

Although no immune complex systems have been identified, two other types of glomerulonephritis present unique characteristics which separate them from the sclerosing form of glomerulonephritis. Chronic membranoproliferative glomerulonephritis (CMPG) or chronic hypocomplementemic glomerulonephritis and membranous nephropathy have certain distinctive features. The CMPG is a slowly progressive disease occurring in children and young adults. It is characterized by asymptomatic proteinuria, hematuria, frequent development of the nephrotic syndrome and hypertension (Gotoff *et al.*, 1965; Cameron *et al.*, 1970; Herdman *et al.*, 1970). The morphological features of the disease include glomerular enlargement with lobulation, marked mesangial proliferation progressing to hyalinization which eventually envelops the glomerulus and nonargyrophilic subendothelial deposits (Gotoff *et al.*, 1965; Cameron *et al.*, 1970; Herdman *et al.*, 1970). Electron-microscopic studies have confirmed the mesangial proliferation and the presence of a dense matrix. Electron-dense deposits appear primarily in a subendothelial location and are associated with splitting of the basement membrane (Herdman *et al.*, 1970). Immunofluorescence reveals a typical lobular, granular, and lumpy deposition of immunoglobulins in about two-thirds of patients, and of C3 in all patients studied. A somewhat lesser incidence of C1q and C4 deposition has been observed in these kidneys (Herdman *et al.*, 1970; Michael *et al.*, 1969, 1971).

The unique features of the disease relate to a variety of serological findings which appear to delineate this entity from the vast majority of idiopathic cases of glomerulonephritis. Depression of total serum hemolytic complement is a frequent feature of the disease, although at certain stages normal complement levels may prevail. The profile of complement depression in contrast to that of SLE shows minimal or no depression of C1, C4, and C2, but C3 is markedly depressed as in SLE (Gotoff *et al.*, 1965; Cameron *et al.*, 1970; Herdman *et al.*, 1970; Michael *et al.*, 1969, 1971).

A series of observations indicate that the mechanism for the depression of complement beginning with C3 may not be related to

antigen–antibody complex activation. Breakdown of β1C *in vivo* has been shown by the presence of α-2D levels in fresh sera of patients with CMPG (West *et al.*, 1967). A heat-labile factor has been found in the serum of these patients which is capable of inactivating one or more of the six terminal components of guinea pig serum (Pickering *et al.*, 1968). This factor has also been found in the serum of patients with acute post-streptococcal glomerulonephritis. A second factor, which appears to be more specific for sera of patients with CMPG, has been designated the C3 nephritic factor (C3Nef) (West *et al.*, 1967; Spitzer *et al.*, 1969; West, 1970; Vallota *et al.*, 1970). The factor, when combined with a serum protein in the presence of magnesium, is able to liberate α-2D from C3. C3Nef appears to differ from the heat-labile factor active in the guinea pig complement system. It is known from experiments *in vitro* that a variety of substances, such as zymosan, endotoxin, and cobra venom factor, may bypass early complement components and inactivate C3 and later components (Götze and Müller-Eberhard, 1971).

Recent studies concerned with the localization of properdin in glomeruli of patients with CMPG have suggested another possible mechanism of C3 deposition. Properdin was localized in a granular pattern along with C3, although in other diseases, such as SLE and membranous glomerulonephritis, deposition of properdin was rare (Westberg *et al.*, 1971). Properdin has been demonstrated to possess the ability to inactivate C3 after complexing with polysaccharides such as zymosan or inulin. At the present time, therefore, there is good evidence that alternative pathways involving the C3 activation system are operative in this form of glomerulonephritis. The nephritic factor appears to activate C3 proactivator and may represent C3 proactivator convertase (West, 1970). It has also been suggested that renal activation of C3 proactivator may induce glomerulonephritis. It is, of course, true that the majority of patients with CMPG have deposits of immunoglobulin, C1q, and C4 in their glomeruli so that antigen–antibody complex deposition cannot be excluded as one of the mediators of glomerular damage. The CMPG, however, remains as a unique entity because of the serological changes related to the complement system and the failure to demonstrate significant γ-globulin deposits in the kidneys of many patients.

Membranous nephropathy is distinguished primarily by morphological and clinical features (Churg *et al.*, 1965; Ehrenreich and Churg, 1968). Nephrotic syndrome with massive proteinuria is frequent, and the incidence of remission following steroid treatment is higher than with other forms of progressive proliferative glomerulonephritis. The microscopic picture shows a diffuse thickening of the basement mem-

brane. Diffuse granular deposits of protein containing γ-globulin and complement are located along the subepithelial aspect of the basement membrane. These deposits are demonstrable with fluorescein-labeled antisera (McCluskey *et al.*, 1966) as well as with special stains such as periodic acid–Schiff. The granular material lies between spikelike projections of the basement membrane which are stained with silver. Both the spikes and intervening proteinaceous deposits are clearly demonstrable by electron microscopy. The immunofluorescent staining pattern suggests deposition of antigen–antibody complexes in a typical subepithelial location. No specific immune complex system has been implicated.

D. Diabetic Glomerulosclerosis

Kidneys obtained from patients with diabetic nephropathy frequently manifest deposits of γ-globulin in the mesangium, diffused in a linear pattern (Gallo, 1970; Burkholder, 1965; Freedman *et al.*, 1960; Moran *et al.*, 1962). Deposition of complement *in vivo* has not been demonstrated in the diffuse lesions, although in the nephrotic syndrome associated with diabetes in children, mesangial deposits of C3 have been reported (Urizar *et al.*, 1969). The linear and the nodular lesions of diabetic glomerulosclerosis do not fix heterologous complement *in vitro,* although, in exudative and arteriolar lesions, heterologous complement fixation in histological sections was noted (Burkholder, 1965). In the absence of significant complement fixation *in vivo,* the latter results are difficult to attribute to fixation by antigen–antibody complexes. Eluates prepared from glomeruli did not contain antibodies reactive with renal basement membranes (Gallo, 1970). Several studies have reported the binding of fluorescein-labeled insulin to renal tissue in diabetic glomerulosclerosis (Burkholder, 1965; Berns *et al.*, 1962a,b; Farrant and Shedden, 1965), but both immunological and disease specificities of this reaction are questionable inasmuch as glomeruli from nondiabetic kidneys were reactive with this reagent (Farrant and Shedden, 1965). Although the nature of the γ-globulin deposition has not been clarified, no direct evidence has been presented supporting the role of immune complexes in the pathogenesis of diabetic glomerulosclerosis.

E. Transplantation

Hyperacute rejection of transplanted kidneys primarily involves injury to the renal vasculature by the interaction of antibodies with endothelial antigens, which are probably related to the histocompatibility system (Kissmeyer-Nielsen *et al.*, 1966). This form of rejection is infrequent and usually occurs in a presensitized patient. With the

advent of immunosuppression and improved surgical techniques, renal allografts survive for considerably longer periods of time and may be subject to a more gradual "rejection" process involving blood vessels and in certain instances glomeruli. The glomerular lesions may reflect either reactivation of a previous glomerulonephritis or reactions involving transplantation antigens (Milgrom *et al.*, 1971). The relative involvement of the renal glomerulus by each of these two processes is difficult to evaluate.

Clinical and experimental observations indicate that reactivation of glomerulonephritis occurs. Eleven of 17 isografts in which no histoincompatibility existed developed recurrent glomerulonephritis (Glassock *et al.*, 1968b). Deposits of γ-globulin and complement were observed along the basement membrane and were seen in both subepithelial and endothelial locations by electron microscopy. A study of the types of glomerulonephritis; i.e., anti-GBM or antigen–antibody complex, in patients receiving renal allografts showed good correlation with the original type of host glomerulonephritis (Dixon *et al.*, 1969). A study of 34 renal allografts 18–31 months after transplantation revealed immunoglobulin deposits in 19 cases; γG-globulin was the predominant immunoglobulin distributed in a granular immune complex pattern in 13 cases, and 4 cases showed linear deposits (McPhaul *et al.*, 1970).

Another mechanism proposed to explain the glomerular lesions involves the interaction of transplantation antigens and antibodies. Transplantation antigens have not been demonstrated on the GBM, although weak reactivity of rat glomerular capillaries with cytotoxic antisera containing antibodies to transplantation antigens has been observed (Sellin *et al.*, 1970). Combination of antibody with transplantation antigens located in the glomerular endothelium might give rise to deposits which had a granular or interrupted linear immunofluorescent pattern. The predominant subendothelial localization of immunoglobulins and complement demonstrable with ferritin-labeled antisera and seen by electron-microscopic study would support this hypothesis (Porter *et al.*, 1968; Andres *et al.*, 1970b). Complexes might also form between soluble transplantation antigen released from the kidney and host antibody. These complexes might then localize in the kidney in the same fashion as other nonglomerular antigen–antibody complexes. Although there is clear evidence that glomerulonephritis recurs in patients with renal grafts, the appearance of glomerular lesions in patients with transplanted kidneys after renal disease of nonimmunological etiology (Porter *et al.*, 1967; Hume *et al.*, 1970; Merrill, 1969) suggests that transplantation antigen–antibody systems play a role in the pathogenesis of the glomerulonephritis in renal allografts.

VII. Vasculitis of Possible Immune Complex Origin in Humans

Certain forms of vasculitis occurring in man are probably associated with the deposition of immune complexes in vessel walls. The nature of the specific immune complexes responsible for the vascular lesions has not been defined in most instances. The presence of immunoglobulins and complement components demonstrable by immunofluorescence provide a basis for classifying vasculitis as antigen–antibody complex mediated. Although this type of vasculitis is usually associated with focal glomerulonephritis, only those diseases with primary vascular involvement will be considered in this section.

Systemic vasculitis involving multiple organs occurs in patients with SLE, although the clinically significant lesions are usually limited to the renal glomeruli. Lesions affecting the vasculature of the central nervous system, however, may result in a variety of neurological manifestations and death (Johnson and Richardson, 1968). Necrosis, thrombi, and proliferative changes involving small blood vessels (Johnson and Richardson, 1968; Berry and Hodges, 1965; Glaser, 1952; Gold and Gowing, 1953; Malamud and Saver, 1954) and capillaries have been associated with the presence of microinfarcts. Larger cerebral vessels are less frequently involved. An immunofluorescent study of the brain tissue from 3 patients dying of SLE did not reveal γ-globulin (Johnson and Richardson, 1968), nor was acute neurological disease found. There have been no reports of immunopathological studies of brain tissue from patients with acute lesions, although the presence of immune complex deposits in vessel walls at other sites suggest a similar pathogenesis for cerebrovascular lesions.

Periarteritis nodosa is a form of necrotizing angiitis which involves medium-sized and small arteries of several organs including kidney, pancreas, testis, gastrointestinal tract, and skeletal muscle. γ-Globulin, complement, and fibrinogen (Mellors and Ortega, 1956; Lachmann *et al.*, 1962; Paronetto and Strauss, 1962; Gitlin *et al.*, 1957) have been demonstrated in areas of vascular necrosis. Recent studies indicate that hepatitis-associated antigen (HAA) may be present in areas of necrosis of the vascular lesions (Gocke *et al.*, 1970). Serological studies indicate that this antigen is demonstrable in certain sera of patients with periarteritis nodosa, raising the possibility that HAA–anti-HAA immune complexes are responsible for the vasculitis (Gocke *et al.*, 1970, 1971). The immunofluorescent studies on blood vessels, however, have been limited, and the role of HAA–anti-HAA complexes has been questioned on the basis of the poor correlation between the occurrence of such complexes and the presence of vasculitis (Prince and Trepo, 1971). The

necrotizing and proliferative glomerulonephritis which occurs during the course of the disease exhibits a typical granular immune complex pattern of γ-globulin and complement deposition (Paronetto and Strauss, 1962). Antigen has not been demonstrated in the renal lesions.

Schönlein-Henoch's syndrome is associated with widespread vasculitis affecting the skin, gastrointestinal tract, and joints. Immunoglobulins and complement were not observed in acute cutaneous vascular lesions with focal necrosis in vessel walls (Koffler *et al.*, 1965; Miescher and Koffler, 1968). Although this would implicate factors other than immune complexes in the pathogenesis of the vascular lesions, it must be remembered that complexes and complement in vessels in experimental, acute, immune complex disease are rapidly degraded by leukocytes. It is, therefore, difficult to eliminate an immune complex etiology. Hypersensitivity reactions, possibly of the delayed type, to bacteria, food, or drugs have been suggested as etiological agents (Miescher and Koffler, 1968). In contrast, renal lesions in patients with Schönlein-Henoch's syndrome may manifest immunoglobulin and complement deposition in mesangial areas as well as in granular and nodular pattern along the GBM (Miescher and Koffler, 1968; Urizar *et al.*, 1968). Early glomerular lesions contain primarily fibrinogen without immunoglobulins or complement. The renal lesions in the syndrome are usually mild and self-limited, although progressive glomerulonephritis has been observed.

VIII. Methods of Detecting Immune Complexes in Serum

One of the earliest and most sensitive experimental methods of detecting circulating immune complexes involved fractionation of globulins with ammonium sulfate (Farr, 1958). Antigen binds to its antibody and then precipitates with the globulin fraction in ammonium sulfate. The globulin-bound antigen offers evidence for the presence of immune complexes. The technique requires that the antigen not precipitate in the absence of antibody at whatever the concentration of ammonium sulfate. One of the commonest uses of this technique has been in detecting circulating immune complexes in acute immune complex disease (serum sickness). Bovine albumin (BSA), labeled with ^{125}I or ^{131}I is injected into rabbits. When antibody is formed it binds to the antigen which, being in excess, maintains solubility of the complexes. The globulin-bound radiolabeled BSA is then precipitated in ammonium sulfate, allowing determination of the percent of BSA in immune complex form (Weigle and Dixon, 1958).

Immune complexes can also be identified by sucrose gradient ultracentrifugation, especially if a readily detected, slowly sedimenting

antigen is employed. Bovine serum albumin-^{125}I bound to antibody in serum has been detected by sucrose gradient ultracentrifugation (Cochrane and Hawkins, 1968), and the technique has provided information on the relative size of the circulating complexes *in vivo* and complexes prepared *in vitro*.

Viral antigens can be detected bound to antibody in the circulation. Antibody, directed to immunoglobulin of the animal infected with virus, is added to a small volume of serum of the infected host. Full precipitation of the host immunoglobulin must occur, bringing with it globulin-bound virus. The washed precipitate can then be assayed for virus (Porter and Larsen, 1967; Oldstone and Dixon, 1969). Antibody to host complement (C3) may also be used to precipitate the complex of virus–antibody–C3.

A variety of sensitive techniques have facilitated the detection of immune complexes in human sera and fluids in the last few years. Analytical ultracentrifugation has been used to detect immune complexes in the sera of patients with rheumatoid arthritis and hypergammaglobulinemic purpura (Kunkel *et al.*, 1961; Capra *et al.*, 1971), although relatively large amounts of complexes are necessary for their demonstration by this method. Agar gel precipitation with C1q globulin has been successfully employed for the detection of γ-globulin complexes in the sera of patients with SLE and in joint fluids obtained from patients with rheumatoid arthritis (Agnello *et al.*, 1970, 1971; Winchester *et al.*, 1970). Rheumatoid factor has also been used for the demonstration of complexes or aggregates of γ-globulin both by precipitation (Winchester *et al.*, 1970) and agglutination-inhibition tests (Winchester *et al.*, 1970; Hannestad, 1967; Oreskes and Plotz, 1965). Monoclonal rheumatoid factors appear to be capable of precipitating with smaller complexes which are more difficult to demonstrate with polyclonal rheumatoid factor or C1q globulin (Winchester *et al.*, 1971). The appearance of a cryoglobulin in serum is a simple test based on the relative insolubility of γ-globulin complexes in the cold. Certain of these higher molecular weight complexes correspond to material reacting with C1q globulin in agar gel diffusion (Agnello *et al.*, 1971) and may be removed by precipitation with excess rheumatoid factor in the cold.

Activation of histamine release from isolated perfused guinea pig lung is a sensitive indicator system for the presence of immune complexes (Baumal and Broder, 1968a). Although several types of macromolecular substances have been shown to induce histamine release, soluble antigen–antibody complexes have been characterized by Sephadex chromatography and have been found in the sera of patients with rheumatoid arthritis, SLE, and anaphylactoid purpura, and in the joint

fluid of patients with rheumatoid arthritis (Broder *et al.*, 1968; Baumal and Broder, 1968b).

Complexes demonstrated by the aforementioned methods have primarily involved the 19 S and 7 S γ-globulin-anti-γ-globulin systems. The contribution of other antigens to these complexes in patients with SLE and rheumatoid arthritis is currently under investigation. There is serological evidence for the presence of complexes of Australia antigen and antibody in the sera of patients with serum hepatitis, on the basis of anticomplementary activity of sera containing both antigen and antibody (Schulman and Barker, 1969). Evidence suggestive of the presence of circulating complexes was obtained in sera of patients with malarial nephrosis by Sephadex G-200 chromatography which demonstrated β1C globulin attached to high molecular weight complexes (Soothill and Hendrickse, 1967). The definition and isolation of circulating complexes by a variety of techniques in disease states where they are present in small quantities should help to delineate a variety of immune complex systems, in addition to those previously cited.

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The Immunopathology of Joint Inflammation in Rheumatoid Arthritis[1]

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The proper study of mankind is man. (Alexander Pope)

I. Introduction

Investigations into the pathogenesis of rheumatoid arthritis have proceeded by fits and starts, often marked by misdirection and shifts in focus. In the 1930s and early 1940s the pioneering efforts of Walter Bauer and his associates began to shed light upon normal articular structure and physiology in an attempt to understand better the alterations represented by joint disease. The introduction of cortisone in the mid-1940s dramatically transformed the study of rheumatic disease. Indeed, it was no coincidence that arthritis and metabolism were wedded at the beginning of the 1950s with the formation of the National Institutes of Arthritis and Metabolic Diseases. During the following decade there was a burst of activity. It was as if rheumatoid arthritis had been discovered anew. Blood, plasma, and urine were minutely dissected in an effort to understand better the endocrine function and immunological status of the afflicted. Epidemiologists sought to capture the essence of the disease in its natural habitat. Studies of the systemic nature of the disorder became fashionable—rheumatoid lung, heart, neurological, and vascular disease were rediscovered and redescribed. The designation

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"rheumatoid disease" began to displace "rheumatoid arthritis." Curiously, little attention was focused on the events in the articular cavity.

It was not until the latter part of the 1950s when, armed with the technology of the immunochemists and the insights of the experimental pathologists, that rheumatologists returned to the joints. In a period of less than 5 years, it was shown that (*1*) the round cells of the rheumatoid synovium produced immunoglobulins and rheumatoid factor; (*2*) within the membrane were complexes of IgG, IgM, and complement; (*3*) complement levels in rheumatoid effusions were significantly lower than in companion serum samples or when compared to synovial fluids from other forms of inflammatory joint disease; and (*4*) granulocytes from rheumatoid synovial fluids contained intracytoplasmic complexes of immunoglobulins, complement and anti-γ-globulin. Shortly thereafter, several schemes were proposed to encompass the available information (Hollander *et al.*, 1965a; Ziff, 1965; Zvaifler, 1965); although differing in emphasis, they were remarkably similar in their conceptualization of the pathogenesis of the articular inflammation in rheumatoid arthritis. They viewed the synovial membrane as an ideal site for an immune response, equipped as it is with a lining layer of phagocytic cells and a vascular stroma adapted for the mobilization and proliferation of lymphocytes. It was proposed that antigens, localized in the articular cavity, stimulate the production of antibody; these combine in the synovial membrane or fluid and activate the complement sequence, thereby generating a variety of biologically active materials, including some with potent chemotactic properties. These latter substances draw polymorphonuclear leukocytes into the joint, where they are attracted to, and ingest, the complexes. During phagocytosis the neutrophiles (and probably also the phagocytic synovial lining cells) discharge from their lysosomal granules a variety of hydrolytic enzymes, and it is these substances that mediate the proliferative and destructive changes characteristic of rheumatoid arthritis (Weissmann, 1972).

The purpose of this review is to present the evidence supporting this pathogenetic concept. Much of it has come to light in the past few years. Limitations of space, an inability to cope with the proliferation of new, and often relevant, information, and the author's own preconceptions have imposed constraints upon the material selected for presentation. There has been no attempt to review or discuss the etiologic factors that initiate rheumatoid arthritis; indeed, one strength of the proposed pathogenetic concept is that it can be dissected, studied, and probably therapeutically manipulated without knowing the cause (or causes) of the disease. Information concerning the structure, function and biochemistry of articular tissues has been limited to those aspects

that seem of immediate relevance to the immunological thesis. Those interested in a more thorough exposition on these subjects are referred to two outstanding recent reviews by Hamerman and his associates (1969, 1970).

In addition, because rheumatoid arthritis is such a uniquely human disorder, greatest emphasis has been placed on information derived from investigations of patients with arthritis; animal studies have been assigned a secondary role. When appropriate, the author, in his capacity as a rheumatologist, has called attention to findings which appear unique to rheumatoid arthritis. All too often in the past, the uninitiated have ascribed differences to rheumatoid arthritis that only reflect the differences between inflamed and noninflamed joints. As an additional control, the findings in rheumatoid arthritis have been contrasted with other joint diseases manifesting similar degrees of synovial inflammation.

One shortcoming of this clinically oriented approach is the tendency to lapse into the jargon of the rheumatologic literature. This is particularly confusing in matters relating to the group of immunoglobulins designated as rheumatoid factors. It is beyond the scope of this presentation to explore the intricacies of this subject. The interested reader should consult the excellent review by Kunkel and Tan (1964). The terms *rheumatoid factor* and *anti-γ-globulin* have been used interchangeably and, unless otherwise stated, rheumatoid factor refers to the conventional IgM anti-γ-globulin detected by agglutination procedures. When known, however, the immunoglobulin class of the anti-γ-globulin has been specified. Patients with seropositive rheumatoid arthritis are those whose serum contains conventional IgM rheumatoid factor.

Finally, the author has elected not to elaborate upon the extra-articular manifestations of rheumatoid arthritis, not because they are unimportant, but rather, from the firm conviction—to paraphrase Alexander Pope—that the proper study of joint disease is joints.

II. Normal Synovial Membrane

A. Fine Structure

The human joint is encompassed by a capsule composed of thick bundles of collagen and fibrous tissue. These structures diminish in size as they descend toward the articular cavity eventuating in a thin lining membrane constructed of a delicate arrangement of fine collagen fibers, a pericellular reticular network, capillaries, and connective tissue cells (Castor, 1960; Barnett *et al.*, 1961). Some (Barnett *et al.*, 1961) consider the synovial membrane to consist of two layers—an intimal lining layer next to the joint cavity and a layer of connective tissue and fat

upon which it lies, termed the *subsynovial layer*. The intima is predominantly cellular with an abundant blood supply, whereas the subsynovial layer is more fibrous and less vascular. The internal surface of the synovial membrane is smooth, moist, and glistening with a few small villi and fringelike folds. It covers all of the intra-articular structures, with the exception of articular cartilage. At its margin the intimal layer is reflected off the capsule and inserts into the periosteum of subchondral bone where the blood vessels of the synovial membrane terminate in the looped anastomoses of the circulus articuli vasculosus—the vascular border of the joint (Barnett *et al.*, 1961).

Hematoxylin and eosin stains of sections of the internal surface of the synovial membrane show large cells with large dark-staining nuclei. The histological appearance of the synovium depends in part on its location within the joint. Three types of synovium are described, named after the underlying tissue. One type is fibrous synovium which appears in areas subjected to great pressure; the connective tissue is well-developed and the lining is often only a single cell layer thick. A second type of synovium, called areolar, is located in areas where the membrane glides freely with little support from the joint capsule, such as in the suprapatellar pouch of the knee. The connective tissue in this type of membrane is loosely structured with delicate fibers, short broad villi, and a surface layer of three or four rows of closely spaced lining cells. A third type of synovial membrane is usually found around fat pads; it is more adipose and has only one to two layers of lining cells (Castor, 1960; Hamerman *et al.*, 1969).

The cellular elements of the joint lining consist of synovial intimal cells (or synoviocytes), unclassified connective tissue cells, such as fibroblasts and histiocytes, and a significant number (approximately 3%) of mast cells (Castor, 1960). Several different oxidative enzymes and a variety of hydrolytic enzyme activities have been demonstrated in synovial lining cells by cytochemical techniques (reviewed in Hamerman *et al.*, 1970). These findings are consistent with the increased synthetic, phagocytic, and digestive functions of synoviocytes as compared with other connective tissue cells.

Superficially, the synoviocytes facing the joint cavity appear to form a continuous uninterrupted layer, but careful light-microscopic examination indicates that they are only loosely arranged in a bed of intercellular ground substance (Castor, 1960). The intimal cells are generally ellipsoidal with cytoplasmic processes extending long distances from the main cell body. No true basement membrane separates them from the underlying supportive connective tissue, rather it is the interdigitation of the cytoplasmic processes that provides the continuity. When

examined under the electron microscope at relatively low magnifications, the synoviocytes can be seen to form a discontinuous margin of cells several layers in depth which gradually merge with the underlying connective tissue. The cytoplasmic processes of the lining cells are usually directed toward the membrane surface, forming a loose network. Occasionally there are interdigitations of the cytoplasmic processes. Among the loosely arranged processes are intercellular spaces which can sometimes be traced to the surface. The intercellular matrix contains amorphous material and numerous thin filaments that lack axial periodicity. In deeper layers, collagen fibers with typical 640-Å. periodicity are seen (Barland *et al.*, 1962).

At higher magnifications, two distinct types of lining cells can be identified—designated as type A and type B cells. In addition there are many cells with features of both types, sometimes called intermediate-type cells. The predominant cell, type A, has many characteristics of a macrophage. Numerous fingerlike projections (filopodia) extend out from the A cells, often enclosing portions of the extracellular matrix. Filopodia can be found over the entire cell surface, although they are most numerous in the distal parts of the cytoplasmic processes. Within the cytoplasmic processes are many vesicles, 0.1–0.3 μ in diameter, which seem to be micropinocytotic vacuoles, and a number of larger vacuoles, 0.4–1.5 μ in diameter, bound by thin single membranes. These larger vacuoles contain dense granular material and are presumed to be lysosomes. Many branched aperiodic fibrils, 50–75 Å. in diameter, are identified in the cytoplasmic ground substance. Type A cells have numerous mitochondria, and a well-developed Golgi apparatus is present in the perinuclear area.

Type B cells are less numerous than the type A and are characterized by abundant, well-developed, rough-surfaced endoplasmic reticulum. Other features are occasional mitochondria, which appear smaller and contain fewer cristae than seen in type A cells, and vacuoles and vesicles, which are less numerous and smaller than in A cells.

A diagrammatic representation of the ultrastructure of the normal human synovial membrane is shown in Fig. 1. Barland and his associates (1962), who conceived this scheme, view the lining cells as an intricate meshwork between the joint cavity and the underlying capillary bed. Direct continuity between the joint cavity and the intercellular spaces is readily observed in thin section and no distinct basement membrane separates the lining cells from the capillaries and other connective tissue cells. The absence of a true physiological basement membrane is of significance and will be considered in greater detail when we deal with the manner in which materials enter and leave the joint.

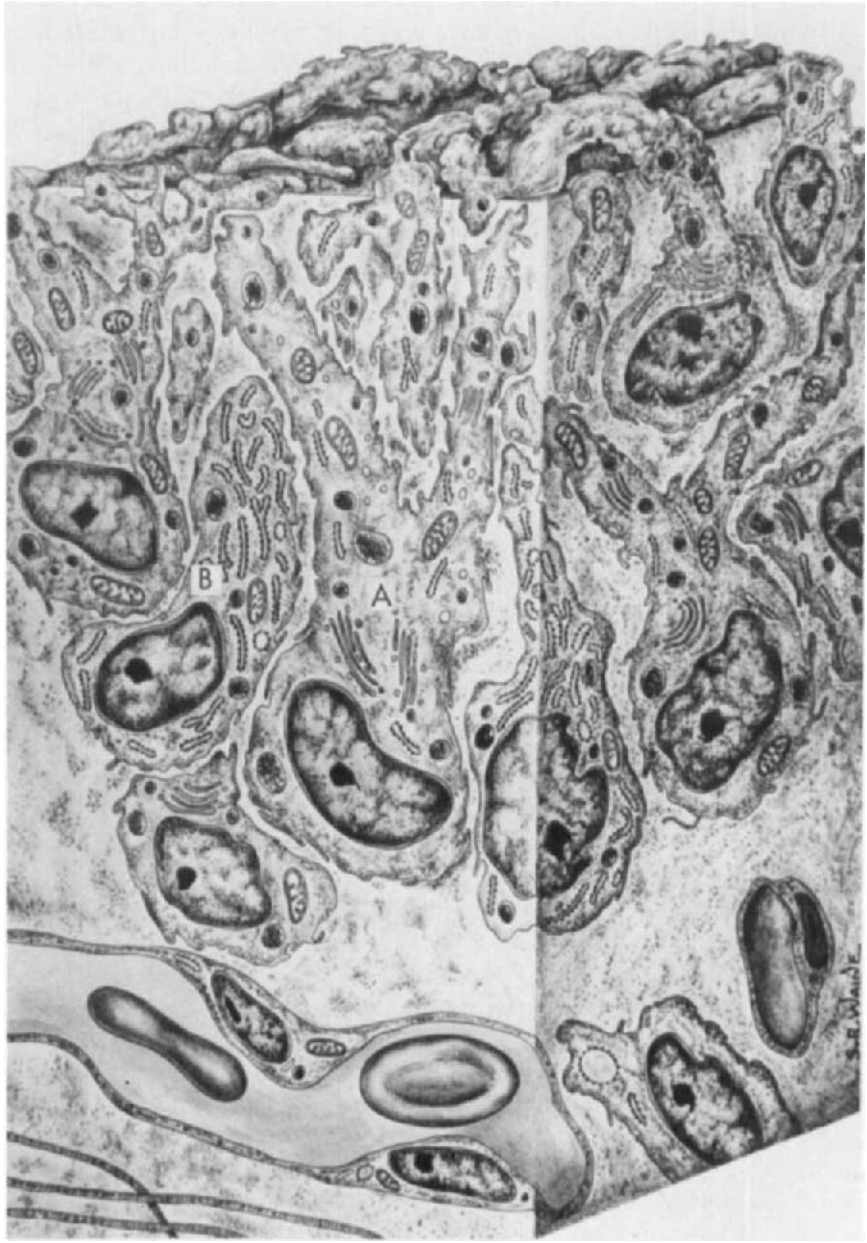

FIG. 1. Schematic representation of normal human synovial membrane, showing the relationship of the lining cells (A and B) to the joint space (at the top) and the underlying capillaries and connective tissue. (From Barland *et al.*, 1962.)

The blood supply of the joint comes from large vessels traversing the capsule. These commonly divide into three branches: one which serves the epiphysis, a second which goes to the joint capsule, and a third which is distributed to the synovial membrane (Barnett *et al.*, 1961; Ham, 1965). The several types of synovium differ somewhat in their vascular patterns. The loose areolar synovium is most vascular, containing parallel plexuses connected by numerous anastamoses. Fibrous synovium has few blood vessels, and adipose synovium is characterized by a vascular network with large vessels near the base. Individual villi have at least one system of vessels consisting of a venule, arteriole, and capillary network. Capillary loops often exhibit distal coil formation not unlike that which is seen in glomeruli (Lindstrom, 1963).

Suter and Majno (1964) investigated the ultrastructure of the capillaries in rat synovium to determine how fluid could pass so easily in and out of joints. They found few vessels in the fibrous synovium and all had a continuous type of vascular endothelium. In contrast, the fat pads from beneath the patellae contained abundant capillaries located immediately beneath the articular surface in a space between the synovial lining cells and the underlying fat cells. The structure of these capillaries was of interest. Typically, the nucleus of the endothelium was on the side away from the synovial cell, whereas the wall of the capillary facing the synovium was extremely attenuated and contained numerous fenestrations closed by diaphragms. Capillaries with attenuated endothelium and rounded fenestrae bridged by a thin membrane are seen in other areas of the body, including the renal glomerulus, ciliary body, choroid plexus, and intestinal villi. The authors suggest that the superficial capillaries in the synovium are specialized microvessels ideally suited for the rapid exchange of water and solutes (Suter and Majno, 1964).

Schumacher (1969) studied the synovial vessels in normal monkey knee joints. The gross anatomy was similar to that of human synovium. Light-microscopic examination disclosed that the superficial capillaries had thin walls, whereas the deeper vessels were characterized by thicker walls and more prominent endothelial cells. In contrast to the rat, the microvasculature of the monkey synovium has prominent fenestrations not only in capillaries but also in larger vessels, presumed to be venules. Vessels of this type were found in the superficial synovium from all parts of the joint. Polarization of capillaries, so that the portion of the wall farthest from the synovial lining contained the nucleus and the nearer part the fenestrae, was a frequent, but not invariable finding. In some instances, fenestrae were not found at all in superficial vessels. The deeper vessels were characterized by an endothelium containing large

numbers of organelles. Active phagocytic capacity of the endothelium was shown by its ability to pick up ferritin and carbon tracers injected intravenously prior to joint biopsy. The monkey synovium appears to be excessively leaky when tested with these tracers. No leakage was seen in capillaries, but carbon was found lying between endothelial cells and pericytes in deeper vessels. This combination of intact capillaries and venular leakage is identical to the vascular permeability induced by chemical mediators, such as histamine and serotonin (Majno *et al.*, 1961). Penetration of vessel walls by carbon particles averaging 250 Å in diameter, without any other morphological alterations, suggested to the author (Schumacher, 1969) that pathogenetic agents, such as bacteria, viruses, or immune complexes might also penetrate these vessels. Carbon and ferritin appeared to leak more from the monkey's synovial vessels than vessels in other areas, such as skin, pericardium, and muscle.

B. Function

Phagocytosis and secretion are recognized functions of the synovial membrane. Light- and electron-microscopic correlations of structure and function have been attempted, but there is no general agreement as to the success. It has long been assumed that the synovial lining cells are the source of synovial fluid hyaluronate (King, 1935; Hamerman and Blum, 1959; Castor, 1960). This concept is further strengthened by *in vitro* evidence that synovial tissues contain the appropriate enzymes to synthesize uridine 5′-diphosphate (UDP) glucuronate from glucose (Bollet *et al.*, 1959) and glucosamine-6-phosphate from glucose-6-phosphate and glutamine (Bollet and Schuster, 1960). Moreover, synovial cells in tissue culture synthesize and secrete hyaluronate into the medium (Castor and Fries, 1961). Incubation of sections of synovial membrane with fluoresceinated antiserum to hyaluronate reveals fluorescence localized in the cytoplasm of the lining cells (Blau *et al.*, 1965). The secretion of hyaluronate would seem to be most reasonably attributed to the B cells, because of their large component of endoplasmic reticulum. However, this is not consistent with the demonstration that the site of hyaluronate synthesis is in the Golgi zone, an organelle which is more common in A cells (Barland *et al.*, 1968).

The phagocytic capacity of the A cell is more easily demonstrated. Intra-articularly injected iron dextran (Ball *et al.*, 1964), Thorotrast (Cochrane *et al.*, 1965), and colloidal gold (Norton and Ziff, 1966; Southwick and Bensch, 1971) can be found localized in type A cells and to a lesser degree in B cells. Protein uptake by the rabbit synovial membrane has recently been examined employing peroxidase as an ultra-

structural marker (Shannon and Graham, 1971). In addition, the use of several species of the protein—horseradish peroxidase (molecular weight 40,000), lactoperoxidase (molecular weight, 82,000), and myeloperoxidase (molecular weight, 160,000)—permitted analysis of the function of molecular size and charge. Following intra-articular injection, the largest portion of protein uptake was accounted for by bulk incorporation through fusion of pseudopods of type A cells. Pinocytotic vesicles formed at the cell membrane in both A and B cells, but fusion of vesicles to form larger vacuoles could only be clearly demonstrated in type A cells. Increasing molecular size of the protein was associated with a definite increase in bulk incorporation. Whether the foreign protein (peroxidase) is handled in an identical manner to the way autologous proteins would be removed from the joint space has not yet been resolved.

Solutes, electrolytes, and some proteins traverse the synovial membrane, but the factors regulating their movement are not well understood. Some controls are operating, however; for instance, normal synovial fluid is conspicuously lacking in plasma proteins of high molecular weight, such as α- and β-macroglobulins, or those of asymmetrical shape, such as fibrinogen. The lining cells themselves are unlikely to be the barrier. They do not form a continuous membrane, neither desmosomes nor adhesion plates are present between the synovial cells, and there is no demonstrable basement membrane beneath the synovial lining. Moreover, there are large gaps between the lining cells, and tracer substances have been found to traverse these spaces passing down into the subsynovial regions (Adams, 1966; Norton and Ziff, 1966; Southwick and Bensch, 1971). Hamerman *et al.* (1969) have proposed that the passage of proteins from the circulation into the synovial fluid is controlled by macromolecules in the intercellular matrix. Macromolecules that have long extended chains can sterically exclude from their diffuse domain the entry of large or asymmetrically shaped proteins. The macromolecule in the joint which shows such an excluded volume effect is hyaluronate (Ogston and Phelps, 1961; Gerber and Schubert, 1964). Furthermore, if hyaluronate is retained on one side of a chamber by a filter and solutes are added to the other side, then the small molecular weight solutes equilibrate throughout the chamber, but proteins are excluded in proportion to their molecular size from the side of the chamber containing hyaluronate (Hamerman *et al.*, 1969). This effect can be increased further by the use of gels of concentrated hyaluronate. When serum is forced to flow through such gels by centrifugation, the emerging proteins resemble closely the pattern of those found in normal synovial fluid (Hamerman *et al.*, 1969). Lastly, it has been proposed that the capillaries immediately beneath the synovial lining control the

diffusion of solutes by adjusting their fenestrations, in a manner similar to the glomerular tuft and the intestinal epithelium.

With the renewed interest in an infectious etiology of rheumatoid arthritis, it is pertinent to consider the manner in which a pathogen might gain access to the synovial membrane. The joint seems to be uniquely predisposed to infection with some bacteria. For instance, the intravenous injection of Type III pneumococci into rabbits is followed within 24 hours by the presence of a culturally proven organism within one or both knees. At the same time the spinal fluid, aqueous humor of the eye, and bladder urine are all sterile. Joint infections could often be demonstrated when blood cultures were negative (Bennett and Shaffer, 1939). In a study of synovitis developing in rabbits during bacteremia, Lewis and Cluff (1965) concluded that regardless of the organism used, that synovitis was a regular accompaniment of bacteremia. They could not determine the mechanism of localization, but suggested that phagocytosis by lining cells, a unique permeability of the blood vessels of the joint to circulating microorganisms, or phagocytosis by the vascular endothelium might all be playing a role. These postulates are all in keeping with known ultrastructural and functional characteristics of the synovium, namely, the phagocytic capabilities of type A cells; the ease with which large-size markers penetrate the synovial vessels and the uptake of particulate materials by the endothelium of synovial blood vessels. In the latter regard, it is interesting to note that phagocytosis by vascular endothelium cells is rare outside the reticuloendothelial system (Cotran and Majno, 1964), but Schumacher (1969) regularly found uptake of marker particles in the deeper vessels in the synovium.

III. Rheumatoid Arthritis

A. Synovial Membrane

1. Histopathology

Rheumatoid synovitis is characterized by a constellation of histological changes which are characteristic but not pathognomonic of this disease (Collins, 1958; Gardner, 1965; Hamerman *et al.*, 1969).

a. Hypertrophy of the synovial lining surface. Grossly, the synovium appears edematous and inflamed and protrudes into the joint space as slender villous projections.

b. Hyperplasia and hypertrophy of the synovial lining cells. The lining cells are multilayered, reaching to a depth of six to ten cells, as

compared to the normal synovial lining which is only one to three cell layers. Cytoplasmic processes from the more superficial cells often extend into the joint space forming a narrow eosinophilic zone along the surface (Kulka *et al.*, 1955; Hamerman *et al.*, 1969). Multinucleated giant cells are common and located in areas of lining cell hyperplasia or in the subintimal layer in a band 25–100 μ below the synovial surface. The giant cells are ovoid, measure approximately 40 μ in diameter and contain as many as ten to fifteen peripherally situated nuclei. The cytoplasm is mildly basophilic and contains eosinophilic granules, many of which stain brilliantly with the periodic acid–Schiff (PAS) test. Analysis of the fine structure of these giant cells suggested to Grimley and Sokoloff (1966) that they were derived from A cells of the synovial lining. Initially, they were thought to be unique to rheumatoid synovitis (Grimley and Sokoloff, 1966; Donald and Kerr, 1968), but similar cells have been observed in other forms of chronic synovitis (Bhan and Roy, 1971).

c. Vascular derangement. Focal or segmental vascular changes are a regular feature of rheumatoid synovitis (Kulka *et al.*, 1955; Kulka, 1966). Varying degrees of venular dilatation, hypertrophy, and hyperplasia of endothelial cells, leakage of erythrocytes and plasma proteins, and capillary obstruction are common. Involvement of arterioles is characterized by cellular infiltration of vessel walls and areas of thrombosis and perivascular hemorrhage.

d. Cellular infiltrates. The connective tissue stroma of the synovial villus is packed with mononuclear cells which are collected into aggregates or follicles, particularly around small blood vessels, but true germinal centers are rarely seen (Gardner, 1965). The predominant cell in these nodules is a lymphocyte, but about the periphery are plasma cells with acidophilic cytoplasm containing abundant endoplasmic reticulum and frequently spherical cytoplasmic inclusions (Russell bodies). The plasma cells are intensely pyroninophilic, suggesting active protein production. Polymorphonuclear leukocytes are occasionally seen but are not a prominent feature of chronic synovitis. Macrophages with iron-containing pigment are found in superficial and deeper layers of the synovium (Muirden, 1966).

It should be appreciated that the so-called typical changes outlined are found in long-standing rheumatoid arthritis. Analysis of these lesions may increase understanding of processes that perpetuate joint inflammation and tissue destruction, but they provide little insight into the initiating events. Investigation of early rheumatoid lesions might be more instructive. Such investigations are uncommon, however, since the disease is most difficult to diagnose at that time, and patients often do

not seek medical attention until joint inflammation has persisted for many months. Furthermore, studies of synovial histology are complicated by the fact that multiple biopsies taken from the same joint, at the same time, may show strikingly different pictures (Wilkinson and Jones, 1963; Hamerman *et al.*, 1969). In general, the changes in early, acute rheumatoid synovitis are confined mainly to the synovial lining layer or the immediate subsynovial zone. As in more chronic synovitis, there is hyperplasia of the lining cells with cellular enlargement and frequently a pattern of disarray. The cytoplasmic processes of the lining cells often extend out into the joint cavity (Hamerman *et al.*, 1969). Another regular feature is a fibrinopurulent exudate, most conspicuous on the synovial lining, but frequently present in the interstitial regions between the lining cells. The nature of these masses of fibrin or fibrinoid material are not well understood. Occasionally the synovial lining cells beneath the fibrinoid material assume a palisading appearance with their cytoplasmic processes extending into the fibrinoid material—a picture very reminiscent of the rheumatoid subcutaneous nodule (Hamerman *et al.*, 1969). Although these fibrin masses are seen frequently in very early and active joint disease, similar histological findings have been reported in tissues from patients whose disease was clinically inactive at the time the tissues were obtained (Zevely *et al.*, 1956; Gardner, 1965).

Edema of the connective tissue stroma beneath the lining cells is a conspicuous feature of the early rheumatoid lesion. The edema is most pronounced in perivascular areas, causing the vessels to stand out when examined microscopically (Hamerman *et al.*, 1969). The small blood vessels immediately beneath the lining cells appear to be increased in number and their lumens may be occluded by hypertrophied endothelial cells (Kulka, 1966). Most of the studies of early rheumatoid synovitis show infiltration of the subsynovial area by inflammatory cells. There is disagreement about the predominant cell type. Lymphocytes are regularly found, particularly in perivascular locations, but the majority of authors (Rodnan *et al.*, 1960; Wilkinson and Jones, 1963; Hamerman *et al.*, 1969) were impressed that polymorphonuclear leukocytes are conspicuous in the cellular infiltrates and were occasionally observed among the proliferating lining cells. On the other hand, Kulka *et al.* (1955) described the infiltrate as predominantly mononuclear in six of eight early lesions. The intensity of the cellular infiltrate varied considerably from case to case and even within sections taken from the same case.

Schumacher and Kitridou (1972) have made an important contribution to our appreciation of the early rheumatoid lesions. They performed

synovial biopsies on 24 patients seen within 30 days of the onset of articular symptoms; 6 of these subsequently fulfilled the diagnostic criteria for definite or classic adult rheumatoid arthritis (Ropes *et al.*, 1958). Vascular changes were prominent, affecting mainly small venules which showed congestion and obliteration of the lumen by inflammatory cells, fibrin, platelets, and enlarged endothelial cells. Perivascular collections of inflammatory cells and erythrocyte extravasation were frequent findings, but cellular infiltration of vessel walls was rare. When seen, it affected both small arteries and veins. Proliferation of synovial lining cells was an invariable finding and even in very early specimens was up to ten cell layers deep. Lymphocytes were the major infiltrating cells; they were mainly perivascular and did not form lymphoid follicles. Plasma cells were not seen, but granulocytes were common and were profuse in the superficial synovium of 2 of the 6 rheumatoid specimens. It is noteworthy, however, that none of the histopathological findings were specific for rheumatoid arthritis, because identical changes were present in biopsies taken during the first month in a variety of other joint diseases.

An important feature of the pathology of rheumatoid arthritis is the formation of pannus, a vascular granulation tissue which extends out from the inflamed synovium onto the cartilage. Histologically pannus is composed of proliferating fibroblasts, numerous small blood vessels, variable numbers of inflammatory cells, and occasional collagen fibers (Hamerman *et al.*, 1969). The initial replacement of articular cartilage occurs at the periphery near the perichondrium, but as the disease progresses more and more cartilage is destroyed and ultimately replaced by fibrous connective tissue. Although cartilage destruction and pannus are almost invariably associated, there is some question about the relationship of one to the other. The prevailing view is that the pannus is responsible for the observed articular cartilage injury. There is no general agreement, however, how this comes about. Several theories have been proposed: (*1*) the hyperemic granulation tissue leeches away the cartilage matrix; (*2*) pannus interferes with cartilage nutrition, which normally depends on percolation of interstitial fluid from below and absorption of synovial fluid from above; and (*3*) pannus is responsible for enzymatic digestion of constituents of cartilage.

The last-mentioned thesis, in particular, has considerable support from experimental observations. The integrity of the physical properties of cartilage, such as elasticity, depends upon its matrix. This matrix is produced by chrondrocytes and consists of high molecular weight, anionic, protein–polysaccharide complexes enmeshed in collagen fibers. Changes in the matrix can be detected by alteration in its physico-

chemical properties. For instance, negatively charged polysaccharides normally bind metachromatic dyes and induce a color change in the dye referred to as metachromasia. Loss of protein polysaccharides from the cartilage matrix results in a failure to stain metachromatically. Solutions of the high molecular weight protein–polysaccharides are highly viscous, and degradation of these molecules causes a loss of viscosity. The integrity of the protein–polysaccharide can be disrupted through cleavage of the polysaccharide chain by enzymes such as hyaluronidase or by proteolytic enzymes that attack the protein portion (Hamerman *et al.*, 1967). The susceptibility of cartilage matrix to hydrolytic enzymes has been demonstrated in a variety of studies. Thomas (1956) observed that when papain, a proteolytic enzyme, was injected intravenously into rabbits, their ears began to droop. The matrix of the rabbit ear cartilage was shown to be depleted, and chondroitin sulfate released from the papain-degraded matrix could be detected in the rabbits' blood and urine (Bryant *et al.*, 1958). Injection of testicular hyaluronidase into a rabbit knee joint followed by vigorous exercise resulted in degradation of the matrix of the articular cartilage (Barnett, 1956). Ziff and his associates (1960) showed that homogenates of rheumatoid synovial membrane decreased the viscosity of solutions of protein–polysaccharide extracted from bovine nasal cartilage. Normal synovial membrane had no such effect. Vitamin A fed to rabbits (Fell and Thomas, 1960) reproduced the cartilage matrix-depleting effect of papain. It was proposed by Fell and Dingle (1963) that vitamin A brought this about by causing an alternation of lysosomal membranes, resulting in release of acid hydrolases, particularly cathepsin, which degraded the protein–polysaccharide. Lysosomal enzymes extracted from polymorphonuclear leukocyte granules were capable of reducing the viscosity of solutions of cartilage protein–polysaccharides. Homogenates of rheumatoid synovial membrane contain larger amounts of cathepsin and acid phosphatase than do normal membranes (Luscombe, 1963).

In studies of the distribution of lysosomes in normal and rheumatoid synovial membranes, using cytochemical staining procedures, it was shown that in rheumatoid arthritis the lining cells contain a greatly increased number and size of lysosomes (Hamerman *et al.*, 1961). Electron-microscopic examination of the same tissue showed numerous residual bodies (Barland *et al.*, 1964). These large altered lysosomes were shown in synovial lining cells overlying eroded articular cartilage. They contained phagocytized material in various stages of digestion, accounting for their varied appearance. This led Barland and his coworkers to propose that following an inflammatory reaction (of unknown

cause) the rheumatoid synovial membrane proliferates and grows over the surface of articular cartilage. Within the proliferating lining cells are an increased number of large and altered lysosomes, with apparent increased lability. Enzymes released from these lysosomes gain access to the articular cartilage causing degradation of the protein–polysaccharide of the cartilage matrix. Possibly some of the matrix breakdown products are phagocytized by lining cells further altering their lysosomes.

Although the weight of evidence would appear to support the concept that cartilage degradation is a function of pannus formation, some new facts cast some doubt on this proposition. Conventionally, it is thought that pannus development occurs late in rheumatoid joint disease, but it is often apparent quite early (Kulka *et al.*, 1955; Gardner, 1965). Studies of articular cartilages from patients with rheumatoid arthritis undergoing arthrotomy have suggested that matrix depletion may develop in areas of cartilage free of any apparent pannus formation. Sometimes loss of matrix metachromasia can be seen before any pannus has been formed (Hamerman *et al.*, 1967). Electron-microscopic examination of pannus-free cartilage shows a process of alteration presumed to be the result of the cartilage being bathed by enzymes present in synovial fluid. Tateishi *et al.* (1971) saw large electron-dense deposits of an amorphous material replacing normal cartilage matrix in areas just below the surface. In several instances depletion of collagen fibers could be observed, whereas in others the fibers appeared to be separated by the matrix deposits. Based on these kinds of evidence, Hamerman and his colleagues (1969) have suggested that articular cartilage matrix depletion may be a very early phenomenon in rheumatoid arthritis. They speculated that "the advancing margin of the pannus is, in reality, the retreating remains of the cartilage in which chondrocytes, no longer trapped in their matrix lacunae and surrounded by residual collagen fibers, have come to resemble fibroblasts." Thus, in this iconoclastic view, pannus is the result, rather than the cause, of cartilage injury.

2. Electron Microscopy

The fine structure of rheumatoid synovium has been examined by a number of investigators (Barland *et al.*, 1964; Hirohata and Kobayashi, 1964; Norton and Ziff, 1966; Wyllie *et al.*, 1966; Ghadially and Roy, 1967; Marin *et al.*, 1969; Neumark and Farkas, 1970; Schumacher and Kitridou, 1972). Disagreement about what constitutes normal variation has produced some differences in interpretation. Also, as a rule, the findings in rheumatoid arthritis have been compared to normal synovium

or tissues from degenerative joint disease, rather than other chronic forms of synovitis. Deservedly, therefore, there is reluctance to accept alterations in rheumatoid synovial ultrastructure as specific, or of pathogenetic significance. Despite these limitations, and the usual problems of sampling errors inherent in electron microscopy, a picture has developed which reinforces the findings on light microscopy.

Deviations from the normal synovium have been identified in synovial lining cells, the intercellular matrix, vascular endothelium, and the subsynovial space.

a. Synoviocytes. There is a striking increase in the number of lining cells; most workers feel that the hyperplasia is predominantly in A cells, but Wyllie *et al.* (1966) and Ghadially and Roy (1967) ascribed much of the increase to B cells. Structural alterations have been described mainly in type A cells. Numerous ovoid, dense bodies are present in the perinuclear cytoplasm. These inclusions are bound by a single membrane and contain homogeneous dense material (lysosomes) or membranous structures (cytolysosomes) or phagocytized material such as "fibrinoid" (phagosomes). Barland and his associates (1964) were the first to identify these large accumulations as residual bodies or altered lysosomes and demonstrated acid phosphatase activity within them. Residual bodies have been a consistent finding in all studies of rheumatoid synovium, but similar lysosomal alterations are seen in traumatic arthritis (Roy and Ghadially, 1966) and osteoarthritis (Roy, 1967), although the degree of change is much less. Ferritin particles are present free in the cytoplasm of A cells and in membrane-bound vacuoles (Hirohata and Kobayashi, 1964; Muirden, 1966). Most workers feel that type B cells are unaltered in rheumatoid synovium. The cytoplasm of normal synovial cells contains a small number of aperiodic filamentous fibers measuring approximately 100 Å. in diameter (Barland *et al.*, 1962). In the rheumatoid synovium there is a consistent increase in the number of cells showing these filaments, which sometimes fill the cytoplasm and displace normal structures (Hirohata and Kobayashi, 1964; Norton and Ziff, 1966; Ghadially and Roy, 1967; Hamerman *et al.*, 1969). There is little information about these fibers and the significance of their presence in increased amounts in rheumatoid synovitis.

b. Intercellular Matrix. The joint cavity is directly connected with the subsynovial space. In rheumatoid synovitis, this intercellular matrix frequently contains amorphous granular deposits of a fine fibrillar material. A similar material is seen on the synovial surface and in synovial lining cells. There is a general feeling that this substance is fibrin or fibrinoid, but the inability to detect a characteristic periodicity has frustrated proper identification. Only Wyllie and co-workers (1966) are

willing to state unequivocally that the fibers in the intercellular space have the well-defined axial periodicity of collagen and fibrin. Norton and Ziff (1966) were impressed that there was less of this fibrinlike material in A cells than they would expect, considering the large amounts present.

c. Blood Vessels. There is major disagreement concerning the presence and extent of vascular abnormalities. Hirohata and Kobayashi (1964) described hyperplasia of the endothelium of small blood vessels as well as occlusions of the lumen, loosening of endothelial cell junctions and deposits of fibrillar material in the cytoplasm of endothelial cells. All subsequent workers concur in the observation of collections of fine filamentous fibers in endothelial cells. The gaps between endothelial cells were specifically looked for by Norton and Ziff (1966) and Branemark *et al.* (1969) but were not found. However, Schumacher and Kitridou (1972) identified them regularly in most cases of early inflammatory synovitis, regardless of its etiology.

d. Subsynovial Space. Electron-microscopic examination of the deeper subsynovial structures confirms the observations of light microscopy. No abnormalities have been noted in the blood vessels, collagen, or cellular elements that comprise the villi, but macrophages and fibroblasts appear to be increased in numbers, as compared to other connective tissue cells. The macrophages have an increase in lysosomal inclusions and overall have the same appearance as the A cells of the synovial lining (Wyllie *et al.*, 1966). Fibroblasts in the deeper areas contain fibrillar material, similar to that seen in the cytoplasm of lining cells.

Round cell accumulation is a consistent finding in the rheumatoid synovium. Similar appearing collections of mononuclear cells are a regular feature of delayed-type hypersensitivity, such as seen in tuberculin reactions. Indeed, in an earlier review of the immunological aspects of rheumatoid disease, it was concluded that "if an immune mechanism underlies the development of rheumatoid disease, it is a mechanism more akin to that responsible for the delayed, than for the immediate, type of hypersensitivity" (Glynn and Holborow, 1960). Additional support for this view came from observations of an increased frequency of a joint disease, similar to rheumatoid arthritis, in children with agammaglobulinemia (Janeway *et al.*, 1956; Good *et al.*, 1957), and the inability to transfer disease passively to normal subjects given serum containing high titers of rheumatoid factor (Harris and Vaughan, 1961).

Tissue injury in delayed hypersensitivity is presumed to occur through antigen stimulation of sensitized lymphocytes. These activated lymphocytes can be directly toxic to target cells, elaborate a variety of nonantibody mediators, which in themselves are cytotoxic, or call forth

macrophages that can produce tissue injury (Bloom, 1971). Ziff has questioned the role of delayed-type hypersensitivity in the pathogenesis of rheumatoid joint injury on the basis of two observations: (*1*) a hallmark of cellular immunity is the transformed lymphocyte, but electron-microscopic examination of the lymphocytes in the rheumatoid synovial membrane shows that less than 5% have undergone blastic transformation (Ziff, 1971); (*2*) mononuclear cells and macrophages are both conspicuous by their absence from lining cells, even in areas overlying dense mononuclear infiltrates. Moreover, on the few occasions where they have been seen in intimate contact with the lining cells, there is no evidence of an adverse effect on these surrounding cells, suggesting that the lining cells are not the target of immunological attack, despite the fact that they are the only cells of synovial tissue not present in other connective tissues (Norton and Ziff, 1966).

A disappointing feature of electron-microscopic observations of rheumatoid synovium has been the inability to identify a definite etiologic agent. Barland *et al.* (1964) suggested that the altered lysosomes in synovial lining cells were similar in appearance to cells infected with viruses. However, they demonstrated no material with the morphological characteristics of a viral agent. Recently, inclusions have been found in the cytoplasm of endothelial cells of small blood vessels in some patients with "collagen" diseases (Györkey *et al.*, 1968; Norton, 1969; Kawano *et al.*, 1969). These inclusions are tubular structures which have an electron-microscopic appearance similar to myxoviruses. Tubular structures have been found regularly in the renal lesions of systemic lupus erythematosus and have also been demonstrated in muscle biopsies from polymyositis (Chou, 1967) and skin biopsies in lupus erythematosus. Schumacher (1970) reported finding similar tubular aggregates lying adjacent to the endoplasmic reticulum in synovial vascular endothelial cells in tissues from a patient with systemic lupus erythematosus and a 13-year-old boy with an undiagnosed, transient, inflammatory synovitis. Similar inclusions were not detected in synovial specimens from 6 early cases of rheumatoid arthritis (Schumacher and Kitridou, 1972).

Rod-shaped bodies have been noted in the endothelial cells of small vessels in the synovium. Highton and his collaborators (1966) suggested that these structures were only found in rheumatoid arthritis and postulated that they represented a possible infectious agent of etiologic significance. Other workers identified these same rod-shaped bodies in normal human synovium, in osteoarthritic synovium, and in synovium from a variety of animals, and feel that they are analogous to the Weibel–Palade bodies (Weibel and Palade, 1964) which are normal

cytoplasmic components of arterial endothelium (Ghadially and Roy, 1967; Schumacher, 1969).

Neumark and Farkas (1970) described nuclear inclusions in synovial membranes from 22 rheumatoid patients. All of the rheumatoid specimens, but none of the controls, showed these characteristic nuclear bodies, measuring approximately 0.4 to 1.6 μ in diameter. The nuclear bodies were detected mainly in endothelial cells of small vessels, in perivascular connective tissue cells (pericytes) and macrophages; they were not seen in synoviocytes, lymphocytes, or plasma cells. The nuclear bodies consisted of groups of filamentous or tubulofilamentous structures. The appearance of the bodies varied, possibly depending upon the manner in which the filaments aggregated or the plane of sectioning, but all of the structural forms encountered closely resembled those seen in known viral infections. These interesting observations have not been confirmed to date.

3. *Immunofluorescence*

Soon after its development, the fluorescent antibody technique was applied to the study of rheumatic diseases (Vasquez and Dixon, 1957). Initial investigations were of fibrinoid, the constituent thought to be a hallmark of the collagen diseases. Immunofluorescent staining of fibrin was demonstrated throughout the synovium in a child with rheumatoid arthritis; of interest, the arthritis was associated with agammaglobulinemia (Gitlin *et al.*, 1957). Subsequent studies employing antisera to fibrinogen disclosed that this protein could be regularly demonstrated in synovial lining cells (Kaplan, 1963; Brandt *et al.*, 1968), in the subsynovium, and in, and around, the walls of the synovial blood vessels (Fish *et al.*, 1966; Bonomo *et al.*, 1968). Fibrin deposition, particularly on the surface of the synovium or in the intercellular spaces between synovial lining cells, has been seen in lesions from joint disease of less than 6 months duration. In these early rheumatoid lesions, fibrin was found consistently in combination with IgG, but IgM staining was sparse (Kaplan, 1963). Diffuse staining of isolated synovial lining cells was obtained with an antifibrinogen antiserum. Fibrinogen was associated with IgG, but the immunofluorescence was less intense than the IgG staining and was found in cells derived from both rheumatoid and nonrheumatoid synovial membranes (Kinsella *et al.*, 1970). It appears that whether one examines synovial membranes or synovial effusions that fibrin or fibrinogen–fibrin degradation products are detected in great abundance. Although the amounts of these proteins may be increased in rheumatoid arthritis, they are by no means specific for

TABLE I
IMMUNOFLUORESCENT STUDIES OF RHEUMATOID SYNOVIAL MEMBRANES

Substance	Lining cells[a]	Subsynovium[a]	Intercellular connective tissue[a]	Blood vessel walls[a]
IgG	+ (1,2,3,5,6,8)	+ (1,6)	+ (2,3,4,6,8)	+ (2,4,6,8)
IgM	+ (3,5[b],8)	+ (6,8)	+ (3[wk],6,8)	+ (4,8)
Anti-IgG	(5[b])		+ (3,4,6[b],7)	+ (6[b],7,8)
C3	+ (2,3,5)		+ (2,3,4,6)	+ (2,4,6)
C4	+ (2)		+ (2)	+ (2)
Fibrin(ogen)	+ (1,5,6)	+ (1,3,6)		+ (7)
Extranuclear nucleoprotein			+ (4)	+ (4)
Albumin	0 (3,5)	0 (3)	0 (3)	0 (3)

[a] Numbers in parentheses indicate the following references:
1. Kaplan (1963)
2. Rodman *et al.* (1967)
3. Fish *et al.* (1966)
4. Brandt *et al.* (1968)
5. Kinsella *et al.* (1969)
6. Munthe and Natvig (1970)
7. Bonomo *et al.* (1968)
8. Kaplan and Vaughan (1959)

[b] Only in rheumatoid factor-positive patients.

this entity, and correlate best with the presence of articular inflammation (Barnhart *et al.*, 1967a).

Immunoglobulins G and M are regularly found in rheumatoid synovium (see Table I). These immunoglobulins have been located singly, or in combination, in lining cells, in the subsynovium, in blood vessel walls, and in the interstitial connective tissue of the synovial membrane (Kaplan and Vaughan, 1959; Kaplan, 1963; Fish *et al.*, 1966; Rodman *et al.*, 1967; Brandt *et al.*, 1968; Kinsella *et al.*, 1969, 1970; Tursi *et al.*, 1970). Certain general characteristics of the synovial IgG deposits have been noted by the majority of authors: (*1*) IgG is present in abundance, whereas IgM is in lesser amounts, and occasionally absent; (*2*) IgG is frequently present in early rheumatoid lesions, in children with rheumatoid arthritis, and in adults with seronegative rheumatoid arthritis, when little or no IgM is detectable; (*3*) whenever complement components (C3 and C4) are identified they are in areas of IgG staining, but not correlated with IgM (complement staining accompanies IgG staining, even in tissue from patients with juvenile rheumatoid arthritis who have rheumatoid factor); (*4*) IgG and complement are often found in extracellular locations as large amorphous deposits, measuring as much as 50–100 μ in diameter; (*5*) IgG and complement are found as deposits in blood vessel walls or immediately about blood vessels.

The features of IgM staining in the synovial membrane can be summarized as follows: (*1*) IgM is associated almost exclusively with

adult, seropositive rheumatoid arthritis; (2) it is present in lesser amounts than IgG; (3) IgM staining is seldom coupled with staining for complement, and at least one author (Fish *et al.*, 1966) felt that, in general, there was an inverse relationship between the amount of IgM staining and the amount of C3 which could be identified.

In a very elegant study, Kinsella and his associates (1969, 1970) isolated synovial lining cells by tryptic digestion of biopsy specimens of synovial membranes. They felt they could easily distinguish the phagocytic type A cells from the type B cells. Immunofluorescent examination of these isolated cells revealed that only A-type cells contained immunoglobulins. Cells from both adult and juvenile rheumatoid arthritics showed a diffuse staining with antisera to IgG and C3. This was not seen in degenerative joint disease or other forms of inflammatory arthritis. Cells obtained from adults with seropositive rheumatoid arthritis showed another distinct pattern of staining. This consisted of large discrete inclusions in the cytoplasm of A-type cells. These inclusions contained mixtures of IgG and C3 or IgG and IgM—the latter usually having anti-γ-globulin activity.

Immunoglobulins are demonstrable in the cytoplasm of the mononuclear cells in the cellular infiltrates of the synovial villi. The majority of the plasma cells in a given section stain with antisera to either IgG or IgM. A minority of these cells also show anti-γ-globulin activity, as detected by reactions with aggregated human IgG or antigen–antibody complexes made with rabbit antibody (Kaplan and Vaughan, 1959; Mellors *et al.*, 1959, 1961). Rheumatoid factor containing plasma cells are present in varying numbers in the inflammatory exudate that forms in the subsynovium and in the cell clusters about small blood vessels. Lymphoid cells do not seem to contain rheumatoid factor. This intracellular localization of anti-γ-globulin has been confirmed in additional studies (McCormick, 1963; Bonomo *et al.*, 1968; Tursi *et al.*, 1970). Rheumatoid factors can be detected in various extracellular sites as amorphous deposits, usually in combination with IgG in the synovial connective tissue (Fish *et al.*, 1966; Brandt *et al.*, 1968; Bonomo *et al.*, 1968; Tursi *et al.*, 1970). Anti-γ-globulin activity localized in the wall of synovial blood vessels is usually associated with alterations of the vascular endothelium and perivascular infiltrations of mononuclear cells (Kaplan and Vaughan, 1959; Bonomo *et al.*, 1968); anti-γ-globulins deposits do not stain with antibodies to complement components.

Extracellular deposits of nucleoprotein have been identified in synovial membranes. Approximately one-half of the rheumatoid samples studied showed extranuclear deposits of nucleoprotein, usually in the same distribution as IgG and C3. These deposits were located in interstitial tissue, in the cytoplasm of synovial lining cells, and in blood vessel walls.

Deposits with a similar appearance and distribution were found in synovial membranes from two subjects with systemic lupus erythematosus but not in the four membranes from patients with Reiter's syndrome or degenerative joint disease (Brandt *et al.*, 1968).

4. Immunoglobulin Synthesis

It was inevitable that the finding of large numbers of mononuclear cells capable of producing immunoglobulins and rheumatoid factor would lead to studies designed to measure the immunoglobulin-synthesizing capacity of the rheumatoid synovial membrane. Rheumatoid synovial tissue was cultured *in vitro* with ^{14}C-labeled amino acids, and the newly synthesized immunoglobulins were isolated and quantified (Smiley *et al.*, 1968). By this technique, it was possible to show that the immunoglobulin-synthesizing capacity of the rheumatoid synovium was similar to that of normal human spleen and lymph node: IgG comprised 79% of the immunoglobulins produced, whereas the remaining 20% was equally divided between IgM and IgA. Less than 10% of the IgM synthesized had anti-γ-globulin activity.

In vivo synthesis of IgG by the rheumatoid synovium was measured by a radioisotope dilution technique (Sliwinski and Zvaifler, 1970; Zvaifler, 1971). Serial specific activities were determined on simultaneously obtained synovial fluid and plasma samples. The specific activity of IgG was identical in the plasma and synovial fluid in subjects with degenerative arthritis and Reiter's syndrome, indicating no production of IgG by their synovium. However, in 5 rheumatoid subjects the synovial fluid IgG-specific activity was consistently less than that of the companion plasma. It was calculated that from 12 to 26% of the IgG in the knee joints studied was produced locally. In these patients, the estimated daily IgG production in a single knee joint ranged from 5 to 95 mg. of IgG.

The ability of the rheumatoid synovium to synthesize specific antibodies has been inferred from the finding of antibodies in synovial fluid, which are not present in companion serum samples (see Section II,B) and the detection of antibody activity in acid eluates from synovial tissues (see below). But, except for anti-γ-globulin activity, there have been no direct measurements of antibody production by the rheumatoid synovium. A comparison of the secondary immune response to tetanus toxoid of peripheral blood lymphocytes and lymphocytes from the synovial membrane has been made in a group of patients undergoing joint surgery (Herman *et al.*, 1971). It could be shown that whereas the rheumatoid synovium synthesized significantly more IgG than did the blood lymphocytes, only negligible amounts of the synovial immunoglobulin were

directed against tetanus toxoid. In contrast, almost half the immunoglobulins of the blood lymphocytes were specifically antitoxoid antibody. The failure of the rheumatoid synovial membranes to produce antitetanus antibody was not due to the route of immunization, since 2 patients had the tetanus antigen injected into the joint 4 or 5 days prior to surgery. The authors concluded that the γ-globulins synthesized in the synovium must represent a prior commitment to some other antigen (Herman *et al.*, 1971). These findings are at variance with earlier studies from the same laboratory (Jasin and Ziff, 1969). In chronic experimental synovitis of rabbits, there is a rapid exchange between circulating blood lymphocytes and the lymphoid cells infiltrating the rabbit synovium. Specific antibody production by the synovium was demonstrated, regardless of whether the synovitis was produced by repeated intra-articular antigen administration, or when the antigen was administered systemically to rabbits with nonspecific synovitis.

5. *Elution Studies*

Complexes of antigen and antibody are dissociated by treatment with acid (pH 2.8–4.5) buffers or concentrated salt solutions. Carefully washed articular tissues, obtained at operation from normal or rheumatoid patients, have been treated in this fashion and antibodies and immunoglobulins eluted from them (Munthe and Natvig, 1970, 1971; Lindstrom, 1970). The major protein constituents of rheumatoid synovial eluates were IgG and albumin. The third component of complement (C3) is regularly found in eluates from both seropositive and seronegative patients. Other proteins which have been detected in trace amounts include C1q, transferrin, haptoglobin, α_1 antitrypsin, and fibrinogen. IgA + IgM were irregularly found and usually only in very minute amounts. When present, IgM was mainly from tissues of seropositive patients. The eluates from normal synovial membrane did not contain detectable IgA or IgM and only small amounts of IgG were present.

Complexes of IgG were regularly detected by precipitation with isolated rheumatoid factor or serum containing high titers of rheumatoid factor, or by reactions that were based on the inhibition of the interactions between rheumatoid factor and aggregated γ-globulin. IgG complexes were found in a greater percent and in larger quantities from seropositive patients, but almost two-thirds of the sero-negative patients showed some evidence for the presence of IgG. None could be demonstrated in eluates from normal synovial tissue (Munthe, 1970). In four of thirty-four eluates from rheumatoid synovium, there was a quantitative difference in the distribution of IgG subclasses when

compared to the corresponding serum sample. The subclasses were detected by a semiquantitative measure of the genetic markers (Gm) which are known to be limited to the various IgG subclasses. These findings suggested restricted types of IgG in some eluates (Munthe and Natvig, 1970, 1971).

The light-chain ratio in IgG eluted from rheumatoid synovial membranes has been studied by quantitative immunodiffusion (Lindstrom, 1970). Light chains of the IgG in the eluates have a different κ and λ composition from the IgG light chains of the peripheral blood. In eight of twenty eluates studied, λ-type chains predominate over κ, and an additional seven eluates had approximately equal amounts of the two classes of light chains. This is in striking contrast to serum, where κ light chains are present in a 2:1 ratio favoring κ. A similar predominance of λ-type light chains has been reported in synovial fluids from patients with rheumatoid arthritis (Epstein and Tan, 1966).

Restricted types of IgG and unusual light-chain distributions both favor the notion that the IgG eluted from the synovium is specific antibody. Discrete antibody activities have been demonstrated in some rheumatoid tissue eluates. Both IgG and IgM rheumatoid factors (anti-IgG's) and antibodies to the Fab_2 portion of IgG (pepsin agglutinators) are present, but only in low titers. Occasional eluates contained antinuclear antibodies, and even a greater number had nuclear antigens. These same eluates did not have demonstrable anti-A or anti-B isoantibodies or heterophile antibodies. An antibody to altered complement components (C3, C4)—immunoconglutinin—was detected in eluates from fifteen rheumatoid synovial tissues. Immunoconglutinin is of interest for two theoretical reasons: first, because it is an antibody to complement factors (C3 and C4) which are fixed to immune complexes, but does not react with free complement components (Lachman, 1967); second, the reaction of immunoconglutinin with complement bound to immune complexes increases complement fixation beyond that of the complex alone. Thus, the detection of immunoconglutinin serves as a marker for the presence of complement-fixing immune complexes, and immunoconglutinin may enhance the pathogenicity of such complexes. The immunoconglutinin activity could only be determined after pepsin digestion of the eluates, suggesting that it was tightly bound and the antibody was found to be of the IgG class (Mellbye and Munthe, 1971).

Currently, in the author's laboratory, eluates from rheumatoid synovial tissue are being examined for viral antigens and antibodies. To date, we have detected no antibodies to a large panel of viral antigens. Neither have these eluates been able to inhibit antibody reactions with this same panel of antigens. Obviously, such negative experiments do not disprove the presence of viral antigen in the rheumatoid synovium.

6. *Physiology*

Vascular abnormalities, a regular feature of chronic rheumatoid synovitis have been correlated with *in vivo* alteration in articular blood flow. A number of studies, measuring the removal of dye [phenolsulfonphthalein (P.S.P.)] or radioisotope washout (^{131}I-labeled protein or ^{24}Na) show increased circulation in rheumatoid joints as compared to controls (Jacox *et al.*, 1952; Ahlström *et al.*, 1956; Harris *et al.*, 1958; Nakamura *et al.*, 1967). It is difficult to quantify results obtained with these techniques, however, because of the recirculation and biological activity of these tracers. These obstacles can be avoided by use of the inert gas, xenon (^{133}Xe) (Dick *et al.*, 1970; Goetzl *et al.*, 1971)—the rate of disappearance of intra-articularly injected ^{133}Xe was found to be monoexponential, reproducible, and allows calculation of a biological half-life ($T_{½}$). Clearance of ^{133}Xe from 43 rheumatoid knee joints—mean $T_{½}$ of 61.2 minutes—was much faster than for 12 normal subjects—mean of 248.8 minutes, but did not differ significantly from other inflammatory joint disease; $T_{½}$ values correlated well with the clinical assessment of knee joint inflammation (Dick *et al.*, 1970). Falchuk, Goetzl, and their associates combined xenon clearances with direct *in vivo* measurements of partial pressure of oxygen (pO_2), carbon dioxide (pCO_2), pH, and lactate concentration of synovial fluids to document a circulatory-metabolic imbalance in the rheumatoid joints (Falchuk *et al.*, 1970; Goetzl *et al.*, 1971). Earlier *in vitro* studies showed markedly increased glucose metabolism, O_2 consumption, and acid production by rheumatoid synovial tissues as compared to normal synovia (Page-Thomas and Dingle, 1955; Dingle and Page-Thomas, 1956; Castor and Fries, 1961; Roberts *et al.*, 1967). Moderate decreases in synovial fluid pO_2 (53–27 mm. Hg) are associated with small falls in pH and minimal elevation of pCO_2 and lactate; greater decrements in pO_2 (27–9 mm. Hg) are accompanied by a profound reduction in pH (mean 7.18) and large increases in pCO_2 and lactate (Falchuk *et al.*, 1970). Similar depressions of synovial fluid pH have been measured in other investigation of arthritic joints (Cummins and Nordby, 1966). The metabolic alterations were not correlated with synovial fluid white blood cell counts but were directly proportional to the extent of synovial cell hyperplasia, leukocyte infiltration, and derangement of the microvasculature in biopsy specimens from the study subjects (Falchuk *et al.*, 1970; Goetzl *et al.*, 1971). The authors' interpreted their findings as follows: the rheumatoid joint has an increase in metabolism and blood flow to the joint, but the overall increase in blood flow is inadequate to meet the metabolic demands of the synovial tissues. They contend that the relative circulatory failure results from the vascular derangements observed histologically.

These physiological measurements may have a direct bearing on observations made by Bitensky and her associates (Chayen and Bitensky, 1971). They found that the permeability of lysosomal membranes is determined by the redox balance of the tissue. Lowering the pH shifts the redox balance toward a more reductive (hydrogen excess) state. Hydrogen excess is associated with an increased permeability of lysosomal membranes and, hence, a reduced latency of the hydrolytic enzymes (Chayen *et al.*, 1969; Poulter *et al.*, 1970).

On the basis of these observations, one could suggest a scheme that would integrate many of the immunopathological, ultrastructural, metabolic, and enzymatic findings in rheumatoid synovial tissues. Namely, compromise of the microcirculation by an immunological reaction, concomitant with an increase in the metabolic needs of synovial tissues, would produce tissue anoxia and acidosis. The resulting change in acid–base balance would shift the redox balance toward a more reductive state, causing the release of hydrolytic enzymes from lysosomes into the cytoplasm of synovial lining cells or into the surrounding microenvironment. The enzymes extruded from synovial cells closely applied to articular structures could produce irreparable tissue injury.

B. Synovial Fluid

1. Proteins

Normal synovial fluid has been characterized as a dialyzate of plasma to which hyaluronate protein has been added (Sandson and Hamerman, 1962). In the normal joint the amount and type of protein present in the synovial fluid is carefully regulated by the synovial membrane: hyaluronate is produced by synovial membrane lining cells, whereas proteins immunologically and electrophoretically identical with the plasma proteins are derived from plasma circulating in the capillaries in the synovial membrane. The way these proteins leave the synovial membrane and enter the joint space is not understood (Hamerman *et al.*, 1969). Normal synovial fluid differs from plasma in having a lower protein content, a higher percentage of albumin, and a smaller amount of α_2-globulin (Schmid and McNair, 1958). Plasma proteins of high molecular weight or asymmetrical shape (fibrinogen) are not detected in normal synovial fluid (Schur and Sandson, 1963). When the synovium becomes inflamed, the plasma proteins in the synovial fluid increase, and the quantity of larger molecular weight proteins is significantly greater than in normal fluid (Makiewicz and Fenrych, 1961; Schur and Sandson, 1963). Glycoproteins behave in a similar fashion (Sundblad *et al.*, 1961). The lipid content of normal human synovial fluid is very low ($\pm$0.2 mg./ml.). In rheumatoid joint disease the total lipid content approximates 40–60% of

the serum concentration, and with inflammation more lipoprotein from the vascular compartment enters the joint space (Bole, 1962; Chung *et al.*, 1962).

The exchange of proteins between plasma and synovial fluid has engaged the attention of investigators for several decades. In early studies, egg albumin was detected in the knee joints of rabbits within 5 minutes after intravenous injection, and Bauer *et al.* (1940) noted that larger molecules, such as horse serum albumin and the euglobulins, appeared somewhat slower. Thereafter, the protein concentration in the joints tended to increase over the next 24 hours (Bennett and Shaffer, 1939). More recently, isolated radiolabeled proteins and antibodies have been used to elucidate the synovial transport, local synthesis, and catabolism of joint fluid proteins. Radiolabeled albumin is detected in synovial fluid a few minutes after intravenous injection, and, by 1 hour, 3.1% of the plasma albumin is present in the knee joint. Complete equilibration occurs in 70 to 90 hours (Brown *et al.*, 1969; Sliwinski and Zvaifler, 1970). The equilibration of IgG is slightly slower than for albumin (Sliwinski and Zvaifler, 1970) and an isolated IgM cold agglutinin was even slower (70–125 hours) (Brown *et al.*, 1969). The rate of decline of radioactivity for each protein in synovial fluid is equal to the half-life of that protein in plasma and the individual proteins are independent of one another (Brown *et al.*, 1969; Sliwinski and Zvaifler, 1970).

A radiolabeled IgM rheumatoid factor was administered intravenously to 5 patients with rheumatoid arthritis (Bluestone *et al.*, 1970). The rheumatoid factor employed did not behave as a homogeneous protein. A part showed avid binding to IgG *in vitro*, and it was rapidly removed from the circulation; whereas, a less avid binding portion equilibrated freely between the joint and intravascular space and behaved like conventional IgM. The rheumatoid synovial membrane appeared to offer no barrier to antiglobulin transport, since both the more and the less avid rheumatoid factors were detected in the synovial fluid. However, the more avid portion was rapidly eliminated from both the joint and the circulation, and the bulk of the radioactivity was detected as free iodine [non-trichloroacetic acid (TCA) precipitable counts]. The less avid rheumatoid factor equilibrated freely between the plasma and synovial compartment and was catabolized in the joint in a manner parallel to IgM in plasma (Bluestone *et al.*, 1970). The authors' interpretation of their findings was that the avid binding portion of rheumatoid factor was complexing to IgG in the plasma and in the joint resulting in rapid clearance by the reticuloendothelial system, in a manner similar to the handling of other large immune complexes (Mannik and Arend, 1971).

The synovial membrane absorbs materials injected into the joint space. Solutions of dyes or pharmacologically active materials, such as

Mecholyl, are detected in the circulation seconds to a few moments after their introduction into the joint (Bauer *et al.*, 1940). Initially, it was thought that proteins were removed only by way of the lymphatics. Bauer *et al.* (1940) observed egg albumin and horse serum albumin in thoracic duct lymph 30 minutes after its intra-articular injection. They detected no protein (using specific antibody) in the blood when communications between lymphatics and the vascular system were obliterated. More recent investigations employing radiolabeled proteins do not entirely support this view. Rodnan and Maclachlan (1960) studied the clearance of radiolabeled albumin and IgG from rabbit and normal human joints. The percent of retained radioactivity was determined by external counting over the joint. Approximately 60% of the injected human serum albumin or IgG remained at the end of 24 hours and 40–46% was still present at 48 hours. Radiolabeled protein appeared in blood samples within 15 minutes after intra-articular injection. Proteins left the joint at an almost identical rate, independent of their molecular size. This finding has been confirmed in other studies. Brown and his colleagues (1969) injected trace-labeled albumin and IgM simultaneously into a human knee joint. Radioactivity was measured every 30 minutes in plasma samples. There was almost an identical egress of the two proteins. Sliwinski and Zvaifler (1969) studied the removal of aggregated and nonaggregated proteins from rheumatoid and osteoarthritic knee joints and found that IgG was removed at the same rate as albumin. Reduction of the IgG with 2-mercaptoethanol (2-ME) did not alter the rate. The rate of clearance of protein was variable from patient to patient, usually the greater the joint inflammation the faster the removal. The percent of radioactivity remaining in the joint at 24 and 48 hours was similar to that detected by Rodnan and Maclachlan (1960). Aggregation of either IgG or albumin resulted in accelerated removal. This could not be accounted for by local degradation of the protein with release of the radiolabel. Unaggregated protein was detected as intact labeled protein in the circulation. With minimal aggregation of IgG, there was a slight increase in the rate of removal, but the blood level of aggregated protein was significantly less than simultaneously administered nonaggregated IgG. Moderate aggregation resulted in an even faster removal from the knee joint, and plasma levels of radioactivity of the aggregated protein were barely measurable. The cumulative urinary excretion of radioactivity was less for the aggregated protein than for the nonaggregated protein. The findings were similar in both rheumatoid and osteoarthritis patients. Sequestration of the aggregated IgG in lymph nodes, liver, and spleen was demonstrated by increased radioactivity in these areas (Sliwinski and Zvaifler, 1969).

It is concluded from these studies that alteration of proteins in the synovial cavity results in their removal from the joint via the lymphatics, in contrast to normal proteins which egress by way of capillaries and small blood vessels. Similar observations were made by Bluestone *et al.* (1970) in their studies of the synovial transport of rheumatoid factor (see above). After intra-articular injection the radiolabel of the less avid binding rheumatoid factor quickly appeared in the plasma, but the more avid rheumatoid factor could not be detected. This was interpreted as sequestration of the more avid rheumatoid factor within the joint. However, if the protein were denatured during isolation, or if the rheumatoid factor were complexed to IgG, then the inability to detect the label of the more avid binding rheumatoid factor in the plasma might be explained by its removal via the lymphatics. An alternate explanation is that all proteins are removed by way of the lymphatics but any denatured material is sequestered in regional lymph nodes.

Although the majority of synovial fluid proteins are thought to be derived from plasma, the amount of IgG in some rheumatoid effusions is greater than would be predicted from the ratio of synovial fluid IgG to plasma IgG as compared to the ratio of albumin in synovial fluid and in plasma (Wilkinson and Jones, 1962). This led to the suggestion of local synthesis of immunoglobulins in the rheumatoid synovium. Ample confirmation has come from both *in vitro* (Smiley *et al.*, 1968) and *in vivo* studies. One *in vivo* method is based on the observation that an approximate straight-line plot can be obtained when the log of the ratio of a protein's concentration in synovial fluid divided by its serum concentration is plotted against the log of the molecular weight of the protein (Kushner and Somerville, 1971). This relationship was true for a number of proteins over a large range of molecular weights: orosomucoid, transferrin, ceruloplasmin, and α_2-macroglobulin as well as for IgG and IgM in osteoarthritic fluids. The values obtained for IgG and IgM in seropositive rheumatoid fluids were significantly greater than predicted; that is, there was more of each of the immunoglobulins in the joint than other plasma proteins, implying local production.

Synthesis of IgG by the rheumatoid synovial membrane was also demonstrated by a radioisotope dilution method employing serial measurements of specific activity from simultaneously obtained synovial fluid and plasma samples (Sliwinski and Zvaifler, 1970). After equilibration of intravenously administered albumin the specific activity was equal in the synovial fluid and plasma in 5 subjects with rheumatoid arthritis, 2 with degenerative arthritis, and 2 with Reiter's syndrome. The specific activity of synovial fluid IgG was identical to plasma IgG in the patients with degenerative arthritis and Reiter's syndrome, indicating no pro-

duction of IgG by their synovium. In the 5 rheumatoid subjects, the synovial fluid IgG-specific activity was consistently less (12–26%) than that of plasma. This difference was felt to represent dilution of the joint fluid IgG by locally produced IgG. Employing calculations based on the concentration of IgG in a synovial fluid of known volume, it was calculated that as much as 95 mg. of IgG could be produced daily by the synovium of a single rheumatoid knee joint.

In the 6 rheumatoid subjects studied by Brown *et al.* (1969), the ratios of the radioactivity of an isolated IgM cold agglutinin in plasma and synovial fluid after equilibration were equal, suggesting, in this instance, that there was no detectable local production of IgM.

2. *Clotting Factors*

Normal synovial fluid does not clot. This has been attributed to the absence of fibrinogen, since this protein is found neither in normal human (Ropes and Bauer, 1953) nor in bovine synovial fluid (Cho and Neuhaus, 1960). When the synovium becomes inflamed, as in rheumatoid arthritis, then fibrinogen passes from the plasma into the joint. There it can be identified as fibrin or fibrinogen–fibrin complexes on the surface of the synovium, in the intercellular matrix of the synovial membrane, as fibrin masses within the cytoplasm of polymorphonuclear leukocytes, or as rice bodies in the synovial fluid (Schur and Sandson, 1963; Caughey and Highton, 1967; Barnhart *et al.*, 1967b; Gormsen *et al.*, 1971).

Plasmin, the enzyme normally involved in fibrin removal, is formed from plasminogen, a β-globulin, which is activated by circulatory and tissue activators. The normal joint contains all components of the fibrinolytic system necessary to produce free plasmin. Plasminogen, has been detected in both normal synovial fluid and synovial membrane (Caughey and Highton, 1967; Barnhart *et al.*, 1967a). Plasminogen is converted to plasmin by either a tissue activator or a proactivator present in plasma. The tissue activator is present in the synovial membrane and the proactivator in normal synovial fluid. Plasminogen activator activity is found localized around blood vessels, in the connective tissue, and at the surface of the synovial membrane (Bach-Andersen and Gormsen, 1970). In addition, plasma thromboplastin antecedent factor (Factor XI) and Hageman factor (Factor XII) are present in normal synovial fluid in concentrations comparable to those found in plasma (Kellermeyer and Breckenridge, 1966). It is known that Hageman factor can activate plasminogen to plasmin as well as initiating the clotting mechanism, activating kallikrein and starting the complement sequence (Ratnoff, 1969). Even free plasmin has been identified in the inflammatory effusions of a few patients with gouty arthritis (Caughey and Highton, 1967).

There is a great deal of interest in the relationship of the fibrinolytic system to chronic articular inflammation. This interest has been provoked by observations that fibrinogen or fibrin breakdown products are present in inflamed synovial fluid in amounts exceeding those present in plasma (Schur and Sandson, 1963; Barnhart *et al.*, 1967a; Gormsen *et al.*, 1971), by the suggestion that fibrin or its degradation products are chemotactic for granulocytes (Riddle *et al.*, 1965), and because fibrin could be altered through *in vivo* proteolysis so as to make it an "autoantigen" which could perpetuate joint inflammation, as has been shown in experimental animal models (Dumonde and Glynn, 1962).

A number of theories have been suggested to explain the large amounts of fibrin and fibrinogen complexes found in inflammatory foci. In general, they postulate an impairment of fibrinolysis brought about by (*1*) an absence of one of the critical components of the fibrinolytic system, (*2*) the existence of an inhibitor of plasmin—antienzymes or antiproteases, or (*3*) the production of degradation products of fibrin or fibrinogen which are unusually resistant to fibrinolysis. As noted above, the first explanation is untenable since the normal joint has all components of the fibrinolytic system in sufficient quantities to produce free plasmin, and in inflamed joints, there are more, rather than less, of these components. Protease inhibitors have been identified in inflammatory synovial effusions–α_1-antitrypsin (Holmes *et al.*, 1935) and α_2-macroglobulin (Albrechtsen *et al.*, 1958); either could impair the digestion of fibrin. Along the same lines, Lack (1959) found plasmin inhibitors in normal synovial fluid, and he states that the increased content of plasmin inhibitor in the synovial tissues in tuberculous joint disease explains the persistence of fibrin in that situation.

Normally, when fibrinogen or fibrin is digested by plasmin there is the generation of a series of well-defined degradation products, differing in molecular weight and electrophoretic mobility from the parent molecule. The fragments formed first are called X and Y and have molecular weights of 240,000 and 155,000, respectively. Further digestion results in two other well-defined fractions called D and E with molecular weights of 83,000 and 50,000. These early (X and Y) and late (D and E) fibrin degradation products differ in their ability to be clotted by thrombin and have distinct anticoagulant properties. The early fibrin degradation products tend to form complexes with soluble fibrin monomers and can be identified by the ease with which they precipitate in solutions of low ionic strength, when exposed to the cold, or when treated with protamine sulfate (Lipinski and Woronski, 1968; Marder *et al.*, 1969; Wegrzynowicz *et al.*, 1971). Schur and Sandson (1963) were the first to note that fibrinogen in some rheumatoid effusions had an unusual

electrophoretic mobility. This observation was confirmed and extended by Barnhart *et al.* (1967a). From immunoelectrophoretic analysis these latter workers suggested that the major fibrinolytic product was the D fragment. Moreover, they showed that there was disparity between the amount of fibrinogen detected in rheumatoid joint fluids by heat coagulation and that which was clottable with thrombin, indicating a change in the molecular architecture of the fibrinogen. Similar electrophoretic abnormalities and impaired responses to thrombin were not found in companion plasma samples.

Gormsen and his collaborators (1971), employing hemagglutination inhibition, immunoassay, radial immunodiffusion, and crossed immunoelectrophoresis, analyzed the synovial fluids from a variety of inflammatory and noninflammatory joint diseases. Inflammatory fluids contained large amounts of breakdown products of fibrinogen or fibrin, mostly in complexes which were not clottable by thrombin, but were precipitated by protamine sulfate. They identified fragments D and E in these complexes. The complexes were resistant to plasmin digestion, even with concentrations of plasmin which in isolated systems could easily digest stabilized fibrin into the final D and E fragments. Noninflammatory synovial fluids showed small amounts of fibrinogen, the majority of which was clottable by thrombin. Distinct D and E fractions were easily obtained by plasmin digestion of the noninflammatory fluids. When considering the resistance of the fibrinogen fragments in inflammatory effusions to plasmin digestion, these authors felt they were observing the effects of large amounts of an antiplasmin, or the formation of complexes with other proteins, possibly by-products of the inflammatory process.

Some investigators have championed the idea that fibrin or fibrinogen degradation products are directly responsible for articular damage (Barnhart *et al.*, 1967a) or serve as a mechanism for the perpetuation of inflammatory joint disease (Glynn, 1968). It is important to point out, however, that none of the reactions relating to fibrin formation or dissolution is unique to the rheumatoid joint. They are a regular finding in all inflammatory effusions, whether self-limited, as in gouty arthritis, or long-standing, as in tuberculous and rheumatoid arthritis.

3. *Kinins*

Increasing attention has been paid to the interrelationships between blood clotting, activation of the complement cascade, and the generation of vasoactive polypeptides, such as kinins. Ratnoff (1969) has reviewed the evidence that Hageman factor occupies the pivotal position linking hemostatic, fibrinolytic, immunological, and inflammatory responses. The

interaction of Hageman factor (Factor XII) with plasma thromboplastin antecedent (PTA; Factor XI) initiates the clotting sequence, resulting in the eventual conversion of fibrinogen to fibrin. Simultaneously, Hageman factor can set into motion the fibrinolytic system, because when activated and combined with a cofactor, it converts plasminogen to plasmin. Another role of Hageman factor is the ability to change prekallikrein to kallikrein by the activation of prekallikrein activator (PKA). Kallikrein, in turn, generates kinin from the plasma α_2-globulin, kininogen. Whether this reaction is accomplished directly by activated Hageman factor or via plasmin is not entirely clear at this time. In like manner Hageman factor is also involved with complement since partially purified kallikrein can markedly enhance the effectiveness of C1 as an activator of the complement sequence (Kaplan *et al.*, 1971). Also, plasmin triggers the elaboration of C1 esterase and the subsequent reaction with the C1 esterase substrates, C4 and C2. Thus, plasmin can initiate the complement sequence in a manner parallel to, but independent from, antigen–antibody reactions.

Because minute amounts of kinins produce vasodilitation, capillary permeability, and pain, these vasoactive polypeptides have been implicated as mediators of inflammatory reactions. It is not surprising, therefore, that kinins were looked for in inflammatory synovial effusions. Melmon *et al.* (1967) demonstrated kinins (polypeptides with oxytocic activity for rat uterine muscle) in synovial fluids of patients with various types of joint disease and from dogs with crystal-induced arthritis. Detectable concentrations of kinin were found in synovial fluid from the knee joints of all patients studied, regardless of the etiology of their arthritis. There was little overall correlation between the amount of kinin and the clinical assessment of the degree of inflammation. Furthermore, they (Melmon *et al.*, 1967) were able to show that the injection of microcrystalline sodium urate into the knee joint of a volunteer was followed by the simultaneous development of acute inflammation and an increase in kinin levels in the joint fluid. The kinin level fell following the institution of colchicine therapy.

A mechanism by which kinins might be implicated in gouty arthritis has been developed from the interesting studies of Kellermeyer and Breckenridge (1965, 1966; Kellermeyer, 1967). Hageman factor, which exists in plasma in an inactive form, is activated by contact with glass and many other negatively charged inert substances. Kellermeyer and Breckenridge (1965) showed that plasma Hageman factor could be activated by the negatively charged crystals of sodium monourate or calcium pyrophosphate but not by amorphous urates. They proposed that the initiation of the inflammatory process in acute gouty arthritis

was the result of activation of Hageman factor by monosodium urate crystals in synovial fluid. This thesis was supported by the subsequent finding that both Hageman factor and PTA (Factor XI) are present in an inactive form in normal human and canine synovial fluid, and they could be readily activated by substances known to activate Hageman factor such as glass, kaolin, and ellagic acid (Kellermeyer and Breckenridge, 1966; Eisen, 1966). Furthermore, normal synovial fluid acquired the capacity to increase vascular permeability when exposed to monosodium urate crystals. The development of this permeability factor could be prevented by preincubating the synovial fluid with an antiserum to Hageman factor. Partial characterization of the permeability increasing factor revealed properties comparable to kinin or kallikrein; permeability factors known to be induced in plasma by activated Hageman factor (Kellermeyer, 1967).

Kinins (or kininlike materials) have been found in rheumatoid synovial fluids, also (Armstrong *et al.*, 1957; Melchiorri, 1963; Melmon *et al.*, 1967). There is no clear mechanism to explain kallikrein activation in rheumatoid synovial effusions. Several recent observations suggest that there may be an analogy between crystal-induced activation of Hageman factor and the ability of certain antigen–antibody complexes to activate Hageman factor. Movat (1967) reported that antigen–antibody complexes accelerated clotting through the involvement of Hageman factor and that kinins were liberated in the process. These experiments are difficult to interpret, however, because there are great technical problems in experiments which use whole plasma or serum. Many simple manipulations, such as contact with glass, pH alterations, or change in temperature will initiate the enzymatic steps leading to kinin production. Therefore, when Epstein and his co-workers (1969) wanted to study a similar problem, they measured the less sensitive depletion of plasmin kininogen. In this system, the addition of a complex of IgM rheumatoid factor and aggregated IgG globulin resulted in approximately a 50% reduction in plasma kininogen, whereas normal IgM, aggregated γ-globulin alone, or a mixture of IgM rheumatoid factor and undenatured IgG did not deplete kininogen. Unfortunately, they have not been able to duplicate these results, measuring kinin formation directly by a sensitive radioimmunoassay procedure (Epstein, 1971). Recently, Kaplan and his associates (1971) were able to show activation of partially purified, unactivated Hageman factor by an immune complex consisting of IgG and a monoclonal, human, IgM anti-IgG. It is noteworthy that these authors were unable to achieve similar results with any other IgM antiglobulins. Moreover, Cochrane *et al.* (1972) have been unsuccessful in their attempts to activate Hageman factor with a variety of antigen–

antibody complexes, aggregates of isolated immunoglobulins of all classes, or a number of complexes containing human IgM anti-γ-globulins. Considering the discrepancy in the results from various laboratories, it seems prudent to withold judgment about the way kinins might be generated in inflammation induced by immune complexes.

4. Complement

In general, total hemolytic complement activity in the serum of patients with rheumatoid arthritis is normal or slightly elevated (Vaughan *et al.*, 1951; Laurell and Grubb, 1958; Ellis and Felix-Davies, 1959). Since the complement system is composed of a group of proteins, nine in number, which are serum globulins, one would expect them to be present in synovial fluid in concentrations similar to other proteins of a similar size. It was significant, therefore, when it was reported, almost simultaneously and quite independently, from two laboratories, that the hemolytic complement activity of joint fluids from patients with rheumatoid arthritis was significantly depressed as compared to the serum complement (Hedberg, 1963, 1964; Pekin and Zvaifler, 1964). In joint diseases other than rheumatoid arthritis the complement activity was not depressed and was proportional to either the leukocyte count, the total protein, or the γ-globulin concentration. This was true regardless of whether the data were analyzed as the ratio of synovial fluid complement (SFC) to any of the parameters studied or as a ratio of hemolytic activity in synovial fluid (SF) and serum compared to any other protein in synovial fluid and serum (SFC/serum C:SF protein/serum protein). Complement levels were most depressed in effusions from rheumatoid subjects with nodules and positive tests for rheumatoid factor and in examples of classic rheumatoid arthritis. The levels were somewhat higher in rheumatoid patients with negative rheumatoid factor tests and highest in nonrheumatoid inflammatory joint diseases such as gouty arthritis, ankylosing spondylitis, ulcerative colitis, or Reiter's syndrome. Repeated observations of the same joint showed a remarkable stability of the level of hemolytic complement activity over months and even years. These initial observations were extended by the original investigators (Hedberg, 1967; Zvaifler, 1968) and have been confirmed in a large number of subsequent reports (Fostiropoulos *et al.*, 1965; Barnett *et al.*, 1966; Peltier *et al.*, 1966; Vaughan *et al.*, 1968a; Ruddy and Austen, 1970; Gligore *et al.*, 1971).

Measurements of individual complement components, performed by the semiquantitative methods available at the time, revealed reductions of the intra-articular concentrations of C1 and C4 (Zvaifler and Pekin, 1963) and C2 (Fostiropoulos *et al.*, 1965). The reductions were pro-

portional to the decrease in total hemolytic complement. These findings, which were consistent with immune activation of the complement sequence, have been confirmed by more quantitative measurements of individual complement components (radial immunodiffusion) and by stoichiometric hemolytic titrations (Ruddy and Austen, 1970; Ruddy *et al.*, 1971; Britton and Schur, 1971). The C1 concentration in the majority of rheumatoid joint fluids is not significantly reduced, although occasional synovial fluids in seropositive patients are very low. One possible explanation for this finding may be the dissociation of C1 from the activating immunoglobulin at physiological ionic strength. Inferential evidence for activation of C1 comes from the observation of a parallel reduction in the intra-articular activity of its two natural substrates, C4 and C2 (Ruddy and Austen, 1970). When expressed as hemolytic activity per gram of synovial fluid protein, Ruddy and Austen found that the mean C4 hemolytic activity of seropositive rheumatoid patients was 700 ± 200 units as compared to a value of 4300 ± 500 units for fluid from seronegative patients and 7800 ± 1100 units in fluids from patients with degenerative arthritis. Synovial fluid C2 activity was similar to C4 in the three groups of patients, although the amount of reduction was not as striking as for C4.

The C3 levels in synovial fluid are decreased proportional to total hemolytic complement when studied by radial immunodiffusion (Zvaifler, 1969a), crossed immunoelectrophoresis (Hedberg *et al.*, 1970), or hemolytic titration (Ruddy and Austen, 1970).

Ruddy *et al.* (1971) found that the serum level of C9 was increased to almost twice normal in patients with both seropositive and seronegative rheumatoid arthritis; however, the synovial fluid C9 levels of seronegative patients were significantly higher than those of the seropositive group. This was interpreted as additional evidence for intra-articular activation and completion of the complement sequence in seropositive rheumatoid arthritis.

The sequential action of $\overline{C1}$ on C4 and C2 generates a new enzymatic activity, C3 convertase (Müller-Eberhard *et al.*, 1967). Activation of C3 by the C3 convertase is responsible for remarkable augmentation of the efficiency of the hemolytic system and, in addition, the generation of several biologically active factors which enhance phagocytosis, have anaphylatoxic and chemotactic activities, and are responsible for the immune adherence phenomenon. These by-products of the conversion of C3 can be detected by immunoelectrophoretic analysis as proteins that share antigenic determinants with C3 but have faster electrophoretic mobilities. Breakdown products of C3 have been detected in synovial fluids from patients with a variety of joint diseases, but a significant

correlation exists between the demonstration of fast-migrating breakdown product of C3 and the diagnosis of rheumatoid arthritis, particularly seropositive rheumatoid arthritis (Zvaifler, 1969a; Hedberg *et al.*, 1970). Similar breakdown products were not found in the serum or plasma of the same patients.

Since activation of the complement sequence is known to result in the production of factors chemotactic for leukocytes, rheumatoid synovial fluids were examined for the presence of chemotactic activity. Approximately two-thirds of rheumatoid synovial fluids (38/54) contained chemotactic activity for rabbit granulocytes. This activity was in large part related to the fifth (C5) and the sixth (C6) components of human complement (Ward and Zvaifler, 1971). Analysis of the effusions by a combination of physical-chemical and immunological techniques demonstrated two discrete mediators of chemotaxis. All but one of the rheumatoid fluids showed a high molecular weight chemotactic factor—the trimolecular complex of $\overline{C567}$. Two-thirds of the fluids also showed a light weight factor, C5a, identified as a cleavage product of C5. In addition, when purified human C5 was added to rheumatoid synovial fluids, approximately one-half were able to generate a chemotactic factor identical to C5a. The enzyme capable of generating C5 leukotactic activity has a molecular weight of approximately 65,000. The addition of various inhibitors to a mixture of isolated human C5 and rheumatoid synovial fluid containing the C5-cleaving enzyme showed a reduction of chemotactic activity of 86% with ϵ-aminocaproic acid (EACA) and 60% inhibition with soybean trypsin inhibitor. Anti-C5 inhibited 72% of the newly formed chemotactic activity, whereas, anti-C3 was without effect (Ward and Zvaifler, 1971). A similar, if not identical, C5-cleaving enzyme was obtained from a large granule fraction of human leukocytes. Chemotactic activity was elaborated when this material was incubated with purified human C5; it had inhibition profiles similar to the enzyme in synovial fluid.

Recently, a new mode of complement activation has been recognized. Immune precipitates of guinea pig γ_1-antibodies consume C3–C9 and yield biologically active by-products without affecting the levels of C1, C2, and C4 (Sandberg *et al.*, 1970). These observations are similar to earlier reports of the preferential inactivation of late-acting complement components by cell wall preparations of yeast (Pillemer *et al.*, 1954) and of gram-negative bacteria (Gewurz *et al.*, 1968). It was postulated that aggregates of certain immunoglobulins and naturally occurring plant or bacterial polysaccharides activate a serum enzyme, distinct from the previously recognized complement components, which can cleave C3. The constituents of this reaction have been described in detail by Götze

and Müller-Eberhard (1971). There is a protein, called C3 proactivator (C3PA), present in human serum which can be activated by treatment with naturally occurring plant or bacterial polysaccharides (i.e., inulin, zymosan, endotoxin) or with aggregates of certain classes of immunoglobulins. The activation of C3PA results in the fragmentation of the C3PA molecule into at least two pieces. One of these, possessing the electrophoretic mobility of a γ-globulin, represents the C3 activator (C3A) which resembles C3 convertase in its enzymatic action on C3. Because this mechanism activates the complement sequence at a later step, it has been called "the C3 activator system" or the "bypass mechanism."

Synovial fluids have been assayed for C3PA, for the presence of C3A, and for their capacity to support conversion (activation) of C3PA to C3A upon incubation with inulin. The average concentration of C3PA in the rheumatoid arthritis group was 79 μg./ml. (51–110 μg./ml.), in the fluids from the joints of patients with infectious arthritis it was 209 μg./ml. (169–242 μg./ml.), and in nonrheumatoid inflammatory joint disease, it was 201 μg./ml. (105–282 μg./ml.). The C3PA concentration correlated well with the average total hemolytic complement level in the synovial fluids of the three groups. The C3 activator was found in 2 of 7 rheumatoid effusions and 3 of the 5 joint fluids from infectious arthritis. It was not present in any of the 8 fluids from other forms of inflammatory, nonrheumatoid arthritis. Addition of inulin to rheumatoid fluids caused no conversion of C3PA, supporting the idea that the "C3 activator system" had been utilized and consumed; it appeared to be intact in the other joint diseases studied (Götze *et al.*, 1972).

The findings of complement depletion in rheumatoid synovial fluids and the pattern of complement component utilization implies intra-articular activation of the complement system by immune complexes or their equivalents. The site and manner of this activation is discussed in more detail in other sections of this review but can be briefly summarized as follows. Complement components can be detected by immunofluorescence in the synovial membrane and in synovial lining cells, usually in combination with IgG. Complement and rheumatoid factor are usually not located together. The leukocytes in synovial fluid have cytoplasmic inclusions containing immunoglobulins and complement components. An inverse correlation exists between the complement level of the fluid and the presence of immunoglobulin inclusion in white blood cells (Vaughan *et al.*, 1968b; Peltier *et al.*, 1967; Britton and Schur, 1971). Noteworthy, however, is that C1q, C4, and C3 are frequently detected in phagosomes in leukocytes from effusions in joint diseases other than rheumatoid arthritis (Vaughan *et al.*, 1968a; Britton and

Schur, 1971). High molecular weight γ-globulin complexes can be precipitated from rheumatoid synovial fluids and the quantity of these IgG complexes is related to the extent of complement depletion in the joint fluid (Hannestad, 1967; Winchester *et al.*, 1970). Cryoprecipitable complexes containing immunoglobulins, deoxyribonucleic acid (DNA), antinuclear factors, and anti-immunoglobulin activity have been detected in a large percentage of fluids from seropositive rheumatoid patients (Marcus and Townes, 1971a; Cracchiolo *et al.*, 1971; Zvaifler, 1973). These cryoprecipitable complexes fix complement (Marcus and Townes, 1971b), and their anticomplementariness is roughly proportional to the depression of hemolytic complement of the fluid from which they are derived (Zvaifler, 1973). Thus, there is evidence for complement consumption in at least three sites in the articular space: the synovium, the synovial fluid leukocytes, and the joint fluid.

Is rheumatoid factor responsible for depletion of synovial fluid complement? The evidence is conflicting. Rheumatoid factor titers, whether serum or synovial fluid, show an overall correlation with the degree of depression of hemolytic activity in the synovial fluid (Hedberg, 1970). However, the isolated anti-γ-globulin complexes from such fluids show *in vitro* complement fixation (Winchester *et al.*, 1970). Furthermore, when normal polymorphonuclear cells are incubated with rheumatoid factor-positive synovial fluids, they develop inclusions that stain for IgG, IgM, and C3, suggesting that complexes of these immunoglobulins and complement exist preformed in such fluids (Hurd *et al.*, 1970). On the other hand, immunofluorescent studies of synovial membrane and granulocytes from rheumatoid joints show that, although both anti-γ-globulins (rheumatoid factors) and complement component are found complexed to IgG, the finding of one usually precludes the other (see Section II, B, 3).

In vitro studies of the complement-fixing ability of isolated IgM rheumatoid factor or serum containing anti-γ-globulins are equally confusing. Early reports suggested that rheumatoid factor could inhibit the fixation of complement to IgG on sheep red cells (Heimer *et al.*, 1962, 1963; Zvaifler and Bloch, 1962), latex particles (Bernhard *et al.*, 1961), or on mitochondria (Davis and Bollet, 1964). Direct testing of the influence of rheumatoid factor on IgG complement fixation reactions awaited the demonstration by Wiedermann *et al.* (1963) that when 7 S IgG antibody or aggregated γ-globulin was reduced with 2-ME, it lost the ability to fix complement but retained the capacity to react with rheumatoid factor. In this manner the anticomplementary nature of the IgG (antigen) was controlled, allowing measurement of complement fixation by the rheumatoid (anti-IgG). Preformed complexes of rheumatoid factor and

reduced, aggregated γ-globulin fix human complement at 37°C. but not at 4°C. Interestingly, preformed complexes did not fix guinea pig complement at either temperature (Zvaifler and Schur, 1968). This requirement for human complement and a 37°C. reaction temperature is consistent with other studies of human 19 S antibodies (Leon, 1957; Ostgard and Orjasaeter, 1962; Cunniff and Stollar, 1968). Early complement components were depleted, suggesting activation through the conventional pathway. The reduction of C2 and C3 was roughly proportional to the decrease in total hemolytic activity (Zvaifler and Schur, 1968).

Incubation of aggregated, reduced γ-globulin with fresh normal serum or serum from seronegative rheumatoid patients results in little or no complement fixation; however, aggregated, reduced γ-globulin fixes complement in seropositive serum. The fixation is proportional to the rheumatoid factor titer of the serum. The actual quantity of complement fixed, however, is only one-half to one-sixth that fixed when nonreduced aggregated γ-globulin is added to fresh normal human serum (Zvaifler, 1969a).

Tesar and Schmid (1970) have shown that the binding of rheumatoid factor to soluble antigen–antibody complexes results in additional complement fixation by the aggregate. Complexes of bovine serum albumin (BSA) and 2-ME-treated anti-BSA antibody fixed little complement at 37°C., but complement fixation was observed following the addition of IgM rheumatoid factor. When an IgG antiglobulin, which had been reduced with 2-ME, was substituted for the IgM rheumatoid factor, there was no fixation. Furthermore, IgM rheumatoid factor lost its ability to fix complement when incubated with the 2-ME treated complexes in the cold. This finding, as noted before, is consistent with the observation that human IgM antibodies dissociate from C1 at this temperature (Leon, 1957). Finally, the effect of rheumatoid factor was inhibited by the addition of an antibody to human IgM which had been treated so as to destroy its own complement-fixing ability. The best overall explanation of these data is that rheumatoid factor blocks the complement-fixing site on IgG by steric hindrance, presumably because the determinant for rheumatoid factor and the site for complement fixation are located close to one another in the Fc portion of the IgG. Therefore, the complement fixation noted is probably due to complement attachment to sites on the IgM rheumatoid factor molecule.

Complement fixation by rheumatoid factor and nonreduced γ-globulins has also been examined. The interaction of IgG anti-herpes-simplex antibody with the herpes virion results in the formation of a virus–antibody complex which retains its infectivity (Notkins, 1971). Such complexes have been recovered from the blood of chronically infected animals.

Neutralization of the infectious virion–antibody complex occurs upon addition of the first three complement components but not with IgM rheumatoid factor. However, if subneutralizing concentrations of complement components are employed, then the addition of rheumatoid factor results in neutralization. It is the author's feeling that neutralization is the result of both anti-immunoglobulins and complement covering the surface of the virion (Notkins, 1971).

The amount of complement fixed by the addition of grossly aggregated (heated) IgG to fresh normal serum or fresh serum containing rheumatoid factor was compared to the amount fixed in the same sera by minimally aggregated γ-globulin. When aggregation of IgG was minimal, sera with high titers of rheumatoid factor fixed more complement than did normal serum. By contrast, half as much complement was fixed in the rheumatoid serum as in the fresh normal serum by the heat-aggregated γ-globulin (Zvaifler, 1969b). In other words, serum containing rheumatoid factor fixed more complement when IgG was minimally aggregated, and less than expected with grossly aggregated IgG. This is in keeping with experiments that show that the addition of purified IgM rheumatoid factor to high concentrations of IgG antibody (hemolysin) bound to red blood cells reduces the amount of complement fixation compared to that observed with the bound IgG alone. Conversely, the addition of a similar amount of IgM rheumatoid factor to low concentrations of the same IgG antibody produced enhancement of complement fixation (Schmid *et al.*, 1970).

This paradoxical response of rheumatoid factor, namely, enhancement of complement fixation at lower concentrations of IgG and inhibition at higher concentrations could have important biological consequences in an extravascular space, such as the articular cavity. In reactions with markedly aggregated γ-globulin, IgM rheumatoid factors may form large complexes of limited toxicity which are removed via the lymphatic system; less aggregated complexes combining with rheumatoid factor may fix complement and stay in the joint to be detected as inclusions in synovial fluid leukocytes. It remains to be seen, however, whether the persistent and remarkable depression of hemolytic complement activity, which is so characteristic of the chronic rheumatoid inflammatory state, can be entirely explained by this mechanism.

5. *Immune Complexes*

Synovial fluids from subjects with seropositive rheumatoid arthritis contain complexes of either aggregated γ-globulin or specific antigen–antibody complexes. Even before their actual demonstration, however,

much circumstantial evidence suggested their presence. This information has been derived from observations of intra-articular complement depletion and utilization (Section III,B,4) and immunofluorescent deposits of immunoglobulins, often in association with complement components, in the synovium (Section III,A,3) and within the phagocytic cells of articular effusions (Section III,B,6). In addition, Broder and his associates, employing the sensitive assay of histamine release from perfused guinea pig lungs to measure antigen–antibody complexes, were able to detect a histamine-releasing factor, or factors, in the synovial fluid and serum of patients with rheumatoid arthritis (Baumal and Broder, 1968; Broder *et al.*, 1969). This material, which they called the *rheumatoid biologically active factor* (BAF), contained IgG and had the biological, biochemical, and immunological properties of soluble immune complexes. It was not an acid-dissociable aggregate, behaved as a macromolecule on Sephadex G-200 and sucrose density gradient ultracentrifugation, and was not affected by reduction and alkylation. Histamine-releasing activity was found in 29 out of 40 rheumatoid synovial effusions, but in none of the 18 effusions from degenerative and nonrheumatoid inflammatory joint diseases (Broder *et al.*, 1969).

The first direct evidence of complexes in joint fluids was reported by Hannestad (1967). He observed the formation of a precipitin when certain rheumatoid synovial effusions were reacted in gel with sera containing high titers of rheumatoid factor. This phenomenon was limited to joint fluids from adult patients with rheumatoid arthritis—the majority had rheumatoid factor activity present in their serum. Some high-titered rheumatoid factors isolated from serum reacted in gel with the IgG from the companion fluid. The reactant material behaved like aggregated γ-G-globulin immunoelectrophoretically and by ultracentrifugation, but, unlike heat-aggregated γ-globulin, it dissociated at low pH. No rheumatoid factor activity could be demonstrated in the sucrose density fractions of 4 of the 11 fluids separated at pH 7.4. However, at pH 3.6, the γ-globulin aggregates dissociated and antiglobulin activity sedimenting in the IgM region was uncovered (Hannestad, 1968).

The γ-globulin complexes of rheumatoid synovial fluids have been further characterized by Winchester and his associates (Winchester *et al.*, 1970, 1971; Agnello *et al.*, 1970). They found the complexes existing as a continuum of high molecular weight components, ranging from 9 S to 30 S. The larger complexes are detectable by precipitin reactions with C1q (Agnello *et al.*, 1970) and purified IgM rheumatoid factors (Winchester *et al.*, 1970). Smaller complexes, or aggregates of γ-globulin, react only with monoclonal rheumatoid factors obtained from patients with lymphoproliferative diseases (Winchester *et al.*, 1971). The complexes isolated

by precipitation with these reagents were readily dissociable in acid or 4–6 *M* urea and consisted primarily, if not entirely, of IgG. No additional antigens have, as yet, been detected. A portion of the IgG complexes was found to be anti-IgG of the 7 S type. A similar 7 S rheumatoid factor has been found in complexes from the serum of some patients with rheumatoid arthritis (Kunkel *et al.*, 1961; Winchester *et al.*, 1970), but in all instances the concentration of complexes in the synovial fluid are greater than in companion serum samples. To date, there is no direct evidence that the 7 S anti-γ-globulins are reacting against a "core" of denatured γ-globulin or that another type of immune complex is present. However, these alternatives are not entirely ruled out.

The synovial fluid complexes fix complement. A direct relationship exists between the amount of γ-globulin complexes present in the joint fluids and the decrease in total hemolytic complement. The reaction of γ-globulin complexes with C1q does not require the presence of IgM rheumatoid factor, since reduction (with 2-ME) of the joint fluid, sufficient to destroy 19 S rheumatoid factor activity, does not alter the amount of precipitation (Winchester *et al.*, 1970). The size of the γ-globulin complex also plays a role in the complement fixation observed. Those fluids with the heaviest and most abundant complexes were anticomplementary when tested with fresh normal human serum at 4°C; smaller complexes reacted better at 37°C. and in the presence of IgM rheumatoid factor. These are the temperature optima for complement fixation with IgG and IgM antibodies, respectively, (Cunniff and Stollar, 1968) and for the complement-fixing reactions of aggregated γ-globulin with rheumatoid factor studied by Zvaifler and Schur (1968) (Section III,B,4). Moreover, addition of joint fluids containing γ-globulin complexes to serum from patients with seropositive rheumatoid arthritis results in an enhancement of the anticomplementary effects of the joint fluid, suggesting that IgM rheumatoid factor in the serum is reacting with the γ-globulin complexes causing increased consumption of complement (Winchester *et al.*, 1970). A similar observation was made by Hurd *et al.*, 1970). When normal leukocytes were incubated with joint fluids from seronegative patients, no inclusions were detected by immunofluorescent staining. However, following the addition of isolated IgM rheumatoid factor, discrete intracellular inclusions developed, which stained for IgG, IgM, and C3.

The serum complexes detected and isolated with monoclonal IgM rheumatoid factors differ in several characteristics from those described in joint fluids. They represent smaller aggregates of γ-globulin. They neither precipitate with C1q nor show anticomplementary effects, and although IgG rheumatoid factor is present, it is not certain that it is a

dominant reactant (Winchester *et al.*, 1971). Their presence in serum is of interest since the larger complement-fixing complexes are infrequently observed. This may be related to the observation (Sliwinski and Zvaifler, 1969) that grossly aggregated γ-globulin is removed from the joint via the lymphatics and does not return to the bloodstream, whereas, minimally aggregated γ-globulin leaves the joint and is detectable in the circulation.

In addition to conventional rheumatoid factors, the serum and synovial fluid from patients with rheumatoid arthritis often contain other anti-γ-globulins (Kunkel and Tan, 1964), among which are antibodies that react specifically with hidden determinants on the Fab_2 portion of the IgG molecule. Since their structures are revealed after pepsin treatment, these antibodies are referred to as pepsin agglutinators. The *in vitro* digestion of human IgG by lysosomal proteases reveals similar antigenic sites, and inflammatory synovial fluids contain proteases with this capacity (Lo-Spalluto *et al.*, 1970). Furthermore, Quismorio and his collaborators (1968) produced a chronic synovitis in rabbit knee joints by repeated intra-articular injections of Fab_2 fragments. They postulated that the γ-globulin fragment was reacting with the naturally occurring antibody (homoreactant) in rabbit serum which is analogous to the pepsin agglutinator. In humans, however, a synovitis has been produced by intra-articular injection of minute amounts of Fc fragments prepared by papain digestion of IgG, whereas only moderate reactions followed 3.0 mg. of the Fab_2 fragment (Quismorio *et al.*, 1968; Hollander and Rawson, 1968).

Pepsin agglutinators have been eluted from rheumatoid synovial tissues (Munthe and Natvig, 1971), and immune complexes containing antibody to the pepsin site of IgG were demonstrated in rheumatoid synovial fluids (Mellbye and Natvig, 1971). The significance of this antibody as a phlogogenic agent is questionable, however, since the addition of pepsin agglutinator to preformed immune precipitates did not increase the amount of complement fixed by the precipitates alone. Nevertheless, complexes of pepsin agglutinator and Fab_2 fragments of IgG prepared *in vitro* do fix small amounts of human complement (Mellbye and Natvig, 1971). This is of interest because of the recent demonstration that the C3 bypass system can be activated by aggregated Fab_2 fragments of guinea pig γ_1 antibody (Sandberg *et al.*, 1970) and the evidence for activation of this alternate complement pathway in rheumatoid synovial effusions (Götze *et al.*, 1972).

Antinuclear antibodies are common in the serum of patients with rheumatoid arthritis, and recent information suggests that they are present in an even higher frequency in joint fluids (Barnett *et al.*, 1966; Elling *et al.*, 1968). Occasionally joint fluids show antinuclear factors when paired serum samples do not (Barnett *et al.*, 1966; MacSween *et al.*, 1968). It has also been reported that the antinuclear activity in rheuma-

toid arthritis is detected with greater frequency when granulocyte nuclei are used as antigen, rather than lymphocytes, thyroid, gastric mucosa, or rat liver, and in a portion of synovial fluids, the antinuclear antibody may be granulocyte-specific (Elling *et al.*, 1968). Deoxyribonucleic acid and soluble nucleoprotein are regularly found in inflammatory articular exudates, probably released from disintegrating granulocytes (Robitaille and Tan, 1972). This has prompted speculation that antinuclear antibodies and nuclear debris may be one of the constituents of the inclusions in the granulocytes of rheumatoid synovial effusions (Zvaifler, 1965; Barnett *et al.*, 1966). The immunofluorescent localization of nucleoprotein in the cytoplasm of synovial leukocytes supports this view (Brandt *et al.*, 1968) as does the findings of antinuclear antibodies concentrated in cell lysates from effusions of adults with rheumatoid arthritis (Zvaifler and Martinez, 1970). In several instances the antinuclear antibody was detected in cell lysates when it was not demonstrated in the corresponding synovial fluid. None of these findings are limited to rheumatoid joint fluids; they are also seen in other forms of synovitis, although less often (see Note Added in Proof No. 1 on p. 336).

Additional information suggesting that DNA is a constituent of immune complexes in rheumatoid effusions comes from the study of cryoproteins. Proteins that precipitate from serum exposed to the cold (cryoglobulins) occur in a wide variety of diseases. They have been the source of a number of studies and reviews, and it is generally agreed that they closely resemble immune complexes (reviewed by Barnett *et al.*, 1970). A recent development is the recognition that almost all synovial fluids from rheumatoid subjects contain proteins that precipitate upon standing at 4°C. (Marcus and Townes, 1971a,b; Cracchiolo *et al.*, 1971). Similar cryoprecipitable proteins are rare in paired serum samples, and they are only very occasionally found in nonrheumatoid inflammatory joint effusions (Marcus and Townes, 1971a; Cracchiolo *et al.*, 1971). They consist, in the main, of immunoglobulins—predominantly IgG and IgM. Fibrinogen or fibrinogen degradation products are regularly found, but they can be removed by repeated washing (Cracchiolo *et al.*, 1971). Deoxyribonucleic acid has been detected in the majority of these cryoprecipitates by the diphenylamine reaction (Marcus and Townes, 1971a; Cracchiolo *et al.*, 1971). Immunochemical identification of the DNA has been less common and is a source of some confusion. Marcus and Townes (1971a) detected DNA very infrequently by a precipitin reaction employing systemic lupus erythematosus (SLE) sera, but they found it regularly by a microcomplement fixation test. However, Cracchiolo *et al.* (1971) using a rabbit antibody to single-stranded DNA could regularly uncover DNA, but only after heating the cryoprecipitate to 100°C.

The biological properties of the cryoprecipitable proteins are of inter-

TABLE II
CRYOPRECIPITATES FROM RHEUMATOID SYNOVIAL FLUIDS

Subject	Proteins (mg %)				Complement[b]			Antibody	
	Total protein	IgG[a]	IgM[a]	IgA[a]	C1q[c]	C3[e]	% C fixed[d]	ANA[b,e]	RF[f]
H.T.	100	5.0	25.0	0	0	+	9	0	0
W.S.	480	31.0	31.0	1.9	0	+	19	0	80
B.S.	47	7.1	8.6	0	0	+	7	±	40
A.R.	92	41.0	18.5	1.1	±	+	55	±	
S.S	60	56.0	7.6	4.5	0	+	64	+	0
B.B	35	5.4	3.0	0	0		39	±	40
J.D.	120	47.0	49.0	1.9	+	+	6	±	80
R.C.	100	32.0	6.6	1.2	0		38	+	
W.A.	34	7.5	0	0	0	0	45		10
H.A.	73	37.0	6.2	2.1	+	+	43	0	0
R.H.	366	4.5	60	15.5	0	+	69	0	160

[a] Single radial immunodiffusion.
[b] 0 = Negative; ± = weak positive; + = strong positive.
[c] Ouchterlony double diffusion in gel.
[d] Percent of 70 to 90 CH_{50} units of guinea pig complement fixed at 37°C. in 45 minutes.
[e] Fluorescent antinuclear antibody (ANA) test on undiluted sample.
[f] Titer of rheumatoid factor (RF) in bentonite flocculation test.

est (see Table II). The majority contain both anti-γ-globulin and antinuclear activity (Marcus and Townes, 1971a; Cracchiolo *et al.*, 1971; Zvaifler, 1973). A significant decrease in the anti-γ-globulin activity follows reduction with 2-ME, suggesting that it is of the IgM type (Cracchiolo *et al.*, 1971; Zvaifler, 1973). The greatest part of the anticomplementary activity of whole synovial fluid resides in the cryoprecipitable fraction (Marcus and Townes, 1971a). The precipitates contained C1q, as determined by a hemolytic assay, and about half had C4 by immunodiffusion but C3 was not detected, although rabbits immunized with the cryoprecipitates produced an antibody to C3. In sorbitol density gradient ultracentrifugation studies, the bulk of the complement-fixing activity resided in fractions greater than 19 S. These also contained variable amounts of DNA. The cryoproteins could be partially dissociated by 15% sodium chloride, and the ability of the dissociated material to react with heat-denatured, calf thymus DNA was studied (Marcus and Townes, 1971b). Individual density gradient fractions were reacted with denatured DNA and 6 to 8 units of human complement from agammaglobulinemic serum. The heavy sedimenting fractions gave the maximum complement fixation; no fixation occurred with native

DNA. Evidence favoring a specific antigen–antibody reaction was that DNA alone fixed no complement, and DNA reacted with heat aggregated γ-globulin fixed no more complement than did aggregated γ-globulin alone. The complement fixation appeared distinct from the anti-γ-globulin activity, which was also located in this region.

Additional observations on complement fixation by synovial fluid cryoprecipitates were made in the author's laboratory (see Table II). They are difficult to compare with the findings of Marcus and Townes (1971a) since the cryoprecipitates were prepared differently and washed more extensively. Greater quantities of precipitate were available because the starting volume of synovial fluid was greater, and cryoprecipitation was allowed to proceed for 72 hours, rather than 18 hours. The precipitates resolubilized completely at 37°C., and C3 was regularly identified by immunodiffusion; C1q was found only infrequently. Generally, the complement-fixing ability of the cryoprecipitate was inversely related to the complement level in the fluid from which it was derived, and roughly proportional to the total protein of the precipitate. Similar amounts of guinea pig and human complement were fixed, and better fixation occurred after overnight incubation at 4°C. than at 37°C. for 45 minutes. Both findings are more consistent with fixation by IgG, rather than by IgM antibody (Zvaifler, 1969b). Complement consumption was unrelated to the concentration of either IgG or IgM in the cryoprecipitate, but was somewhat proportional to the ratio of the concentration of IgG to IgM (Fig. 2). Whether this represents a balance between IgG fixation and inhibition by IgM rheumatoid factor is not yet known, but the available data is consistent with that hypothesis.

The biological activities of the synovial fluid cryoprecipitates, their content of anti-γ-globulin and antinuclear activity and the similarity to serum cryoprecipitable proteins all suggest that they represent immune complexes that have been separated in the cold. This is reinforced by the occasional finding in the cryoprecipitates of antibody activity not present in the accompanying serum samples nor in the fluid from which the precipitates were derived (Cracchiolo *et al.*, 1971).

The reason why these precipitates are so readily detected in rheumatoid synovial fluids, when they are absent from the accompanying serum, is not known. Possibly, the solubility of the proteins has been altered by reactions with enzymes present in the inflammatory exudate. Alternatively, the antibodies in the precipitate may be immunoglobulins manufactured by the synovial membrane which combine with their antigens in the joint and thereby do not gain access to the serum. Most significantly, it should be recognized that it is incorrect to compare synovial fluid to serum, since the fluid in an inflamed joint is really analogous to

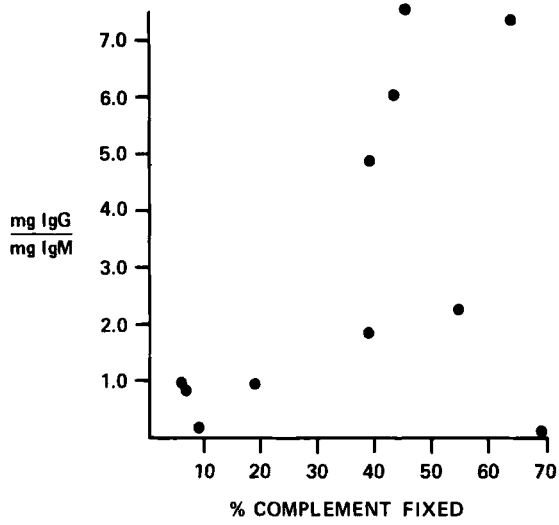

FIG. 2. Cryoprecipitates from rheumatoid synovial effusions. Relationship of the ratio of the IgG and IgM concentrations (determined by single radial immunodiffusion) to their ability to fix complement (percent of 70 to 90 CH_{50} units of guinea pig complement fixed at 37°C. in 45 minutes).

plasma. Therefore, it is more appropriate to contrast the rheumatoid synovial fluid cryoprecipitates with proteins precipitated from cooled plasma. Such precipitates are called *cryofibrinogens*. These plasma cryoprecipitates have been observed in a number of diverse clinical conditions including metastatic malignancy, acute suppurative diseases, pregnancy, and occasionally connective tissue disorders. In one study, 9 of 10 rheumatoid subjects showed plasma cryoprotein precipitates (Zvaifler, 1972). The total protein of the precipitates obtained from 6 cc. of plasma ranged from 0.26 to 0.88 mg./ml. with an average of 0.56 mg./ml. The major constituent of the precipitates was fibrinogen; small amounts of IgG (0.08–0.14 mg./ml.) were detected but no IgM or IgA. Unlike the synovial fluid cryoprecipitates, the rheumatoid plasma cryoprecipitates had no antinuclear activity, and, although rheumatoid factor was present in 7 of the 9 plasma samples, only 3 of the cryoprecipitates had anti-γ-globulin activity, and in low titers (1:20 or less). The plasma cryoprecipitates showed neither C1q nor C3 on immunological analysis and there was no complement fixation after incubation with fresh guinea pig complement (Zvaifler, 1972a).

Rheumatoid effusions characteristically contain large amounts of fibrinogen and fibrinogen degradation products; the antithrombin activity of the latter probably accounts for the fact that they clot poorly, if at

all (Gormsen *et al.*, 1971). Soluble complexes of fibrin monomers and fibrinogen degradation products are remarkably insoluble in the cold (Shainoff and Page, 1962; Wegrzynowicz *et al.*, 1971). It is likely that when rheumatoid synovial fluids are kept at 4°C., the mixture of fibrin and fibrinogen degradation products becomes increasingly insoluble and precipitates from solution. In the process they could trap preexisting soluble immune complexes of the type previously reported by Hannestad (1967) and Winchester and associates (1970). Thus, the synovial fluid cryoprecipitates may result from a fortuitous combination of physical-chemical events—cold-insoluble by-products of the digestion of fibrinogen forming in the presence of soluble immune complexes.

6. Cells

Articular effusions in rheumatoid arthritis are characterized by large numbers of white cells, the majority of which are polymorphonuclear leukocytes. Cell counts range from 2500 to 25,000/mm.^{3}, and higher counts are occasionally found. The effusions tend to be chronic and show remarkable stability of the cell population over months and years (Bertino *et al.*, 1963) and even within several joints in the same individual (Pekin and Zvaifler, 1964). Synovial fluid leukocytes are derived from the bloodstream (Bertino *et al.*, 1963) and the half-life of granulocytes in an inflammatory synovial effusion is approximately 4 hours (Hollingsworth *et al.*, 1967). Only a small proportion of the original cells remain after 24 hours, indicating a rapid destruction of exudate granulocytes regardless of the inflammatory stimulus. It has been estimated that in a modest 20-ml effusion containing 25,000 granulocytes/mm.^{3} the daily turnover of cells in the joint space might exceed a billion cells (Hollingsworth *et al.*, 1967).

The predominant cell in rheumatoid synovial fluids is a mature polymorphonuclear granulocyte, accounting for about 75 to 90% of all nucleated cells. The remainder consist of lymphocytes, 5–10%, monocytes, occasional macrophages, and synovial lining cells (Kling, 1938; Ropes and Bauer, 1953; Malinin *et al.*, 1967). The polymorphonuclear leukocytes differ from those seen in the blood due to their striking loss of granules, vacuolated appearance, and hypersegmentation of the nuclear chromatin. A small percentage of cells show pyknotic chromatin (Bodel and Hollingsworth, 1966; Malinin *et al.*, 1967). These findings are common to inflammation and can be seen in granulocytes from a variety of articular diseases. Somewhat more specific for rheumatoid arthritis are oval, basophilic, Feulgen-staining globular particles. These DNA globules appear to represent nuclear debris from degenerating cells and are found free in the synovial fluid, adherent to the membrane of polymorpho-

nuclear cells, and in pseudopod-like projections of the cytoplasm from healthy granulocytes. The striking difference in the Feulgen staining of the cell nucleus and the DNA globules in the cytoplasm suggested that the DNA material is being phagocytized by, rather than extruded from, these cells (Pekin *et al.*, 1966; Malinin *et al.*, 1967). A cell quite unique to rheumatoid effusions is a granulocyte with peripherally displaced nuclear chromatin, reminiscent of a small lupus erythematosus (LE) cell. But, instead of containing a hemotoxylin body, the center of the cell is occupied by either a large Feulgen-staining globule or a proteinaceous mass. About 1% of the granulocytes in rheumatoid effusions show such changes (Pekin *et al.*, 1966; Malinin *et al.*, 1967). Nuclear displacement by a protein-staining mass was encountered only in cases of rheumatoid arthritis and is remarkably similar to the "rheumatoid cell" reported by Williamson and Ling (1965).

Electron-microscopic examination of the neutrophiles in rheumatoid synovial fluid reveals large numbers of cytoplasmic bodies (Fig. 3). Within them are subcellular structures, such as mitochondria, granules, and nuclear material, in various stages of digestion. Similar cellular debris is present in the surrounding media. Also found within limiting membranes are small amounts of a fibrinlike material, a significant amount of amorphous proteinaceous material, and an unidentified gray globular structure (Zucker-Franklin, 1966). From morphological consideration and acid phosphatase staining, it has been concluded that these cytoplasmic bodies represent secondary lysosomes or phagolysosomes. Their appearance is determined by the stages of degradation of the ingested material (Zucker-Franklin, 1966; Coimbra and Lopes-Vaz, 1967). The gray globular structures do not contain acid phosphatase but do take Sudan stains, suggesting that they are lipid in origin. Similar morphological findings are present in synovial fluid leukocytes from a variety of other inflammatory forms of arthritis; however, the number of cells showing cytoplasmic abnormalities and the number of cytoplasmic inclusions per cell is significantly greater in granulocytes from rheumatoid synovial effusions (Zucker-Franklin, 1966).

Synovial fluid obtained from rheumatoid effusions of short duration have a variable appearance. Some authors report low white cell counts with a predominance of lymphocytes or large mononuclear cells (Kulka *et al.*, 1955; Schumacher and Kitridou, 1972); others find mainly polymorphonuclear cells even in "acute rheumatoid arthritis" (Ropes and Bauer, 1953). Cytoplasmic inclusions, seen by electron microscopy, were much less common, and mainly limited to mononuclear cells. The "gray globular bodies" described by Zucker-Franklin (1966) in chronic rheumatoid synovitis were an infrequent finding in early effusions. Other phagocytized matter, including fibrin, lipid, vesicular and membranous struc-

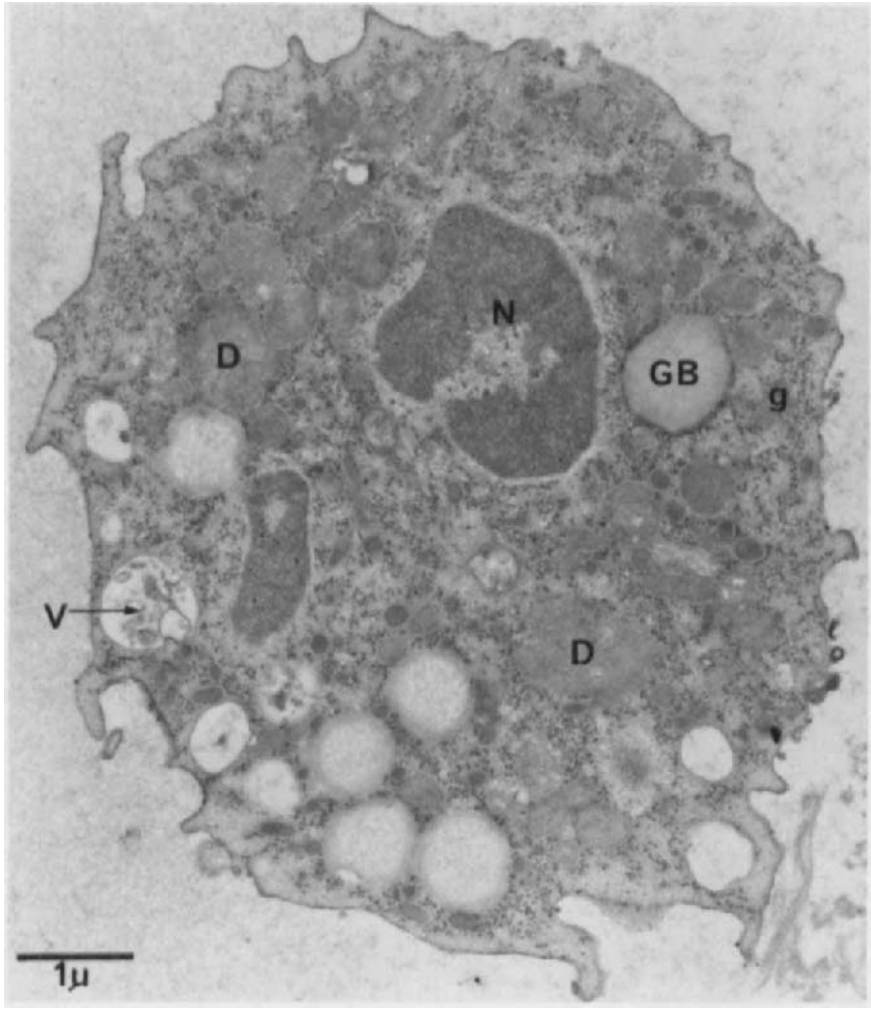

FIG. 3. Thin section of an intact polymorphonuclear neutrophile leukocyte in an effusion from a rheumatoid knee joint. The section demonstrates three types of inclusion bodies within the cell: the so-called gray bodies (GB), a variety of dense inclusions which may represent immune complexes (D), and some debris containing vacuoles (V). A portion of the nucleus (N) is depicted. A fair number of neutrophile granules (g) are still present. (This electron micrograph was generously provided by Peter Henson.)

tures, and cell debris were identified in acute synovitis of various causes and was not unique to early rheumatoid inflammation (Schumacher and Kitridou, 1972).

Wet, unstained preparations of polymorphonuclear cells from freshly aspirated rheumatoid synovial fluid contain numerous dark cytoplasmic granules which are distinctly larger than the normal granules. These granules are shiny, dense, and hyaline in appearance, measuring from 0.5 to 1.5 μ in diameter. Initially, they were considered quite specific for rheumatoid arthritis (Delbarre *et al.*, 1964; Hollander *et al.*, 1965a), but subsequent studies have failed to support their uniqueness (Astorga and Bollet, 1965; Hollander *et al.*, 1965b; Malinin *et al.*, 1966; Willkens and Healey, 1966; Sones *et al.*, 1968). It is generally agreed, however, that cells fitting the description of "ragocytes" (Delbarre *et al.*, 1964) or "inclusion body cells" (Hollander *et al.*, 1965a) are present in a higher percentage in effusions from rheumatoid arthritis than in any other joint disease and that the number of cells containing inclusions are greater in this disease, particularly when the patient has rheumatoid factor. The initial presumption that the inclusions were the morphological expression of phagocytosis was supported by the demonstration that disrupted leukocytes from rheumatoid effusions released rheumatoid factor into the supernatant fluid. In some instances anti-γ-globulin activity was released from cell homogenates when it was not demonstrable in the accompanying serum or synovial fluid (Astorga and Bollet, 1965; Hollander *et al.*, 1965a,b). This latter finding has been challenged by Sones and co-workers (1968) who felt that rheumatoid factor is seldom present in the synovial fluid when absent from the serum and is only demonstrable in cell lysates from seropositive synovial fluids. They concluded that the conflicting results could be explained by the variable concentrations of cells employed by others and by the fact that the latex slide test appeared to be affected by nonimmunological variables when applied to synovial fluid.

The recognized phagocytic capacity of neutrophiles, the morphological appearance of the cells, and the release of a 19 S immunoglobulin with anti-γ-globulin activity all suggested that the neutrophiles in rheumatoid effusions were taking up immune complexes. A number of investigators have further defined the cytoplasmic inclusions by immunofluorescent techniques (Rawson *et al.*, 1965; Willkens and Healey, 1966; Zucker-Franklin, 1966; Barnett *et al.*, 1966; Barnhart *et al.*, 1967b; Vaughan *et al.*, 1968a,b; Britton and Schur, 1971). Their conclusions can be summarized as follows: (*1*) IgG, IgM, complement components, and rheumatoid factor are present in varying combinations in the cells from rheumatoid effusions; (*2*) IgG and complement components are frequently found in the cells from nonrheumatoid inflammatory effusions; (*3*) anti-γ-globulin inclusions are correlated with rheumatoid factor in the serum or synovial fluid and a low level of joint fluid complement; (*4*) the quantity of a complement component within cells is inversely

proportional to the concentration of the same component in the accompanying fluid; (5) the first (C1q) and fourth (C4) components of complement are detected in most of the cells from patients with seropositive rheumatoid arthritis, but, they are also found frequently in synovial fluid leukocytes from patients with seronegative rheumatoid arthritis; (6) immunofluorescent staining for IgM or C3 correlates closely with seropositive rheumatoid arthritis.

That the changes in rheumatoid cells relate to phagocytosis of immune complexes is also supported by experiments in which similar light, fluorescent, and electron-microscopic changes can be produced in normal cells by the ingestion of preformed complexes of rheumatoid factor and heat-aggregated γ-globulin (Parker and Schmid, 1962; Astorga and Bollet, 1965; Williamson and Ling, 1965; Zucker-Franklin, 1966). Moreover, the injection of IgG, isolated from the serum of rheumatoid patients, into the clinically uninvolved knee joints of rheumatoid volunteers results in an acute inflammatory reaction. The leukocytes from these induced effusions have IgG and IgM complexes in their cytoplasm (Restifo *et al.*, 1965). In addition, when normal polymorphonuclear cells are exposed to rheumatoid factor-positive synovial fluids, they develop cytoplasmic inclusions containing IgG, IgM, and C3. This does not occur when normal polymorphonuclear cells are exposed to rheumatoid factor-negative fluids; if IgM rheumatoid factor is added to these fluids, then complexes containing immunoglobulins and complement are detectable in the leukocytes. No such inclusions are formed if IgM without anti-γ-globulin activity is substituted (Hurd *et al.*, 1970).

Extranuclear deposits of nucleoprotein have been identified by immunofluorescent techniques in synovial fluid leukocytes. These inclusions are in the same distributions as IgG and C3. Similar cytoplasmic deposits of nucleoprotein are found in leukocytes from abscesses, suggesting that this may be a common accompaniment of significant inflammation of any origin (Brandt *et al.*, 1968).

Bodel and Hollingsworth (1966) assessed the physiological function of leukocytes from the joint fluids of patients with rheumatoid arthritis. Despite remarkable vacuolation and degranulation, synovial fluid leukocytes can exclude vital dyes—a sensitive measure of cell viability. The respiration of joint fluid leukocytes was less than half that of plasma leukocytes from the same subject, but the changes in respiration could not be correlated with the degree of morphological change. Most joint fluid cells show a normal increase in respiration following phagocytosis. The ability of leukocytes from rheumatoid synovial exudates to phagocytize staphylococci was examined, and they were found to be deficient, as compared to blood leukocytes. The fault was not with the cells, how-

ever, since the phagocytic capacity of both joint fluid and blood leukocytes was decreased equally when they were suspended in joint fluid. Synovial fluid leukocytes placed in plasma regained phagocytic function. Incubation of plasma leukocytes for prolonged periods in joint fluids from patients with seropositive rheumatoid arthritis did not produce morphological or respiratory changes in these cells but they did depress phagocytic capacity (Bodel and Hollingsworth, 1966).

7. *Enzymes*

Granulocytes appear to play a central role in the induction of acute articular inflammation and structural damage to joint tissues (reviewed in detail by Weissmann and Dukor, 1970; Chayen and Bitensky, 1971). These effects are mediated by the hydrolytic enzymes of the cell which are normally sequestered in a latent form within a semipermeable membrane (lysosomes). Polymorphonuclear leukocytes extrude a portion of their sequestered enzymes when engulfing particulate matter and other biologically active materials, such as bacteria or immune complexes. It is not too surprising, therefore, that hydrolytic enzymes of lysosomal origin are found in rheumatoid synovial effusions. In recent years a number of these enzymes have been identified in synovial fluid including: acid phosphatase (Smith and Hamerman, 1962; Lehman *et al.*, 1964; Caygill and Pitkeathly, 1966; Kerby and Taylor, 1967; Wegelius *et al.*, 1968; Jasani *et al.*, 1969; Beckman *et al.*, 1971), β-glucuronidase (Jacox and Feldmahn, 1955; Bartholomew, 1968; Jasani *et al.*, 1969), muramidase (Kerby and Taylor, 1967; Pruzanski *et al.*, 1970), β-acetylglucosaminase (Caygill and Pitkeathly, 1966; Bartholomew, 1968), and β-galactosidase (Bartholomew, 1968). Although the results vary somewhat for individual enzymes, there are some consistent features. Lysosomal enzymes are found in very low concentration in the cell-free supernatants of synovial fluids from normal joints or noninflammatory joint diseases, such as degenerative and traumatic arthritis. With increasing degrees of articular inflammation, there is a corresponding increase in the various enzymes. The levels attained in the joint fluid are much higher than those in companion serum samples. In general, there is a rough correlation between the total white blood cell count of the synovial effusion and the enzyme content, but significant deviations have been noted. The effusions of rheumatoid arthritis and infectious arthritis have the highest concentration of lysosomal enzymes. Consistently smaller amounts are found in other inflammatory, nonrheumatoid, joint diseases such as gout, psoriatic arthritis, and the arthritis of ankylosing spondylitis.

To support the idea that the enzymes in rheumatoid synovial effusions are actively released following ingestion of immune complexes, one would

like to see evidence that they are derived from phagocytic synoviocytes or white blood cells and that they represent a response to endocytosis rather than cell death and disorganization. This latter point is emphasized by the detection of nonlysosomal (cytoplasmic) enzymes in most synovial effusions. Lactic dehydrogenase (LDH) has been studied extensively (Vesell *et al.*, 1962; West *et al.*, 1963; Cohen, 1964; Kerby and Taylor, 1967; Jasani *et al.*, 1969; Veys *et al.*, 1970). Large amounts of catalase, another cytoplasmic enzyme, are found in inflammatory joint diseases, and the catalase activity is roughly proportional to the total white cell count in the various joint diseases studied (Arai, 1967).

Information concerning the derivation of the synovial fluid enzymes has come from characterization of their isozymes. In the case of LDH, it has been demonstrated that the isozyme distribution in inflammatory effusions is distinct from that of companion serum samples (Vesell *et al.*, 1962; Cohen, 1964). There is a consistent and striking increase in the percentage of the LDH-5 isozyme in rheumatoid and other inflammatory synovial fluids. Moreover, Vesell *et al.* (1962) showed that the isozyme patterns of leukocytes and synovial tissues are similar to each other and to that found in the fluid, suggesting that the elevated LDH activity may have its source in either, or both, of these tissues. Likewise, Bartholomew (1968) has presented evidence that β-*N*-acetylglucosaminadase, a lysosomal enzyme, is derived from two sources. When rheumatoid synovial fluid was chromatographed on Sephadex G-100 the enzyme activity was identified in two distinct peaks: the first corresponded to the β-*N*-acetylglucosaminidase obtained from a preparation of synovial tissue; the second peak corresponded to the elution pattern for the enzyme isolated from white cells of peripheral blood. Findings of a higher level of a specific enzyme in synovial fluid than in paired plasma samples and the demonstration that transaminase concentrations in the joint are independent of blood levels (West *et al.*, 1963; Cohen, 1964; Jasani *et al.*, 1969) support the argument that a significant portion of the enzymes in joint effusions are derived from the white cells of the exudate or from inflamed synovial tissues. The acid and alkaline phosphatases of synovial fluid are of interest in this regard. A comparison of serum and synovial fluid levels, the distribution of cell-free and bound activity, and isozyme patterns suggest different origins for the two enzymes. Alkaline phosphatase (a nonlysosomal enzyme) occurs free in the synovial fluid, and its concentration and isozyme pattern parallels serum levels (Lemperg *et al.*, 1971). Acid phosphatase (a lysosomal enzyme) is present in the synovial fluid in greater concentration than in serum, is predominantly cell bound, and the isozyme characteristics are not those of the serum isozymes (Beckman *et al.*, 1971). Thus, synovial fluid, alkaline phos-

phatase is predominantly derived from serum, whereas acid phosphatase has a local origin from synovial lining cells or leukocytes.

Although many of the enzymes outlined above are of interest as markers for the extrusion of lysosomal contents, they do not necessarily participate in the induction of joint inflammation or cause structural injury to articular tissues. Nevertheless, inflammatory effusions do contain many enzymes with these properties. Neutral proteases extracted from human polymorphonuclear leukocytes and from rheumatoid synovial membrane are able to hydrolyze bovine chondromucoprotein and a neutral-acting protein–polysaccharidase has been found in rabbit polymorphonuclear cell granules (Ziff *et al.*, 1960; Weissmann and Spilberg, 1968; Davies *et al.*, 1971). A similar, chondromucoprotein-degrading, neutral protease was identified in rheumatoid synovial effusions by Wood and his collaborators (1971). They found that cell extracts from the majority of rheumatoid effusions produced a marked fall in the specific viscosity of a chondromucoprotein preparation of bovine nasal cartilage. One or more enzymes were responsible for this effect and had optimum enzyme activity at a pH near 7.5. But, the pathological significance of the chondromucoprotein-degrading enzyme was questioned, because a potent inhibitor was present in almost all of the inflammatory fluids studied. Kerby and Taylor (1967) also reported that lysozyme from the large granule fraction of rheumatoid synovial leukocytes was inhibited by cell-free synovial fluid. They also observed a partial inhibition of LDH activity by the same fluids.

Articular cartilage degradation has been produced by an acid protease of lysosomal origin (Dingle, 1962) and a neutral protease fraction of human leukocyte lysosomes (Janoff and Blondin, 1970). The neutral protease has an elastinolytic capacity, demonstrated by its ability to cleave the elastase substrate [*t*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester (NBA)] and release protein from rabbit articular cartilage with a resultant loss of matrix metachromasia. These same authors (Janoff and Blondin, 1970) found a heat-labile material with NBA esterase activity in more than half of the rheumatoid effusions they studied. The enzyme was not well correlated with the number of white cells in the effusion and at least one fluid from an osteoarthritic knee joint contained a significant quantity of enzyme. A cathepsin-like protease with a pH optimum around 2.8 has also been identified in rheumatoid effusions (Ennis *et al.*, 1968), but no biological significance has been ascribed to it.

Enzymes able to act on undenatured collagen were first reported by Gross and Lapiere (1962). Collagenase activity was detected by the ability of tissue explants to solubilize glycine ^{14}C-containing peptides from collagen gels. This sensitive assay system has been used to study

synovial tissues. It was demonstrated that rheumatoid synovium in tissue culture synthesizes and releases a collagenase capable of degrading native collagen at 37°C. and neutral pH. Although the enzyme was most abundant in membranes from rheumatoid arthritis, collagenolytic activity was also found in cultures of biopsy material from nonrheumatoid joint diseases, in small amounts from synovium of degenerative joint disease, but not in normal synovium (Evanson *et al.*, 1968; Lazarus *et al.*, 1968a; Harris *et al.*, 1969a). Collagenase extracted from tissue cultures was partially purified and characterized. It is inhibited by ethylenediaminetetraacetic acid (EDTA) and γ-globulins of serum and has negligible nonspecific proteolytic activity. It degrades purified collagen substrates, both in solution and in the native fibril form, cleaving the molecule in a manner similar to tadpole collagenase (Gross and Lapiere, 1962).

Another collagenolytic enzyme has been isolated from the large granule fraction of human leukocytes (Lazarus *et al.*, 1968b). The granulocyte collagenase differs from synovial collagenase in that it works best on soluble collagen and only poorly on collagen in fibrillar form. Moreover, its activity is not inhibited significantly by serum proteins. Since collagen does not exist in solution *in vivo*, there has been some reluctance to accept the hypothesis that this enzyme could be playing a significant role in collagen resorption (Harris *et al.*, 1970). However, Wegelius and his collaborators recently (1970) reported that suspensions of synovial fluid cells obtained from 6 patients with rheumatoid arthritis were capable of lysing acetic acid-soluble gels. A similar activity was not obtained from comparable numbers of buffy coat leukocytes in the blood of the same patients. There is, as yet, no information concerning the characteristics of this enzyme or the manner in which it cleaves collagen, except that polyacrylamide gel electrophoresis of the digestion products gives a pattern similar to that produced by a commercial collagenase (Wegelius *et al.*, 1970).

Evidence that collagenase is responsible, at least in part, for the tissue destruction in rheumatoid arthritis comes from the observation that partially purified collagenase obtained from cultures of rheumatoid synovium is capable of degrading cartilage collagen (Harris *et al.*, 1970). In addition, synovial tissue collagenase activity appears to be related to the amount of local and systemic disease activity (Harris *et al.*, 1969a; Lazarus *et al.*, 1968a).

Although little or no collagenolytic activity has been found free in homogenates of fresh rheumatoid tissues, synovial collagenase has been detected regularly in rheumatoid synovium and joint fluid using a monospecific antiserum to purified human synovial collagenase. This immunological method has the advantage that it can measure collagenase in the

absence of demonstrable enzyme activity, thus circumventing the effects of inhibitory proteins (Bauer *et al.*, 1971). The theoretical objection to a major pathogenetic role for synovial collagenase in rheumatoid arthritis comes from the observation that almost all human sera and most synovial fluids inhibit collagenolytic activity *in vitro*. These serum antiproteases consist of α_1-antitrypsin, α_2-macroglobulin, and other α-globulins (Eisen *et al.*, 1970; Hawley and Faulk, 1970). As a group, however, rheumatoid synovial fluids have less inhibitory capacity per unit protein than do fluids from degenerative or other nonrheumatoid inflammatory joint diseases (Harris *et al.*, 1969b, 1970). In addition, occasional (4 of 27) rheumatoid synovial fluids contain free collagenolytic activity. In 1 fluid this was present in two forms: one resembled the synovial tissue collagenase and the other had characteristics reminiscent of the collagenase derived from leukocyte granules. The collagenase activity was not associated with the joint fluid white count, and it was an irregular finding in this 1 patient (Harris *et al.*, 1969b). Further studies are obviously in order to clarify the role of this important enzyme in inflammatory joint disease.

A great number of mediators of the inflammatory response are present in rheumatoid effusions—kinins, pyrogens, and leukotactic factors have all been identified. Kinins have been discussed in detail in Section III,B,3. Leukocytes obtained from joint fluids of patients with inflammatory arthritis do not release pyrogen after incubation in saline. Following phagocytosis, they produce less pyrogens than do granulocytes from blood. Bodel and Hollingsworth (1968) interpreted these findings to mean that the granulocytes in joint effusions have previously released their pyrogens *in vivo*, especially since they found that cell-free synovial fluid from these same patients contained a rapidly acting, endogenous pyrogen.

Rheumatoid synovial fluids contain complement-derived factors which influence the migration of leukocytes (see Section III,B,4). In addition, Stasny and Ziff (1971) have identified two fractions in the majority of fluids from chronic synovitis which will inhibit the migration of guinea pig macrophages: one, a dialyzable fraction, is derived from granulocytes; the other is nondialyzable and appears to be produced by mononuclear cells. It is still not clear that the latter factor is the same as the classic migration inhibitory factor (MIF) (Bloom, 1971).

Willoughby *et al.* (1962) have isolated a material from aqueous extracts of sensitized or normal lymphocytes that they call *lymph node permeability factor* (LNPF). When injected intradermally, it produces a profound increase in vascular permeability, as measured by skin blueing, causes immigration of mononuclear cells, and brings about the deposition of an eosinophilic staining material resembling fibrinoid. The LNPF can

be distinguished from other permeability factors, such as histamine, 5-hydroxytryptamine, kallikrein, and bradykinin by muscle strip contractions, blood pressure measurements, and the use of appropriate antagonists. Chemical fractionation of lymphocyte extracts containing LNPF reveals that the vascular activity is associated with more than one protein fraction, suggesting that LNPF may not be a macromolecule but rather a small substance that absorbs noncovalently to a variety of serum and tissue proteins (Meacock and Willoughby, 1968). Permeability factors similar to LNPF have been detected in fluid removed from the knee joints of patients with a variety of arthritic conditions (Spector and Willoughby, 1968). It remains to be seen, however, if the LNPF is simply another lymphokine, the term used to describe collectively the various nonantibody mediators of cellular immunity, or whether it is a separate factor.

IV. Role of Rheumatoid Factors in Rheumatoid Joint Inflammation

Interest in rheumatoid factors has waxed and waned over the past several decades. Any initial enthusiasm, based on the presumption that the cause of rheumatoid arthritis had been found, was quickly dispelled by the demonstration of rheumatoid factors in a variety of nonarticular diseases and its absence in otherwise typical rheumatoid arthritis. Subsequently two alternative hypotheses were considered: (*1*) rheumatoid factor mediates the pathogenesis of rheumatoid arthritis, and (*2*) rheumatoid factor is merely an interesting by-product of rheumatoid arthritis—a response to whatever causes the disease, but not involved in its pathogenesis (a relationship similar to the Wassermann antibody and syphilis). After reviewing these possibilities, an active worker in the field (Christian, 1961) rejected the first and chose the latter, because at that time there was no direct evidence that rheumatoid factor was responsible for the articular manifestations of rheumatoid arthritis.

What has happened to change this view? It is now recognized that anti-γ-globulins are detectable in the serum of many adults and children with what was originally called seronegative rheumatoid arthritis. Anti-γ-globulins which are absorbed to, and then eluted from, insolubilized human IgG can be quantified by radial immunodiffusion against specific antisera. IgM, IgG, and IgA anti-γ-globulins have been detected in this manner, in contrast to the conventional IgM rheumatoid factor which is demonstrated by agglutination tests (Torrigiani and Roitt, 1967; Torrigiani *et al.*, 1969, 1970; Panush *et al.*, 1971). Furthermore, although rheumatoid factor is noted in a variety of clinical situations, when seen in the context of rheumatoid arthritis, it is associated more often with an unremitting disease course, limited functional capacity,

greater joint destruction as assessed radiologically, and systemic manifestations of rheumatoid disease, such as nodules, vasculitis, neuropathy, leg ulcers and cardiovascular complications (Weintraub and Zvaifler, 1963; Sharp *et al.*, 1964; Bland and Brown, 1964; Sievers, 1965; Mongan *et al.*, 1969). Whereas these correlations have, in general, been applied to serum measurements of conventional IgM rheumatoid factors, it is becoming abundantly clear that similar correlations exist with anti-γ-globulins of other classes, both in the serum and synovial fluid (Panush *et al.*, 1971). This observation is of particular import if one views rheumatoid factor as having its pathogenetic effects in an extravascular tissue space, such as the joint.

In what ways could rheumatoid factors mediate the pathogenesis of rheumatoid arthritis? If the scheme outlined in the Introduction to this review is correct, then there are several points in the cycle of intra-articular events where rheumatoid factors could operate to mediate or enhance joint inflammation. Two have already been discussed in detail—namely, participation in immune complex formation and in the activation of the complement sequence. In the final analysis, however, it is the phagocytic process and the subsequent release of biologically active material from polymorphonuclear leukocytes or synovial lining cells which provokes joint inflammation and tissue injury. It is, therefore, important to consider how rheumatoid factors might influence the manner in which cells are brought into an inflammatory focus (chemotaxis), their interaction with and ingestion of immune complexes (phagocytosis), and the subsequent extrusion of lysosomal contents (enzyme release).

The contribution of various complement components to chemotaxis is well-established (Ward, 1970), and if, as has been suggested, rheumatoid factors, either directly or indirectly, fix complement, then the mobilization of leukocytes and their appearance in synovial fluid might be explained. Direct measurements of the effects of rheumatoid factors on chemotaxis and phagocytosis are limited, however. Immunoglobulin M rheumatoid factor was found to decrease significantly the generation of chemotactic activity from fresh serum by immune complexes (Horwitz *et al.*, 1971). However, this inhibition could be abolished by increasing the incubation time of the complexes with fresh serum or by increasing the amount of serum in the reaction mixtures. In the *in vivo* situation it is unlikely that the constraints of limited amounts of serum would have an important effect on rheumatoid factor activity. Immunoglobulin M anti-γ-globulins have also been shown to inhibit the opsonic action of 7 S γ-globulin bound to bacteria. This antiopsonic effect was independent of the ability of the 7 S antibody to potentiate agglutination of the bacteria; rather, it was felt to be owing to the fact that, when the anti-γ-

globulin fixes to the Fc portion of the IgG molecule, it sterically blocks the structures responsible for attachment of the IgG to specific receptors on the surface of neutrophiles (Messner *et al.*, 1968; Williams, 1971). Again, it is important to note that the antiopsonic effects of rheumatoid factor were best demonstrated in the absence of serum, and several preparations of anti-γ-globulin factors did not block phagocytosis when tested in the presence of fresh whole serum. This suggested to the authors that the labile complement components were capable of overcoming the anti-γ-globulin effects (Messner *et al.*, 1968).

The interaction of leukocytes with immune complexes has been studied in detail by Henson (1971a). The initial reaction is adherence of the complex to the leukocyte membrane. The third component of complement is a major contributor to this reaction, and it appears that both polymorphonuclear and mononuclear cells contain a surface membrane receptor for C3. In addition, polymorphonuclear cells have receptors for immunoglobulins which are distinct from the C3 receptor. Aggregates of various classes and subgroups of immunoglobulins have been reacted with leukocytes *in vitro*. There appear to be surface receptors for all the subgroups of IgG and for immunoglobulins of the IgA class, but not for IgM or IgD. Following fixation to the cell membrane, complexes are ingested and, under appropriate circumstances, extrude lysosomal, but not cytoplasmic enzymes (selective enzyme release) (Movat *et al.*, 1964; Pruzansky and Patterson, 1967; Tew *et al.*, 1969; Hawkins and Peeters, 1971; Weissmann *et al.*, 1971; Henson, 1971a).

The release of intracellular enzymes appears to depend on the particulate nature and size of the complex. When neutrophiles were reacted with latex particles having IgG on their surface, they were induced to release β-glucuronidase—the larger the particle the greater the enzyme release (Henson, 1971b). Aggregated IgG of all four subclasses and aggregated IgA caused the release of enzymes from washed human neutrophiles. Five hundred micrograms was an effective stimulus, but no release was obtained with 50 μg. Immunoglobulin M aggregates did not adhere to the leukocyte membrane nor did they induce the release of β-glucuronidase at any concentration studied (Henson, 1971a). The particulate nature of the aggregate is critical for efficient enzyme release. When aggregates of immunoglobulins were made more soluble, by using less bisdiazotized benzidine for cross-linking, there was a several-fold reduction in the amount of β-glucuronidase released. Precipitates of immune complexes made at equivalence, or in slight antigen or antibody excess, are effective releasing agents. However, highly soluble complexes (100 times antigen excess) induce no release of lysosomal enzymes. The exact point at which immune complexes become sufficiently soluble so that

they no longer cause enzyme extrusion is not clear. Hawkins and Peeters (1971) found that complexes made at 20 times equivalence were as effective as insoluble complexes. They used rabbit peripheral blood polymorphonuclear leukocytes as the phagocytic cell. Ward and Zvaifler (1973) and Henson (personal communication) employing rabbit polymorphonuclear leukocytes from peritoneal exudates, did not effect release of lysosomal enzymes with complexes prepared beyond 10 times equivalence.

It is tempting to speculate that the most significant pathogenetic role of IgM rheumatoid factors is in the area of phagocytosis of immune complexes and subsequent lysosomal enzyme release. Several pieces of evidence support this notion. Heat-aggregated γ-globulin is more readily phagocytized from fresh serum containing rheumatoid factor than from fresh normal serum (Parker and Schmid, 1962). When polymorphonuclear leukocytes are incubated with preformed complexes of rheumatoid factor and heat-aggregated IgG, they ingest the complexes and selectively release lysosomal enzymes without suffering cellular injury (Astorga and Bollet, 1965; Weissmann *et al.*, 1971). It is important to note, however, that all of these *in vitro* experiments used particulate complexes—a situation not likely to occur *in vivo*. An important observation in this regard is the finding that normal polymorphonuclear cells do not show evidence of ingestion of immunoglobulins or complement after incubation with fresh synovial fluid from patients with seronegative rheumatoid arthritis. When isolated rheumatoid factor is added to these same fluids, however, cytoplasmic inclusions develop in the leukocytes that stain for IgM, IgG, and C3 (Hurd *et al.*, 1970).

Three possible mechanisms are envisioned whereby IgM rheumatoid factor could enhance the interaction of immune complexes with phagocytic cells: (*1*) by converting soluble complexes into more particulate and less soluble complexes, (2) by increasing the size of an existing immune complex, (3) by converting soluble, non-complement-fixing complexes to complement-fixing ones. There is some *in vitro* evidence to support each of these proposals. For instance, antigen–antibody complexes formed in the presence of rheumatoid factor are less soluble at equivalence and in 3 to 5 times antigen excess than similar complexes formed in the absence of rheumatoid factor (Tesar and Schmid, 1970; Horwitz *et al.*, 1971). In addition, there is a significant increase in size of aggregates formed in antigen excess when formed in the presence of rheumatoid factor (Tesar and Schmid, 1970). Furthermore, preformed complexes reacted with rheumatoid factor are much more difficult to solubilize completely than the complexes alone, even in profound (greater than 100 times) antigen excess (Ward and Zvaifler, 1973). Antigen antibody complexes formed in great antigen excess fix little or no complement. Tesar and

Schmid (1970) have shown, however, that the addition of IgM rheumatoid factor to complexes formed in great antigen excess not only results in their aggregation but also converts them into complement-fixing units. It is easy to see a possible extrapolation of these observations to the *in vivo* situation. In the synovial fluid, IgM rheumatoid factor converts soluble complexes into larger, less soluble complexes, which when phagocytized cause the release of proportionally larger amounts of lysosomal enzymes. The ability of the IgM rheumatoid factors to fix complement may allow these IgM-containing complexes to bind to the leukocyte membrane at the C3 receptor. The complement-fixing ability of rheumatoid factor may also result in enhanced phagocytosis of these large complexes in a manner similar to the enhancement which is noted when complement is added to latex particles or to antigen–antibody complexes adherent to nonphagocytosable surfaces (Henson, 1971c) (see Note Added in Proof No. 2 on p. 336). Current investigations in several laboratories are examining these questions, and definitive answers should be forthcoming in the near future.

V. Conclusions

Having observed the spectacular progress in our understanding of the pathogenesis of joint inflammation in the past few years, it is difficult not to speculate about the developments that will follow. Several things appear clear. One is that the articular cavity will continue to be an ideal location for the *in vivo* dissection of the multiple, interlocking events of the inflammatory response. It appears that this anatomically confined, extravascular space has a limited capacity for clearing the reactants and by-products—both stimulating and inhibitory—of inflammation. Thus we are permitted a slow motion, or stop-action, view; one which is not possible in the circulation. It can be anticipated that the lessons learned in the joint space will soon be applied to other body cavities.

A second observation, and one which requires little perspicacity, is that the search for the immediate cause(s) of rheumatoid arthritis must focus on the synovial membrane, regardless of whether one believes that the etiologic factor(s) is continually disseminated to the joint via the bloodstream or that seeding of the articular cavity has occurred at some earlier time. The nature of this putative primary antigen is, of course, unknown; so is the way in which it sets into motion the immunological reactions that eventuate in the chronic articular inflammation which we recognize as rheumatoid arthritis. But the detection of viral materials in the pathological lesions of certain human diseases which are closely akin to rheumatoid arthritis, such as the Australia antigen in polyarteritis and

anti-DNA and anti-RNA complexes in SLE, suggests that a search for similar agents in rheumatoid arthritis will be profitable.

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Notes Added in Proof

(1) Employing a sensitive radioimmunoassay capable of detecting either soluble nucleoprotein antigen (sNP) or sNP antibody, Robitaille and his collaborators (1973) found sNP antigen in 18 of 31 (58%) synovial fluids of patients with rheumatoid arthritis and in 14 of 23 (61%) fluids from nonrheumatoid patients with inflammatory synovitis. More interesting, however, was the finding that 8 of the 31 (26%) rheumatoid synovial fluids contained antibody to nuclear antigens, whereas, with only one exception, antibody could not be detected in 23 nonrheumatoid synovial effusions. The presence of free sNP antigen or its homologous antibody in synovial fluid of RA patients suggested that at certain times immune complexes might form and contribute to synovial injury.

(2) This second mechanism of neutrophile degranulation (called "frustrated phagocytosis") may have important implications in chronic inflammatory joint disease. When neutrophiles encounter immune complexes along a nonphagocytosable surface, they discharge their constituents without ingesting the complexes. Only minute amounts of antibody are needed; for instance, to achieve 13% release of β glucuronidase requires less than 2 μg. of bound antibody as compared to 31 μg. antibody in a particulate complex (Henson, 1971c). Consider this in the light of the recent observation that, in the chronic experimental arthritis produced by injection of antigen into the knee joints of previously immunized rabbits, the inducing antigen persists for long periods of time complexed to antibody in the surface layers of articular cartilage, menisci, and ligaments (Cooke *et al.*, 1972). In this situation, where antigen and antibody are chronically sequestered, neutrophiles may be continually discharging their enzymes in immediate proximity to articular cartilage in response to minute doses of immune complexes that cannot be removed by conventional phagocytic mechanisms.

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