ADVANCES IN IMMUNOLOGY

Volume 18

F. J. Dixon &
Henry G. Kunkel

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Immunology

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PREFACE

The reviews in Volume 18 deal with subjects ranging from the basic physicochemical changes induced in antibodies by antigens to two important aspects of the control of the immune response: genetic background of the host and antigenic competition to the cellular and humoral events determining immunologic cytotoxicity. The broad scope of this subject matter reflects the ever expanding horizons of immunologic interest and research which continue to contribute importantly to almost every field of biology and medicine.

David L. Gasser and Willys K. Silvers present in the first article a thorough and authoritative consideration of the genetic determinants of immunologic responsiveness. They review both the subject of the Ir genes' determination of specific antibody responses and also less specific genetic influences on general immunoresponsiveness. Finally, the association of various genetic factors with susceptibility to particular diseases, specifically autoimmune disorders and tumors, is critically discussed.

In the second paper, the several forms of cell-mediated cytotoxicity are discussed by Jean-Charles Cerottini and K. Theodore Brunner, whose studies in this field have contributed greatly to our current understanding. The antibody-independent action of sensitized cytotoxic T cells, the antibody-dependent killing by nonsensitized lymphoid cells bearing receptors for the Fc piece, the specific cytotoxicity of macrophages armed by either cytophilic antibody or products of T cell interaction with specific antigen, and, finally, the nonspecific cell killing by macrophages activated by a variety of nonspecific stimuli are presented in terms of the extensive *in vitro* experimental data available. The possible roles of these several mechanisms in the complex processes of *in vivo* rejection of allografts and tumor immunity are discussed.

The important problem of interference and enhancement between two different antigenic challenges is presented by Hugh F. Pross and David Eidinger in the third review. Survey of the literature suggests that a variety of mechanisms may operate at different points in the immune response to produce what the authors term "antigen-induced suppression" and "antigenic promotion." One attractive hypothesis explaining many of these observations is that they result from nonspecific manifestations of normal immunoregulatory phenomena and that products of T cells may well be among the more important immunoregulatory substances which determine the number of antigen-reactive units.

The nature of the critical change in the properties of antibodies upon combination with antigen is discussed by Henry Metzger in the fourth

article. Against a background of information on the structure of intact immunoglobulins and their degraded products, on the characteristics of antigen–antibody complexes, and on the requirements for antigen-induced antibody function, the allosteric and distortive models for antigen action are found wanting and an associative model based upon an approximation of Fc regions is favored. This presentation brings into sharp focus one of the most important steps in the sequence of events leading to significant biological consequences of immunologic reactions.

As a result of the investigations of Karl Erik Hellström and Ingegerd Hellström, it is now clear that the final effect of an immune response on a tumor depends upon the interaction of both cellular and humoral components, which often behave antagonistically. In the fifth paper these authors discuss the evidence for cell-mediated antitumor responses in man and animals in terms of *in vitro* destruction of tumor cells and the means by which tumor cells can escape from immunologic destruction as a result of blocking factors present in the serum. The potential benefit of manipulation of these two immune responses in order to maximize cellular cytotoxicity as a therapeutic approach is considered. The simultaneous operation of cellular cytotoxic mechanisms and humoral blocking factors is not limited to neoplastic disease but apparently is a general phenomenon also occurring in pregnancy, allografts, and chimeras.

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FRANK J. DIXON
HENRY G. KUNKEL

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Genetic Determinants of Immunological Responsiveness

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I. Introduction

The study of genetic differences in the ability of animals to produce specific immune responses has attracted a great deal of attention from geneticists and immunologists for several reasons: first, the possibility exists that these studies will play a key role in understanding the generator of diversity: second, studies on genetic differences in the ability of immunocompetent cells to respond to specific antigens may help

elucidate the steps involved in the differentiation of these cells; and third, the fact that some of the genetic determinants of the immune response seem to be intimately associated with histocompatibility antigens could be of clinical significance.

To focus attention on the activity in this field, various genetically determined immune response systems are listed in Table I. Our failure to discuss some of these reports in what follows is not to minimize their significance. After perusing the table, one cannot help but be impressed by the number and variety of specific immune responses which have been subjected to genetic analysis.

II. Some General Genetic Considerations

The genetic control of the immune response is a subject that has attracted much greater interest among immunologists than among geneticists. Because of this, some individuals with the greatest interest in this field are not conversant with some of the basic genetic concepts which need to be employed. What follows is a brief description of a few of these concepts for the benefit of readers whose background in genetics is not extensive.

Of primary importance in analyzing the factors involved in immune responsiveness is the necessity to make a distinction between variability which is genetic and that which is nongenetic in origin. In many cases the response to a given antigen will be determined by both genetic and nongenetic factors. It has been the experience of many investigators that highly inbred strains may show considerable variability in their responses to some particular antigen. Unless the strain is not really inbred, this variability must be considered nongenetic. Any variability in an F_1 generation obtained by mating two fully inbred strains must likewise be considered nongenetic. However, the variability appearing in F_2 and backcross generations arises from both genetic and nongenetic components, although in some cases one or the other of these components could be insignificant.

If intrastrain variation is observed in the response to some particular antigen, an experimenter can determine whether this variability is genetic and the strain is not genetically uniform or whether it is nongenetic by making assortative matings within the strain or between F_1 hybrids derived from it. High responders should be mated with high responders and low responders with low responders and the offspring immunized. If parent–offspring correlation is not observed in such an experiment, then one can assume that all the intrastrain variation is nongenetic. An example of how this was applied to an F_1 generation is shown in Table II. Most YBR mice were responders to Ea-1, most B10 mice were non-

responders, and two-thirds of the (YBR × B10)F_1 hybrids were responders. If these results occurred because one or both of the parental strains were not fully inbred or possessed mutations affecting this response, the F_1 responders would differ genetically from the F_1 nonresponders, and the two types of matings would produce significantly different proportions of responders. This experiment demonstrated, however, that the responders and nonresponders from the (YBR × B10)F_1 generation did not differ genetically with respect to their antibody responses to Ea-1 antigens. The importance of genetic factors was demonstrated by making assortative matings with F_2 and F_3 mice as parents (Gasser, 1970). Since there was phenotypic variation within each parental strain and the F_1 generation and since this could not be attributed to genetic heterogeneity within either of the parental strains, the genes for high response and low response are, therefore, said to be "incompletely penetrant."

The problem of incomplete penetrance, the phenomenon by which an organism does not express a genetically endowed trait, is an old one in genetics and arises from the fact that the expression of some genetic traits can be influenced by environmental factors. In a classic paper on polydactyly in guinea pigs, Wright (1934) demonstrated that the age of the mother influenced the expression of genes controlling the number of toes in her offspring. Any number of nongenetic factors could conceivably affect the expression of an immune response (Ir) gene. The important distinction for an investigator to make is to assess properly the degree to which such factors are influencing the data.

If a significant degree of nongenetic variability is present in the parental strains, then this variation must also be taken into consideration in all generations derived from these strains (e.g., F_2 and backcross generations). Several methods have recently been published which allow an experimenter to evaluate genetic hypotheses when the results are confounded by significant amounts of nongenetic variation (Mode and Gasser, 1972; Birnbaum, 1972; Elston and Stewart, 1973).

Having established that one is dealing with a genetically determined immune response, a decision which most investigators attempt to make is whether this response is controlled by a single gene or by more than one gene. If an unambiguous distinction can be made between high responders and low responders (or responders and non-responders), then the F_2 and backcross generations should furnish data which either do or do not conform to Mendelian expectations. Even if a single-gene hypothesis is supported by such data, it is still possible that other genes could be involved. In the course of his studies on the inheritance of polydactyly in guinea pigs, Wright (1934) showed that data

TABLE I
VARIOUS GENETICALLY DETERMINED IMMUNE RESPONSES[a]

Antigen	Host	Reference	Comments
Hapten–PLL conjugates, BSA, HSA, GA, GT	Guinea pig	Section III	Linked to major histocompatibility locus
Glucagon–PLL	Guinea pig	Cuatrecases *et al.*, 1971	Linked to major histocompatibility locus
Hapten–guinea pig albumin conjugates	Guinea pig	Section III	Linked to major histocompatibility locus
Insulin specificities	Guinea pig	Section VIII	
Diphtheria toxin	Guinea pig	Section III	
Hydralazine	Guinea pig	Section III	Not linked to major histocompatibility locus
(T,G)-A- -L, (H,G)-A- -L, (P,G)-A- -L	Mouse	Section IV	*Ir-1;* Linked to *H-2*
TNP–MSA	Mouse	Rathbun and Hildemann, 1970	*H-2* Linked; low response dominant
GAT_{10}	Mouse	Section IV	*H-2* Linked
GLA_5	Mouse	Section IV	
(T,G)-Pro- -L	Mouse	Section IV	*Ir-3;* Not linked to *H-2*
Ovalbumin and ovomucoid	Mouse	Vaz *et al.*, 1971	Single dominant gene; *H-2* linked
Poly-Ser, poly-Ala	Mouse	Bosma *et al.*, 1972	Poly-Ser-specific clones associated with C3H allotype; poly-Ala-specific clones with C57BL allotype
H-Y histocompatibility antigen	Mouse	Section V	Probably more than one gene involved, one of which is *H-2* linked
H-2.2	Mouse	Stimpfling and Durham, 1972	*H-2* Linked; probably more than one gene
H-2.2	Mouse	Lilly *et al.*, 1970	Not linked to *Ir-1* or *Ir-2*
θ Thymic antigen	Mouse	Fuji *et al.*, 1972	*H-2* Linked; may be more than one gene
γG_2a	Mouse	Kindred and Weiler, 1971	Polygenic; ability to respond was recessive.
IgA and IgG myeloma proteins	Mouse	Section V, I	*H-2* Linked
BSA	Mouse	Section V	
H-3	Mouse	Berrian and McKhann, 1960	*H-2* Incompatibility increases response to *H-3*[a]

H-13	Mouse	Snell *et al.*, 1967; Graff *et al.*, 1973	*H-3*^{a} mice can recognize *H-13* incompatibility; *H-3*^{b} mice cannot
SRBC	Mouse	Section V	Polygenic
SRBC	Mouse	Sabolovic *et al.*, 1971	Importance of *H-2* was demonstrated
SRBC	Mouse	Gottlieb *et al.*, 1972	The degree to which AKR spleen cells could proliferate in response to SRBC in various irradiated hosts was determined by the genotype of the host, with C3H being a good host and AKR a poor one.
Ea-1 Mouse blood groups	Mouse	Section V	
Escherichia coli Vi antigen	Mouse	Gaines *et al.*, 1965	Strain differences in formation of complete antibodies were demonstrated; responder and nonresponder strains could both produce incomplete antibodies.
Bacteriophage fd	Mouse	Section V	
Influenza viruses	Mouse	Lennox, 1966	
S-III pneumococcal polysaccharide	Mouse	Section VI	X-Linked
Salmonella lipopolysaccharides	Mouse	DiPauli, 1972	Strain differences in the magnitude of response as well as specificity of antibodies produced were observed.
Streptococcal group A carbohydrate	Mouse	Braun *et al.*, 1972	BALB mice not only produced a higher response than other strains, but also showed a high degree of restriction; effect was thymus-dependent; not linked to *H-2* or *Ig*.
Tetanus toxoid	Mouse	Section V	
Lysozyme	Mouse	Mozes *et al.*, 1971	Strain differences were observed in the production of antibodies to a portion of the lysozyme molecule attached to a synthetic polymer.
Thyroid antigen	Mouse	Section X	*H-2* Linked

(*Continued*)

TABLE I (*Continued*)

Antigen	Host	Reference	Comments
DNP–RSA	Mouse	Weinstein *et al.*, 1972	Strain-related differences in the number of antibody-combining sites that could be labeled with bromoacetyl derivatives were observed.
Dextran specificities	Mouse	Section VIII	
p-Aminobenzoic acid and sulfanilic acid	Mouse	Škárová and Říha, 1969; Říhová-Škárová and Říha, 1972a,b	
T blood group	Mouse	Stimpfling and McBroom, 1971	*H-2* Incompatibility between donor and host enhanced response to T.
GLϕ	Mouse	Merryman *et al.*, 1972	*H-2* Linked
SRBC	Mouse	Silver *et al.*, 1972	After repeated injections of SRBC, A/J mice switch from 19 S to 7 S production; C57BL/10 continues to make predominantly 19 S; F_1 hybrids respond like C57BL/10; multigenic.
Keyhole limpet hemocyanin	Mouse	Cerottini and Unanue, 1971	Quantitative differences observed in serum antibody titers; dominant gene or genes
Mammary tumor virus	Mouse	Section VII	
Lymphocytic choriomeningitis (LCM) virus	Mouse	Oldstone and Dixon, 1968, 1969	Strain differences in the ability to make anti-LCM antibody lead to varying degrees of severity of disease. Glomerulonephritis is most severe in those strains with the best antibody response.
O antigen of *Rhizobium meliloti*	Mouse	Sobey and Adams, 1961	Heritability of response was estimated to be 0.78.
Egg albumin	Mouse	Treadwell, 1969	Susceptibility to anaphylactic shock was inherited as a polygenic trait.
Dextran	Rat	Harris and West, 1961, 1963; Harris *et al.*, 1963	Failure to form anaphylactoid reaction determined by autosomal recessive gene.

$Glu^{52}Lys^{33}Tyr^{15}$	Rat	Simonian *et al.*, 1968; Gill *et al.*, 1970, 1971; Gill and Kunz, 1971; Sloan and Gill, 1972	Quantitative difference in antibody titer controlled polygenically.
(T,G)-A- -L	Rat	Section V	Linked to *Ag-B*
DNP-BGG	Rat	Lamelin and Paul, 1971	A strain difference in the rate of increase of antibody affinity under certain conditions of immunization was demonstrated.
H-Y histocompatibility antigen	Rat	DiMarco *et al.*, 1972	Females that normally do not reject male skin can be made to do so if the Y antigen is on the same tissue as Ag-B incompatible antigens.
GAT_{10}	Rat	Maurer *et al.*, 1972	Two of nine strains tested were nonresponders; no association with *Ag-B* observed.
Spinal cord	Rat	Section XI	Susceptibility to experimental allergic encephalomyelitis is controlled by *Ag-B* or a closely linked gene.
Lactic dehydrogenase	Rat, rabbit	Section V	
Hg blood group specificities	Rabbit	Cohen, 1962	Antibodies to determinants of the complex *Hg* blood group locus are most easily produced when donor and recipient are incompatible for all known Hg specificities. When donor and recipient share an Hg specificity, it is less likely that an antibody to another Hg determinant can be produced.
Groups A and C streptococcal vaccines	Rabbit	Eichmann *et al.*, 1971	The magnitude and degree of heterogeneity of the antibody response are genetically determined and were shown to be independent variables.
Tobacco mosaic virus	Rabbit	Sang and Sobey, 1954	Heritability of antibody titer was estimated as 0.876.

(*Continued*)

TABLE I (*Continued*)

Antigen	Host	Reference	Comments
Bovine serum albumin	Rabbit, mouse	Section V	
Graft-versus-host reactions	Chicken	Longenecker *et al.*, 1972	Blood group *B* locus determined magnitude of response.
Blood group A	Chicken	Schierman and McBride, 1967	Response was enhanced by incompatibility at the strong blood group *B* locus.
Sheep O blood group	Cattle	Section V	
Protein antigens	Man	Cooke and Vander Veer, 1916	The importance of heredity in various kinds of sensitivities was recognized.
Rh blood group	Man	Litwin, 1972, 1973	Suggestive evidence was obtained for anti-Rh V-region genes linked to *IgG1* allotype locus.
ABO agglutinins	Man	Section V	
Ragweed	Man	Section VIII	

[a] Abbreviations: PLL, poly-L-lysine; BSA, bovine serum albumin; HSA, human serum albumin; G, L-glutamic acid; A, L-alanine; T, L-tyrosine; L, L-lysine TNP, trinitrophenol; MSA, murine serum albumin; DNP, dinitrophenyl; SRBC, sheep red blood cells; BGG, bovine γ-globulin; Ig, immunoglobulin, (T,G)-A--L, poly(Tyr, Glu)-poly-DL-Ala--poly-Lys; H, histidine; P, phenylalanine; RSA, rabbit serum albumin.

TABLE II
POSITIVE ASSORTATIVE MATINGS OF (YBR × B10)F_1 RESPONDER AND NONRESPONDER PARENTS[a]

Mating	F_2 Responders (No.)	F_2 Nonresponders (No.)	% Responders
(a) F_1 responder × F_1 responder	14	24	37
(b) F_1 nonresponder × F_1 nonresponder	13	26	33
Contingency $X^2 = 0.009$, $p > 0.90$			

[a] From Gasser, 1970.

on F_2 and backcross generations obtained from the inbred strains D and 2 were compatible with single-gene segregation, but that this mode of inheritance was not substantiated when second and third backcross generations were examined. A similar situation concerning the anti-allotype response of mice was recently reported by Kindred and Weiler (1971).

If an investigator has identified a single gene involved in a specific immune response, he may attempt to do linkage analyses. Genetic linkage is an important exception to Mendel's second law—the law of independent assortment. If two genetic loci are situated on the same chromosome and are not too distant from one another, they will not assort independently. The frequency with which they recombine is determined by the distance that separates them. Genetic linkage, however, does not necessarily imply any functional relationship between two genes. For example, the genes for albinism and hemoglobin beta chains are closely linked in mice, but there is no apparent functional relationship between these traits. Furthermore, if two loci are linked, it makes no difference as to which alleles are present at these loci. For example, the *H-2K* locus is known to have at least eight alleles. The *T1a* locus is linked to *H-2K* regardless of which allele is present at this locus in any given experiment. The only requirement for demonstrating linkage is that one parent must be heterozygous at both loci. A different situation arises when a seemingly unrelated biological effect is known to be associated with a given allele at some genetic locus. It is fairly well established that the *A* allele of the *ABO* blood group locus in man is associated with a greater frequency of stomach cancer than the other alleles at this locus (Aird *et al.*, 1953; Vogel, 1970). Unless a closely linked gene is responsible for these cancers, which is highly unlikely in a random breeding population, this situation is not the same as genetic linkage. Rather, the *A* allele for

some unknown reason causes an individual to be somewhat more susceptible to gastric cancer than the other alleles.

In studies on the genetics of the immune response, it has frequently been observed that the possession of certain alleles at a major histocompatibility locus is correlated with a characteristic level of response to some particular antigen. There are two reasons why this could occur, and they are very different from one another: (*1*) there could be an association between the possession of certain histocompatibility antigens and the range of antigenic diversity to which an organism is capable of responding or (2) there could be a separate genetic locus, an Ir gene, which is closely linked to the histocompatibility locus. If possibility 1 is correct, then what appears to be an Ir gene is actually a histocompatibility locus. If possibility 2 is correct, then the Ir gene could conceivably have no functional relationship at all to histocompatibility antigens.

III. Specific Immune Response Genes in Guinea Pigs

The discovery of the first specific Ir gene in guinea pigs was stimulated by investigations on the nature of antigenicity. Kantor *et al.* (1963) reported that good immune responses could be obtained in some random bred guinea pigs after they were injected with dinitrophenyl (DNP)–poly-L-lysine (PLL) or a DNP copolymer of lysine and glutamic acid (GL). The responding animals demonstrated both immediate, and delayed hypersensitivities and produced appreciable amounts of antibody, whereas animals with negative skin reactions did not seem to be immune to the injected antigen, since there was no sign of anaphylaxis upon intravenous challenge with DNP–guinea pig albumin. This striking all-or-none difference among the guinea pigs seemed to reflect constitutional differences which were presumed to be genetically determined. This assumption was verified by breeding experiments, in which it was shown that the immune response difference could be explained by the segregation of two alleles at a single locus (Levine *et al.*, 1963a; Levine and Benacerraf, 1965). Ten matings consisting of nonresponders crossed with heterozygous responders produced 31 progeny, 14 of which were responders, so that the trait appeared to be determined by a single gene (Levine and Benacerraf, 1965).

All guinea pigs of strain 2 were responders to DNP–PLL and DNP–GL, no animals of strain 13 were responders, and the (2 × 13)F_1 hybrids gave a response which is the same as that of strain 2, so that responsiveness is inherited as a completely dominant trait (Ellman *et al.*, 1970a).

In order to determine whether this gene had specificity for the carrier or the hapten, Levine *et al.* (1963b) immunized a group of 33 Hartley

guinea pigs simultaneously with four different PLL conjugates: benzylpenicilloyl–PLL, DNP–PLL, *p*-toluene sulfonyl–PLL, and dimethylaminonaphthalene sulfonyl–PLL. Every guinea pig in the group either responded to all four of the non-cross-reacting haptens or to none of them. This demonstrated that the specificity of the gene was associated with the PLL carrier rather than with any hapten attached to it, and the gene was subsequently referred to as the *PLL* gene.

Genetic nonresponder guinea pigs (e.g., strain 13) could be induced to produce low levels of anti-DNP antibodies if repeatedly immunized with DNP–PLL mixed with 10 mg/ml. *Mycobacterium tuberculosis*. Apparently the mycobacteria were able to function as a carrier for the DNP–PLL (Green *et al.*, 1969a,b). Nonresponder animals immunized by this method, however, failed to develop delayed hypersensitivities to DNP–PLL or DNP–GL.

The antibody produced in genetic nonresponders immunized with DNP–PLL mixed with *Mycobacterium tuberculosis* belonged only to the γ_2 class. That no γ_1 antibodies were detected in these sera contrasted sharply with the results obtained with genetic responder guinea pigs, in which high concentrations of γ_1 antibodies were generally found (Green *et al.*, 1969a).

Levine (1969) reported that PLL nonresponders also failed to respond to DNP conjugates of poly-L-arginine, poly-L-homoarginine, and poly-L-ornithine, which suggested that the same gene controlled the response to all four conjugates of these basic polyamino acids. Similar findings were reported by Green *et al.* (1969b), who discovered that the delayed hypersensitivity response to some of these antigens was not controlled by the *PLL* gene as was the case with the antibody response. Levine also observed an interesting correlation between the rank order of immunogenicity of DNP conjugates of the four homopolyamino acids and the rank order for the hydrolysis of these conjugates by trypsin, but the significance of this correlation remains to be clarified (Levine, 1969).

The ability of guinea pigs to respond to low doses of bovine serum albumin (BSA) is also inherited as a dominant genetic trait. No recombinants were observed between this gene and the *PLL* gene among $(2 \times 13)F_1 \times 13$ backcross animals, and all PLL responders among Hartley guinea pigs were high responders to low doses of BSA. However, results with the Hartley PLL nonresponders were somewhat different in that some of these animals produced substantial amounts of antibody when immunized with low doses of BSA, but always less than what was observed in responder animals (Green *et al.*, 1970). Perhaps the *PLL* gene is associated with response to one determinant of BSA, and some PLL nonresponders possess another gene associated with recog-

nition of another BSA determinant. Further breeding tests and studies on the specificities of the antibodies could determine whether this is the case.

Genetic control of the response to low doses of human serum albumin (HSA) was found to parallel the situation with BSA (Green and Benacerraf, 1971). Because the ability to respond to limiting doses of HSA and BSA was not restricted to PLL responder Hartley guinea pigs, it would appear that there is at least one gene controlling responses to BSA and HSA which is not the same as the *PLL* gene.

Perhaps the most interesting experiments on responses to hapten–serum albumin conjugates are those involving the use of guinea pig albumin (GPA) as a carrier (Green and Benacerraf, 1971; Green *et al.*, 1972; Davie *et al.*, 1972). When immunized with DNP_6–GPA, strain 13 guinea pigs developed significantly higher anti-DNP serum antibody levels than strain 2 animals, even when the GPA was obtained from strain 13 guinea pigs. The gene controlling this response is linked to the major histocompatibility locus and appears to have specificity for the carrier rather than the hapten (Green *et al.*, 1972). Presumably the carrier is modified to some extent by the addition of DNP, but the fact that an Ir gene with specificity for a self-protein, even if a slightly modified self-protein, may exist is most intriguing.

In order to determine how Ir genes exert their effects, it is necessary to identify the cell population in which the processes controlled by these genes are expressed. Green *et al.* (1967) demonstrated that passive transfer of delayed sensitivity to DNP–PLL and DNP–GL among Hartley guinea pigs could usually be successfully accomplished only by transferring cells from immunized responder animals into other genetic responder guinea pigs. These investigators suggested that a cell type other than an immune lymphocyte (e.g., a macrophage) was also essential in producing this response and that genetic nonresponders were deficient in some way with respect to this cell type. It was subsequently shown, however, that if irradiated genetic nonresponders were reconstituted with bone marrow cells from responders, most of them could develop delayed hypersensitivity when challenged with DNP–PLL after sensitization with this antigen (Foerster *et al.*, 1969). Lymph node and spleen cells were even more effective than bone marrow in achieving this result. It was concluded that the process controlled by the *PLL* gene finds expression in some type of lymphoid cell rather than in monocytes or macrophages. The discrepancy between the findings of Green *et al.* (1967) and Foerster *et al.* (1969) has not been entirely resolved but may suggest that donor macrophages histocompatible with recipient lymphocytes are required for an effective interaction to occur (E. M. Shevach and A. S. Rosenthal, personal communication).

The discovery by McDevitt and Tyan that the *Ir-1* gene of mice is closely linked to the *H-2* histocompatibility locus (see Section IV,A) stimulated Benacerraf and his colleagues to search for evidence of linkage between the *PLL* gene and the major histocompatibility locus of guinea pigs. Among 18 backcross progeny of the generation $(2 \times 13)F_1 \times 13$, the 9 animals that were responders possessed the $(2 \times 13)F_1$ histocompatibility type, whereas the 9 animals that were nonresponders possessed the strain 13 histocompatibility type, thus demonstrating that the *PLL* gene is, indeed, linked to the guinea pig's major histocompatibility locus. Typing for the major histocompatibility locus was accomplished by both the mixed leukocyte culture test and ^{51}Cr release from lymph node cells exposed to strain 13 antiserum directed against strain 2 antigens (Ellman *et al.*, 1970b).

This linkage was confirmed by the use of a third technique, that is, susceptibility to L_2C leukemia, a lymphatic leukemia that arose spontaneously in a strain 2 guinea pig. When 15 $(2 \times 13)F_1 \times 13$ backcross animals were inoculated with 2×10^6 L_2C cells, all 9 PLL responders were dead within 44 days, whereas all 6 PLL nonresponders were alive and free of leukemia at the end of 3 months (Ellman *et al.*, 1971a).

Two random-bred lines of guinea pigs, the Hartley and NIH strains, were tested for major histocompatibility antigens and PLL responsiveness. Among 78 Hartley guinea pigs (42 PLL responders and 36 nonresponders) and 14 NIH multipurpose guinea pigs (1 PLL responder), the lymphocytes of all PLL responders were susceptible to lysis by cytotoxic antistrain 2 antiserum (Ellman *et al.*, 1971a). The complete correlation between PLL responsiveness and possession of the strain 2 specificity suggests that no recombination had occurred between the major histocompatibility locus and the *PLL* gene among these random-bred stocks.

Bluestein *et al.* (1971a) reported that a random copolymer of L-glutamic acid and L-alanine (GA) was immunogenic in strain 2 but not in strain 13 guinea pigs, whereas a copolymer of L-glutamic acid and L-tyrosine (GT) was immunogenic in strain 13 but not in strain 2. The $(2 \times 13)F_1$ hybrids responded to both GA and GT. These observations suggested that two separate genes were associated with response to GA and GT and that both were dominant over the alleles for nonresponsiveness. Among random-bred Hartley guinea pigs, most animals capable of responding to GA could respond to PLL and most nonresponders to GA were nonresponders to PLL; but several exceptions to this rule suggest that the *GA* and *PLL* genes were nonidentical (Bluestein *et al.*, 1971b). When 9 progeny of the backcross generation $(2 \times 13)F_1 \times 13$ were simultaneously immunized with GA and DNP–PLL, 4 responded to both GA and PLL, whereas 5 responded to neither GA nor PLL. The 4 GA

and PLL responders were shown to possess the major histocompatibility type of strain 2, whereas the 5 nonresponders all lacked the major strain 2 histocompatibility specificities (Bluestein *et al.*, 1971c). These observations suggested not only that the *GA* and *PLL* genes were linked but that both genes were linked to the major histocompatibility locus. Among 17 progeny of the reciprocal backcross generation, $(2 \times 13)F_1 \times 2$, 9 animals were responders to GT and all possessed the strain 13 major histocompatibility specificities, whereas the remaining 8 guinea pigs that were nonresponders to GT were shown to lack these specificities. This observation confirmed that the locus controlling GT responsiveness was linked to the major histocompatibility locus of the species (Bluestein *et al.*, 1971c). Further evidence for this linkage was obtained when it was shown that among random-bred Hartley guinea pigs, all GT responders possessed one of the major strain 13 histocompatibility specificities (Bluestein *et al.*, 1971d).

Since separate genes were shown to control the immune responses to GA and GT, it was of great interest to study guinea pigs immunized with a random polymer of L-glutamic acid, L-alanine, and L-tyrosine (GAT). Bluestein *et al.* (1972) found no cross-reactivity between delayed hypersensitivity skin reactions specific for GA and GT. All inbred strain 2 and 13 guinea pigs developed delayed hypersensitivity and circulating antibodies directed against GAT. However, the anti-GAT sera from strain 13 guinea pigs (GA nonresponders) were not able to bind GA–HSA–^{125}I, whereas anti-GAT sera from strain 2 guinea pigs (GA responders) were able to bind significant amounts of this ligand. The *GT* gene did not have a similar effect on the specificity of anti-GAT sera. The demonstration that the specific Ir gene, *GA*, can affect the specificity of an animal's response to a complex antigen is similar to work done in mice on the response to (Phe,G)–Pro--L (see Section IV,B) and is reminiscent of studies on the response of guinea pigs to insulin (Arquilla and Finn, 1963, 1965). An important implication of these studies is that the immune response to a complex antigen may be controlled by more than one Ir gene, with each Ir gene being associated with the response to one determinant of the complex antigen.

The response of guinea pigs to immunization with hydralazine in Freund's complete adjuvant (CFA) was investigated by Ellman *et al.* (1971b). These authors observed that strain 13 and Hartley guinea pigs were able to mount a vigorous immune response to this drug, but that strain 2 guinea pigs were nonresponsive to the same antigen. The ability to produce this response was inherited as an autosomal dominant trait unlinked to the major histocompatibility locus and, therefore, not linked to the *PLL* gene.

One of the most incisive experiments on the cellular effects of Ir

genes was recently reported by Shevach *et al.* (1972). Alloantisera against guinea pig histocompatibility antigens were produced, and the effects of these heat-inactivated sera on lymphocytes stimulated by GL and GT were studied. Peritoneal exudate lymphocytes were incubated with antigen for 30 minutes, washed 3 times to remove excess antigen, and cultured for 72 hours. Four hours prior to harvesting, tritiated thymidine was added to each culture. The amount of tritiated thymidine incorporated into cellular deoxyribonucleic acid (DNA) was used as a measure of the degree of antigenic stimulation. Strain 2 lymphocytes incubated with DNP–GL incorporated an average of 13,409 c.p.m., but if strain 13 anti-2 sera were included in the media, only 380 c.p.m. were recorded. Strain 13 lymphocytes responded to GT with 3,612 c.p.m., but if strain 2 anti-13 sera were included in the media, only 320 c.p.m. were recorded. In order to determine whether this represented a specific inhibitory effect or a nonspecific blocking effect, this experiment was repeated by using lymphocytes from $(2 \times 13)F_1$ guinea pigs. These lymphocytes are capable of responding to both GT and GL and possess the histocompatibility antigens of both parental strains, 2 and 13. Responses to phytohemagglutinin (PHA) were included as a specificity control. Because in all cases the proliferative response to PHA was less inhibited than the response to antigen, the results were expressed as the ratio of response to antigen to the response induced by PHA. The response to purified protein derivative (PPD) was included for comparison, since this response was not known to be genetically determined.

Representative results from this truly elegant experiment are shown in Table III. There can be no doubt that strain 13 anti-2 sera markedly

TABLE III

INHIBITION OF LYMPHOCYTE PROLIFERATION IN F_1 ANIMALS[a]

	Sera[c]			
Stimulant[b]	Normal	13 Anti-2	2 Anti-13	2 Anti-13 + 13 anti-2
None	3,499	1,479	1,484	1,494
DNP-GL	68,361 (1.21)	1,231 (0.0)	46,385 (1.69)	2,178 (0.02)
GT	17,070 (0.25)	12,147 (0.25)	1,770 (0.01)	3,793 (0.07)
PPD	20,049 (0.31)	12,086 (0.25)	15,159 (0.51)	4,344 (0.09)
PHA	56,887 (1.00)	44,281 (1.00)	28,094 (1.00)	34,940 (1.00)

[a] From Shevach *et al.*, 1972.

[b] Abbreviations: DNP, dinitrophenyl; G, L-glutamic acid; L, L-lysine; T, L-tyrosine; PPD, purified protein derivative; PHA, phytohemagglutinin.

[c] Results are expressed as counts per minute per tube; each value is the mean of three determinants. Fraction of PHA response is given in parentheses.

inhibited the response to DNP–GL, whereas the same effect occurred on the GT response when strain 2 anti-13 sera were used.

To rule out the possibility that inhibition was caused by blocking cell surface immunoglobulins, the alloantisera were absorbed with γ-globulin of the opposite strain. No decrease in the inhibitory activity of either of the alloantisera was observed after these absorptions. It was concluded that Ir genes produce a cell surface-associated product and that this product plays a role in the mechanism of antigen recognition by the T lymphocytes.

In an interpretation which was at variance with that of Green and his colleagues, Levin *et al.* (1971) have suggested that bone marrow-derived cells may be critically involved in the action of the *PLL* gene. By fluorescence quenching, these authors studied the exact specificity of anti-DNP antibody produced by Hartley guinea pigs immunized with a series of defined α,DNP– and ε,DNP–oligolysines. When the antigens were incorporated into an adjuvant containing *Mycobacterium tuberculosis*, both responders and nonresponders produced anti-DNP antibodies, whereas only responders developed delayed sensitivities as in the experiments of Green *et al.* (1969a). However, Levin and his colleagues discovered an important difference in the anti-DNP antibody produced under these conditions. All responder animals made anti-DNP antibodies that recognized the precise chain length, ± one lysyl residue, of the DNP–oligolysines used to induce the immune response as measured by an increase in binding energy for that antigen. In contrast, antibodies made by nonresponder animals to the identical antigens showed no greater binding energy with the immunizing antigen than for DNP–oligolysines of other chain lengths. These authors concluded that antigen recognition by the antibody-forming cell is directed not only to the hapten but to the local environment of the hapten as well, and that the *PLL* gene exerts its control in the antibody-producing cell (Levin *et al.*, 1971).

More recently, Schlossman and his colleagues have used the method of isoelectric focusing to examine the anti-DNP-oligolysine antibodies produced by responder and nonresponder guinea pigs. This method is the most sensitive technique presently available to identify the products of individual antibody-producing clones. Every clone which Schlossman observed in more than one animal was seen in both responder and nonresponder guinea pigs. One clone was seen ten times in individual responder guinea pigs and the same clone was observed seven times in nonresponder animals. These results strongly suggest that there is no detectable difference between responder and nonresponder B cell populations. Although the results reported by Levin *et al.* (1971) remain

enigmatic, Schlossman interprets his most recent findings as supporting the hypothesis that the *PLL* gene is expressed exclusively in T cells (Schlossman, 1972).

IV. Responses of Mice to Synthetic Polypeptide Antigens

A. Responses to (T,G)-A--L, (H,G)-A--L, and (P,G)-A--L: The *Ir-1* Gene

A very important series of experiments was initiated by the observations of McDevitt and Sela (1965) on the responses of inbred mice to the branched multichain synthetic polymer, poly(Tyr,Glu)-poly-DL-Ala--poly-Lys[(T,G)-A--L]. These authors demonstrated that when strains CBA and C57 were given footpad injections of (T,G)-A--L in Freund's adjuvant followed 5 weeks later by another injection of the antigen in saline, the antigen-binding capacity of the C57 sera was approximately 10 times the antigen-binding capacity of the CBA sera. The dosage used for demonstrating this effect was 10 μg. in both the primary and secondary injections, but essentially the same result was obtained when the dosage was increased to 100 μg. This quantitative difference in antibody production was inherited as a dominant trait, and breeding experiments suggested that a single gene was largely responsible for this capacity. When these same strains were immunized with (H,G)-A--L, a synthetic polypeptide similar to (T,G)-A--L except for the substitution of tyrosine by histidine, CBA mice gave a good response whereas C57 mice gave no response at all (McDevitt and Sela, 1965). When (CBA $\times$ C57)F_1, $F_1 \times$ CBA, and $F_1 \times$ C57 mice were immunized with 100 μg. (H,G)-A--L, boosted 5 weeks later and tested 10 days after receiving the booster, it was apparent that the difference in the ability of CBA and C57 mice to respond to (H,G)-A--L was under genetic control. High response is dominant over low response, and more than one genetic factor seems to be involved (McDevitt and Sela, 1967). As discussed in Section II, it is sometimes difficult to assess the number of genes involved in the determination of a quantitative trait. The suggestion of McDevitt and Sela (1967) that high response to (H,G)-A--L was largely determined by a single gene was borne out by studies on congenic strains (McDevitt and Chinitz, 1969) despite indications than an unknown number of modifier genes could be involved (McDevitt and Sela, 1967).

The *PLL* gene of guinea pigs was shown to have specificity for the carrier rather than the haptenic portion of the immunizing antigen (Section III). To determine whether the same principle applied to *Ir-1*, as this Ir gene was named (Herzenberg *et al.*, 1968), Mozes and McDevitt (1969) immunized CBA and C57 mice with lightly substituted

(L.S.) or heavily substituted (H.S.) DNP–(T,G)-A--L and DNP–(H,G)-A--L. When sera from these mice were assayed for anti-DNP antibodies, the high responders to (H,G)-A--L (CBA) produced better responses to DNP–(H,G)-A--L than the low responders to (H,G)-A--L (C57), when either L.S. or H.S. carriers were used. The high responders to (T,G)-A--L (C57) produced better anti-DNP responses to DNP–(T,G)-A--L than the low responders to (T,G)-A--L (CBA) when L.S. carriers were used. Since substitution with many DNP groups changes the antigenic determinants on the carrier, it was not surprising that the same correlation was not observed when the immunizing antigen was DNP–(T,G)-A--L, H.S. These data are shown in Table IV. It was shown in control experiments that CBA and C57 responded equally well to DNP when the immunizing antigen was DNP–BSA. Thus Mozes and McDevitt have demonstrated that the effect of *Ir-1* is primarily associated with carrier recognition. This conclusion is also supported by McDevitt's demonstration that the difference between responders and nonresponders can be partially eliminated if mice are injected with (T,G)-A--L complexed with methylated BSA (McDevitt, 1968).

McDevitt and Tyan (1968) demonstrated that the ability to respond well to (T,G)-A--L could be passively transferred from (C3H × C57)F_1 donors to irradiated C3H hosts with spleen cells. In an attempt to minimize the problems of radiation deaths and rejection of injected cells,

TABLE IV

RESPONSE TO DINITROPHENYL AFTER IMMUNIZATION WITH DINITROPHENYL CONJUGATES OF SYNTHETIC POLYPEPTIDES[a,b]

	Strain			
	CBA		C57	
Immunizing antigen[c]	Antigen bound[d] (average %)	Range	Antigen bound[d] (average %)	Range
DNP-(T,G)-A--L, L.S.	1	0–4	11	6–26
DNP-(H,G)-A--L, L.S.	52	47–68	2	0–5
DNP-(T,G)-A--L, H.S.	9	6–23	2	0–4
DNP-(H,G)-A--L, H.S.	18	13–28	4	0–8

[a] From Mozes and McDevitt (1969).

[b] Iodinated dinitrophenyl (DNA)–bovine serum albumin (BSA) was used for the titration. The DNP-BSA-^{125}I was 93% precipitable by trichloroacetic acid and 70% precipitable by excess specific antibody.

[c] Abbreviations: (T,G)-A--L, poly(Tyr,Glu)-poly-DL-Ala--poly-Lys; H, histidine; L.S., lightly substituted; H.S., heavily substituted.

[d] Antiserum dilution 1/50.

they examined congenic pairs of mice differing only at the *H-2* locus, the genetic region that specifies the major histocompatibility antigens of mice. This experiment led to the discovery that the gene associated with response to (T,G)-A--L is closely linked to *H-2* (McDevitt and Tyan, 1968). Thus it was shown that when 38 (CBA × C57)F_1 × CBA mice were immunized with (T,G)-A--L and tested for *H-2* type, 14 of 15 responders were $H\text{-}2^b/H\text{-}2^k$ and 22 of the 23 nonresponders were $H\text{-}2^k/H\text{-}2^k$. Although this proved that *Ir-1* is very closely linked to *H-2*, it was later shown that this gene actually maps within the *H-2* region. The linkage between *Ir-1* and *H-2* was confirmed by McDevitt and Chinitz (1969) after extensive testing of congenic pairs of mice. These investigators reported that a characteristic pattern emerged when 16 strains of eight different *H-2* types were immunized with three synthetic antigens: (T,G)-A--L, (H,G)-A--L, and (P,G)-A--L, the last-named antigen being similar to (T,G)-A--L except for the substitution of tyrosine by phenylalanine. The two $H\text{-}2^s$ strains responded poorly to all three antigens, DBA/1 responded well only to (P,G)-A--L, and all other strains responded well to two out of three of the antigens used, the pattern of responses being the same for all strains of any given *H-2* genotype (McDevitt and Chinitz, 1969).

The exact position of *Ir-1* within the *H-2* region has been the subject of a great deal of careful investigation. Examination of nine *H-2* recombinant strains permitted McDevitt *et al.* (1969) to localize *Ir-1* in the middle of the *H-2* complex, lying between the *Ss* gene and the genes coding for *H-2* histocompatibility specificities 11 and 13 (McDevitt *et al.*, 1969). Since 1969, information about *H-2* has increased a great deal; a new model for the organization of this genetic region has been developed (Shreffler *et al.*, 1971; Klein and Shreffler, 1972), and the position of *Ir-1* within this region has been established (McDevitt *et al.*, 1972). It has been proposed that the *H-2* complex has undergone one or more duplications during evolution so that the present *H-2* chromosome consists of two genetic regions, *H-2D* and *H-2K*, which code for histocompatibility antigens. Between these two regions are found the *Ss-Slp* and *Ir-1* genes as shown in Fig. 1. Since there is no evidence for any functional relationship between the *Ss-Slp* and *H-2* traits, it is quite possible

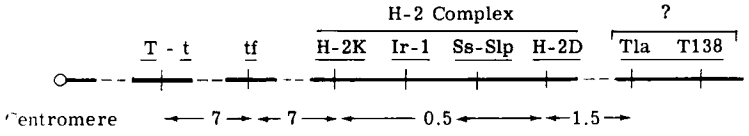

FIG. 1. A simplified map of linkage group IX of the mouse. (From Klein and Shreffler, 1971.)

that *Ir-1* is also functionally unrelated to *H-2*. Whether or not this is the case remains to be determined.

Studies on the nature of the lesion in the *Ir-1* nonresponder have led to some interesting findings. McDevitt (1968) initially reported that CBA and C57 mice failed to show any simple qualitative difference in the antibodies which they produced against (T,G)-A--L when immunized with antigen in CFA. In order to avoid complications caused by the effects of adjuvant on lymphoid tissue and by the possibility of persistent release of low doses of antigen from a CFA deposit, mice were immunized and subsequently boosted with (T,G)-A--L in saline alone (Grumet, 1972). This immunization protocol revealed qualitative differences between responders and nonresponders that were not evident from previous studies. It was shown that both responder and nonresponder strains produced equally good immunoglobulin M (IgM) responses to (T,G)-A--L, but that only responder strains were able to produce a 7 S immunoglobulin G (IgG) response to this antigen. Both responder and nonresponder strains produced brisk primary responses consisting of IgM antibody during the first week after immunization. With subsequent immunization, only the high-responder mice showed immunological memory and produced high titers of IgG antibody. It was suggested that the *Ir-1* gene exerts its effect on the immune response at the time of switchover from IgM to IgG antibody production (Grumet, 1972).

Although the nature of the lesion in the *Ir-1* nonresponder has not yet been determined, two possibilities have been ruled out. One possibility is that there is cross-tolerance between (T,G)-A--L and the histocompatibility antigens of the nonresponder. Because high responder lymphoid cells that were tolerant of low-responder tissues retained the high-responder phenotype, the cross-tolerance hypothesis does not appear to be tenable (Chesebro *et al.*, 1972). A second possibility is that the inability of low responders to produce high antibody response is due to a defect at the level of the antibody-producing cell. Since low responders to (T,G)-A--L can produce a good anti-(T,G)-A--L response when injected with (T,G)-A--L coupled to methylated BSA (MBSA) (McDevitt, 1968), this possibility would likewise appear to be ruled out.

Mitchell *et al.* (1972) made the important observation that adult thymectomy blocked the IgG response to (T,G)-A--L in responders, but did not block the IgM response. Thymectomized responders and nonresponders therefore produced the same response to (T,G)-A--L, which consisted of IgM antibody. They also showed that adult thymectomy ablated the otherwise high response of nonresponder mice to MBSA–(T,G)-A--L in adjuvant. These experiments suggested that nonresponder mice possess a functional or cognitive lesion in their T-cell population

with respect to the carrier moiety of the (T,G)-A--L immunogen. The *Ir-1* gene effect mediated via T cells would presumably act during the stage of induction of IgG antibody formation.

Experiments by Tyan and McDevitt (1970) have established that the *Ir-1* genotype of the thymic epithelial reticulum is not reflected in the antibody response to (T,G)-A--L and (H,G)-A--L. Thymectomized, lethally irradiated mice which normally respond well to either (T,G)-A--L or (H,G)-A--L were protected with fetal liver cells and a fetal thymus graft from donor mice of known *Ir-1* genotypes. Although the thymus was essential for a detectable response to occur, the phenotypic expression of the response was characteristic of the genotype of the injected liver cells and was independent of the genotype of the thymic epithelial reticulum.

The various cell populations capable of responding to (T,G)-A--L were analyzed by Tyan (1972) in an *in vitro* system. Blood leukocytes, spleen, bone marrow, and fetal liver cells from high responders or low responders were cultured in the presence of antigen, and proliferative responses were measured by incorporation of thymidine-^{3}H. An antiserum to the θ antigen greatly reduced the responses to (T,G)-A--L of blood, spleen, and marrow cells but had no effect on the response by fetal liver cells. Furthermore, the proliferative responses of fetal liver cells from low-responder or high-responder embryos were best increased when the cells were incubated with spleen cells from irradiated high-responder mice previously given syngeneic thymus cells. These experiments demonstrated that the response to (T,G)-A--L by both high-responder and low-responder mice was quantitatively dependent on the function of thymus-passaged cells.

Further studies on the role of thymus-derived cells in the *Ir-1* response were reported by Ordal and Grumet (1972). These authors demonstrated that a graft-versus-host reaction induced by injection of nonresponder lymphoid cells into heterozygous nonresponder recipients at the time of primary challenge with (T,G)-A--L elicited the production of both IgM and IgG anti-(T,G)-A--L antibodies. It appeared that nonresponder T cells activated against host histocompatibility antigens could substitute for (T,G)-A--L reactive T cells to bring about an antibody response normally observed only in responders.

B. Responses to the (Phe,G) and Pro--L Determinants

The synthetic polypeptide antigen poly-L-(Phe,Glu)-poly-L-Pro--poly-L-Lys [(Phe,G)-Pro--L] possesses some interesting immunogenic characteristics. When mice of strain DBA/1 were injected with this antigen, the antibody reacted almost exclusively with the (Phe,G) sequence,

whereas SJL mice produced antibody primarily directed to the Pro--L portion of the antigen (Mozes *et al.*, 1969a). Immunizations of (DBA/SJL) × SJL mice indicated that the response to the (Phe,G) portion of the molecule was determined by a gene linked to the *H-2* locus. The response to the Pro--L determinant appeared to be genetically distinct from the (Phe,G) response, since no linkage could be demonstrated between *H-2* type and response to Pro--L in the (DBA/SJL) × DBA population. The gene determining the response to the Pro--L specificity was designated *Ir-3*, but it was evident from the segregation data that these results could not be explained solely by a single gene (Mozes *et al.*, 1969b). In subsequent experiments utilizing younger mice than those used initially, data were obtained which more nearly approximate single gene segregation (Shearer, 1972).

When F_1 anti-(Phe,G)-Pro--L antisera were tested for their ability to bind either (T,G)-Pro--L or (Phe,G)-A--L in the presence of a 100-fold excess of the other antigen, there was no inhibition of binding, indicating that the two populations of antibodies were separate and distinct (Mozes *et al.*, 1969b).

The limiting dilution technique was used to compare the numbers of immunocompetent precursors sensitive to (T,G)-Pro--L in spleen cell suspensions from the low-responder DBA/1 and the high-responder SJL strains (Mozes *et al.*, 1970). Recipient SJL or DBA/1 mice, heavily irradiated to destroy their own immune systems, were injected with graded numbers of spleen cells from immunized syngeneic donors along with antigen. Approximately 60% of SJL sera were positive when 1×10^6 spleen cells were injected, but $2\text{–}4 \times 10^7$ spleen cells were required for an equivalent proportion of DBA/1 sera to be positive. These results suggest that SJL spleens contain approximately 24 times as many (T,G)-Pro--L precursor cells as DBA/1 spleens. A significant 4.5-fold difference was observed in the number of splenic antigen-sensitive precursors from nonimmunized donors, suggesting that the defect is expressed even before immunization (Mozes *et al.*, 1970).

Since SJL and DBA/1 mice each recognize different determinants on (Phe,G)-Pro--L, this type of analysis has been extended to this immunogen as well. Graded and limiting inocula of spleen cells from SJL, DBA/1, and F_1 donors were injected into X-irradiated, syngeneic recipients along with (Phe,G)-Pro--L (Shearer *et al.*, 1971a). Nonimmunized DBA/1 mice possessed 1 antigen-sensitive unit specific for (Phe,G) in 8.5×10^6 spleen cells and 1 Pro--L-sensitive precursor in 38×10^6 cells. Nonimmune SJL mice were found to have 1 (Phe,G) precursor per 20×10^6 spleen cells and 1 Pro--L-sensitive unit per 3.4×10^6 spleen cells. The numbers of cells sensitive to (Phe,G) and to Pro--L in (SJL × DBA)F_1 spleens were approximately the same. These results

demonstrate that the genes controlling responses to (Phe,G) and to Pro--L are both associated with varying frequencies of immunocompetent precursors and that two different populations of spleen cells are involved in the response to these determinants (Shearer *et al.*, 1971a).

Mozes and Shearer (1971) have demonstrated that the response to (Phe,G)-Pro--L requires cooperation between thymocytes and bone marrow cells. A nonlimiting number of thymocytes was mixed with limiting numbers of bone marrow cells and transferred into syngeneic irradiated recipients. In SJL mice, about 5 times as many marrow precursors were detected for Pro--L as for (Phe,G), whereas, in DBA/1 mice, about 5 times as many (Phe,G) marrow precursors as Pro--L precursors were estimated.

When a large number of bone marrow cells was mixed with graded numbers of syngeneic thymocytes and injected with antigen into heavily irradiated recipients, no significant differences were observed between SJL and DBA/1 mice in their responses to the (Phe,G) and Pro--L determinants (Mozes and Shearer, 1971). This conclusion was modified in a subsequent paper, however, in which it was reported that only the response to Pro--L was reflected solely by frequency of bone marrow cell precursors. The response to (Phe,G) involved frequency differences in both bone marrow and thymus cell populations (Shearer *et al.*, 1972).

The response to the Pro--L determinant can be enhanced in low responders by injection of peritoneal cells or poly AU,[1] but this is not the case with the response to (Phe,G) (Shearer *et al.*, 1971b). Since it was shown that poly AU increased the number of bone marrow precursors in DBA/1 mice, these observations are compatible with the interpretation that nonresponse to Pro--L represents a B-cell defect and nonresponse to (Phe,G) involves a defect in T-cell function.

C. Responses to GLA_5 and GAT

In studies on the antigenicity of synthetic polypeptides, Pinchuck and Maurer (1965a) observed that antibody responses could not be obtained when mice were injected with copolymers of only two amino acids, but the introduction of as little as 4 mole % of a third amino acid resulted in an immunogenic molecule, regardless of the nature of the third amino acid. Depending on the composition of the injected antigen, variable proportions of random-bred Swiss mice produced antibodies that were detected by passive hemagglutination of antigen-coated, tanned, red blood cells. The polymer $Glu^{51}Lys^{38}Ala^{5}$ (GLA_5) was immunogenic in three inbred strains and did not elicit antibody response in four strains (Pinchuck and Maurer, 1965b). Forty-seven percent of the Swiss mice

[1] Polyadenylic-uridylic acid.

responded to this antigen, and matings were set up between male and female responders and male and female nonresponders. All progeny of the nonresponder matings were nonresponders, and most of the progeny of the responder matings were responders. It was suggested that a single dominant Mendelian factor determined the ability to respond to GLA_5.

Strain C57BL/6J mice normally respond very poorly to $Glu^{60}Ala^{30}$-Tyr^{10} (GAT), whereas C3H/HeJ and DBA/2 strains respond very well to this polymer. Pinchuck *et al.* (1968) studied the possible role of macrophage ribonucleic acid (RNA) in the determination of this difference. When RNA, extracted from rabbit or mouse macrophages which had been incubated with antigen, was injected into either primed or unprimed C57BL/6J recipients, a number of these mice produced anti-GAT antibodies. When rabbit macrophages were incubated with Glu^{36}-$Lys^{24}Ala^{40}$ (GLA) and injected into recipient mice, anti-GLA antibodies could be detected in all cases, but anti-GAT antibodies could not be detected at all, even if the RNA was incubated with GAT prior to injection. Although these studies are very interesting, their significance is unclear since they have not been followed up by further work.

The immunogenicity of GAT in a number of inbred and congenic strains of mice was examined by Martin *et al.* (1971), who showed that the ability to produce anti-GAT antibody correlated with the possession of any one of four alleles at the *H-2* locus. Mice possessing any one of seven other *H-2* alleles did not respond at all to this antigen, and the pattern of reactivity of inbred strains was different from that observed with any previously studied antigen (Merryman and Maurer, 1972). When this antigen was complexed to MBSA, nonresponder mice were capable of producing as much anti-GAT antibody as responder mice (Gershon *et al.*, 1972; Dunham *et al.*, 1972). Since there was no significant difference in the number of lymphocytes from unsensitized responders and nonresponders capable of binding antigen (Dunham *et al.*, 1972), these two observations suggest that antigen-sensitive precursor cells of the nonresponders were not defective. It was concluded that the inability of nonresponder mice to produce anti-GAT antibodies must be attributed to a genetic defect in the thymus-derived cell population (Dunham *et al.*, 1972).

One possible explanation for the mode of action of this Ir gene is that T cells of nonresponders are able to recognize GAT but are easily made tolerant of it. When lethally irradiated nonresponders were inoculated with syngeneic thymocytes and GAT, they made good primary responses as measured by DNA synthesis, but were unable to respond when confronted with GAT a second time. When intact responders and nonresponders were injected with GAT and then challenged with GAT/

MBSA, the anti-GAT response of nonresponders was abolished, but responders produced normal secondary responses (Gershon *et al.*, 1972, 1973).

V. Genetics of Response to Naturally Occurring Antigens

A. Responses of Rabbits and Mice to Bovine Serum Albumin

Sang and Sobey (1954) observed that some of the rabbits which they injected with BSA were complete nonresponders to this antigen, that breeding tests showed this to be a genetically determined trait, and that responsiveness is associated with a number of recessive genes (Sobey, *et al.*, 1966).

Similar experiments were extended to mice, in which 1 animal out of 50 injected with BSA produced no detectable circulating antibody and continued to show circulating BSA (Sobey *et al.*, 1966). The offspring of this mouse were used to initiate a selection experiment in which male and female nonresponders to BSA were mated to produce the next generation. As in the case with rabbits, the results of this experiment did not support a single-gene model (Sobey *et al.*, 1966).

Subsequent experiments showed that with low doses of BSA, the putative nonresponder or Sobey mice were as reactive as responder Swiss white mice. However, when antigen doses greater than 500 μg. were injected, Sobey mice frequently became tolerant whereas Swiss white mice did not (Hardy and Rowley, 1968). The genes for unresponsiveness to BSA, therefore, appear to be involved in lowering the threshold of tolerance induction. These genes have recently been transferred from Sobey mice to a BALB/c genetic background (Hardy and Kotlarski, 1971).

B. Responses to Diphtheria Toxin and Tetanus Toxoid

By employing diphtheria toxin as the antigen, Fjord-Scheibel (1943) attempted to develop lines of good and poor antitoxin producers in guinea pigs. Within one generation, over 90% of the pigs selected for good production were antitoxin producers, but five generations of selection were required to develop a line that did not produce antitoxin. As pointed out by Carlinfanti (1948), this observation suggests that the factors responsible for antitoxin production are inherited as recessives.

Ipsen (1959) examined the immunizability of four inbred mouse strains with tetanus toxoid. After injections of varying doses of toxoid, mice were given challenge doses of toxin and were scored according to how soon death occurred, or, in the case of those that survived, whether or not the mice had tetanus after 168 hours. These experiments demon-

strated significant differences in the ability of members of each of the four strains to become immunized and also showed that the same differences occurred when the mice were given a secondary immunization.

C. Responses to Human A_1 and B Antigens

Carlinfanti (1948) examined the anti-A_1, anti-A_2, and anti-B agglutinins in fifty-one families with 159 children and looked for parent–offspring correlations in the titers of these antibodies. The following values of the linear coefficient of correlations were obtained:

anti-A_1

fathers–children, 0.55 ± 0.11
mothers–children, 0.52 ± 0.15

anti-B

fathers–children, 0.41 ± 0.09
mothers–children, 0.36 ± 0.08

The theoretical value expected if agglutinin titers were determined solely by heredity in the absence of dominance is 0.5, and dominance would lower this value to approximately 0.3. Therefore the values obtained suggest that the agglutinin titers to the A_1 and B antigens are to a large extent genetically controlled. This problem ought to be reexamined in the light of more recent information. It is quite possible that, if donors were typed for HL-A haplotypes, a significant correlation between HL-A type and anti-A_1 and anti-B titers could be demonstrated within families such as these.

D. Response of Cattle to the O Blood Group of Sheep

In the J–Oc blood group system of cattle, animals can be classified according to four phenotypes: J, JOc, Oc, and "–" (Sprague, 1958). Some cattle of the "–" phenotype were shown to possess a naturally occurring antibody with specificity for the O blood group of sheep. Among cattle matings consisting of "–" × "–" crosses, production of the naturally occurring anti-O antibody was shown to be transmitted by a single dominant gene (Sprague, 1958). Although this system is potentially just as interesting as any of the others that have been studied, unfortunately no additional work has been done on it.

E. Polygenic Control of the Murine Response to Sheep Red Blood Cells

A two-way selection experiment for high and low antibody response was undertaken by Biozzi *et al.* (1968a,b, 1970a,b, 1971a). Sixty-two

random-bred Swiss mice were initially immunized against sheep red blood cells (SRBC) and were then assortatively mated—high responders with high responders and low responders with low responders. This type of selection was continued for six generations, at which time it was observed that high responders to SRBC were also high responders to pigeon red cells, and low responders to SRBC were low responders to pigeon red cells, despite the fact that no cross-reactivity was observed between these two types of erythrocytes (Biozzi *et al.*, 1970a). It was also observed that antibody production against SRBC was inhibited by maternally transmitted antibodies. For subsequent generations of selection, mice were therefore immunized with pigeon red cells in alternate generations. By the ninth generation of selection the two lines were completely separated in the ranges of antibody titer produced in response to SRBC, with a thirty-fold difference between the mean agglutinin titers attained (Biozzi *et al.*, 1968b). The two lines were also separated with respect to their antibody responses to the O and H antigens of *Salmonella typhi* (Biozzi *et al.*, 1970b), hen ovalbumin (Prouvost-Danon *et al.*, 1971), pneumococcal polysaccharide SIII (Howard *et al.*, 1972), DNA hapten (Del Guercio and Zola, 1972), and T4 bacteriophage (Lieberman *et al.*, 1972a).

After thirteen generations of selection, the levels of serum immunoglobulin were determined in low and high responder lines. It was observed that (*1*) immunoglobulin levels in the high responders before immunization were about double those in the low responders, except for IgM where the difference was much smaller, and (2), after immunization, low responders showed little increase in immunoglobulin levels, whereas high responders showed a large increase (Biozzi *et al.*, 1970a). When individual mice were typed for immunoglobulin allotypes, there was considerable variation in the phenotypes expressed in the high line, but all mice of the low line exhibited the identical phenotype $G^{3,5,7,8}H^{9,11}$-A-F^{f} (Biozzi *et al.*, 1970a). Apparently this breeding program selected for genes concerned with genetic regulation of immunoglobulin synthesis irrespective of antibody specificity, and in the case of the low line, for a heavy chain linkage group associated with depressed immunoglobulin levels.

That the two populations did not differ in thymus-derived cell functions was suggested by the observation that the high and low lines behaved alike in graft-versus-host reactions (Byfield and Howard, 1972). There was no difference in the rejection time of interline skin grafts exchanged between high and low responders, but the high line produced a much higher titer of cytotoxic antibodies than did the low line (Biozzi *et al.*, 1971b). When mice of the two lines were injected with 100 or

200 μg. of benzopyrene, the incidence of tumors was higher in the low line for both doses (Biozzi *et al.*, 1971b). Since the two lines did not appear to differ in cell-mediated immunity, the greater tumor resistance of the high line seemed to be based on humoral rather than cellular immunity.

When Sarcoma 180 grafts were implanted subcutaneously into low responders and high responders, the tumors completely regressed in the low line in less than 40 days but they grew progressively larger in the high line (Biozzi, 1972). Apparently, tumor growth in this case was facilitated by the production of enhancing antibodies by the high responder mice, whereas failure to produce such antibodies had a protective effect in the low responder mice.

Evidence has been reported suggesting that the genes by which these two lines differ are involved in regulation of the rate of multiplication and differentiation of the antibody-producing cells (Biozzi *et al.*, 1972). Before immunization, the two lines of mice had similar numbers of rosette-forming cells (RFC) and plaque-forming cells (PFC); after immunization, the high line possessed 11 times as many PFC per spleen as the low line. The RFC doubling time for the high line was calculated to be 11 hours as compared with 19 hours for the low line. The PFC doubling times in the high and low lines were estimated as 5 hours and 11 hours, respectively (Biozzi *et al.*, 1972).

In order to evaluate the role of the heavy chain linkage group in this genetic system, mice of the high and low lines were crossed to obtain F_1, F_2, and backcross individuals. When these mice were immunized against SRBC, a significant correlation was observed between their antibody responses and their immunoglobulin allotypes. For example, in the F_2 generation, mice that were homozygous for the allotypes of the high line showed a significantly greater response to SRBC than did mice that were homozygous for the allotypes of the low line, with the heterozygous mice showing an intermediate level of response. These results suggest that one of the genes involved in the response to SRBC is linked to the immunoglobulin, heavy chain, allotype loci (Lieberman *et al.*, 1972a).

Playfair (1968a) observed differences among three inbred strains of mice, NZB, C57BL, and BALB, in their PFC responses to SRBC, and it was suggested that at least three genes were responsible for these differences. Neonatal thymectomy reduced the response of all three strains to the same low level. When irradiated (NZB × C57BL)F_1 and (NZB × BALB)F_1 hybrids were given mixtures of parental and syngeneic cells, it was shown that the high NZB response to SRBC was characteristic of newborn liver rather than of thymus cells (Playfair, 1968b).

The NZB thymus cells were shown to be more resistant to tolerance

induction by high levels of SRBC than the BALB thymus cells (Playfair, 1971). This was demonstrated by measuring spleen PFC responses in irradiated (NZB × BALB)F_1 mice that had received syngeneic marrow cells, various doses of SRBC, and thymus cells from either NZB, BALB, or F_1 donors. In each case the "thymus effect" was determined by subtracting the PFC obtained with bone marrow and SRBC alone from those obtained with marrow, SRBC, and thymus. The peak thymus effect for BALB cells occurred at a much lower dose of SRBC than for NZB cells. It was suggested that the increased propensity of NZB mice to develop autoimmunity (Howie and Helyer, 1968) could in part result from a raised threshold of NZB T cells for both immunity and tolerance (Playfair, 1971).

F. Genetics of Response to the Ea-1 Blood Group Antigens

The Ea-1 blood group system was discovered in wild populations of the house mouse, *Mus musculus* (Singer *et al.*, 1964). Three alleles, *Ea-1*^{a}, *Ea-1*^{b}, and *Ea-1*^{o}, are determined by a single locus which is linked to the *Es-1* serum esterase locus in the eighteenth linkage group (Foster *et al.*, 1969). Despite the fact that all inbred strains which have been typed so far are *Ea-1*^{o}, most of these strains are not capable of producing agglutinating antibodies to Ea-1^a or Ea-1^b antigens.

The inbred strain which initially was observed to be the best responder to Ea-1 antigens was YBR. When YBR was crossed with either of two nonresponding strains, BALB or CBA, responsiveness behaved as a recessive trait determined by a single gene. This locus was named *Ir-2* and was shown to be linked to agouti in the fifth linkage group. Since *H-3* and *H-6* are also linked to agouti with recombination frequencies comparable to that occurring between *Ir-2* and agouti, it was suggested that *Ir-2* could be closely linked or identical to one of these loci (Gasser, 1969).

It seemed unlikely that the nonresponders were tolerant or "cross-tolerant" of Ea-1 antigens because (*1*) *in vivo* and *in vitro* absorption experiments failed to detect any Ea-1 antigens in nonresponder tissues, (*2*) some mice of the nonresponding BALB strain were capable of responding to Ea-1 when intensively immunized, and (*3*) the Ea-1 locus is carried in the eighteenth linkage group whereas *Ir-2* maps in the fifth linkage group (Gasser, 1969).

When YBR mice were mated with C57BL/10 (B10), a strain in which approximately 80% of the mice are nonresponders to Ea-1, responsiveness was inherited as a dominant trait and a significant nongenetic effect was observed (Table II, Section II). In order to demonstrate the importance of genetic factors, assortative matings were made between male and

female responders and male and female nonresponders to obtain F_2, F_3, and F_4 generations. The F_4 generation obtained in this way included 81% responders in the line selected for response, and the line selected for nonresponse did not include any responders (Gasser, 1970).

It was suggested by Gasser and Shreffler (1972) that these results could be explained by the segregation of two genetic loci, one of which was closely linked or identical to the *H-2* locus. Two independent types of evidence were obtained in support of this hypothesis: (*1*) when (YBR × B10)F_2 mice were tested for *H-2* type and for response to Ea-1 antigens, a significant correlation was observed between these two characteristics; and (*2*) data obtained from F_2 and backcross generations derived from YBR, an $H\text{-}2^d$ strain, and B10, an $H\text{-}2^b$ strain, did not support single-gene determination of response to Ea-1, but when YBR was crossed with B10.D2, an $H\text{-}2^d$ strain with the B10 genetic background, response to Ea-1 segregated as a single-gene trait.

Work is in progress which hopefully will provide further information about the gene by which YBR and B10.D2 differ. There can be no doubt, however, that the nonresponder gene possessed by CBA and BALB is different from either of the nonresponder genes possessed by B10, since a single dose of the *Ir-2* nonresponder gene causes a mouse to be unresponsive to Ea-1.

G. Responses to Lactic Dehydrogenase Subunits

Some very interesting immunogenic characteristics of the enzyme lactic dehydrogenase (LDH) have been described by Rajewsky and his colleagues (Rajewsky *et al.*, 1967; Rajewsky and Rottländer, 1967; Armerding and Rajewsky, 1970). Lactic dehydrogenase is a tetramer that can exist in all possible combinations of two subunits, A and B (A_4, A_3B, A_2B_2, AB_3, and B_4). In some rabbits this enzyme behaved like a hapten-carrier system in that the haptenic B subunits induced antibody formation only if combined with carrier A subunits. In other rabbits the B subunits alone were immunogenic, and the ability to respond to B_4 was inherited as a genetic trait (Rajewsky and Rottländer, 1967). Hyporesponsiveness to the A_2B_2 enzyme could be induced by injection of LDH-A_4 into newborn rabbits. Both anti-A and anti-B titers were equally depressed. The response to A_2B_2 was not depressed by neonatal injections of LDH-B_4. The carrier property of the A subunit was therefore evident in the induction of both immunity and tolerance to LDH-A_2B_2 (Rajewsky *et al.*, 1967).

The situation in rats differed in that Sprague-Dawley and Wistar rats responded equally well to LDH-B_4 but they differed drastically in their

response to LDH-A_4. At low doses of antigen, only Sprague-Dawley rats produced a detectable response to LDH-A_4, but with increasing doses of antigen, Wistar rats could also be stimulated (Armerding and Rajewsky, 1970). Since there is some evidence that carrier specificity can be partially overridden by high doses of antigen (Brownstone *et al.*, 1966), these results were compatible with the interpretation that the difference between these two strains involved a difference in carrier recognition.

Breeding experiments showed that the difference in response to LDH-A_4 was determined by a single gene (Armerding and Rajewsky, 1970), and it was subsequently shown that this gene was closely linked to the major histocompatibility locus of rats, *H-1* or *Ag-B* (Würzburg, 1971). Further evidence that the *Ag-B* region of rats controls immunological reactivity is provided by the demonstration that the response to (T,G)-A--L is also associated with this locus (Günther *et al.*, 1972).

H. Genetics of Response to the H-Y Antigen

Males of all inbred strains of mice which have been appropriately tested are known to possess a histocompatibility antigen lacking in females (reviewed in Gasser and Silvers, 1972). Females of some inbred strains rapidly reject skin grafts from males of the same strain, whereas females of other strains reject such grafts very slowly or not at all. It has been demonstrated that the *H-2* region is of primary importance in determining the ability of female mice to make this response (Gasser and Silvers, 1971a; Bailey and Hoste, 1971), but other genetic factors also appear to be involved (Gasser and Silvers, 1971b). It has also been demonstrated that C3H and DBA females that cannot reject male skin are, nevertheless, capable of making good serum antibodies to the Y antigen (Goldberg *et al.*, 1972).

Evidence suggests that a specific site within the *H-2* region controlling the response to H-Y is near *H-2K* and could be identical to *Ir-1* (Bailey, 1971b; Stimpfling and Reichert, 1971). The only evidence which is at variance with this conclusion is the observation of Bailey (1971b) that HTG females reject male skin in a manner comparable to females of *H-2*^{b} strains. Strain HTG mice possess the *H-2*^{g} allele which is a recombinant chromosome possessing the *b* specificities in the *H-2D* region and the *d* specificities in the *H-2K* region (Klein and Shreffler, 1971). The *b*-like response of HTG mice suggests that either this response is determined by a non-*H-2* gene or that there is a gene in the *H-2D* region that is responsible for this effect. Experiments by Gasser and Shreffler are in progress which hopefully will determine which of these alternatives is correct.

I. Antibody Response to Immunoglobulin A and G Allotypes of Mice

Lieberman and Humphrey (1971) studied antibody responses of inbred and congenic strains of mice to allotypic determinants on IgA myeloma proteins. These authors observed that mice possessing the *H-2* alleles, *a*, *k*, *s*, and *r*, were good responders whereas those possessing alleles *b*, *d*, *q*, *l*, and *v* were poor responders or nonresponders. Two strains possessing *H-2* alleles derived by recombination between $H\text{-}2^a$ and $H\text{-}2^b$ chromosomes were immunized, and the results suggested that the gene controlling response to IgA mapped between the genetic determinants of *H-2* specificities 2 in *H-2D* and 22 in *H-2K*.

Lieberman and Humphrey (1972) demonstrated that the response to IgG γ2a allotypes was also determined by an *H-2*-linked gene. Evidence that this gene was different from *Ir-IgA* was obtained by immunizing five strains possessing *H-2* alleles derived by recombination between $H\text{-}2^a$ and $H\text{-}2^b$. Strain $H\text{-}2^a$ mice are high responders to IgA but not to IgG allotypes, and $H\text{-}2^b$ mice are high responders to IgG but not IgA allotypes.

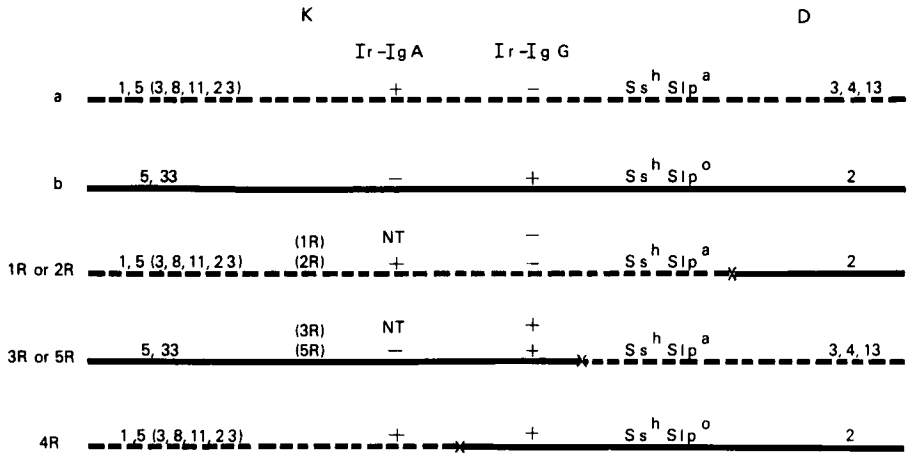

Fig. 2. Schematic representations are shown of the chromosomes of B10.A ($H\text{-}2^a$), B10 ($H\text{-}2^b$), and of the $H\text{-}2^a/H\text{-}2^b$ crossover chromosomes of B10.A (1R, 2R), B10.A (3R, 5R), and B10.A (4R). The region of crossover in the generation of each recombinant chromosome is indicated by an X. It has previously been established that B10.A (4R) chromosome resulted from a crossover event between the *K* region of *H-2* and *Ss-Slp* genes. Data presented elsewhere indicate that the crossover separates the *Ir-IgA* and *Ir-IgG* genes occurring in this region, and, thus, *Ir-IgA* and *Ir-IgG* are at separate loci. The 1R and 2R chromosomes are combined as the *H-2* products have not yet been distinguished; this is also true of 3R and 5R. (From Lieberman *et al.*, 1972b.)

Of the five recombinant strains tested, one was a high responder to both IgG and IgA, which established the genetic distinction between *Ir-IgG* and *Ir-IgA* genes (Lieberman *et al.*, 1972b). The results of testing the various recombinants are summarized in Fig. 2, in which one can observe the type of recombination between the a and b chromosomes that would be required in order to derive the 4R chromosome. Genes *Ir-IgA* and *Ir-IgG* thus appear to map in approximately the same position as *Ir-1* (Fig. 1). Evidence was obtained suggesting that *Ir-IgG* and *Ir-1* are distinct from one another, but the question of whether *Ir-IgA* and *Ir-1* are at different loci has not yet been resolved (Lieberman *et al.*, 1972b).

J. Murine Response to Bacteriophage fd

The antibody response to the bacteriophage fd was analyzed in several inbred strains of mice by Kölsch *et al.* (1971). Strain CBA/MH was found to be a high responder, AKR/HO was a low-responder, and (CBA × AKR)F_1 hybrids behaved like the high-responding parental strain over the whole dose range tested. Dominance in the F_1 is therefore complete, but among 45 F_2 animals, only 29% were high responders. Despite the authors' interpretation to the contrary, the response to fd bacteriophage does not appear to be determined by a single gene, since 75% of the F_2 generation would be expected to be high responders if this were the case.

Kölsch and Diller (1971a) observed that partial low zone tolerance to phage fd could be induced in the high-responding C57BL/6J strain as well as in the low-responding AKR/J strain. Partial tolerance was more readily established in the C57BL/6J strain, however, suggesting that receptor antibodies in the low-responding strain differed from those present in high-responding mice. Experiments utilizing acid dissociation of antigen–antibody complexes provided evidence that antibodies from low-responder mice showed a more restricted heterogeneity than antibodies obtained from high responders (Kölsch and Diller, 1971b).

VI. Genes on the X Chromosome Influencing Immune Responses

The first evidence that the immune response is controlled in some way by a gene on the X chromosome stemmed from the description by Ogden Bruton in 1952 of a sex-linked agammaglobulinemia. This disease, now known as "infantile sex-linked agammaglobulinemia" (Seligmann *et al.*, 1968), is chiefly one of the immunoglobulin-producing system. Although an affected child has a normal-appearing thymus, produces normal amounts of blood lymphocytes, and is therefore able to exhibit delayed hypersensitivity and homograft reactivity, he has virtually no

capacity to form plasma cells and γ-globulin. Indeed, many patients with this syndrome have never produced detectable antibodies.

Patients with another X-linked disease, the Wiskott-Aldrich syndrome (Aldrich *et al.*, 1954; Cooper *et al.*, 1968) or immune deficiency with thrombopenia and eczema (Seligman *et al.*, 1968), possess immunoglobulins of all major classes, but serum IgM levels are usually low. These patients respond to certain antigens with normal IgM and IgG antibody synthesis, but their antibody responses to several polysaccharide antigens are deficient. There are indications that a third disease, primary lymphopenic immunological deficiency, may also be sex-linked (Gitlin and Craig, 1963; Seligman *et al.*, 1968). Whatever the underlying mechanisms of these disorders might be, it is clear that genes on the human X chromosome are involved in humoral immune functions and that mutant forms of these genes are associated with immunological deficiencies.

Levels of serum IgM immunoglobulin were observed to be higher in XXX and XXXY individuals than in normal XX women and XXY persons, whereas normal XY males had the lowest IgM levels of all of these groups (Rhodes *et al.*, 1969). The serum levels of IgG and IgA were not correlated with the number of X chromosomes. These results suggested that at least one gene on the X chromosome is involved in IgM production and that this gene is not subject to "Lyonization" (Lyon, 1961).

Further evidence for the existence of such a gene was obtained by Grundbacher (1972), who showed that the best parent–offspring correlation in serum IgM levels was obtained when a parent was compared with a child to whom an X chromosome was transmitted. Thus a much closer correlation was shown between boys and their mothers than between boys and their fathers, and the serum IgM concentrations of girls were more closely correlated with those of their fathers than with those of their mothers. These correlations were not observed with respect to IgG or IgA levels.

The abilities of inbred strains of mice to produce serum antibody and antibody PFC's in response to Type III pneumococcal polysaccharide (SSS-III) were studied by Braley and Freeman (1971) and Amsbaugh *et al.* (1972). Both groups observed significant differences among strains and reported that these differences were genetically determined. Amsbaugh *et al.* (1972) obtained evidence that an X-linked gene was involved in this response. When females of the low-responding strain CBA were mated with male BALB high responders, the male offspring gave a low response to SSS-III whereas females from the same mating gave an intermediate response. The intermediate response of the females was explained on the basis of the Lyon hypothesis (Lyon, 1961). When mice

of the F_2 and backcross generations were tested, the results were compatible with the hypothesis that a single gene on the X chromosome of CBA mice was associated with low responsiveness. For example, there were no low responders among the females of the (♀ [♀ CBA × ♂ BALB]F_1 × ♂ BALB) backcross generation, but 7 of 11 males in this generation were low responders.

Most of the antibody to SSS-III was of the IgM class, and the low-responding CBA strain also gave extremely low or undetectable IgM antibody responses to SRBC, SSS-I, SSS-II, and *Escherichia coli* lipopolysaccharide. The gene or genes controlling the SSS-III response, and perhaps these other responses, could well be homologous to the human X-linked genes described above.

VII. Tumor Susceptibility and Immune Response Genes

Eight steps that take place in the viral induction of tumors have been outlined by Lwoff (1971), and genetic factors could conceivably influence any of these. Several of these steps could also be influenced by an immune response, so genetic factors controlling such a response would be likewise reflected in inherited differences in tumor susceptibility. This subject has been reviewed recently (Lilly, 1970a, 1971a, 1972), and in the present article only a few aspects of the role of Ir genes in tumor susceptibility will be discussed.

One of the most important observations on the role of genetic factors in tumor susceptibility was the discovery that susceptibility of mice to the Gross leukemia virus is strongly influenced by their *H-2* genotype (Lilly *et al.*, 1964). That susceptibility or resistance could have an immunological basis was suggested by Aoki *et al.* (1966), who reported that many mice of the $H\text{-}2^b/H\text{-}2^b$ or $H\text{-}2^k/H\text{-}2^b$ genotypes, which are resistant to the Gross virus, possessed a natural antibody to the Gross leukemia antigen, but mice of the susceptible genotype $H\text{-}2^k/H\text{-}2^k$ did not possess this antibody.

Further evidence for the primary importance of *H-2* in determining susceptibility to the Gross virus was obtained by the examination of congenic mouse strains. In each case, on a given genetic background, changes of *H-2* type produced a significantly different level of susceptibility to Gross virus infection (Lilly, 1970b). The locus determining the response to the virus was designated *Rgv-1* and was shown to be located near the *K* end of the *H-2* region (Lilly, 1970a). It was proposed that a second locus unlinked to *H-2*, *Rgv-2* is also involved in determining resistance to the Gross virus (Lilly, 1970a, 1971b).

The possible mechanisms by which *Rgv-1* could determine susceptibility or resistance to the Gross virus have been discussed by Lilly (1971a)

and include: (*a*) the functioning of the *H-2* antigens as virus receptor sites, (*b*) an interaction between an *H-2* antigen and a virus-induced antigen which leads to the expression of a new antigen necessary for the development of neoplastic properties of the cell, and (*c*) an effect of *Ir-1*, which determines the host's ability to respond immunologically to virus-induced antigens and thereby its capacity to reject the infected cells.

Resistance to the Friend leukemia virus is determined by a number of loci in mice (Lilly, 1972): *H-2* or a closely linked locus (Lilly, 1968), *Fv-1* (Lilly, 1970b; Rowe, 1972; Rowe and Hartley, 1972), *Fv-2* (Odaka, 1970; Lilly, 1970b), *f* (Axelrad and van der Gaag, 1968), *W* (Steeves, 1971), and *Sl* (Bennett *et al.*, 1968). In addition, it was demonstrated by Axelrad (1966) that congenic pairs of mice differing at the *H-7* locus show significant differences in susceptibility to the Friend virus. It is now known that *H-7* and *Fv-2* are either linked or identical (Lilly, 1972), which would explain this finding. Three of the above genes (*f*, *W*, and *Sl*) are known to affect erythropoiesis and apparently increase resistance to the virus by decreasing the availability of target cells for it (Lilly, 1972). The mechanism by which *Fv-1* affects tumor susceptibility has not been conclusively established, but experiments by Rowe and Hartley (1972) are compatible with the interpretation that this locus contains virus genetic determinants.

Experiments by Dietz and Rich (1972) confirmed that regression of Friend virus occurred primarily in mice homozygous for the $H\text{-}2^k$ allele and did not occur in two $H\text{-}2^d$ strains. But the failure of leukemia regression in one $H\text{-}2^k$ strain and the occurrence of regression in one $H\text{-}2^d$ strain suggested that *H-2* itself was not responsible for these differences. Lilly's results could therefore be explained either by the occurrence of a non-*H-2* gene closely linked to *H-2*, or by the fact that resistance required the combined influence of *H-2* plus one or more other genes.

The susceptibility of four inbred and four congenic strains of mice to the BALB/Tennant lympholeukemogenic virus was studied by Tennant and Snell (1968). Strain BALB/c mice were 100% susceptible to this virus, whereas 39% of the B10 mice developed this leukemia. Different alleles at the *H-2* locus were shown to predispose to both different incidences and different rates of development of leukemia. Different allelic combinations behaved differently: the heterozygote sometimes resembled the susceptible parent, sometimes the resistant parent, and sometimes it was intermediate. These results cannot be explained by molecular mimicry, since this hypothesis would predict complete dominance of susceptibility (Tennant and Snell, 1968).

Lilly (1966) reported suggestive evidence that *H-2* was involved in

determining susceptibility to the Bittner mammary tumor virus (MTV). Convincing evidence for this association was subsequently reported by Mühlbock and Dux (1971), who observed that *H-2*^{h} mice were more resistant to MTV than mice with the *H-2* alleles *a*, *d*, *f*, *k*, or *m*.

Genes at the agouti locus of mice have been known for many years to be involved in some way with tumor susceptibility. The lethal yellow (*A*^{y}) gene is associated with relatively high susceptibility to induced pulmonary tumors (Heston, 1942), spontaneous pulmonary tumors (Heston and Deringer, 1947), induced skin tumors (Vlahakis and Heston, 1963), mammary gland tumors and hepatomas (Heston and Vlahakis, 1961), and reticular neoplasms (Deringer, 1970). The viable yellow gene (*A*^{vy}) is associated with susceptibility to mammary tumors (Heston and Vlahakis, 1968; Vlahakis *et al.*, 1970), hepatomas (Heston and Vlahakis, 1968), and spontaneous cholangiomas (Vlahakis and Heston, 1971). Nonagouti (*aa*) mice, on the other hand, are more resistant than *A*^{y}*a* or *A*^{vy}*a* mice to all of the above tumors. The reason for these inherited differences in susceptibility could be quite complicated, but the possibility that there might be an immunological basis was explored by Gasser and Fischgrund (1973). The cellular immune reactivity of nonagouti mice was compared with that of *A*^{y}*a* and *A*^{vy}*a* mice by means of the graft-versus-host assay. Spleen cells from nonagouti mice were shown to be significantly more reactive in this assay than cells from congenic *A*^{y}*a* or *A*^{vy}*a* animals. These results suggested that reduced cellular immune responsiveness may be involved in the tumor susceptibility of *A*^{y}*a* and *A*^{vy}*a* mice (Gasser and Fischgrund, 1973).

VIII. Histocompatibility Locus HL-A and Disease Susceptibility

During the past few years it has become increasingly evident that there is a statistical correlation between the possession of certain HL-A antigens and the occurrence of certain diseases, usually either neoplastic or of a suspected autoimmune etiology (Walford *et al.*, 1971a; McDevitt and Bodmer, 1972). This suggests that susceptibility to certain diseases is genetically controlled and, more specifically, that some of the important genetic factors involved are closely associated with the major histocompatibility locus of the species, namely HL-A. As in the case of the *H-2* locus of mice this locus is more accurately defined as a chromosomal region since there is evidence that each HL-A chromosome (haplotype) carries two subloci and can determine a maximum of two antigens (one by each sublocus) (Walford *et al.*, 1970b; Klein and Shreffler, 1971).

The possible mechanisms which might lead to an association between a histocompatibility locus and some diseases in inbred mice were men-

tioned in Section VII. In the case of HL-A, however, an additional complication must be considered since human populations are randomly bred. Consequently, if there are genetic loci in man similar to *Ir-1* which are closely linked to HL-A, this linkage would only be demonstrable within individual pedigrees unless linkage disequilibrium prevailed.[2] Barring the possibility of linkage disequilibrium, if possession of a given HL-A allele is associated with some disease at the population level, it is most plausible that it is because the HL-A allele itself causes the individual to be somewhat more susceptible to the disease. This could come about because of molecular mimicry and cross-tolerance, or perhaps because the HL-A antigens play a direct role in the physiology of the patient and allelic variation leads to certain pathological consequences.

The initial evidence that there might be an association between a particular HL-A specificity and some disease was presented by Amiel (1967), who found that HL-A antigen 4c occurred in 51% of 41 patients with Hodgkin's disease as opposed to its incidence of 13% in the French population. This association has been confirmed by a number of subsequent investigations (van Rood and van Leeuwen, 1971; Zervas *et al.*, 1970; Thorsby *et al.*, 1971; Bertrams, 1971; Bertrams *et al.*, 1972; Kissmeyer-Nielsen *et al.*, 1971; Falk and Osoba, 1971; Forbes and Morris, 1970, 1972). (*1*) van Rood and van Leeuwen found that there was an increased frequency of W5, a 4c-related antigen, in 98 patients with Hodgkin's disease. (2) Zervas *et al.* (1970) compared the incidence of HL-A5, an antigen now known to be included in 4c, in 27 patients with Hodgkin's disease, with 20 normal, unrelated control subjects from the same geographic area: of the patients with the disease, 63% were HL-A5 positive, as compared with an incidence of 20% among the controls. (3) Forbes and Morris (1970) in Australia have reported more than twofold increased frequency of W5 in 110 patients with Hodgkin's disease and, more recently (1972), have confirmed this association by determining the HL-A phenotypes of 127 additional patients. They also found the disease to be associated with a significant increase in HL-A11. (*4*) Falk and Osoba (1971) have reported an increased incidence of HL-A1, HL-A8, and HL-A5 in a population of 112 patients with Hodgkin's disease. Interestingly HL-A8 was increased in frequency only in those having the disease for more than 5 years, whereas the frequencies of HL-A1 and

[2] Linkage disequilibrium refers to the situation in which specific alleles at two linked loci are associated with one another in a population despite the fact that recombination would be expected to dissociate them. This could come about by selection for a specific combination of alleles at the two loci. In the absence of such selection, and given enough time, however closely two loci are linked, they will not show their linkage at the population level (Bodmer, 1972).

HL-A5 were high regardless of the duration of the disease (Falk and Osoba, 1971). (5) A significant association between HL-A5 and Hodgkin's disease has also been confirmed by Rege and his associates (1972). Indeed, their study suggests that this association may extend to other types of lymphomas as well.

Although these independent observations strongly suggest that there is at least a bona fide association between antigens of the 4c group and Hodgkin's disease, evidence to the contrary has also been forthcoming. Thus, a Dutch study (Sybesma *et al.*, 1972) on 35 patients with Hodgkin's disease, 23 patients with other lymphomas, and 26 control subjects failed to reveal any differences in the frequency of HL-A5 among these groups. Similar findings, based on 44 patients, have been reported from Stanford (Coukell *et al.*, 1971).

The fact that Hodgkin's disease can be histologically divided into different types as well as the possibility of sampling error may be responsible for some of the reported discrepancies.

Studies have also been undertaken to determine if there is an increased incidence of any HL-A specificities in individuals with acute lymphoblastic leukemia. In Los Angeles, Walford *et al.* (1970a) tested 10 children with this disease, their parents, and 13 siblings. They found a significant positive association between the disease and the HL-A2/HL-A12 haplotype. Moreover, none of the patients possessed HL-A1. Similar results have also been reported by Svejgaard *et al.* (1971) for a Scandinavian population, although these investigators found no deficiency of HL-A1 in the affected group. These findings have likewise been confirmed by Sanderson and Mahour (cited by Walford *et al.*, 1971b), who phenotyped 43 youngsters with acute childhood leukemia and found an excess of HL-A2 (+) 12 (+) individuals. Indeed, 40% of the patients, as compared with only 6% in a control population of 180 persons, possessed these specificities. An increase in the frequency of the haplotype HL-A2,12 in children with acute lymphoblastic leukemia has also been reported by Thorsby and Lie (1971) and Thorsby *et al.* (1971). These investigators also observed an increase in the haplotype HL-A1,8, consistent with the report of Singal *et al.* (1971).

In a subsequent analysis, Walford and his colleagues reported that, although children with acute lymphatic leukemia displayed an increased frequency of HL-A12, the authors were unable to confirm their original finding of a statistically significant increase of the HL-A(2,12) haplotype in this disease (Walford *et al.*, 1971b).

However, Rogentine and his colleagues (1972) have recently reported a significant association between acute lymphocytic leukemia and HL-A2 but *not* HL-A12. Their analysis was based on typing 50 Caucasian chil-

dren with the disease, as well as 219 members of their families and a panel of 200 normal Caucasians.

In contrast to these results are a number of reports which failed to detect any disturbance in the distribution of HL-A antigens in patients suffering from lymphoblastic leukemia. In fact, the initial study in this area by Kourilsky and his associates (1968) was in this negative category. These investigators tested 102 cases of acute lymphoblastic leukemia and concluded that the reaction frequencies of the HL-A antigens in the patients did not differ from those in normal controls. Similar results have also been obtained by Lawler *et al.* (1971) who studied 58 Caucasian patients, by Batchelor *et al.* (1971) who typed 17 similar patients, by Harris and Viza (1971) who studied 20 children with hematologically proven acute lymphoblastic leukemia in remission, and by Thorsby *et al.* (1969).

It seems evident that at present the association between HL-A and acute lymphatic leukemia is less convincing than that for Hodgkin's disease. Most frequently, HL-A2 and HL-A12 have been implicated, and some of the variations observed could be related to the fact that, like Hodgkin's disease, "acute leukemia" undoubtedly lumps together a variety of conditions of perhaps different etiologies.

A number of reports have also been published indicating associations between distinct HL-A specificities and other malignant conditions. These include: (*1*) a significant association between HL-A12 and malignant lymphoma (excluding Hodgkin's disease), particularly follicular lymphoma (Forbes and Morris, 1971); (2) a higher frequency of HL-A3 in chronic (Degos *et al.*, 1971) and perhaps acute myeloid leukemia (Pegrum *et al.*, 1970), and (3) an association between carcinoma of the breast and HL-A7 (Patel *et al.*, 1972).

A correlation between specificities W17 and HL-A13 and psoriasis seems particularly significant inasmuch as this association was established in two completely independent investigations. Russell *et al.* (1972) phenotyped 44 unrelated psoriatic patients and found that HL-A13 was present in 12 of them as opposed to only 3 of 89 controls; W17 was also present in 10 of the patients as well as in 17 family members with the disease. This association between HL-A13 and W17 and psoriasis was confirmed by White *et al.* (1972), who also found a decrease in HL-A12 among patients with the disease. They present evidence that, whereas psoriasis associated with W17 or HL-A12 appears to have a distinct mode of inheritance, HL-A13-associated psoriasis does not.

Susceptibility to ragweed hay fever and its relationship to HL-A haplotypes were studied by Levine *et al.* (1972). Each of seven families was analyzed in terms of whether family members possessed or did not

possess the HL-A haplotype observed in another family member with ragweed hay fever. Of 26 family members having the hay fever-associated haplotypes, 20 members (77%) had ragweed hay fever and intense skin reactivity to antigen E, a purified protein derived from ragweed pollen extract. By contrast, clinical, ragweed hay fever did not occur in any of the 11 family members who had the other haplotypes of the propositi and lacked the hay fever-associated haplotype. These results suggest that an Ir gene, designated "Ir-antigen E," is closely linked to the HL-A locus. Preliminary data suggest that both IgE and IgG responses to antigen E were controlled by this gene (Levine *et al.*, 1972).

A possible correlation between the possession of certain HL-A alleles and susceptibility to systemic lupus erythematosus (SLE) has been reported. Grumet *et al.* (1971) observed a significantly greater frequency of HL-A8 and W15 (LND) among patients with SLE than in controls, and similar findings were reported by Waters *et al.* (1971). This disease is especially interesting since a good animal model is available. Strains NZB and NZB/NZW mice spontaneously develop an illness that resembles SLE (Howie and Helyer, 1968). It was shown that these mice readily produced anti-DNA antibodies when injected with polyinosinic–polycytidylic acid and that patients with SLE had antibodies with similar specificities (Steinberg *et al.*, 1969). Strain differences in the anti-DNA response are discussed in Section XI. Further work is needed to clarify the genetic basis of anti-DNA antibody production and the relationship of these antibodies to disease in both species.

Analyses of HL-A specificities have been carried out on individuals suffering from a number of other pathological conditions resulting in some positive indications. Thus chronic glomerulonephritis (Patel *et al.*, 1969; Mickey *et al.*, 1970), multiple sclerosis (Naito *et al.*, 1972), autoimmune, active chronic hepatitis (Mackey and Morris, 1972), adult celiac disease (Stokes *et al.*, 1972), asthma (Thorsby *et al.*, 1971; Thorsby and Lie, 1971), dermatitis herpetiformis (Katz *et al.*, 1972), and gluten-sensitive enteropathy (Falchuk *et al.*, 1972) have all been associated with one or more HL-A determinants.

One of the most striking associations between an HL-A antigen and a disease has recently been reported in two different geographical locations. Thus, Schlosstein and his associates (1973) at the University of California found that 35 out of 40 patients with ankylosing spondylitis, a rheumatoid disease, possessed HL-A antigen W27, and, in London, Brewerton *et al.* (1973) found this same antigen in 72 of 75 similarly affected individuals. The patients tested were all Caucasians, in whom the frequency of the W27 antigen is normally less than 10%. It remains to be determined whether this highly significant correlation is the result of

very close genetic linkage of an Ir gene to HL-A, and in particular with a strong linkage disequilibrium with W27, or, to the strong immunologic cross-reactivity between W27 and the etiologic agent.

Finally, it should be noted that among the diseases associated with an increase in more than one HL-A specificity are a number of conditions associated with an increase in HL-A1 and HL-A8. These include Hodgkin's disease (Kissmeyer-Nielsen *et al.*, 1971), autoimmune, active chronic hepatitis (Mackay and Morris, 1972), childhood asthma (Thorsby *et al.*, 1971), myasthenia gravis (Engelfriet, cited by Mackay and Morris, 1972), and celiac disease (Stokes *et al.*, 1972). Moreover, there are some indications that incompatible renal transplants to HL-A1,8 recipients may be more likely to suffer severe rejection than transplants to recipients of other HL-A types (Mickey *et al.*, 1971). To account for the increase of these antigens in patients with diseases of suspected autoimmune etiology, as well as to account for the fact that HL-A1,8 is the most commonly occurring HL-A haplotype among Caucasians, Mackay and Morris (1972) have proposed that this haplotype may be "associated, either by genetic or phenotypic processes, with change in the point of balance between proliferative and tolerance responses in specifically stimulated antigen-reactive cells." Thus, although the HL-A1,8 phenotype might be more prone to develop autoimmune conditions, he might have a stronger immune response against childhood infections.

It seems obvious that only the surface has been scratched in correlating specific HL-A determinants with specific pathological conditions. Consequently, we can anticipate that many new associations will undoubtedly be revealed, and others will "fall by the wayside." The most intriguing aspect of these associations is, of course, their etiological relationships, and as specific associations become better documented, hopefully, these relationships will become more apparent.

IX. Inherited Differences in Antibody Specificity

The study of immunoglobulin structural genes has in the past been primarily based on two types of analyses: the inheritance of allotypic differences and the amino acid sequences of immunoglobulins (reviewed by Cohen and Milstein, 1967; Kelus and Gell, 1967; Potter and Lieberman, 1967; Herzenberg *et al.*, 1968; Milstein and Munro, 1970; Gally and Edelman, 1972). There is a growing body of evidence, however, that a third type of genetic analysis is feasible, namely the study of the inheritance of antibody-combining sites as reflected either in idiotypes or differences in antibody specificity.

The responses of inbred guinea pigs to antigenic determinants on insulin have provided an important model system for studying the ge-

netics of antibody specificity. It was reported by Arquilla and Finn (1963) that strain 2 guinea pigs produced antibodies to portions of the insulin molecule to which strain 13 guinea pigs could not respond. Insoluble insulin complexes were saturated with antibodies from one animal, and the precentage of antibodies from another animal which could be bound to these complexes was measured. By means of this test, antibody variation within either strain 2 or strain 13 could not be demonstrated, but when the two strains were compared with one another, remarkable differences in specificity were observed. For example, when insulin complexes were saturated with one particular rabbit antiserum, 22.9–99.5% of the strain 2 antibodies were bound, whereas only 0.5% of the antibody activity from strain 13 guinea pigs could be bound to the same aggregates.

When antibodies from individual $(2 \times 13)F_1$ guinea pigs were tested, there were no differences between F_1 specificities and specificities of either strain 2 or 13 which could consistently be demonstrated (Arquilla and Finn, 1965). When sera from individual $(2 \times 13)F_2$ guinea pigs were tested, some of the sera had specificities characteristic of strain 13, others of strain 2, and still others had specificities not observed in either parental strain (Arquilla and Finn, 1965).

In subsequent work it was demonstrated that strain 2 guinea pig antisera reacted preferentially with the N-terminals of the A and B chains of insulin, whereas strain 13 antisera reacted better with the C-terminals of the A and B chains (Arquilla *et al.*, 1967).

A search for genetic differences in the fine specificity of anti-DNP antibodies has been undertaken by Yoshida *et al.* (1970) and Paul *et al.* (1970a). The fine specificities of anti-DNP antibodies from four mammalian species were quantitated by the use of ligand inhibition of the binding of DNP-^{3}H–ϵ-aminocaproic acid. Interspecies differences were demonstrated which could be accounted for neither by the amount nor by the affinity of these antibodies (Yoshida *et al.*, 1970). When inbred strains of mice were examined by the same techniques, clear and reproducible differences in the fine specificities of anti-DNP antibodies produced by several strains were demonstrated (Paul *et al.*, 1970a). The strain differences in the anti-insulin responses of guinea pigs described above were shown to stem from differences in the nature of the antigenic determinant to which the animal makes its most vigorous response (Arquilla *et al.*, 1967), whereas the anti-DNP variations involve fine specificity differences of antibodies with the same general specificity.

A myeloma protein was described which was capable of binding α-1,3-dextran (Weigert *et al.*, 1970) and which had an idiotypic specificity to which the anti-idiotype antibody reaction could be inhibited by

a disaccharide with the α-1,3 linkage (Blomberg *et al.*, 1972). It was proposed that the antibody-combining site could be coded for by two germ-line variable-region genes: one for the λ-chain variable region and one for a heavy chain variable region. If this were so, it was suggested that germ-line polymorphism in either V_λ or V_H might render mice unresponsive to α-1,3-dextran, and that responsiveness would show dominant inheritance. Mice were, therefore, immunized with dextran, and antibody responses were tested in a modified Jerne plaque assay. Most of the cells from BALB/c mice with anti-α-1,3-dextran antibody produced antibody of the lambda class which possessed the idiotypic specificity described above. Mice of the other strains, such as C57BL/6, however, produced anti-α-1,3-dextran antibody mainly in the kappa light-chain class and without the test idiotype. The response produced by BALB/c mice ranged from 1000 to 2800 PFC/10^6 cells, whereas the response for C57BL/6 mice ranged from 50 to 160 PFC/10^6 cells. The ability to respond well was dominant, as the (BALB/c × C57BL/6)F_1 hybrids produced a mean response of 1140 PFC/10^6 cells (Blomberg *et al.*, 1972).

Linkage relationships of the gene involved in the dextran response were explored by testing seven recombinant inbred strains derived from (C57BL/6 × BALB/c)F_2 mice (Bailey, 1971a). These results suggested that the immune response locus was linked to the genes controlling the heavy chain constant regions (Blomberg *et al.*, 1972).

Evidence for a similar genetic difference involving the antibody response to the phosphorylcholine determinant was reported by Sher and Cohn (1972). The immunoglobulin products of a series of BALB/c plasmacytomas were shown to precipitate with the phosphorylcholine determinant of the pneumococcal C-carbohydrate (Cohn *et al.*, 1969; Potter and Lieberman, 1970; Leon and Young, 1971). It was demonstrated that seven of the eleven immunoglobulins in this group possessed an idiotypic determinant (S107) which could be identified by mouse antisera. Since it seemed most improbable that somatic mutation would result in the production of antiphosphorylcholine sequences in seven independently derived plasmacytomas, the possibility was explored that this sequence was encoded in germ-line V genes. Inbred strains of mice were accordingly immunized with C-carbohydrate, and immune responses were measured in an agar-slide plaque assay. Strains BALB/c and A/J both produced good antiphosphorylcholine responses, but the BALB/c response could be specifically inhibited by anti-S107 serum, whereas the A/J response could be only slightly inhibited by this antiserum.

Various inbred strains were then immunized against the C-carbohydrate, and antiphosphorylcholine responses were classified as *idiotype*

positive (inhibitable by anti-S107) or *idiotype negative* (not inhibitable by anti-S107). None of the inbred strains that were tested gave intermediate levels of inhibition (21–91%).

In the (BALB/c × A/J)F_1 × A/J backcross generation, 15 of the 17 idiotype-positive mice proved to possess the BALB/c IgA allotype and only 1 of the 11 idiotype-negative mice possessed this determinant. Nine of the mice exhibited intermediate levels of inhibition in this generation.

These results suggest that a heavy chain germ-line V gene codes for an antiphosphorylcholine sequence and that this sequence possesses the S107 idiotype. In order to explain the occurrence of intermediate levels of inhibition in the backcross generation, it was suggested that a second genetic factor, possibly linked to *H-2*, was also involved in this response (Sher and Cohn, 1972).

X. Genetic Control of the Mixed Leukocyte Culture Reaction

A. Introduction

It was shown by Bach and Hirschhorn (1964) and by Bain and Lowenstein (1964) that when blood leukocytes from two unrelated individuals are mixed and maintained in culture for several days, a mutual proliferative response occurs which is characterized by the emergence of blast cells and increased DNA synthesis. The evidence that this reaction occurs in response to immunological incompatibility is fairly strong (Wilson, 1971): (*1*) the responding cells in the mixed leukocyte culture (MLC) reaction are lymphocytes—cells known to be of primary importance in immune phenomena; (*2*) the magnitude of the proliferative reaction in the MLC depends on the degree of immunogenetic disparity of the two leukocyte codonors (Bain *et al.*, 1964); (*3*) the immunoproliferative behavior of lymphocytes is similar in the graft-versus-host reaction and the MLC reaction (Wilson and Elkins, 1969); (*4*) alloantigens of the major histocompatibility loci of man (Amos and Bach, 1968), the mouse (Rychlikova and Iványi, 1969), and the rat (Silvers *et al.*, 1967) appear to stimulate proliferation in culture; (*5*) cells that respond are thymus-derived lymphocytes of the circulating lymphocyte pool (Johnston and Wilson, 1970); and (*6*) cells from immunologically tolerant animals do not respond to the H isoantigens of which the donors have been made tolerant (Wilson *et al.*, 1967).

Silvers *et al.* (1967) demonstrated that among genetically defined backcross populations of rats, mixed lymphocyte reactivity occurred only when leukocyte donors differed at the *Ag-B* locus, which is the major histocompatibility locus of rats. The major histocompatibility locus in man is the HL-A locus. It was shown by Bach and Amos (1967) that

leukocytes from sibling pairs possessing similar HL-A types did not stimulate one another in culture.

Because of all of the above observations, it was widely believed that mixed lymphocyte stimulation was directly controlled by the major histocompatibility locus of each species. It has been necessary to modify this belief, however, after several types of observations were described: (*1*) some reports suggest that the combined effects of multiple minor histocompatibility loci may induce proliferation in MLC's (Dutton, 1966; Colley and DeWitt, 1969; Häyry and Defendi, 1970); (*2*) a growing body of evidence suggests that a locus or loci closely linked to the major histocompatibility locus are of primary importance for proliferation in MLC's in man and in the mouse (Amos and Yunis, 1971; Yunis and Amos, 1971; Rychlikova *et al.*, 1971; Klein *et al.*, 1972; *Bach et al.*, 1972); (*3*) a single genetic locus has been identified in mice which is not linked to *H-2*, but which controls mixed leukocyte reactions (MLR's). Allelic differences at this locus lead to MLC reactions equivalent to those occurring when *H-2* incompatible cells are cultured together (Festenstein *et al.*, 1972).

B. Studies in Man

It was proposed by Yunis and Amos (1971) that MLR's are determined by two loci, a stimulator locus (MLR-S) and a response locus (MLR-R) which is analogous to an Ir gene, and that both of these loci are closely linked to HL-A. According to this hypothesis, differences at the MLR-S locus would lead to a MLR even in the absence of a major HL-A difference.

In order to prove this hypothesis, it would be necessary to demonstrate that, in certain cases, (*a*) there are HL-A identical individuals whose leukocytes stimulate in culture, and (*b*) there are individuals whose leukocytes do not stimulate in culture despite the fact that these individuals differ at the HL-A locus. At least six reports providing evidence for condition *a* have recently been published: (*1*) Kissmeyer-Nielsen *et al.* (1970) studied 4 HL-A identical unrelated pairs and found all 4 of them to be stimulatory in mixed cultures; (*2*) Koch *et al.* (1971) reported that only 2 of 9 HL-A identical unrelated pairs of individuals did not stimulate in MLC's (that the other 7 pairs stimulated in culture suggests the existence of a separate MLR locus); (*3*) Yunis and Amos (1971) reported studies on one family in which 2 siblings, A and B, were HL-A identical and 2 other siblings, C and D, were also HL-A identical but differed from A and B. All four siblings showed stimulation with an unrelated subject who was phenotypically identical for the HL-A antigens of C and D; (4) Johnston and Bashir (1971) studied MLC re-

actions in 13 pairs of HL-A identical unrelated individuals and found that in all combinations stimulation occurred, and it was frequently of a magnitude similar to that observed when HL-A nonidentical subjects were tested; (5) Eijsvoogel *et al.* (1971) studied 26 HL-A identical unrelated pairs of individuals and found that only 3 of these pairs did not stimulate; and (6) Mempel *et al.* (1972) found one family in which 1 sibling showed definite stimulation with 3 HL-A identical siblings and, conversely, a clear nonstimulation with a sister who differed by one haplotype.

Evidence of nonstimulation between HL-A nonidentical individuals has also been reported for unrelated people. Pentycross *et al.* (1972a) studied 2 unrelated individuals, 1 of type HL-A1, HL-A9, HL-A8, W5 (donor 1, D1) and the other of type, W19, HL-A12 (donor 2, D2). The lymphocytes of these donors failed to stimulate one another in culture, even though their cells responded normally to PHA. Further studies were performed utilizing cells from a third donor (D3) of type HL-A9 (W24), HL-A11, W14, W15 (LND*), a full sibling of D1 (S1) of type HL-A2, W10, W15 (LND*) and a full sibling of D2 (S2) of type HL-A1, HL-A2, HL-A8 (Pentycross *et al.*, 1972b). The cells of S2 stimulated the lymphocytes of D1, D2, D3, and S1, giving transformation scores of 3% or over in each case. But when the cells of S2 were the stimulated ones in the MLC, the transformation score was never more than 1%. Since the S2 cells responded normally to PHA, it was concluded that S2 is a nonresponder in the MLC. It was suggested that these results could be explained by the hypothesis of Yunis and Amos (1971), but there are some difficulties with this explanation. Yunis and Amos proposed that any 2 individuals possessing different alleles at the MLR-S locus should stimulate in culture. Thus, S2 appears to differ at the MLR-S locus from D1, D2, D3, and S1 because this donor could stimulate all of these cells. Therefore, the failure of S2 to respond to these donors cannot be attributed to compatibility at the MLR-S locus. One could argue that S2 possesses a mutation at the MLR-R locus, but no evidence has been presented that this is the case. As far as present evidence is concerned, it would appear that the genetic or nongenetic factors causing the unresponsiveness of S2 could be totally unrelated to both the MLR-S and MLR-R loci.

If there is an MLR locus or loci genetically separable from the loci coding for HL-A antigens, it would be of great interest to understand the linkage relationships of these loci. Yunis *et al.* (1971) have proposed the tentative order HL-A first series—HL-A second series—MLR. This order was suggested because one family (Duke family 0189) included a probable recombinant who seemed to have kept the second series and

MLR locus of the C haplotype, but lost the first series from this chromosome.

DuPont *et al.* (1971) tested for MLC reactions among 7 siblings, 1 of whom was a recombinant between the LA (first) and Four (second) series. Isolated LA antigen differences always failed to stimulate, whereas isolated Four antigen differences gave unequivocally positive responses. These findings are in agreement with those of Yunis *et al.* (1971) in placing the MLR locus closer to the second series than the first.

Eijsvoogel *et al.* (1972) have provided additional evidence that antigens of neither the LA nor the Four series are responsible for MLC activation. Their crucial finding was a child who was MLC positive when tested with cells from 2 siblings known to possess the *same* LA and Four antigens, and yet was MLC negative when tested with cells from other siblings who were known to be HL-A incompatible. Their results suggest that a recombinant chromosome derived by crossing-over between the MLR locus and the HL-A Four locus occurred and that the proposal of Yunis *et al.* (1971) that the genes are arranged in the order LA–Four–MLR is correct.

C. Studies in Mice

The availability of a large number of inbred mouse strains, some of which possess recombinant *H-2* chromosomes, has greatly facilitated the study of this problem. Rychlikova *et al.* (1971) studied a series of mouse recombinant strains that differed only for the *H-2D* region or *H-2K* region and other inbred strains that differed at both the *H-2D* and *H-2K* regions. Their findings suggested that difference for the *H-2D* region alone did not result in MLC reactions, but that *H-2K* differences, either alone or in combination with *H-2D* differences, resulted in positive MLC reactions. These studies were repeated by Klein *et al.* (1972) who confirmed that *H-2K* differences were associated on the average with stronger MLC stimulation than *H-2D* differences but also showed that some *H-2D* region differences by themselves do result in significant stimulation in MLC. Bach *et al.* (1972) have proposed that in addition to the serologically defined (SD) antigens coded by the *H-2D* and *H-2K* loci (Fig. 1), the major histocompatibility complex of the mouse determines other antigens. Although these lymphocyte-defined (LD) antigens cannot be detected serologically, they can cause stimulation in MLC. Direct evidence has also been obtained for the existence of LD differences that cannot be detected by skin grafting. Thus cells of 4R mice respond to stimulating cells of 2R mice despite the fact that skin grafts between 2R and 4R mice are not rejected (Stimpfling and Reichert, 1970; Bach *et al.*, 1972). The exact map position of the genes for LD differences

has not been determined, but evidence has been reported suggesting that in some cases LD differences map genetically with the immune response loci between *H-2D* and *H-2K* (Bach *et al.*, 1973; Meo *et al.*, 1973). The response of 4R lymphocytes to 2R cells is especially interesting in this respect, since these strains differ at the *Ir-IgG* locus (Fig. 2; Lieberman *et al.*, 1972b).

Meo *et al.* (1973) tested for MLR's among a number of strains possessing intra-*H-2* recombinant chromosomes. In combinations differing only in *H-2K*, *H-2D*, *Ss-Slp* or any two of these together, the reaction either was weak or not different from controls. In combinations differing in the *Ir* region, both with or without accompanying differences in other regions, the reaction always was very strong. These results clearly demonstrate that MLR's are induced by incompatibilities in the *Ir* region and not by the serologically detectable differences at the *H-2D* and *H-2K* loci. It is, therefore, possible that the product of the *Ir-1* gene is an LD antigen which can be studied in the MLC reaction.

XI. Genetic Control of Autoimmune Reactions

Although genes for high immune response would be advantageous in resisting infections and tumor growth, some of these same genes could be deleterious insofar as autoimmunity is concerned. Ir genes concerned with autoimmunity have now been described and are very similar to those genes that regulate responses to synthetic or environmental antigens.

When the immune responses of NZB/W mice, which are notoriously susceptible to autoimmune disease, were compared with those of several other strains, it was observed that NZB/W's were relatively high responders to some antigens and low responders to others. When these same strains of mice were injected with DNA–MBSA, the titer of antinuclear antibodies was much greater in NZB/W mice (252) than in four other strains (32, 4, 4, 1). It was concluded that, although NZB/W mice do not possess a uniform immune hyperresponsiveness, they do have a genetically determined hyperreactivity to some antigens and particularly to the antigens involved in their autoimmune disease (Lambert and Dixon, 1970).

Vladutiu and Rose (1971) studied the induction of autoimmune thyroiditis in thirty-three inbred and congenic strains of mice and demonstrated the importance of *H-2* in determining susceptibility to this condition. The animals were injected twice with murine thyroid extract emulsified in CFA and killed 4 weeks after the first injection. The titer of thyroid antibody and degree of thyroid pathology were recorded in each case. There was a remarkable uniformity among all strains of a

given *H-2* type in both the antibody titer and its degree of thyroid pathology. That the severity of autoimmune thyroiditis was dependent on *H-2* rather than the genetic background was best demonstrated by studies on congenic strains. In every case the relative severity of the disease correlated with that characteristic of other strains of the same *H-2* type and was independent of the genetic background. Responsiveness to thyroid antigens was inherited as a dominant trait, since F_1 hybrids derived by crossing good responders with poor responders developed antibody titers and thyroid pathology similar to those observed in the good responder (Vladutiu and Rose, 1971).

The genetic basis of susceptibility to experimental allergic encephalomyelitis (EAE) in rats was first explored by Kornblum (1968), who observed that EAE could easily be induced in most Lewis and DA rats by injecting guinea pig spinal cord in CFA. The same treatment failed to induce EAE in any of the BN rats tested, but 64% of the (Lewis × BN)F_1 hybrids were susceptible to the disease. Evidence suggesting that susceptibility to EAE is determined by a locus closely linked to the *Ag-B* histocompatibility locus was obtained by Gasser *et al.* (1973). It was observed that all Lewis rats tested were susceptible to EAE, that no BN rats were susceptible, and that all of the (Lewis × BN) F_1 hybrids could develop the disease. Among 26 (Lewis × BN)F_1 × BN backcross individuals, 11 rats were homozygous for the *Ag-B* allele possessed by strain BN and 15 were heterozygous. None of the 11 homozygotes developed EAE, but 12 of the 15 heterozygotes were susceptible.

That the locus determining EAE susceptibility is not the *Ag-B* locus itself was suggested by results obtained with the BN.B4 strain. BN.B4 is a congenic strain which has the genetic background of strain BN but is homozygous for the $Ag\text{-}B^4$ allele of strain DA. Since DA is susceptible to induction of EAE, BN.B4 should likewise be susceptible if the *Ag-B* locus determines susceptibility. When 14 BN.B4 rats were tested, 13 were completely resistant to the induction of EAE, and one was scored as "questionable." Assuming that the susceptibility of DA rats is determined by the same locus as is the case with Lewis animals, the results with BN.B4 demonstrate that the locus determining susceptibility to EAE is distinct from *Ag-B*. Whether this assumption is valid is being tested by experiments now in progress.

XII. Genetic Control of Nonspecific Immunological Function

Most of the Ir genes described in this article were discovered by observations concerning deficiencies in specific immune responses which were not known to affect overall immunological function. Nevertheless,

genetic factors have been studied which are known to have nonspecific effects on the immune system. Examples of such genes include those responsible for (Bruton-type agammaglobulinemia and the Wiskott-Aldrich syndrome (Section VI) as well as some of the genes that responded to selection in Biozzi's experiments (Section V,E). In this section, other genetic factors known to affect generally *in vivo* and *in vitro* manifestations of immunological reactions will be considered.

In the course of an experiment in which lines of mice were selected for large and small size, Chai (1957) observed that the line selected for small size was leukopenic. The average white blood cell count for the small line was 2320 leukocytes/mm.^{3}, as compared with an average count of 8380 cells/mm.^{3} in the animals selected for large size. When these two lines were crossed to produce F_1 and F_2 populations, variance estimates indicated that approximately 50% of the variance could be attributed to genetic factors (Chai, 1957).

A two-way selection experiment was then initiated to study the genetic control of leukocyte production (Chai, 1970). The initial population was established by intercrossing six inbred strains to provide a wide spectrum of genes for selective breeding. One line (HLC) was selected for high leukocyte count, another (LLC) for low leukocyte count, and a random line derived from the same population was maintained as a control. After eighteen generations of selection, the mean leukocyte count in the HLC line was about 4 times as great as that of the LLC line (24,510 versus 5760 for females and 26,080 versus 6090 for males). Selection for high leukocyte count had the effect of increasing the percentage of lymphocytes in the blood, whereas the proportion of neutrophils was correspondingly decreased.

Thymus, spleen, and adrenal weights were recorded for animals of the eighteenth generation. The mean thymus weight of the HLC mice was larger than that of the LLC mice (52.4 mg. versus 26.3 mg.), but the spleens were smaller in the HLC mice (85.2 mg. versus 119.6 mg.), and the adrenals likewise were smaller (4.7 versus 5.9 mg.). Genes associated with low leukocyte counts were dominant over those for high counts, and it was suggested that only a few genes with rather large effects were responsible for these differences (Chai, 1970).

Selection of mice for high and low leukocyte counts over a period of twenty-five generations was also conducted by Weir and Schlager (1962). These authors concluded that the heritability of leukocyte count was approximately 20%. The mice with high leukocyte counts were more resistant to irradiation than the animals with low leukocyte counts, but no differences were observed in resistance to *Salmonella typhimurium.*

Strain differences in the susceptibility of guinea pigs to anaphylaxis were explored by Stone *et al.* (1964). These authors observed that strain 2 guinea pigs were considerably more resistant than Hartley animals to the acute (bronchospasm) phase of analphylactic shock. Intravenous injections of histamine revealed no differences between these two lines of animals in histamine sensitivity. However, when the histamine content of various tissues was determined by bioassay, these authors observed a threefold difference in lung histamine between strain 2 (10.5 ± 4.3 μg./gm.) and Hartley (31.2 ± 15.8 μg./gm.). It was concluded that susceptibility to acute analphylaxis is related to the quantity of liberatable histamine available for release in the lung.

During the course of their studies on the mechanisms of asthma sensitivity, Takino *et al.* (1969) designed a method for selecting a sensitive and nonsensitive bronchial walls in guinea pigs. Animals were placed in a 10-liter glass container into which was blown an atomized aqueous solution of 1.5% acetylcholine or 0.5% histamine. The length of time before the animals fell with dyspnea was regarded as an expression of the sensitivity of the bronchial walls or the irritability of bronchial vagi. After eight to ten generations of selection, the line selected for sensitivity consisted of 26 sensitive and 2 nonsensitive animals, whereas the line selected for insensitivity consisted of 26 nonsensitive and 1 sensitive guinea pig. The length of time before the animals fell with dyspnea due to acetylcholine averaged 1 minute and 50 seconds for the sensitive guinea pigs, but it was over 5 minutes for most of the nonsensitive animals. The time for standing dyspnea due to histamine likewise showed wide divergence between the two lines. It was subsequently demonstrated that guinea pigs sensitive to acetylcholine and histamine were also sensitive to pilocarpine, serotonin, and bradykinin, whereas animals not sensitive to acetylcholine were not sensitive to these other chemicals (Takino *et al.*, 1971).

The sensitivities of tracheobronchial strips from 6 sensitive and 6 nonsensitive guinea pigs were compared by stimulating these strips with acetylcholine while they were weighted with 1.0 to 1.5-gm. weights. When the 2.0-gm. weight was used, five strips from the sensitive animals produced a strong contraction and the remaining one produced a moderate reaction, whereas none of the nonsensitive strips produced a strong contraction and only one produced a moderate reaction. When the experiment was repeated using ileum strips, no significant differences in contraction could be demonstrated. The two groups did not differ significantly in the amounts of salivation produced in response to pilocarpine injections nor in the degree that their blood pressure was reduced in response to acetylcholine.

When sensitive and nonsensitive animals were intensively immunized with egg albumin before inhaling acetylcholine, no decreases were found in the length of standing dyspnea. These results demonstrated that genetic factors were selected which were not concerned with antigenic stimulation but rather with the activity of nonspecific mediators. The interpretation favored by Takino *et al.* (1971) was that these differences resulted from variations in the peripheral bronchial vagi.

For a number of years the proliferative response of lymphocytes stimulated by PHA has been used as a measure of the overall immunological reactivity of an organism. This test has recently been recommended as a means of assessing the activity of the thymus-derived lymphocyte population (Fudenberg *et al.*, 1971). Genetic differences in the level of this response have recently been described in mice and rats, but it is not clear how these differences might be reflected in immunological functions.

Williams and Benacerraf (1972) described heritable differences among inbred strains of mice in the *in vitro* response of spleen cells to PHA and concanavalin A (con A). These responses were controlled by more than one autosomal dominant gene, and no linkage to *H-2* could be demonstrated. Since responses to PHA and con A are predominantly properties of thymus-derived cells, these authors compared the frequency of θ-positive cells in the high- and low-responding strains. No significant correlation could be demonstrated between the proportion of θ-positive cells and the peak level of responses to con A or PHA.

Quantitative differences in the responses of peripheral blood leukocytes from inbred rat strains were reported by Newlin and Gasser (1973). The response of Lewis lymphocytes was approximately 5 to 7 times as great as that of BN lymphocytes when these cells were cultured in the presence of 0.7 μl. PHA/ml. culture medium. The response of (Lewis × BN)F_1 hybrid leukocytes was similar to that of Lewis cells, and all data obtained from F_2 and backcross generations were compatible with single gene segregation. Thus the (Lewis × BN)F_2 generation included 37 high responders and 7 low responders, the (Lewis × BN) × Lewis generation included 12 high responders and no low responders, and the (Lewis × BN) × BN generation included 24 high responders and 21 low responders. When high responders were selected from this last backcross and mated with BN rats to obtain a second backcross generation, the results were again compatible with single gene segregation (Newlin and Gasser, unpublished observations). In all generations tested, the response to con A correlated fairly well with the response to PHA, and the gene controlling these responses was not linked to the major histocompatibility locus, *Ag-B* (Newlin and Gasser, 1973).

XIII. Discussion and Conclusions

For a number of years the literature concerning Ir genes was extremely puzzling and difficult to interpret. Although we are not claiming that this era is over, enough important advances have now been made to justify formulating some tentative conclusions.

The first conclusion we feel justified in making is that the immune response to a very large number of antigens is under some type of genetic control. There are at least four reasons why this genetic control may be difficult to demonstrate. If one is not successful in finding genetic differences in the response to some particular antigen, the reason could be that the antigen has a number of determinants and a given strain may be a nonresponder to only one of these. As long as the assay system depends on the use of an antigen that includes a determinant which the nonresponder can recognize, this type of Ir gene will not be detected. An excellent example of this principle is the response of mice to lysozyme (Mozes *et al.*, 1971). It was observed that most mouse strains responded well to immunization with lysozyme, but only certain strains could produce antibodies against a conjugate consisting of the loop region (residues 60-83) of lysozyme attached to a synthetic polymer.

A second reason why an Ir gene may escape detection is that many genetically controlled immune responses are dose-dependent. This type of Ir gene will never be detected unless an investigator uses a sufficiently low dose of antigen for sensitization.

Third, in many immunization procedures, some unsuspected molecule may act as a carrier. In the experiments of Green *et al.* (1969a,b), *Mycobacterium tuberculosis* was apparently capable of acting as a carrier for DNP–PLL. In the chicken's response to the weak antigens of the A blood group system, incompatibilities at the strong B blood group can provide an "adjuvant effect," which is perhaps the same as a carrier function (Schierman and McBride, 1967). The *H-2* incompatibilities in mice produce a similar effect on the response to *H-3* (Berrian and McKhann, 1960) and to the blood group antigen T (Stimpfling and McBroom, 1971).

Fourth, an Ir gene may escape detection because the individual components of the antibody response are not analyzed separately. For example, two strains may appear to respond similarly to a given antigen, when, in fact, one of the strains is responding with both IgG and IgM production whereas the other is producing only IgM. The *Ir-1* gene is known to affect IgG and not IgM (Mitchell *et al.*, 1972), and the response of mice to SRBC involves a similar effect. The response of

C57BL/10 mice to SRBC consists largely of 19 S antibody, whereas strain A/J produces both 19 S and 7 S responses (Silver *et al.*, 1972).

An important question with respect to any Ir gene is what cell type is primarily involved in the expression of that gene. A considerable body of evidence has now been accumulated implicating the thymus-derived lymphocyte as being directly involved in the expression of a number of Ir genes. The following are examples: (*1*) both the *PLL* and *Ir-1* genes appear to involve carrier recognition, since nonresponders in these systems can be made to respond if the antigen in question is complexed with an immunogenic carrier (Green *et al.*, 1966; McDevitt, 1968); (*2*) in the case of the *PLL* gene, neither the use of an immunogenic carrier (Green *et al.*, 1966) nor the use of an adjuvant containing *M. tuberculosis* (Green *et al.*, 1969a) induces nonresponder guinea pigs to express delayed hypersensitivity reactions to DNP–PLL; (*3*) adult thymectomy eliminates the phenotypic difference between responders and nonresponders to (T,G)-A--L (Mitchell *et al.*, 1972); (*4*) the *Ir-1* locus has no effect on IgM production (McDevitt *et al.*, 1971; Grumet, 1972), which is in accord with the observation that IgM responses tend to be independent of the thymus (Katz and Benacerraf, 1972); and (*5*) activation of T cells by a graft-versus-host reaction (the allogeneic effect described by Katz *et al.*, 1971) enables "nonresponders" to react to (T,G)-A--L in a manner normally observed only in responders (Ordal and Grumet, 1972).

The function of T cells in T–B cell interactions remains enigmatic, although some important contributions have been made in this area (e.g., Katz *et al.*, 1970, 1971; Paul *et al.*, 1970b; Mitchison, 1971a,b,c; Boak *et al.*, 1971; Britton *et al.*, 1971). Mitchison (1971a) has suggested that T cells function primarily to concentrate antigen. If an immunogen consists of repeating antigenic subunits, the response to it may be independent of T cells (Möller, 1971; Möller and Michael, 1971). However, there are antigens that consist of repeating determinants but are still dependent on thymus-derived cells to elicit immunity. Such antigens are apparently metabolized very rapidly, so that T cells are required for antigen concentration. It was shown by Sela *et al.* (1972) that rapidly metabolized enantiomorphs are thymus-dependent immunogens, whereas slowly metabolized optical isomers are thymus-independent immunogens.

A growing body of evidence suggests that T cells possess immunoglobulin receptors on their surfaces (Basten *et al.*, 1971; Greaves and Hogg, 1971; Cone *et al.*, 1972). The important observations of Shevach *et al.* (1972) also suggest that some type of receptor is directly or indirectly controlled by Ir genes. Present evidence does not allow us to

discriminate between the two alternative hypotheses: (*1*) that an Ir gene in some way modifies the activity of the immunoglobulin receptor or (*2*) that an Ir gene produces a T-cell receptor which is functionally distinct from the immunoglobulin receptor. Evidence suggesting that the *Ir-1* gene product is responsible for stimulation in MLC's (Bach *et al.*, 1973; Meo *et al.*, 1973) is germane to this point. Lymphocyte stimulation *in vitro* promises to be a powerful tool in elucidating the nature of the T-cell receptor or receptors.

Immune response genes have also been identified which appear to concern primarily B cells. The response to Pro--L is probably in this category (Section IV,B) as well as at least some of the genes that responded to selection in Biozzi's experiment (Section V,E). The most direct evidence for genetic factors concerned with B-cell function is the demonstration that a specific Ir gene is closely linked to the immunoglobulin allotype loci. Two important examples of such genes have now been reported: the responses to α-1,3-dextran and to phosphorylcholine (Section VIII). It is necessary to keep in mind, however, that the occurrence of an allotypic and idiotypic determinant on the same molecule does not necessarily imply genetic linkage. If an antibody molecule consisted of a constant portion and a variable portion coded by two unlinked genes, it would be possible to demonstrate the occurrence of an Ir gene that was reflected in an idiotypic specificity but was not genetically linked to the allotype loci. In the experiment by Sher and Cohn (1972), the idiotype and allotype loci were shown to be linked, but the possible existence of V-region genes not linked to allotype loci should not be overlooked.

There is obviously more than one mechanism by which a B-cell dependent Ir gene could function. One type of gene could code for the V region of an antibody molecule, as discussed in the foregoing, whereas other B-cell-dependent Ir genes could regulate the division rate of antibody-producing cells, as seems to be the case with the murine response to SRBC (Biozzi *et al.*, 1972).

The activities of macrophages are undoubtedly controlled genetically, but in very few cases has there been conclusive evidence for genetic variation in macrophage function. One excellent example involves a difference in susceptibility to arbor B virus which is controlled by a single autosomal dominant gene (Goodman and Koprowski, 1962a,b). It was shown that macrophage cultures from susceptible mice supported the growth of the virus *in vitro*, whereas macrophage cultures from resistant mice did not (Goodman and Koprowski, 1962b).

Another conclusion difficult to escape is that the massive efforts that have been expended on the genetics of the immune response are being

rewarded by the advancement of clinical knowledge. The correlations between HL-A type and disease susceptibility discussed in Section VIII cannot be fully explained at the present time, but the fact that such correlations exist can be useful clinical information. It is quite possible that on the basis of histocompatibility typing, clinicians will eventually be able to predict with some degree of accuracy the profile of diseases to which a given patient will be most susceptible. In some cases this knowledge might have rather somber overtones. For example, many persons who possess HL-A 4c might not wish to be informed as to the implications of possessing this antigen. But in many cases there could well be significant preventative measures that could be used on patients known to have an inherited predeliction toward the development of some diseases.

The point has been stressed by other reviewers that the immune response is a complex process which must be regulated at many levels by genetic factors (McDevitt and Benacerraf, 1969; Benacerraf *et al.*, 1971; Benacerraf and McDevitt, 1972). Fortunately, experimental systems involving genetic variation at many of these levels are receiving increasing attention so that within a few years we should have a much better understanding of the genetic determinants of immunological responsiveness.

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Cell-Mediated Cytotoxicity, Allograft Rejection, and Tumor Immunity

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I. Introduction

Allograft rejection and tumor immunity are generally considered as manifestations of cell-mediated immunity. This concept is based on a great many observations showing that both phenomena are regularly transferred from immune donors to normal recipients with lymphoid cells but not with serum. However, the actual mechanism by which transfer of immune lymphoid cells leads to cell destruction is still poorly understood. Due to the complexity inherent in studies *in vivo*, much effort has been devoted during the last decade to establishing *in vitro* models for allograft and tumor rejection. The various *in vitro* models have been extensively reviewed by Perlmann and Holm (*1*). As a result of these studies, it became apparent that lymphoid cell populations from immune donors were often capable of destroying target cells bearing surface antigens to which the donors were sensitized. Evidence was obtained that these lytic reactions could be distinguished from those brought about by antibody and complement, and the term *cell-mediated cytotoxicity*

was thus proposed to account for immune lysis requiring the participation of lymphoid or nonlymphoid cells.

A major advance in immunobiology was the recent demonstration that lymphocytes could be divided into two general types: (1) T cells or lymphocytes that have differentiated under the influence of the thymus, and (2) B cells or lymphocytes that have not been directly influenced by the thymus. It is generally accepted that both types of lymphocytes contain antigen-reactive cells which undergo proliferation and differentiation upon antigenic stimulation. Although it is well established that B cells differentiate under appropriate conditions into antibody-secreting cells and are thus responsible for humoral immunity, antigenic stimulation of T cells leads to the formation of effector cells with different specific functions.

1. They have a fundamental regulatory influence in the development of humoral immunity, although they are not capable of secreting humoral antibodies.

2. They are responsible for mediating delayed hypersensitivity reactions presumably by virtue of releasing various factors.

3. They play a crucial role in resistance to infection by intracellular microorganisms. [The extensive literature on these three topics has recently been well reviewed by Katz and Benacerraf (2), Bloom (3), and Mackaness (4).]

4. When antigen is a normal or abnormal constituent of a cell membrane, antigen-stimulated T cells differentiate into specific cytotoxic lymphocytes that are able to destroy directly target cells carrying the sensitizing antigen. Sensitized T cells are thus the effector cells involved in some *in vitro* models of cell-mediated cytotoxicity. With appropriate methods, the activity of the cytotoxic T lymphocytes can be very accurately quantitated *in vitro,* in contrast to the other functions of sensitized T cells.

Evidence has recently been obtained that several mechanisms including different cell types are involved in cell-mediated cytotoxicity *in vitro.* By using defined cell populations, it has been possible to demonstrate at least two different pathways by which lymphocytes exert direct cytotoxicity *in vitro.* The first one requires the participation of T cells from immune donors. These cells interact directly with membrane-associated antigens by virtue of specific receptors. Humoral antibodies are not involved or may even inhibit the effect of the cytotoxic T lymphocytes. In contrast, the second pathway depends on the presence of immunoglobulin G (IgG) antibody bound to target-cell antigens and involves the participation of lymphoid cells present in normal donors and carrying receptors for the Fc portion of IgG molecules. Although present

evidence indicates that T cells are not involved in antibody-dependent cytotoxicity, the true nature of the lymphoid effector cell remains to be established. In addition to direct cytotoxicity mediated by lymphocytes, it has been shown that specific cytotoxicity *in vitro* can be obtained with macrophages carrying cytophilic antibodies or specific factors released by sensitized T cells. It is thus evident that the mechanisms of cell-mediated cytotoxicity *in vitro* are numerous and involve different effector cells according to the *in vitro* models studied.

In the present review, we first describe the experimental data recently accumulated concerning the nature of the effector cells and the mechanisms underlying the various pathways of cytotoxicity *in vitro*. In the last part, the relevance of the *in vitro* findings to the rejection of allografts or tumors *in vivo* will be briefly discussed. To avoid overlapping with two recent reviews of this series (*1*, *3*), this review will primarily include experimental work performed during the last 3 years.

II. Methodology

As stated in Bloom and Glade (*5*), understanding of the basic principles of biological phenomena can proceed no further than the methods by which they are studied will permit. This remark is particularly pertinent to the studies dealing with cell-mediated cytotoxicity *in vitro* since most of the methods used are subject to serious limitations that are often underestimated. Detailed descriptions of the various *in vitro* assay systems have been published (*5*), and critical discussion of their applicability and limitations can be found in several recent articles (*5–7*).

In this review, we will mainly discuss the results obtained from measuring direct cytotoxicity by release of radioactive chromium (^{51}Cr) from labeled target cells (*8–10*). Such an assay is simple, sensitive, precise, quantitative, reproducible, and independent of target cell multiplication. Its sensitivity and precision allow detection of cell-mediated cytotoxicity in appropriate model systems as early as 15 minutes after the sensitized lymphocytes and target cells are mixed.

Studies of cell-mediated immunity are severely limited by the absence of assays for detecting individual effector cells as is the case for antibody-producing cells. A promising approach to this question is the development of a virus plaque assay which appears to enumerate a certain category of sensitized T cells (*11*, *12*). Although release of ^{51}Cr per se is not an assay for individual effector cells, its use under appropriate conditions provides opportunities for the relative estimation of cytotoxic cell frequency in different cell populations. Such an estimation is based on the existence of a quantitative relationship between the degree of cytotoxicity and the concentration of effector cells. Analysis of

dose–response relationship, i.e., the number of labeled target cells lysed by varying numbers of lymphoid cells from immune donors within a few hours, has demonstrated that specific cytotoxicity varies linearly with the logarithm of the number of immune lymphoid cells, at least over cell concentrations causing more than 15 and less than 75% lysis (*13*). In addition, it was observed that dose–response curves obtained with different immune lymphoid cell populations resulted in parallel lines, as illustrated in Fig. 1. Empirical estimation of the relative frequency of effector cells in different populations can thus be made by comparing the number of cells required to achieve a fixed value of cytotoxicity (*13*). Experimental evidence supporting the validity of this procedure for quantitative estimates of effector cell frequency has been presented recently (*14*). Practical use of the ^{51}Cr assay for quantitation of cytotoxic lymphocytes will be presented in Section III.

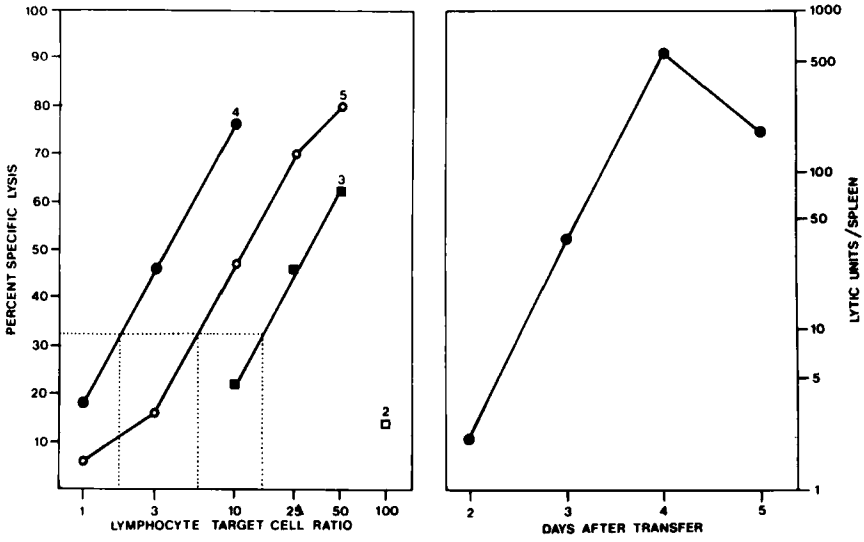

FIG. 1. Relationship between effector cell concentration and target cell lysis *in vitro*. Strain C57BL/6 spleen cells were immunized against DBA/2 alloantigens by transfer into lethally irradiated DBA/2 recipients (see Section III). The lymphoid cells present in the recipient spleens were collected from days 2 to 5 after transfer. Each cell population was assayed *in vitro* by using varying numbers of lymphocytes and a constant number (25×10^3) ^{51}Cr-labeled P-815 (DBA/2) target cells. Specific target cell lysis was determined after 6-hour incubation at 37°C. On the left side, the results are plotted on a semilogarithmic scale. The numbers refer to the time (days) of spleen cell collection after transfer. From these plots, the lymphocyte–target cell ratio necessary to obtain 33% lysis is graphically determined (dashed lines). The number of lymphocytes corresponding to this ratio is arbitrarily taken as 1 lytic unit (LU). The number of LU's present in recipient spleens is then calculated and the results are plotted on a semilogarithmic scale (right side).

Studies of the specificity of cell-mediated cytotoxicity can be performed by using a variety of target cells bearing related or unrelated membrane antigens. However, the results of direct cytotoxicity tests may be difficult to interpret because of differences in target cell susceptibility to lysis. Kinetic studies of ^{51}Cr release from various normal or tumor cells of the same H-2 genotype in the presence of the same population of immune lymphoid cells revealed considerable differences (*15*), the nature of which is still poorly understood. Selection of appropriate target cells is thus of primary importance, as exemplified by recent studies concerned with the detection of cytotoxic lymphocytes formed during the mixed leukocyte reaction (MLC) in man (*16*). It was found that the sensitivity of the assay was increased manyfold by using phytohemagglutinin-induced lymphoblasts instead of normal lymphocytes as the source of target cells. Recently, an inhibition assay using a mixture of ^{51}Cr-labeled and unlabeled target cells was proposed for the study of the specificity of cell-mediated cytotoxicity (*17*). Competitive inhibition of lysis of labeled target cells by immune lymphoid cells was noticed in the presence of unlabeled target cells bearing the sensitizing antigens, whereas lysis was unaffected by addition of target cells lacking the relevant antigens. Further studies are needed to establish the applicability of this method to various systems (see Section III,C,2).

One difficulty often encountered with the ^{51}Cr assay is related to the spontaneous release of the isotope label which varies from 0.2%/hour with chicken red cells to 1–2%/hour with established cell lines, but may reach unsuitable levels with freshly explanted tumor cells. Another limitation of the ^{51}Cr assay is the requirement of relatively large numbers of lymphoid cells. With the specific activity of ^{51}Cr presently available, a minimum number of 5 to 10×10^3 labeled target cells per individual tube is required to obtain a measurable reaction. Because excess lymphocytes are needed to obtain significant lysis, therefore a limited supply of lymphocytes may restrict the use of such a method, especially in man.

In contrast to the short incubation time needed with the ^{51}Cr assay, the other commonly used assay method, based on visual assessment of destruction of target cell monolayers, requires incubation of target cells with lymphocytes for 48 to 72 hours (*18*). This assay, which can be performed in microtitration plates, is limited to the use of target cells grown in monolayer cultures since it relies on the detachment of injured cells from the monolayer. It is thus evident that the actual target-cell counts at the end of the incubation period result from a combination of direct lysis, detachment, and proliferation. The same problems apply to modifications of the cell-counting method in which target cells are labeled with radioactive thymidine (*19, 20*) or iododeoxyuridine (*21*), since both compounds are incorporated into the target-cell deoxyribo-

nucleic acid (DNA) and are not released from damaged cells unless treatment with trypsin is used (*22*).

Extensive comparison of the results obtained with the ^{51}Cr and the microplate assay methods has not been done. There are situations in which discordant results have been obtained suggesting that the two methods do not necessarily detect the same basic phenomenon (see Section III). A clear relationship between *in vitro* lysis and growth inhibition of target cells has been established in a few instances (*23*), but this may not be the general rule. In view of the complexity of the factors that can influence the final results in the microplate assay, the results obtained with this method will not be discussed in this review unless they extend observations made with the ^{51}Cr assay method.

III. Cytotoxicity Mediated by Specifically Sensitized T Cells

Following the observation of Govaerts (*24*), several studies demonstrated that lymphoid cells from individuals immunized against normal or tumor allografts exerted a cytotoxic activity *in vitro* on target cells bearing the transplantation antigens to which the donors were sensitized (for references, see Ref. *1*). Cytotoxicity was specific, depended on close contact between reacting cells, and needed no added complement. It was assumed that antibody was not involved in the lytic process since addition of alloantibody directed against the target cells to the reaction mixture inhibited rather than increased cytotoxicity (*25, 26*). The validity of this assumption, however, was questioned by the observation of a temporal relationship between development of cell-mediated cytotoxicity and appearance of serum alloantibody (*23*). Not until 1970 was direct evidence obtained that cell-mediated cytotoxicity in mouse allogeneic systems was directly caused by specifically sensitized T cells, the activity of which was independent of antibody, normal T or B cells, antibody-producing cells, and/or macrophages.

A. Identification of Cytotoxic Lymphocytes as Sensitized T Cells

The first direct evidence for mediation of cytotoxicity by sensitized T cells was provided by studies of the formation of cytotoxic lymphocytes (CL) and cytotoxic alloantibody during graft-versus-host (GVH) reactions (*27*). These investigations were facilitated by the development of two quantitative *in vitro* assay systems allowing the independent detection of CL and of alloantibody plaque forming cells (PFC) with the same target cells (*28*). Thus, lethally irradiated mice were injected intravenously with spleen, thymus, or bone marrow cells from allogeneic donors. Five days after transfer of spleen cells, the vast majority of the

cells present in the recipient spleen were found to be of donor genotype and contained CL and alloantibody PFC directed against the recipient alloantigens. In contrast to the results obtained with spleen cells, thymus cells sensitized by inoculation into irradiated allogeneic recipients produced only CL, but no alloantibody PFC. Transferred bone marrow cells showed little cell-mediated cytotoxicity, with the exception of one strain combination (C57BL/6 bone marrow cells injected into 800 R-treated DBA/2 mice) whereby evidence was obtained that the relatively high degree of cytotoxicity was related to the presence of contaminating T cells in the bone marrow preparations (*27*).

Subsequent studies by Cerottini *et al.* (*29–31*) showed that specific elimination of T cells from spleen cells containing both CL and alloantibody PFC (obtained either from immune donors or by transfer into irradiated allogeneic recipients) abrogated cell-mediated cytotoxicity but had no effect on plaque formation. Elimination of T cells was achieved by treatment with either anti-θ serum or rabbit serum against mouse T cells and complement (*29–32*). Treatment of the same immune spleen cell populations with rabbit serum against mouse B cells or plasma cells and complement had no effect on cell-mediated cytotoxicity but completely inhibited the formation of PFC (*32*).

These results, which were confirmed in several other studies (33–39), made it clear that sensitized T cells were responsible for cell-mediated cytotoxicity observed in allograft system and in GVH reactions in mice. As will be detailed in the following, similar conclusions have been obtained concerning the nature of mouse CL formed *in vitro* during the MLC. In other species, direct identification of CL involved in allogeneic systems either *in vitro* or *in vivo* still awaits the demonstration of specific markers for T and B cells. From the increasing body of evidence suggesting that such markers exist, especially in man (*40*), the role of T cells in cytotoxic reactions detected in allogeneic systems should be clearly defined in the very near future.

In contrast to the clear evidence for the formation of cytotoxic T cells in allograft immunity, studies of cytotoxic lymphoid cells appearing in xenograft systems provided opposite results. On the one hand, Beverley and Simpson (*41*) showed that anti-θ treatment of spleen and lymph node cells from mice immunized with hamster tumor cells abolished their cytotoxic activity as assessed in a microplate assay. Similarly, indirect evidence for the participation of cytotoxic T cells was obtained in studies of rat lymphocytes sensitized *in vitro* on mouse embryo fibroblasts (*42*). On the other hand, MacLennan and his associates (*43, 44*) demonstrated that cell-mediated cytotoxicity observed with lymphoid cells from rats immunized against a human tumor cell line did not in-

volve the participation of T cells but required the presence of antibody to the target cells and of normal lymphoid cells (see Section IV). A similar mechanism was suggested by Chapuis and Brunner (*45*) in studies of *in vitro* cytotoxicity of spleen cells from guinea pigs immunized against mouse tumor cells.

Much less information is available in syngeneic tumor systems. Evidence for the participation of T cells in cell-mediated cytotoxic reactions against syngeneic tumor cells has been suggested by the results of a few investigations testing the effect of treatment with anti-θ serum and complement on the lytic activity of immune lymphoid cell populations (*46*). Since the results were essentially negative, i.e., anti-θ-treated cells showed little cytotoxic activity, the possibility could not be ruled out that T cells were not the actual effector cells, but that their presence was required for the effector cells to become functional, as suggested in other systems (see Section V). Recently, Wagner and Röllinghof (*47*) reported the formation of cytotoxic lymphocytes in *in vitro* cultures of mouse thymus cells with irradiated syngeneic plasmocytoma cells. Additional support for the formation of cytotoxic T cells in syngeneic tumor systems was provided by studies on the nature of the cytotoxic cells detected in spleen cells from mice bearing Moloney sarcoma virus (MSV)-induced tumors. Leclerc *et al.* (*48*) found that treatment of the immune spleen cells with anti-θ and complement abrogated their cytotoxic activity, as measured by the ^{51}Cr assay method, whereas removal of B cells or addition of anti-immunoglobulin to the reaction mixture had no effect. Using the microplate assay method, Lamon *et al.* (*49*) studied the same system and found cytotoxic T cells in spleens and lymph nodes taken just prior to tumor development and soon after regression. In addition, these authors found that a non-T cell subpopulation was also cytotoxic *in vitro*. This non-T-cell-mediated activity, which was detectable prior to tumor development, was particularly evident following tumor regression. Similarly, studies by Perlmann *et al.* (*50*) on the cytotoxicity of blood lymphocytes taken from bladder carcinoma-bearing patients suggested the presence of T as well as non-T effector cells.

To sum up, in the mouse, clear evidence has been obtained that cell-mediated cytotoxicity of lymphocytes sensitized against alloantigens is primarily a T-cell function. Further work is needed to confirm the validity of this finding in other species. Conflicting results have been reported in xenograft systems. Cytotoxic T cells have been described in a few syngeneic tumor systems, but extensive investigation is still required to assess their role critically in the increasing number of studies demonstrating the presence of reactive lymphoid cells in tumor-bearing animals and patients (see Section VI,B).

B. Formation of Cytotoxic T Lymphocytes

1. During Host-versus-Graft Response

a. Primary Response. With the development of quantitative assay methods, it has been possible to follow the appearance of CL in recipients of normal or tumor allografts. In mice injected intraperitoneally with a single optimal dose of living allogeneic tumor cells, lytic activity in spleen cells was detectable on days 3–4, rose rapidly to a maximum on days 10–11, and then fell gradually over the ensuing weeks (*15, 51*). Detectable activity was still present in spleen cells taken 2–3 months after a single immunization. Comparative studies of CL appearance in spleen and blood suggested a 48-hour delay between formation of active cells in lymphoid organs and their subsequent release into circulation (*15*). At peak activity, dose–response curves demonstrated that the highest concentration of CL was present at the rejection site, the peritoneal cavity in this instance, and then in the blood, thoracic duct, spleen, and lymph nodes (*15*, 52). Negligible cytotoxicity was found in the thymus.

Such observations indicated that at least some of the CL belonged to the circulating pool of lymphocytes. Similar conclusions were reached by Sprent and Miller (*53*) in extensive studies of the fate of CL formed during GVH reactions (see below).

In certain instances, kinetic studies of mice immunized with tumor allografts detected the sequential appearance of two peaks of cytotoxic activity, the first near day 10, and the second near day 25 (*54, 55*). Physical characterization of CL during the development of the immune response indicated important differences in density and size between the effector cells obtained early or late after immunization (*56–58*). Although the detailed mapping of a lymphocyte differentiation pathway is difficult in whole animal studies, the separation data, taken together, suggest the following changes during CL formation *in vivo:* (1) the CL progenitor in the normal mouse spleen is a very dense, small lymphocyte; (2) early effector cells are extremely light in density and very large and correspond to blast cells; and (3) as the response proceeds, the CL appear to pass through a series of maturation steps reflected by increase in cell density and decrease in size. One month after immunization, the effector cells have the density and sedimentation properties of small lymphocytes. Whether or not these cells, after ceasing their cytotoxic activity, can act as memory cells, i.e., they differentiate into cytotoxic blast cells upon secondary stimulation, remains to be established.

It is well established that cytotoxic antibody is also produced in allograft immunity. In the studies cited above, the peak of cell-mediated

cytotoxicity coincided very closely with the peak of serum IgM alloantibody (*23, 59*). Moreover, the development in time of alloantibody PFC followed very closely that of CL in spleens of mice immunized with tumor allografts (*28, 60*). Ample evidence has been obtained, however, that CL and PFC differ as regards their origins, physicochemical properties, and mechanisms of action. Some of the differences between the two types of effector cells have been reviewed recently (*61*). In addition, clear dissociation of the formation of CL and PFC has been achieved in certain instances (*60*).

Besides CL and alloantibody PFC, another function of effector cells has been demonstrated in allograft immunity. As first shown by Al-Askari *et al.* (*62*), inhibition of macrophage migration, an *in vitro* assay system widely used in studies of cell-mediated immunity (*3*), can be applied to detection of allograft immunity. In a comparative study of the cytotoxic and macrophage migration inhibitory activities of spleen cells during the primary response to alloantigens, Brunner and Cerottini (*60*) found that both activities reached peak values at the same time and were abrogated by treatment of spleen cells with anti-θ serum and complement. Whether or not the lymphocytes responsible for cytotoxicity and for inhibition of macrophage migration are identical remains unsettled.

These studies indicate that the development of effector cells of both cellular and humoral responses to transplantation antigens can be measured independently in a quantitative fashion. It is thus feasible to assess critically the immunogenicity of different alloantigen preparations according to source, dose, and route of administration. Examples of such studies can be found in recent publications (*51, 63*).

Formation of CL has also been followed after skin allografting in mice (*64, 65*) or in rats (*66*). Cytotoxic activity, as assessed by the ^{51}Cr assay method, was detected several days before graft rejection first in draining lymph nodes, then in spleens, blood, and contralateral lymph nodes. Peak activity usually coincided with rejection of the allogeneic skin and was more prominent in draining lymph nodes than in spleens. In contrast to the results obtained with living or irradiated tumor allografts, no CL were detected 2 weeks after skin graft rejection.

b. Secondary Response. The question of memory has often been raised in transplantation immunity. Since immune responses to allografts are characterized by the accelerated rejection of a second graft of the same specificity, it may be assumed that this corresponds to a secondary type of cellular immune response involving an increased number of antigen-reactive cells. Another explanation of the second-set rejection phenomenon is based on the persistence of effector cells in the primed host. The latter possibility, however, appears very unlikely in the light

of the recent demonstration of a typical secondary formation of CL (*51, 52*). Comparative studies clearly established the parallelism between earlier appearance, accelerated formation, and higher peak of both CL and alloantibody PFC during the secondary response to allogeneic tumor cells (*67*).

These results suggest that memory is a property of both T and B cells. This conclusion has been clearly established in other systems of immune responses, as recently reviewed by Katz and Benacerraf (*2*). In the case of T cells including CL, it is unclear whether memory is related to an increase in the number of antigen-reactive cells or involves qualitative changes as well. Dose–response analysis of CL obtained at peaks of primary and secondary responses showed no striking difference (*67*), but further refinement in methodology is needed to exclude qualitative changes in these cell populations.

2. *During Graft-versus-Host Reaction*

It is generally assumed that the GVH reaction represents an immune response called forth by the introduction of antigen-reactive cells into a host carrying different transplantation antigens and incapable of rejecting the grafted cells (*68, 69*). The validity of this assumption has been confirmed by the demonstration of the formation of both CL and alloantibody PFC after transfer of mouse spleen cells into lethally irradiated, allogeneic recipients (*27, 31*). It seems clear that these effector cells derive from precursor cells present in the transferred spleen cells which undergo proliferation and differentiation on contact with the recipient alloantigens. In this respect, the immune process taking place during the GVH reaction is similar to that occurring in allograft immunity.

Quantitative assessment of the formation of CL after transfer of 50×10^6 spleen cells into heavily irradiated allogeneic recipients is illustrated in Fig. 2. Cytotoxic activity was first detected on day 2, then rose sharply and reached a peak on day 4. Formation of CL was prevented by injection of mitotic inhibitors, such as vinblastine, indicating that the exponential rise in cytotoxic activity was related to proliferation of the transferred reactive cells (*32*).

Subsequent studies established the usefulness of the transfer system to analyze the distribution, the relative number, and the physical characteristics of CL progenitors in different lymphoid organs. In addition, such studies provided further insight into the differentiation process triggered by exposure of T cells to histocompatibility antigens. Finally, elucidation of the mechanism of cell-mediated cytotoxicity was facilitated by the availability of almost pure populations of sensitized T cells in certain instances.

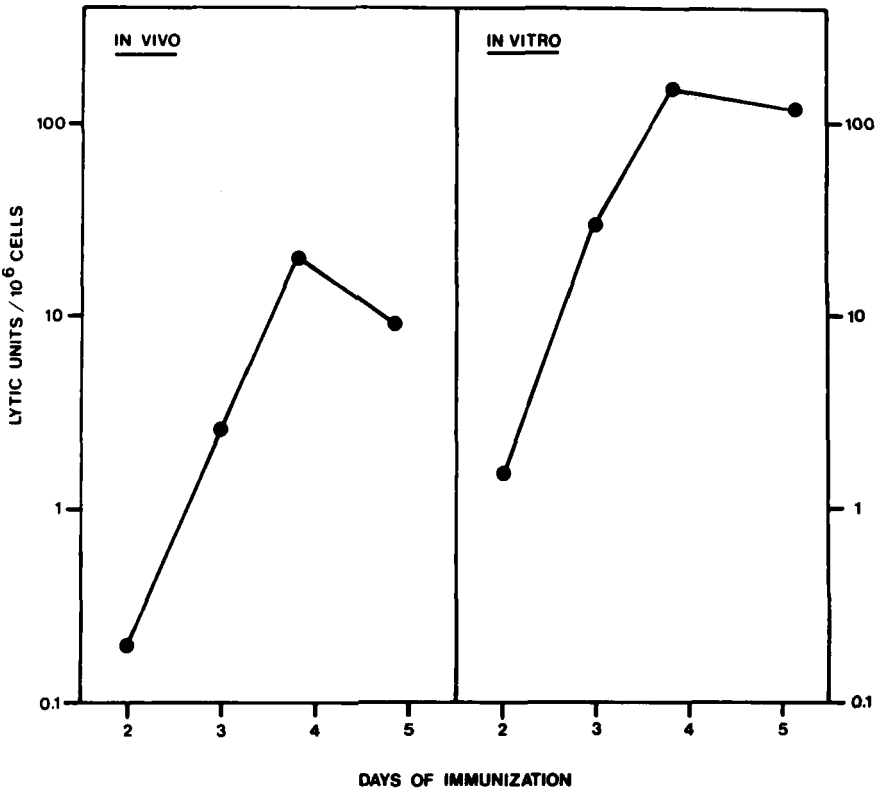

FIG. 2. Kinetics of cytotoxic lymphocyte formation during graft-versus-host (*in vivo*) or mixed leukocyte (*in vitro*) reactions. Strain C57BL/6 spleen cells were immunized against DBA/2 alloantigens either *in vivo* (by transfer into lethally irradiated DBA/2 recipients) or *in vitro* (by incubation with irradiated DBA/2 spleen cells). At different times thereafter, the lymphoid cells were assayed *in vitro* for cytotoxic lymphocytes by using ^{51}Cr-labeled P-815 (DBA/2) cells as target cells. The number of lytic units present in individual cell populations was calculated as discussed in the legend of Fig. 1.

Evidence has been obtained that CL progenitors are present in spleen, lymph node, blood, thoracic duct, and thymus (*27*, *31*, *32*, *53*). Pretreatment of lymphoid cells with anti-θ serum and complement prior to transfer into heavily irradiated, allogeneic hosts prevents formation of CL (Table I). Since CL are T cells, as cited in the foregoing, these results clearly demonstrate that CL progenitors belong to the T-cell population. Bone marrow cell preparations in mice are usually deficient in CL progenitors, but strain differences exist, as exemplified by the relatively high cytotoxic activity observed after transfer of C57BL/6 bone

TABLE I

ROLE OF T AND B CELLS IN THE FORMATION OF CYTOTOXIC LYMPHOCYTE AND ALLOANTIBODY PLAQUE-FORMING CELLS DURING THE GRAFT-VERSUS-HOST REACTION[a]

Transferred cells	LU/10^6	LU/spleen	PFC/10^6	PFC/spleen
50×10^6 Spleen cells	3.5	35	16.1	161
25×10^6 θ-Negative spleen cells	<0.03	<0.3	5.9	41
25×10^6 θ-Negative spleen cells + 25×10^6 thymus cells	2.4	27	8.1	91
25×10^6 Thymus cells	4.5	27	<0.1	<0.6

[a] Strain C57BL/6 normal spleen cells, spleen cells treated with anti-θ and complement, or thymus cells were injected into lethally irradiated DBA/2 mice. On day 5, the lymphoid cells in the recipient spleens were assayed for the presence of cytotoxic lymphocytes (CL) by using ^{51}Cr-labeled P-815 (DBA/2) tumor cells, and of alloantibody plaque-forming cells (PFC) with the same target cells. Lytic units (LU) were calculated as illustrated in Fig. 1.

marrow cells into irradiated DBA/2 mice. Even in the latter case, however, pretreatment of bone marrow cell preparations with anti-θ serum and complement abolished their ability to form CL on transfer (*32*). Evidence for the presence of T cells in bone marrow cell preparations from certain mouse strains has also been reported in other studies (*70–72*).

Estimation of the relative number of CL progenitors in different lymphoid organs has been made feasible by using the quantitative method described in Section II. Measurement of the relative amount of effector cells in spleens of irradiated hosts inoculated with graded numbers of lymphoid cells indicated that the frequency of CL progenitors was not very different in blood, lymph nodes, and spleen when calculated on the basis of number of T cells present in these lymphoid cell preparations (*32*). In the thymus, CL progenitors were 10–20 times less frequent than in the spleen. Further studies demonstrated that these progenitors belonged to a minor population of thymus cells characterized by relative resistance to cortisone (*55*), high levels of H-2 antigens, low levels of θ antigen (*57*) and, in appropriate strains, absence of the thymus leukemia (TL) antigen (*32*). Such results are in agreement with the demonstration of a small subpopulation of thymus cells carrying all of the GVH reactivity of the thymus (*73, 74*).

The antigenic properties of the CL progenitors in the thymus are very similar if not identical to those of peripheral T lymphocytes (*75*). Shortman *et al.* (*57*), however, found a striking difference in the physical characteristics of CL progenitors in thymus and spleen. Density dis-

tribution analysis in continuous gradients of albumin showed that CL progenitors in the thymus were characterized by a relatively narrow distribution in the light-density regions, whereas the typical thymus, small lymphocytes were completely inactive. In contrast, the reactive cells in the spleen were much denser than those in the thymus, and their density distribution corresponded to the region of typical small lymphocytes. These results were interpreted as indicative of further differentiation or maturation of CL progenitors occurring after they leave the thymus until they seed into the peripheral lymphoid tissues.

Some understanding of the events leading to the formation of CL during the GVH reaction has been obtained by studying the fate of thymus or thoracic duct cells inoculated into irradiated histoincompatible recipients. It is well known that T cells migrate to the lymphoid tissues after intravenous injection and localize predominantly in the thymus-dependent areas (*76*). When the recipient is histoincompatible with the T-cell donor, the reactive cells present in the transferred lymphocyte population undergo blast transformation and cell proliferation (*77, 78*). By measuring the amount of radioactivity in spleens of irradiated recipients of allogeneic T cells following a single injection of tritiated thymidine, Sprent and Miller (*79*) showed that peak incorporation occurred 3 days after thymus cell injection and 4–5 days after injection of thoracic duct lymphocytes. Subsequent studies revealed that the reactive cells eventually left the lymphoid organs after 4 days and entered the recirculating pool of lymphocytes from which they could be recovered by thoracic duct cannulation (*36*). The cells which first appeared in lymph were mostly rapidly dividing blast cells, followed later by predominantly small lymphocytes. Nearly 100% of these cells were derived from the original inoculated lymphocytes and had the characteristic features of T cells. When assayed *in vitro,* the lymphocyte population showed a high specific cytotoxicity aaginst target cells bearing the alloantigens of the recipient host (*36*).

It is thus clear that CL progenitors, during the GVH reaction, respond to the histocompatibility antigens by blast transformation and cell proliferation. Two questions are raised by these observations. First, what is the source of the immunogenic stimulus in the irradiated recipient host. Using two different approaches to answer that question, Sprent and Miller (*79*) concluded that the reticular framework of the recipient lymphoid tissue was not involved in the stimulation and suggested that the effective stimulus might be provided by relatively radioresistant cells derived from the bone marrow. A second question relates to the role of interaction between different cell types for optimal formation of CL. Whereas the regulatory influence of T cells on B-cell immune response

is well established [as recently reviewed by Katz and Benacerraf (*2*)], the existence of analogous interactions in the formation of effector T cells is less clearly defined. Evidence has been obtained, however, for a lack of influence of B cells on the formation of CL during the GVH reaction. As already cited, both alloantibody PFC and CL were formed after transfer in irradiated allogeneic recipients, whereas only CL appeared after transfer of thymus cells. Spleen cells treated with anti-θ serum and complement produced PFC only upon transfer, although in a reduced number as compared to untreated spleen cells. Addition of θ-negative spleen cells, as the source of B cells, to thymus cells, as the source of T cells, had no influence on the amount of CL recovered 5 days after inoculation (Table I). It may be noted that the production of PFC, in this system, was relatively thymus-independent and, hence, was only slightly affected by the presence of T cells.

Evidence for interaction between two types of T cells in the production of GVH has been reported (*80, 81*). The maximal degree of amplification observed with appropriate mixtures of T cells did not exceed 2.5-fold, an amount much lower than that observed in studies of T–B cell cooperation in humoral responses. More recent studies suggest the presence of two cell populations: the first one containing progenitors of effector cells and the second one, cells able to amplify the activity of the former cells (*82*). In addition, in the same GVH system, suggestive evidence was obtained that different effector functions, namely the abilities to produce either death or splenomegaly, involve distinct types of precursor cells (*82*).

No synergistic effect was noticed by Shortman *et al.* (*57*) in transfer experiments using mixtures of reactive and nonreactive subpopulations of thymus cells prepared by centrifugation to equilibrium in continuous albumin gradients. As discussed below, suggestive evidence for cooperative interaction among subsets of T cells has been recently provided in *in vitro* studies. More investigations employing refined separation procedures and quantitative assay methods are needed before any firm conclusion can be drawn as to the necessity of two functionally distinct T-cell types for optimal *in vivo* formation of effector cells.

3. In Vitro

Further understanding of the cellular events involved in CL formation has been provided by studies of *in vitro* induction of such effector cells. Following the work of Ginsburg and his associates (*83, 84*), who developed a xenogeneic system using normal rat lymphocytes cultures on mouse fibroblast monolayers, several investigators reported successful induction of CL in allogeneic systems (*14, 85–90*). In the mouse, a criti-

cal observation was made by Häyry and Defendi (*85*) who first noticed the appearance of CL in unidirectional MLC. Subsequent quantitative studies established the usefulness of the MLC system in providing cell populations with greater cytotoxic activity than those obtained after *in vivo* immunization (*90*).

Several systems of *in vitro* induction of CL have been proposed, but it appears that the most effective one consists of culturing normal lymphocytes with allogeneic lymphoid cells, the mitotic activity of which has been abolished by treatment with X-rays or mitomycin C. Elaborate culture conditions, as devised for obtaining PFC responses *in vitro* (*91, 92*), have been used in some studies (*88, 90*), but simpler culture conditions appear equally effective in supporting CL formation, provided that critical ingredients are added to the culture medium (*32*).

In one of the most sensitive systems, cytotoxic activity was first detected on day 2, then rose sharply and reached a peak on day 4. It thus appeared that the kinetics of CL formation *in vitro* was very similar to that observed *in vivo* during the GVH reaction, although a tenfold difference in relative frequency of CL existed between *in vitro* and *in vivo* sensitized spleen cell populations (Fig. 2). Such quantitative studies suggest that *in vitro* formation of CL is related to the cell proliferation taking place during the MLC (for references, see Ref. *93*). By using agents that damage proliferating cells during a short-time exposure, several investigators, working independently, presented evidence for proliferation in the formation of CL (*14, 94, 95*). Kinetic studies revealed that no significant proliferation occurred during the first 24 hours of culture. Between 24 and 48 hours, however, all precursor cells appeared to be dividing (*14*). Later in the response, dissociation between proliferation and formation of CL has been reported by Wagner (*96*), who found a 48-hour delay between the peak of cell proliferation and that of cytotoxic activity, suggesting that some differentiation process took place after cell proliferation. Essentially identical observations were reported by Häyry *et al.* (*95*) who also found little cell proliferation at the time of peak cytotoxic activity. Any interpretation of these results should take into account the recent suggestion that the majority of cells undergoing division during the MLC reaction might be different from those differentiating into CL (*96a*).

Because the MLC is also marked by the appearance of blast cells, attempts have been made to characterize the physical properties of CL. Velocity sedimentation analysis of the lymphoid cells obtained at 4 to 6 days of MLC demonstrated that CL were a relatively heterogeneous, rapidly sedimenting population, whereas very little activity was obtained in the small lymphocyte fractions (*14, 95*). These results strongly sug-

gest that CL were indeed blast cells, but the authors give no information as to the actual percentage of blast cells that are cytotoxic. When the blast cells are separated by velocity sedimentation and kept in culture on syngeneic fibroblast monolayers for several days, they revert in size to small lymphocytes (*95*). These findings therefore support the general pathway of CL differentiation suggested by the studies of CL formation *in vivo*. In the mouse, compelling evidence for the T-cell nature of *in vitro* formed CL has been reported. First, no CL appeared in MLC using lymphoid cells deprived of T cells as the source of responding cells. The cell populations tested included bone marrow cells (*14*), spleen cells treated with anti-θ serum and complement (*14, 97*), spleen cells from adult thymectomized, irradiated, bone marrow-reconstituted animals, or spleen cells from congenitally athymic (nude) mice (*98*). More convincing was the demonstration that nearly pure populations of T cells, such as thymus cells (*99, 100*) or spleen cells depleted of B cells by different procedures (*14, 95, 97*) were able to generate CL in MLC. Consistent with the results obtained in experiments *in vivo* (*55*) was the observation that progenitors in the thymus were concentrated in the cortisone-resistant population (*97*). These results were confirmed by the demonstration that the cytotoxic activity of *in vitro* sensitized lymphoid cells was abolished by treatment with anti-θ serum and complement (*14, 97*).

Subsequent studies revealed that adherent cells, presumably macrophages, were required for the *in vitro* induction of CL (*14, 101, 102*). A similar requirement for adherent cells had been demonstrated previously in the initiation of some humoral responses and of the proliferative response in MLC, as recently reviewed by Unanue (*103*). In the latter case, the role of macrophages appeared to be nonspecific, since macrophages syngeneic to either responding or stimulating cells could be used (*104*). Identical observations were made by MacDonald in studying the formation of CL *in vitro* (*14*). Several mechanisms of macrophage function during the induction phase of the immune response have been postulated (*103*). In addition to these mechanisms, the possibility cannot be excluded that the presence of small numbers of macrophages merely provides optimal culture conditions as suggested by observations of successful substitution of macrophages by macrophage culture medium (*105*) or mercaptoethanol (*106*).

Cooperation between T and B cells has been recently described in an *in vitro* model for graft rejection (*107*). Several observations, however, are inconsistent with this concept. As mentioned before, cortisone-resistant thymus cells or B-cell-depleted spleen cells are perfectly able to generate CL *in vitro*. Quantitative comparison of the relative frequency

of effector cells formed in MLC with unseparated spleen or B-cell-depleted spleen cells as the source of responding cells revealed a three-fold enrichment on a cell-to-cell basis in the latter case (*97*). In another study, cell separation experiments in which B-cell-rich fractions were mixed with T-cell-rich fractions failed to demonstrate a synergistic effect (*58*). These results are in agreement with the observations cited previously in the GVH system and indicate that formation of CL occurs independently of the presence of B cells.

Evidence for T–T cell interaction in the *in vitro* generation of CL has been suggested by a recent study demonstrating that limited numbers of either thymus or lymph node cells, although unable to generate high CL activity when incubated with allogeneic irradiated spleen cells, produced large numbers of CL when mixed together at the onset of the MLC (*107a*). In addition, the results of this study indicated that peripheral T cells provided the major source of CL precursors, whereas thymus cells acted mainly as helper cells during the MLC.

Using a different approach, Bach *et al.* (*96a*) and Alter *et al.* (*107b*) also suggested the participation of two subsets of lymphocytes in the formation of CL during the MLC. These studies were related to the recent demonstration of a third (or several) locus in the genetic region of the major histocompatibility complex (*107c–107e*). In contrast to the first two loci, which control serologically detectable antigens, the third one appears to be responsible for differences which lead to cell proliferation in MLC but are not detectable serologically. For that reason, the terms serologically defined (SD), or lymphocyte-defined (LD), differences are used to designate the products controlled by the first two loci, or the third one, respectively. Both in man and in the mouse, clear evidence has been provided for the existence of such LD differences. The basic finding has been the occurrence of a proliferative response of lymphocytes in MLC tests despite identical SD histocompatibility antigens on both responding and stimulating cells (*107c*). Since the three loci are linked with a low recombination frequency, and because of the rarity of recombination in a family, human siblings identical for SD antigens are also likely to have identical genotype at the third locus. By contrast, at the population level, crossing-over is frequent, and accordingly the lymphocytes of the majority of unrelated individuals with the same HL-A phenotype do stimulate each other in MLC tests because they presumably differ at the third locus (*107f*). The same observations have been made in mice using recombinants which are identical at the *H-2K* and *H-2D* loci (*107e*, *107g*). Similar studies have indicated that in certain strain combinations which are SD identical but different for LD, no CL are formed despite intensive proliferation (*107b*). Moreover,

it has been shown that neither proliferation nor CL activity is detectable in strain combinations which are LD identical but different for SD. However, when responding cells are incubated with two different stimulating cell populations, one differing from the responding cells by LD and the other differing from the responding cells by SD, CL able to destroy target cells carrying the SD antigens are then formed. Whether or not the lymphocytes responding to LD differences correspond to the helper cells, as described by Wagner (*107a*), which can amplify the generation of CL directed against SD antigens remains to be determined.

The source of stimulating cells appears to play a role in the optimal generation of CL *in vitro*. From the data available in identical systems, it appears that lymphoid cells stimulate better than nonlymphoid cells such as fibroblasts. In a parallel study, identical stimulation by either T-cell depleted spleen cells or cortisone-resistant thymus cells was noticed, whereas normal thymus cells appeared less effective (*32*). Similarly, von Boehmer *et al.* (*108*) found that both T and B cells could stimulate allogeneic MLC. Tumor cell lines could be substituted for normal lymphoid cells in some instances (32). Recently, Wagner (*109*) reported that inhibition of the metabolism of the stimulating cells decreased their ability to elicit the formation of CL. As a tentative explanation, this author suggested the existence of a relationship between the turnover of surface proteins or transplantation antigens of living cells and their immunogenicity. The question remains whether the immunogenic stimulus is provided by direct contact of the stimulating cell with the responding cell or by soluble transplantation antigens released from the surface of the stimulating cells. Indeed, *in vitro* induction of CL by subcellular alloantigen preparations has been reported (*110*, *111*), although the very low degree of cytotoxic activity achieved suggests a rather weak immunogenicity of the soluble material as compared to intact living cells. As more purified preparations of soluble transplantation antigens become available, further studies on their immunogenicity *in vitro* should clarify this point. It is obvious that these studies should take into account the necessity of the presence of both SD and LD antigens for CL formation *in vitro*.

The cellular events leading to the formation of CL *in vitro* may be separated into two categories according to the requirement of antigen or not. Lohmann-Matthes *et al.* (*112*) demonstrated that lymphoid cells, after 48 hours of contact with allogeneic fibroblasts, were able to generate CL 3 days later even after transfer onto syngeneic fibroblasts. These results, which corroborated the findings of Cohen and Feldman (*113*) in the xenogeneic, rat antimouse system, suggest that the proliferation and/or differentiation phases could proceed even in the absence

of antigen once antigen-dependent triggering had occurred. However, the possibility of antigen transfer has not been ruled out completely.

A useful extension of these experimental studies has been suggested by similar findings in humans. The first observation was made by Solliday and Bach (*87*) who showed that human blood lymphocytes, when cultured with lymphoblast cell lines, preferentially destroyed lymphoblasts to which they had been sensitized. However, in this study, as in other similar investigations (*114*), a nonspecific component of cytotoxicity could not be ruled out since target cells syngeneic to the responding cells were not available. This difficulty was circumvented by the use of lymphocytes transformed into blasts by treatment with phytohemagglutinin as the source of target cells (*16*). As compared to normal lymphocytes, such blasts were found to be more susceptible to humoral and cell-mediated cytotoxicity, while retaining the original antigenic specificity. Although direct evidence of the participation of T cells in this system has not been obtained, the results in mice and other data in man (*115*) strongly support this view.

4. During Induction of Tumor Immunity

As mentioned before, very few studies have dealt with the formation of cytotoxic T cells in syngeneic tumor systems. One such study has been recently reported by Leclerc *et al.* (*48*, *116*) who followed the appearance of CL in spleen and lymph node cells from mice bearing MSV-induced tumors. Cytotoxicity was first detected 7 days after virus inoculation, when the tumor became visible. Peak activity was reached at 13 to 15 days, i.e., at a time when tumor regression took place in adult mice. In these animals, the cytotoxic activity then declined slowly to become hardly detectable after 40 days. In contrast, CL disappeared within a few days in younger mice which were unable to reject their tumor. It may be noted that the evolution of cytotoxicity, as assessed by the ^{51}Cr assay method, is not in agreement with the persistence of cytotoxic effector cells in mice with progressively growing sarcomas as demonstrated with the colony inhibition assay (*117*).

C. Mechanism of Cytotoxicity

Despite extensive studies, our understanding of the mechanism of T-cell cytotoxicity in molecular terms is very limited. It is clear that a specific contact is required between CL and the relevant target cell, and yet the true nature of the receptors on the effector cells and of the corresponding antigenic determinants on the target cells remains to be established. Following contact, irreversible changes of the membrane permeability of target cells occur very rapidly, but the actual pathway leading to these changes is unknown.

1. Characteristics

With refined techniques, it has been possible to demonstrate that lymphocyte-mediated lysis may proceed at a rate almost as fast as that observed with antibody and complement. Also, with enriched sources of CL, the number of lymphoid cells required to achieve significant lysis has been considerably reduced as compared to earlier studies. A typical example is illustrated in Fig. 3. It is shown that (1) cytotoxicity proceeds linearly as a function of time, and (2) the extent of lysis is dependent on the concentration of lymphocytes. The data also indicate that one lymphocyte can kill more than one target cell since the number of cells lysed may be greater than the number of lymphocytes (7.5×10^3 target cells are killed after 4-hour incubation with 3×10^3 lymphoid cells). As

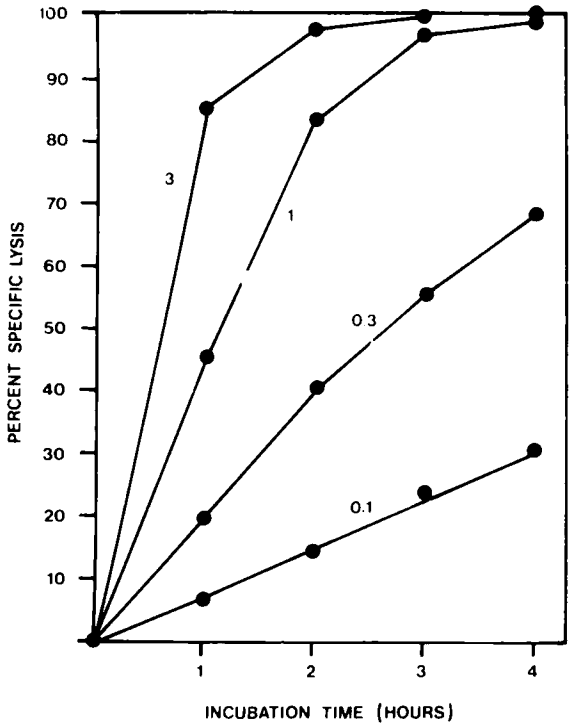

FIG. 3. Time kinetics of cytotoxic lymphocyte-mediated cytotoxicity. Spleen cells from C57BL/6 mice injected 6 months earlier with 30×10^6 P-815 (DBA/2) tumor cells were incubated *in vitro* with irradiated DBA/2 spleen cells. After 4 days in culture, the lymphoid cells were assayed for *in vitro* cytotoxicity by using 30×10^3 ^{51}Cr-labeled P-815 cells and varying incubation time periods. The numbers above the lines refer to the various lymphocyte–target cell ratios used in the cytotoxicity assay.

similar results have been obtained with CL formed either *in vivo* (*118, 119*) or *in vitro* (*32*), these findings do not support the view expressed by Henney (*63*) that the effector cell is inactivated as a result of its cytotoxic activity but provide direct evidence for multiple contacts of CL as suggested previously by Brunner *et al.* (*15*).

The activity of CL is temperature-dependent, as shown by the complete absence of lysis at 4°C. and the reduced cytotoxicity noticed at room temperature (*32, 120, 121*). According to Berke *et al.* (*121*), interaction of CL with target cells is followed by an intermediate phase which is less temperature-dependent than the lytic process itself. Further experiments are needed to clarify this point.

It is well established that CL must be alive and metabolically active in order to exert cytotoxicity. Inhibitors of ribonucleic acid (RNA) and protein synthesis (*9, 122*) impair cytotoxicity to a greater or lesser degree. Brunner *et al.* (*9*) reported that cycloheximide, a reversible inhibitor of protein synthesis, reduced but did not abolish, cytotoxicity. Recently, evidence for complete inhibition of cytotoxicity by an irreversible inhibitor of protein synthesis has been reported (*123*). One interpretation of these results, among others, would be to postulate the synthesis of an effector protein following lymphocyte–target cell interaction, as is the case for some mediators of cellular immunity (*3*). It should be noted, however, that in most of these studies the inhibitors were present during the whole incubation period and, therefore, a possible effect on the target cells could not entirely be ruled out. Furthermore, the relatively low degree of cytotoxicity exerted by the lymphoid cell populations tested required a prolonged incubation in order to reach significant values. The results cited in the foregoing should be confirmed with enriched CL populations in short-term experiments before any valid interpretation can be made.

Cytotoxicity is unaffected by high dose of γ radiation. This radioresistance is particularly evident with CL obtained relatively early (around day 10) after immunization with tumor allograft (*54, 124*) or on day 5 in MLC (*14*). In the latter case, a quantitative assessment of CL activity after varying doses of γ radiation showed an exponential decrease in cytotoxicity with increasing dose. Between 5500 and 7000 rads were required to reduce the relative effector cell frequency to 37% of the control value (*14*). As already mentioned, kinetic studies of the formation of CL in mice immunized with tumor allografts showed, in certain instances, a biphasic curve with two peaks of activity. Denham *et al.* (*54*) reported that the activity of CL collected during the second peak (day 21) was very radiosensitive as assessed by a 48-hour growth inhibition technique. Substantial reduction of activity was observed

after 100 rads, whereas 500 rads completely abrogated the effect of the immune spleen cells. The latter findings have not been confirmed in two other studies using the ^{51}Cr assay system and short-term experiments (*14, 124*). It is possible that these conflicting results reflect a fundamental difference between the phenomena measured by the two techniques, the ^{51}Cr method detecting only the actual effector cells, whereas the 48-hour assay also measures the activity of memory cells differentiating into CL during the incubation period.

An early stage in the lytic process requires the presence of calcium and, to a lesser degree, magnesium ions, as shown by complete inhibition of cytotoxicity by ethylenediaminetetraacetate (EDTA) (*125*). The inhibitory effect of EDTA was found to be reversible and was mainly observed when the chelating agent was added at the onset of the incubation period. When the addition of EDTA was delayed for 45 minutes, the rate of ^{51}Cr release was unchanged during the next 60 minutes, suggesting that the stage inhibited by the chelating agent preceded isotope release by 1 hour. Although the point of action of EDTA has not been established, it is very unlikely that inhibition of complement components is involved. Evidence against the participation of complement components includes (1) the demonstration of cytotoxicity in serum-free medium (*124*), (2) the failure of added complement to enhance cytotoxicity (*126*), (3) the absence of effect of several complement inhibitors such as carrageenan, cobra venom factor (*126*), and antibody to C_2, C_3, or C_5 (*127*). It is impossible, however, to rule out the participation of late complement components synthesized or carried by the effector cells or other lymphoid cells.

Inhibition of cytotoxicity has also been observed in the presence of cytochalasin B (CB). Cerottini and Brunner (*128*) showed that the drug completely abrogated CL activity when added at the onset of the incubation period. The inhibitory activity of CB was rapidly and completely reversible. Moreover, addition of the drug 1 hour after the beginning of incubation had only a slight effect on ^{51}Cr release during the next hour, suggesting that CB, like EDTA, acted on an early stage of the lytic reaction. Successful dissociation of the stages inhibited by either EDTA or CB has been reported (*129*), but conflicting results have been obtained in another study (*129a*). Although CB interferes with numerous biological functions, its site of action in the cell is unknown. No appreciable effect on DNA, RNA, or protein synthesis has been reported. Several studies have demonstrated a correlation of the effects of CB with disruption of the contractile system of microfilaments present in virtually all cells (*130*). Moreover, resumption of normal function and restoration of microfilament integrity closely followed removal of the drug. Wessells

et al. (*130*) suggested therefore that if a cell property was affected by CB, then this might be evidence for the presence of microfilaments. Along this line, it may be more than fortuitous that immune cell populations enriched in CL contain T lymphocytes characterized by a striking accumulation of filamentous network analogous to the microfilament system found in other cells (*131*). Recent reports, however, have questioned the strict dependence of CB activity on disruption of microfilaments (*132, 133*). It has been established that transport of glucose and glucosamine, but not of leucine, into cells was inhibited by the drug (*134–136*). These findings suggest that the primary action of CB may be at the cell membrane, leading to blockade of certain transport systems with resulting metabolic changes in the cell. As to the mechanism responsible for inhibition of lymphocyte-mediated cytotoxicity by CB, the possibility exists that alteration in membrane properties of CL may prevent their interaction with the relevant target cells, as recently suggested by Henney and Bubbers (*137*). Whatever the mechanism, the inhibitory activity of CB on CL cytotoxicity in immune lymphoid cell populations may provide an interesting tool for parallel investigation of the production of cytotoxic humoral antibody against the same target cells. As it has been shown that CB affects neither synthesis and secretion of antibody by antibody-forming cells nor complement-dependent antibody lysis (*128, 138*), measurement of ^{51}Cr release after incubation of two identical lymphoid cell–target cell mixtures, the first one in normal medium without complement and the second in CB-containing medium with complement, allows independent detection of cytotoxicity mediated by CL or by antibody and complement, respectively.

Convincing evidence has been obtained that there is a direct correlation between the intracellular level of cyclic adenosine-3′,5′-monophosphate (cAMP) and the cytotoxic activity of CL (*139, 140*). Significant inhibition of cytotoxicity was observed in the presence of cAMP or its dibutyryl derivative, and of substances that increase cAMP intracellular level either by stimulation of adenyl cyclase activity (isoproterenol, histamine, and prostaglandins E_1 and E_2) or by inhibition of phosphodiesterase activity (theophylline). Such studies established that the time course of the increase in cAMP content of the lymphoid cells following exposure to drugs closely agreed with the time course of inhibition of cytotoxicity. Subsequent studies showed that exposure of lymphoid cells alone to cholera toxin, a potent and permanent activator of adenyl cyclase, prior to incubation with the target cells, caused a significant reduction in cytotoxicity (*141*). In contrast, cholinergic agents, such as acetylcholine and carbamylcholine, produced augmentation of cytotoxicity at low optimal concentrations. This augmentation was abolished

by atropine, suggesting the presence of functional cholinergic receptors on lymphoid cells. Taking into account the results of other studies, Strom *et al.* (*141*) suggested that the effect of cholinergic agents on CL activity was mediated by elevation of intracellular cyclic guanosine-3',5'-monophosphate (cGMP). However, these findings deserve further investigation since increased cytotoxicity after cholinergic stimulation was not observed in two other independent studies (*32, 141a*). It is of some interest that another manifestation of the immune response, namely release of histamine from sensitized cells challenged with antigen, is also decreased by elevation of intracellular cAMP level (*142*). Whether lymphocyte-mediated cytotoxicity also involves a secretory process modulated by intracellular cAMP and cGMP is still speculative. Another interesting, but unexplained observation has been the increased cytotoxic activity observed after treatment of CL with interferon preparations (*143*).

2. *Specificity*

It is well established that the *in vitro* cytotoxicity of lymphocytes from mice immunized with allogeneic cells is specific (for references, see Ref. *1*). All studies, with a few exceptions, have demonstrated that CL destroy allogeneic target cells carrying transplantation antigens identical to or cross-reacting with the sensitizing antigens but have no effect on either syngeneic target cells or allogeneic target cells that do not carry cross-reacting antigens. It should be stressed, however, that this is a demonstration of relative rather than absolute specificity since measured cytotoxicity is dependent on the susceptibility of a particular target cell to lysis. This restriction is particularly important to keep in mind regarding syngeneic tumor systems, which are characterized by low levels of cytotoxicity. In these cases, determination of a specificity index, i.e., the ratio between the minimal number of immune lymphocytes required for significant lysis of a given target cell and the maximal number of normal or irrelevant immune lymphocytes producing no lysis of the same target cell, may provide a more precise evaluation of specificity. The activity of CL induced *in vitro* during MLC also appears very specific (*14, 32, 97*), although, with the high level of cytotoxicity observed in these systems, a slight effect on syngeneic target cells may be found at high lymphocyte–target cell ratios.

Confirmation of the specificity of CL activity has been provided by the results of inhibition experiments in which unlabeled target cells were added to a mixture of CL and labeled target cells. Under appropriate conditions, a linear decrease of ^{51}Cr release from labeled target cells was observed in the presence of increasing numbers of identical un-

labeled target cells, whereas addition of a thirty-fold excess of irrelevant unlabeled target cells had very little effect on CL activity.

The demonstration of a high degree of specificity in the activity of immune lymphocytes does not necessarily imply the presence of specific effector molecules. By analogy with the process of complement-dependent antibody-mediated lysis, lymphocyte-mediated cytotoxicity may be viewed as a two-step phenomenon, including first specific recognition and then lysis by a nonspecific process. It is clear, however, that the latter step is not mediated by a diffusible nonspecific factor, such as the lymphotoxin described in other systems (*144, 145*). Release of nonspecific cytotoxic substances into the medium has been ruled out in mixed target cell experiments whereby syngeneic target cells had been incubated together with immune lymphocytes and the relevant allogeneic target cells. With CL formed either *in vivo* (*29, 55, 126, 146*) or *in vitro* (*14, 95, 147*), and target cell suspensions, no cytotoxic effect on syngeneic target cells was noticed despite extensive lysis of the allogeneic target cells present in the same reaction mixtures. Nonspecific lysis, however, has been found in studies using fibroblast monolayers as the source of target cells (*148, 149*). These conflicting results can tentatively be explained by assuming that nonspecific cytotoxicity may be manifested only under conditions in which the geometry of cell association enables a single CL to establish a simultaneous contact with specific and nonspecific target cells. Such conditions would be feasible with fibroblast monolayers at high density but very unlikely with cell suspensions.

It should be emphasized that the failure to demonstrate the presence of nonspecific cytotoxic factors in the medium does not exclude the participation of substances released at the site of contact and active within a very short range.

3. *Receptors*

Specificity of cytotoxicity strongly suggests the existence of specific receptors on the surfaces of CL, complementary to determinants on the surfaces of target cells. Support for this interpretation has been provided by Brondz *et al.* (*150, 151*) who demonstrated that an immune lymphoid cell population could be depleted of CL by incubation on cell monolayers syngeneic to the relevant target cells. Subsequent studies showed that specific adsorption of CL on relevant monolayers was responsible for this depletion since CL could be recovered by trypsinization of the adherent lymphoid cells (*152, 153*). It should be noted that no estimate of the proportion of CL could be provided by these adsorption experiments since many irrelevant cells, up to 50% in some experiments, were adsorbed on either relevant or irrelevant monolayers.

Another consequence of nonspecific adsorption was the failure to obtain cell populations highly enriched in CL by trypsinization of the adherent cells.

By using such adsorption experiments, it was clearly shown that, in animals immunized against two different sets of histocompatibility antigens, there were two populations of CL each of which carried only one type of receptors (*152*). These results, as well as others (*154*), exclude the possibility of the existence of multipotential receptors on the surface of CL. However, the presence of receptors with more than one specificity on the same cell cannot be entirely ruled out.

Since very little specific adsorption occurs at 4°C. (*152*), it can be speculated that temperature-dependent surface changes taking place on the receptor-bearing CL are a prerequisite for their specific interaction with target cells. That the temperature-dependent events are unrelated to the metabolic activity of the adsorbing fibroblasts have been clearly shown in experiments using glutaraldehyde-fixed monolayers (*155*).

Several other observations are relevant to the question of specific receptors on CL. Studies using enzymes to remove surface constituents from the lymphoid cells suggest but do not prove that proteins are involved in the recognition process. Thus pretreatment of immune lymphoid cell populations with proteolytic enzymes, such as trypsin (*125, 146*), pronase (*156*), or papain (*67*), caused a reversible inhibition of their cytotoxic activity. Moreover, adsorption of CL on relevant monolayers was prevented by pretreatment of the lymphoid cells with pronase (*157*). It is thus tempting to postulate that the specific receptors on CL membrane were degraded by the treatment with proteases. If this is true, the reversion of the inhibitory effect within a few hours suggests a rapid reappearance of the missing moiety on the lymphocyte surface. Moreover, this process appears to depend, at least partially, on protein synthesis. Thus, trypsin-treated lymphocytes incubated with target cells in the continuous presence of cycloheximide, an inhibitor of protein synthesis, did not recover full activity as was the case in the absence of the drug (*125*).

In contrast to the inhibitory activity of proteolytic enzymes, treatment of CL with neuraminidase increased their cytotoxic activity (*157, 158*). Adsorption experiments suggested that this augmentation was related to an increased rate and/or number of interactions between CL and target cell following removal of sialic acid (*157*).

The exact nature of the specific receptors on CL is still controversial. It is not within the scope of this review to assess the validity of observations providing evidence for the presence of immunoglobulin receptors on T cells. It is clear that immunoglobulins cannot be detected on the

surfaces of normal T cells by direct techniques (*159, 160*). Similar negative results have been obtained with pure populations of sensitized T cells containing a high frequency of CL (*32*). In the latter situation, as in others, any interpretation of positive findings should take into account the recent demonstration of receptors for antigen–antibody complexes on such cells (*161*). More germane to the question are the results of the studies using anti-immunoglobulin antisera for inhibition of T-cell activity. In contrast to certain observations demonstrating the inhibition of some T-cell functions by antisera against the light and heavy chains of immunoglobulins, similar studies, performed independently in several laboratories, have consistently failed to demonstrate any effect of such antisera on the cytotoxic activity of CL (*31, 45, 126, 127*). The negative results do not rule out, however, the possibility of the existence of buried immunoglobulin receptors with a rapid turnover rate. Using another approach, Cone *et al.* (*162, 163*) have recently described the isolation of labeled IgM molecules in the supernatant of tissue cultures of radiolabeled T cells sensitized against histocompatibility antigens. Binding experiments suggested a preferential reactivity of the labeled immunoglobulins for thymus cells carrying the immunizing histocompatibility antigens. Because similar attempts, using an identical technique for identification of immunoglobulin molecules on the surface of normal T cells, have given completely opposite results (*164, 165*), confirmation of the foregoing data is needed before definite conclusions can be drawn on this crucial point.

In addition to the demonstration of specific receptors on the surface of CL, recent studies provide evidence for the existence of such receptors on CL progenitors. Using the xenogeneic system studied by Berke *et al.* (*166*), two groups of investigators, working independently, showed that a normal rat lymphoid cell population could be depleted of CL progenitors by adsorption on mouse fibroblast monolayers (*167, 168*). By using monolayers of different genotypes and repeated adsorptions, it was possible to eliminate completely the CL precursors directed against the antigens of the adsorbing monolayers, without affecting the capacity of the lymphoid cells to form CL following incubation on antigenically unrelated monolayers. Conversely, a relative enrichment in CL could be achieved by this method since the adsorbed lymphoid cells were found to detach from the monolayers during the sensitization process.

Such studies, which have been confirmed in mouse allogeneic systems (*149*), establish the existence of specific receptors on the surface of CL progenitors. Further support for this concept has been provided by the observation that specific adherence of the precursor cells to the

fibroblast monolayers decreased in the presence of either particulate or soluble membrane antigens of the same specificity (*149*). Additional experiments indicated that, as was the case for CL themselves, adsorption of CL progenitors was an energy-dependent process since it did not take place at 4°C. nor at 37°C. in the presence of metabolic inhibitors, such as dinitrophenol or sodium azide. On the other hand, specific depletion of reactive cells could be obtained even in the cold after treatment of the lymphoid cells with neuraminidase. As a tentative explanation, Feldman *et al.* (*149*) suggested the requirement of an energy-dependent step for the exposure of cryptic receptors on the surface of the reactive T cells.

In this system, too, anti-immunoglobulin antisera failed to inhibit the specific adherence of either normal or neuraminidase-treated lymphoid cells (*149*). In contrast, significant inhibition was achieved in the presence of antibody directed against the transplantation antigens of the lymphoid cells. This observation is consistent with the results reported by Ceppellini (*169*) showing inhibition of MLC in man by addition of serum containing antibodies against HL-A antigens of the responding cells. On the contrary, the same antiserum had no inhibitory effect on the lymphoid cells once they had been sensitized (*170*). The latter results are in agreement with the results obtained in the mouse by Brunner *et al.* (*9, 31*) (see also Tables II and III), who demonstrated that the activity of CL was unaffected by the presence of antibody against CL alloantigens. These findings deserve further investigation since they suggest a fundamental difference in the recognition process effective in the induction phase and in the effector phase of sensitized T cells. Indeed, it is quite possible that the antisera which inhibit the induction phase of MLC contain, in addition to anti-HL-A antibodies, antibodies directed against LD antigens or other surface structures which are directly or indirectly involved in the proliferative response of lymphocytes to the major histocompatibility complex.

The presence of specific receptors on the surfaces of CL necessarily implies complementary determinants on the surfaces of the target cells. Since histocompatibility antigens have been defined by serological tests, the question whether these antigens are also those recognized by specifically sensitized T cells is of considerable interest. Several approaches have been tried to answer this question. First, Brondz and his colleagues (*150, 151*) attempted to adsorb lymphoid cells from animals immunized against an H-2 antigen complex on a mixture of macrophage monolayers each carrying only a selection of the immunizing complex specificities. Since such a mixture of adsorbing macrophages was much less effective than macrophages bearing the whole set of specificities, it was con-

cluded that the specificity of CL was directed against the whole antigen complex rather than against individual specificities. Subsequent studies, however, indicated that such conclusion could be applied only to the antigenic specificities controlled by one of the two loci of the major histocompatibility complex. Thus, in the mouse, where the *H-2* genetic region is separated by unrelated genes into two major loci, the *H-2K* and the *H-2D* loci (*171*), differences for the two *H-2* loci between donors and recipients of tumor cells led to the formation of two populations of CL, each reacting with only one of the two sets of antigens (*154*). These results are in agreement with the serological demonstration of at least two different H-2 alloantigen molecules on the cell surface, each representing the product of one locus (*172*).

In another approach, the ability of alloantibody directed against the target cell alloantigens to inhibit the activity of CL was used. It has been repeatedly shown that the presence of relatively large amounts of anti-target cell antibodies throughout the incubation period resulted in a strong inhibition of lymphocyte-mediated cytotoxicity (*9, 26, 31, 126, 173*). The specificity of this inhibitory effect has been supported by the demonstration that antibodies directed aginst one set of the antigenic specificities carried by the target cells were unable to prevent the lytic effect of lymphocytes sensitized against another set of antigens present on the same target cells. Thus, when $(A \times B)F_1$ hybrid target cells were used, A anti-B antiserum inhibited the activity of A anti-B lymphocytes, but had no effect on target cell lysis by B anti-A lymphocytes and conversely (Table II). These results can be adequately explained by a mechanism of competition of antibody with the lymphocyte for the same antigenic determinants on the target cell surface. However, it is impossible to exclude the possibility that the determinants are different but are present on either the same molecule or on separate, closely associated molecules. The definite answer to that question depends on the availability of purified soluble histocompatibility antigens which could be used in competition experiments. In this context, it should be mentioned that attempts to inhibit lymphocyte-mediated cytotoxicity by addition of soluble, serologically active, antigenic preparations have been unsuccessful except in one recent study (*111*); even in the latter, the degree of inhibition achieved was rather low.

Any interpretation of these results should take into account the recent demonstration of SD and LD loci in the genetic region of the major histocompatibility complex (*107c–107e*). All available evidence suggests that SD differences, and not LD differences, are the complementary structures involved in lymphocyte-mediated cytotoxicity (*107b, 170*). According to the present concept, the proliferative response of lymphocytes in MLC tests would depend only on LD differences,

TABLE II
INHIBITORY EFFECT OF ALLOANTIBODY ON TARGET CELL LYSIS BY IMMUNE T CELLS

Immune T cells[a]	Target cells[b]	Percent inhibition of target cell lysis in presence of antiserum[c]	
		H-2^b anti-*H-2*^{d}	*H-2*^{d} anti-*H-2*^{b}
H-2^b anti-*H-2*^{d}	*H-2*^{d}	68	0
	(*H-2*^{b} × *H-2*^{d})F_1	67	0
H-2^d anti-*H-2*^{b}	*H-2*^{b}	3	88
	(*H-2*^{b} × *H-2*^{d})F_1	0	99

[a] Immune T cells were obtained 4 days after transfer of spleen cells from C57BL/6 (H-2^b) or DBA/2 (H-2^d) mice into lethally irradiated H-2^d or H-2^b recipients.

[b] Target cells included P-815 (H-2^d) tumor cells, EL-4 (H-2^b) tumor cells, or peritoneal macrophages from (H-2^b × H-2^d)F_1 hybrid mice.

[c] An amount of 2.5×10^6 lymphocytes and 25×10^3 ^{51}Cr-labeled target cells was incubated at 37°C. for 6 hours in the presence of alloantiserum (final dilution 1:10). Percent inhibition of target cell lysis was calculated relative to control cell mixtures without alloantiserum.

whereas once the sensitization process has been achieved, cytotoxicity would only be related to SD differences. However, the possibility exists that the antigenic determinants recognized by CL are determined by genes closely linked, but not identical, to those determining SD antigens. Obviously, much more information is needed to substantiate these findings and delineate the relationship of the LD differences to the immune response gene(s) as well as its phenotypic expression on the cell surface. Further experimentation is also required to determine whether the SD antigens are indeed the structures recognized by CL or act only as markers to linked antigenic determinants.

4. Cooperation with Other Cells

From the evidence cited above, it is clear that immune T lymphocytes are cytotoxic. The question whether they are also able to confer cytotoxic activity to normal cells is less clearly understood, at least as far as macrophages are concerned. Using a pure population of immune T cells, we have been unable to demonstrate any augmentation of cytotoxicity following addition of normal T and/or B cells (55). Similarly, Golstein *et al.* (*37*) found that thymus cells sensitized by transfer into irradiated allogeneic hosts had the same cytotoxic activity after removal of B cells. Using a cell population highly enriched in CL by velocity sedimentation, MacDonald (*14*) found no increase in cytotoxicity in the presence of normal B cells.

These results do not support the hypothesis of T–T or T–B cell co-

operation in cytotoxicity (*174*). Further attempts to demonstrate cooperation between immune T cells and macrophages for direct cytotoxicity have usually been unsuccessful. Other studies, however, suggest that immune T cells may render normal macrophages specifically cytotoxic by releasing cytophilic factors upon incubation with specific antigen. This aspect will be considered in more detail in Section V. These investigations revealed that the use of widely accepted methods for separation of lymphocytes from macrophages have certain pitfalls which may lead to erroneous conclusions. When immune spleen cells were filtered through glass bead columns, it was found that the unretained population was virtually free of macrophages and, yet, had an increased cytotoxicity, on a cell-to-cell basis, as compared to the unseparated lymphoid cells (*52*). However, only 60% of the total activity was recovered in the column-filtered cell population, suggesting the retention of some adherent effector cells on the column beads. Elution of the adherent cell population confirmed the validity of that assumption and showed, in addition, that the adherent effector cells were T cells (*175*). This observation is very pertinent to the controversy concerning the nature of the effector cells found in the peritoneal cavities of mice inoculated by this route with allogeneic cells. It is a common finding that such a population, collected at about 10 days, is characterized by a very high cytotoxic activity, several-fold higher than that of spleen cells (*52, 146, 176, 177*). Separation of the immune peritoneal cells into adherent and nonadherent cells indicated that cytotoxic activity was present in both populations, although at a lower degree in the adherent cells (*52*). Treatment with anti-θ serum and complement abolished the cytotoxicity of the adherent cells, whereas treatment with antimacrophage serum and complement had no effect (*52*). Essentially identical results have been reported by other investigators (*146, 177*). In addition, the relative frequency of effector cells in the peritoneal cell population increased about twofold after the phagocytic cells had been removed magnetically following injection of carbonyl iron (*32*). These results, taken together, indicate that (1) some CL behave as adherent cells, and (2) the high cytotoxic activity exerted by immune peritoneal cells taken during primary immunization may be related to an accumulation of CL at the site of allograft rejection rather than to a participation of macrophages in the lytic process. Although these observations have been made primarily in allogeneic systems, the data available at the present time suggest a similar behavior of CL in syngeneic tumor systems. At this point, we wish to make it perfectly clear that the observations just cited are not interpreted by us to rule out the existence of macrophages with a specific or nonspecific cytotoxic potential, as will be discussed in Section V.

IV. Antibody-Dependent Cytotoxicity Mediated by Normal Lymphoid Cells

Following the original observation of Möller (*178*), it has been repeatedly shown that target cells coated with appropriate antibody may be specifically lysed *in vitro* in the presence of lymphocytes from normal individuals. This subject has been extensively reviewed by Perlmann and Holm (*1*). Cytotoxicity is independent of added complement and, therefore, is sometimes referred as lymphocyte-dependent antibody (LDA) activity. In this review, we prefer to use the operational term, antibody-dependent (AD) lymphocyte-mediated cytotoxicity.

Most studies have used heterogeneous combinations of target cells, antibody, and lymphoid cells from different species, such as chicken red blood cells (RBC), rabbit antibody, and human lymphocytes (*179*), sheep fibroblast monolayers, rabbit antibody, and human lymphocytes (*180*), or Chang liver cells (a human cell line), rabbit antibody, and rat lymphocytes (*181*). Recently, evidence has been provided for a similar phenomenon in allogeneic systems. Bubenik *et al.* (*182*) demonstrated that normal blood lymphocytes from an inbred strain of ducks were able to destroy erythrocytes or embryonic fibroblasts from another inbred strain in the presence of alloantibodies against the target cells. In our own laboratory, lysis of DBA/2 tumor cells coated with alloantibodies and incubated with normal mouse spleen cells has been observed under certain conditions (*32*). A similar effect has been reported by several investigators using human blood lymphocytes or blasts coated with anti-HL-A antibodies in the presence of normal human blood lymphocytes (*170, 183, 184*). In syngeneic tumor situations, there have been two reports of AD cell-mediated cytotoxicity in the MSV systems (*185, 186*), but both studies have used the microplate assay for the assessment of cytotoxicity, and these results have not been confirmed by Leclerc *et al.* (*116*) who employed the ^{51}Cr assay method.

As will be discussed in the following, the lymphoid effector cell involved in the AD cytotoxicity is characterized by surface receptors directed against the Fc portion of IgG. It is clear that Fc receptors are by no means an exclusive property of certain lymphoid cells since they are also present on nonlymphoid cells, such as polymorphonuclear (PMN) cells, monocytes, and macrophages (*187–189*). In addition, several studies have demonstrated that these nonlymphoid cells may be responsible for specific or nonspecific destruction of target cells *in vitro* (*1*). Thus, Lundgren *et al.* (*190*) reported that contamination of a lymphocyte population with more than 1% PMN cells and monocytes could give rise to a nonspecific detachment of skin fibroblast monolayers. Also, phagocytosis and/or lysis of antibody-coated RBC by macrophages

or PMN cells are well documented. It has been clearly shown that the presence of 5% contaminating monocytes and PMN cells in a lymphocyte preparation had a profound effect on the initial rate of lysis of antibody-coated chicken RBC or, to a lesser degree, of Chang cells (*179*). Identical results have been reported recently by Gelfand *et al.* (*191*) who showed that the addition of as few as 0.5% phagocytic cells to a purified lymphocyte population produced a significant change in the lytic rate of antibody-coated chicken RBC. As emphasized by all these observations, any study of AD lymphocyte-mediated cytotoxicity should only be performed with highly purified populations of lymphocytes. Unfortunately, such precautions have not always been taken in recent investigations. It is therefore not too surprising that the present literature on this subject is somewhat confusing. Also great caution should be exercised in extrapolating the results from one system to another.

A. Nature of Effector Cells

The first evidence of the role of lymphocytes in lysis of antibody-coated chicken RBC has been provided by Perlmann and Perlmann (*179*) who used human blood lymphocytes prepared by gelatin sedimentation and filtration on nylon fiber columns. In subsequent studies, further purification was achieved by centrifugation on Ficoll-Hypaque medium, followed by overnight incubation in tissue culture flasks (*192*). During that period, the remaining monocytes became adherent, whereas the few PMN cells left over after the first separation procedure died.

In the rat, AD effector cell activity has been shown to be greatest in peritoneal exudate cells, second in spleen cells, moderate in blood lymphocytes, relatively low in lymph node cells, and undetectable in thymus or thoracic duct cells (*193, 194*). A participation of macrophages has been excluded in this system since removal of phagocytic or adherent cells from the peritoneal cell population had no effect on cytotoxicity. From the distribution of the effector cells in the lymphoid organs, it seems unlikely that they were T cells. More direct evidence was provided by Harding *et al.* (*44*) who showed that spleens from T-cell-depleted rats retained normal numbers of cytotoxic cells. In the mouse, treatment of spleen cells with anti-θ serum and complement had no effect on their ability to lyse antibody-coated sheep RBC (*195*) or alloantibody-coated tumor cells (*32*). By using human blood lymphocytes, Wigzell *et al.* (*115*) found that a population of presumably pure T cells had no activity on antibody-coated chicken RBC. In contrast to these results, Gelfand *et al.* (*191*) reported that rabbit thymus cells, even after removal of adherent cells, caused a relatively low but significant cytotoxicity. In the absence of antigenic markers for T cells in

the rabbit, it is impossible to exclude the possibility that contaminating non-T cells in the rabbit thymus are responsible for this lytic activity.

The results cited above strongly suggest that the effector cell is a non-T cell. In addition, participation of cells producing antitarget cell antibody in AD cytotoxicity appears unlikely, since only low activity against Chang cells was demonstrated in lymphocytes from rat lymph nodes producing large amounts of anti-Chang cell antibodies (*196*). Moreover, effector cell activity returned before antibody-forming capacity in the lymphoid organs of irradiated rats (*197*).

It is thus tempting to speculate that B cells are the effector cells in the AD cytotoxicity. Several findings, however, do not support this interpretation. As already mentioned, no activity was detected in rat thoracic duct cells, which are known to contain precursors of antibody-producing cells, i.e., B cells (*198, 199*). Opposite results, however, have been reported recently by Fakhri and Hobbs (*200*). Other evidence against the participation of conventional B cells in AD cytotoxicity has been provided by Wislöff and Fröland (*201*) who demonstrated that lymphocyte populations from patients with severe hypogammaglobulinemia, although devoid of Ig-carrying cells, had a normal effector activity after removal of monocytes and PMN cells. Identical results have been obtained by two other groups of investigators (*194*). In addition, removal of Ig-carrying lymphocytes, monocytes, and PMN cells by filtration of normal human blood lymphoid cells on nylon–wool columns had no effect on cytotoxicity (*201*). These results are in apparent disagreement with two other observations supporting the concept that B cells are the effector cells. First, it has been shown that treatment of lymphoid cells with anti-Ig antiserum and complement abrogated their cytotoxic potential (*127, 195*). Second, Perlmann *et al.* (*192*) found that effector cells were retained on a column of Ig–anti-Ig coated beads (*115*). These conflicting results could be reconciled, however, if one assumes that certain human lymphocytes may carry either surface immunoglobulin or Fc receptors, but not necessarily both. As will be shown, inhibition of cytotoxicity by anti-Ig serum appears to reflect a blocking of Fc receptors rather than a direct effect on surface immunoglobulin. Also, in the fractionation procedure using Ig–anti-Ig coated beads, cell retention may be due to column binding of cells with surface immunoglobulin and/or Fc receptors. In a recent study, Wigzell *et al.* (*202*) found that effector cells, in contrast to Ig-bearing cells, were not retained on a column of beads coated with Ig-$F(ab')_2$ anti-Ig. Taken together, the results cited above suggest that effector cell activity is a property of lymphocytes with Fc receptors but not of those with surface immunoglobulin. The possibility that effector cells also carry receptors for the third component of com-

plement (*187, 203*) appears likely in view of recent findings of Perlmann *et al.* (*192*). Extensive use of fractionation procedures based on the various surface properties of lymphocytes should provide further insight into the existence of lymphocyte populations with different membrane characteristics.

Another suggestion has been made by Allison (*204*) who found that the effector cells in mouse peritoneal exudate cells could be separated from T and B cells by velocity sedimentation. These cells were adherent but nonphagocytic, and, although they appeared like medium lymphocytes or small macrophages after conventional staining, they could be distinguished from both cell types by certain distinctive histochemical and ultrastructural features. Another property of these cells is the presence of Fc receptors on their surface, as demonstrated by rosette formation with antibody-coated red cells. These rosettes, however, are not engulfed by these cells, in contrast to the rapid phagocytosis which follows attachment of opsonized red cells to macrophages. In a recent study, Greenberg *et al.* (*205*) also suggested that the effector cells in mouse spleen were non-T, non-Ig-bearing cells, and indeed belonged to the monocytic series. Whether these cells are identical with the lymphoid effector cells present in human blood which form typical uropods upon attachment to antibody-coated target cells (*206, 207*) remains to be established. From the data available, this seems unlikely because the two categories of cells appear to have different adherence properties and Fc receptors with different affinity for the various IgG subclasses (see following).

B. Nature of Antibody

It is well established that the structural requirements on immunoglobulin molecules necessary for cell-mediated cytotoxicity are different from those involved in complement-dependent lysis. These differences are probably related to different structures on the complementary molecules with which antibody interacts during the lytic process, namely with the Fc receptor on the lymphocyte surface, on the one hand, and the first complement component, on the other. The observations that have led to this conclusion are briefly summarized.

1. It has been a common finding that the amounts of antibody required for cell-mediated cytotoxicity are far below those needed for complement-dependent lysis. Thus, antiserum dilutions as high as $1:10^7$ were sufficient for inducing significant lysis of target cells in the presence of normal lymphoid cells (*208–210*). Indeed, lysis of target cells coated with as few as 100 antibody molecules has been reported (*192*).

2. Lymphocyte-mediated cytotoxicity is induced by IgG antibody

but not by IgM antibody (*179, 211*), whereas both classes fix complement (*212*).

3. Inhibition studies using aggregated IgG myeloma proteins of different subclasses suggest that the receptors on the effector cells do not distinguish between IgG subclasses, with the possible exception of IgG_4 (*192, 194*). This finding contrasts with the demonstration of little or absent C1 fixation by IgG_2 or IgG_4, respectively (*212*). More important, the effector cell receptors have a different specificity than that of macrophages; the latter react with IgG_1 and IgG_3 subclasses only (*213, 214*). Since contamination of lymphoid cells by PMN cells has not always been ruled out, it is worth noting that the latter cells also appear to react with all four IgG subclasses (*215*). It should be stressed that these results have been obtained in inhibition experiments, and they should be confirmed by direct studies of the activity of antibody of a given subclass. Such a study in guinea pigs demonstrated that IgG_2 antibodies could sensitize chicken RBC to the lytic action of normal, guinea pig lymphoid cells, whereas IgG_1 antibodies, despite similar hemagglutinating activity, had no effect in this system (*45*).

4. Although the Fc portion of IgG antibody is required for both interaction with lymphocyte receptors and fixation of the first complement component (*210, 212, 216*), it appears that distinct structures on the IgG molecule are involved in each phenomenon as suggested by the results of studies using plasmin-digested rabbit antibody (*194*).

The interaction between the effector cell receptor and the complementary structure on IgG is probably rather weak. This is suggested by the finding that AD lymphocyte-mediated lysis is inhibited much more efficiently by soluble antigen–antibody complexes or aggregated IgG than by monomer IgG (*217*). As for other interaction systems involving the Fc portion of IgG (*218*), it is likely that this difference reflects the much higher avidity of lymphocyte receptors for complementary sites of antibody molecules that are held together by antigen or by physicochemical aggregation rather than in free solution. It can be inferred that the antibody responsible for lymphocyte-mediated cytotoxicity is not cytophilic for lymphocytes. Thus, pretreatment of lymphocytes with antiserum has been found to be 10,000-fold less efficient for inducing cytotoxicity than pretreatment of the target cells (*179, 208*). It is, therefore, very unlikely that certain lymphocytes from immune donors may adsorb enough antibody *in vivo* to exert cytotoxicity *in vitro*. The possibility is not excluded, however, that such an effect could be induced by some lymphocytes carrying antigen–antibody complexes formed *in vivo*. Thus, Perlmann *et al.* (*209*) found that normal lymphocytes pretreated with antibody-coated chicken RBC or soluble antigen–antibody com-

plexes had acquired a specific cytotoxic potential as shown by their lytic effect on chicken RBC in the absence of added antibody. It is obvious that this effect is likely to depend critically upon the relative proportions of antigen and antibody. When adsorbed on lymphocytes, only antigen–antibody complexes in antibody excess can make the cells reactive with the relevant target cells in the absence of added antibody, whereas lymphocytes carrying immune complexes in excess of specific or unrelated antigens, or aggregated IgG, will be prevented from reacting with target cells, even when the latter are coated with antibody. A diagrammatic representation of these opposite effects of antigen–antibody complexes has appeared in a recent paper from Allison (*204*). Inhibition of AD lymphocyte-mediated cytotoxicity by unrelated antigen–antibody complexes will be discussed in more detail in Section IV,C,1.

C. Mechanism of Cytotoxicity

It is well established that contact between effector cells and antibody-coated target cells is a prerequisite for subsequent lysis (*1*). Contact is followed by an energy-dependent activation of the lymphoid cells, as reflected by uropod formation and increased mobility of lymphocytes incubated with antibody-coated target cells (*206, 207*). Although phagocytosis is not involved when the effector cells are purified lymphocytes, the exact mechanism of lysis has not been elucidated.

1. Characteristics

In general, cytotoxicity is detectable within 1 hour and then proceeds linearly as a function of time (*183, 192*). The initial rate of lysis, however, may be influenced by the degree of contamination of lymphocytes with nonlymphoid cells, such as monocytes, macrophages, and PMN cells (*179*). It is worth noting that the cytotoxic activity of nonlymphoid cells has a very rapid onset but ceases after a few hours, whereas lymphocytes exert continuing cytotoxicity during several days in culture, although at a slower rate than nonlymphoid cells (*191*).

Under optimal conditions, complete lysis of antibody-coated chicken RBC may be achieved within 10 to 20 hours (*192*). In other systems, however, the cytotoxic reaction did not reach completion until the fourth day of incubation (*210*). The extent of lysis appears to depend on the concentration of lymphocytes. In two instances, analysis of dose–response curves has demonstrated that cytotoxicity varied with the logarithm of the number of normal lymphoid cells incubated with antibody-coated target cells (*183, 194*). As mentioned earlier, the same dose–response relationship has been found in cytotoxic reactions involving immune T lymphocytes. Thus, estimation of the relative frequency of

effector cells in different cell populations is also feasible in the system of AD lymphocyte-mediated cytotoxicity by direct comparison of their dose–response curve.

These studies also indicated that significant lysis of antibody-coated chicken RBC or lymphocytes could be detected at lymphocyte-to-target cell ratios of 1:1. At this ratio, 5–7 lymphocytes were required to lyse one target cell (*192*). Whether one effector cell can kill more than one target cell has not yet been proven in this system.

All available evidence indicates that the effector cells must be alive in order to be cytotoxic. Antimycin A, which inhibits cell respiration, inhibits cytotoxicity (*1*). Protein synthesis, however, may not be required, as shown by the failure of puromycin (*193*) or cycloheximide (*183*) to affect AD cell-mediated cytotoxicity. Further investigation using irreversible inhibitors of protein synthesis is needed to clearly establish this point. Conflicting results have been reported concerning the effect of CB. Perlmann *et al.* (*192*) showed a profound inhibitory effect of the drug on lysis of antibody-coated chicken RBC by normal human blood lymphocytes. These results have been confirmed in our own laboratory by using the same system (*219*) and another system of alloantibody-coated tumor cells and normal mouse spleen cells (*128*). In contrast, Henney *et al.* (*123*) found no effect of CB on lysis of sheep RBC by mouse spleen cells. Whether or not this negative result reflects the participation of CB-insensitive nonlymphoid cells remains unclear at the present time. In this context, it is worth noting that CB appears to increase rather than to inhibit the release of enzymes from PMN cells confronted with antigen–antibody complexes (*220, 221*).

Inhibition by anti-Ig antiserum is a characteristic feature of AD cell-mediated cytotoxicity (*45, 127, 195, 222, 223*). In general, the anti-Ig antiserum has been added to the mixture of antibody-coated target cells and lymphocytes. Pretreatment of lymphocytes with antiserum, followed by washing, also resulted in complete inhibition of cytotoxicity (*127, 195*). These results thus suggest that the inhibition is related to an effect of the anti-Ig antiserum on the lymphoid cells rather than on the antibody itself. Direct evidence for this hypothesis was provided by experiments in which anti-Ig antiserum and antitarget cell antibody were of the same species. Thus, human blood lymphocytes incubated with rabbit antihuman immunoglobulin serum exerted no cytotoxicity on chicken RBC coated with rabbit antibody (*192*). This finding raised the question whether surface immunoglobulin on lymphocytes had any functional significance in the lytic reaction. If this were the case, one would expect a preparation of $F(ab')_2$ antibody against immunoglobulin to be as effective as the native IgG antibody, because pepsin antibody fragments

are known to bind to antigen with the same avidity as the undigested molecules. By using this approach, Perlmann *et al.* (*192*) found no inhibition of cytotoxicity by rabbit $F(ab')_2$ antibody against human Fab, whereas the undigested antibody was very effective. It is thus likely that inhibition by anti-Ig antiserum reflects the formation of Ig–anti-Ig complexes that bind to the Fc receptors on the effector cells, thus preventing any interaction with the antibody-coated target cells. To explain this inhibitory effect, it is not necessary to postulate that Ig-carrying cells and effector cells are identical. Indeed, the possibility exists that Ig-carrying cells coated with anti-Ig antibody cause a blocking of the effector cell receptors either by behaving as antibody-coated target cells or by releasing Ig–anti-Ig complexes into the medium.

These results illustrate the great caution which should be exercised in trying to identify the nature of effector cells by inhibition studies using anti-Ig antiserum. The same consideration seems to apply to the use of antisera against other surface constituents of lymphocytes. Thus, MacDonald (*219*) demonstrated that mouse spleen cells incubated with antibody directed against their H-2 antigens exerted no cytotoxicity on chicken RBC coated with rabbit antibody. Moreover, he found that addition of anti-θ serum without complement to the cell mixtures completely inhibited lysis. Since the effector cells in this system are θ-negative cells, it is clear that the inhibitory effect of anti-θ serum is not due to the specific binding of antibody to the effector cells. The reverse situation exists also since it is known that addition of anti-θ serum does not inhibit the cytotoxicity of immune T cells, whereas pretreatment of spleen cells with anti-θ serum and complement abrogates T-cell activity (Table III).

Direct evidence for the inhibitory effect of unrelated immune complexes on AD cell-mediated lysis has been reported by MacLennan (*217*). By using antibody-coated Chang cells and rat spleen cells, this investigator found that the degree of inhibition produced by complexes of human serum albumin (HSA) and rabbit anti-HSA serum was critically related to their physical configuration, the most effective complexes being small and soluble. Because complexes of this type are known to circulate in the blood without being removed by the reticuloendothelial system, it is crucial to analyze their inhibitory activity further, especially *in vivo*.

2. *Specificity*

It has been repeatedly shown that the specificity of AD cell-mediated lysis is identical to that of the antibody responsible for its induction (*1*). Furthermore, the possible participation of diffusible cytotoxic factors in the reaction has been excluded by the results of mixed target cell ex-

TABLE III
EFFECT OF ADDED ALLOANTISERUM ON T-CELL-MEDIATED LYSIS AND ON ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY[a]

Serum (final dilution in reaction mixture)	Percent inhibition of lysis of	
	P-815 (DBA/2) tumor cells[b]	Antibody-coated chicken RBC[c]
Normal C57BL/6 (1:10)	0	0
DBA/2 anti-C57BL/6 (1:10)	0	96
DBA/2 anti-C57BL/6 (1:100)	0	92
C57BL/6 anti-DBA/2 (1:10)	90	20
C57BL/6 anti-DBA/2 (1:100)	44	4
AKR anti-θ C_3H (1:10)	0	99
AKR anti-θ C_3H (1:100)	0	54

[a] Spleen cells from C57BL/6 mice injected 11 days earlier with 30×10^6 P-815 (DBA/2) tumor cells were used as the source of immune T cells and of normal non-T effector cells mediating antibody-dependent lysis.

[b] An amount of 3×10^6 lymphocytes was incubated with 30×10^3 ^{51}Cr-labeled P-815 (DBA/2) tumor cells at 37°C. for 5 hours.

[c] An amount of 3×10^6 lymphocytes was incubated with 100×10^3 ^{51}Cr-labeled chicken red blood cells (RBC) and rabbit serum antichicken RBC (final dilution 1:5000) at 37°C. for 20 hours.

periments. When mixtures of antibody-coated target cells and non-cross-reacting antibody-free target cells were incubated together with normal lymphocytes, no significant lysis of the bystander target cells was observed (*179*, *210*).

Very few attempts have been made to compare the antigenic specificity of antisera as determined by lymphocyte- or complement-mediated lysis. In one of these studies, Hersey *et al.* (*184*) investigated the specificity of anti-HL-A antisera by using normal blood lymphocytes or phytohemagglutinin-transformed blasts as target cells. The results obtained with the two assay methods showed a good correlation, although the antiserum lytic titers were much higher in the lymphocyte than in the complement assay method. Moreover, additional antigenic specificities were revealed by AD cell-mediated lysis. Further studies are needed to establish the usefulness of this assay method for tissue typing.

D. Cytotoxicity of Lymphoid Cells from Immune Donors

The foregoing studies have mainly dealt with the mechanism of AD cell-mediated cytotoxicity using antibody-coated target cells and normal lymphoid cells. In view of the demonstration of *in vitro* lysis of untreated target cells by lymphoid cells from immune donors, it is pertinent to ask

whether such a mechanism may be responsible for this effect. In other words, Is it possible to demonstrate this phenomenon with immune lymphoid cell populations without added antibody? From the data on the nature of effector cells and of antibody involved, it is clear that such immune lymphoid cell populations should contain non-T lymphoid cells with Fc receptors and antitarget cell IgG antibody-producing cells. Alternatively, according to the results presented, the presence of lymphoid cells carrying antigen–antibody complexes in antibody excess should be sufficient to induce lysis of target cells carrying the same antigen. In order to investigate this, the studies performed so far have concentrated on the demonstration of the prominent role of non-T lymphocytes in the cytotoxic activity of immune lymphoid cells. Evidence for such a participation in spleens of mice immunized with sheep or chicken RBC has been suggested by several studies, although the effector cell activity has been attributed either to B lymphocytes (*127*, *195*), to macrophages (*224*), or to PMN cells (*14*). Removal of B lymphocytes on Ig–anti-Ig columns has been used by Lamon *et al.* (*49*) to study the nature of effector cells in immune spleen and lymph node cells from mice bearing MSV-induced tumors. The same approach has been used by Perlmann *et al.* (*50*) in a study of the activity of blood lymphocytes from bladder carcinoma-bearing patients. In both instances, the participation of B or T cells in *in vitro* cytotoxicity, as assessed by the microplate assay, was variable in lymphoid cells taken at different times after tumor induction in the animal or from different patients.

Another observation suggests that antibody-producing cells are required for the manifestation of AD cell-mediated lysis by immune lymphoid cells. Thus, MacLennan and Harding (*193*) showed that addition of puromycin to cultures of spleen cells from rats immune to Chang cells inhibited their cytotoxicity on uncoated Chang cells but had no effect on antibody-coated Chang cells. Because puromycin, an inhibitor of protein synthesis, has been shown to inhibit antibody production (*225*) but not effector cell activity (*193*), these findings are consistent with but do not prove the hypothesis that the mechanism of *in vitro* cytotoxicity by immune lymphoid cells may involve (*a*) the production of antibody by antibody-forming cells, (*b*) the binding of antibody to target cells, and (*c*) the destruction of antibody-coated target cells by normal non-T lymphocytes carrying Fc receptors.

In summary, two different mechanisms have been demonstrated to account for *in vitro* lymphocyte-mediated cytotoxicity.

1. Immune T lymphocytes, carrying membrane-bound receptors directed against surface antigens, specifically interact with the related antigenic target cells and destroy them in the absence of added comple-

ment. The lytic process is dependent on protein synthesis, but does not involve the release of diffusible nonspecific cytotoxic factors. Cytotoxicity may be inhibited by addition of antibodies directed against the relevant target cell antigens, but is unaffected by anti-Ig antiserum, by antibodies against effector cell transplantation antigens, nor by unrelated antigen–antibody complexes.

2. Normal lymphocytes, carrying membrane-bound receptors directed against the Fc portion of IgG, specifically interact with immunoglobulin antibody-coated target cells and destroy them in the absence of added complement. The lytic process does not involve diffusible nonspecific factors. Cytotoxicity is unaffected by addition of antitarget cell IgG antibody but may be inhibited by anti-Ig antiserum, by antibodies against lymphocyte transplantation antigens, or by unrelated soluble antigen–antibody complexes.

V. Cytotoxicity Mediated by Macrophages

In recent years, the role of macrophages in cell-mediated immunity has been investigated in great detail. The basis for such interest has been the demonstration of a cooperation between immune T cells and macrophages in the effector phase of some immune reactions. Thus, it is clearly established that acquired resistance to intracellular parasites involves a two-step mechanism whereby immune T cells, when confronted with specific antigen, elaborate various factors that activate macrophages. As a consequence of this activation, macrophages acquire an increased nonspecific microbicidal activity (*4*, *226–229*). The concept of macrophages being the effector cells of cell-mediated immunity has been extended to a variety of immune reactions, including allograft rejection and tumor immunity. This subject has been extensively reviewed by Nelson (*230*, *231*) and by Pearsall and Weiser (*232*). Following the observations of Bennett (*176*) and Granger and Weiser (*233*, *234*), several investigators have demonstrated that macrophages may exert specific or nonspecific cytotoxicity *in vitro*. The conclusions of some of these studies, however, could be seriously questioned since no attempt was made to rule out the participation of lymphocytes in the lytic process. As already mentioned (Section III,C,4), certain cytotoxic T cells have adherence properties similar to those of macrophages and are particularly abundant in the peritoneal cell populations which have been used as the source of macrophages.

Recently, better evidence for the role of macrophages in *in vitro* cytotoxicity has been provided by the finding that normal macrophages could be made specificially cytotoxic by contact with immune lymphoid cells or cell-free supernatants from cultures of immune lymphoid cells

incubated with antigen (*235*, *236*). Suggestive evidence has been obtained that the cell-free supernatant contains a specific factor elaborated by immune T cells which is cytophilic for macrophages. In addition to the specific activity of macrophages just mentioned, several studies have shown that macrophages from immune and/or chronically infected animals exerted a nonspecific cytotoxic effect *in vitro*. In this section, both aspects of macrophage-mediated cytoxicity will be briefly discussed. For the sake of simplicity, we adhere to the terminology proposed by Evans and Alexander (*237*) to refer to the two functional categories of effector cells. *Armed* macrophages are specifically cytotoxic to the immunizing target cell, whereas *activated* macrophages do not discriminate among different target cells. The two properties are not mutually exclusive, and armed macrophages, following specific interaction with target cells, become activated and kill in a nonspecific way.

A. Specific Cytotoxicity of Armed Macrophages

In 1970, Evans and Alexander (*235*) reported that peritoneal cells from mice immunized by repeated injections of X-irradiated syngeneic lymphoma cells were able to inhibit the growth of the same tumor cells *in vitro*. Spleen cells from these immune animals had no detectable inhibitory activity *in vitro,* although they were capable of arresting tumor growth when injected together with the lymphoma cells into normal mice. In order to rule out the participation of lymphocytes so far as possible, the peritoneal exudate cells were seeded into tissue culture dishes and the nonadherent cells removed by repeated intensive washings until the cell monolayers contained less than 3% lymphocytes. Subsequent studies (*237–239*) indicated that (*a*) the growth-inhibitory effect was immunologically specific since the macrophages from mice immunized against allogeneic lymphomas had no effect on syngeneic tumor cells and vice versa, (*b*) growth inhibition was reversible during the first 24 hours in the syngeneic system, whereas most of the lymphoma cells were dead after 24 hours in the allogeneic system, (*c*) the effector cells, after exposure for 12 to 24 hours to the tumor cells were unable to inhibit the growth of a second inoculum of lymphoma cells but retained full activity when incubated for 48 hours without lymphoma cells, and (*d*) direct contact between macrophages and tumor cells was necessary, as shown by normal growth of lymphoma cells when separated from the effector cells by a Millipore filter.

The specificity of growth inhibition strongly suggested the involvement of specific recognition structures on the surfaces of armed macrophages. However, the nature of these structures has not been clearly established. One likely possibility is the presence of cytophilic antibodies

on the macrophage membrane. As mentioned earlier, macrophages have surface receptors for the Fc portion of IgG molecules (*187*, *189*). As is the case for other cells with Fc receptors, the energy of binding of IgG antibody to macrophages is very much increased when antibody is complexed to antigen (*218*). In the mouse, macrophages apparently possess surface receptors also for IgM molecules (*240*) and an α_1-globulin having the characteristics of a cytophilic antibody (*241*). Further details on cytophilic antibodies can be found in two recent reviews (*103*, *230*). Several attempts have been made to attribute the specificity of macrophage-mediated cytotoxicity to cytophilic antibodies carried on macrophages. Thus, Granger and Weiser (*234*) showed the presence of cytophilic antibody on the surfaces of armed macrophages. The antibody could be eluted by heating at 56°C. for 30 minutes. Trypsin treatment of immune macrophages abrogated their ability to destroy the relevant target cells. However, trypsin-treated macrophages regained activity after exposure to immune serum or heat eluate from immune macrophages (*242*). Recently, Evans and Alexander (*239*) have also studied the effect of trypsin on growth inhibition by armed macrophages. In their hands such a treatment had no effect, except at relatively high concentrations of the enzyme (10 mg./ml.). When affected, macrophages did not regain activity in culture. A more disturbing finding was reported by Lohmann-Matthes *et al.* (*243*) who tested the cytotoxicity of armed macrophage monolayers on ^{51}Cr-labeled target cells at different time intervals after trypsin treatment. No cytotoxic activity was detectable during the first 6 hours after treatment, but the monolayers regained full activity after 48 hours, although all contaminating lymphocytes had been released from the monolayers by trypsin treatment. Indeed, another striking feature of this system was the stability of macrophage cytotoxicity which could be demonstrated over a period of 15 days in culture, despite repeated addition of target cells. This finding is in contrast to the observation, already mentioned, of Evans and Alexander that macrophage activity disappeared 24 hours after incubation with target cells.

These rather confusing results do not allow any conclusion as to the nature of the recognition structures on armed macrophages. Similarly, the demonstration of large amounts of immunoglobulin on the surfaces of cytotoxic macrophages provides no conclusive evidence for involvement of cytophilic antibodies since growth inhibition was found to be unaffected by anti-Ig serum (*239*).

Further studies indicated that normal macrophages could be rendered specifically cytotoxic after incubation with either lymphoid cells from repeatedly immunized mice or cell-free supernatants from cultures consisting of *in vivo* or *in vitro* sensitized T cells and specific target cells.

Because the mechanism of arming appears to be different in the two situations, they will be considered separately.

As already mentioned, spleen and lymph node cells from hyperimmune mice had no growth-inhibitory activity in the syngeneic tumor system studied by Evans and Alexander (*235*). When monolayers of normal macrophages were incubated with these immune lymphoid cells for 24 hours and then extensively washed, they became able to inhibit the growth of specific target cells. Direct contact between hyperimmune lymphoid cells and normal macrophages appeared necessary for arming, since neither supernatants from cultures of hyperimmune spleen cells, nor hyperimmune spleen cells separated from macrophages by a Millipore membrane were able to produce this effect (*238*, *239*).

In contrast to the arming activity of hyperimmune lymphoid cells, spleen cells obtained after a single immunization had no effect on normal macrophages. When these cells, however, were incubated with specific target cells for 24 hours, the cell-free supernatants of such cultures were able to render normal macrophages specifically cytotoxic to target cells (*236*, *238*, *239*). The factor responsible for this effect has been called *specific macrophage-arming factor* (SMAF). Recent studies (*244*) suggest that this factor possesses a cytophilic moiety for macrophages as well as a specific recognition site for target cells. On fractionation, SMAF appears to consist of two major components, one of a molecular weight greater than 300,000, the other between 50,000 and 60,000.

This factor appears to be produced by immune T cells, since no factor was detected when immune spleen cells treated with anti-θ serum and complement were incubated with specific target cells (*244*). Conversely, cortisone-resistant thymus cells, when sensitized *in vitro* in MLC, liberated SMAF incubation with the relevant target cells (*245*).

Several questions are raised by these findings. The first concerns the nature of the cytophilic factor produced by T cells. In view of its specificity for antigen, it is tempting to speculate that SMAF corresponds to specific T-cell receptors which are shed during the incubation of immune T cells with antigen, as also suggested by the studies of Feldmann (*246*) on the mechanism of the helper function of T cells in the humoral response.

The relationship of SMAF to the specific T-cell receptor described by Cone *et al.* (*162*) or the specific migration inhibition factor reported by Amos and Lachmann (*247*) is not yet known. Another question raised by these findings concerns the relationship between the immune T cells producing SMAF and the cytotoxic T cells since, in some studies, the immune lymphoid populations used for the production of cytophilic

factor also contained large numbers of CL. In other experiments, however, SMAF-like material could be obtained from cell populations not directly cytotoxic toward specific target cells (*236*, *248*). Whether or not these findings reflect quantitative rather than qualitative functional differences remains to be determined.

B. Nonspecific Cytotoxicity of Activated Macrophages

When studying the specific growth-inhibitory effect of armed macrophages, Evans and Alexander (*237*) found that during contact with specific target cells the effector cells acquired the capacity to inhibit the growth of susceptible target cells in a nonspecific way. Such nonspecific activity was detectable after 4 hours of incubation of macrophages with specific target cells. An identical pattern of behavior was shown by normal macrophages which had been armed *in vitro* by incubation with immune spleen cells. Moreover, macrophages from mice immunized with bacille Calmette-Guérin (BCG) vaccine became nonspecifically cytotoxic following *in vitro* incubation with specific antigen, the purified protein derivative (PPD). These results suggested that a two-step mechanism was involved in the activity of macrophages from immune animals, including first an interaction of armed macrophages with specific antigen leading to their activation into nonspecific effector cells.

This concept shows some analogy with the mechanism put forward to account for the increased nonspecific bactericidal activity of macrophages from immune animals (*4*). There is, however, an important difference between the two model systems. In the experiments cited in the foregoing activation of macrophages *in vitro* has been achieved by direct exposure of macrophages to the specific antigen, whereas the studies on cellular resistance to microorganisms have clearly shown that activation of macrophages *in vitro* involved the participation of mediators released by immune lymphoid cells following interaction with antigen (*3*, *227*). Along the same line, it should be noted that normal macrophages exposed to the supernatants of cultures consisting of immune lymphoid cells and specific antigen did not exert nonspecific cytotoxicity when directly challenged with unrelated target cells (*236*). Whether different mechanisms of activation or different factors are involved is unknown.

Other studies indicated that normal macrophages became nonspecifically cytotoxic after exposure *in vitro* to various agents such as endotoxin, double-stranded RNA isolated from fungal viruses or poly I·poly C (*249*). As in the case of armed macrophages, growth inhibition of target cells was reversible during the first 24 hours of incubation with treated macrophages. Similarly, the growth-inhibitory activity of such

macrophages was lost after exposure to a first population of target cells. Phagocytosis was not involved in the process of growth inhibition. Macrophages activated *in vivo* either by chronic infection with Protozoa, infection with intracellular bacteria or Metazoa, or injection with complete Freund's adjuvant were also cytotoxic to tumor cells (*250–252*).

These results thus suggest that macrophages activated in one way or another are deleterious to cells in culture. Whether these activated macrophages can discriminate between normal and abnormal cells is less clearly defined. Recently, Hibbs *et al.* (*250, 252*) have suggested that the *in vitro* cytotoxicity of activated macrophages, although nonspecific in an immunological sense, was, nevertheless, discriminatory insofar as only target cells with abnormal growth characteristics were selectively destroyed. Thus, macrophages from mice chronically infected with intracellular Protozoa or from mice injected with complete Freund's adjuvant destroyed *in vitro* various primary or established cultures of tumor cells but had no effect on mouse embryonic fibroblast strains. Moreover, when the same fibroblasts became transformed, as happened spontaneously within 3 months of culture, they not only acquired abnormal growth characteristics but also became susceptible to the cytotoxic effect of activated macrophages (*253*). Further experiments are needed to verify the validity of this interesting concept.

VI. Relevance of Cell-Mediated Cytotoxicity to Allograft Rejection and Tumor Immunity

From the various models just presented, it is clear that cell-mediated cytotoxicity *in vitro* is by no means the expression of a single mechanism. Different cell categories (T cells, B cells, monocytes, macrophages, PMN cells) may be involved, and their relative contribution may vary according to various factors such as the type of target cell, the presence of antibody, the source of effector cells, or the cytotoxic assay system. In view of this complexity, it appears difficult to appreciate the significance of *in vitro* activities of heterogeneous cell populations in terms of actual correlates of *in vivo* phenomena. Indeed, the possibility exists that some mechanisms found *in vitro* have no corresponding reality *in vivo*. However, with the recent development of knowledge on the physicochemical and antigenic characteristics of the cells involved in the immune response, it becomes possible to design experiments in which the various mechanisms demonstrated *in vitro* may be put under test *in vivo*. One approch is the analysis of the activity of well-defined cell populations following transfer into animals which are normally or artificially depleted of a given cell type.

In analogy with the criteria used to ascertain the role of antibody in

some pathological reactions (*254*), several conditions should be fulfilled in order to establish a direct relationship between *in vitro* and *in vivo* effector cells: (*a*) reproduction in immunologically inert recipients of the *in vitro* reactions; (*b*) dose dependence of the *in vivo* activity of transferred cells; (*c*) absence of effect *in vivo* after specific removal of the effector cells active *in vitro;* and (*d*) identification of effector cells at the site of the reaction. It is obvious that some of these requirements are still difficult to achieve, but they may become feasible in the near future in view of the current improvement in cell separation techniques.

In this section, we will review recent experiments undertaken to determine the relative contribution of the mechanisms found *in vitro* to the phenomenon of allograft rejection and tumor immunity. We will describe the available evidence that (1) immune T cells, enriched in CL, play a major role in the rejection of normal or tumor allografts, at least in the mouse, (2) macrophages are not always an absolute requisite for allograft rejection, although they may amplify the activity of immune T cells in certain instances, and (3) cooperation between immune T cells and macrophages may be more evident in syngeneic tumor systems.

A. Allograft Rejection

As mentioned earlier, quantitative studies of lymphocyte-mediated cytotoxicity in lymphoid cells from animals that had received normal or tumor allografts showed an excellent degree of correlation between the time course of CL formation and graft rejection (*15, 51, 64–66*). However, in the instances where the humoral response was tested in parallel, it was found that a correlation also existed with development of alloantibody-forming cells (*28, 60*) and circulating alloantibodies, particularly of the IgM class (*23, 59*). These findings made it difficult to draw any firm conclusion as to the actual effector cells involved in studies of adoptive transfer of transplantation immunity with immune lymphoid cells (*255–258*).

With the development of appropriate methods for obtaining defined cell populations containing either immune T cells or B cells and alloantibody-forming cells, it has been possible to demonstrate the predominant role of the former cells in allograft rejection. The first evidence for *in vivo* activity of immune T cells was provided by Sprent and Miller (*53, 259*) who showed accelerated skin graft rejection in neonatally thymectomized CBA mice following intravenous injection of a pure population of CBA T cells immunized against the graft alloantigens. This population was obtained by injection of CBA thymus cells into irradiated recipients bearing the graft alloantigens. As discussed earlier (Section III,B,2), some of the transferred thymus cells became sensitized

against the recipient alloantigens and could be recovered by thoracic duct cannulation in an almost pure form. The same population of immune T cells was also able to inhibit tumor growth when mixed together with tumor cells carrying the relevant alloantigens before intraperitoneal injection into appropriate recipients (*51*, *259*). Since the lymphoid cell population contained a large proportion of CL, as assessed *in vitro* by the ^{51}Cr assay method, it was tempting to attribute graft rejection to the direct activity of the latter cells. The results, however, did not exclude the possibility, although unlikely, that the immune T cells functioned as helper cells for the production of alloantibody which caused rejection in the presence of complement or non-T effector cells. Alternatively, the effector phase could be mediated by macrophages armed or activated by factors released by the immune T cells. In order to approach this question, we studied the tumor growth-inhibitory activity of different immune lymphoid cell populations injected into heavily irradiated syngeneic recipients (*260*, *261*). When these recipients were reconstituted with normal spleen cells, intraperitoneal inoculation of allogeneic tumor cells resulted in rapid tumor growth followed by death within 10 to 20 days. In contrast, complete protection against tumor growth was achieved by intravenous or intraperitoneal injection of spleen cells from syngeneic mice immunized against the same allogeneic tumors. When the immune spleen cells were treated with anti-θ serum and complement prior to inoculation into the irradiated recipients, no protection occurred, indicating that B lymphocytes, including IgM or IgG alloantibody-forming cells, were ineffective in that system. Moreover, a pure population of T cells, immunized by transfer into relevant allogeneic irradiated recipients, was perfectly able to prevent tumor growth in the irradiated hosts. The same experiments were repeated in lethally irradiated mice inoculated subcutaneously with allogeneic tumor cells, protected against radiation death with bone marrow cells, and injected with immune T cells. At no time was any tumor detectable, whereas control mice injected with normal thymus cells died of disseminated tumor within 2 weeks. These findings clearly indicated that pure populations of immune T cells could effectively protect heavily irradiated mice against subcutaneous growth of allogeneic tumor cells.

Although these results have ruled out the possible role of AD cell-mediated lysis in this system, the question remains whether immune T cells acted alone as effector cells or in conjunction with macrophages. Several points are worth considering in this respect.

1. The immune T cells responsible for tumor growth inhibition *in vivo* carry specific receptors for the relevant alloantigens. Thus, Berke and Levey (*153*) showed that preincubation of immune spleen cells on fibroblasts carrying the immunizing alloantigens removed not only the

lymphocytes effective *in vitro*, as mentioned earlier in Section III,C,3, but also those inhibiting tumor growth *in vivo*. Furthermore, recovery of the fibroblast-adsorbed lymphocytes provided a cell population specifically efficient *in vivo* and *in vitro*.

2. The immune T cells accumulate preferentially at the specific graft site as recently suggested by Lance and Cooper (*262*), who used deoxyuridine-^{125}I-labeled spleen and lymph node cells from mice bearing skin allografts. In similar unpublished experiments, we found that the number of ^{51}Cr-labeled immune T cells injected into heavily irradiated hosts 5 days after subcutaneous inoculation of allogeneic and syngeneic tumor cells was four- to eightfold greater at the site of the allograft carrying the immunizing alloantigens than at the site of syngeneic or unrelated allogeneic graft. These results, which are in conflict with previous reports indicating no preferential accumulation of effector cells in the specific graft site (*263–266*), can be explained by the relative enrichment in effector cells achieved by means of immunization by transfer into irradiated allogeneic recipients, the unreactive T cells being diluted by the rapidly multiplying antigen-reactive lymphocytes.

3. The specificity of allograft rejection speaks against the participation of nonspecific factors or effector cells. In the experiments cited above (*261*), injection of immune T cells had no effect on the *in vivo* growth of bystander tumor cells in the presence of total destruction of the related tumor cells. These results confirm the exquisite specificity in the effector phase of rejection of normal or tumor allografts (for references, see Refs. *12* and *267*).

4. The necessity of an amplifying mechanism by macrophages may vary according to the system studied. On the one hand, in studies demonstrating prevention of subcutaneous growth of tumor cells in heavily irradiated recipients after intravenous injection of immune T cells (*261*), it appears unlikely that a large number of monocytes could accumulate at the graft site since these cells are known to derive from rapidly dividing radiosensitive precursors (*268*). On the other hand, Rouse and Wagner (*269*) found that immune T cells caused no rapid rejection of skin graft in irradiated recipients when injected at the time of irradiation, whereas the same cell population was effective in thymectomized, lethally irradiated hosts grafted 4 weeks after bone marrow reconstitution. These results are very reminiscent of observations made in adoptive transfer studies of delayed skin reactions or immunity to *Listeria* organisms—two phenomena involving the participation of both immune T cells and monocytes (*4*, *270*, *271*). Clearly, further reconstitution experiments using purified populations of potential effector cells are needed to assess their relative roles.

Recent studies indicate that T cells immunized *in vitro* are also able

to prevent tumor growth or to induce skin graft rejection in immunologically inert mice. Thus, Cohen *et al.* (*272*) showed that spleen cells incubated with allogeneic fibroblasts for 5 days inhibited tumor growth when mixed with the related allogeneic sarcoma cells before intramuscular injection into 550 R-treated recipients. Curiously, the same spleen cells showed no detectable cytotoxicity *in vitro*. Direct evidence for the role of immune T cells was provided by Rouse *et al.* (*269, 273*) who used cortisone-resistant thymus cells immunized *in vitro* in MLC. These cell populations, which are highly cytotoxic *in vitro*, caused skin graft rejection or tumor growth inhibition when injected into T-cell-deprived animals. Quantitative estimation of the *in vivo* activities of immune T cells immunized either *in vivo* or *in vitro* suggested, however, lower effectiveness of *in vitro* immunized T cells, despite similar or even higher *in vitro* cytotoxic activity as compared to *in vivo* immunized T cells. One possible explanation for this difference may be the lower ability of *in vitro* immunized T cells to recirculate and, hence, to accumulate in the graft site (*269*).

Several studies have shown that peritoneal cells from immune animals were able to confer *in vivo* resistance against the related allogeneic tumor cells (for references, see Ref. *231*). These results have been interpreted as evidence for the predominant role of macrophages in tumor allograft rejection. In some instances, however, no attempt has been made to rule out the participation of immune lymphocytes which are known to accumulate at the site of a specific or nonspecific inflammatory reaction (*52, 146, 274*). When highly purified macrophages were used in adoptive transfer studies, it was found that these cells had a suppressive effect only on tumor cells injected at the same site, whereas the lymphoid cells from the same immune animals were able to inhibit the growth of tumor cells inoculated at a different site (*275*). It thus appears that armed macrophages do not extensively recirculate. Further work is needed to define the role of monocytes that sometimes, but not always, comprise a high proportion of the cells infiltrating the graft during rejection (*276–278*). Such studies should also establish whether or not the immune T cells that release macrophage-specific factors are identical to the effector cells exerting direct cytotoxicity. Also the participation of antibody-dependent lysis by non-T lymphoid cells remains to be clarified, particularly in view of the demonstration of graft survival rather than rejection in animals passively administered with IgG alloantibody (for references, see Ref. *279*) or actively producing cytotoxic alloantibody (*280, 281*).

When considering the *in vivo* effect of defined cell populations following transfer into immunologically manipulated recipients, one should

not forget that the conclusions drawn from such studies are not necessarily consistent with the results obtained by microscopic examination of the graft during rejection in intact animals. This possibility has been best illustrated by Gershon and his colleagues (*282, 283*) who investigated the role of macrophages in the rejection of allogeneic lymphoma cells in the hamster. In this model system, animals injected subcutaneously with these lymphoma cells developed a state of concomitant immunity, i.e., they became resistant to challenge with a second dose of 10^5 times the number of tumor cells required to produce tumors in normal hamsters. Morphological evidence strongly suggested that macrophages of such animals played the major role in the rejection of second grafts since they were the only cells that consistently invaded the graft. Despite this striking morphological evidence, attempts to demonstrate a crucial role of macrophages, by means of transfer experiments, were unsuccessful. To the contrary, such studies clearly indicated that immune lymphocytes played a major role in the rejection process. Thus, the ability of immune lymph node cells to prevent subcutaneous growth of the tumor cells, either when mixed with them or after intravenous injection into thymectomized and irradiated recipients was affected very little by reduction of the number of available macrophages or by local introduction of large numbers of macrophages from nonspecifically induced peritoneal exudates (*283*).

B. Tumor Immunity

The considerations just mentioned certainly apply, even to a greater extent, to the mechanism of immunity in syngeneic tumor systems. As already mentioned, very few studies have dealt with the characterization of the effector cells responsible for *in vitro* cytotoxicity of lymphoid cells from normal or immune tumor-bearing animals. Moreover, when the activity and the origin of effector cells have been extensively investigated, as in the MSV system in mice, conflicting results have been obtained according to the assay method used for assessing *in vitro* cytotoxicity. Using a colony inhibition technique, Hellström and Hellström (*117*) regularly found cytotoxic lymphoid cells in animals with progressive tumors, whereas Leclerc *et al.* (*116*) reported a very sharp decline in CL, as measured by the ^{51}Cr assay method, as tumor growth proceeded. A somewhat similar observation was made by Lamon *et al.* (*284*) using the microplate assay. Moreover, different results as to the nature of the effector cells have been reported, since only T cells were involved in the ^{51}Cr assay, whereas T and non-T cells contributed to the effects measured by the microplate assay (*48, 49*). Studies of the effect of sera from tumor-bearing mice on the *in vitro* activity of lymphoid cells

have also provided confusing results, varying from no detectable effect (*116*) to complete inhibition of lymphocyte activity (*117, 285*). In one study, pretreatment of target cells with a large amount of antiserum often resulted in an increased cytotoxicity by lymphocytes, whereas lower concentrations of the same antiserum often produced inhibition (*185*). To the contrary, Pollack *et al.* (*186*) reported that AD lysis by normal lymphoid cells could be demonstrated at dilutions of antiserum much higher than that necessary for inhibition of immune lymphocytes.

In view of these conflicting observations, all made in the same system, it is difficult to evaluate the numerous reports on *in vitro* cytotoxicity of lymphoid cells from tumor-bearing animals or patients in terms of the actual effector cells *in vivo* (*286–295*). The same difficulty of interpretation applies to the demonstration of various blocking or unblocking factors in sera of the same donors (*296–299*). Clearly, comparative studies of the nature of lymphoid effector cells detected in different phases of tumor growth by the various assay methods as well as precise immunochemical characterization of serum factors are urgently needed before any conclusion as to the significance of *in vitro* findings for surveillance of tumor growth can be made.

Only few attempts have been made to identify the nature of lymphoid cells responsible for transfer of adoptive tumor immunity. In his study of the development of tumors in adult-thymectomized, anti-lymphocyte serum-treated mice infected with polyoma virus, Allison (*300*) found that tumor formation could be prevented by transfer of syngeneic lymphoid cells from specifically immunized donors. Pretreatment of the lymphoid cells with anti-θ serum and complement, however, abolished their ability to prevent tumor development. Rouse *et al.* (*301*) found that spleen cells from mice immunized against a syngeneic plasma cell tumor, after treatment with anti-θ serum and complement, were no longer able to inhibit tumor growth in sublethally irradiated mice, whereas treatment of the same spleen cells with rabbit antiserum against mouse immunoglobulin light chains in the presence of complement, which specifically killed B cells, had no effect. Similar results have been found with lymphoid cells from mice immunized with syngeneic cells transformed by the papovavirus SV40 (*302*). The latter studies, however, suggested that inhibition of tumor growth by admixed immune lymphoid cells was dependent on the presence of radiosensitive effector cells, presumably monocytes, provided by the recipient mice (*303*). This conclusion was based on the observation that (1) tumor growth inhibition by immune lymphoid cells was less efficient in irradiated than in normal hosts or in normal recipients treated with silica particles, and that (2) bone marrow cells, once they had differentiated for several days in

irradiated hosts, provided the effector cells necessary for tumor growth inhibition.

The possible participation of macrophages in syngeneic tumor rejection has been suggested by several observations. This aspect has been reviewed recently by Allison (*300*). The role of macrophages activated by immune T-cell products has been recently strengthened by the demonstration of increased resistance to autochthonous and transplanted tumors in animals chronically infected with intracellular Protozoa or bacteria (*304–306*) or infected with Metazoa (*307*). As mentioned earlier, macrophages from such animals were nonspecifically cytotoxic *in vitro*. Although it is tempting to attribute the increased antitumor resistance to the activated macrophages, the question remains whether these cells are mainly involved in actual destruction of tumor cells or, alternatively, in stimulation of the specific immune response. The same dilemma applies to the various examples of tumor regression after nonspecific stimulation with local or systemic adjuvants (for references, see Ref. 3). Whatever the mechanism, it is clear that the effect of adjuvants involves complex interactions with many variables such as tumor type, species and age of animals used, and timing of adjuvant administration. It is, therefore, not surprising that conflicting results have been obtained in studies on the effect of adjuvants on tumor resistance (*308*).

VII. Summary

The term cell-mediated cytotoxicity applies to lytic reactions that require the participation of lymphoid or nonlymphoid cells, but not of added complement. It has been clearly established that several pathways including different cell types are involved in cytotoxic reactions *in vitro*. With membrane-associated antigens, such as transplantation and tumor-associated antigens, the cytotoxic effect of effector cells on adequate target cells is most often highly specific and requires intimate contact rather than release of diffusible toxic factors. Specific cell-mediated cytotoxicity *in vitro* can be divided into three categories according to the nature of the effector cells.

1. Cytotoxic T cells (CL) are derived from antigen-reactive precursors which have proliferated and differentiated during the immune response to transplantation antigens, either *in vivo* or *in vitro*. These effector cells are equipped with specific receptors, the nature of which is still controversial. Under optimal conditions, target cell lysis reaches completion within less than 1 hour. One effector cell can kill several target cells. The lytic process is temperature- and energy-dependent, is reversibly inhibited by EDTA or CB, and is modulated by the intracellular level of cAMP. Operationally, any antibody directed against

target cell antigens identical or closely related to those complementary to the effector cell receptors inhibits cell-mediated cytotoxicity, whereas anti-Ig serum has no effect.

Cytotoxic lymphocyte progenitors belong to the category of small lymphocytes. Early after antigenic stimulation, the effector cells are large and correspond to blast cells, whereas they appear as small lymphocytes later in the immune response. Memory exists, but it is unclear whether it implies quantitative and/or qualitative changes.

2. Antibody-dependent cell-mediated cytotoxicity is due to effector cells with surface receptors for the Fc portion of IgG molecules which interact with IgG antibody bound to target cells. The exact nature of these effector cells remains to be established, but T cells are not involved. They are present in unimmunized individuals and may represent a subpopulation of B cells. Nonlymphoid cells, such as monocytes, macrophages, or PMN cells may also be involved.

Under optimal conditions, target cell lysis reaches completion within 10 to 20 hours. Operationally, the lytic process is inhibited by anti-Ig serum, antibody against surface antigens of the effector cells or unrelated lymphoid cells, aggregated IgG, or soluble antigen–antibody complexes.

3. Armed macrophages can also be responsible for specific cell-mediated cytotoxicity. Their surfaces appear to be equipped with either cytophilic antibodies or cytophilic factors produced by immune T cells following contact with specific antigen. When armed macrophages are incubated with the specific target cells, they become activated and also exert a cytotoxic effect on irrelevant target cells. Macrophages activated by a variety of procedures may also be nonspecifically cytotoxic.

The relevance of *in vitro* findings to the effector mechanisms operating in allograft rejection and tumor immunity is not yet clearly defined. Adoptive transfer studies suggest that immune T cells play an important role in allograft rejection, but the question remains open as to what extent their direct activity is amplified by the release of factors acting on macrophages or other cells. In syngeneic tumor systems, the information available is neither extensive nor conclusive. The experimental results are still confusing and poorly quantitated. Another source of conflict is the finding that the different assay methods for measuring *in vitro* cytotoxicity do not necessarily detect the same mechanisms of cell destruction. It is thus difficult to evaluate the significance of *in vitro* variations in effector cell activities in terms of *in vivo* surveillance of tumor growth. Similarly, the synergistic or antagonistic effect of antibody, antigen, or antigen–antibody complexes remains to be clarified. The development of cell separation techniques, based on physicochemical and immunological

characteristics of lymphoid cells, as well as the isolation of transplantation or tumor-associated antigens and the corresponding antibodies, should be helpful for a better understanding of the delicate interplay among these various components of the immune response.

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Antigenic Competition: A Review of Nonspecific Antigen-Induced Suppression

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I. Introduction

In 1902, the German immunologist Michaelis (*156*) first described a phenomenon in which the immune response to one antigen was suppressed by the response to a second, unrelated antigen. This phenomenon, known as competition of antigens, was the subject of a great deal of research in the first half of this century, principally oriented toward determining the practical importance of the observation with respect to polyvalent vaccines and immunization schedules. The early literature has been reviewed previously by Adler (*6*, *7*) and will not be discussed further in this paper. The present review article will deal with progress which has been made in the last decade, especially with respect to the mechanism of antigenic competition. An attempt will be made to correlate the large amount of recent and diverse information in this field with current concepts of cellular immunology.

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In 1955, Jerne (*130*) postulated the natural selection theory of antibody formation—later modified by Burnet (*41*)—and now generally accepted as a useful model of antibody formation. In Burnet's clonal selection theory, it is postulated that many different antibody-forming cell precursors are present in the adult animal, each with receptor molecules on the surface capable of specifically reacting with potential antigens, i.e., the specificity of the clone is genetically determined before contact with antigen. Although many immunological phenomena could be explained by this theory, the observation of antigenic competition between unrelated antigens represented a serious challenge to its validity and, for this reason, antigenic competition became the subject of renewed interest.

Until recently, experiments on antigenic competition were largely descriptive in nature and established a number of parameters concerning antigenic competition which are now generally, but not universally, accepted.

a. Antigenic competition *can* be demonstrated when (1) the first antigen is thymus-dependent (most publications) or independent (*68, 72, 143*), particulate or soluble protein, polypeptide, or glycopeptide (*7, 14, 27, 37, 69, 108, 173, 190, 197, 211*), and in some circumstances, polysaccharide (*68, 72, 141, 143*); (2) the response to the first antigen is a primary or secondary response as well as following hyperimmunization (*7, 14, 27, 37, 69, 72, 107, 108, 173, 190, 197, 211*); (3) the animal is tolerant (*68, 146, 211*), in the process of tolerance induction (*143, 145, 148*), or genetically unresponsive to the first antigen (*2, 27, 28*; cf., however, *212, 238, 250*); (4) the antigens are injected simultaneously (*6, 7, 27, 37, 211*) or up to several weeks apart (*6*); and (5) the antigens are injected into the same or separate sites (*5, 72, 76, 237*; cf., however, *36*).

b. Antigenic competition is *best* evoked when (1) the antigens are injected into the same site (*5, 72*); (2) a time interval of 1 to 7 days separates the injection of the two antigens (*6, 13, 36, 69, 108, 173, 190, 197*); (3) the dosage of the first antigen is high relative to the second (*7, 35, 62, 72, 140*); and (4) the first antigen is thymus-dependent (*68, 72, 172, 229*).

c. Antigenic competition can cause suppression of both immunoglobulin (Ig) M and IgG antibody formation (most publications), delayed hypersensitivity (*27, 67, 148, 212*), graft-versus-host reactions (*144, 147, 158*), graft rejection (*69, 105, 138*), and experimental autoimmune lesions (*73, 152*).

d. Suppression caused by antigenic competition is not merely a lag in the onset of antibody formation but represents a quantitative decrease

in the immune response and the establishment of immunological memory (*69, 72, 108, 250*).

e. Localization of antigens in the responding lymphoid organ is the same in responses suppressed by antigenic competition as it is in the normal immune response (*107, 203*).

f. The sequential administration of two unrelated antigens may lead to *enhancement* of the immune response to the second antigen, or to antigenic competition, depending upon the experimental protocol used (*7, 9, 153, 195, 196, 206, 207, 244, 248, 255*).

Although these observations are extremely informative with respect to the characteristics of antigenic competition, for the most part they are of limited assistance in evaluating the mechanism of the phenomenon. To do this, it is first necessary to understand the cellular events surrounding the normal immune response—a subject that is far from being completely delineated.

II. Nature of the Antigen-Reactive Unit

The antigen-reactive unit (ARU) can be defined as the minimum complex of cells and factors necessary to produce a detectable immune response in reaction to antigenic stimulation. In recent years, at least three cell types (*67*) have been implicated in the events leading to antibody formation to thymus-dependent antigens: the macrophage, accessory or A cell (*81, 176, 178, 219, 220, 221*), the thymus-derived or T cell (*51, 52, 162, 204, 239*), and the bone-marrow derived, bursal-equivalent, or B cell (*51, 52, 162, 204, 239*). In the mouse, the synthesis and release of antibody has been shown to be a function of the B lymphocyte (*128, 161, 181*). With certain antigens, the proliferation of these lymphocytes apparently requires their interaction with thymus-derived lymphocytes (*51, 52, 162, 239*), each cell type recognizing and reacting with different determinants on the antigen (*201*). This function of the T cell is radioresistant (*74, 122, 136*) and can be transferred by culture supernatants (*61, 97*). Indirect evidence suggests that both T (*26, 98–100*) and B (*1, 3, 26, 98, 224, 225, 252, 253*) lymphocytes bear specific Ig receptors that recognize either the carrier determinants (T cells) or the haptenic determinants (B cells) (*160, 170, 199*), and the antibody that is produced is similar in avidity (*18*), specificity (*251*), and class (*247*) to the B cell receptors.

At least three models have been suggested to explain the mechanism of thymus–bone marrow cell interaction and the way in which this interaction triggers cellular proliferation, differentiation, and antibody formation. One of these models, suggested by Mitchison (*171*), pos-

tulates that the T cell acts as an antigen-concentrating device to present the determinants to the B cell, and to allow the stimulation of B-cell precursors with receptors of relatively low avidity. This type of model is supported by the observation that polymeric antigens, such as pneumococcal polysaccharide, polyvinylpyrrolidone (PVP), or polymerized flagellin (POL), which consist of repeating identical subunits presumably in high local concentration, do not require T-cell helper activity (*19, 21, 58*). On the other hand, as pointed out by Dutton *et al.* (*64*), the need for T–B cell cooperation *in vitro* (*113, 122, 245*), where antigen concentration can be extremely high at the cellular level, would appear to mitigate against an antigen-concentrating mechanism as being the *only* function of T cells.

A second model, that of Bretscher and Cohn (*33*), attempts to explain both tolerance induction and T–B cell cooperation. These authors postulate that the initial step in antibody induction and paralysis is the interaction between antigen and receptor antibody, which is recognized by an "interaction-sensing unit." The antigen-sensitive cells for both carrier and ligand have a similar system of receptor molecules and interaction-sensing units, and the induction of antibody synthesis on the part of the B cell requires the recognition of two determinants (carrier and hapten) so that the two types of cells are involved in associative interaction. Receptor stimulation in the absence of associative interaction leads to tolerance induction and is similar in mechanism for both the T and the B cell and for high and low zone tolerance (*168*).

The third model of T–B cell interaction, postulated by Dutton *et al.* (*64*), is somewhat less complex than that of Bretscher and Cohn, although similar in some respects. These authors also postulate that the induction of the B cell requires two signals—one from its own receptor molecules and one from a T cell that has also been stimulated by an antigen determinant. The model differs from that of Bretscher and Cohn in that the authors suggest that the T cell reacts to stimulation by the elaboration of a diffusible chemical mediator capable of activating the B cell. Thus, nonlinked recognition is possible in this model, i.e., the carrier and haptenic determinants need not necessarily be confined to the same molecule, although linked recognition is obviously more efficient because the cells are held in close proximity. This hypothesis has recently received support from experiments apparently showing cooperation between cells separated by nucleopore membrane (*79, 80*).

These models are further complicated by recent observations suggesting that subpopulations of both T (T_1 and T_2) (*22, 42, 43, 116, 200*) and B cells (B_1 and B_2) (*89, 116, 193*) can be identified on a functional basis. At present it is debatable whether these cell types represent dif-

ferent stages in the process of differentiation or whether they are distinctly separate subpopulations.

The role of the macrophage in the immune response is also a matter of considerable debate at the present time. It has been demonstrated by Mosier (*176*) and confirmed by other investigators (*65, 114, 134, 178, 191, 235*) that a cellular component of normal spleen cells that adheres to plastic or glass is necessary for an immune response to sheep red blood cells (SRBC). This function is radioresistant (*65, 178*), can be replaced by supernatant fluid from 24-hour cultures of peritoneal exudate cells (*123*), and is inhibited by rabbit, antimouse macrophage serum (*221*). That this cell is important *in vivo* has been postulated by Gorczynski *et al.* (*96*) who obliterated the ability of irradiated mice to support the response of normal spleen cells to SRBC by inoculation with 10^{10} horse red blood cells (HRBC) 24 hours prior to irradiation. The distinction between this experiment, antigenic competition, and reticuloendothelial blockade is not clear.

There are a number of mechanisms by which the macrophage may facilitate the immune response: (*a*) the processing of antigen to supply specific lymphocytes with informational ribonucleic acid (RNA) (*8, 53, 81, 82*) or a highly immunogenic antigen–RNA complex (*23, 86, 88*), (*b*) the localization and concentration of antigen for more effective interaction with T and B cells (*178, 192, 214, 221*), and (*c*) the prevention of antigen overloading at the lymphocyte level (*214*). The data supporting these contentions are beyond the scope of this review and, at the present time, the only unequivocal statement which can be made is that spleen cells depleted of adherent cells do not produce an immune response to certain antigens *in vitro*.

Throughout this discussion of cellular cooperation, reference has been made to thymus-dependent antigens. By using mice that had been neonatally thymectomized, Miller (*159*) was the first to show that a number of antigens evoked normal immune responses in spite of the absence of thymus-derived lymphocytes (reviewed in *163*). Because of the possibility that T cells had already been seeded to the peripheral organs prior to thymectomy (*111*), it was necessary to remove the T cells by anti-θ serum (*198, 202*) or to perform experiments on adult thymectomized, lethally irradiated, bone-marrow reconstituted mice to establish which antigens were truly T-independent. These antigens, notably PVP (*19*), pneumococcal polysaccharide (*58*), and POL (*21*) are characterized by a repetitive polymeric molecular structure. This structure supposedly results in similar antigenic determinants being presented to the B cell in a concentration sufficient to stimulate the cell to proliferation and antibody formation. The property of thymus independence has

proven a useful tool in assessing the importance of the T cell in various immunological phenomena.

III. Mechanisms of Antigenic Competition

The complexity of these cellular interactions and cooperative functions has made the study of antigenic competition extremely difficult, although it has served to expand the number of possible mechanisms available for study. Some of these mechanisms can be enumerated as follows:

1. The antigens compete with each other for a cell type occurring in limiting frequency (*7, 13, 14, 17, 35, 36, 55, 62, 69, 107–109, 140, 141, 143, 146, 150, 190, 196, 212, 256*).

2. The response to the first antigen causes the depletion of nutrients necessary for the response to the second.

3. The response to the first antigen leads to partial tolerance to the second antigen.

4. The antibody produced in response to the first antigen suppresses the response to cross-reacting determinants on the second antigen by specific feedback inhibition.

5. The increased number of cells arising in response to the first antigen interferes physically with the response to the second, either by crowding out the cells at the follicular level (*93*) or by blocking thymus–bone marrow cell interactions (*135*).

6. The presence of thymus cell-derived helper molecules attached to the surface of the macrophage and acting to present antigen to specific B cells interferes with the action of other helper molecules (*78, 238, 240*). This interference may be due to competition for the macrophage attachment sites (*78, 238, 240*) or to steric hinderance imposed by the specific interacting B cells, preventing the interaction of other B cells with their respective helper molecules and antigens (*240*).

7. Antigenic competition occurs between antigens bearing cross-reactive determinants (*63, 211*). This may result in B-cell competition for the same carrier-specific T cell, to the disadvantage of B cells with low affinity receptors or occurring in low frequency (*63*).

8. The immune response to the first antigen results in the elaboration of inhibitory cells or humoral factors which suppress the response to the second antigen (*76, 77, 90, 91, 172, 173, 197, 227, 229, 248*).

9. Other mechanisms have been postulated which are even less well understood. It has been suggested that macrophages, triggered by initial antigen and thereby activated, catabolize the second antigen more vigorously and render it less immunogenic (*129*). In other systems, particularly involving states of anergy in clinical infectious states, in

addition to circulating inhibitory factors (*47*, *104*), the initial immunogenic challenge may deplete the host of effector cells for the delayed response (*194*).

With most of these mechanisms, the expected experimental observation is an apparent decrease in the frequency of antigen-reactive units specific for the second antigen. This is also true of mechanism 8, provided that the inhibitor acts to some extent by precursor inactivation and not merely by inhibition of cell proliferation and differentiation. On the basis of limiting dilution studies *in vivo* (*14*) and *in vitro* (*196*), it has been demonstrated that such a decrease in antigen-reactive units specific for the second antigen does occur. The failure to demonstrate this on the part of some investigators (*173*) lies in the fact that only one cell dose was used in these experiments, and this does not constitute a limiting dilution assay (*133*, *218*). On the other hand, an interesting experimental observation by Sjöberg and Britton (*229*), using *in vitro* techniques, argues against a decrease in the frequency of antigen-sensitive units specific for unrelated antigens. In these experiments, spleen cells taken from mice hyperimmune against HRBC were normal in their response to SRBC and demonstrated suppression of the anti-SRBC response only if HRBC were also present in the culture. Thus, the observation that there is a decrease in the frequency of antigen-reactive units specific for the second antigen depends upon the experimental protocol used.

IV. Antigenic Competition for a Limiting Cell Type—Role of the Macrophage

The possibility that antigenic competition is the result of true competition for a common cell type has been discounted by some authors (*173*) on the grounds that a time interval is generally required following injection of the first antigen (see Section I). This reasoning is not necessarily correct, however, as competition may take place for early pluripotential precursor cells and hence require several days before the effects of this preemption are observed. Alternatively, processed antigen may be more effective as a competitor than the native antigen, because a time interval is required to generate a sufficient quantity of this material to interfere with the immune response to other antigens.

Of the three cell types believed to be involved in the immune response, the macrophage may well be considered the most likely target cell of antigenic competition on theoretical grounds. However, the results of a number of experiments are contrary to this conclusion. By using rabbit Ig and rabbit serum as the antigens, Adler (*5*) demonstrated that the response of the rabbits to the globulin was reduced when these

antigens were injected into different sites. This has been repeated in rabbits using trinitrobenzene sulfonic acid (TNBS) or *p*-arsanilic acid conjugated to keyhole limpet hemocyanin (KLH) (*76*) and in mice using goose and rat red blood cells (*72*) as well as SRBC and HRBC (*186*). On the other hand, using dinitrophenylated bovine γ-globulin (DNP–BGG) and arsanilic acid–azo-BGG, Brody and Siskind (*35*, *36*) could not show suppression of the immune response of rabbits to either antigen when the antigens were injected into different footpads. All of the experiments cited, except the ones performed in mice (*72*, *186*), made use of complete Freund's adjuvant to render preparations more immunogenic. Because the cells responding to a footpad injection of particulate antigens are localized to the draining lymph nodes (*70*, *112*), it is unlikely that reticuloendothelial (RE) blockade can account for the suppression observed, and it is equally unlikely that antigenic competition involves fixed phagocytic cells.

In another type of experiment, Liacopoulos *et al.* (*143*) examined the competitive effects of high- and low-dose tolerance to bovine serum albumin (BSA) on the immune response to ovalbumin. These authors found greater suppression of the antiovalbumin response during low-dose tolerance induction to BSA than was observed during high-dose tolerance induction. This observation was also taken as evidence against the involvement of the macrophage in antigenic competition since a much larger dose of antigen was used to bring about high-dose tolerance. Other indirect evidence against RE blockade as the mechanism of competition is the fact that RE blockade requires large doses of antigen, and the effects of the blockade are comparatively short-lived (*208*). It has also been documented using isotope-labeled antigens (*107*) and fluorescent labeling techniques (*203*) that antigen localization is the same whether or not the response to the antigen is normal or suppressed by antigenic competition. Although these results argue against antigenic competition involving the phagocytic function of macrophages, it is still possible that these cells are the source of inhibitory factors postulated to cause antigenic competition (*228*).

V. The Lymphocyte as the Target of Antigenic Competition

To postulate that antigenic competition acts at the level of the antibody-forming cell precursor or the antigen-reactive cell implies either a lack of specificity of these cells or an undetectable cross-reactivity between the antigens at the level of either the carrier or the haptenic determinant. Such cross-reactivity would render acceptable theories involving partial tolerance, inhibition by specific antibody, or competition

for cells believed to have specific receptors, as well as reconciling antigenic competition with the clonal selection theory. However, the initial assumption of cross-reactivity between competing antigens has been rendered untenable by the experiments of Ben-Efraim and Liacopoulos (*27*) using the chemically defined antigens 252, poly (Tyr, Glu, Lys) and DNP–poly (Lys), or 28, poly-DL-Ala–poly (Tyr, Glu)--poly-Lys. These experiments demonstrated that antigenic competition could occur between antigens that were structurally unrelated and for which no possibility of cross-reactivity could exist at the B-cell level. Furthermore, it has been shown by Brody and Siskind (*35*) and ourselves (*72*) that the affinity of antibody produced under conditions of antigenic competition is similar to that of control sera and is not increased or decreased, as would be predicted from a model based upon specific feedback inhibition (*226*) or partial tolerance (*241*).

In contrast, Harel *et al.* (*109*) demonstrated a *decrease* in the affinity of antibody suppressed by competition and observed (*110*) a similarity between the characteristics of this antibody and that produced after the injection of a large dose of specific antigen. The antigens used, DNP–BSA and BGG, do not cross-react, and the authors interpreted their data as indicating that antigenic competition affected the antibody-forming cell. The majority of investigators in the field of antigenic competition have ruled out serological cross-reactivity between the antigens that they have used, and such cross-reactivity as a *necessary* facet of antigenic competition is unlikely.

Also against antigenic competition being due to cross-reacting haptenic determinants are the experiments of Brody *et al.* (*37*), Gershon and Kondo (*91*), and Taussig and Lachman (*238*). In each of these studies, antibody specific for the suppressing antigen was administered in a system designed to show antigenic competition. In every case the effect of the antibody was to reduce the suppressive effects of antigenic competition—the reverse of what would have been observed if competition was due to feedback inhibition by antibody specific for the suppressing antigen.

An alternative explanation is that the carrier-specific lymphocytes recognize cross-reacting determinants on the carrier molecules not detected at the level of antibody formation (*75*, *124*). This has been investigated by Brody and Siskind (*35*) and by Fauci and Johnson (*76*) using haptens conjugated to the same or different carrier molecules. In their experiments, antigenic competition was observed in either case, in contrast to the results of Schecter (*211*) in which antigenic competition occurred only if the haptens were conjugated to the same carrier. It is difficult to account for this discrepancy. All of the experiments were

done using rabbits, and, in each case, competing antigens were injected simultaneously in complete Freund's adjuvant. The only obvious difference was the antigens used. Competition could be shown between DNP–egg albumin and arsanilate–azo rabbit γ-globulin (*35*) and between arsanilate–KLH and TNBS–BGG (*76*) but not between poly-DL-phenylalanyl rabbit serum albumin and poly-DL-alanyl ribonuclease (RNase) or between poly-DL-phenylalanyl RNase and poly-DL-alanyl human serum albumin (*211*). It is possible that the use of a different dose combination in the latter case (*211*) would have allowed the demonstration of antigenic competition, since it is well established that the phenomenon is strongly dependent on the dosage of the antigens used (see Section I).

Lymphocytes that are experimentally observed as being B antibody-forming cells or T antigen-sensitive cells are believed to be derived from the same pluripotential stem cells (*83, 95, 175*). These cells migrate to the bone marrow and the thymus from the fetal liver and yolk sac (*84, 175, 187*). After birth, certain B cells pass through the thymus and become altered (*234*) in such a way as to become T cells (*83, 95*). By adoptively transferring graded numbers of B cells in the presence of an excess of thymocytes, Miller and Cudkowicz (*157*) found that the precursor units of the antichicken red blood cells (CRBC) and anti-SRBC responses acted independently of each other, and the anti-CRBC responder unit was unaffected by priming with SRBC. A similar result was obtained *in vitro* by Osoba (*185*) using CRBC and SRBC, and by Dutton and Mishell (*66*) using burro red blood cells and SRBC.

The specificity of the T cell has been documented at the level of T–B interaction (*51, 167, 217, 239*) and in tolerance induction (*49, 239*). In both cases, the altered cellular reactivity induced by the antigen was found to be specific for that antigen. Furthermore, by using a technique of "antigen suicide" with highly radioactively labeled antigen (*4*), Basten *et al.* (*26*) showed that T and B cells could be specifically killed without affecting the response to unrelated antigens.

In contrast, recent experiments, both *in vivo* and *in vitro,* have suggested that cross-reactivity at the T-cell level may not reflect that observed at the B-cell level (*75, 124*). Falkoff and Kettman (*75*) observed that priming with SRBC gave substantial enhancement of the response to trinitrophenol (TNP) evoked by a subsequent injection of TNP–burro red blood cells, even though the burro cells and SRBC show little cross-reactivity at the plaque-forming cell (PFC) level. Parallel results were obtained by Hoffmann and Kappler (*124*) who restimulated the primed cells *in vitro.* It should be kept in mind that these data do not indicate a lack of specificity on the part of T cells but rather that T-cell specificity may not reflect that demonstrated by B cells. It is also possi-

ble that the results are due to specifically induced mediators with nonspecific effects (*64*).

The results of experiments using certain drugs have also been used as evidence in favor of a cellular mechanism of antigenic competition. Dukor and Dietrich (*62*) have reported the effects of cyclophosphamide and cortisone on antigenic competition between SRBC and HRBC, given 2 days apart, in mice. If 100 mg./kg. of cyclophosphamide was administered 1 day after the first antigen (SRBC), the effects of antigenic competition were abolished and a normal antibody response was obtained to HRBC. In contrast, if 100 or 500 mg./kg. of cortisone was given 1 day after SRBC, the suppression of the response to HRBC was more marked. The authors suggested that the effect of cyclophosphamide was to interfere with proliferation in response to the first antigen, and, from a number of possibilities listed, it was postulated that antigenic competition involves the preemption of multipotential B precursors by antigenically stimulated T cells. The interpretation of the potentiation of competition by cortisone is less clear, however, and it was postulated that the drug acts at the same site as antigenic competition either on the B cell or at the level of antigen clearance.

The effect of actinomycin D on antigenic competition has also been studied by a number of authors (*16*, *68*, *256*). Wust and Hanna (*256*) observed that if 12 μg. of actinomycin D was administered to mice 7 hours before the first antigen (SRBC), antigenic competition of the response to the second antigen (rat red blood cells) was abolished. It was speculated by the authors that one side effect of actinomycin D was to interfere with multipotential cells becoming committed to the first antigen and, hence, leaving an adequate residual of immunocompetent cells capable of responding to the second antigen. This interpretation was subsequently challenged by Ambrose (*16*) whose experimental results led him to postulate that actinomycin D prevents the synthesis of an inhibitory factor responsible for antigenic competition (see Section IX).

Because almost every mechanism postulated to account for antigenic competition is dependent upon cellular proliferation in response to the first antigen, experiments invoking cytotoxic drugs are difficult to interpret and still leave a large number of possibilities open to consideration.

In conclusion, it appears that the immediate precursors of the T and the B cell are restricted in their specificity for antigens at this stage and are unlikely to be preempted by antigens that do not bear the appropriate determinants. If such a preemption does occur, it must be at a very early stage in the differentiation of the precursor, because, at some point, the precursor cell must be pluripotent. This theory is supported

by the work of Perkins and Makinodan (*190*), using the adoptive transfer system. On the basis of these experiments, the authors concluded that there exists two sequential compartments of precursor cells, the first made up of multipotent cells capable of differentiating along several paths, depending upon the stimulus, whereas the cells of the second compartment are differentiated to the extent of being insensitive to erythropoietin or unrelated antigens. This concept has recently been applied to antigenic competition by Liacopoulos *et al.* (*142*). By using mice immunized to both SRBC and pigeon red blood cells, these authors found a significant proportion (up to 10%) of spleen cells that formed double rosettes. These rosettes appeared on day 3, peaked on day 5, and began to disappear on day 10. If SRBC were injected 1–3 days before the pigeon red blood cells, then the number of double rosettes was fewer and their appearance of briefer duration than with simultaneous antigen injection. After showing that the antigens do not cross-react, these observations were taken to mean that the early antibody-forming precursor cell (AFPC) is unrestricted with respect to specificity until differentiation in the presence of antigen leads to the more commonly observed restricted specificity of the antibody-forming cell (*185*, *188*, *216*). Theoretically, the pluripotential cell could be diverted toward a certain specificity at the expense of the potential response to another unrelated antigen injected at a later date.

If this theory is the true explanation for antigenic competition, it is necessary to postulate the same effect at the level of the T cell, since it is well established that cell-mediated immunity can also be influenced by immune responses to unrelated antigens. Against the theory of AFPC preemption are the experiments of Möller and Sjöberg (see Section IX) (*173*) in which transferred normal or antigenically stimulated spleen cells could be inhibited by immunization of the host with an unrelated antigen prior to irradiation. Also opposing the concept that antigenic competition depends on AFPC multipotentiality are the experiments of Monier and Salussola (*174*) and of Gershon and Kondo (*90*) who demonstrated that there was no antigenic competition in the absence of T cells. Because there is a small response on the part of B cells in the absence of T cells, this response should also have been affected by antigenic competition if the B-cell precursor were indeed responsible for this phenomenon.

VI. Antigenic Competition for Essential Nutrients

Since the ARU can be defined as the minimum complex of cells and factors that interact to produce a detectable immune response, a defect in the ARU may actually only reflect a depletion of nutrients or factors necessary for the optimum response of the cells reacting to the second

antigen. It is unlikely, however, that antigen-induced suppression (AIS) is caused by a nutritional deficiency because the use of *in vitro* systems still allows the demonstration of suppression (*55*, *196*, *227*, *229*), and the medium from "suppressed" cultures is still capable of supporting normal immune responses upon transfer to other cultures (*195*, *227*, *229*).

VII. Thymus-Bone Marrow Cell Interference

The fifth theory of antigenic competition which should be reviewed in the light of current data is the interaction interference hypothesis of Kerbel and Eidinger (*135*). According to their theory, the proliferation and nonspecific inflammatory cell invasion occurring in the spleen following antigenic stimulus results in interference with the T and B cell interactions that are necessary for the immune response to a second antigen. Thus, any data demonstrating a decrease in the frequency of ARU specific for the second antigen (*14*, *196*), indirectly supports this theory by confirming the fact that marked cellular proliferation occurred in response to the first antigen. Other support in favor of the theory lies in the fact that in almost all the examples of antigenic competition studied *in vivo*, including models involving tolerance to the first antigen, suppression correlated well with the size of the spleen at the time that the second antigen was injected (*135*). One exception is the use of endotoxin as the first antigen, which results in marked cellular proliferation without competition. Since endotoxin is a potent adjuvant (*189*), it is difficult to evaluate this observation.

Against the theory is the observation that antigenic competition *in vitro* did not always occur 4–5 days following KLH immunization (*196*), although there was a greater number of nucleated cells per spleen and, presumably, a greater dilution of the ARU's specific for the second antigen by cell proliferation in response to the first. The crucial experiments to test this theory involve the ability of different antigens to suppress responses that do not involve T–B interaction, e.g., the response to PVP (*19*). Experiments performed by Möller (referred to in *172*) and Sjöberg and Britton (*229*) indicate that suppression of the response to *Escherichia coli* can be easily accomplished. In this laboratory we have found that suppression by antigenic competition of the response to T-independent antigens is only irregularly produced (e.g., PVP) (*68*, *72*). Thus, the validity of this hypothesis awaits experimental confirmation and a greater knowledge of the normal immune response.

VIII. Involvement of Helper Molecules in Antigenic Competition

It has recently been postulated that antigenic competition involves helper molecules that mediate T–B cell interaction (*63*, *78*, *238*, *240*). In this model, competition occurs between specific thymus-cell derived

helper molecules for binding sites on the macrophage (*78, 238, 240*), or it is due to steric hindrance between cells attempting to gain access to these molecules and the antigen that is bound to them either on the macrophage (*240*) or on the thymus cell (*63*). The theory is indirectly supported by data showing abrogation of antigenic competition by the induction of tolerance to the first antigen (*212, 238, 250*). A theory based on steric hindrance is compatible with data showing that competition can be demonstrated when the haptens are on the same carrier (*211*), but both aspects of this theory are difficult to reconcile with the fact that thymus-independent antigens can be shown to cause antigenic competition (*68, 72, 143*). Also data against this theory are to be found in the large number of papers describing antigenic competition in spite of tolerance to the first antigen (*68, 146, 211*), or unresponsiveness to the first antigen (*2, 27, 28*). In addition, in the case of macrophage mediation of antigenic competition (*78, 238, 240*), the experiments described previously (Section IV) would appear to show that the macrophage is unlikely to be involved in this phenomenon (*5, 72, 76, 107, 143, 203*). This mechanism of antigenic competition could still be supported if it is first shown that tolerance is associated with the production of helper molecules specific for the tolerogen and that helper molecules are produced in sufficient amounts to affect the lymph node cells draining the contralateral footpad.

IX. Role of an Inhibitory Factor

The existence of a functional deficit at the level of the ARU or inhibition of the proliferative capacity of the reacting cells has been supported vigorously in a number of recent publications citing evidence for an active inhibitory factor or cell as the cause of antigenic competition (*16, 77, 90, 91, 172, 173, 197, 227, 229, 246, 248*). This concept was first advanced by Radovich and Talmage (*197*) who demonstrated the suppression by HRBC of the response to SRBC of 10×10^6 normal spleen cells transferred to irradiated syngeneic mice. Because the degree of suppression was increased when 50×10^6 spleen cells were transferred, the experiments were taken as evidence that a humoral inhibitory factor was elaborated by the cells in response to the first antigen, although the authors have subsequently stated that a cellular interpretation is possible (*236*). The experiments of Möller and Sjöberg (*173*), confirmed by Waterston (*248*) and by Gershon and Kondo (*90*), have also lent convincing support to some form of inhibitory factor as mediating antigenic competition. In these experiments, using lethally irradiated mice, the response of transformed normal spleen cells to one antigen could be significantly suppressed by pretreating the recipient mice with another

unrelated antigen prior to irradiation. Furthermore, as discussed previously, it has been demonstrated that the injection of competing antigens need not be into the same site (*5*, *72*, *76*, *186*, *237*), also implying the presence of a circulating inhibitory factor.

Assuming that such a factor does exist, its nature and origin have defied experimental analysis. Möller (*172*) and Sjöberg and Britton (*229*) believe that it is the product of stimulated T cells and cite experiments showing that the T-independent antigen, *E. coli*, is poor at suppressing the immune response to HRBC. Gershon and Kondo (*90*) have also obtained evidence that the thymus cell is fundamental to antigenic competition. Although thymectomized, irradiated, and spleen cell reconstituted mice do not normally show antigenic competition, they do so if thymocytes are provided, and the resultant suppression is increased if a greater number of thymocytes is inoculated. Similarly, neonatally thymectomized mice fail to demonstrate antigenic competition between HRBC and SRBC (*174*).

Other evidence for the inhibitory effects of T cells has recently been advanced by Haskill and Axelrad (*115*). Following low-dose immunization with SRBC, a spleen cell fraction could be isolated by velocity sedimentation which was strongly inhibitory of the response of the other fractions to SRBC. This fraction was sensitive to treatment with anti-θ antiserum, and presumably contained inhibitory T cells. Inhibition by unstimulated thymus cells has also been shown in this laboratory (*71*) using both irradiated recipients and *in vitro* techniques. In these experiments it was found that only $0.5–1.0 \times 10^6$ cortisone-resistant thymus cells could inhibit the response of 12×10^6 normal spleen cells *in vitro*, that the inhibition was present on each day of culture from day 3 to day 5, and that the degree of suppression obtained was reduced by irradiation of the thymus cells (*194*).

The importance of T-cell inhibition has also recently been stressed by Okumura and Tada (*183*). It had previously been shown by these authors that the production of homocytotropic antibodies in the rat against dinitrophenylated extract of *Ascaris suum* was greatly enhanced and sustained if the animals were irradiated with 400 R at the time of immunization (*233*). In their most recent experiments (*183*), these authors report that thymocytes and spleen cells from rats hyperimmunized with the hapten–carrier conjugate, or with just the carrier, inhibited anti-DNP antibody production within 2 days of inoculation into the irradiated and immunized rats.

Thus T cells appear to demonstrate both inhibitory and helper functions, although it has not been established whether one or two subpopulations of T cells are involved (*15*). At the present time, it is also not

known whether the inhibitory effects are mediated via soluble factors or by cell-to-cell contact. For the reasons outlined in the foregoing, a number of investigators favor the interpretation that the T cells elaborate an inhibitory factor and that this factor is responsible for antigenic competition.

X. Nature of the Inhibitory Factor

A number of possibilities exist as to the nature of the postulated inhibitory factor. In 1963, Mowbray (*179*) reported that an α_2-globulin fraction from either human, ox, or rabbit serum could inhibit antibody formation against a number of different antigens. This fraction could also suppress allograft rejection (*137*), phytohemagglutinin (PHA) responsiveness, and the *in vitro* proliferative response of sensitized cells to antigen (*54*). An apparently different factor has been described by Thomson and Fishel (*242*) who extracted an inhibitory substance from normal mouse tissues such as lung, spleen, and thymus. Because normal mouse serum produced no inhibition, the authors concluded that the factor was different from the α-globulin of Mowbray.

By using a system somewhat more similar to antigenic competition, Ambrose (*16*) found that an antibody-inhibitory material (AIM) was produced in cultures of lymph node fragments from immunized rabbits. This material inhibited the immune response in culture to other unrelated antigens and was apparently not specific antibody. Recently, the possibility of a mouse serum inhibitory factor has been revived by Veit and Michael (*246*) and by Bullock and Möller (*40*). Veit and Michael (*246*) have described an inhibitory effect of normal mouse serum which could be detected by incubating the spleen cells for 4 hours at 37°C. in 18% mouse serum. This effect was increased with immunization and was not diminished by absorption of the serum with the test antigen. The degree of suppression could be reduced by the addition of thymus cells, and thus these cells are apparently the site of action of the factor. The factor described by Bullock and Möller (*40*) inhibited the spontaneous production of anti-4-hydroxy-3,5-dinitrophenylacetyl PFC in mouse spleen cell cultures when 10% normal mouse serum was added to the cultures.

The target of these factors may well be cellular proliferation in general. The proliferation of normal spleen cells, without antigen, in SRBC-primed, lethally irradiated hosts was only 50% of that observed in mice that had not received a prior injection of SRBC (*172*). In a similar model, Hanna (*106*) observed an inhibition of the colony-forming ability of normal mouse bone marrow and embryonic liver cells up to 3 weeks

after the last of three weekly injections of SRBC given prior to irradiation.

Furthermore, the effects of antigenic competition on delayed hypersensitivity (*27*, *67*, *148*, *212*) and the graft-versus-host reaction (*144*, *147*, *158*) suggest that the target of the postulated inhibitory factor is at least the T cell, and this is supported by reconstitution experiments of antigenic competition *in vitro* (*195*). The observation of Veit and Michael (*246*) that thymus cells partially overcome the suppressive effects of their serum inhibitory factor also suggests that this cell may be the target of antigenic competition.

Recent evidence also suggests that the site of action of antigenic competition is at a level preceding antibody production. Using lethally irradiated mice, Mitchell and Humphrey (*165*) studied the response to DNP–ovalbumin of spleen and lymph node cells transferred from DNP–squid hemocyanin-primed and ovalbumin-primed mice. This response was inhibited if the mice were also injected with DNP–lysine conjugated to pneumococcal polysaccharide Type III (DNP–lysine–SIII), and the suppressing antigen could be given up to 3 days after transfer of the cells. In a further study, Mitchell *et al.* (*166*) performed isoelectric focusing on the anti-DNP antibody produced by mice in which the response had been only partially suppressed by DNP–lysine–SIII. The strong intensity of the individual bands of antibody was taken as indicating that the progeny of B cells that escape suppression produce unaltered amounts of antibody. Whether or not antigenic competition directly affects the B cell has not been established. Thus far, suppression by antigenic competition of the response to T-independent antigens has been difficult to achieve, although not impossible (*72*), and it has also been reported by Möller (*172*), and by Sjöberg and Britton (*229*).

In experiments performed in this laboratory (*196*), a single preimmunization with KLH resulted in suppression of the *in vitro* immune response to HRBC. In this model, true competition between the antigens did not occur, although limiting dilution assays indicated a decrease in the frequency of ARU specific for HRBC. On mixing normal and KLH-primed spleen cells *in vitro*, a synergistic effect was observed, in contrast to suppression of the normal cells by the primed cells. This effect could not be duplicated by adding B cells to the suppressed cultures (*195*).

These limiting dilution and cell mixing experiments are not incompatible with the concept of an inhibitory factor mediating antigenic competition but do provide strong evidence that the effect of such a factor, if it exists at all, is to cause an apparent cellular defect. These experiments also indicate that this factor is not produced in effective

amounts *in vitro*. In contrast, by using spleen cells that had been subjected to a graft-versus-host reaction, Sjöberg (*227*) observed that the *in vitro* response to SRBC was markedly suppressed in comparison to the normal spleen cell controls. Furthermore, by mixing the suppressed spleen cells with normal spleen cells, the response of the mixture was less than expected. Similar results have also been reported by Sjöberg and Britton (*229*), using cultures of spleen cells from mice hyperimmunized with HRBC, although neither Sjöberg (*227*) nor Sjöberg and Britton (229) could transfer the suppression using culture fluid from suppressed cultures. The difference between these results and our own may simply reflect the fact that the graft-versus-host response and hyperimmunization with HRBC are more potent stimuli than primary immunization with dissociated KLH.

The failure to transfer antigenic competition with medium or serum has made isolation of the putative inhibitory factor impossible. Other inhibitory substances, such as the AIM of Ambrose (*16*), Mowbray's α-globulin factor (*179*), and the inhibitor of Veit and Michael (*246*) may be implicated as the cause of antigenic competition, but the evidence to support this concept is only indirect. Braun and Ishizuka (*32*) maintain that the inhibitory substances responsible for AIS are actually nonspecific stimulators, such as the homopolymeric nucleotide duplex, polyadenylate-uridylate[poly(Au)] or cyclic adenosine 5′-monophosphate (AMP), which, in excessive amounts, can be shown to inhibit immune responses *in vivo* and *in vitro*. Further studies on this system (*151*) have shown that the suppressive effects of antigenic competition can be overcome by administering poly(AU) or poly(AU) plus theophylline at the time of immunization with the second antigen.

Although interesting from a theoretical point of view, these many demonstrations that a substance or cell can suppress an immune response or overcome antigenic competition are only indirect evidence as to the mechanism of the phenomenon.

XI. Antigenic Competition and Heterologous Enhancement

A number of recent reports have described enhancement of immune responses under conditions which might otherwise be expected to demonstrate antigenic competition (*7*, *9*, *34*, *153*, *195*, *196*, *206*, *207*, *244*, *248*, *255*). This phenomenon has been termed antigenic promotion by Wu and Cinader (*255*) and specific heterologous enhancement by Rubin and Coons (*206*). In the latter studies, mice were primed with tetanus toxoid (TT), ovalbumin (OA), or burro erythrocytes and, 30–60 days later, the spleen cells were cultured with SRBC, with or without the priming anti-

gen or another unrelated antigen. In those cultures containing the primed spleen cells, the presence of the specific priming antigen resulted in an increased immune response to SRBC. These authors (*207*) have recently demonstrated enhancement of the response of normal spleen cells to SRBC by graded doses of syngeneic thymocytes primed against tetanus toxoid. This enhancement only occurred if the priming antigen were also present in the culture, and was not observed using normal syngeneic thymus cells. The effect of the addition of 1 ng. of TT on day 2 on cultures of 10×10^6 primed thymocytes and 20×10^6 normal spleen cells was a 56% enhancement of the response to SRBC. However, the effect of the primed thymocytes on spleen cells cultured with TT and SRBC was only about 12% enhancement of the response to SRBC as compared to cultures without added thymocytes. The magnitude of this enhancement depends on the way in which the data is examined, and the thymic dependency of the enhancement, although an attractive possibility, remains to be unequivocally proven.

Other experiments involving heterologous enhancement have been done by Hartmann (*113*) and Vann and Kettman (*245*) using "educated" T cells and B cells. It was found by both groups that B cells mixed with educated T cells would respond to a heterologous antigen in culture, provided that the antigen used for education was also present in the culture. These data would also imply that the T cell is responsible for specific heterologous enhancement.

Other information which may be pertinent to this discussion of heterologous enhancement comes from the experiments of Katz *et al.* (*131*) demonstrating the allogeneic effect. In these studies, immunocompetent lymphoid cells from allogeneic donor guinea pigs stimulate the synthesis of anti-DNP and anti-OA antibodies by recipients previously primed with DNP-OA. The reaction does not occur if F_1 hybrid cells are injected into primed parental hosts. The authors postulate that the stimulus caused by the attack of the allogeneic cells on the host spleen cells renders the B cells among them more sensitive to antigenic stimulation, i.e., the helper effect of T cells can be bypassed by a nonspecific mechanism.

In another system, using rabbits as the experimental animal, Wu and Cinader (*255*) have observed that the antihapten response to hapten–human serum albumin conjugates was enhanced when the animals were previously injected with one of a number of unrelated macromolecules including KLH. This enhancing effect was not observed if the rabbits were made tolerant to the first antigen or following a secondary response to this antigen.

In the experimental model used by us to study antigenic competition

in vitro (*196*), preimmunization of mice with KLH *in vivo* regularly resulted in suppression of the *in vitro* response of spleen cells from these animals to HRBC. If microgram amounts of KLH were added to these cultures, marked enhancement of the response to HRBC was observed (*195*). Other antigens such as TT or chicken γ-globulin had no effect on these cells when added to the cultures, and the addition of KLH had no effect on the *in vitro* response of normal spleen cells to HRBC. The observations are analogous to those of Rubin and Coons (*206*), except that heterologous enhancement was observed in a population of cells which demonstrated antigenic competition if the priming antigen was not added *in vitro*. In this system, it was also more difficult to obtain heterologous enhancement following a secondary immunization with KLH, but this could be partly overcome by increasing the dose of KLH used *in vitro*.

In some respects the ability to elicit enhancement parallels the proliferative capacity of T cells described by Davies (*58*). In these experiments, it was observed that the proliferative response of T cells to SRBC was greatest after primary and secondary stimulation, and less after subsequent immunization, depending on the route, timing, and dosage of antigen. Whether or not specific heterologous enhancement is mediated by proliferating T cells awaits further experimental confirmation.

In some respects, the paradox of antigenic competition *in vitro*, described by Waterston (*248*), may be partly explained by these results. In this type of experiment, there is an enhancement of the response to SRBC in cultures of spleen cells from mice primed with pig red blood cells (*248*) or HRBC (*196*), antigens that suppress the response to SRBC *in vivo*. It is possible that determinants shared by the two antigens (*197*) may be sufficient to restimulate the cells primed to the first antigen, with the concomitant enhancement of the response to the determinants that are not shared, i.e., the cross-reacting determinants are acting in the same way that KLH does in our system and represent restimulation *in vitro* with the priming antigen.

The importance of studying more than one parameter of immune responsiveness in studies of antigenic competition and enhancement has recently been emphasized by Merchant and Liacopoulos (*153*). Following repeated immunization with hemocyanin, the hemolysin response of mice to SRBC was markedly reduced whereas there was an increased number of anti-SRBC PFC in the spleen. This observation was attributed to the recruitment by hemocyanin of cells reactive to antigens only peripherally related to SRBC, with a resultant immune response of very low avidity antibody. These low efficiency antibodies would react poorly in a hemolysin titration, whereas the PFC could be still detected.

It has been suggested by a number of investigators (*64*, *92*, *206*) that the enhancement phenomenon may result from the release, by the primed cells, of a nonspecific stimulatory factor, similar to those described in cell-mediated immunity (*139*). Alternatively, the effect may be due to an increase in the number of helper cells (*169*), such as carrier-specific T cells (*170*) or macrophages coated with cytophilic antibody (*31*), with receptors of broad specificity since many of the antigen pairs used do not cross-react detectably. From our work, described in the foregoing (*195*), the evidence is against the latter hypothesis for the following reasons. First, antigenic enhancement and antigen-induced suppression can be demonstrated in the same KLH-primed spleen cells, depending on the presence or absence of KLH *in vitro*. It is difficult to reconcile the observation of suppression of the response to HRBC with the hypothesis that there is an increase in the number of cells capable of helping this response unless, under certain conditions, an excess of helper T cells mediates nonspecific inhibition. Second, if the helper cells that have arisen as a result of priming with KLH are also capable of reacting to the HRBC, the receptors on these cells should be blocked by the addition of more molecules of KLH *in vitro*. In a system such as ours, it would be predicted that competition between the added KLH and the HRBC for helper cell receptors would result in a decreased response to the HRBC. In actual fact, the opposite result is obtained, suggesting that some alternative explanation is required.

Although there is no direct evidence to support the hypothesis, the possibility that a chemical mediator is released by the primed cells upon restimulation is an attractive one at the present time. To some extent, the data support the model proposed by Ekpaha-Mensah and Kennedy (*74*), Dutton *et al.* (*64*), and Feldmann and Basten (*79*, *80*) for the mechanism of thymus–bone marrow cell interaction. As described in Section II (model 3), there is not an absolute requirement for linked recognition between the T and B cells, although linked recognition is possible and would be more efficient because the cells are closer together.

We have to the present dealt with mechanisms underlying antigenic competition when assayed under selected circumstances in the experimental animal. It would seem relevant to pose the question as to whether the phenomenon is manifested also in the human and, finally, to consider whether antigenic competition can exert effects that may be detrimental to the host.

XII. Antigenic Competition in Humans

A remarkable paucity of data exists as to the role of antigenic competition in humans. In 1954, Chen and colleagues undertook a study of

the immune response to diphtheria–pertussis–tetanus (DPT) combined vaccine in Taiwanese children (*48*). Children were given a combined DPT vaccine in a standard regime. The immune responses were evaluated in children who had no preformed antibodies to any of the antigens as compared to those who had evidence of prior exposure to diphtheria toxin. The results of the study indicated that children between the ages of 9 months and 2 years with latent diphtheria immunity exhibited deficient responses to the tetanus and pertussis components of the vaccine, in comparison to the group who had no preformed antibodies to diphtheria. These studies demonstrated further that diphtheria antitoxin levels of 0.01 unit per milliliter represented the level of latent immunity beyond which significant interference of immune responsiveness occurred. This observation has not received the attention it has deserved at a clinical level, perhaps because the level of latent immunity to diphtheria in the general North American population is significantly lower than that which might be expected to occur in Southeast Asia.

With the recent advent of numerous additional combination vaccines, it would appear to be essential to reevaluate immune responsiveness in individuals immunized with such mixtures. In a recent study carried out in Nigeria as reported by Foster (*85*), Ruben and colleagues have investigated the immune response following combined immunization with DPT given in association with smallpox, measles, and yellow fever immunization. Responses to diphtheria, pertussis, tetanus, yellow fever, and smallpox antigens were designated as satisfactory; however, measles immunity was significantly reduced in the groups receiving only smallpox, measles, and yellow fever antigens. It is possible that in the absense of the adjuvant effect of the pertussis component of the vaccine, the response to measles vaccine can be suppressed by smallpox and yellow fever antigens.

These results suggest that some caution is needed in evaluating immune responses to multiple vaccine. It is probably insufficient to simply record the immune response to multiple vaccines in selected groups within limited geographic areas, because it is clear that the immune response to vaccines is in no small measure related to the immunological memory of the population.

These limited observations indicate clearly that the phenomenon of antigenic competition to vaccine antigens is manifested in the human. That these observations are not exclusively observed in the human is evident from the work of Barr and Llewellyn-Jones (*24*, *25*). These investigators studied the immune responses to combined vaccines in the guinea pig and found that this animal species also exhibits antigenic competition when immunized with a multiple vaccine following prior exposure to one of the antigens of the mixture.

XIII. Antigenic Competition in Viral Oncogenesis and Infection

Nowhere is the multiplicity of mechanisms underlying antigenic competition more clearly evident than under circumstances of immunosuppression associated with viral oncogenesis and viral, bacterial, or parasitic infections. Many investigators have evaluated the characteristics and mechanisms underlying immunodepression associated with viral oncogenesis. Immunodepression is particularly evident in association with Friend (*44, 50, 120, 182, 209, 249*), Rauscher (*50, 117, 222*), Maloney (*50, 56, 57*), lymphocytic choriomeningitis (*164*), Gross (*60*), and other oncogenic as well as nononcogenic viruses (*126, 155, 184, 243*). Immunodepression also has been observed during the course of infection with *Plasmodium* (*101–103, 210*) and *Toxoplasma gondii* (*232*), and finally, in animals bearing tumors of variable etiology (*10, 29, 30, 159*).

Antigenic competition has been considered as a possible mechanism underlying immunodepression under the above circumstances (*44, 243*), although it appears to be held in little favor (*59, 243*). The main reasons for rejection of antigenic competition as a possible mechanism are threefold: first that an immune response to many of the viral agents has not been demonstrated to any significant extent (*59*); second that immune responses to viral antigens in the absence of viral replication is often not associated with immunosuppression (*249*); and third that the timing is not generally conceded to be optimal for antigenic competition in the classic sense in that maximum immunosuppression is frequently obtained at long intervals after induction of the viral infection (*117, 209*) although much shorter time intervals for suppression have been noted (*182*). The problems in interpretation may be in part related to the unequal latent periods for oncogenesis with the various viral agents. However, certain observations which have been made during the course of investigation of oncogenic events lead us to believe that antigenic competition is, indeed, important as an underlying mechanism for immunosuppression.

It has been demonstrated frequently that the spleen of the mouse is the most important organ for primary immune responses to particulate antigens (*205, 254*). Numerous groups of investigators have also commented on the splenomegaly at times to extreme magnitude, which occurs as a consequence of viral oncogenesis and infection with murine *Plasmodium* (*102, 120, 243*). Several groups have commented on the relationship of magnitude of splenomegaly with degree of immunosuppression (*102, 120*). The splenomegaly and immunodepression associated with viral oncogenesis is reminiscent of experiments by Hunter and colleagues who observed the suppression of the graft-versus-host reactivity and antibody formation in mice challenged with PHA (*127*). In their experiments, the degree of suppression of graft-versus-host response

could also be correlated with the degree of splenomegaly. Based on the observation that PHA is antigenic, these workers concluded that the immunosuppression was due to antigenic competition.

These results are similar to those of other investigators, who have also attempted to define further the nature of the cellular deficit. Biano and colleagues demonstrated that spleens of 4-week-old AKR mice bearing the Ridgeway osteogenic sarcoma were defective in generating primary responses to sheep erythrocytes *in vitro* (*29*). The defect was diminished by the addition of nonadherent lymphocyte populations. They concluded that the defect was analogous to that in neonatally thymectomized animals in which nonadherent lymphoid populations of spleen cells also reconstituted the deficit of responsiveness *in vitro*. Adler and colleagues showed that animals bearing methylcholanthrene tumors exhibited a defect in PHA responsiveness, also pointing to a deficit in the T-cell population (*10*). Finally, in similar reconstitution experiments in lethally irradiated mice, Ceglowski and Friedman demonstrated that the defect was also present in the B-cell population (*45*).

Several groups of investigators have studied the converse of the above experiments, namely, the effect of nonspecific immunization on the induction of oncogenesis (*20, 154, 213*). Metcalf (*154*) demonstrated that the C3H mice developed an increased incidence of reticular tumors following repeated immunization with bovine serum albumin. Schwartz and colleagues (*20, 213*) have also demonstrated the development of reticulum cell sarcomas in mice chronically affected with graft-versus-host reactivity. It would appear that nonspecific immunization in most instances can enhance the development of oncogenic alteration suggesting perhaps an influence on the potentiality for immune surveillance. This would appear, however, not to be invariably the case, since Siegel and Morton (223) have shown that mice stimulated with horse erythrocytes over a prolonged period exhibited a retarded development of murine viral leukemogenesis with Rauscher leukemia virus. Perhaps this is due to an adjuvant effect on the RE system produced by prolonged immunization. The complexity of this problem is further emphasized by the work of Jennings and Oates (*129*) who implied that antigenic competition could in fact, be due to the enhanced RE cell activity following administration of the initial antigen. The greater number of more active macrophages following initial immunization or administration of materials, such as PHA and endotoxins, might provide large numbers of cells that nonspecifically take up the second antigen which is thereby quickly metabolized. In these instances, enhanced phagocytosis would appear to be a prerequisite for antigenic competition. Further work is indicated to evaluate macrophage function during various manifestations of

administration of antigens or during infections with viral agents, particularly when these agents are oncogenic.

It may be tentatively concluded that most of the studies of immunosuppression under the circumstances defined in the foregoing have in common the development of splenomegaly. In the work by Kerbel and Eidinger (*135*), a model system was proposed in which *one* of the mechanisms underlying antigenic competition was considered to be due to the development of splenomegaly arising as a consequence of immunization with an initial antigen. The increased number of cells in the spleen was postulated to interfere with the essential cell cooperative events needed for an immune response to a second unrelated antigen. (See section VII.) Mosier has drawn attention to the requirement for specific cell clusters in immune responses to thymus-dependent antigens *in vitro* (*177*). It is not too far-fetched to believe that similar cooperative events requiring similar cell clusters are needed for immune responsiveness *in vivo*. Indeed, the early work of Kennedy and colleagues (*133*) employing the focus assay and the investigations of Celada and Wigzell (*46*) have drawn attention to the focal nature of immune responsiveness in the spleen. The number of these foci is apparently proportional to the number of specific antigen-reactive T cells, whereas the size of an individual focus is dependent on the number of specific B cells with which they interact (*167*). It is not unreasonable to suppose that anything that interferes physically with cell migration or with cell–cell interaction may result in a decrease in the size of these foci and, hence, a decrease in the amount of antibody formed per focus.

Thus, it may be concluded that, under selected circumstances, splenomegaly, whether produced by virus, antigen, or mitogen, may cause an interference with cell cooperative events. It is essential to stress that the term "antigenic competition" restricts the phenomenon to circumstances in which splenomegaly occurs as a result of immune responsiveness. In a broader sense, however, RE cell proliferation, the inflammatory response, or any other material or agent that crowds out those cells essential for immunological reactivity, may result in the development of manifestations best referred to as nonspecific agent- or antigen-induced suppression. Thus, true antigenic competition, if indeed it exists, would be a subtype of a much broader group of entities which should be properly referred to as nonspecific agent- or antigen-induced suppression. Whether or not one believes that splenomegaly is an important factor in creating the immunodepression may depend on the outcome of the experiments to demonstrate whether close cell contact is needed for cell cooperation or whether cell cooperation can, in fact, take place at a distance (*64*, *79*, *80*).

XIV. Immunosuppression in Chronic Infectious and Tumor-Bearing States in Humans

We have to the present reviewed evidence upon which two different mechanisms underlying induced suppression are based, namely, evidence for an inhibitory factor and evidence for interference of cell cooperation essential for immune reactivity. We may now consider whether these or other mechanisms are operative under certain selective circumstances in humans.

It has been demonstrated frequently that patients with Hodgkin's disease (*11*, *12*, *38*, *47*, *119*, *125*, *132*, *180*), leprosy (*39*, *215*), Boeck's sarcoid (*47*, *87*, *121*), and others (*104*, *230*, *231*) are anergic when measured in terms of skin reactivity to certain immunogens which otherwise elicit delayed hypersensitivity. The anergy is expressed not only during induction but also during the effector phase of the response. These manifestations of immunosuppression can occur in the presence of normal or above normal humoral antibody formation. With regard to the underlying mechanisms, some evidence exists for a humoral inhibitory factor underlying the deficient response (*47*, *104*). It is also evident that the cells themselves are immunologically unreactive (*11*, *39*, *119*, *125*, *132*, *180*, *215*). However, it should be emphasized that the cellular unresponsiveness could be mediated by cellular inactivation via an inhibitory factor.

A third mechanism, one which has been given very little attention, may be the exhaustive utilization of mononuclear cells as effector cells in the immune response to the inciting agent. For example, Sheagren and colleagues have shown that patients with leprosy have deficient expression of delayed hypersensitivity, both of the cutaneous type as well as when measured by *in vitro* tests of blastogenesis (*215*). The susceptibility to murine *Plasmodium* and to murine leukemogenesis is enhanced in the presence of a previous exposure to an infectious agent (*102*). A possible interpretation of these and other similar situations may be the defect in numbers or function of mononuclear cells occurring as a consequence of the intense response to the infectious agent, thereby creating a deficiency in delayed response to an unrelated immunogen.

Evidence that a deficiency in a population in effector cells in cutaneous responses of delayed hypersensitivity could occur was sought employing the following animal experimental model (*194*). Mice were lethally irradiated and reconstituted with a syngeneic mixture of thymus and bone marrow cells sufficient to reconstitute animals for delayed responsiveness to methylated human serum albumin. Nine days after reconstitution and sensitization, the animals were challenged either with a single dose of

methylated human serum albumin or with two separate doses. The degree of response in the doubly challenged group was significantly less than in the singly challenged group. This deficiency could be overcome in part by greatly increasing the numbers of bone marrow cells in the reconstitution population, a result interpreted on the basis of the greater numbers of mononuclear cells emanating from bone marrow precursors (*149*) acting as effector cells for the delayed response (*67*). Because the effector stage of delayed hypersensitivity depends on the recruitment of nonspecific cells (*67*, *149*), inhibition of skin reactivity may thus occur in response to both the initial suppressing antigen and to unrelated antigens by the same mechanism.

XV. Hypothesis

Many data presented in this review are compatible with the following somewhat speculative model of antigenic competition and heterologous enhancement. Variations of this model have been suggested by a number of other authors.

Antigenic competition and heterologous enhancement represent nonspecific manifestations of normal immunoregulatory phenomena. In response to stimulation by a thymus-dependent antigen, the triggering of T cells results in the elaboration of a diffusable mediator capable of stimulating B cells as part of the normal immune response. At a later stage in the development of these T cells, or, alternatively, with the later development of another population of T cells, a substance is produced that is capable of inhibiting precursor cell differentiation and proliferation, designed to prevent unlimited progression of the immune response. Under most experimental conditions the effect of this regulation is not observed because of concomitant antibody formation and *specific* feedback inhibition. This T-cell regulation is nonspecific, however, and can effect the antigen-reactive cells specific for other antigens (antigenic competition).

In parallel with these events, the process of priming to the first antigen leads to a greater number of T cells capable of specifically reacting to this antigen. Restimulation with the priming antigen then results in the release of sufficient amounts of the diffusable stimulator to augment a normal secondary response to the priming antigen and, incidentally, to enhance a concurrent immune response to unrelated antigens whose antigen-reactive cells are in close proximity (heterologous enhancement).

Whatever the basic mechanism of these phenomena may be, we feel that the term antigenic competition is misleading and possibly implies

an incorrect mechanism. We have recently (*135*) adopted the term nonspecific antigen-induced suppression (AIS) as a more accurate phrase and this has met with some acceptance in the literature (*151*). It can also be said that "antigenic promotion" (*255*) is a more suitable term than "specific heterologous enhancement" because of possible confusion with the enhancing factors observed in tumor immunology (*118*).

XVI. Conclusion

The number of conflicting reports that now exist on AIS suggests that no single hypothesis can be applied to this phenomenon. It is probable that the suppression observed is the end result of a heterogeneous group of phenomena varying with the antigens used, the experimental animal, and the time sequence of immunization. It is also possible that, at any particular time, more than one of these mechanisms is operative in the experimental animal.

Note Added in Proof

Subsequent to the submission of this review article for publication, a number of papers have appeared referring to antigen-induced suppression. It has been pointed out by Taussig *et al.* (*238a*) that many of the inconsistencies of "antigenic competition" can be resolved by subdividing the phenomenon into intra- and intermolecular competition, depending upon whether the competing antigenic determinants are on the same or different carrier molecules. This concept has been discussed in detail by Taussig (*237a*) in a subsequent article.

In a recent paper by J. W. Schrader and M. Feldmann (*211a*), the *in vitro* anti-DNP response of normal mouse spleen cells to DNP-fowl gamma globulin (DNP-FγG) was compared with that of spleen cells from mice injected 2 days before with donkey red cells. This work confirmed a number of observations made by ourselves using a similar model (*196*) and extended them as follows: (*a*) although the response to DNP-FγG was suppressed in "competed" spleen cells, there was a normal response to DNP coupled to the thymus-independent carrier *Salmonella flagella;* (*b*) trypsinization of the "competed" spleen cells before culture restored the anti-DNP response to DNP-FγG to normal values; and (*c*) the addition of normal or anti-θ treated peritoneal exudate cells to the suppressed spleen cells restored the response. These data were taken as evidence that "antigenic competition represents competition for sites on the macrophage surface by antigen-T-cell-immunoglobulin complexes, a macrophage-bound matrix of such complexes being necessary for B-cell activation."

Using a somewhat similar system, Sjöberg (*229*) observed that spleen

cells from mice undergoing a GVH reaction are deficient in their responses to sheep RBC and *Escherichia coli* lipopolysaccharide *in vitro*. In this system the "GVH spleen cells" suppressed the *in vitro* response to SRBC of normal syngeneic spleen cells if mixed with them. This effect was not affected by anti-θ treatment of the "GVH spleen cells," but was abolished if the cells were subjected to carbonyl iron and magnetism treatment. The author postulates that activated T cells in turn activate macrophages to produce some type of inhibitory factor.

Finally, Katz *et al.* (*131a*) have studied the effect of simultaneous supplementary immunization with unrelated carriers on secondary anti-DNP responses in the guinea pig. Although the details of this work are beyond the scope of this note-in-proof, the authors conclude that T lymphocytes can exert either a positive or negative effect on antibody production, antigen-induced suppression being an example of such a negative effect.

These recent articles again point out the heterogeneity of antigen-induced suppression and emphasize the possibility that more than one mechanism is responsible for this phenomenon.

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Effect of Antigen Binding on the Properties of Antibody

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I. Introduction

When antigens bind to antibodies they frequently initiate one or more physiologically significant reactions. For example, when the antibodies are situated on the surface of an antibody-forming precursor cell, antigen may induce such cells to differentiate into antibody-secreting cells. Alternatively, the result of such interactions may be to make such cells refractive to a subsequent antigenic challenge. When antigens combine with antibodies on the surface of tissue mast cells or circulating basophiles the reaction may trigger degranulation with release of histamine, slow-reacting substance of anaphylaxis, and eosinophile-chemotactic factor. Antigens reacting with humoral antibodies can promote the binding of C1q, which is the initial event in the activation of the complement melange.

How do antigens trigger these events? One possibility is that the structure of the antibody changes by virtue of the noncovalent interactions between the antigenic determinants and the amino acid side chains in the antibody-combining sites. The transformed antibody could then more avidly bind complement or plasma membrane components. I shall refer to this proposal as the *allosteric model* of antigen action.

A second possibility is that the distribution of the antigenic deter-

minants in space is a critical factor. If a bi- or multivalent antibody became stretched when binding to two or more appropriately spaced antigenic determinants, the strain might reveal or cover, activate or deactivate critical sites on the antibody molecule. I shall refer to this as the *distortive model* of antigen action.

A third possibility is that antigens act by aggregating antibodies. I shall refer to this suggestion as the *associative model* of antigen action. Such aggregation can have two effects. It can cause preexisting sites on individual molecules to be approximated; that is, to the "eye" of some third component, a unifunctional substrate has been converted into a multifunctional one. Alternatively, new sites may result (or preexisting sites may disappear) as a consequence of the polymerization of the antibody.

One purpose of this review is to evaluate critically the available experimental data to see if they are adequate to permit us to choose which model (or combination of models) is most correct. There are several sources of such data: determinations of the shape and flexibility of antibodies; measurements of antigen-binding rates and equilibrium constants; comparisons of the structure of complexed with uncomplexed antibodies; observations on the functional properties of such complexes.

After reviewing these findings it is clear that no final conclusions can be arrived at as yet. Although the associative model adequately accounts for many of the data, the alternative ways by which aggregation produces an effect remain difficult to choose between. Moreover, it is unclear whether such a relatively simple model is sufficient to explain a variety of complex phenomena related to the immune response.

A second purpose of this review is to document what we know about antibodies as proteins in regard to their potentialities and limitations as biological transducers. Any general theory of immune regulation must take these properties into account. The writing of this review was in part prompted by the rather different approach of some "cis-immunologists" (Jerne, 1967) who, in trying to answer the difficult questions raised by certain complex biological phenomena, have (to my mind) rather facilely attributed the events to all sorts of specific discriminatory contortions of antibodies. I hope this review will be helpful in setting some constraints on such Rube Goldberg (1968) inventions.

II. Antibodies as Proteins

A. Intramolecular Specialization

It is now well recognized that antibodies interact with antigens by way of combining sites located in the Fab regions. Interactions with all

other biological components such as complement and the plasma membranes of cells occur by way of the Fc regions. Considerable experimental effort has been expended in order to localize the sites of these interactions more precisely.

With regard to the antigen-combining sites, these efforts have been highly successful. The sites (1 per Fab fragment) result from the folding of the amino-terminal variable regions of the light and heavy chains, and site-labeling studies have shown that the hypervariable segments contribute contact amino acid side chains. The rough outlines of such a site has been directly visualized by X-ray diffraction studies (Poljak *et al.*, 1972), and it appears to be of a size consistent with the predictions made from antigen-binding studies (Kabat, 1968; Schechter, 1971).

The sites at which immunoglobulins (Ig) interact with nonantigenic components (complement, cell membranes) are not nearly as well defined, but this area of investigation is progressing rapidly.

There is increasingly good evidence that C1q interacts with the region known as C_H2 [second homology region of the constant region of γ heavy chains—residues 234–341 using the numbering for the Eu IgG protein (Edelman *et al.*, 1969)]. Connell and Porter (1971) were able to cleave selectively rabbit IgG at positions 326 and 327 yielding an Fabc fragment consisting of an $F(ab')_2$ fragment with a more-or-less complete C_H2 region, i.e., a fragment that can be represented as $(V_L,C_L;V_H,C_H1,C_H2)_2$. It retained 60% of the complement-binding activity. Ellerson *et al.* (1972) isolated a more-or-less intact dimeric C_H2 region from a human G_1 myeloma protein which contained the $222(2 \times 111)$ NH_2-terminal amino acids of a plasmin-produced Fc region. This fragment (which contains the hinge region) was ~50% as active in binding complement as intact Fc fragments. These results lend support to the earlier study of Kehoe and Fougereau (1969) who demonstrated complement-binding activity in a 7000-mol. wt. cyanogen bromide fragment derived from the C_H2 region. On a molar basis the activity of this fragment was, however, only 3% of that shown by Fc fragments.

Reiss and Plescia (1963) and Schur and Becker (1963) showed that some residual complement-binding activity was retained by $F(ab')_2$ fragments. It now appears that the latter activity is not related to C1q binding but to the binding of C3 (Sandberg *et al.*, 1971; Reid, 1971). Activation of the latter provides an alternate pathway toward activation of the subsequent complement components.

Neither the C_H2 region from IgG (Ellerson *et al.*, 1972) or an apparently similar fragment from IgE (Ishizaka *et al.*, 1970) possess cytotropic activity. Recent data on isolated C_H3 regions suggest that these regions may mediate this function (Minta and Painter, 1972) despite

earlier failures to detect activity in such fragments (Irimajiri *et al.*, 1968; Prahl, 1967; Utsumi, 1969; Frommel and Hong, 1970). When rabbit lymphocytes are studied with anti-immunoglobulin antisera (Pernis *et al.*, 1971), immunoglobulins can be detected with antisera directed to the $A_{11,12}$ allotype specificities—γ-chain markers that correlate with a methionine–threonine interchange at position 219 (Prahl *et al.*, 1969). Antisera to more distal (COOH-terminal) regions are ineffective. Similarly, only the C-terminal half of the Fc of IgG appears to be bound on human B-lymphocytes (Fröland and Natvig, 1972).

Despite the fragmentary nature of these data, it is clear that any hypothesis concerning how antigens induce important biological changes via their interaction with antibody must explain how reactions in the Fab regions are "recognized" in the Fc regions. A model that assigns simply an associative function to the antigen has no difficulties in this respect: as long as the Fc regions are covalently attached to the Fab regions, the former will perforce aggregate when the latter interact with multifunctional antigens. On the other hand, if antigens act as allosteric modifiers, there must be sufficiently intimate contact between the Fab regions and the Fc regions for the signal to be transmitted. Such an interface has been called a *domain of bonding* (Monod *et al.*, 1965). It, therefore, becomes pertinent to review what is known about the extent of interaction between the Fab and Fc regions.

Evidence about such interactions can be obtained from studies on the overall shape of immunoglobulin molecules, from studies on the flexibility of the molecules, and from comparisons between the structural and functional properties of isolated Fab and Fc regions and the properties of these regions as they exist in the intact molecule.

B. Shape of Immunoglobulins

Data on the shape of immunoglobulins have been reviewed relatively recently in these volumes (Dorrington and Tanford, 1970; Green, 1969), and I shall therefore limit my own discussion of this point. Electron-microscopic data on both isolated and complexed IgG have clearly shown the overall validity of the Y-shaped model first clearly formulated by Noelken *et al.* (1965). Similar Y-shaped structures combine to form the polymeric IgA and IgM (reviewed in Feinstein *et al.*, 1971). What remains uncertain are the precise dimensions of the Fab and Fc arms, the angle between the arms, and the degree and ease with which the arms of the Y can move relative to each other. In addition, the relative mobilities of the Y-shaped subunits within the IgA and IgM polymers remain unclear from these studies. Certainly great variability in the disposition of the Fab and Fc regions has been visualized but to

what extent these reflect strains on the molecule imposed during preparation of the specimens is uncertain.

Pictures of uncomplexed rabbit IgG showed an average center-to-center distance between the Fab and Fc fragments of 60 to 65 Å. and an average inter-Fab angle of 60° (Green, 1969). The corresponding values for IgM (from a variety of species) and IgA (mouse) have been estimated as between 55 to 70 Å. and 30° to 110°, respectively (Feinstein *et al.*, 1971). Electron-microscopic pictures of rabbit IgG show a rather compact hinge region. Since an analysis of the hydrodynamic behavior of such IgG suggests a somewhat larger Fc to Fab center-to-center distance (Charlwood and Utsumi, 1969), this may mean that the hinge region has collapsed somewhat in the electron-microscopic preparations.

The X-ray diffraction data of Davies *et al.* (1971) are consistent with at least four different Y- or T-shaped models for the human IgG myeloma protein which they studied. Provisionally, they felt that their model IV, a T-shaped structure, to be most probable. Recently, some preliminary data on this same protein suggest that it may have an aberrant hinge region (Lopes nad Steiner, 1973) so that it seems unwise to generalize from these initial X-ray results.

Pilz *et al.* (1970) examined the fully sequenced human IgG myeloma protein Eu, its tryptic Fab and Fc fragments, and its peptic $F(ab')_2$ fragment by small-angle X-ray scattering. The scattering of the Fab fragment was equivalent to that expected for an elliptic cylinder with axes of 22 and 56 Å. and a length of 98 Å. The corresponding figures for the Fc fragments were 21, 63, and 99 Å., respectively. The authors computed the scattering curves that would be generated by various combinations of two such Fab regions and one Fc region. The model whose computed curve was most similar to the experimental scattering curve was one in which the inter-Fab angle was 180° and in which the Fc was composed of two contiguous ellipsoids. All of the ellipsoids overlap in the hinge region in this model. Since the Fab and Fc dimensions are "equivalent" dimensions and do not necessarily correspond to a physical reality, the model must be considered simply as consistent with the data. The authors in their summary quite properly refrain from giving a specific model and simply conclude that their data show that "the Fab and Fc regions of the γG immunoglobulin molecule are relatively compact but that the whole molecule has an extended structure in solution."

Cathou and O'Konski (1970) examined heterogeneous rabbit anti-dinitrophenyl (anti-DNP) antibodies by the method of transient electric birefringence. The birefringence is due to the asymmetry of the immunoglobulin molecules which become oriented in an electric field if their charge distribution is asymmetric. If the molecule binds a highly charged

ligand, there should be a measurable change in the permanent dipole. The magnitude of the latter depends on the net charge of the bound ligand and its distance from the center of rotation of the molecule. With both binding sites occupied, one expects a restoration of the initial charge asymmetry if the sites are at both ends of an elongated rod. With a Y-shaped molecule the magnitude of the dipole contribution would depend on the angle between the two Fab fragments and the distance between the combining sites and the center of rotation. The authors used an antibody preparation with a mean association constant of 10^8 M^{-1} so that, at an antibody concentration of 4×10^{-5} M, "all" of any added hapten would be bound. The hapten they used was dinitrophenyl–glutamyl–aspartate which has three fully ionized carboxylate groups in the pH 8.2 Tris buffer they employed. With 1 or 2 moles of hapten per mole antibody, no significant alteration in the dipole moment was observed. Each of the rather lengthy list of assumptions necessary to interpret their results is defensible, but their model (a Y-shaped structure with an Fab region 60 Å. in length and an inter-Fab angle of 130° to 150°) can only be considered reasonable, not definitive. It is worth noting that one of their required assumptions is that the bound hapten does not affect the overall shape of the molecule.

C. Flexibility of Immunoglobulins

The flexibility of immunoglobulins potentially can be measured directly by the method of depolarization of fluorescence. This method has been rather widely used for a number of years, but many of the early results now appear to be uninterpretable because they involved unwarranted assumptions and inadequate methods (Brochon and Wahl, 1972). It appears now that nanosecond pulse techniques provide the most reliable data but even so the interpretation of these data is by no means straightforward.

Yguerabide *et al.* (1970) measured the rotational motions of rabbit IgG antibodies which had been induced by 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) conjugates of hemocyanin and whose combining sites were saturated with dansyl lysine. The plot of the anisotropic decay with time was linear for the Fab fragments, yielding a single rotational correlation time of 33 nsec. This value is consistent with what would be expected for a 50,000-mol. wt. fragment in the shape of a rigid prolate ellipsoid with an axial ratio of about 2 (Pilz *et al.*, 1970) and with a reasonable amount of hydration (0.32 ml./gm.). The decay curve for the $F(ab')_2$ fragments was complex, and the experimental curve could not be duplicated by any computed curve for a rigid ellipsoid of appropriate size and hydration. Because their data suggested

that the Fab fragments were rigid, they reasoned that the complex curve for the $F(ab')_2$ was due to a combination of motions: the independent motion of the Fab's and a global motion of the $F(ab')_2$. Similar reasoning was used to explain the complex decay curve of the intact IgG. By assuming that the latter curve was contributed to by the independent motions of the Fab and the global motion of the IgG, a value of 168 nsec. for the rotational correlation time of the latter was calculated. Their data could not distinguish between rotations about the axis joining the Fab regions to the Fc region and rotations perpendicular to these axes. If both rotations are possible the angular range of motion in the nanosecond range would be 33°; if only one kind of motion is permitted, the angular range would be greater.

Brochon and Wahl (1972) were unable to confirm the model of Yguerabide *et al.* The French workers studied the decay of fluorescence anisotropy of dansylated rabbit IgG and its Fab, Fc, and $F(ab')_2$ fragments. After subtracting out that part of the decay likely to be due to local motion of the dye molecule, a longer decay process remained which they felt was due to the global motion of the molecule. With suitable adjustments, their data are not very different from those of Yguerabide *et al.*, and it appears that at this time an unambiguous interpretation of the observations is impossible. Both groups must make some assumptions in order to interpret the complex decay curves. I do not feel competent to choose between these formulations, and it seems wiser to let those who are experts in what has become a highly complex field iron out their differences. Some comments seem appropriate, however. (*1*) The use of fluorescent *ligands* for these analyses (Yguerabide *et al.*, 1970), although having the advantage that the fluorescent probes have little or no independent motions themselves, assumes that no changes occur in the antibody structure in the presence of ligand. Much of the data reviewed here are consistent with this assumption, but it would be useful if the conclusions could be verified using fluorescent conjugates. Russian workers have, in fact, claimed that changes may occur. Tumerman *et al.* (1972) measured the polarization of fluorescence of rat (antidansylated bovine IgG) antibodies in the presence of dansyl lysine and compared it to IgG conjugated with dansyl chloride. The latter preparation gave almost identical relaxation times for the intact molecule and the isolated Fab regions. On the other hand, whereas the liganded Fab values were the same as the conjugated Fab results, the liganded IgG was calculated to have a significantly longer relaxation time than the conjugated IgG. The authors suggest this could either be due to interaction of the bound ligands with each other or due to an induced decrease in the flexibility of the IgG in the presence of ligands in the combining sites. In view of

the questions raised by Brochon and Wahl about the validity of steady-state depolarization measurements, the correctness of Tumerman *et al.*'s interpretation is uncertain. (2) Although both the studies of Yguerabide *et al.* and Brochon and Wahl show *some* limitation of intramolecular motion in the IgG, it should be stressed that this refers only to the nanosecond time scale. It is fair to say that, if such rapid mobility exists, then it is likely that the interactions between the moving parts are very weak. However, even if these molecules are rigid over this time scale, rotations with longer time constants are not precluded. Presumably such distortions would require proportionately larger energy inputs.

An interesting study by Werner *et al.* (1972) also suggests incomplete freedom of motion of the Fab regions. These workers prepared hybrid rabbit IgG antibodies in which one active site bound an energy donor, dansyl lysine, and the other an energy acceptor, fluorescein. The fluorescence lifetime of the bound dansyl groups will decrease if there is energy transfer between them and the bound fluoresceins. The extent of transfer is determined by the distance and orientation between the transition dipole moments of the pair, by their spectral properties, and by the refractive index of the medium through which the transfer occurs. The spectral properties (including the overlap integral of the donor–acceptor pair and the quantum yield of the donor) can be measured and a reasonable value for the refractive index determined. By assuming a random orientation of the dipole transition moments with respect to each other, the distance between them at which 50% of the excitation energy of the donor will be transferred can then be estimated. Using the known uncertainties of the lifetime measurements, it could then be calculated that some energy transfer should have been observed if the distance between the chromophores would have been less than 82 Å. for an appreciable period of time. Other considerations might increase this value somewhat. That no decrease was observed suggests that the chromophores spent little of the time closer to each other than these distances. Taking the length of an Fab region as 70 Å., the minimum angle between the Fab regions was calculated to be at least 80° to 95°. For these results to be indicative of the properties of the native immunoglobulin, one must assume first of all that the preparation of the hybrids (which involved two exposures to acid pH values) did not produce irreversible structural changes. Their hapten-binding data were consistent with some loss of sites, and other structural changes at these pH values can be anticipated although most of these are probably reversible (Doi and Jirgensons, 1970). A second assumption is that there was sufficient segmental flexibility or differences in combining site orientation or both so that the transition dipole moments of the donor–acceptor pair were more-or-less

randomly disposed. As indicated above, the extent of such flexibility is uncertain so that some ambiguity with regard to this point remains. Finally these studies assume that the donor and acceptor haptens did not alter the structure of the hybrid antibody.

Green and co-workers (Valentine and Green, 1967; Hyslop *et al.*, 1970) and Schumaker and associates (Green *et al.*, 1972) have studied the complexes formed between bifunctional dinitrophenyl ligands and rabbit IgG antibodies. Provided that the functional groups were adequately far apart so that intramolecular bridging was possible, only monomeric complexes should have been observed since such internal circular complexes would be by far the most stable (Crothers and Metzger, 1972). Because there is some ambiguity as to whether any of the ligands were sufficiently long, we shall leave this point aside. The second most stable complex would be a dimer—two bifunctional ligands bridging two antibody molecules to form a bimolecular circular complex. The concentration of different types of complexes can be estimated as follows (Schumaker *et al.*, 1973). The concentration of each type of complex (H_mA_n) is determined by the mass law relation

$$(H_mA_n) = k(m,n)(H)^m(A)^n$$

where H refers to the bivalent hapten, A to bivalent antibody, and m and n to the number of each in the complex. The equilibrium constant for each such complex, $k(m,n)$, may be written as equivalent to

$$k(m,n) = S(m,n) \exp[-(b\,\delta F + \xi(m,n))/RT]$$

where $S(m,n)$ is a statistical factor equal to the number of distinct ways such structures may be formed, b is the number of antibody sites occupied in each type of complex, $\xi(m,n)$ is the thermodynamic equivalent, in calories, of all "strains" in the H_mA_n complex and δF is the average free energy of binding for the univalent hapten-combining site interaction. If $\xi(m,n)$ for the fully saturated circular monomer (H_1 A_1) is set sufficiently high so that it will not form (experimentally up to 80% of antibody molecules are found as dimer and higher polymers at 1:1 hapten antibody ratios), the relative ratios of various polymers can be calculated. The results show that only by inserting relatively large "strains" for dimer formation can the experimentally observed concentrations of higher polymers be predicted by the computations. By similar reasoning, it would be expected that the avidity of the divalent ligand would be measurably greater for antibodies participating in such circular complexes. The expectation is not borne out by the experimental data. Fluorescence quenching analyses of the binding parameters failed to

1971). These observations suggest that there is an energy barrier to forming such complexes, i.e., the Fab arms are incompletely free to move with respect to each other. That with time the higher polymers gradually transform into dimers (Hyslop *et al.*, 1970) is consistent with this interpretation of the data.

Other observations that are pertinent here include the functional pentavalence of IgM molecules (and monovalence of their subunits, IgMs) with large antigens even though these molecules are known to have the expected number of combining sites when the isolated Fab regions are examined separately (Metzger, 1970a). A recent study by Arend *et al.* (1972) suggest that even with IgG, in great antigen excess (where no cooperative interactions play a role) the effective valence may be 1 rather than 2 as had been previously thought. Although these findings also suggest limited flexibility of the immunoglobulin molecules, they suggest that the Fab regions are most relaxed when they are close together. This is in direct contradiction to the model suggested by Werner *et al.* (1972), Cathou and O'Konski (1970), and Pilz *et al.* (1970) and with the studies on the divalent ligands referred to in the foregoing.

Many of these studies leave the role of Fc–Fab interactions undefined since they could in whole or in part reflect Fab–Fab interactions primarily. It would probably be worthwhile to compare the $F(ab')_2$ and whole immunoglobulins in several of these systems to see if there are important differences. It would also be useful to select the antibodies to be examined more carefully. It has been observed that, among antibodies of comparable binding affinity and valence, there are some which tend to form intramolecular circular complexes with multifunctional ligands readily and hence do not precipitate, whereas others preferentially form intermolecular aggregates (Klinman and Karush, 1967; Warner and Schumaker, 1970a). These variations probably reflect differences in the flexibility of the molecules. Such distinct classes would be more usefully explored separately.

D. Structure of Isolated Immunoglobulin Fragments

An alternative method for assessing the degree to which the Fab and Fc regions interact is to compare the properties of these regions as they exist within the intact molecule with their properties when they are separated. The early studies, especially those on papain cleavage of rabbit IgG, demonstrated that limited proteolysis produced Fab and Fc fragments whose individual properties were largely intact. Subsequent work on both IgG and IgM showed that the cleavage of single peptide bonds in each of the heavy chains was adequate to dissociate the Fab and Fc regions (Hill *et al.*, 1967; Putnam *et al.*, 1971). The fragments

thereby produced show no tendency to remain associated with each other.

Optical studies have consistently shown that the optical rotations of the separate fragments completely account for the rotatory spectrum of the intact immunoglobulin (Steiner and Lowey, 1966; Dorrington and Tanford, 1968). This may be contrasted with the interaction between the heavy and light chains. The chains remain strongly associated even in the absence of covalent bonds, and when forcibly wrenched apart, changes in the tertiary structure [as assessed by optical (Dorrington *et al.*, 1967) or immunochemical techniques (Rivat *et al.*, 1969)] become obvious.

Sequence studies and proteolytic digestion experiments have indicated that the individual chains are composed of homology regions: two in the light chain, and four to five in the heavy chains (Edelman and Gall, 1969). Furthermore, strong noncovalent interactions appear to be primarily perpendicular to the axis of pseudosymmetry which exists between the heavy and light chains in the Fab regions (Singer and Thorpe, 1968) and between the axis of symmetry which exists between the heavy chains in the Fc region (Davies *et al.*, 1971). Proteolytic fragments that contain the $V_L + V_H$ homology regions (Inbar *et al.*, 1972), the $C_L + C_H1$ regions (Gall and D'Eustachio, 1972) and the dimer of C_H3 (Turner and Bennich, 1968; Utsumi, 1969) have been isolated. Only the C_H2 regions show relatively little tendency to associate (Ellerson *et al.*, 1972), but these are usually covalently bound by one or more disulfide bonds in the hinge region immediately NH_2-terminal to these regions. These results are consistent with the "domain hypothesis" of immunoglobulin structure (Edelman and Gall, 1969). Recent X-ray diffraction data of Poljak *et al.* (1972) convincingly support such a model. A schematic representation of Poljak *et al.*'s view of an IgG molecule is shown in Fig. 1.

E. Functional Properties of Isolated Immunoglobulin Fragments

Since significant interactions parallel to the axes of symmetry (i.e., between domains) appear to be absent, it is difficult to see how reactions in the combining site can be sensed more distally. Most of the functional data on intact immunoglobulins and on the isolated Fab and Fc regions reflect this isolation of the domains.

Most binding studies have failed to detect differences in the antigen-binding properties of the isolated Fab regions compared to the intact molecule. If the allosteric model of antigen action were correct this result would not be anticipated. That is, if upon binding antigen, non-

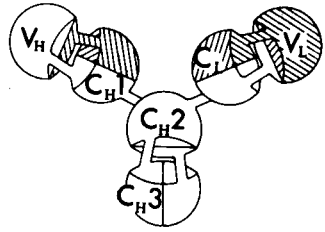

FIG. 1. Schematic representation of typical IgG molecule. This model is an extrapolation from X-ray diffraction data on isolated Fab fragments, and from a variety of other data which suggest that the molecule is composed of six relatively independent globular moieties. As is evident, strong interactions between polypeptide segments are thought to be chiefly perpendicular to the long axes of the molecule. (From Poljak *et al.*, 1972, by kind permission of the authors and publishers.)

covalent bonds in the Fc region were made or broken this should be reflected in the free energy of binding of the antigen. When the Fab regions are isolated such Fc changes would not contribute as would be evidenced by a different binding constant.

In this respect it would be interesting to know if in the study by Tumerman *et al.* (1972) referred to in the foregoing, differences in the binding of the dansyl groups by the Fab fragments and intact molecules could be detected. If their hypothesis of a ligand-induced change in IgG flexibility is correct, one would surely expect to see such differences.

Studies on heterogeneous antibody populations (Karush, 1959; Nisonoff *et al.*, 1960; Velick *et al.*, 1963) can be criticized as being inadequate because changes in the average binding constants might be difficult to detect in the presence of binding-constant heterogeneity. Studies on homogeneous immunoglobulin preparations have so far been limited to investigations of myeloma proteins which were more or less fortuitously found to bind certain ligands (Eisen *et al.*, 1968; Ashman and Metzger, 1969; Pecht *et al.*, 1972). These may be inadequate for the following reason: antigen–antibody combining site complementarity may be a necessary but not sufficient requirement for allosteric changes to occur. With conventional antibodies there is a biological relationship between the antigen and antibody—the former leading to the production of the latter. In this situation, one can expect to see the hypothetical changes in antibody structure in the presence of ligand. This does not necessarily hold true in the case of a myeloma protein and a ligand that it may bind. Only in a few such cases can one intuit that the appropriate antigen is being examined (Metzger, 1969; Potter, 1972). In most of the studies on myeloma proteins, it is unlikely that the ligands represent the "true" antigens. The increasing availability of homogeneous induced

antibodies (Krause, 1970) now permits one to avoid these ambiguities, and it would be useful to obtain some very precise binding data on such systems.

The rapid kinetics of antibody–antigen reactions have been cited as evidence against significant conformational sequelae to the binding reaction (Day *et al.*, 1963). Most protein conformational changes have rate constants in the range of 10^2–10^4 M second^{-1} (Hammes, 1968). Since the "on" reaction of ligands with antibodies is so fast, no conformational change is likely to be required for (or result from) the binding of ligand. These studies have largely involved hapten-binding experiments, and the kinetics closely approximated those expected for diffusion controlled reactions (Froese and Sehon, 1965; Froese, 1968). The study of Pecht *et al.* (1972) is particularly significant in this respect. They followed the quenching of tryptophan fluorescence in a nitrophenyl-binding mouse myeloma protein by a DNP–lysine ligand. Since in this case the sites are homogeneous, any secondary conformational changes which might have had different time constants were potentially observable. None were observed; the Fab fragments and intact molecules had the same single-valued forward rate constant which was close to that predicted for a diffusion-controlled reaction. Levison *et al.* (1971) also failed to observe differences in the rate constant for fluorescein binding to either intact IgG or Fab fragments from rabbit antifluorescein antibodies.

The kinetics of binding also have been examined with antigens (e.g., proteins) which are multifunctional with respect to a heterogeneous population of antibodies. Dandliker and Levison (1968) studied the kinetics of reaction between fluorescein-labeled ovalbumin (45,000 mol. wt.) and rabbit antiovalbumin antibodies by the technique of depolarization of fluorescence. The initial rate followed a simple bimolecular rate law suggesting that the primary binding reaction rather than secondary effects was being studied. The forward rate constant was 2×10^5 M^{-1} second^{-1}. Sachs *et al.* (1972) studied the rate of antibody-induced enzyme inactivation using rabbit antibodies to staphylococcal nuclease (mol. wt. 18,000). Their specifically absorbed antibody preparation probably reacted with only one site on the nuclease. The maximum rate constant for the antigen–antibody reaction was estimated to be 8×10^5 M^{-1} second^{-1}. Noble *et al.* (1972) studied the binding of Fab fragments from rabbit antibodies directed to discrete determinants on cytochrome c (mol. wt. 11,500) and hemoglobin S (mol. wt. 68,000). The forward rate constants were 4.5×10^5 and 5.8×10^6 M^{-1} second^{-1}, respectively. In view of the similarity of the rate constants and the substantial differences in molecular weight for the interacting species, it is

unlikely that all these reactions are diffusion-controlled reactions. However, no rigorous calculation of the theoretical (diffusion-controlled) rate constant has been presented for any of these systems. The absolute values of these constants cannot, therefore, be used to come to any conclusions regarding the presence or absence of conformational changes in the antibodies during the binding reaction. The rate of reaction may be slowed not only by potential conformational changes but also by electrostatic repulsions or local transport of solvent effects, and these kinetic studies have not yet been very helpful in resolving the question of how antigens affect the structure of antibodies.

A few studies have given anomalous results. Kelly *et al.* (1971) measured the rate and equilibrium constants for the reaction between rabbit antidinitrophenyl antibodies and either DNP–lysine or 1-hydroxy-4(2,4-dinitrophenylazo)-2,5-naphtholdisulfonate (DNP–dye). Binding was assessed either by fluorescence quenching or spectral changes using temperature jump and stop-flow techniques. Intact IgG or peptic Fab′ fragments were used. Small but apparently reproducible increases in the association constant were observed when Fab′ fragments and intact molecules were compared. The forward rate constants showed larger differences; the value for the Fab′ was twice that observed with the whole molecules for both the DNP–lysine and the DNP–dye. This difference is much greater than would have been expected simply on the basis of the difference in the diffusion constants for the Fab′ and intact molecules. The authors consider various explanations. They feel that a conformational change is unlikely since only one relaxation effect was observed in the temperature-jump method. They raise the possibility that the two sites in the intact antibody may be so close that ligand binding at one site might interfere with binding at the second site. They cite as evidence the results of Warner and Schumaker (1970a,b) which suggested that the two sites might be bridged by short bifunctional ligands such as di-DNP–cystine and α,ϵ-DNP–lysine. Such a model is certainly in conflict with the conclusions of Werner *et al.* (1972) (Section II,C). Kelly *et al.* raise the alternative possibility that cleavage of the molecule into Fab′ fragments resulted in some slight conformational change. In view of this possibility, it is unfortunate that the $F(ab')_2$ fragments were not studied simultaneously.

The results of Kelly *et al.* suggested the possibility of negative cooperativeness. Positive cooperativeness has also been observed. Zimmering *et al.* (1967) studied the binding of steroids to steroid-specific sheep IgG antibodies using equilibrium dialysis. In some instances they observed what could be interpreted as cooperative binding effects. The data appeared to indicate that this was only true of a small percentage

of the antibody population and, surprisingly, only of the most weakly binding sites. Similar effects might be seen if the ligand tended to dimerize and, although this was excluded for aqueous solutions of the ligands, it is not impossible that the bound ligand might undergo such a dimerization. Alternatively, it might be possible that with a heterogeneous antibody population these relatively large ligands are bound by more than one molecule of antibody at a time, in which case cooperativeness might be observed for other reasons (see in the following). Unfortunately no studies on Fab fragments were performed so that further speculation as to the nature of these effects is fruitless.

Cooperative binding phenomena were observed by Matsukura *et al.* (1971) and confirmed for the same system by Matsuyama *et al.* (1971). The system involves a radioimmunoassay of guinea pig antibodies directed toward adrenocorticotropic hormone (ACTH). Ordinarily the ratio of bound to free labeled ligand falls as increasing unlabeled ligand is added. In this instance the ratio first rose several-fold before a decline was observed. In these studies the properties of Fab fragments were not examined. Weintraub *et al.* (1973) detected a similar phenomenon in studying guinea pig antibodies to human chorionic gonadotropin. Their data were consistent with 10% of the antibodies expressing cooperativeness. The Fab fragments did not show these anomalous effects.

Both ACTH and the gonadotropin can be expected to be multifunctional with respect to a heterogeneous population of antibodies. The addition of small amounts of antigen may cause immune complexes to form to which the labeled ligand may bind cooperatively and, therefore, more avidly than to uncomplexed antibodies. Such a phenomenon would yield binding data indistinguishable from those observed. Further experiments will be required before the mechanism of these effects is resolved. It may be noted that, even if such binding could be shown to represent a true homotropic allosteric phenomenon, there is no reason from these results to invoke changes in the Fc regions.

On the other hand, Marrack and Richards (1971) describe some cooperative effects which they believe cannot be explained simply by cooperative binding. They studied the rate of aggregation of rabbit antibodies to lysozyme and bovine serum albumin by light scattering. By measuring the intensity of light scattered at 90° and the ratio of the intensities of light scattered at 45° and 135°, they hoped to assess the size and structure of aggregates prior to gross flocculation. They observed that under conditions where the rate of aggregation was slow (with low antibody concentrations, with $F(ab')_2$ fragments, or with acetylated albumin) the time curve for aggregation was sigmoidal. For the first few minutes there was a slow rise in the scattering intensity

then a more rapid phase and then a slower phase again. Significantly, their calculations showed that at the end of the first slower phase the antigen and antibodies had already formed aggregates with possibly as many as 10^3 molecules per aggregate. Because the number of particles per unit volume was drastically reduced and the number of vacant combining sites and free antigenic determinants markedly diminished, they reasoned that the increase in the rate of aggregation was not due to the formation of additional antigen–antibody bonds. Instead they suggested that "a change in the structure of antibody molecules or antigen molecules or both increases the number of collisions that result in combination." Clearly, there are too few data to explain this phenomenon. I am uncertain to what extent similar curves might be generated by any substance that would aggregate proteins, e.g., cross-linking reagents or precipitants such as ammonium sulfate. Ascribing the phenomenon to antigen-induced changes via an allosteric or distortive mechanism seems to me premature. It is notable that the $F(ab')_2$ fragments showed these effects, thus suggesting that they were not necessarily related to changes that might have occurred in the Fc regions.

I have already cited some of the data indicating that the binding sites in the Fc region remain intact when this region is cleaved from the rest of the molecule (see Section II,A). Further data will be cited in subsequent sections. However, none of these data is sufficiently precise to rule out even moderate differences between the sites as they exist on the separate fragments and on the intact molecule.

III. Structure of Antigen–Antibody Complexes

The most straightforward way of studying the effect of antigens on antibodies is to study the structure of the complexes themselves. Here we face a serious dilemma. Complexes with monofunctional ligands are studied relatively easily but such complexes rarely if ever exhibit important biological activities (Section V). On the other hand, whereas multifunctional antigens do induce the biological changes in which we are interested, their complexes with antibodies are difficult to study rigorously. These considerations limit the interpretability of this direct approach to the problem.

A. Complexes with Monofunctional Ligands

1. Physicochemical Studies

Monofunctional ligand–antibody complexes have been examined by a variety of techniques. Both heterogeneous antibodies and homogeneous immunoglobulins have been examined by techniques that measure the

optical anisotropy of the molecules. Ishizaka and Campbell (1959), studying rabbit antibenzene arsonate antibodies, Steiner and Lowey (1966) and Cathou *et al.* (1968), studying rabbit anti-DNP antibodies, and Ashman *et al.* (1971), studying a human nitrophenyl-binding Waldenström macroglobulin, failed to detect changes in the presence of ligand. Holowka *et al.* (1972) studied three homogeneous antibody preparations from a single rabbit which had been immunized with S3 polysaccharide. In the presence of a hexasaccharide hapten, changes in the circular dichroism spectrum between 260 and 310 nm. which were unique to each antibody were observed. Since the changes were distinctive for each antibody the authors consider it likely that the ligand-induced changes were limited to the antibody-combining site. Hopefully more direct evidence will be sought to establish this point.

Warner and Schumaker (1970b) and Warner *et al.* (1970) studied rabbit IgG antibodies by a differential sedimentation velocity technique (Schumaker and Adams, 1968). In these studies the reference solution, nonspecific rabbit IgG, is added to one ultracentrifuge cell containing a 1° positive wedge window, whereas the experimental sample, antibody with or without ligand, is in a second cell in the same rotor containing a 1° negative wedge window. Control runs and suitable corrections permitted the authors to detect changes in sedimentation coefficients which were greater than 0.0168. Changes were not observed with anti-DNP antibody with either DNP–glycine or DNP–lysine at hapten concentrations that were adequate to saturate the combining sites (Warner and Schumaker, 1970b). With antilactoside antibodies (Karush, 1957), changes were observed with both lactose and *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside (Lac dye) (Warner *et al.*, 1970). Although sufficiently high concentrations of lactose and Lac dye were present to assure site saturation, a larger change was observed with the more tightly bound Lac dye ($\delta S°_{20,w} + 0.18_0$) than with lactose ($\delta S°_{20,w} + 0.05_6$). The increase in sedimentation coefficient can be interpreted as due to a decrease in the frictional coefficient, implying that the ligand-saturated antibody molecule becomes more compact. The authors conclude that their data are not consistent with haptens inducing a "click-open" distortion in the IgG molecule (Feinstein and Rowe, 1965). Unfortunately no similar experiments have been reported on the isolated Fab fragments, so that is uncertain whether the observed changes reflect changes in the Fab regions alone or in the $F(ab')_2$ region or in addition in the Fc regions. The greater diffusion constant of the Fab fragments makes it much more difficult to achieve comparable precision in the sedimentation measurements for the smaller fragments.

Ohta *et al.* (1970) measured the volume changes occurring when

solutions of antigens and antibody are mixed. Such changes result from a change in the amount of bound water around polar groups (by electrostriction) and around hydrophobic groups (by clathrate formation). Substantial changes were observed when rabbit anti-DNP IgG antibodies were mixed with DNP–lysine. In comparing preparations having different mean association constants, it was found that the more avid antibodies showed larger changes. For example, a preparation with an average K_0 of 3.1×10^6 M^{-1} showed a maximum volume change of 3.65×10^{-1} M^{-1}, whereas antibodies with a K_0 of $\sim 1 \times 10^9$ released 8×10^{-1} M^{-1}. Solutions of Fab fragments showed volume changes almost exactly half as large, leading the authors to conclude that all of the changes were occurring in the Fab regions. They reasoned that in view of the negative findings by circular dichroism (already mentioned), it was unlikely that there were conformational alterations large enough to account for these substantial volume changes. Because charge neutralization would also lead to only small changes, they concluded that hydrophobic interactions between the ligand and combining site residues were largely responsible for the release of bound water. These workers also studied the interaction of these antibodies with multifunctional antigens, and the results are discussed in Section III,B,1.

Decreases in the susceptibility of rabbit antibenzene arsonate and trimethylanilinium antibodies to chymotryptic digestion in the presence of the appropriate ligands were documented by Grossberg *et al.* (1965). As discussed elsewhere (Metzger, 1970b), their data are not adequate to allow one to determine if these effects were limited to the Fab fragments or not. Our own studies (Ashman and Metzger, 1971) utilizing the Waldenström macroglobulin referred to previously, showed quite dramatic inhibition of proteolysis in the presence of hapten. The effects were clearly limited to the Fab fragments and could have resulted either from an overall stabilization of the fragments or from shielding of critical residues by the bound ligands or both. As part of that study the rate of cleavage of the inter-heavy-chain disulfide bond in $F(ab')_2$ fragments was compared in the presence and absence of ligand. No change was observed.

The same nitrophenyl-binding IgM was studied by the technique of hydrogen exchange (Ashman *et al.*, 1971). Native protein was incubated with tritiated H_2O, and the rates of exchange out of the tritium were assessed in the presence and absence of ligand. Although there were relatively large variations in the data, the results were consistent with an inhibition of exchange out in the presence of ligand. Again, changes observed with the isolated Fab fragments fully accounted for the results on the whole molecule.

2. Other Studies

Changes in the antigenic determinants of antibodies in the presence of monofunctional ligands have been observed. In the best-documented cases, these involve an inhibition of binding by antibodies that are directed to idiotypic determinants in the ligand-binding antibody (Brient and Nisonoff, 1970). These idiotypic determinants must be in the variable regions so that these changes are clearly limited to the Fab fragments. The inhibition is probably due to direct blocking by the bound ligand.

Many of the changes induced by ligands can be explained by a stabilization of the Fab regions. Direct evidence for such stabilization is provided by studies in which the presence of hapten (*a*) inhibited chain dissociation (Metzger and Singer, 1963), (*b*) promoted chain reassociation (Metzger and Mannik, 1964), and (*c*) inhibited the denaturing effects of guanidine–HCl as measured by circular dichroism (Cathou and Werner, 1970). Since heavy- and light-chain variable regions both contribute to the combining sites, the formation of additional noncovalent links between the chains via interaction with the ligand makes such stabilization a thermodynamic necessity.

B. Complexes with Multifunctional Ligands

1. Physicochemical Studies

Robert and Grabar (1957) measured free sulfhydryl groups in ammonium sulfate fractions of equine antihuman serum albumin and in anti-*Salmonella* polysaccharide antisera by amperometric titrations. Free SH groups were not observed with the native preparations but five to ten titratable groups per 10^5 gm. protein became available upon denaturation with urea or guanidine. Small numbers of SH groups (0.05–0.1 mole/10^5 gm. protein) were reproducibly detected when antigen–antibody complexes were examined. The authors stated that these findings may represent conformational changes in the antibody when reacting with antigen, but considerably more data are required before these tenuous findings can be meaningfully interpreted in the context of our current knowledge of immunoglobulin structure.

Ishizaka and Campbell (1959) prepared soluble bovine serum albumin–rabbit antibovine serum albumin complexes by dissolving equivalence precipitates with excess antigen. Free boundary electrophoresis analyses at both acid and neutral pH values permitted them to determine the overall antigen-to-antibody ratio as well as the antigen-to-antibody ratios in the different complexes. The optical rotation of the solutions was measured at the sodium D line and compared to the rotation calculated

for mixtures containing equivalent amounts of antigen and nonantibody IgG. At progressively higher antibody-to-antigen ratios, progressively greater differences were observed between the calculated and experimental values. The enhanced levorotation correlated with the amounts of those complexes showing relatively high antibody-to-antigen ratios. No excess levorotation could be attributed to those complexes thought to represent antibody saturated with 2 moles of antigen.[1] These workers obtained similar results with complexes formed from antibenzene arsonate and a trisubstituted resorcinol azophenylarsonate dye. This finding suggested that with the albumin complexes also, the calculated changes were in the antibody and not simply in the antigen. Similar studies were performed by Henney and Stanworth (1966) using rabbit antibovine serum albumin–albumin and antiferritin–ferritin complexes. Although the influence of the different types of complexes was not assessed, it is clear from their data that larger changes were seen with solutions containing higher antibody-to-antigen ratios. The latter probably explains their calculation that the ferritin–antiferritin complexes showed greater changes since complexes having equivalent weight ratios of antigen-to-antibody were compared. Ferritin has a molecular weight of 750,000, i.e., 11 times that of serum albumin. Therefore when complexes of equivalent *weight* ratios of antigen to antibody are compared, the ferritin–antiferritin complexes would have a *molar* antibody-to-antigen ratio 11 times as high as the corresponding albumin–antialbumin complexes. Their data recalculated on this basis appear to show that, if anything, ferritin complexes show a smaller effect.

The results of Ishizaka and Campbell and Henney and Stanworth have recently been challenged. Ross and Marrack (1972) examined rabbit IgG antibovine serum and its peptic $F(ab')_2$ fragments in the presence of antigen with the more sophisticated instrumentation now available. They were unable to confirm any differences in the specific rotation of the complexes at the D line. Changes were observed at wavelengths below 300 nm., but, because of large differences between successive estimates of the optical rotation on the same solution at these wavelengths, the magnitude of the changes is difficult to assess. The changes were greater than the specific optical rotation of the antibody at these wavelengths, and the antibody constituted only one-half of the protein in solution, so they concluded that the changes are mainly due to alterations in the antigen. Although measurements were made at wavelengths down to 190 nm., their inability to obtain control data for these wave-

[1] The study of Arend *et al.* (1972) (Section II,B) suggests these may have been 1:1 complexes.

lengths made it impossible for Ross and Marrack to determine if changes in this spectral region had occurred in the presence of antigen. Overall, their rotatory spectra showed no dramatic differences from what would have been anticipated from other data on uncomplexed immunoglobulins.

Ross and Marrack also examined the $F(ab')_2$ fragments of equine antidiphtheria toxin–toxin complexes. The largest differences between the calculated rotations for the individual components and the observed rotations of the complexes were in the 210–400 nm. range and occurred in the mixture with the highest antitoxin-to-toxin ratio. There is no way of determining the relative contributions of the antitoxin and toxin to the changes observed.

In all these instances the optical rotatory changes appear to correlate with the degree of antibody aggregation rather than with antibody site saturation. Even if some of these changes could be assigned to changes in the antibody, whether the differences are due to changes in the environment of amino acid side chains in the regions of contact between adjacent antibody molecules or due to changes in antibody conformation secondary to such contacts cannot be determined from these results. The data provide no direct evidence that the antigen acts as an allosteric effector.

I have already referred to the dilatometry studies of Ohta *et al.* (1970) (Section III,A,1). Those studies included experiments with anti-DNP antibody–dinitrophenylated bovine IgG complexes. Interestingly, the maximal volume changes seen with the protein conjugate were quantitatively identical to the changes seen with the monofunctional DNP–lysine. Since the latter changes could be fully accounted for by changes in the Fab regions, it might at first be assumed that the changes with the protein conjugate–antibody complexes had a similar explanation. As the authors point out, however, the situation is not so simple. As increasing amounts of the conjugate were added to the antibody solutions—to a point beyond equivalence—the volume changes decreased even though the degree of antibody site saturation presumably remained constant. It appears that there may be a number of competing changes going on in this system so that these data cannot be usefully interpreted.

A careful study on an unusual antibody–antigen system has been performed by Liberti *et al.* (1972a,b). They studied the hydrogen exchange properties of sheep antibodies reacting with calcium-dependent antigenic determinants on poly($Glu^{60}Ala^{30}Tyr^{10}$). Two effects were observed. With isolated Fab fragments, interaction with antigen resulted in a trapping of 23 to 24 hydrogens per molecule of Fab. Their analysis suggested that all or most of these were blocked from exchange by the direct presence of ligand (shielding) rather than indirectly due to some

conformational change produced by the antigen. Additional changes were observed with the $F(ab')_2$ fragments. As complexes with higher antibody-to-antigen molar ratios were approached, certain hydrogens seemed to be preferentially released. The changes in the $F(ab')_2$ fully accounted for the changes observed with intact antibody. It thus appears that either hydrogens normally "buried" via Fab region contact or hydrogens directly involved in the hinge region became accessible to solvent when the antibody reacted with antigen. This is a provocative study, and it will be most interesting to see if the site(s) of these alterations can be localized. If one can extrapolate from these findings on sheep IgG to other antibodies where the sites of complement (C1q) fixation and cytotropic activity have been better localized (see Section II), it would appear that those regions were not participating in the changes observed by Liberti and co-workers.

2. *Electron Microscopic Studies*

The electron microscopic studies of Valentine and Green (1967) on rabbit anti-DNP IgG antibodies complexed to bifunctional DNP ligands have already been mentioned (Section II,B). Several additional points should be noted: (*1*) although specifically sought for, no changes were observed in the Fc regions of such complexes, and (*2*) complexes that had been treated with pepsin and were therefore missing the Fc regions appeared otherwise similar to the untreated complexes. If the Fc regions had an important influence on the mobility of the Fab regions through a "domain of bonding," a redistribution of complexes might have been expected.

In these studies as in the earlier studies of Feinstein and Rowe (1965), the latter using ferritin–antiferritin complexes, the average angle between the Fab regions on the complexed antibodies was somewhat larger than that observed in uncomplexed antibodies. It has not been excluded that this is an artifact of preparation. In all these studies individual molecules show greatly variable inter-Fab angles.

Two types of structure have been seen with antigen-complexed IgM (reviewed in Feinstein *et al.*, 1971). In studies on IgM complexed to *Salmonella* flagella the familiar starfish shape of IgM has been observed cross-linking the strands. This structure is comparable to the structures seen with uncomplexed IgM. In addition, "staple" forms have been seen with "a prominent bar parallel to the long axis of the flagellum attached by less prominent rods" (Feinstein *et al.*, 1971). Others have seen similar "multilegged table" forms (Humphrey and Dourmashkin, 1965; Svehag and Bloth, 1967). For entropic reasons, these staplelike complexes should be extraordinarily more stable than other configurations (Crothers and

Metzger, 1972) and, at low IgM-to-antigen ratios, should be the form seen exclusively. That just the opposite is true as the IgM-to-antigen ratios are varied (Feinstein and Munn, 1966; Svehag and Bloth, 1967) strongly suggests that the staple form is energetically unfavorable because of intramolecular strains. I shall return to this point again (Section V,A,2).

3. *Antigenic Determinants*

Milgrom *et al.* (1956) found that some human sera contained antibodies reacting with antigen-complexed but not free homologous antibodies. Similar antibodies were later discovered by Milgrom (1962) in rabbit sera. In both cases it was postulated that these antibodies were directed against determinants that resulted from a change in antibody structure during the course of an occult *in vivo* serological reaction. The rabbit "anti-antibodies" were examined further by Fudenberg *et al.* (1964). They could be absorbed by complexes of any rabbit antibody–antigen system but not when the antigen was a simple mono- or bifunctional hapten. Immunoglobulin G aggregated with ethanol or bisdiazotized benzidine was also an effective absorbent. Significantly the antibodies were found to be directed against determinants in the Fab fragments and not in the Fc fragments.

In experiments of Henney and co-workers (1965; Henney and Stanworth, 1966) such anticomplex antibodies were detected after two immunizations with soluble bovine serum albumin—antialbumin complexes. The appropriately absorbed antisera reacted with dimeric rabbit IgG, but on Ouchterlony analysis the reaction with the complexes gave a slight spur over the line formed by the naturally occurring 9–10 S material. Preliminary data were said to have indicated that the new determinants were on the Fd regions, but no supporting evidence was provided. Henney and Ishizaka (1968) prepared similar antibodies in guinea pigs with aggregated human or rabbit IgG. Absorbed sera reacted only with the Fc regions of aggregated IgG of the homologous species. The complexes were active regardless of the method used to aggregate the IgG (NaOH, sodium dodecyl sulfate, heat, bisdiazotized benzidine, antigen). Also aggregates of each of the subclasses of human IgG contained the equivalent determinants. These workers observed that the antibodies would react with complexed human IgG when the latter served as the antibody (in A substance–human anti-A substance complexes) but not when it served as antigen (in human IgG–rabbit anti-human IgG complexes). With regard to this result they commented, "The finding that the characteristic antigenic determinants are revealed only in the antibody molecule when γG is used as both antigen and antibody

suggests that polymerization alone is not sufficient to induce the antigenic determinants and that certain structural alteration requirements must be met." Other explanations come to mind. When human anti-A substance antibodies combine with antigen, there is ample opportunity for the Fc regions to approach each other and they will tend to be exposed on the surface of such aggregates. In contrast, when human IgG is complexed by the rabbit antibody the Fc's of the former are surrounded by molecules of the latter and there will be less opportunity for Fc–Fc aggregation and exposure to the surface. Both types of complexes contain multiple molecules of human IgG but only the one would be expected to contain exposed aggregated human IgG.

Although often quoted as evidence for antigen-induced conformational changes, these data permit no such direct interpretation. The reaction with the aggregate-specific antibodies could have resulted (Fig. 2) (*a*) from determinant completion, i.e., by the formation of new determinants arising by the approximation of two molecules containing preexisting partial determinants, (*b*) from determinant polymerization and thereby amplifying the avidity of antibodies having a low affinity for monomeric IgG, and (*c*) from determinant transformation—the formation of new determinants due to the aggregation. It would be interesting to know if the Fab fragments of the specific anticomplex antibodies would react with the complexes; if they did it would tend to rule out the second alternative.

It is unlikely that the determinants studied by Henney and Ishizaka were related to the sites of complement fixation or cytotropic activity

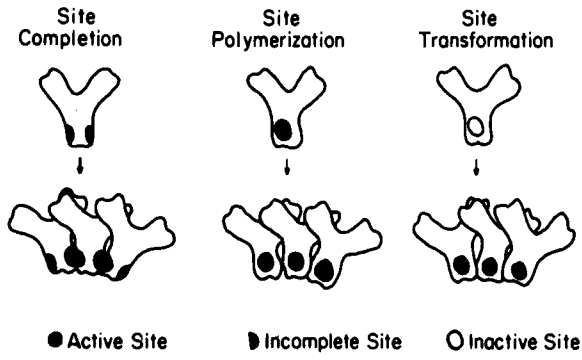

FIG. 2. Effect of immunoglobulin aggregation on Fc sites. Three alternative mechanisms by which the Fc sites become activated are illustrated. The site polymerization mechanism is the minimal model because it involves no changes in the structure of the relevant sites.

for two reasons: (*1*) the new determinants showed complete species specificity whereas this is not true for the functional sites referred to; and (*2*) all human subclasses of IgG were shown to have the equivalent determinants and this also is not true for the functional sites.

IV. Functional Properties of Antigen–Antibody Complexes

A. Complement Fixation

1. Immunoglobulin G

Uncomplexed monomeric human IgG may bind the first component of complement although the reaction is weak (Augener *et al.*, 1971), and in general it has not been possible to detect such binding with monomeric rabbit IgG (Ishizaka *et al.*, 1965; Hyslop *et al.*, 1970). Active binding is observed with aggregated IgG. The method used to aggregate the IgG appears irrelevant; heating, or cross-linking with bisdiazotized benzidine or with antigen all seem more-or-less equivalently effective. The isolated monomeric Fc fragments bind complement more or less as well as intact monomeric IgG (Augener *et al.*, 1971), and there is a corresponding increase in activity with aggregation.

Several studies have shown that two *adjacent* IgG molecules are required for activation (Borsos and Rapp, 1965a,b; Humphrey and Dourmashkin, 1965; Cohen, 1968), thereby confirming the more indirectly arrived at conclusions of Weigle and Maurer (1957). Cohen (1968) used IgG whose complement-binding activity was destroyed but whose antigen-binding activity remained intact (Cohen and Becker, 1968) in a particularly elegant study. The complement binding of mixtures of native and altered IgG declined much more sharply than would have been anticipated from the amount of native IgG in the mixtures. An analysis of the data indicated that they were consistent with a requirement for two adjacent intact IgG. This result graphically demonstrates that antigen alone is insufficient (if, indeed, required at all) to activate the IgG optimally for complement binding and that aggregation is a requirement. Although there seems to be general agreement on this latter point the mechanism by which aggregation causes optimal complement binding remains uncertain. I have already discussed some alternative ways by which "new" antigenic determinants can arise via aggregation (see Fig. 2 and Section IV,B,3). Exactly similar proposals can be suggested for how complement-fixing sites arise. For example, Henney and Ishizaka (1968) analyzed the earlier work of Ishizaka *et al.* (1963)

in much the same way as they did the data on the antigenic determinants. The earlier work involved the use of rabbit IgG, which binds guinea pig complement, and chicken IgG which does not. It was observed that rabbit antichicken IgG–chicken IgG complexes bound complement but that chicken antirabbit IgG–rabbit IgG complexes did not. Again it was reasoned that some specific denaturation was required to reveal the chemical groupings necessary for complement binding and that this could only occur when the IgG was acting as antibody and not as antigen. As discussed in the foregoing, the two types of complexes are not necessarily equivalent and the requirement for two adjacent (and exposed) IgG may simply not be fulfilled when the rabbit IgG is complexed by the chicken antibody.

Whole complement and C1 fixation by rabbit anti-DNP antibodies in the presence of bis-DNP octamethylenediamine was studied by Hyslop *et al.* (1970). They found that only those Sepharose 4B fractions containing predominantly tetrameric and higher polymers were active. Significantly a plot of the amount of C1 fixed by varying concentrations of an active fraction was linear, and fractions containing predominantly dimers were inactive over a ten-fold range of concentrations. As the authors point out, both these observations make it unlikely that C1 fixation is occurring simply to random aggregates. Instead these studies suggest that the ability to bind C1 is determined either solely by the number and relative positions of the Fc regions or by a structural change in the Fc region which occurs only in the higher polymers. The only structural difference between inactive and active complexes that they observed was in the angle between the Fab arms of each IgG. In the inactive complexes, this angle was less than 60°, whereas in the active polymers, it was between 90° and 180°. It seems to me possible that the cyclic structures demonstrated in the beautiful electron micrographs might not be active in the planar state and that it is the greater ability of the higher polymers to form structures very similar to the IgM staple form (described in the foregoing) which accounts for their enhanced activity.

Thompson and Hoffmann (1971) performed a detailed analysis of complement (C1) binding by IgG antierythrocyte antibodies of rabbits and other species (Thompson and Hoffmann, 1972). Their basic observation is that once a small amount of complement is bound, further complement binding is enhanced. When the data are plotted in an r/c versus r plot (Scatchard, 1949), the data points appear to fall on an upward-pointing convex curve. By what appears to me a somewhat subjective procedure the data points from samples at high complement inputs are analyzed as representing a linear component in order to estimate (a) the total C1-fixing sites (by extrapolation) and (b) the dissociation

constant (from the slope of the line). These values can then be inserted into the equation for homotropic cooperative reactions:

$$\bar{Y} = \frac{\alpha(1 + \alpha)^{N-1} + LC(1 + \alpha C)^{N-1}}{(1 + \alpha)^{N} + L(1 + \alpha C)^{N}}$$

This equation (Monod *et al.*, 1965) describes the behavior of a system which in the absence of a ligand is in a taut (poorly binding) state but which is shifted into a relaxed (high binding) state upon binding the first molecule of ligand. The dissociation constant of the binding sites in the taut state is denoted as K_T, and in the relaxed state as K_R. In the preceding equation, $\bar{Y}$ is the average proportion of binding sites occupied by the ligand in equilibrium with a free ligand concentration (c); α equals c/K_R; L equals the ratio of the taut-to-relaxed forms in the absence of ligand; N equals the number of binding sites per system; and C is the ratio K_R/K_T. In the Thompson–Hoffmann analysis, a cluster of IgG is considered the system, and C1 is considered the ligand. By using their values of $\bar{Y}$ and α, a series of Scatchard plots can be computed for different values of N, L, and C with the foregoing equation. The values of N, L, and C that yield the best fit for the experimental data are accepted. In this way they estimate that the critical cluster size contains twenty binding sites for complement, that L is approximately 3–4, and that C is approximately 0.002. In brief, they hypothesize that, when IgG becomes aggregated, subsequent conformational changes occur in concert for each such cluster. When C1 becomes bound, the cluster is shifted into an active state thereby increasing the clusters' avidity for additional molecules of complement. In the later studies (Thompson and Hoffmann, 1972) they show that their complicated experimental procedure is not itself responsible for the observed cooperativeness. They also show that a variety of other explanations based on (*1*) heterogeneity of antibody sites for C1, (*2*) heterogeneity of C1-binding sites, and (*3*) binding of unbound C1 to bound C1 are inadequate to explain the results without additional *ad hoc* assumptions. Leaving the question of the ambiguity of the actual data aside (which would tend mainly to influence the accuracy of their estimates of N, L, and C), I feel that an important alternative explanation has not been adequately considered. That is, if Clq is multivalent with respect to binding sites for IgG (Müller-Eberhard and Calcott, 1966; Shelton *et al.*, 1972; Svehag *et al.*, 1972; Yonemasu and Stroud, 1972) and IgG has more than one binding site for C1q [the activity of monomeric C_H2 fragments (Ellerson *et al.*, 1972) supports this possibility], then the first molecule of bound complement would stabilize an IgG aggregate in a configuration favoring the binding of additional C1q. This explanation is similar to Thompson and Hoffmann's

but has the critical differences that (*a*) multivalence of IgG for complement and vice versa is required and (*b*) no change in the binding sites on IgG for C1q is postulated.

Their binding data suggests that L, the ratio of taut-to-relaxed forms, is surprisingly small. If this value also applied to uncomplexed antibody, one would expect to see appreciable C1 binding by uncomplexed IgG. Also the requirement for a cluster of IgG is unexplained. Hoffmann (1972) postulates that the low L is only for antigen-bound forms and that, in the absence of antigen, L is considerably larger; that is, the antigen is considered to be a heterotropic allosteric effector. Space does not permit a more complete account of Hoffmann's proposal which is a well worked out idea fitting many of the data and making several testable predictions. There is so far no independent evidence to support the hypothesis.

Henney and Ishizaka (1968) interpret the fact that complement fixation and the production of new antigenic determinants can arise regardless of the method used to aggregate the Fc regions as evidence that a structural change occurs in the Fc. Augener *et al.* (1971) reached the opposite conclusion from the same data! Although I am more sympathetic to the latter logic, it is clear that more facts are needed.

2. *Immunoglobulin M*

Monomeric IgM uncomplexed to antigen binds complement weakly (Augener *et al.*, 1971; MacKenzie *et al.*, 1971); nonspecific aggregates or soluble IgM–antigen complexes bind C1 somewhat more avidly (Augener *et al.*, 1971; Ishizaka *et al.*, 1968); and IgM complexed to particulate antigens binds C1 most tightly (Ishizaka *et al.*, 1968). Dose–response curves indicate that single molecules of particulate-bound IgM are capable of binding complement (Borsos and Rapp, 1965a,b; Ishizaka *et al.*, 1968). These observations provide some of the more convincing evidence that the IgM immunoglobulin must undergo some conformational change in order that the sites which will bind complement are activated. However, it must be remembered that IgM is itself an aggregate of IgMs subunits. It seems possible that just as the IgG circular complexes of Hyslop *et al.* (1970) (already described) must have a specific topological arrangement of the Fc regions for full activity, so the activity of the IgM may also be related to its conformation. That is, it may be that when the IgM forms a staple conformation upon binding to a particulate antigen the binding sites for C1q are then brought into an appropriate spatial relationship. With such a mechanism, no change in the tertiary structure of the sites on the IgMs would be necessary. If there is more than one Clq-binding site per IgMs, then once one mole-

cule of C1q was bound it would stabilize the energetically unfavorable staple conformation of the IgM making the binding of an additional molecule of C1q more favorable. Thus, the apparent cooperativity of C1 binding observed by Thompson and Hoffmann for both IgG and IgM could be explained without evoking an allosteric mechanism.

There is one study which is difficult to reconcile with the hypothesis that no changes of the complement-binding sites on IgM are required. Plaut *et al.* (1972) studied the fixation of human and guinea pig complement by human IgM and its Fc fragments. They observed that the isolated $(Fc\mu)_5$ fragment was considerably (7–19 times) more active than the parent IgM on a molar basis. These fragments were produced by briefly digesting the IgM with trypsin at 65°C. It is uncertain what the activity of their IgM would have been had trypsin been omitted, but it was remarkable (and unexplained) that the unfractionated digest was considerably less active than the isolated $(Fc\mu)_5$. The structure of these fragments is undoubtedly altered from what exists in the native state since electron micrographs of material prepared by the foregoing procedure show rather unusual spiral rod forms quite unlike what one would anticipate from the circular structure of the Fc in the intact molecules (E. Shelton and H. Metzger, unpublished observations, 1970). One of the most interesting observations of Plaut *et al.* is that reduction and alkylation of the pentameric Fc (which leads to the formation of $Fc\mu$, mol. wt. 67,000[2]) did not diminish the specific complement-binding activity. These results suggest that a conformational change in the region of the complement-fixing sites may be important and that the high-temperature tryptic proteolysis has fortuitously induced a similar distortion.

B. Antigen–Antibody Complexes on Cell Surfaces

1. Nonreaginic Cytotropic Antibodies

Systemic or localized anaphylactic reactions can be triggered by antigen when the appropriate tissues are sensitized with IgG antibodies. It has been known for almost 50 years (Landsteiner, 1924) that, with rare exceptions, haptens will not only fail to elicit such reactions but will actually inhibit them. With avid antibodies, bifunctional ligands are adequate (Parker *et al.*, 1962; Hurlimann and Ovary, 1965; Levine, 1965b; Mancino *et al.*, 1969). If bifunctional ligands with two different determinants are used the animal must be sensitized with the antibodies directed against both functional groups (Levine, 1965a). Similarly in reversed anaphylaxis (in which it is the antigen, e.g., human IgG, which

[2] Not mol. wt. 34,300 as stated in Plaut *et al.* (1972).

becomes bound to the tissues), a reaction is elicited only by bivalent antibodies or antibody fragments (Ovary and Taranta, 1963). These results suggest that aggregation or cross-linking of the cell-bound immunoglobulin is a critical step. Indeed, if the capacity of a series of homologous bifunctional ligands to elicit passive cutaneous anaphylaxis (PCA) reactions (Levine and Redmond, 1968) and their ability to polymerize IgG (Green *et al.*, 1972) are compared (Fig. 3), there is quite a good correlation. It is apparent exceptions that "prove" (i.e., test) a rule, and this is certainly a valid criterion in the present case. Several studies have shown that some apparently monofunctional ligands are capable of eliciting a PCA response (Amkraut *et al.*, 1963; Frick *et al.*, 1968; Green *et al.*, 1970; Rosenberg *et al.*, 1971). For example, Frick *et al.* (1968) studied PCA reactions in guinea pigs using anti-DNP antibodies. Whereas α-butanoyl, α-hexanoyl, and α-hexahydrobenzoyl analogs were inactive, the α-benzoyl, α-carbobenzoxy, α-octanoyl, and α-decanoyl derivatives worked. In earlier studies it had been shown that different sensitizing sera gave distinctive reactions (Amkraut *et al.*, 1963). A variety of control experiments have made some of the most obvious explanations [aggregation of the ligands and binding of the

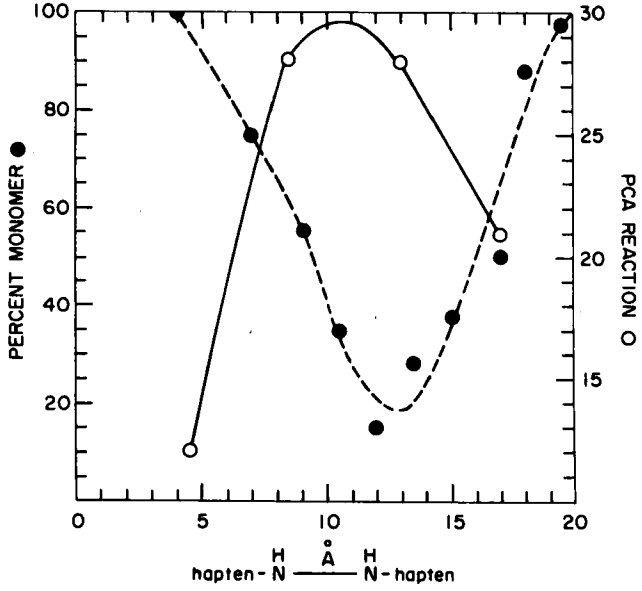

FIG. 3. Effect of distance between determinants on a divalent ligand and (*1*) immunoglobulin aggregation (Green *et al.*, 1972) and (*2*) passive cutaneous anaphylaxis (PCA) response (Levine and Redmond, 1968). In the latter case averages of all the data are given in arbitrary units.

ligands to serum proteins (Amkraut *et al.*, 1963; Frick *et al.*, 1968)] unlikely, but the explanation offered by de Weck *et al.* (1973) is a serious possibility. These workers showed that agarose beads coated with monofunctional ligands are capable of eliciting PCA reactions even though the unbound ligands inhibit rather than elicit reactions. The bead-bound ligands are rather inefficient, but this is also true of those monofunctional ligands that give PCA reactions. Orders of magnitude higher concentrations of these ligands are required to give reactions comparable to those produced by the multifunctional analogs. De Weck *et al.* point out that most of the univalent eliciting haptens possess hydrophobic side chains which would be likely to stick to cell membranes. They suggest that "cell membranes might provide the matrix on which apparently monovalent reagents will bind and form a multivalent binding antigen."

Another instance which at first sight appears to contradict the associative model for antigen action in these cases is the effectiveness of nonprecipitating antibodies in sensitizing tissues. Such antibodies fail to cross-link antigens and appear to form cyclic complexes with multifunctional antigens (Klinman and Karush, 1967). From a functional point of view, they behave like univalent antibodies (although with small monofunctional ligands they appear to have two perfectly normal combining sides). Such antibodies can sensitize tissues (Kabat and Benacerraf, 1949; Kuhns and Pappenheimer, 1952; Ovary and Biozzi, 1954; Margni and Binaghi, 1972) often as efficiently as precipitating antibodies. I think that the explanation here may be rather simple. Such populations are typically heterogeneous so that when the tissue mast cells are sensitized, antibodies directed against a variety of determinants on the antigen would be expected to bind to any individual cell. Therefore, even though lattice formation would not be possible, that is, no molecule of antibody can link two molecules of antigen, molecules of antigen can bridge two or more molecules of antibody.

2. *Reaginic Antibodies*

Although some IgG antibodies may exhibit reaginlike properties (Parish, 1973), the significant immunoglobulin involved in these reactions is IgE. Monomeric IgE binds firmly to the surface of peripheral basophiles or tissue mast cells but does not by itself cause histamine release. When cross-linked by antigen, anti-IgE or anti-light-chain antibodies, or any other means, histamine release can occur (Ishizaka and Ishizaka, 1971). Isolated Fc fragments of IgE also bind to the cells and can also trigger cell degranulation when cross-linked by antibodies or chemically. Univalent Fab fragments of anti-IgE antibodies are incapable

of stimulating these cells. These observations suggest that bridging of two or more IgE molecules is essential for these reactions (Ishizaka and Ishizaka, 1971). At high levels of anti-IgE, histamine release is inhibited. A ready explanation for this finding is that 1:1 complexes of anti-IgE and IgE are formed so that bridging does not take place. Recent experiments in our laboratory suggest that this explanation may be too facile. Sullivan *et al.* (1971) and Becker *et al.* (1973) observed that the surface IgE became aggregated into large surface patches and caps when reacted with anti-IgE, similar to the redistribution of surface immunoglobulin on lymphocytes in the presence of anti-Ig's (Taylor *et al.*, 1971; Loor *et al.*, 1972). The Fab fragments fail to produce such a redistribution of surface IgE regardless of the dose used (Becker *et al.*, 1973), suggesting that patching and capping requires bridging of the surface IgE. Surprisingly, such patching and capping were almost exclusively observed at high levels of anti-IgE when histamine release is inhibited (Sullivan *et al.*, 1971; Becker *et al.*, 1973). These results suggest that, although IgE bridging is required to trigger degranulation, some forms of aggregation can be inhibitory. The molecular mechanisms involved can only be guessed at this time, but it is fascinating that these results in many ways paralleled the results obtained with B lymphocytes exposed to antigens of varying determinant densities (Feldman, 1972). Excess antigen added to sensitized cells can also cause inhibition of degranulation, but, under these circumstances, no redistribution of surface IgE was observed. Only a small proportion of the surface IgE is likely to be capable of binding the specific antigen in this situation and here excess antigen may lead to a failure of bridging due to the formation of 1:1 antigen–IgE complexes.

3. *Antibodies of Lymphocytes*

a. B Cells. Antisera to immunoglobulins can stimulate the incorporation of thymidine by lymphocytes (Sell and Gell, 1965). Since with few exceptions anti-Ig fail to detect immunoglobulins on T cells, the reaction in question can be attributed to B cells. When rabbit lymphocytes and antirabbit immunoglobulin antisera are employed, the stimulation can be observed with antisera specific for *b*-locus markers (on the constant region of the light chain), *a*-locus marker (probably on the variable region of the heavy chain), and class-specific markers (on the constant region of the heavy chains) (Sell, 1967; Sell and Gell, 1965). It would be remarkable, indeed, if antibodies directed to these different regions on the receptor molecules could all bring about the same conformational change at some distant site in the Fc region. It seems more likely that the common feature is the capacity of all the antisera to cross-link the surface immunoglobulin. There is still some controversy

regarding the capacity of Fab fragments from certain anti-Ig antisera to mediate these effects (Sell *et al.*, 1970; Fanger *et al.*, 1970). There seems no doubt, however, that bivalent antibodies are considerably more active. Although these experiments show that the cells are stimulated (transformed), it is unclear to what such reactions are analogous in a normal immune response. Much more needs to be learned about the functional aspects of these reactions.

When antigens are used in such studies the situation becomes much more complicated. It is known that B-cell stimulation can involve multicell interactions (T cells, macrophages), and it is uncertain at this time to what extent these other cells play a role in antigen-induced lymphocyte stimulation. In a system that apparently is measuring only B-cell–antigen interactions, it was noted that cell stimulation required multifunctional antigens. Notably this was true for cell stimulation which ultimately led to either tolerance or immunity. Monofunctional antigens produced neither effect (Feldman, 1972). If an associative action of antigen is required to signal these cells (each of which is coated with homogeneous receptors), it is clear why globular proteins, which lack repeating determinants, would be ineffective and would require help. Such help could be provided by a multivalent antibody or the surface of a second cell either of which would convert the antigen to a multifunctional one. This can be contrasted to the situation on the surface of mast cells and basophiles where the heterogeneity of the adsorbed antibody assures that even antigens without repeating determinants are multifunctional.

b. T Cells. Antigens interact with T cells, but it remains to be more fully documented that this interaction is mediated by surface immunoglobulin. It is known that seemingly monofunctional ligands can stimulate such cells. For example, cells from animals stimulated by various monodinitrophenyl oligolysine analogs will respond to these latter compounds (Schlossman, 1972). It seems possible that the same mechanism as proposed by de Weck *et al.* (1973), by which monofunctional ligands can stimulate cytotropic antibody-induced histamine release, might be relevant here. Indeed, Waldron *et al.* (1973) have recently obtained data that suggest that the antigens must first interact with macrophages. It may be, therefore, that the monofunctional ligands are presented to the appropriate lymphocyte in the form of cell-bound multifunctional antigens.

V. Summary and Conclusions

In this review I have described the currently available data on the structure of intact immunoglobulins and their degradation products, on the characteristics of antigen–antibody complexes, and on the require-

ments for antigen-induced antibody function in complement fixation and stimulation of immunoglobulin-bearing cells.

The structural data indicate that immunoglobulins are organized into domains consisting of strongly interacting homology regions. The domains appear to interact relatively little. Similarly the evidence is good that the Fab and Fc regions behave quite independently of each other. What lack of flexibility exists in the molecule is more likely secondary to steric inhibition of movement rather than strong interactions at "domains of bonding." Thus, from structural considerations alone, it seems unlikely that antigens reacting with the combining site at the NH_2-terminal end of the molecule could induce changes in the more distal regions. Studies of antibodies complexed to monofunctional ligands have failed so far to provide evidence for such distal changes. All the evidence suggests that those changes that do take place are due mainly to stabilization of chain structure and shielding. Small configurational changes have yet to be convincingly documented. The circular dichroism changes observed by Holowka *et al.* (1972) could reflect such alterations, but it will have to be seen whether these changes occur outside the Fv region. Those studies purporting to show negative (Kelly *et al.*, 1971) or positive (Zimmering *et al.*, 1967) cooperative binding phenomena require further data before we can interpret them.

The studies of antibodies complexed with multifunctional ligands have evinced changes in antigenic determinants, complement-binding sites, and, most significantly, stimulation of a variety of cells. It is unlikely that these result from a distortive action of antigen. The most likely area on the immunoglobulin molecule where antigens would produce changes is in the region of the inter-Fab angle, but we know of no important binding sites in those regions.

Thus the weight of the evidence speaks against both the allosteric and distortive models for antigen action. We are left with the associative model. I know of no data that are inconsistent with this model (although the promotion of complement binding by antigen binding in IgM requires that in this case the association be an intramolecular one), and there is much evidence in favor of it. What remains obscure is the mechanism by which the approximation of the Fc regions leads to the changes in which we are interested. On the face of it, the valence polymerization model (see Fig. 2) seems simplest. It makes two predictions, one of which may be experimentally testable in the near future. The first prediction is that the critical sensor for antigen–antibody interaction will itself be polyvalent. In the case of complement activation this appears to be true, although the multivalency of C1q requires further substantiation. In the case of cell-bound sensors, either the receptors for

the immunoglobulin or molecules interacting with these receptors would have to be polymeric. It may be quite some time before such molecules are available for detailed study. The site polymerization model also predicts that a monovalent subunit of such a sensor will not bind any more strongly to aggregated immunoglobulin than to the uncomplexed monomeric antibody. Work on C1q is proceeding apace and this experiment may be feasible before too long. Both the site completion and site transformation models make the opposite prediction with regard to such an experiment. That is, if a monovalent subunit of C1q became available, it should show an increased affinity for aggregated versus monomeric antibody if the latter models are correct.

Our understanding of the immune response has exhibited a circadian rhythm—there are intervals of light and darkness. Complex interactions of a variety of cells and humoral factors appear to be involved, and the effect of antigen on antibody appears to play a key role. The complexities of even a relatively simple system such as the function of the IgE receptors on basophiles (Section V,B,2) teaches us that much remains to be learned.

My own conclusion from the data presented in this review is that postulating a variety of specific conformational contortions on the part of immunoglobulins is not one of the more promising paths out of the woods.

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Lymphocyte-Mediated Cytotoxicity and Blocking Serum Activity to Tumor Antigens[1]

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I. Introduction

It is more or less accepted today that most tumors in experimental animals, as well as in man, have antigens that at times are tumor-specific

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and in other cases, are better characterized as tumor-associated because they are also present in small amounts in certain normal tissues (Old and Boyse, 1964; Sjögren, 1965; Hellström and Möller, 1965; G. Klein, 1966, 1969, 1971; Southam, 1967; Alexander, 1968; Smith, 1968, 1972; Haughton and Nash, 1969; Fisher, 1971; Cinader, 1972; Oettgen and Hellström, 1973). It is also accepted by most investigators that immune cells play a major role in the host's immunological defense against neoplasms (Klein *et al.*, 1960; K. E. Hellström and Hellström, 1969; Smith, 1968; McKhann and Jagarlamoody, 1971; Oettgen and Hellström, 1973).

Lymphocytes from both animals and human patients cured of tumors can often destroy cultivated cells from the respective neoplasms. The same is also true in many cases when the lymphocyte donors have a growing tumor mass. The latter observation raises the question why the immune lymphocytes are not able to eradicate all growing neoplastic cells *in vivo*. An answer to this question might lead to the development of better tumor therapy.

There are probably many mechanisms by which antigenic tumors can escape from immunological destruction by host lymphocytes. Serum factors (antigen–antibody complexes, antigens, antibodies) capable of blocking the cytotoxic effect of sensitized cells *in vitro* appear to provide one of the more important of these escape mechanisms.

In this article, we will discuss papers dealing with the *in vitro* destruction of animal and human tumor cells by immune lymphocytes, the detection of blocking serum factors capable of abrogating the cytotoxic lymphocyte effect, and the possible *in vivo* roles of these parameters. We have not attempted to give a complete coverage of the literature but will concentrate on work performed during the last few years. For more background in this area, we recommend several relatively recent reviews such as by K. E. Hellström and Hellström (1969, 1970, 1972), Klein (1971), McKhann and Jagarlamoody (1971), Fisher (1971), Cinader (1972), Feldman (1972), and Oettgen and Hellström (1973).

We want to make clear from the onset of this article that host reactivity to a growing tumor is a much more complex event than can be properly understood by just studying whether or not cultivated tumor cells can be killed by the tumor-bearing individual's blood lymphocytes (or lymph node cells) and whether or not tumor-bearer serum can interfere with the lymphocyte effect—the two aspects of the immune response that are discussed here. For example, rejection of an antigenic neoplasm is mediated not only by specifically cytotoxic lymphocytes (and macrophages), the activities of which can be demonstrated *in vitro* with techniques commonly employed today but also by originally noncommitted lymphocytes that are recruited into the reaction and by various

inflammatory cells including macrophages. One has to expect, therefore, that there is a multitude of escape mechanisms by which the process that leads to rejection can "go wrong," making it possible for an antigenic tumor to persist.

One should also keep in mind when reading this article that humoral antibodies may play an important role in counteracting tumor growth *in vivo* by destroying neoplastic cells in the presence of complement (Lewis *et al.*, 1969; Hellström *et al.*, 1968c, 1969b; Wood and Morton, 1970) in addition to influencing tumor growth by the mechanisms we will discuss here. The relative roles of such cytotoxic antibodies and specifically sensitized cells in tumor immunity are not well known.

II. Evidence for Role of Immune Lymphocytes in Rejection of Antigenic Tumor Cells

We will first recapitulate some of the evidence which has led to the conclusion that the rejection of antigenic tumors is to a large extent mediated by cells (lymphocytes and macrophages). For further discussion of this topic, see Gowans and McGregor (1965), Wilson and Billingham (1967), Perlmann and Holm (1969), K. E. Hellström and Hellström (1969), Smith (1968, 1972), Smith and Landy (1971), Burnet (1971), McKhann and Jagarlamoody (1971), and Oettgen and Hellström (1973).

Mitchison showed in 1955 that immunity to tumor allografts containing foreign H-2 antigens could be adoptively transferred with lymphoid cells but not with serum. Winn (1959) developed a neutralization test with which he found that the outgrowth of tumor cells in syngeneic hosts was inhibited if the cells were inoculated together with lymphocytes from allogeneic donors previously immunized against the alloantigens of the tumors. Serum did not appreciably inhibit the tumor takes, except when leukemias were used as targets. Analogous results were obtained by Klein and Sjögren (1960).

Klein *et al.* (1960) demonstrated that tumor-specific immunity, as allograft immunity, is mediated by cells, and work by others has supported this notion (Old and Boyse, 1964). Tumor immunity can be adoptively transferred by lymph node and spleen cells and admixed lymphoid cells from mice immunized to specific antigens of syngeneic tumors can inhibit the outgrowth of the respective neoplasms in neutralization tests.

Immunological resistance to tumors is similar to allograft immunity, because it involves reactions of the delayed hypersensitivity type. This can be exemplified by the experiments of Bloom *et al.* (1969), which showed that soluble antigens prepared from chemically induced tumors of inbred guinea pigs could elicit delayed hypersensitivity reactions when

inoculated intradermally into syngeneic animals previously immunized against the same tumors. The immunized guinea pigs were found to be resistant to challenge with other tumors lacking the relevant antigen. Similar studies have demonstrated delayed hypersensitivity reactions against chemically induced mouse sarcomas (Halliday and Webb, 1969). Both in guinea pigs and in mice, the tests for delayed hypersensitivity often become negative when there are massive growths of tumor.

Another line of evidence pointing to the importance of cell-mediated immune reactions against neoplasms comes from work showing that procedures depressing such reactions can precipitate tumor development, both in animals and in man. Mice and rats, neonatally thymectomized or given immunosuppressive drugs or antilymphocytic serum, develop more primary tumors after infection with certain oncogenic viruses than do control animals, and they are more susceptible to certain—but not to all (Wagner and Haughton, 1971)—forms of chemical carcinogenesis (Ting and Law, 1967; Vandeputte, 1969; Allison, 1970; McKhann and Jagarlamoody, 1971). Human patients who have been immunosuppressed so as to be able to accept foreign organ grafts have a much increased risk of developing malignancies, particularly of the lymphoid system (Penn *et al.*, 1971; Good, 1972). Likewise, patients with immunological deficiencies involving cell-mediated immune reactions get more tumors than do normal subjects (Good, 1972). This kind of evidence is in agreement with the postulate by Thomas (1959) and by Burnet (1965, 1971) that cell-mediated immune mechanisms can provide a surveillance against neoplasia but does not at all prove it (Editorial, 1972).

Further support for the concept that host reactivity to tumors plays a role in guarding against neoplasia *in vivo* might be derived from work showing that agents, such as Bacillus Calmette–Guérin (BCG), capable of nonspecifically increasing cell-mediated reactivity, when properly given, can also prolong the latent period before antigenic primary tumors appear and prevent such appearance altogether, at least in some systems (Old *et al.*, 1961; Weiss *et al.*, 1961; Ankerst and Jonsson, 1972), as well as having an immunotherapeutic effect *in vivo* (Zbar *et al.*, 1970, 1972; Mathé, 1971; Bartlett and Zbar, 1972).

III. Techniques for Studying Lymphocyte-Mediated Cytotoxicity and Blocking Serum Activity *in Vitro*

In vitro assays have been established to measure various functions of sensitized lymphoid cells. The effects of lymphoid cells on target cells (destruction or damage) and the responses of lymphoid cells (stimulation of deoxyribonucleic acid (DNA) synthesis and release of soluble mediators such as lymphotoxin and macrophage migration inhibitory

factor) have been studied. These assays have been helpful also when one looks for blocking serum factors. We will briefly present some of the assays in order to make the studies to be discussed more understandable.

The present tests of tumor cell destruction by immune lymphocytes have developed from cruder techniques introduced in the early 1960s, according to which the effects of immune lymphocytes (or macrophages) on neoplastic cells were demonstrated as microscopically detectable cytopathic changes in growing tumor cell monolayers (Granger and Weiser, 1964) or as decreased numbers of tumor cells still alive according to supravital staining (Rosenau and Moon, 1961; Takeda, 1969).

The colony inhibition (CI) assay was introduced to provide a more quantitative as well as a more sensitive test for cellular immunity (Hellström, 1967; Hellström and Hellström, 1971). Cells to be used as targets (normal or neoplastic) are first explanted and maintained *in vitro* for at least a few days prior to the tests. Trypsinized suspensions of cultivated target cells are then prepared, seeded into 50-mm. petri dishes, and allowed to attach to their surfaces. The following day, the culture medium is removed and the diluted serum to be checked for blocking activity added (the same dilution of control serum is added to other petri dishes). The target cells are incubated with the serum for 45 minutes; the serum is then removed; the dishes are washed and lymphocytes added. Blood lymphocytes, lymph node cells, or spleen cells from specifically immune (and control) donors are used in doses of 1 to 5×10^6 lymphocytes per petri dish. The dishes are incubated for 3 to 7 more days, after which the lymphocytes are removed by washing and the target cells stained. By plating in agar, one can study target cells not able to attach to plastic as well (Heppner and Kopp, 1971; Hewetson *et al.*, 1972). The tests are scored by counting the number of colonies growing out from the plated cells in experimental groups as compared to controls. The ability of a serum to abrogate the inhibitory effect of immune lymphocytes on colony formation is measured.

A "microcytotoxicity" assay was developed from the original CI test (Takasugi and Klein, 1970; Hellström and Hellström, 1971). The principle of this assay is to seed tumor cells into the wells of Microtest plates instead of adding them onto petri dishes and to count the number of cells remaining attached to the bottom of the wells following exposure to immune lymphocytes (rather than counting the number of colonies). The ability of a serum to block lymphocyte-mediated destruction of the target cells is, as in CI assays, generally measured by exposing the target cells to the serum before the lymphocytes are added. The preferred size of the plates in which the tests are performed has varied among different investigators; some use a plate in which the bottom surface of

the wells is 2 mm. (Takasugi and Klein, 1970), whereas others prefer wells with 7-mm. bottom surface (Hellström and Hellström, 1971). The size of the wells does not appear to have any appreciable effect on the findings obtained, but the advocates of large wells emphasize greater ease in keeping the tumor cells at a suitable pH when ratios between lymphocytes and target cells are large.

The microcytotoxicity assay has to a great extent replaced the original CI test, since it is technically simpler, takes less time, and works with smaller amounts of target cells, lymphocytes, and sera. The results of the CI and microcytotoxicity assays have been in good agreement when the two techniques have been compared (Hellström *et al.*, 1971a).

Rather than count visually the number of target cells surviving exposure to lymphocytes, one can label them with an isotope, such as ^{51}Cr (Brunner *et al.*, 1968), tritium-labeled proline (Bean *et al.*, 1973), or ^{131}I-labeled deoxyuridine (Cohen *et al.*, 1971, 1972), which is released upon cell damage or one can assay for target cell utilization of some compound, such as tritium-labeled thymidine (Jagarlamoody *et al.*, 1971). One advantage of labeling is that one can perform competitive experiments by adding nonlabeled cells (of the same or different specific antigenicity), which makes it possible to work also with cells that do not grow well *in vitro* (de Landazuri and Herberman, 1972c). If a ^{51}Cr label is used, the time span of the test is commonly a few hours, because longer incubation periods allow release of ^{51}Cr from viable cells. As will be discussed later, this short incubation period may influence the findings when compared to those obtained with the standard (30–40 hr) microcytotoxicity assay.

Two other techniques, although they do not measure lymphocyte-mediated cytotoxicity, will be taken up here as well, since results obtained with these techniques have corroborated data obtained in assays of tumor cell destruction (and are referred to below). These are the lymphocyte transformation technique and the macrophage migration inhibition test.

The former technique is based on the transformation of lymphocytes into lymphoblasts when stimulated with antigen; the increased DNA synthesis by the stimulated lymphocytes is generally measured (Bach and Voynow, 1966; Adler *et al.*, 1970). This technique has been used extensively in typing human donors for tissue grafting. It has been employed to some extent in studies on tumor immunity (Stjernswärd *et al.*, 1970; Forbes *et al.*, 1973). Blocking activity can be also assessed (Oppenheim, 1972; Vanky *et al.*, 1971) as decreased stimulation of DNA synthesis in the presence of the blocking serum.

The macrophage migration inhibition test (David, 1971; Bloom, 1971)

has also been used to study cell-mediated tumor immunity and its blocking by serum. The migration assays are performed, as a rule, by enclosing peritoneal cells from sensitized animals (or blood cells from immune human patients) in capillaries that are placed in culture medium to which the specific antigen (or control material) is added. The extent to which the migration of the macrophages out of the capillaries is inhibited upon their contact with antigen is determined. This inhibition is probably due to the production of macrophage migration inhibitory factor (MIF) by sensitized lymphocytes. When the assay is used to search for blocking serum factors, the material to be tested for blocking is added to the medium in which the capillaries are placed (Bernstein and Wright, 1971; Halliday, 1971, 1972).

Another assay, the leukocyte-adherence inhibition test, has been recently described (Halliday and Miller, 1972). This test is based on the finding that leukocytes from immune donors adhere less to a glass surface in the presence of the specific antigen. It has been reported that tumor-specific immunity, as well as its blockade with serum, can be detected this way, but it remains to be seen whether this assay provides a sensitive and reproducible way of measuring cell-mediated immunity to tumor antigens.

For a further (and much more detailed) description of various techniques by which to study cellular immunity, see Bloom and Glade (1971) and, for a discussion of the usefulness (and limitations) of the *in vitro* tests of tumor immunity commonly employed, see Oettgen *et al.* (1972).

IV. *In Vitro* Demonstration of Cell-Mediated Immunity to Tumors

The development of *in vitro* assays by which various aspects of cell-mediated immune reactions to tumors can be analyzed has made possible some understanding of the role of such reactions to tumors in animals and in man. Before discussing findings obtained in this area, we want to point out, however (as will be discussed in section V), that our knowledge of the cell types involved in the reactions studied is very limited and that killer cell populations referred to as "lymphocytes" often contain other cells as well, including macrophages.

A. Animal Tumors

One of the first reports of lymphocyte-mediated immunity to tumors was published by Yoshida and Southam (1963), who presented suggestive, but not definite evidence that spleen cells from mice sensitized to a chemically induced sarcoma had a cytotoxic effect on cultivated cells from the same sarcoma. Similar findings were obtained by Takeda (1969). Rosenau and Morton (1966) showed more conclusively that

mice sensitized to transplanted methylcholanthrene-induced sarcomas had lymphocytes capable of destroying cultivated cells from the respective tumors. Analogous findings could be obtained with the CI assay which permitted better quantitation of the effects obtained (Hellström and Hellström, 1967; Hellström *et al.*, 1968a). With that assay it was possible to show specific destruction of cultivated tumor cells by lymphocytes also in the autochthonous situation, when lymphocytes and tumor were both taken from the host in which the tumor had originated. This finding was important because it indicated that the host possessed the immunological ability to react against its own neoplasm (without further immunization), although this ability might not suffice for producing tumor rejection.

Subsequent studies from many laboratories have demonstrated cell-mediated cytotoxic reactions against a large variety of tumors in various experimental animals. The list of tumors investigated includes Moloney virus-induced sarcomas (I. Hellström and Hellström, 1969; Lamon *et al.*, 1972a; Leclerc *et al.*, 1972); spontaneous mouse mammary carcinomas (Heppner and Pierce, 1969; Heppner and Kopp, 1971); Shope virus-induced rabbit papillomas and carcinomas (Hellström *et al.*, 1969a); polyoma virus-induced tumors in mice and rats (Sjögren and Borum, 1971; Datta and Vandeputte, 1971); adenovirus 12-induced sarcomas in mice (Hellström and Sjögren, 1967; Ankerst, 1971); Schmidt–Ruppin–Rous virus-induced sarcomas in mice (Sjögren and Jonsson, 1970); Rous virus-induced sarcomas in chicken (Jonsson, 1969) and in Japanese quails (Hayami *et al.*, 1972); SV 40 virus-induced sarcomas in hamsters (Dierlam *et al.*, 1971); Gross virus-induced lymphomas in rats (Wright *et al.*, 1973); chemically induced sarcomas in mice and rats (Hellström *et al.*, 1970c; Kikuchi *et al.*, 1972); chemically induced hepatomas in rats (Baldwin and Embleton, 1971; Baldwin *et al.*, 1971); chemically induced sarcomas in guinea pigs (Cohen *et al.*, 1972); sarcomas induced by implantation of plastic discs in mice (Hellström *et al.*, 1968a).

Cell-mediated tumor immunity can commonly be detected at least as early as 5 to 7 days after transplantation of syngeneic tumor grafts (Hellström *et al.*, 1970c). This finding parallels the demonstration of so-called concomitant tumor immunity to a second tumor transplant as early as 4 days after animals have received a first tumor graft, the concomitant immunity being detected as rejection of the second graft (Zarling and Tevethia, 1973a).

It is significant that two independent *in vitro* assays of tumor immunity have, to the extent they have been utilized, given results in good agreement with those obtained with the microcytotoxicity test. Inhibition of macrophage migration has been detected following exposure of peri-

toneal cells from immune animals to antigens from the respective tumors (Bloom *et al.*, 1969; Halliday and Webb, 1969; Tompkins *et al.*, 1970; Churchill *et al.*, 1972; Meltzer *et al.*, 1972; Vaage *et al.*, 1972; Blasecki and Tevethia, 1973; Smith *et al.*, 1973), and studies performed with the lymphocyte transformation technique have demonstrated specific stimulation of DNA synthesis in lymphocytes from immune animals upon contact with tumor antigens of the respective types (Forbes *et al.*, 1973). The latter findings are particularly interesting. It was observed that regional lymph node cells from mice grafted with a syngeneic methylcholanthrene-induced sarcoma, underwent transformation following exposure to antigen preparations from the same sarcoma, but not when exposed to antigens from other sarcomas, during the first 5–14 days following tumor grafting. Subsequently, reactivity developed to antigens from other methylcholanthrene-induced sarcomas, suggesting that some degree of cross-reactivity between them may have existed (although other explanations of these findings may be possible).

As a rule, data obtained from *in vitro* studies on cell-mediated cytotoxicity have correlated with the *in vivo* situation, in that tumors cross-reacting *in vivo* have done so when studied *in vitro,* whereas, to the extent this has been studied, tumors not cross-reacting *in vivo* have not cross-reacted *in vitro,* either. There is one possible exception to this rule, however, at least if tumor antigenicity is determined by standard transplantation tests (Prehn and Main, 1957; Sjögren, 1965) in which the ability of an immunized animal to reject a syngeneic tumor transplant is measured. Brawn (1970), Dierlam *et al.* (1971), and Baldwin *et al.* (1972a) have shown that lymphocytes from repeatedly pregnant animals, as well as from animals immunized against fetal tissues, are cytotoxic to cultivated neoplastic cells from various sources (including chemically induced sarcomas) but not to cultivated cells of normal origin. Before one argues, however, that the immunity so detected is ineffective *in vivo,* it is important to point out that a decrease in the frequency of primary SV 40-induced tumors has been detected in hamsters immunized against the antigens of normal fetal cells (Coggin *et al.*, 1971; Dierlam *et al.*, 1971). For further discussions as to the existence of embryonic antigens in neoplastic cells and of their role as targets for an immunological defense, see Dierlam *et al.* (1971) and Alexander (1972).

Studies on tumors of the urinary bladder of rats and mice should also be commented on in this context. As will be discussed in Section IV,C, human tumors of the same histological type, e.g., bladder carcinomas, cross-react antigenically so that lymphocytes from one patient with a given tumor are cytotoxic not only to autochthonous tumor cells but also to allogeneic tumor cells of the same type. In an attempt to find

an animal model for a human tumor type which has such common antigens, a study was made of urinary bladder carcinomas of rats and mice and of bladder papillomas of rats (Taranger *et al.*, 1972). It was found that lymph node cells and blood lymphocytes from rats that carried either growing papillomas or carcinomas of the bladder or from which such a tumor had been removed could destroy cultivated cells from the same tumor; cells from other bladder tumors, papillomas, and carcinomas were also found to cross-react. Cross-reactions were found among the bladder tumors, independently of whether they were induced by methylcholanthrene or had arisen following the insertion of a silastic foreign body into the bladder (Taranger *et al.*, 1973). Other types of neoplasms were not destroyed by lymphocytes reacting against bladder tumor cells. Sera from rats with growing bladder tumors could block the lymphocyte effect, further indicating that it was specific. Preliminary experiments have shown delays in the appearances of primary papillomas of the rat bladder following immunization of the animals with bladder papilloma tissue before a tumor-inducing methylcholanthrene pellet was inserted into the bladder (Taranger *et al.*, 1972). These experiments might thus indicate that an immunity to antigens common to bladder tumors was effective *in vivo*. One needs to study this system further, however, to find out whether the common antigens detected *in vitro* can be demonstrated by standard transplantation tests *in vivo* together with individually unique antigens, which these tumors are likely also to have as the chemically induced neoplasms they are.

A provocative observation was recently made. It was found (Prehn, 1972; Medina and Heppner, 1973) that, in some systems, small ratios between lymphocytes and tumor cells could stimulate tumor growth, as detected with neutralization tests *in vivo* as well as by assays performed with the microcytotoxicity technique. This stimulation shows the same specificity as does the immunological destruction of tumor cells. The concept of immunostimulation of neoplastic growth may be an important one, both for understanding mechanisms facilitating tumor development *in vivo*, and, from the practical point of view, when trying to establish procedures for immunotherapy (Prehn and Lappé, 1971).

B. Lymphocyte-Mediated Cytotoxicity in Relation to Tumor Load

In most experimental systems investigated, lymphocytes have been found to be cytotoxic to cultivated cells from the respective tumors even when derived from animals with growing neoplasms (Allison, 1964; Rosenau and Morton, 1966; Hellström *et al.*, 1968a; Sjögren and Borum, 1971; Datta and Vandeputte, 1971). This *in vitro* finding seems to parallel

the *in vivo* phenomenon of concomitant tumor immunity, defined as the ability of a tumor-bearing animal to reject a second graft of the same tumor (Gershon *et al.*, 1967).

In some cases, the degree of cytotoxicity has been found to be approximately equal to that seen in animals with tumors that have been removed, whereas in other systems it is less in the tumor bearers, particularly if the tumor load is large. In order to get meaningful information on this point, titrations of the minimum lymphocyte dose capable of producing significant killing are useful. The fact that reactivity is commonly found in animals with tumors, even if at low levels, does not support the idea that complete immunological nonresponsiveness against the growing tumor exists. This theory was popular a few years ago, but its scientific support is vanishing following a variety of observations with respect to both cell-mediated and humoral immune reactions against growing neoplasms (Smith and Landy, 1971).

There are exceptions, however, in which no *in vitro* cytotoxicity has been detected with lymphocytes from animals bearing tumors. One of the first of these exceptions came when it was shown that regional lymph node cells from rabbits with Shope virus-induced carcinomas lacked cytotoxic effects on cultivated Shope papilloma and carcinoma cells, whereas lymph node cells from rabbits with persistent papillomas (or with papillomas that had regressed) were cytotoxic (Hellström *et al.*, 1969a). An earlier observation of possibly the same phenomenon had been described by Mikulska *et al.* (1966), who showed that spleen cells from rats with tumors could not inhibit the outgrowth of cells from the same tumor in neutralization tests, but an inhibitory capacity developed about 2 weeks after tumor removal. Because the studies of Mikulska *et al.* were performed *in vivo* with the neutralization technique, it could not be excluded, however, that cytotoxic lymphocytes had been actually present in the tumor-bearing animals' spleens but were somehow prevented from destroying the neoplastic cells.

Data analogous to those obtained with Shope carcinomas have been reported by Barski and Youn (1969). They found that peritoneal lymphocytes from mice bearing sarcomas did not affect the respective neoplastic cells in CI tests, but CI activity was detected both very early after tumor transplantation, when the growing tumor mass was quite small, and about 2 weeks after tumor removal. Similar results were obtained by Le François *et al.* (1971) and by Belchradek *et al.* (1972). Likewise, cell-mediated immunity to mouse mammary carcinomas is lower in tumor-bearing animals than in animals with tumors that have been removed (Heppner, 1972).

Studies by Hayami *et al.* (1972) are interesting in this respect. They

have shown that spleen cells from Japanese quails, which had carried Rous virus-induced sarcomas that had spontaneously regressed (regressors), were cytotoxic to cultivated Rous sarcoma cells, whereas spleen cells from tumor-bearing quails (progressors) were not. When the latter type of spleen cells was mixed with spleen cells from the regressors, the cytotoxic effect of the regressor cells was abolished. The suppressor effect of spleen cells from tumor-bearing quails may have resulted either from some cellular interaction, for which living progressor cells were needed, or from the passive release of blocking factors into the culture medium. Sera from progressor quails, but much less regularly from regressors, were found to have blocking activity (Hayami *et al.*, 1973).

Results similar to those of Hayami *et al.* were obtained by Halliday (1971, 1972), who used the macrophage migration inhibition assay to study sarcomas induced in mice with either the Moloney sarcoma virus or with methylcholanthrene. Peritoneal cells were tested from mice with growing tumors that had been removed and from mice with growing tumors (progressors). Cells from the former group were specifically inhibited when brought in contact with antigen preparations from the respective sarcomas, and progressor sera from mice with the same sarcomas could block that effect. No evidence of reactivity was obtained, however, when progressor cells were studied from mice with the respective tumors, and the data thus agreed with those of Barski and Youn (1969) and of Hayami *et al.* (1972). Furthermore, in accord with Hayami's findings, it was possible to detect a suppressor effect of peritoneal cells from progressors; when these were mixed with otherwise reactive peritoneal cells from tumor-free mice, migration inhibition was suppressed, i.e., the macrophages migrated out of the capillaries as in the controls. This effect was specific for the given tumor studied.

In the system studied by Halliday, it appeared likely that a soluble factor was responsible for the suppression observed, since culture medium from peritoneal cells harvested from tumor-bearing mice, but not from immune, tumor-free or from untreated control mice, could also suppress the migration inhibition seen with cells from immune mice. It was shown, furthermore, that the addition of sera from mice with Moloney sarcomas that had regressed, could bestow a specific reactivity upon nonreactive peritoneal cell populations from progressor mice. The latter observation might suggest that some sensitized lymphocytes were present also in the tumor-bearing animals but were prevented from reacting there by blocking factors released from other cells within the same population and that the action of the blocking factors was abolished by an "unblocking" effect (Section VIII) of the regressor sera.

Lymphocytes from tumor-bearing animals are often unreactive when tested with a ^{51}Cr assay, which allows only 3 to 6 hours incubation of the

lymphocytes and target cells (de Landazuri and Herberman, 1972a,b), and the same is sometimes true even when lymphocytes are tested from specifically immunized, tumor-free donors. Reactivity detected with the ^{51}Cr assay typically shows a sharp peak 5–30 days after primary inoculation with tumor cells, whereas reactivity measured with the microcytotoxicity test is consistently of a greater duration. There are several possible explanations for the apparent discrepancy. The two assays may differ in their sensitivities or they could measure different functions of the same cells. Furthermore, the two assays could reflect the functions of different cell populations. One may speculate that the ^{51}Cr assay picks up the "top of the iceberg," consisting of those cells that are capable of immediately destroying their targets. However, during the longer time span of the microcytotoxicity test, recruitment of noncommitted cells into becoming cytotoxic as well as expression of a cytotoxic potential of cells that have less reactivity, or are suppressed from reacting, may occur.

It is important to remember that the reactivity monitored by assaying for cytotoxic cells by the standard ^{51}Cr test does not appear to be the only one important *in vivo:* lymph node and spleen cells from rats in which Gross lymphomas have regressed, and which have strong transplantation immunity to Gross lymphoma cells, do not react in ^{51}Cr tests, whereas these cells do react in standard (24–48 hours) microcytotoxicity assays (Wright *et al.*, 1973a).

The findings obtained with the ^{51}Cr test and with the standard microcytotoxicity assay may be reconciled by the demonstration that lymphocytes from immune animals, which do not express any cytotoxic activity when tested with the ^{51}Cr test, commonly become cytotoxic when incubated for 12 to 24 hours in culture medium prior to the test (de Landazuri and Herberman, 1972a; Wright *et al.*, 1973a). This finding indicates that some originally nonreactive lymphocytes do, indeed, have the potential to kill but that this potential is, somehow, blocked when the cells are taken directly from the animals. The nature of the blocking mechanism is unclear. So far, no one has succeeded in demonstrating any significant blocking in the ^{51}Cr assay by adding sera taken from rats at time points when the lymphocytes do not react *in vitro,* but this issue may need more investigation. Findings similar to those of de Landazuri and Herberman (1972a) were described by Canty *et al.* (1971), who studied immunity to normal alloantigens. These investigators, however, commonly had to incubate the lymphoid cells with their respective targets to turn on a cytotoxic response.

It is also interesting in this context that studies performed with the lymphocyte transformation technique have demonstrated that mice carrying methylcholanthrene-induced syngeneic sarcomas have lymph node and spleen cells reactive to the antigens of their growing tumors

(Forbes *et al.*, 1973). When the tumor is large, reactivity in the regional node is depressed, as tested *in vitro*, but improves following washing and incubation of the lymphoid cells before they are exposed to the antigen.

The fact that specifically cytotoxic lymphocytes can be detected in most tumor-bearing animals must not lead to any simplistic conclusion about tumor bearers having normal cell-mediated reactivities against their neoplastic cells and to the idea that only the extent to which a cytotoxic lymphocyte effect is blocked by serum factors is important. Quantitative differences in the amounts of specifically reactive cells and in the amounts (and reactivity) of various inflammatory cells needed for tumor rejection, etc., may exist. This view is compatible with data from titrations of the amount of lymphocytes needed to produce significant target cell killing (Hellström and Hellström, 1973), as well as with findings from tests performed with relatively small lymphocyte doses (Lamon *et al.*, 1972a; Leclerc *et al.*, 1972). It may be well to remember that specifically cytotoxic lymphocytes have been detected in some mice and rats neonatally made tolerant to allografts, in spite of the fact that such animals are known to be defective in some thymus-dependent immunological responses against tolerated antigens (Section XII,C). One may conclude from those findings that the presence of an immunological reactivity in some cell populations or to some antigens by no means excludes a concomitant immunological unresponsiveness in other cell populations or to other antigens.

C. Cell-Mediated Immunity to Human Tumors

Many human tumors are infiltrated with lymphoid cells, and the degree of such infiltration sometimes shows a favorable correlation with tumor prognosis (Black *et al.*, 1955). Likewise, sinus histiocytosis in lymph nodes draining mammary carcinomas has been reported to be associated with a favorable clinical course (Black and Speer, 1958; Black, 1969). Because of findings of this type, some pathologists postulated, long ago, the existence of immunological defense mechanisms against neoplasms (MacCarty, 1922). The fact that tumor cells often localize in lymph nodes without evidence of being destroyed there, led other pathologists, however, to discard the possibility of an immune response to tumor antigens; rather than doing this, one should, however, be aware that tumor cells destroyed by the host are less likely to be detected in the nodes, whereas cells escaping destruction may be seen.

In order to study cell-mediated immunity to human tumor antigens more quantitatively and sensitively than can be done by just employing histological techniques, several *in vivo* and *in vitro* assays have been utilized. These assays have to a large extent been used previously in animal systems.

One of the first investigations of cellular reactivity to human cancer was made by Southam (1967). He inoculated patients with their own neoplastic cells with or without added autochthonous lymphocytes and found that a large dose of tumor cells was generally needed for a tumor transplant to develop into a palpable nodule. This was interpreted to suggest that many patients have a certain level of immunological reactivity against their cancers. However, the approach used by Southam was not easy to control, and the findings obtained could thus be open to alternative interpretations.

Another way to search for human tumor immunity is to try inducing delayed skin reactions to intradermally inoculated antigen prepared from autologous tumors. Such reactions have been reported for a number of human cancers, including malignant melanoma, Burkitt's lymphoma, acute leukemia, sarcomas, and carcinomas of the breast, colon, lung, stomach, and ovary (e.g., see Hughes and Lytton, 1964; Stewart and Orizaga, 1971; Fass *et al.*, 1970a,b). Recent work with this technique has shown that patients with carcinomas of the colon generally give a positive skin test to extracts of fetal human intestine but not to similar extracts from adult colon (Hollinshead *et al.*, 1972). The antigens involved in these reactions appear to be distinct from the carcinoembryonic antigen of Gold and Freedman (1965), which does not induce delayed hypersensitivity when purified (Hollinshead *et al.*, 1972) nor does it stimulate lymphocyte transformation (Lejtenyi *et al.*, 1971).

A general assessment of the validity of the skin tests so far reported is difficult because one must have adequate controls to exclude reactivity that is nonspecific and is caused, for example, by some contaminant of the tumor (such as a bacterial product or a passenger virus) or by just the amount of foreign protein injected. Furthermore, skin tests are difficult to quantitate. "Criss-cross" experiments, in which patients with two different types of tumors are tested with extracts from each of the two tumors, seem to be much needed when trying to elucidate the specificity of the findings obtained. Information is also needed as to what extent delayed hypersensitivity to tumor antigens correlates with the clinical course of the disease. Some evidence for such correlations has been published from work with Burkitt's lymphoma (Fass *et al.*, 1970a) and with malignant melanoma (Fass *et al.*, 1970b), although the evidence for a correlation in melanomas became less clear on further studies (Bluming *et al.*, 1972). Unfortunately, the number of patients was quite small in all three studies. It would also be important to know whether tumor preparations inducing delayed hypersensitivity *in vivo* would stimulate lymphocytes from tumor patients to react *in vitro* (or would block their reactivity), for this may help bridge the gap between *in vivo* and *in vitro* observations on human tumor immunity.

Still another way to follow human tumor immunity has been suggested by Black and Leis (1971). These authors have reported that sections of breast cancer tissue, placed on cover slips which are then applied as skin windows, show heavy accumulations of inflammatory cells when the patients have a small tumor load. No such accumulation is seen when benign breast tissue is put on the cover slips, and neither is it found when cover slips with breast carcinoma sections are placed on patients with large tumor loads. So far, patients have been studied only for reactivity against their autochthonous neoplasms. This approach is potentially interesting and merits further study; it also relates to the existence of cross-reactivity among histologically similar tumors.

The development of *in vitro* assays has offered new possibilities for investigating the extent to which lymphoid cells from cancer patients can recognize tumor cells as foreign and destroy them under appropriate conditions.

Chu *et al.* reported (1967) that lymphocytes from patients with nasopharyngeal carcinomas were more cytotoxic to autochthonous tumor cells than to normal skin fibroblasts from the same patients. The effects observed were fairly small, however, and it could not be excluded that they resulted from a greater sensitivity of tumor than normal cells to a nonspecific cytotoxic effect of certain lymphocyte preparations.

Neuroblastoma was the first human neoplasm with which a good indication for cell-mediated tumor immunity was obtained by using *in vitro* techniques (Hellström *et al.*, 1968b, 1970a; Jagarlamoody *et al.*, 1971; Kumar *et al.*, 1972). This tumor is particularly interesting, since there is some suggestive evidence for tumor immunity operating *in vivo*. Spontaneous regressions of neuroblastomas have been reported (Everson and Cole, 1966) and so-called neuroblastomas *in situ* have been detected much more frequently than clinically overt neuroblastomas in young children (Beckwith and Perrin, 1963). Furthermore, neuroblastoma cells grow well in culture and maintain a characteristic morphology. With cultivated neuroblastoma cells as targets, it was shown that peripheral blood lymphocytes from children who either had actively growing neuroblastomas or who were in remission (or cured) decreased the plating efficiency of both autochthonous and allogeneic neuroblastoma cells. Normal skin fibroblasts from the donors of the tumor cells or other types of neoplastic cells were not affected. Lymphocytes from healthy subjects or from patients with tumors other than neuroblastomas did not influence the plating efficiency of neuroblastoma cells, except occasionally when the same lymphocyte suspensions were toxic both to the tumor cells and the normal cells from the same patients. Lymphocytes from some healthy relatives of patients with neuroblastomas, particularly from the mothers

of many patients, were inhibitory to neuroblastoma cells, indicating that either a genetic factor or a transmissible agent may have been involved (Hellström *et al.*, 1968b, 1970a).

A large number of other human tumors has since been studied. One of the most striking observations made during these studies was that histologically similar tumors cross-reacted, so that lymphocytes from one patient having, e.g., a colonic carcinoma could destroy colonic carcinoma cells from not only the same but also from other patients but did not affect neoplastic cells of other types (e.g., breast carcinomas). The following are some of the tumor groups within which cross-reactions have been detected: carcinomas of the colon (Hellström *et al.*, 1968c, 1970b, 1971a; Nairn *et al.*, 1971; Baldwin *et al.*, 1973a,b), breast (Andersen *et al.*, 1970; Hellström *et al.*, 1971a; Fossati *et al.*, 1972; Segall *et al.*, 1972), kidney (Stjernswärd *et al.*, 1970; Hellström *et al.*, 1971a; Bubenik *et al.*, 1971; Currie and Basham, 1972), ovary (Hellström *et al.*, 1971a; DiSaia *et al.*, 1971), and urinary bladder (Bubenik *et al.*, 1970, 1973; O'Toole *et al.*, 1972a,b; Bean *et al.*, 1973), Wilms' tumor (Hellström *et al.*, 1968c; Diehl *et al.*, 1971; Kumar *et al.*, 1972), endometrium (Hellström *et al.*, 1971a), cervix uteri (Hellström *et al.*, 1971a), malignant melanoma (Hellström *et al.*, 1968c, 1971a; Fossati *et al.*, 1971; de Vries *et al.*, 1972; Segall *et al.*, 1972; Heppner *et al.*, 1973), gliomas (Levy *et al.*, 1972a; Levy, 1973; Kumar *et al.*, 1973; Wahlström *et al.*, 1973), and sarcomas (Hellström *et al.*, 1971a; Vanky *et al.*, 1971, 1973; Sinkovics *et al.*, 1972a,b; Cohen *et al.*, 1973). The degree of cross-reactivity varies among different tumor groups, however, and it is not absolute for some of them (Segall *et al.*, 1972; also see below).

Lymphomas and leukemias have been studied less, and it is still unclear to what extent specific lymphocyte-mediated cytotoxicity can be detected against them. Several authors have, however, presented evidence that a patient's lymphocytes, taken during remission, can recognize the same patient's blast cells in lymphocyte transformation assays (e.g., see Fridman and Kourilsky, 1969; Gutterman *et al.*, 1973). Findings by Leventhal *et al.* (1972) indicate that blood lymphocytes from leukemic patients, but also from a large number of healthy controls, are capable of destroying ^{51}Cr-labeled leukemic cells but do not destroy normal lymphoid cells from the same patients. Lymphocytes from leukemia patients who have identical twins are cytotoxic to the respective patient's leukemic cells but not to the twin's normal cells (McCoy *et al.*, 1973).

Golub *et al.* (1972a,b) have used a modified CI assay (Hewetson *et al.*, 1972) to search for cell-mediated immunity to Burkitt lymphoma cells. Lymph node cells taken from patients with a Burkitt tumor had, in the few experiments conducted, a CI effect on cell lines of Burkitt origin,

whereas peripheral blood lymphocytes from the patients had no such effect. Blood lymphocytes were, however, found to undergo transformation to lymphoblasts when brought in contact with Burkitt tumor lines, and such "educated" lymphocytes could then inhibit colony formation by Burkitt cells. The CI effect appeared to be specific for cells of Burkitt lymphoma origin, but more studies are needed before this is proven and before information on cell-mediated immunity to Burkitt tumors can be related to the extensive data on humoral immunity to these neoplasms (G. Klein, 1971).

One problem when studying lymphocyte-mediated immunity to human tumors concerns the nature of the target tumor cells (see discussion in Oettgen *et al.*, 1972). The fact that the cells studied have grown out from a tumor explant does, of course, not necessarily mean that they are tumor cells, since various types of normal stroma cells may grow as well *in vitro* as do the neoplastic ones. This problem emphasizes the need to work with relatively fresh explants (which are generally less overgrown by stroma cells), as well as with cell lines that have been well characterized with respect to various signs of neoplastic behavior (Bubenik *et al.*, 1973).

Another important problem when studying cell-mediated immunity to human tumors is to decide which controls are most adequate (see Oettgen *et al.*, 1972), particularly since (varying among different laboratories) lymphocytes from 5 to 15% of normal healthy subjects have been found to be cytotoxic to normal and neoplastic cells alike. The meaning of this nonspecific toxicity is unclear. It might be fairly trivial due to sensitization to some component in the culture medium or to a lymphocyte-mediated immunity to target cell alloantigens, but it may also have a wider biological significance, e.g., indicating the presence of activated macrophages. The presence of this nonspecific cytotoxicity points toward the importance of performing experiments in a criss-cross pattern with the same lymphocyte suspensions being tested against two different tumors at the same time as well as against normal cells (since tumor cells may be more sensitive than normal cells to the toxic effect). It is obvious that without controlling for this nonspecific toxicity, the results obtained, positive or negative, will be very difficult to evaluate. Procedures recently worked out for the selective absorption of specifically reactive lymphocytes (Brondz, 1968; Brondz and Snegiröva, 1971; Lonai *et al.*, 1972; Clark and Kimura, 1972) may be helpful when trying to analyze the specificity of the cytotoxic effects as may also procedures by which lymphocyte-mediated cytotoxicity on isotope-labeled cells can be competitively inhibited by adding nonlabeled target cells of the same antigenic specificity (de Landazuri and Herberman, 1972c).

To what extent apparently healthy subjects possess lymphocytes specifically reactive against a particular type of tumor has not been adequately studied. Existing data indicate that this is a relatively rare event. However, a weak, specific reactivity in many "normal" controls cannot be excluded and neither can a strong reactivity in a small number of normal donors: most tests simply show that the experimental group's lymphocytes (from autochthonous and/or allogeneic patients with the same type of tumor) are more reactive than are those from the controls (from healthy subjects as well as from patients with other types of tumor). Of interest in this respect is the recent demonstration that lymphocytes from many healthy black (North American Negro) donors are specifically reactive on melanoma target cells and that sera from some blacks are "unblocking" (see Section VIII) in the melanoma system (Hellström *et al.*, 1973a). Whether these findings are due to sensitization to a normal skin antigen that is more prevalent in blacks or whether the antigen reacted against is tumor-specific and many blacks have incipient melanoma, is not known (the former alternative appears to be the more likely one).

In contrast to most other investigators of human tumor immunity, Currie *et al.* (1971) were initially unable to find any cross-reactions when studying cell-mediated immunity to melanomas. Their findings gave evidence only for reactions against individually unique antigens of melanoma cells, and these reactions were seen only in a few patients, all of whom had a very small tumor load. Similar evidence for unique antigens in melanomas and lack of evidence for any common antigens have been presented by Lewis *et al.* (1969), who searched for humoral antibodies with the membrane immunofluorescent assay and with complement-dependent cytotoxicity tests. Lewis' findings, however, were in contrast to membrane immunofluorescent studies by Morton *et al.* (1968), who showed cross-reactivity.

According to a more recent report, Currie and Basham (1972) can now detect cell-mediated reactivity against a variety of human neoplasms, and they also find cross-reactions among histologically similar tumors, including melanomas (although the degree of cross-reactivity among melanomas is somewhat less than that demonstrated, e.g., by Hellström *et al.*, 1971a). The earlier failure of Currie *et al.* to detect any reactivity in patients with growing tumors and the lack of cross-reactivity in their previous tests have been attributed by them to the fact that they washed the lymphocytes less extensively in their early work than did, e.g., Hellström *et al.* (1971). The poorly washed lymphocytes may, therefore, have been blocked by serum factors bound to them.

The work of Lewis *et al.* (1969) as well as the early studies by Currie

et al. (1971) indicate that melanomas have individually unique tumor antigens, and findings by several other groups of investigators (as well as the later study by Currie and Basham) indicate that such tumors possess cross-reacting antigens. Thus, there is evidence for both common and individually unique tumor antigens in melanomas. Evidence for the presence of more than one antigen in melanomas has been also obtained by Fossati *et al.* (1971), who found that some patients' lymphocytes were more cytotoxic to certain melanoma lines than to others, whereas a reversed pattern of reactivity was obtained with lymphocytes from other melanoma patients. Similar findings have been described by de Vries *et al.* (1972) and by Hellström and Hellström (1973). Likewise, D. L. Morton (personal communication), who recently tested sera for cytotoxic antibodies to melanoma cells, found evidence for both common and individually specific melanoma antigens. One may speculate that a similar reactivity pattern may be detected with other human tumors as well, as these are investigated more extensively, and that the same tumor cell may contain one, two, three, or more sets of different tumor-specific (and/or tumor-associated) antigens. Studies on spontaneous mammary carcinomas of mice may support this notion. These tumors have both common antigens, associated with the mammary tumor virus (Morton, 1962, 1969; Weiss *et al.*, 1964) and individually unique antigens, specific for each tumor (Vaage, 1968; Morton, 1969; Heppner and Pierce, 1969), and it depends very much on the way the tumors are studied which type of antigen is detected.

It remains to be studied what the roles are, *in vivo*, of individually unique tumor antigens as compared to common ones as inducers and targets of an immune response. We know extremely little about this important issue at the present time; from what we know about animal tumors of "spontaneous" as well as chemical etiology it seems unwise to discard an important role of individually unique antigens of human tumors (in addition to their common ones), in spite of the fact that the overwhelming amount of *in vitro* data concern the common human tumor antigens, so far. Another important question concerns the stability of the common vs. unique antigens (further discussed in Section XI,C,3); one may expect that fluctuations in the amounts of various tumor antigens may occur, including antigenic losses, and that the ease by which this occurs may vary among different antigens.

The fact that histologically similar human tumors cross-react has raised doubts in some investigators' minds as to the possible *in vivo* relevance of the *in vitro* reactivity to human neoplasms, as studied so far. We will now discuss two explanations of the common antigens, which the critics of the concept of their biological importance, have provided.

First, one may argue that the common antigens detected are just normal alloantigens, to which some lymphocyte donors happen to be immune. All the findings on cell-mediated human tumor immunity can obviously not be dismissed this way, since large numbers of patients have been studied and found to be reactive also against their own, autochthonous tumors (when alloantigenic differences are excluded). However, studies on autochthonous tumors do not exclude that the reactivity of allogeneic lymphocyte donors is due to alloantigenic differences. There is experimental evidence, however, strongly arguing against the common tumor antigens being alloantigens. One, extensive experiments have been performed in criss-cross patterns with two different tumor types being tested at the same time and with the same lymphocyte suspensions being used as experimental material with one set of tumor cells and as control material with the other. A tumor group-specific reactivity pattern was found also under these conditions. Two, to be discussed further, the cytotoxic effect of lymphocytes can be blocked by sera from various patients having the same type of tumor as the respective target cells, but not with sera from patients having different types of tumors (Hellström *et al.*, 1971b). Such blocking can be seen in the autochthonous situation, as well, when sera are used from either the autochthonous patient or from allogeneic patients with the same tumors (but not when the sera come from patients with tumors of different types). Three, cell membrane preparations from tumors of the target cell type, but not from other types of tumors or from normal tissues, can block lymphocyte-mediated cytotoxicity (Perlmann *et al.*, 1973; Baldwin *et al.*, 1973a).

A second possibility is that the common human tumor antigens are just normal organ- (or tissue-) specific antigens. Although normal colon epithelial cells are not destroyed by lymphocytes destroying colon carcinomas, nor fibroblasts destroyed by lymphocytes destroying sarcomas (Hellström *et al.*, 1971a), the possibility that the antigens involved are of normal origin still remains. One may just argue that some particular tissue specific antigens are present in larger amounts in neoplastic cells than in their normal counterparts or that they are, for some other reason, not detected in cultivated normal cells the way these have been tested (the normal cells may, e.g., be less immunosensitive). The lack of reliable methods to date for selective absorption of lymphocytes sensitized to tumor antigens makes it difficult to discard this theory completely. It is significant, however, that the common tumor antigens seen in human neoplasms behave as if they are targets for a clinically important immunological defense against neoplasia: a good correlation has been detected between immune responses to the common antigens *in vitro* and clinical courses of tumor patients *in vivo* (see below). It also seems

relevant that, in preliminary studies in an animal model with common tumor antigens of tissue-specific papillomas and carcinomas of the urinary bladder, tumor induction can be delayed by immunizing the animals against common tissue-specific tumor antigens, as already referred to (Taranger *et al.*, 1972); these findings need more follow-up work. Furthermore, as already pointed out, lymphocyte mediated cytotoxicity on colon carcinoma cells can be blocked with cell membrane preparations from colon carcinoma but not with such preparations from normal colon mucosa (Baldwin *et al.*, 1973a).

In carcinomas of the colon, there is an indication that the common antigen detected upon *in vitro* testing is present in fetal but not in adult colon (Hellström *et al.*, 1970b). These data are interesting in view of the previously discussed work by Hollinshead *et al.* (1972) on delayed hypersensitivity *in vivo*. More studies of this type, preferably with absorption techniques, are needed.

The relation between human cell-mediated tumor immunity and tumor load seems to be similar to that seen in animal systems. Lymphocytes from patients with growing neoplasms are commonly reactive *in vitro* as are lymphocytes from patients whose tumors have been removed (Hellström *et al.*, 1971a), whereas titrations of the minimum dose of lymphocytes needed to produce significant kill (Hellström and Hellström, 1973) as well as studies performed with small ratios between lymphocytes and target cells (O'Toole *et al.*, 1972a,b) have shown that patients with large tumor loads are less reactive than are patients with small amounts of tumor. Earlier controversies over this issue probably arose because some investigators worked with large ratios between lymphocytes and target cells and others with relatively small ratios, so that the quantitative differences were either obscured or believed to be qualitative.

Detectable cell-mediated immunity following tumor removal is long lasting in many, but not in all, patients. For example, some patients who had recovered from neuroblastoma were found to have reactive lymphocytes, whereas others did not (Hellström *et al.*, 1970a). Some patients with melanomas and colonic carcinomas have been shown to retain lymphocyte reactivity many years after the last clinical signs of tumor—the longest interval observed was in a patient whose colonic carcinoma occurred 29 years before the test (Hellström *et al.*, 1971a). In patients with bladder carcinoma, on the other hand, lymphocyte reactivity was reported to be lost often within a few months after tumor removal (O'Toole *et al.*, 1972a,b). Because the bladder tumor investigation was performed with relatively low ratios between lymphocytes and target cells, as compared to the other cited work, the possibility remains that the differences in how long the immunity lasts were quantitative rather than qualitative.

A potentially important finding was recently described by Cheema and Hersh (1972). These authors reported that lymphocytes from tumor patients often retarded neoplastic growth when inoculated into the patients' subcutaneous tumor nodules, particularly when the lymphocytes had been exposed first to phytohemagglutinin. Although several explanations of this phenomenon are possible, it is interesting that lymphocytes found to be tumor-cytotoxic *in vitro* may, indeed, have an antitumor effect when directly tested *in vivo*.

V. Cell Types Involved in Host Defense Reactions against Cancer

One of the least understood areas in tumor immunology is that of the cell types involved in various host responses to tumor antigens. Lymphocytes (possibly with the aid of macrophages) are the cells recognizing tumor antigens as foreign, after having been presented with a proper dose of the antigen, and at least part of the recognition mechanism may be studied by performing lymphocyte transformation assays. Lymphocytes recognizing the antigens as foreign may then develop into killer cells and/or they may stimulate other cells to become killer cells both specifically, e.g., by producing antibodies that can "arm" bone marrow-derived (B) lymphocytes and macrophages, and nonspecifically by releasing various substances that can activate other cells as well as help to destroy the tumor by a cytotoxic effect. The *in vivo* role of the latter nonspecific component is probably prominent, but exactly how great is not known.

The fact that neonatal thymectomy increases the frequency of several types of experimentally induced primary tumors (Ting and Law, 1967) indicates that thymus-derived (T) lymphocytes play a significant role in the defense against tumors. This role may be important with respect both to the sensitization process and to the effector mechanism.

The kind of cytotoxic cells detected *in vitro* may vary for different tumor systems, as well as with the time points during the tumor course and with the techniques used. On the basis of studies performed with respect to allograft immunity, one may expect the most important killer cells to be T cells (Cerottini *et al.*, 1970), but it is possible that B killer cells are equally important or even more so under some conditions. According to a recent report (Lamon *et al.*, 1972b), the cytotoxic cells seen in mice with Moloney virus-induced sarcomas that have regressed are non-T cells, whereas T-cell cytotoxicity is detected only at the time of regression. It seems important, at this time, to keep an open mind as to the individual roles of T and non-T (B?) cells as killers in tumor immunity, and not prematurely ascribe all significance to the T cells, on the basis of studies done with respect to immunity to H-2 antigens in mice.

According to other reports, macrophages are the predominant killer cells *in vitro* and perhaps even more so *in vivo* (Tsoi and Weiser, 1968; Evans and Alexander, 1970, 1972). Both a specific killing effect of armed macrophages and a nonspecific cytotoxic effect of activated macrophages have been described (Evans and Alexander, 1972). Tumor cell neutralization by immune lymphoid cells has been found to occur less effectively in mice treated with silica, a macrophage toxin (Zarling and Tevethia, 1973b). It is interesting, furthermore, that tumors that grow poorly *in vivo* are often more infiltrated with macrophages than are tumors that grow well (Evans, 1972). Also neoplastic cells may have a greater sensitivity than their normal counterparts to the nonspecific cytotoxic effect of activated macrophages (Hibbs *et al.*, 1972). Once again, the need to use proper controls when studying tumor immunity is thus emphasized, and the role of nonspecific mechanisms in tumor rejection should not be underestimated.

The *in vivo* situation is likely to be complex. Recruitment of originally uncommitted lymphocytes by various mechanisms, including arming of B lymphocytes and of macrophages as well as attraction and activation of various inflammatory cells (including macrophages) and release of various substances, such as lymphotoxin and MIF, may all be important, and misfunctions with respect to any of these events may provide the tumors with an "escape" from surveillance.

The cell types involved in the production of unblocking serum factors and of the antibody part of blocking factors (see Sections VII and VIII) have not been identified. An interesting observation, the meaning of which is still unclear, is that antisera to antigens specific for plasma cells of the mouse have been found to be capable of depressing humoral antibody synthesis (Harris *et al.*, 1972) and of counteracting the growth of both chemically induced sarcomas and Moloney sarcomas in mice (Jagarlamoody and McKhann, 1972). The hypothesis is that the sera have acted by depressing the formation by plasma cells of the antibody component of blocking factor (see discussion in the following), but there is no direct experimental support of this hypothesis.

In conclusion, we want to point out that no statements are warranted about T cells as being invariably "good" and B cells as "bad" in tumor immunity. Procedures selectively depleting B cells would probably not be beneficial (but, rather, harmful) in individuals with cancer. It is too early to evaluate the potential value of antisera to plasma cells. One may expect, however, that the immunological manipulations needed to improve upon the host's defense to cancer will have to be more specific than can be obtained by deleting one population of immunologically competent cells or by stimulating another. These treatments must be based on the

nature of the immunological defect that exists in a particular patient, be it lack of adequate sensitization, lack of appropriate effector cells (with specific and/or nonspecific action), high production of blocking serum factors, or something else. Before real progress will be made in the immunological manipulation of tumor growth, a better understanding of the mechanisms of cellular immunity to tumor antigens is needed.

VI. Escape Mechanisms of Growing Tumors from Immunological Destruction

That tumors can grow progressively and kill, and yet are sensitive to *in vitro* destruction by lymphocytes from the tumor bearers might seem paradoxical. Several possible explanations of this situation exist (Old and Boyse, 1964; Deichman, 1969; K. E. Hellström and Hellström, 1969; G. Klein, 1969, 1971; Smith and Landy, 1971; Smith, 1972).

One explanation, which probably holds true for many cases, is that the degree of lymphocyte-mediated reactivity is not strong enough to cope with a rapidly growing tumor mass. Nevertheless, it may be sufficient for the destruction of isolated neoplastic cells, and this may account for the "concomitant" tumor immunity which is often, but not always, seen in tumor-bearing individuals (Gershon *et al.*, 1967; Southam, 1967; Deckers *et al.*, 1971; Lausch and Rapp, 1972; Zarling and Tevethia, 1973a). The growth of an established tumor mass may be further facilitated by the fact that the local lymph nodes draining a growing tumor are often prevented from releasing specifically cytotoxic cells (Alexander *et al.*, 1969) as well as by a high local concentration of blocking factors (see Section VII).

Another circumstance contributing to progressive tumor growth *in vivo* may be that the destruction of antigenic tumors not only involves the specifically sensitized lymphocytes commonly studied *in vitro* but also needs an amplification mechanism to which various cells contribute, including macrophages and originally uncommitted lymphocytes that are recruited into the reaction. It is conceivable, therefore, that even when sensitized lymphocytes are present and are capable of destroying antigenic tumor cells *in vitro*, a defect can exist *in vivo* with respect to the mobilization and/or action of some other essential effector cell type.

Experiments of Bernstein *et al.* (1971a, 1972) may point in that direction. Lymphocytes capable of transferring tumor immunity to normal guinea pigs were found not to do so when given to guinea pigs with large tumors; the depressive effect of the growing tumor was immunologically nonspecific. These animals also had impaired delayed hypersensitivities to a protein antigen, although their lymphocytes were responsive to the same antigen *in vitro*. Attempts to induce inflammation by exposing their

skins to turpentine showed a defect in the accumulation of inflammatory cells.

It is also noteworthy that patients with carcinomas of the head and neck can be divided, more or less, into two categories, with respect to their abilities to form delayed hypersensitivity reactions to cutaneously applied dinitrochlorobenzene (DNCB): patients who respond fairly vigorously and those who do not respond at all (Eilber and Morton, 1970). This distinction is seen also when patients who have undergone surgery and have no clinically detectable remaining tumors are tested. The patients not responding to DNCB almost invariably have poor clinical outcomes, whereas patients responding to DNCB have a much higher probability of long-term tumor-free states. The findings indicate that mechanisms involving the development of delayed hypersensitivity reactions are important to fight growing neoplastic cells.

Another escape mechanism from immunological control is apparent from the evidence that certain molecules, e.g., sialomucin, are often bound to the surfaces of tumor cells and are able to block the tumor antigens from being fully exposed *in vivo* as well as to repel sensitized lymphocytes (Apffel and Peters, 1969; Watkins *et al.*, 1971). It is unknown how significant this masking of tumor antigens is *in vivo* for the tumor's escape from immunological control. The facts that host reactivity to tumor antigens, as studied *in vitro,* correlates well with tumor growth *in vivo* and that an immunotherapeutical effect can be seen in at least some systems indicate that the masking cannot be absolute.

There is also evidence that individuals with cancer have serum factors that depress lymphocyte response to phytohemagglutinin and reactivity in mixed leukocyte cultures (Whittaker *et al.*, 1971; Brooks *et al.*, 1972). This may be a very important observation, indeed, since it may help to explain why cancer patients have decreased abilities to mount delayed hypersensitivity reactions to various antigens. It is also quite possible that the (so far unknown) molecules capable of nonspecifically depressing immunological reactivity against various antigens have depressing effects on the patients' abilities to react against their tumors. Antigenic modulation, as well as simple losses of tumor antigens (or decreases in their amounts), are two additional escape mechanisms, further discussed in Section XI,C,3.

Specific blocking serum factors provide still another escape mechanism from immunological surveillance (and this escape mechanism is the one we have elected to deal with primarily in this article). There is evidence that the *in vivo* role of the blocking factors is an important one.

VII. Role of Blocking Serum Factors in Host–Tumor Interactions

Although there had been much speculation over the past 15 years that enhancing antibodies might protect tumors from immunological

destruction (Kaliss, 1958, 1962, 1967, 1970; Batchelor, 1968) and although enhancement of syngeneic tumor growth had been obtained with hyperimmune sera against their specific antigens (Möller, 1964; Bubenik and Koldovsky, 1964; Attia and Weiss, 1966; Batchelor, 1968), it was not until the late 1960s that experimental evidence to that effect was forthcoming in the autochthonous situation. The first demonstration that sera from tumor-bearing individuals could block cellular cytotoxicity came from studies in mice with progressively growing sarcomas (progressors), induced by the Moloney sarcoma virus (Hellström *et al.*, 1969b; I. Hellström and Hellström, 1969). It was shown that progressor serum could block the destruction of cultivated Moloney sarcoma cells by sensitized lymph node cells, as compared to sera taken from normal syngeneic mice or from mice carrying tumors with antigens that did not cross-react with those of Moloney sarcomas (spontaneous mammary carcinomas and sarcomas induced by methylcholanthrene). The blocking effect was not regularly seen with sera from mice with Moloney sarcomas that had regressed, when tested either in the same dilution as the progressor sera (1:5) or in higher dilutions (up to 1:40). When Maloney sarcomas started to regress (which happened in most adult mice given the virus), the blocking serum activity, initially present, commonly disappeared (I. Hellström and Hellström, 1970).

Findings similar to those made in the Moloney sarcoma system were obtained in rabbits with papillomas induced by the Shope virus (Hellström *et al.*, 1969a). Rabbits carrying persistent papillomas (persistors), as well as rabbits with papillomas that had regressed (regressors) had regional lymph node cells cytotoxic to cultivated Shope tumor cells, but only the former group of rabbits had any detectable blocking serum activity. These findings were interesting, since previous studies by Evans and Ito (1966) had demonstrated a difference in the *in vivo* immune status between the two groups of rabbits. Deoxyribonucleic acid from the Shope virus could induce new Shope papillomas in the persistors (as it could in untreated rabbits with lymphocytes that were not cytotoxic to Shope tumor cells), but it did not induce papillomas in the regressor rabbits; this difference does not appear to be explainable by differences in the titers of antiviral antibodies. Thus, the Shope findings indicate that the blocking phenomenon, as measured *in vitro*, is an important correlate of the host response to tumors *in vivo* (K. E. Hellström and Hellström, 1970).

Blocking serum activity, similar to that demonstrated in the Moloney and Shope tumor systems, was detected in mice with spontaneous mammary carcinomas (associated with the mammary tumor virus), protecting tumor cells from destruction by lymphocytes sensitized against the individually unique antigens of such tumors (Heppner, 1969, 1972; Heppner and Kopp, 1971). Similar findings were later made with a

variety of other neoplasms as well: polyoma virus-induced sarcomas in mice and rats (Sjögren and Borum, 1971; Sjögren and Bansal, 1971), Schmidt–Ruppin–Rous virus-induced sarcomas in mice and rats (Sjögren and Jonsson, 1970), adenovirus 12-induced sarcomas in mice (Ankerst, 1971), chemically induced sarcomas in mice, rats (Hellström *et al.*, 1970c), and guinea pigs (Cohen *et al.*, 1972), and chemically induced hepatomas in rats (Baldwin and Embleton, 1971; Baldwin *et al.*, 1971, 1973c,d). Furthermore, blocking activity has been detected in the sera of patients with a variety of neoplasms (Hellström *et al.*, 1971b; Bubenik *et al.*, 1970; de Vries *et al.*, 1972; Currie and Basham, 1972; Heppner *et al.*, 1973; Cohen *et al.*, 1973; Levy, 1973) and shows the same specificity as do lymphocytes from these patients (see Section VII,A).

It is interesting that a blocking effect can be demonstrated also with the macrophage migration inhibition assay, an *in vitro* correlate of delayed hypersensitivity (Halliday, 1971, 1972). Since antigenic extracts were used in these studies, they excluded that the blocking phenomenon —at least as studied with this technique—was due to antigenic modulation (Old and Boyse, 1964).

In human patients with sarcoma, blocking serum effects have also been detected by using the lymphocyte transformation test (Vanky *et al.*, 1971).

Correlations between *in Vitro* and *in Vivo* Findings

The question is no longer whether blocking serum factors exist but whether they play a role in facilitating tumor growth *in vivo* or are detectable as just another sign of a growing tumor. One should realize, however, that even if the latter alternative were true, monitoring serum blocking factors might still provide diagnostically and prognostically useful information.

The data obtained in the Shope system (Hellström *et al.*, 1969a) argue against the role of the blocking factors being trivial, but a more detailed analysis, utilizing both *in vitro* and *in vivo* assays, is needed to clarify what *in vivo* significance blocking serum factors might have. Part of this analysis has been done and will now be discussed.

Stutman (1971) studying chemically induced sarcomas in mice, Pierce (1971) and Pearson *et al.* (1973) investigating sarcomas induced in mice with the Moloney sarcoma virus, and Bansal *et al.* (1972) working with polyoma virus-induced sarcomas in rats could all show that sera of the type that blocks *in vitro* (and which were actually demonstrated to do so in the experiments of Bansal *et al.*) could enhance the growth of transplanted tumors of the respective types *in vivo*. The blocking factors thus

appeared to mediate the phenomenon of immunological enhancement, studied by Kaliss (1958, 1962, 1967, 1970) and others with allogeneic tumor grafts.

It is significant that the studies by Bansal *et al.* (1972) were so carried out that rats were given serum in sufficient quantities to possess blocking activity detectable upon *in vitro* testing of sera from the inoculated animals. Rats, which after receiving serum had detectable blocking titers, developed polyoma tumors earlier from a test graft with syngeneic tumor cells than did rats receiving control serum. No difference was detected in the establishment of cell-mediated immunity to polyoma tumor antigens in the two groups, indicating that the enhancing serum effect detected in this case was more likely of an efferent or central type than of an afferent one. This finding is different from observations made in several other systems, where antibodies to alloantigens mediated an afferent enhancement, delaying the onset of cell-mediated reactivity (e.g., see Peter and Feldman, 1972). Three to five days after the rats were first given blocking serum, followed by tumor cells, the differences in blocking activities between experimental and control groups disappeared —sera from rats belonging to the control groups were blocking as well. An active form of "self-enhancement" then may have taken place, as seen in isografted but not otherwise treated rats.

In the experiments listed, although enhancement had been observed following inoculation of sera from tumor-bearing animals, it was rather unimpressive in degree compared to the enhancement seen in studies with tumor allografts. This may be due to rapid elimination of the blocking factors inoculated—perhaps explainable if they are antigen–antibody complexes (see Section X)—as well as to the quick appearance of blocking factors in control animals (mediating self-enhancement and obscuring the differences between the experimental groups and the controls). The latter explanation is the more likely one in the experiments of Bansal *et al.* (1972).

Vaage (1973) was unable to detect any enhancing effect at all following inoculation of sera from tumor-bearing mice. The lack of data as to whether the animals inoculated with sera developed detectable blocking activity, as compared to the controls, makes it difficult to interpret this finding, however. It is interesting that tumor antigen inoculated in the same experiments did, indeed, facilitate tumor growth *in vivo*. Whether this occurred by desensitizing lymphoid cells, by inducing an active form of immunological enhancement, by combining with antibodies to form blocking complexes, or by some other mechanism, is not known.

Blocking activity can be eluted from polyoma tumors taken from the

body, cut into small pieces, repeatedly washed, and then incubated at pH 3.1 (Sjögren and Bansal, 1971; Bansal *et al.*, 1972); similarly prepared eluates from normal tissues taken as controls from the same tumor bearers showed no blocking activity. Blocking factors eluted from rat polyoma tumors were found to enhance polyoma tumor growth *in vivo*, similar to that seen after inoculation of serum (Bansal *et al.*, 1972). Analogous findings have been obtained in systems in which no *in vitro* measurements on blocking activity were done. Eluates of SV 40 tumors in hamsters were found to contain antibodies to the cell surface antigens of SV 40 tumor cells (Sobszak and De Vaux Saint Cyr, 1971); immunoglobulins (Ig) of the IgG_2 variety were found in eluates from chemically induced mouse sarcomas (Ran and Witz, 1970), and mice injected with such eluates showed enhanced tumor growth (Ran and Witz, 1972). The degree of specificity of the enhancement obtained was lower, however, than found when the same type of tumor was studied for specific transplantation antigenicity (implying, either, that the tumors shared antigens to which enhancement could be detected or that the *in vivo* effect of inoculating tumor eluates was partially nonspecific). No nonspecific effect was seen when eluates from rat sarcomas induced by either the polyoma virus or methylcholanthrene were tested in parallel (Bansal *et al.*, 1972).

Blocking activity, similar to that seen with several animal tumors, can be eluted from human neoplasms as well (Sjögren *et al.*, 1972) and shows the same degree of specificity as detected when studying the cytotoxic effect of lymphocytes from the respective tumor patients. These data may be analogous to the demonstration that eluates of Burkitt lymphoma cells at a low pH often contain IgG with specificity for the cell surface tumor antigens of the Burkitt lymphoma (G. Klein, 1971).

The fact that blocking factors can be eluted from both animal and human neoplasms taken directly from the body is significant, since it implies that at least some of the antigens involved in the reactions studied *in vitro* are expressed on at least some of the neoplastic cells growing *in vivo*. It also indicates that the level of blocking activity is likely to be higher at the tumor site than in the whole organism, since blocking factors bound to the tumor are present at the tumor site, in addition to those occurring in the bloodstream. This is likely to lead to less effective cell-mediated tumor immunity locally than elsewhere in the organism and may, in turn, help to explain the phenomenon of concomitant tumor immunity (Gershon *et al.*, 1967). Because animals (and human patients) with growing tumors have circulating cytotoxic lymphocytes as well as blocking serum factors, one may expect concomitant immunity to occur when the circulating blocking factors cannot fully abrogate the lympho-

cyte reactivity, whereas no concomitant immunity is to be expected when the blocking serum activity is relatively stronger than the lymphocyte reactivity. If this reasoning is correct one may expect, furthermore, that the degree of concomitant tumor immunity should vary at different sites in the tumor-bearing host (as a result of the local supply of reactive cells and available blocking factors) as well as among different neoplasms, being very prominent with some and virtually absent with others. One must remember, in this context, that in the classic studies on enhancement of allogeneic tumor grafts, concomitant immunity to transplanted cells was almost regularly found in animals bearing enhanced grafts of the same neoplasms (Kaliss, 1962; Zimmerman and Feldman, 1970). The belief (held by some) that the phenomenon of concomitant tumor immunity disproves an *in vivo* tumor enhancing role of blocking serum factors is thus unfounded.

Further evidence that a blocking serum effect detectable *in vitro,* has relevance *in vivo* comes from studies by Sjögren and Bansal (1971; Bansal and Sjögren, 1973). Rats were first transplanted with syngeneic polyoma tumors, and their levels of transplantation resistance to second polyoma tumor grafts *in vivo* as well as their lymphocyte-mediated cytotoxicity and serum blocking activity *in vitro* were measured. It was shown that lymphocytes from rats carrying growing polyoma tumors, as well as from rats carrying such tumors that were subsequently removed, were cytotoxic to polyoma tumor cells and that the degree of lymphocyte reactivity in the two groups of rats was about the same (unless the tumor was very large—it then was less). Only those rats that had growing tumors had blocking serum activity, except in cases where the tumors were not completely removed but reappeared and a blocking effect was regularly detected. Rats with blocking sera (and cytotoxic lymphocytes) were significantly more sensitive to polyoma tumor test grafts than were rats with cytotoxic lymphocytes and no blocking activity; the rats with cytotoxic lymphocytes and blocking sera were approximately as susceptible to polyoma tumors as controls never exposed to polyoma tumors and lacking detectable lymphocyte cytotoxicity (indicating that no significant concomitant immunity was seen in this particular system). The findings indicated that the blocking serum activity, as measured *in vitro,* was, indeed, related to the degree of effective host response to the tumors *in vivo.*

An equally striking correlation between the finding of blocking serum activity *in vitro* and facilitated tumor growth *in vivo* has been obtained in studies on human cancer patients. A large number of such patients has been tested, representing a variety of different neoplasms (Hellström *et al.,* 1971b). Findings obtained by studying patients with malignant

melanomas appear representative and will, therefore, be dealt with in some detail. Among a group of patients with melanomas, the following patterns were identified (Hellström *et al.*, 1973b). First, patients were observed in whom primary melanomas, not metastasized, had been surgically removed and in whom no tumor recurrency was detected during the observation period. Lymphocytes from these patients had a strong cytotoxic effect on autochthonous and allogeneic melanoma cells and sera from the same patients had no regular blocking activity after the tumors had been removed. Second, of patients who had widespread disease when first seen, most died from their melanomas within the observation period. Sera from these patients were almost invariably blocking. Furthermore, the degree of lymphocyte-mediated cytotoxicity was less than that seen in the first group. Third, of patients whose clinical course changed remarkably during the observation period, some developed metastases subsequent to their original examinations. Blocking serum activity commonly antedated the presence of metastases by several months and could be seen in all patients when metastases were clinically evident. There were also patients in whom blocking serum activity was found to disappear in conjunction with the surgical removal of small metastatic masses and in whom tumor-free states (lasting from a few months to more than 2 years) were then observed. No blocking activity was detected after these patients became clinically tumor free, nor was any blocking effect seen in one patient whose melanoma underwent spontaneous remission; rather, this patient's serum potentiated the cytotoxic activity of lymphocytes from other patients with melanomas.

Fluctuations in cell-mediated antitumor reactivity correlating with clinical status have been demonstrated in patients with carcinomas of the bladder (O'Toole *et al.*, 1972a,b). Lymphocytes from patients with small tumor loads were, as a rule, found to react to bladder tumor cells, whereas lymphocytes from patients with large tumors reacted much less frequently. Increased reactivity was often seen in the latter group following treatment that reduced the tumor size. No tests for serum blocking activity were performed in these studies.

A blocking effect with sera from patients with growing neoplasms has been detected also when using the lymphocyte transformation assay, and, at least in some instances, a correlation was seen between the blocking activity and the clinical course (Vanky *et al.*, 1971). Tumors taken directly from the patients did not stimulate lymphocyte transformation, as a rule, whereas stimulation was seen after incubation of the tumors at pH 3.1 so as to elute blocking factors; addition of the eluates blocked lymphocyte transformation (Vanky *et al.*, 1973). These findings agree with the data obtained with the microcytotoxicity test that showed binding of blocking factors to the tumor cells.

The finding that the cytotoxic effect of lymphocytes from human patients can be specifically blocked by the same patient's serum achieves importance not only by indicating that blocking activity may contribute to facilitated tumor growth *in vivo*, but also by providing a way to study the tumor specificity of the reactions measured (see foregoing discussion). The specificity of the blocking effect suggests that the lymphocyte reactivity is more likely directed against antigens that are tumor-specific (or, at least, tumor-associated) than against normal alloantigens.

VIII. Unblocking Serum Factors

Sera from mice with Moloney sarcomas which have regressed (or have started to regress) have been shown not only to lack blocking activity but to cancel the blocking effect of sera from mice that have growing such tumors (I. Hellström and Hellström, 1970). This serum effect has been denoted as "unblocking" (or deblocking), an operational term that states nothing about the mechanisms of the phenomenon. It shows the same specificity as the blocking activity and the cell-mediated reactivity.

An unblocking effect has been detected in other systems as well. For example, sera from rats with polyoma tumors that have been successfully removed are unblocking in the polyoma tumor system, as are sera from rats, first given BCG and then transplanted, 14–16 days later, with a polyoma tumor syngeneic graft and tested after 4 to 8 days (Bansal and Sjögren, 1971, 1972, 1973).

Two patients in whom spontaneous tumor remissions were seen as well as several apparently cured from neoplasia by surgery have been found to have unblocking sera (Hellström *et al.*, 1971c). As in the animal systems, the unblocking effect was specific; for example, sera unblocking in the colon carcinoma system could not unblock in the melanoma system, and vice versa. So far, no unblocking serum activity has been detected in patients with clinically overt tumors.

It is likely that the unblocking serum factors are related to humoral antibodies detected by the membrane immunofluorescent assay (G. Klein, 1971; Morton *et al.*, 1968, 1969) and with assays for tumor cell cytotoxicity in the presence of complement (Wood and Morton, 1970). Patients with Burkitt's lymphoma have antibodies to surface antigen(s) of these tumor cells more frequently when the tumor loads are small than when they are large (Klein *et al.*, 1966; G. Klein, 1971), and antibodies to the Burkitt, tumor cell, surface antigens have been found to disappear preceding tumor relapses in patients who had been in remission (G. Klein, 1971). Analogous findings have been reported from studies on human sarcomas with membrane immunofluorescent, complement fixation, and serum cytotoxicity tests (Morton, 1971). Regression of tumors induced by feline sarcoma virus has been shown to

correlate with increased titers of antibodies to the cell membrane antigens of such neoplasms (Essex *et al.*, 1971). All these findings imply that generalized statements about humoral antibodies being invariably bad in cancer patients (which have been seen in the literature) are unwarranted. They indicate, instead, that humoral antibodies, at least in some situations, are beneficial to the host. If the hypothesis that blocking factors are antigen–antibody complexes (and occasionally free antigens) is correct (see Section X), one would expect that antibodies to the antigens involved in such complexes, if present in sufficient amounts, should be unblocking.

The regular occurrence of blocking serum factors in patients with growing tumors and the presence of an unblocking serum activity in many patients who are clinically tumor free indicate that monitoring patients for unblocking serum activity may provide clinically useful information; the reason for concentrating on unblocking rather than blocking serum effects is that the disappearance of unblocking serum factors may occur before the blocking ones are detected.

IX. Potentiating and Arming Serum Effects

Certain sera taken from either animals or human patients during the period of tumor remission have been found to increase the cytotoxic effect of immune lymphocytes (Hellström *et al.*, 1971c, 1973b,d). This has been referred to as "potentiation." A similar effect has been described by Skurzak *et al.* (1972), who reported that the serum dilution was critical as to whether potentiation or blocking of the lymphocyte effect was seen and who found potentiation even with some sera from tumor-bearing animals (if the dilution was right). The mechanisms of the potentiating effect are unknown as are the molecules mediating it. One may speculate that potentiation is related to the unblocking phenomenon and operates by removing (or neutralizing the action of) blocking serum factors present in the lymphocyte suspensions tested, but other mechanisms, including arming of nonsensitized cells, are equally possible.

It has also been demonstrated that certain immune sera, particularly in experimental animal tumor systems, are capable of "arming" lymphoid cells from nonimmune syngeneic donors so that they become cytotoxic (Pollack *et al.*, 1972; Pollack, 1973; Skurzak *et al.*, 1972). The cytotoxic effect is specific and appears to be analogous to that previously shown with respect to immunity to alloantigens (MacLennan *et al.*, 1969; Perlmann *et al.*, 1969) that is mediated by B (or so-called K) lymphocytes. One common feature of the arming effect is that even very dilute antisetra can arm, whereas dilution of sera quickly diminishes their unblocking activity. The *in vivo* role of the arming phenomenon remains to be studied. No obvious correlation has been detected, as yet, between

the presence of arming factors *in vitro* and the degree of effective tumor immunity in the animal, but this does not at all have to mean that the arming factors are not important *in vivo*. The arming phenomenon may, indeed, have a potential for tumor therapy which needs to be investigated (Hersey, 1973).

X. Nature of the Blocking Phenomenon as Revealed by Tumor Studies

The nature of the blocking serum factors and their mode of action are not well known. We will try to discuss the sketchy information available. Another review article in this area was recently published by Feldman (1972).

First, however, we want to point out that it has been well documented that enhancement of mouse tumor allografts in the classic models, studied *in vivo*, can be mediated by an immunoglobulin, commonly of the IgG_2 variety (Takasugi and Hildemann, 1969; Safford and Tokuda, 1970). To the extent this has been studied, the immunoglobulins eluted from syngeneic mouse tumors at low pH have been of the IgG_2 type (Ran and Witz, 1970, 1972). Reports have been published, however, that other immunoglobulins, including IgG_1 (Chard, 1968), may also enhance.

It is also important to keep in mind that *hyperimmune* sera (in which there is no evidence of free antigen being present) to target cell alloantigens can block lymphocyte-mediated cytotoxicity (Möller, 1965; Brunner *et al.*, 1968; Takasugi and Klein, 1971; Peter and Feldman, 1972), as well as lymphocyte transformation in mixed leukocyte culture tests (Cepellini *et al.*, 1971; Oppenheim, 1972). Likewise, blocking can be detected with hyperimmune sera directed against tumor-specific antigens (Ankerst, 1971; Sjögren and Borum, 1971). The blocking effect of hyperimmune sera has been detected both in standard microcytotoxicity tests (Takasugi and Klein, 1971) and in the short-term ^{51}Cr assay. In all these cases, a blocking effect is seen after incubation of the target cells with the blocking serum, followed by washing, but it is generally not observed after a similar incubation of the lymphocytes (Peter and Feldman, 1972). It would be surprising if a similar type of strictly efferent blocking does not occur also with respect to tumor-specific immunity. It is not clear, however, the extent to which such an efferent blocking occurs, as compared to a more central one (acting on the lymphocytes). As will be discussed in the following, the central type of blocking appears as the more important one in tumor immunity.

The blocking factors detected *in vitro* when testing sera from individuals bearing autochthonous or syngeneic tumors have several characteristics in common with antibodies. Target cells incubated with blocking serum and washed are protected from destruction by immune lymphocytes (I. Hellström and Hellström, 1969), although their sensi-

tivity to lymphocyte-mediated cytotoxicity recurs within approximately 6 hours (Hellström *et al.*, unpublished findings). The blocking activity can be removed from tumor-bearing individuals' sera by absorption with neoplastic cells of the respective types, but not with control cells with antigens that do not cross-react with those of the experimental group, target, tumor cells (I. Hellström and Hellström, 1969), and they can be eluted back from the tumors used for absorption (Sjögren *et al.*, 1971). The fact that blocking serum activity can be removed by absorption is true also when the blocking is measured by incubating the effector cells (rather than the target cells) with tumor-bearer serum (Hayami *et al.*, 1973). Blocking factors commonly belong to the 7 S fraction upon Sephadex filtration, and antisera to mouse IgG can abrogate the blocking effect of sera from mice with growing Moloney sarcomas (I. Hellström and Hellström, 1969).

There is also evidence that blocking factors from tumor-bearing individuals (as studied *in vitro*) may contain antigen, in addition to their antibody component. Sera from mice with either Moloney virus-induced sarcomas or methylcholanthrene-induced sarcomas can be separated into two components by ultrafiltration at pH 3.1: one component with a molecular weight higher than 100,000 and another component with a molecular weight between 10,000 and 100,000 (Sjögren *et al.*, 1971). When assayed for blocking activity (by incubation with the target cells followed by removal), neither component blocks alone, while significant blocking is seen when the two components are mixed. The smaller fraction (mol. wt. 10,000–100,000) alone can block only when it is added to the lymphocytes rather than the target cells (as in the standard tests). On the other hand, no blocking has been seen with the larger component, known to contain antibodies, when added to either the lymphocytes or the target cells. This argues against the blocking seen in these experiments with sera from tumor bearers being caused by simple blindfolding of target cell antigens, the mechanism believed to be the most important one (I. Hellström and Hellström, 1969) before these experiments had been carried out and before it had been shown that Moloney regressor sera (which also bind to target cell antigens) did not regularly show blocking activity (I. Hellström and Hellström, 1970). It certainly does not exclude, however, the possibility that blocking *can* occur by a blindfolding mechanism under conditions when the amounts of antibodies are right for efficient covering of target cell antigens.

There is more support for the idea that the blocking factors, as regularly tested (by incubation of the target cell with blocking material, followed by its removal and addition of lymphocytes), are complexes of antigens, released from the tumors, and antibodies, formed by the host. When a known tumor antigen and specific antibodies are mixed, the

mixtures block, but neither antigen nor antibody blocks alone when tested under the standard conditions (Baldwin *et al.*, 1972b). If, however, the antigen is added to the lymphocytes, it can block as well (Baldwin *et al.*, 1973d). Before accepting the antigen–antibody complex hypothesis as more than a tentative explanation of the blocking phenomenon, a good chemical analysis of the molecules involved is needed, however. We need to know better, e.g., that the blocking factor really is a complex, and, if so, what kind of complex and the exact nature of the antigen and antibody involved.

Findings that may be analogous to those obtained when studying tumor-specific immunity have been reported from investigations of enhancement of tumor allografts: incubation of target cells with antibodies to their H-2 antigens was found to release a factor that could make lymphocytes nonreactive when added to them (Amos *et al.*, 1970; W. J. Klein, 1971). This factor may be a complex of enhancing antibodies and released H-2 antigen or some other molecule with an immunosuppressive effect formed by the tumor cells, the lymphocytes, or both. These findings suggest that even (some of?) the blocking effect of hyperimmune sera added to target cells may not be due to a simple blindfolding of target cell antigens but rather involves the cytotoxic lymphocytes.

Suggestive evidence that antigen alone may block was first reported by Brawn (1971). He found that lymphocytes immune to H-2 alloantigens of cultivated normal fibroblasts could be specifically prevented from killing these if incubated with normal mouse sera from the same strain as the target cells or with semiallogeneic F_1 hybrid sera (but not with sera from the lymphocyte donor strain), and he attributed this effect to alloantigens present in the sera. The blocking effect on the lymphocytes was found to last only briefly after serum removal (Brawn, 1972). The reason why an effect could be observed after incubation of the lymphocytes with serum in Brawn's work, although no such effect had been previously seen (I. Hellström and Hellström, 1969), was probably that the earlier work was done with the CI assay. Three to five days elapsed between incubation of the lymphocytes with a blocking serum and termination of the CI experiments; thus the reactivity of the lymphocytes may have recovered, even if it was initially depressed. In later studies on tumor immunity, blocking could, indeed, be detected also by incubating the effector cells with tumor-bearing serum (Baldwin *et al.*, 1973d; Hayami *et al.*, 1973; Currie and Basham, 1972) as well as with preparations presumed to contain tumor-specific antigen (Sjögren *et al.*, 1971; Perlmann *et al.*, 1973; Baldwin *et al.*, 1973a,d).

It appears that antibodies per se present in individuals with growing tumors or who previously had tumors may not be the important blocking factors of cell-mediated cytotoxicity *in vitro*, neither in con-

centrated nor in diluted form, unless the antibodies have attached antigen, or are able to release antigen from the target cells. They may still, of course, depress the development of strong cell-mediated tumor immunity by a feedback inhibition mechanism (Uhr and Möller, 1968). One may take issue with this view, however, on the basis of experiments showing that some sera from rats with Moloney sarcomas that had regressed did block when the sera were tested at some, but not at other dilutions (Skurzak *et al.*, 1972). Although a dilution effect of this type obviously can occur, it is important to realize that blocking could not be regularly demonstrated with murine Moloney regressor sera by simply diluting them (I. Hellström and Hellström, 1969); neither was it regularly seen with sera from Japanese quails with Rous sarcomas that had regressed (Hayami *et al.*, 1973). It is also important to realize that even in systems where a serum that originally does not block and after dilution does block, the finding of a dilution effect does not contradict the notion that the blocking is mediated by complexed tumor antigens and antibodies or sometimes, perhaps, by just free antigen. The sera may contain mixtures of molecules with blocking and unblocking effects in various proportions as expected if the tumors release antigens, the host forms antibodies, and the "right" combination between the two, as well as free antigen, can block. This point of view is supported by recent data showing that diluted regressor sera occasionally block in the Rous sarcoma system, and that their blocking activity appears to be due to the release of antigens from the tumor cells, interacting (in the form of a complex?) with the lymphocytes (Hayami *et al.*, unpublished findings).

A model of the blocking phenomenon which we find attractive is one suggested from experiments by Feldmann and Diener (1971; Diener and Feldmann, 1971), who studied the *in vitro* induction of immunological unresponsiveness in spleen cells exposed to *Salmonella* antigen. These authors found that exposure of spleen cells to a high dose of antigen could induce nonreactivity (akin to high zone immunological tolerance), as could exposure to much lower doses of antigen complexed with antibodies. The latter situation might represent low zone tolerance. The primary role of the antibodies was believed to be in cross-linking antigens to their postulated receptor sites at the lymphocyte surface; in the presence of large amounts of antigen, such cross-linking antibodies are not needed for the lymphocytes to be blocked.

It is important to remember, however, when trying to understand the blocking seen in tumor systems, that the response studied by Diener and Feldmann was one of originally nonsensitized B lymphocytes against a relatively simple antigen. Bearing this in mind one may postulate, nevertheless, that the primary effect of the blocking factors seen in tumor

bearers is on the lymphocytes by blocking their receptor sites with antigen and that the antibody part of blocking antigen–antibody complexes acts by carrying the antigen to the lymphocytes as well as by cross-linking it to the lymphocyte receptor sites. This may be needed, particularly when the amount of free antigen is relatively small. A simple explanation of the unblocking effect would then be that it is mediated by antibodies that bind to the antigenic sites of blocking complexes, as well as to antigen molecules, in such a way that there is no longer any free antigen to interact with the lymphocytes and to block their cytotoxic activity. Under still other conditions, however, an efferent blockade may, indeed, be possible, and one could envision that a normally unblocking serum may, occasionally, mediate efferent enhancement by blindfolding target antigens.

An alternative explanation of the blocking phenomenon, which may be worthy of consideration for experimental analysis, is that tumor-bearing individuals form antibodies to the receptor sites of their cytotoxic lymphocytes, thereby specifically blocking their reactivity. This explanation, based on the work of Ramseier and Lindenmann (1972), may hold true for some situations, but we consider it a less likely one than the hypothesis that the blocking factors are commonly antigen–antibody complexes as well as free tumor antigens.

If the assumption about the blocking factors being antigen–antibody complexes (as well as, sometimes, free antigen) is correct, it follows that the release of antigen from a growing tumor could be of fundamental importance for its escape from immunological surveillance, and one may speculate that one way tumors become independent of immunological surveillance is by the appearance of cell variants with great abilities to release antigen. Such antigen can then stimulate immunologically competent cells to form the antibody component of blocking complexes. Furthermore, the antigen might be capable of abrogating lymphocyte cytotoxicity even when no antibodies are present (if the amount of free antigen is sufficient).

An observation, which may deserve comment in this context, was recently made on rats bearing enhanced kidney allografts. It was found that sera from rats with enhanced kidneys could block lymphocyte reactivity, as tested with the microcytotoxicity test (Stuart *et al.*, 1971; Biesecker *et al.*, 1973b) but not as tested with the ^{51}Cr assay (Biesecker *et al.*, 1973a). A possible explanation of these findings is that the blocking factors present in certain sera primarily act on cells that are in the process of developing a cytotoxic effect (as they may do during a 36-hour microcytotoxicity test) and that they are much less (if at all) effective on lymphocytes ready to kill their targets within a few hours

(as in the ^{51}Cr assay). If this speculation is valid and if it also applies to the tumor system, it would follow that lymphocytes taken from tumor-bearing individuals and triggered to become active killer cells may not be inhibited from reacting when put back *in vivo* by blocking factors present there.

For a better understanding of the blocking phenomenon, more knowledge is needed about the nature of the killer cells effective in various systems, about how they have been armed or activated, about their modes of action, and about the blocking molecules involved. *A priori*, it is possible that molecules blocking one cell population may act differently on another cell population. Studies on the effect of blocking factors on various functions of T and B cells and of macrophages have not even begun.

XI. Possible Clinical Implications of Findings on Cell-Mediated Tumor Immunity (and Its Blocking by Serum)

A. Diagnosis

The first utilization of immunology for diagnosis of tumors has been made with hepatomas (Abelev, 1971) and with carcinomas of the colon (Gold, 1970), but at least in the latter case, the specificity of the findings is not clear (Lo Gerfo *et al.*, 1972). The fact that human tumors of the same histological type cross-react antigenically, as detected by studying, e.g., lymphocyte-mediated cytotoxicity and serum blocking activity, suggests possible ways for an immunological diagnosis of tumors by searching for circulating tumor antigens in the serum, for antibodies to such antigens (when they occur), and for lymphocytes sensitized to the antigens. To what extent an immunological diagnosis will replace more conventional techniques by screening large patient populations and by providing earlier detection is as yet uncertain. Much more needs to be known about the frequency of false positive and of false negative reactors.

It seems likely that assays of blocking factors in serum will be of prognostic value when following individual patients with respect to tumor recurrency after surgery and when monitoring the effects of chemotherapy. Work in this direction would be greatly helped if the blocking molecules were better characterized, since radioimmunoassays might then be employed.

From the practical point of view, it seems relatively difficult to monitor cytotoxic antibodies and, even more so, cell-mediated tumor immunity in large patient groups, but replacement of present techniques with simpler and more precise assays may change this situation.

B. Prevention

Whether the common antigens of histologically similar human tumors will ultimately form the basis for vaccination against neoplasms is quite uncertain at the present time. Before meaningful conclusions can be made to this end, more knowledge is needed as to how immune reactions to common antigens of the tissue-specific type influence neoplastic growth *in vivo* and whether such reactions impair normal tissues (as might be expected if these have small amounts of tissue-specific tumor antigens). It may be significant that vaccination of mice with an antigen prepared from spontaneous mouse mammary carcinomas has been reported to decrease the frequency of such primary tumors (Irie and Irie, 1971), that the appearance of primary chemically induced bladder papillomas in rats can be delayed by immunization against the common antigens of such tumors before the rats are exposed to the carcinogen (Taranger *et al.*, 1972), and that the frequency of methylcholanthrene-induced sarcomas in mice has decreased following vaccination of the mice with an antigen prepared from leukemic mouse cells (Whitmore and Huebner, 1972). However, any similar attempts to vaccinate healthy human subjects against malignancies would most definitely not be justified at the present time. In addition to the great probability that nothing beneficial would be achieved, the risks for inducing either tumor enhancement or autoimmune diseases (or both) would be prohibitive at the present state of knowledge.

C. Therapy

From the therapeutic point of view, it appears that procedures capable of increasing the tumor-destructive part of cell-mediated immune reactions (without increasing blocking serum activity), as well as procedures leading to a decrease of serum blocking factors, may contribute to a more effective defense against neoplastic cells. Direct studies in man may show whether this is possible. One may argue, however, that no immunotherapy of human neoplasms should be tried at the present time, since too little success has been obtained with primary animal tumors to justify the potential risks of a human trial. If, nevertheless, the decision is made to proceed on a limited number of human patients (and we feel that such a decision is justified), it seems essential that the following criteria are met. First, the patients selected must have poor prognoses. Second, of that group an immunotherapeutic trial would be meaningful only in those with relatively small tumor burdens. The need for choosing patients with relatively little tumor is clear. For example, Bernstein *et al.* 1972), studying guinea pigs with large, growing tumors, could not transfer

to these animals any effective immunological reactivity against a small number of grafted neoplastic cells carrying either the same antigens as those of the growing tumor or different ones. Third, the patients should be carefully followed with *in vitro* assays that can yield information relevant to their immunological responses (cell-mediated antitumor immunity, blocking, unblocking, cytotoxic serum activities, and general ability to mount delayed hypersensitivity reactions).

With this background, we will now discuss some ideas for human immunotherapy. We will intentionally limit our discussion, however, to approaches directly related to the findings covered in the previous sections of this article, and, therefore, we do not go into the whole area of tumor immunotherapy, a subject reviewed, e.g., by Alexander (1968), Mathé (1971), Motta (1971), Fefer (1971a), Hellström and Hellström (1972), Smith (1972), and Oettgen and Hellström (1973).

1. Attempts to Increase Cytotoxic Effect of Patient's Killer Cells

One way to go about the problem of tumor immunotherapy may be to find some vaccine that could actively stimulate cell-mediated antitumor immunity as well as the level of unblocking and cytotoxic antibodies without stimulating the development of tumor-enhancing serum factors. In the long run this approach may be the most meaningful. Unfortunately, however, no such vaccines are readily available, either against animal or human tumors. Tentative means for developing them (if at all possible) may involve the use of chemically modified tumor antigens, tumor cells treated with neuraminidase (Simmons *et al.*, 1971; Bekesi *et al.*, 1972), tumor cells attenuated by growth in tissue culture, etc. One way to vaccinate patients against their growing neoplasms may be to inoculate the antigenic material intradermally, alone or mixed with an adjuvant such as BCG, a procedure that has met with some success in experiments carried out in guinea pigs in which regression of small established tumors has been achieved (Kronman *et al.*, 1970; Zbar *et al.*, 1970, 1972; Bartlett and Zbar, 1972). Intradermal inoculation of melanoma cells has, indeed, been claimed to lead to some regressions in human patients (Krementz, 1970). It is, however, questionable, how much practically applicable information will appear along any of these lines until more is known about the nature of the human tumor antigens and about the best ways to achieve strong cell-mediated tumor immunity (without blocking serum activity) in animal models.

Another approach, which has been tried, is to stimulate nonspecific host reactivity by inoculating BCG (Mathé, 1971), following animal studies in which an immunotherapeutic effect was achieved this way (Mathé, 1971; Zbar *et al.*, 1970, 1972). There is suggestive evidence that

treatment of humans with BCG (in combination with antigenic tumor cells) might be therapeutically beneficial, especially in patients with leukemia who have been first given vigorous chemotherapy to reduce the remaining tumor mass, but it is still debated to what extent the immunotherapy which has been used is better than the most successful modes of combination chemotherapy. In a few patients with melanomas, complete tumor remission has been seen following BCG inoculation into subcutaneous metastases (Morton, 1971, 1972). One must bear in mind, however, that remissions are sometimes seen in patients with melanomas even without therapy (Everson and Cole, 1966). Since there is evidence that BCG treatment of animals with solid tumors can enhance tumor growth (Piessens *et al.*, 1971), caution is needed before applying this mode of therapy to solid tumors. When BCG is tried, it may be advisable to watch for increases in blocking factors during therapy; there are claims that such increases may occur (Levy *et al.*, 1972b).

One might consider, first as a project for animal experimentation, combined therapy with BCG and unblocking antibody inoculation (Bansal and Sjögren, 1973). By such a combination, it might be possible to achieve the stimulatory effect of BCG on cell-mediated reactivity and, at the same time, to counteract its possible stimulatory effect on the formation of blocking factors (as well as the blocking serum activity already present in the treated individuals).

It would probably also be worthwhile to put more effort into investigating nonspecific immunostimulants in addition to BCG (Yashpe, 1971) and to reassess the potential value of Coley's toxin, which was used with some claimed success in the early part of this century but the real therapeutic effect of which remains obscure.

During the last few years, a way of treating skin carcinomas (and cutaneous metastases from some other tumors as well) has met with remarkable success; it probably involves an immunological component (Ed. Klein, 1969). Tumors are exposed to the focal application of chemical agents, such as DNCB, that can elicit delayed hypersensitivity reactions, and this has led to disappearance of both the exposed tumors, and, occasionally, of distal tumor nodules of the same type. Similar treatment of subcutaneous metastases from breast carcinomas has, likewise, led to regression of many of the treated tumor nodules, although the systemic effects have been modest (Stjernswärd and Levin, 1971). Most likely, the tumor regressions obtained in the studies by Ed. Klein and by Stjernswärd and Levin were the outcome either of a specific lymphocyte–tumor cell interaction or of a nonspecific destruction of tumor cells accompanying a specific reaction of lymphocytes with the sensitizing antigen in close proximity to the neoplastic cells. The latter explanation is supported by

experiments performed in guinea pigs with transplanted hepatomas; there is evidence that at least some of the therapeutic effect of BCG on tumors is best explained this way, too (Zbar *et al.*, 1972).

Inoculation of transfer factor (Lawrence, 1969) might be worth considering, since transfer factor may be able to increase cell-mediated immunity without increasing the formation of tumor-enhancing antibodies. Of course, more knowledge about the nature of transfer factor, about the specificity of its effects, and about its mode of action would help in evaluating the merits of this approach. There are no good animal models available, so that the experiments must be carried out directly in man. It would probably be best to give transfer factor prepared from patients whose own tumors have either regressed spontaneously or been eliminated by conventional therapy. Active immunization of patients (or healthy persons) to form transfer factor is not advisable because of the potential hazards involved in inoculating tumor cells (or extracts) into human subjects.

The adoptive inoculation of intact lymphoid cells into allogeneic recipients seems less meaningful: if not rapidly eliminated by host immunity (and in such a case doing the patients no good), the cells may induce potentially fatal graft-versus-host disease. In syngeneic recipients, however, this approach can, indeed, lead to tumor regression under favorable circumstances (Alexander, 1968; Hellström *et al.*, 1969c; Fefer, 1971a,b; Borberg *et al.*, 1972). A related approach may very much be worth considering, however. If lymphocytes that have been triggered by antigen so that they have become effective killer cells are relatively unaffected by the blocking activity of serum factors, as suggested by findings (Biesecker *et al.*, 1973b) discussed above, *in vitro* culturing of patients' own lymphocytes may establish large numbers of specifically sensitized, highly cytotoxic and nonblockable cells. Finally, one should consider attempts to increase cell-mediated cytotoxicity by giving immune sera capable of arming nonimmune lymphocytes or potentiating the reactivity of immune ones, as described in Section IX.

2. *Attempts to Decrease Blocking Serum Activity*

The finding that blocking serum factors, detectable *in vitro*, may play an important role in facilitating the growth of antigenic tumor cells *in vivo*, suggests that procedures decreasing blocking serum activity, without concomitantly impairing cell-mediated immunity, may be therapeutically beneficial. Several such procedures will now be discussed.

Since the release of antigen from neoplastic cells appears to be a prerequisite for blocking factors to be formed, one may expect that any procedure capable of interfering with this release would decrease blocking serum activity and, therefore, make the tumor host more resistant to growth of the neoplastic cells. Theoretically, the simplest approach for achieving this seems to be the physical removal of as many tumor cells as

possible (surgery) (Hellström *et al.*, 1970c; Heppner, 1972) or their destruction by radiation or chemotherapy.

Another apparently straightforward approach decreasing blocking serum activity is to develop ways of physically removing the blocking factors, e.g., by performing plasmaphoresis and replacing blocking plasma with plasma that does not block. Recently developed techniques involving the cannulation of the thoracic duct, removal of the lymph, and separation of the lymph plasma from its cells may be helpful in this respect, since they allow removal of large amounts of blocking plasma, followed by reinoculation of washed lymph cells together with nonblocking plasma (S. Rose, personal communication). By performing splenectomy in conjunction with this therapy and thus eliminating blocking complexes which would not end up in the thoracic duct, one might possibly remove most circulating blocking factors shortly after their formation. One may, however, raise objections to this approach. For example, the removal of antibodies may stimulate their increased synthesis by decreasing feedback inhibition (Uhr and Möller, 1968); this would counteract the purpose of the study, that is, the removal of antibodies taking part in the formation of blocking complexes. Furthermore, the concept of removing *antibodies* may not be good at all, because of the evidence that the antigen part of blocking factors may be the crucial one and that antigen alone can also block. The same reservations can be made, irrespectively of whether one attempts to remove blocking factors from blood plasma or from lymph. If procedures can be worked out, however, by which both freely circulating *antigen* and antigen–antibody complexes can be removed (using, e.g., immunoadsorbants to which free antigen as well as the antigen part of circulating complexes would bind), the justification for this approach may increase.

Another way of decreasing blocking serum activity in tumor-bearing individuals may be to use drugs capable of suppressing antibody formation more than affecting cell-mediated immunity, since less blocking factor may be formed if most blocking factors contain antibody and antibody formation is interfered with. Griswold *et al.* (1972) and Heppner and Calabresi (1972) have reported that cytosine arabinoside can have such an effect: mice with spontaneous mammary carcinomas, treated with small doses of this drug were found to have a lower blocking serum activity than controls, whereas their cell-mediated tumor cytotoxicity remained intact (it even increased). The treated mice lived longer than the controls, and their tumors grew less well. A large dose of cytosine arabinoside, on the other hand, which also depressed cell-mediated tumor immunity, did not depress tumor growth in this system. Decrease in blocking serum activity also has been seen in some human patients treated with cytosine arabinoside (Sinkovics *et al.*, 1972b), but the number of patients studied is too small to allow any conclusions as to the possible clinical effect (or lack of such) of this mode of therapy. A problem with

this approach for decreasing blocking serum activity is that it is based on the idea that humoral antibody formation in cancer patients is invariably bad, an idea which is contradicted, e.g., by the demonstration of unblocking antibodies in cancer patients who do clinically well (see Section VIII).

The finding that decreased protein intake depresses blocking serum activity as well as tumor growth (Jose and Good, 1971) is extremely interesting but its practical usefulness for tumor therapy is as yet difficult to evaluate.

Another approach is to base one's therapy on the demonstration that certain immune sera in animals, as well as in man, are unblocking. Experiments have been carried out, both in mice and in rats, that attempt to affect tumor growth by decreasing blocking serum activity through the administration of such unblocking sera. Because of the relationship of this approach to the scope of the article, we will dwell more on it than the success achieved, so far, may actually justify.

It was first shown that mice with small primary Moloney sarcomas, in about one-third of cases, rejected their tumors following inoculation of sera from mice in which Moloney sarcomas had regressed (Hellström *et al.*, 1969c); therapeutic and tumor preventive effects of such regressor sera have been detected in independent studies as well (Fefer, 1971a,b; Pearson *et al.*, 1973). Although the sera inoculated in these experiments were not tested for unblocking activity, similar sera have regularly been shown to be unblocking (I. Hellström and Hellström, 1970). Inoculation of lymph node and spleen cells from regressor mice was equally effective in inducing tumor regression (Fefer, 1971a,b). It was not possible to make any conclusions, however, whether the regressions observed were caused by unblocking, cytotoxic, arming, or potentiating serum factors (or production of such by the inoculated cells). A more trivial explanation, which could not be dismissed, was that antiviral antibodies, known to be present in regressor sera and probably produced by transferred lymphoid cells as well, prevented the recruitment of normal cells into becoming tumor cells following infection by free virus. In order to exclude this possibility, it was felt that tumors other than Moloney sarcomas had to be studied, because in some tumors release of infectious oncogenic virus does not play an important role (K. E. Hellström and Hellström, 1970).

One such system is polyoma tumors in rats, and these tumors have recently been investigated with respect to the potential value of therapy with unblocking serum (Bansal and Sjögren, 1971, 1972, 1973). Both primary and transplanted syngeneic polyoma tumors have been studied. Unblocking rat sera have come from two different sources: (*1*) from animals with polyoma tumor grafts that had been surgically removed

when they were approximately 10 mm. in diameter and the animals bled 6 to 8 days subsequently; (2) from rats inoculated with BCG, given polyoma tumor grafts 14–18 days later, and bled and tested after 5 to 7 days. For other experiments, unblocking sera were derived from rabbits, immunized according to the second of the two protocols for rats. The rabbit sera were extensively absorbed before being tested. The absorptions were first carried out *in vitro* with rat spleen, liver, and kidney cells from the strain of rats to be used for the *in vivo* experiments, and this was followed by *in vivo* absorptions, in which normal rats of the same strains were inoculated with serum and bled 4–6 hours later. The sera so prepared were unblocking in the polyoma tumor system, and they were also cytotoxic to polyoma tumor cells in the presence of complement.

Unblocking sera were inoculated both into rats with syngeneic polyoma tumor transplants and into rats with primary polyoma virus-induced sarcomas of the kidneys. In the former case, treatment started immediately upon the subcutaneous inoculation of tumor cells, whereas the rats with primary tumors were not treated until the neoplasms were of a fairly large size (more than 6–8 mm. in diameter). In experiments with primary polyoma tumors, the rats were splenectomized, in addition to receiving unblocking serum, because splenectomy could decrease blocking serum activity (Hellström *et al.*, 1970c).

Two major findings were made. First, it became apparent that the blocking effect seen in rats with polyoma tumors could be decreased by inoculation of unblocking serum (as verified by testing serum samples taken at various time points from the treated rats). Second, the growth of tumor in the treated animals was often arrested, at least temporarily, and regressions of established tumors were observed. The antitumor effect was most apparent in rats with transplanted polyoma tumors, four of five tumors regressed, but it was seen in primary tumor-bearing rats as well. In 2 of 11 rats receiving unblocking serum, complete regressions occurred, and in 7 of the remaining rats tumor growth was significantly slower (and survival longer) than in rats receiving the same amounts of control serum. *In vitro* measurements of blocking activity in the treated rats correlated well with the *in vivo* findings of tumor regression: sera from rats with tumors that regressed did not block, whereas the blocking activity was virtually unchanged in 2 rats with tumors that grew equally as well as those in controls.

The finding that changes in blocking serum activity in treated rats correlated with host resistance against the tumors indicates that the blocking serum activity, as measured *in vitro*, may, indeed, influence the effectiveness of the antitumor response *in vivo*. One must keep in mind,

however, that there may be alternative explanations for the *in vivo* changes in tumor growth which were observed, including a cytotoxic effect on tumor cells by antibodies and complement, arming of non-immune lymphocytes and/or macrophages so they became cytotoxic (Section IX), potentiation of the cytotoxic effect of immune lymphocytes, and activation of macrophages. Nonimmunological effects of the serum transfusions, which secondarily affected the immunological parameters studied, cannot be excluded either.

Procedures leading to the active production of unblocking (arming, potentiating, cytotoxic) antibodies would be much more efficient (and convenient) from the therapeutic view than passive administration of large amounts of serum with such properties over a long period of time. However, no such procedures have been worked out as yet, and we do not know to what extent they are feasible.

3. *Possible Reasons why Immunotherapy May (Sometimes, Often?) Not Work*

Even if it were possible to increase cell-mediated immunity and to remove (or cancel the mode of action of) blocking serum factors, an established tumor mass still might not be eliminated and might even continue to grow.

First, the release of tumor antigen from the neoplastic cells is likely to lead to a much higher concentration of blocking factors locally than in the bloodstream (as shown in the elution experiments discussed in Section VII) so that lymphocytes within tumors (and in their close proximity) may be prevented from reacting even when there is no detectable blocking activity in the serum. We consider this possibility a very real one, indeed. The presence of nonspecific blocking factors, either in serum or at the tumor site, may also hamper immunotherapy.

Second, antigenic modulation may occur, i.e., some tumor antigens would not be expressed in the presence of antibodies to them (Old *et al.*, 1968). Antigenic modulation has been clearly demonstrated for TL antigens of mouse leukemias, although it does not appear to play any important role with respect to some other tumor antigens such as those of polyoma tumor cells (Sjögren, 1965). To what extent antigenic modulation of human tumor antigens occurs is unknown. It is interesting, however, that most cells from primary Burkitt lymphomas, directly studied *in vitro* with the membrane immunofluorescent technique, possess the surface antigens characterizing this type of tumor (Klein *et al.*, 1966), which indicates that at least some cells within a primary tumor biopsy have not been modulated.

Third, losses of the tumor antigens or development of a high degree

of immunoresistance may occur. These events, if frequent, may provide the greatest obstacle to effective immunotherapy. It is poorly understood to what extent tumor-specific (associated) antigens may be lost. It has been reported, however, that metastases of SV 40 virus-induced hamster sarcomas are less immunogenic (and immunosensitive) than primary SV 40 tumors (Deichman, 1969), and Moloney lymphoma lines with decreased immunosensitivities have been isolated following immunoselection (Fenyö *et al.*, 1968). There is even evidence that one tumor line capable of nonspecific growth in many mouse strains, the Ehrlich ascites carcinoma, produces a substance that makes the Ehrlich cells immunoresistant and that the production of this substance is genetically dominant, as detected by cell hybridization experiments (Klein *et al.*, 1972).

On the other hand, it has not been possible, in spite of prolonged attempts at immunoselection, to isolate *in vivo,* from polyoma tumors, any cell variants that lack the polyoma virus-induced transplantation antigen (Sjögren, 1965). The degree of tumor antigenicity and/or immunosensitivity, as detected by transplantation tests with chemically induced mouse sarcomas, has been reported to decrease, as these are propagated by transplantation (Prehn and Main, 1957), but the frequency at which their antigens are actually lost or the tumor cells decrease in immunosensitivity is not well known.

Whether the common tumor antigens, detected among histologically similar human neoplasms, are stable or can be lost and whether individually unique tumor antigens, observed in some human neoplasms (and perhaps occurring in all), behave similarly to the common ones remain to be studied. One may speculate that the common antigens reflect a more important property of the neoplastic cells than do the individually unique ones (which may just be the outcome of a genetic variation among various tumor cell clones) and that the common antigens are, therefore, more stable; but this is, of course, just a speculation, and we do not know anything about the effectiveness of common versus unique antigens as targets for an immune response *in vivo.* The fact that neoplastic cells cultivated from metastases (including pleural effusions and ascites) are sensitive as targets in lymphocyte-mediated cytotoxicity assays for common tissue-specific tumor antigenicity (Hellström *et al.*, 1971a) indicates that at least some cells from advanced tumors retain their common antigens. This conclusion is supported by the observation that blocking factors can be isolated from primary and metastatic human neoplasms, taken directly from the body and subjected to elution at a low pH (Sjögren *et al.*, 1972). However, these findings tell nothing about how many cells within a tumor may lack the common

antigens or about how many nonantigenic cells would have been present if an effective tumor immunity had been established which could have selected for nonimmunogenic and/or immunoresistant cells.

XII. Lymphocyte Cytotoxicity and Blocking Serum Activity in Allograft Immunity

Findings similar to those described with tumors (coexistence of cytotoxic lymphocytes and blocking serum activity detectable *in vitro*) have been made in some systems not involving neoplasia. Although these findings have been reviewed recently (K. E. Hellström and Hellström, 1970; Feldman, 1972), a few of them will be summarized in this section because of our belief that information obtained by studying nonneoplastic tissues may elucidate the tumor field. However, we make no attempt to go into depth in any of the three areas covered in this section, since we feel this is beyond the scope of a review on tumor immunity.

A. Pregnancy

There is evidence that a female pregnant by an allogeneic male can be sensitized to his antigens. Lymph node and spleen cells from female mice which have undergone repeated pregnancies produced by allogeneic males have been reported to be more apt than control cells to induce graft-versus-host reactivity when inoculated into mice containing the paternal antigens (Sörén, 1967). Lymph nodes in rats to which the uterus drains (the para-aortic nodes) have been found to increase in size following allogeneic (but not syngeneic) pregnancies (Beer and Billingham, 1971). Pregnant mice often have antibodies to the mate's H-2 antigens, which can be detected by hemagglutination tests (Herzenberg and Gonzales, 1962). Sera from mice that have undergone repeated pregnancies can sometimes enhance the growth of tumors from the strain of the mate (Kaliss and Dagg, 1964).

The placenta probably plays the major role in protecting the fetus from immunological destruction (Currie and Bagshawe, 1967; Currie, 1968). This does not explain, however, why mothers, on one hand, become sensitized to paternal antigens of the fetus, and why, on the other hand, mice that have undergone pregnancies are generally less capable of rejecting skin or tumor allografts from the strain of the mate (Breyere and Barrett, 1963). The latter phenomenon, which is often referred to as parity tolerance, is most commonly seen with respect to weak (non-H-2-determined) histocompatibility antigens and after several pregnancies, but it has also been observed for H-2 antigens and after only one pregnancy.

In an attempt to clarify the immunology of pregnancy, studies were

performed with the CI technique where specific cell-mediated immunity and blocking serum activity in pregnant mice were sought; the experiments were modelled on the work with tumors (K. E. Hellström *et al.*, 1969). It was shown that female mice impregnated by allogeneic mates had lymph node cells that were specifically cytotoxic to cultivated fibroblasts from the male strain, and it was also demonstrated that sera from the same pregnant mice specifically abrogated this cytotoxic effect. These sera did not abrogate the cytotoxic effect of lymphocytes immune to unrelated antigens, such as the tumor-specific antigens of Moloney sarcoma cells. Cell-mediated reactivity was detected after one pregnancy, although it was less than after repeated pregnancies. The blocking activity was found to behave similarly: sera from repeatedly pregnant mice were regularly blocking, whereas sera from only some of the mice pregnant for the first time were blocking (under the conditions of the tests).

The finding of a blocking serum activity during pregnancy has been confirmed in studies performed in man by using the mixed leukocyte culture technique. Sera taken during pregnancy or shortly afterward can most commonly block reactivity between the mother's lymphocytes and cells from either the baby or the father (Ceppellini *et al.*, 1971). Furthermore, blocking serum factors have been eluted from the placenta, suggesting that their concentration there might be particularly high. Women who have been pregnant repeatedly are sensitized to their husband's alloantigens, as detected by macrophage migration inhibition assays (Rocklin *et al.*, 1973), and the reactivity seen in these tests can be blocked by the respective mother's serum (Youtananukorn and Matangkasonbut, 1973). Factors nonspecifically depressing mixed leukocyte reactivity have also been detected in sera from pregnant women, however (Kasakura, 1971; Buckley *et al.*, 1972; Jenkins and Hancock, 1972).

An *in vivo* role of blocking serum factors in pregnancy remains to be established. For example, it is not known whether there are any shifts in the blocking activity accompanying delivery or how the phenomenon of parity tolerance *in vivo* correlates with blocking serum activity, as measured *in vitro*. Studies on these questions may be helpful also in establishing whether an organism's ability to form the antibodies involved as at least part of the blocking factors is under endocrine control and whether the blocking effect seen in pregnancy is related to the fact that a fetus, like a growing tumor, is likely to give off small, steadily increasing amounts of antigens into the bloodstream. Information on these points may be useful not only for a better understanding of the immunology of pregnancy, but also with respect to the field of cellular immunology in general.

B. Allogeneic Organ Grafting

There is an apparent similarity between the fact that a tumor can grow progressively, in spite of its being antigenic, and the fact that allogeneic organ grafts, most notably kidneys, can escape from immunological destruction (if proper immunosuppressive treatment is given). For this reason, techniques employed to study the immunological interaction between the host and its growing tumor have been used to investigate recipients of allogenic kidney grafts. The questions asked were whether any evidence of cell-mediated reactivity against the grafted organ could be detected and whether there was any blocking serum activity similar to that seen in individuals with tumors. The finding that allogeneic rat kidney grafts could be made to take by passive and active immunological enhancement (Stuart *et al.*, 1968; French and Batchelor, 1969) stimulated this approach.

Patients receiving allogeneic kidney grafts were studied, including those who had carried their grafts for more than a year, and several of these patients were followed sequentially (Quadracci *et al.*, 1970; Pierce, 1971). Two patterns of reactivity could be identified: about one-third of the patients with healthy grafts had no evidence of cell-mediated reactivity against donor tissues, at least not with the lymphocyte doses tested, whereas the other patients with healthy grafts showed specific cell-mediated cytotoxicity and blocking serum activity with respect to the graft donors. The individual roles of the two mechanisms need to be studied, and one would like to know if patients who had no detectable immunity against donor tissues had more favorable clinical courses.

A situation similar to that encountered in humans with kidney allografts occurred in rats transplanted with allogeneic or F_1 hybrid kidneys (Stuart *et al.*, 1971). In rats, grafted kidneys are permanently accepted without continuous administration of immunosuppressive agents if the animals are pretreated with hyperimmune sera directed against the foreign antigens of the grafts, either alone or in combination with spleen cells from the kidney donors (Stuart *et al.*, 1968; French and Batchelor, 1969). Rats receiving hyperimmune sera and which *accepted* kidney grafts and rats receiving control sera and which *rejected* the grafts were both shown to possess blood lymphocytes cytotoxic to fibroblasts of donor origin, but only the rats given immune sera and accepting their grafts had a regular blocking serum effect. Furthermore, blocking activity could be eluted from the enhanced kidney grafts. These findings suggest that blocking activity detected *in vitro* was, indeed, related to the prolonged survival of the grafts *in vivo*. Evidence that rats bearing enhanced kidney grafts are able to recognize graft donor antigens as foreign was also provided by the demonstration that rats bearing enhanced kidney grafts

could form antibodies, detectable by their cytotoxic effect (in the presence of complement) against lymphocytes of the donor strain (French and Batchelor, 1969). It is interesting, however, that neither cellular reactivity nor a blocking serum effect could be detected when rats with enhanced kidney grafts were tested with the ^{51}Cr assay for cellular immunity (Biesecker *et al.*, 1973a); parallel studies with the microcytotoxicity test confirmed the original findings of Stuart *et al.* (1971; Biesecker *et al.*, 1973b).

Subcutaneous grafts of allogeneic hearts will take almost regularly in rats that have been impregnated recently by a mate from the donor strain (Heron, 1972a,b). There is evidence that blocking serum factors play an important role in the acceptance of these heart grafts. Sera from rats bearing grafts can enhance the take of similar grafts in other rats. Skin from the donor of the heart is rejected, however, by rats bearing healthy heart transplants.

If blocking serum factors are, indeed, important for the acceptance of allogeneic organ grafts, at least two conclusions can be drawn. First, techniques by which the activity of such factors can be monitored may offer some possibilities to learn more about how to use immunosuppressive treatment of graft recipients and when to discontinue such treatment. Second, if the blocking serum activity plays an important role in facilitating graft acceptance, the passive transfer of blocking factors and (even more so) the induction of their synthesis may provide more specific and complete immunosuppression (with less risks of unwanted side effects) than that achieved by using only nonspecific immunosuppressive agents.

C. Blocking Serum Factors in Chimeras and in Allograft Tolerance

Owen (1945) was the first to show that nonidentical twin cattle often are chimeric with respect to their blood groups, red cell populations from both twins coexisting in the same animal. This finding indicated that some mechanism must exist by which the immunological system can be prevented from eliminating antigenically foreign cells (Burnet and Fenner, 1949) and stimulated work which led to the discovery of immunological tolerance (Billingham *et al.*, 1953).

A crucial question is why permanent chimeras can be established experimentally in which the inoculated genetically foreign cells do not develop graft-versus-host reactions killing the recipient of the grafts. At least two different types of explanations can be entertained. One may postulate that lymphoid cell clones in the graft would have been able to react against the recipient antigens but have been eliminated (e.g., see

Wilson and Billingham, 1967), or one can hypothesize that some mechanisms exist by which reactivity of the grafted cells against their host is suppressed, without any lymphoid cell clones having to be killed (Thompson *et al.*, 1969; Voisin *et al.*, 1968; K. E. Hellström and Hellström, 1970). Of course, a combination of these two mechanisms is possible as well: Some reactive lymphoid cell clones may be killed while others remain but are suppressed from reacting against their host. The first hypothesis, selective depletion of reactive lymphocyte clones, has gained wide but not universal acceptance.

In the last few years, studies with CI and microcytotoxicity assays have indicated that lymphoid cells (of donor origin) capable of killing cultivated fibroblasts of host origin, often exist in radiation-induced chimeras, and the cytotoxic effect can be blocked by serum factors present in the chimeric individuals. The authors have thus argued against the idea that all reactive lymphocytes are specifically deleted.

The first system investigated was one in which dogs were given a lethal dose of X-rays, followed by allogeneic bone marrow transplants (bone marrow cells from dogs matched with respect to the major histocompatibility antigens as well as from unmatched dogs were used) (Hellström *et al.*, 1970d). Although repopulation with bone marrow resulted in fatal graft-versus-host disease in a relatively high proportion of the dogs, some dogs did not develop this disease but remained healthy following repopulation and lived a normal life-span as stable chimeras. Chimerism was confirmed in two ways: blood group markers identified the erythrocytes of the engrafted dogs as being of donor origin, and sex chromosome markers showed the blood leukocytes to be of donor origin as well. Blood lymphocytes from the graft recipients and from unrelated dogs (as controls) were studied for their abilities to destroy cultivated skin fibroblasts from the host. Furthermore, the blocking ability of recipient sera was assessed. It was shown that lymphocytes from most chimeras had a specific cytotoxic effect on recipient fibroblasts and that recipient sera could block the cytotoxicity. Sera taken from unrelated dogs did not block.

Studies similar to those performed with chimeric dogs have been conducted with mouse chimeras as well (Hellström *et al.*, 1973c). Mice were given lethal doses of X-irradiation, following which they were inoculated with allogeneic bone marrow cells. Several strain combinations were studied, but most experiments were performed with T6 mice getting BALB/c bone marrow. The *in vitro* tests were set up so that both donor and recipient strain fibroblasts were used as targets, and sera were tested from the chimeric recipients as well as from untreated mice of the host and donor strains. The data showed that lymph node cells

from the murine chimeras had a specific cytotoxic effect on recipient (but not on donor) strain target cells, and that sera from the chimeras (but not from control mice) could specifically block this effect. In some of the mice there was evidence of graft-versus-host reactivity (weight loss and ruffled fur). Sera from these mice did not block, which supports the interpretation that the blocking activity detected in the healthy chimeras might play a role in counteracting the development of graft-versus-host reactions.

Three human patients, who had been reconstituted with foreign bone marrow following immunosuppression, were tested and found to react similarly to the chimeric dogs and mice in that each patient's blood lymphocytes (of donor origin) were cytotoxic to his own fibroblasts and his serum could block this effect (Jose *et al.*, 1971). Removal of the immunoglobulin fraction from the serum by passing it through a column charged with antibodies to human immunoglobulin, removed the blocking activity, which could be recovered by eluting the columns. The blocking activity had a certain degree of specificity, but it was not completely specific, which might have been due to some overlapping of the HLA antigenicity of the patients studied.

The phenomenon of allograft tolerance has been investigated by using a similar approach. Mice and rats rendered neonatally tolerant by inoculation of allogeneic lymphoid cells, as well as tetraparental (allophenic) mice, have been tested to find out whether their lymphoid cells have any detectable reactivity *in vitro* against the tolerated tissues and whether they have any blocking serum factors that can interfere with a cellular reactivity. Some evidence in favor of such a view was first presented by Voisin *et al.*, who showed that sera from some mice neonatally inoculated with allogeneic bone marrow, and so rendered tolerant to skin grafts of the allogeneic strain, contained antibodies detectable with hemagglutination assays. They demonstrated classic enhancement of allogeneic tumor transplants by using sera from mice considered to be tolerant to skin grafts (Voisin *et al.*, 1968, 1972).

Experiments were performed by using the standard microcytotoxicity technique to study CBA mice which had been neonatally inoculated with A cells, as well as A mice neonatally given CBA cells (Hellström *et al.*, 1971d). Some of the animals so treated were skin grafted and found to be capable of permanently accepting the grafted skin; the mice were, therefore, considered to be tolerant. Other animals from the same group, not skin grafted, were used for the *in vitro* assays. Experiments were performed, in which cultivated A fibroblasts were exposed to lymph node cells from CBA mice tolerant to A, as well as to cells from nontolerant CBA and A mice. The reciprocal type of test was performed as well. A

specific cytotoxic effect of the lymphoid cells on cultivated fibroblasts from the tolerated strain could be demonstrated. It could also be shown that serum from the tolerant strain abrogated the cytotoxic effect. The blocking effect was specific, e.g., sera from CBA mice tolerant to A abrogated destruction of target cells from A (but not from CBA) by specifically reactive lymphocytes. These findings then indicated that the tolerant animals were specifically reactive against cells to which no reactivity was detected *in vivo* and they also showed that this reactivity could be blocked by tolerant serum.

One might argue, however, that the mice studied were not completely tolerant after all and that just a case of neonatally induced enhancement had been investigated. For this reason, it became important to perform similar studies on allophenic (tetraparental) mice, which had been established as chimeras by joining embryos at the eight-cell stage, i.e., long before any immunological system had been established. Determinations using hemoglobulin markers, found to correlate well with those using immunoglobulin markers and with karyotypic studies on the distribution of lymphoid cells characteristic for each of the two strains from which chimeras had been established (Gornish *et al.*, 1972), showed that the mice studied were true chimeras. According to Mintz and Silvers (1967), such animals were tolerant to skin grafts from the strains represented in the chimeras. It was found, in agreement with the data from mice neonatally inoculated with foreign cells, that lymph node cells from tetraparental chimeras were cytotoxic to cultivated fibroblasts from either of the two strains making up the chimeras and that sera from the same mice could block the lymphocyte effect (Wegmann *et al.*, 1971). These findings go further than those obtained by using neonatally inoculated animals in suggesting that the blocking phenomenon may be relevant for establishment of long-lasting allograft tolerance.

Another indication that blocking factors occur in tetraparental mice comes from work done with the mixed leukocyte culture technique. It was shown by Phillips *et al.* (1971) that sera from some (C57BL-SJL), but not all (e.g., not from C3H-C57BL) tetraparental chimeras could block mixed leukocyte reactivity, as compared to sera from the parental strains or their F_1 hybrids (or to mixtures of parental strain sera). The blocking activity could be removed by passing the sera through a column containing antibodies to mouse IgG. Furthermore, it was demonstrated that spleen cells from tetraparental mice could specifically suppress the reactivity in mixed leukocyte tests of spleen cells from either of the parental strains to each other, although they did not suppress reactivity to third-party cells (Phillips and Wegmann, 1973). The cell-mediated suppression has had the same degree of specificity as that detected by studying blocking serum factors; it is not known whether it is mediated

by soluble factors (similar to those in the serum) or by some other mechanism (e.g., suppressor cells).

Studies performed with rats, in which tolerance was induced by neonatal inoculation of allogeneic bone marrow, have supported the data from mice (Bansal *et al.*, 1973a). In sequential experiments on individual animals, B/N rats were inoculated with W/Fu bone marrow, and W/Fu rats were given B/N marrow. The following reactivity patterns could be observed. First, there were rats that were not tolerant and rejected skin grafts (from the strain to which tolerance induction had been attempted) within the normal time span (9–11 days). The blood lymphocytes of these rats were cytotoxic to cultivated fibroblasts carrying alloantigens of the type inoculated at birth, and the sera did not block the cytotoxic reactions. Second, there were rats that, when tested *in vivo,* were tolerant, i.e., they accepted skin grafts for more than 100 days (they also accepted second grafts from the respective strains). Lymphocytes from these rats were cytotoxic *in vitro* to tissues having alloantigens to which tolerance had been established, and sera from the same rats could block this cytotoxicity. The lymphocyte reactivity declined as the rats grew older, and approximately one-third of the rats had no detectable reactivity to tolerated tissues when they had carried the foreign skin grafts more than 100 days. Third, there were rats which may be characterized as temporarily tolerant. These rats rejected their skin grafts within 30 to 90 days. Their lymphocytes were cytotoxic to approximately the same extent as in the former group, when studied at the same time intervals after grafting. It is interesting that the blocking serum activity changed in these rats, so that sera taken when the rats carried healthy grafts were blocking, but blocking activity disappeared within approximately 7 days prior to graft rejection. Tolerant W/Fu rats receiving large doses of immune (W/Fu anti-BN) or nonimmune (W/Fu) lymphocytes lost tolerance and rejected their BN skin grafts. The blocking serum activity was regularly lost before or during the time of graft rejection (Bansal *et al.*, 1973b).

The data summarized support the notion that blocking serum factors play a role in the development and/or maintenance of allograft tolerance. There is a considerable amount of other data, however, which does not seem to agree with this idea. Mixed leukocyte culture assays have detected a specific lack of cellular reactivity in tolerant animals (Wilson *et al.*, 1967; Schwartz, 1968; Elkins, 1972), suggesting that potentially reactive lymphocyte clones may have been deleted (or are permanently suppressed). Attempts at passive transfer of tolerance with serum have failed (e.g., see Brent *et al.*, 1972), and attempts to induce classic immunological enhancement by using sera from tolerant donors have succeeded only in Voisin's experiments. Parabiosis of tolerant mice with

syngeneic, nontolerant mice did not transfer tolerance, and neither did it transfer any increased reactivity (Brent *et al.*, 1972). Mixtures between tolerant and nontolerant cells tested for induction of graft-versus-host reactivity were found to behave as nontolerant, i.e., they reacted, while tolerant cells tested alone did not (Atkins and Ford, 1972). On most occasions, hemagglutinating or cytotoxic antibodies have not been detected in tolerant mice and rats (Brent *et al.*, 1972).

It is also important to point out that even the fundamental question as to whether any blocking factors can be demonstrated in tolerant animals has received different answers in different laboratories that use slightly different techniques. Although blocking factors have been regularly found in studies performed by Hellström *et al.* (1971d), by Bansal *et al.* (1973), and also by Wood *et al.* (1972), they were seen only in so-called partially tolerant mice by Beverley *et al.* (1973), the partially tolerant animals accepting foreign skin grafts for a prolonged period of time but not indefinitely; in completely tolerant mice, neither lymphocyte cytotoxicity nor blocking serum activity was detected. Furthermore, no suppressor cells were seen in B/N rats made neonatally tolerant to the Lewis strain (Elkins, 1972). After tolerance had been broken by inoculation of nontolerant, syngeneic spleen cells, suppressor cell activity was detected also in the rat system, however, both by the mixed leukocyte culture technique and by assays for graft-versus-host reactivity *in vivo*.

One may argue that, in defense of the hypothesis that blocking serum factors play an important role in allograft tolerance, the failure to detect them in the experiments listed may have reflected a lack of sensitivity of the techniques used rather than a true absence of blocking factors. One may also argue that the only way one can establish whether blocking factors, as detected *in vitro*, play any role *in vivo*, is when the experiments are carried out so that sera (from the animals tested for tolerance *in vivo*) are really checked for blocking activity. If such *in vitro* checks for blocking activity are performed, one may be able to find out whether, for example, the failure to transfer tolerance with serum is simply due to the amounts of serum transferred being too small, the transfers being made too infrequently to maintain a blocking serum activity, the appearance of unblocking serum factors counteracting the blocking ones, or some other explanation. Better knowledge as to the nature of the blocking factors may also help to clarify apparent contradictions. If, for example, the blocking factors in tolerant animals are antigen–antibody complexes as suggested by recent findings (Wright *et al.*, 1973b), it would not be surprising if they are not detectable with immunological assays (such as hemagglutination tests) that have been developed to show antibodies.

Even if the failure to detect important *in vivo* effects of blocking

serum factors in allograft tolerance has a simple technical explanation (such as lack of sensitivity of the assays used or too little serum inoculated), it seems likely that the specific nonreactivity, which can be observed *in vivo* as tolerance to allogeneic grafts, has more than one explanation and that blocking serum factors provide one, but not the only explanation. One might speculate that the most important role of the blocking serum factors is to act as a back-up mechanism under conditions when all potentially reactive lymphocyte clones are not irreversibly suppressed (or eliminated). It is not known whether the absence of detectable lymphocyte reactivity in these systems is due to the presence of blocking factors bound to the lymphoid cells in a very stable fashion, or whether some lymphocyte clones with crucial functions, e.g., in graft-versus-host assays, have been actually deleted. Neither does one know whether the lymphocyte reactivity that can be detected in tolerant animals by using the microcytotoxicity assay is due to the continuous appearance of a small number of "forbidden" lymphocyte clones from stem cells, which remain for a short time before they are deleted. Furthermore, it is not known whether the reactive cells in tolerant animals are of T or B origin.

It also remains to be studied whether a complete immunological nonreactivity or a cell-mediated immunity counteracted by blocking serum factors is the preferred mechanism for establishment of self-tolerance to tissue-specific antigens, and whether the induction of complete unresponsiveness to allogeneic organ grafts renders the recipient better capable of accepting the grafts than the induction of a blocking serum activity (coexisting with cellular reactivity). Before this is known, valid conclusions as to the roles, *in vivo*, of complete or partial tolerance are difficult to draw.

XIII. Concluding Remarks

We have reviewed evidence for cell-mediated immune reactions against tumors in animals and in man, particularly reactions that can lead to destruction of neoplastic cells cultivated *in vitro*. Various mechanisms by which tumor cells can escape from immunological destruction have been discussed, most notably one involving blocking factors, present in the serum. Findings analogous to those obtained when studying tumor immunity (coexistence of cell-mediated reactivity and blocking serum activity) have been summarized from three other areas (pregnancy, allografting, and chimeras).

The studies reviewed have given a relatively large amount of evidence for various cell-mediated and humoral immunological reactions against growing tumors, and at least some of the reactions observed *in vitro* appear to be able to influence tumor growth *in vivo*. One is struck, how-

ever, by the lack of knowledge on certain crucial points: What cell types are involved in the reactions? What are their individual roles and how do they interact? Which are the molecules acting as antigens, blocking factors, arming factors, unblockers? What are their mechanisms of action and on what cells?

More rapid and more precise tests to study the various phenomena quantitatively and a much firmer knowledge of the cells and the molecules involved are needed. When we have these, better ways to manipulate the immune response to tumor antigens may evolve.

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Author Index

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