ADVANCES IN IMMUNOLOGY

VOLUME 29

ADVANCES IN

Immunology

VOLUME 29

CONTRIBUTORS TO THIS VOLUME

KLAUS BENDTZEN
DIRK GREINEDER
DAVID H. KATZ
MARGUERITE M. B. KAY
AUSTIN E. LARSEN
TAKASHI MAKINODAN
HANS J. MÜLLER-EBERHARD
MICHAEL B. A. OLDSTONE
DAVID D. PORTER
HELEN G. PORTER
ROSS E. ROCKLIN
ROBERT D. SCHREIBER
J. G. PATRICK SISSONS

ADVANCES IN
Immunology

EDITED BY

HENRY G. KUNKEL
The Rockefeller University
New York, New York

FRANK J. DIXON
Scripps Clinic and Research Foundation
La Jolla, California

VOLUME 29

1980

ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

New York London Toronto Sydney San Francisco

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 61–17057

ISBN 0–12–022429–1

PRINTED IN THE UNITED STATES OF AMERICA

80 81 82 83 9 8 7 6 5 4 3 2 1

CONTENTS

LIST OF CONTRIBUTORS ix
PREFACE xi

Molecular Biology and Chemistry of the Alternative Pathway of Complement
HANS J. MÜLLER-EBERHARD AND ROBERT D. SCHREIBER

I. Introduction 2
II. The Proteins 2
III. The C3b-Dependent Feedback and Amplification 15
IV. Regulation 17
V. The Metastable Binding Site 17
VI. Initiation 19
VII. The Alternative Pathway: Molecular Events 27
VIII. The Cytolytic Alternative Pathway 30
IX. Generation of Bactericidal Activity and Bacteriolysis 32
X. Lysis of Nucleated Cells 35
XI. Inactivation of Viruses 37
XII. Effects on Host–Parasite Relationship 38
XIII. Reaction Products Influencing Cellular Functions 40
XIV. Nephritic Factor: Autoantibody to C3 Convertase 42
XV. Conclusion 45
References 46

Mediators of Immunity: Lymphokines and Monokines
ROSS E. ROCKLIN, KLAUS BENDTZEN, AND DIRK GREINEDER

I. Introduction 56
II. Lymphokines 58
III. Cell Types Involved in Lymphokine Synthesis 101
IV. Regulation and Pharmacologic Modulation of Lymphokine Production and Action 103
V. Antibodies to Lymphokines 109
VI. *In Vivo* Significance of Lymphokines 112
VII. Clinical Relevance of the Measurement of Lymphokines 114
VIII. Monokines 115
IX. Biologic Significance of Monokines 124
X. Conclusion 125
References 127

Adaptive Differentiation of Lymphocytes: Theoretical Implications for Mechanisms of Cell–Cell Recognition and Regulation of Immune Responses
DAVID H. KATZ

I. Introduction 138
II. Basis for Genetic Restrictions on Cell Interactions 142
III. The Concept of Adaptive Differentiation 144
IV. Recent Experiments with Bone Marrow Chimeras 145
V. Theoretical Considerations on the Mechanisms of Adaptive Differentiation 147
VI. Extrathymic versus Intrathymic Influences on Adaptive Differentiation... 160
VII. The Concept of Environmental Restraint and Possible Explanatory Mechanisms 167
VIII. The Case for B Cell Adaptive Differentiation and Pertinence to the Concept of Interacting "Partner Cell Sets" 184
IX. Relevance of the Proposed Mechanisms of Adaptive Differentiation to Certain Unresolved Immunologic Puzzles 192
X. Conclusions 201
References 203

Antibody-Mediated Destruction of Virus-Infected Cells
J. G. PATRICK SISSONS AND MICHAEL B. A. OLDSTONE

I. Introduction 209
II. Viral Antigen Expression and the Antibody Response 211
III. Effect of Antibody Alone on Virus-Infected Cells 215
IV. Antibody- and Complement-Mediated Lysis of Virus-Infected Cells 229
V. Antibody-Dependent Cell-Mediated Cytotoxicity of Virus-Infected Cells 245
VI. Conclusions 251
References 255

Aleutian Disease of Mink
DAVID D. PORTER, AUSTIN E. LARSEN, AND HELEN G. PORTER

I. Introduction 261
II. Biology and Immunobiology of Mink 262
III. Aleutian Disease Virus 263
IV. The Host Immune Response to Infection 267
V. The Pathogenesis of Lesions 273
VI. Disease Control 281
VII. Discussion 282
References 284

Age Influence on the Immune System
TAKASHI MAKINODAN AND MARGUERITE M. B. KAY

I. Introduction 287
II. Age-Related Changes in Immune Functions 288
III. Mechanisms of the Age-Related Alterations in Normal Immune Functions 295
IV. Prevention and Restoration 312
V. Concluding Remarks 321
References 322

SUBJECT INDEX 331
CONTENTS OF PREVIOUS VOLUMES 335

This Page Intentionally Left Blank

LIST OF CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

KLAUS BENDTZEN, *Department of Medicine, Tufts University School of Medicine, New England Medical Center Hospital, Boston, Massachusetts 02111* (55)

DIRK GREINEDER, *Harvard Medical School, Robert B. Brigham Hospital, Boston, Massachusetts 02115* (55)

DAVID H. KATZ, *Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037* (137)

MARGUERITE M. B. KAY, *Geriatric Research, Education and Clinical Center (GRECC), Veterans Administration Wadsworth Medical Center, and the Department of Medicine, University of California, Los Angeles, California 90073* (287)

AUSTIN E. LARSEN, *Department of Cellular, Viral and Molecular Biology, University of Utah College of Medicine, Salt Lake City, Utah 84112* (261)

TAKASHI MAKINODAN, *Geriatric Research Education and Clinical Center (GRECC), Veterans Administration Wadsworth Medical Center, and the Department of Medicine, University of California, Los Angeles, California 90073* (287)

HANS J. MÜLLER-EBERHARD, *Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037* (1)

MICHAEL B. A. OLDSTONE, *Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California 92037* (209)

DAVID D. PORTER, *Department of Pathology, UCLA School of Medicine, Los Angeles, California 90024* (261)

HELEN G. PORTER, *Department of Pathology, UCLA School of Medicine, Los Angeles, California 90024* (261)

ROSS E. ROCKLIN, *Department of Medicine, Tufts University School of Medicine, New England Medical Center Hospital, Boston, Massachusetts 02111* (55)

ROBERT D. SCHREIBER, *Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037* (1)

J. G. PATRICK SISSONS, *Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California 92037* (209)

PREFACE

One of the inevitable consequences of the scientific explosion of our era has been ultraspecialization with the emergence of communication barriers even among the disciplines of the biological sciences. However, opposing this trend, the science of immunology has represented a unique unifying force. This has become ever more apparent in the last few years with the remarkable advances of immunological research techniques, making them essential tools of virtually all biological research. The hybridoma system producing monoclonal antibodies is the most obvious example, but there are many others. The lymphocyte, because of its availability, ease of differentiation, and ready long-term propagation, also has become a model cell for a wide assortment of eukaryotic studies. Some of these diverse forefronts are subjects of this volume. Even the enigmatic topic of aging is not exempt from the influence of immunology.

The first chapter by Müller-Eberhard and Schreiber is a very complete review of the recent work on the alternative pathway. The confused state of this subject just a few years ago has been replaced by a remarkably well-defined system whose component parts are clearly established. The six proteins of the alternative pathway have been isolated, an accomplishment in which the authors played a key role. These proteins and their special properties as enzymes, enzyme substrates, and regulators are presented in detail in this review. The participation with the classical pathway components, C5–C9, in membrane attack is considered but the details are left to a forthcoming review. The surprising discovery of the activation of the alternative pathway by many systems other than antibody is emphasized as is the key role played by C3 and its component parts.

The second chapter, on mediators of immunity, is written by Rocklin, Bendtzen, and Greineder. These authors have very successfully covered the very broad array of lymphokines and monokines that have marked biological effects on a variety of cell types, including B and T lymphocytes, macrophages, and other cells. The migration inhibitory factors, the chemotactic factors, the mitogenic factors, the helper and suppressor factors, the lymphotoxins, and growth-promoting factors are all considered in detail. Very few of these substances have been isolated but the progress being made and the acquisition of specific antibodies to individual factors are well covered. Evidence is presented for single factors with multiple activities, a topic that has

plagued investigators in this field. The biological significance of some of the factors is evident; in other cases it clearly remains to be proved.

In the third chapter David Katz reviews in detail our current knowledge of cell–cell interactions in immune responses and their relationship to Ir and other major histocompatibility complex genes. This is done primarily from the theoretical standpoint in an attempt to understand these relationships. After reviewing various previous theories, he presents his own concept involving CI or cell interaction molecules of primary importance in self-recognition which he envisions as the products of Ir genes. It is an attractive theory which he feels might explain the high degree of polymorphism among the MHC genes and the frequency of lymphocytes in any one individual that appear to be specific for histocompatibility antigens of other individuals. Such CI molecules and their anti-CI receptors have not as yet been identified but the ready explanation of self-recognition phenomena offered by such molecules should stimulate a search for at least some kind of analogous system.

In the fourth chapter, Sissons and Oldstone describe the effect of antibody on virus-infected cells with special reference to the human system. The destruction by antibody of the infected cells through the mediation of complement is described in detail based in considerable part on the contributions of the authors. Activation of the alternative pathway by the various infected cells is of special interest. The interesting effect of the ADCC system involving viral antigens in cell killing is also presented. Multiple additional topics are also covered, such as the effect of antibody on the expression of viral proteins both on the surface of the cell and intracellularly. Multiple potential roles of these antibody-mediated reactions in altering systemic viral infections are evident from this review.

The immunological parameters of the Aleutian disease of mink is covered in the fifth chapter of this volume. Drs. Porter, Larsen, and Porter have had wide experience with this disease and its strikingly increased immune response. The disease is of special interest because it serves as an excellent model of immune complex disease in which the antigen involved in the immune complexes is known. Viral infection produces a uniquely great antibody response with marked accumulations of plasma cells in the lymph nodes, spleen, kidney, and liver along with marked hyperglobulinemia. Immune complexes are readily demonstrable even by analytical ultracentrifugation, and viral antigen has been demonstrated in the complexes. Severe kidney disease develops in some animals, and viral antibody and antigen have been eluted from the kidney. The glomerular lesions are typical of an immune complex-mediated injury with fine granular deposits along

the glomerular basement membrane which can be stained for Ig, C3, and viral antigen.

The final chapter of this volume is devoted to the question of perturbations in the immune system during aging. Drs. Makinodan and Kay have reviewed in detail the many observations indicating a decline in immune function with aging both in experimental animals and in humans. Many of the results are conflicting, a problem which for a long time has plagued this field, but the authors have analyzed these observations in detail and extracted the justified conclusions. It is evident that a decline in T cell function gradually occurs and this affects the entire immune system. Antibody formation and B cell function appear to be secondarily altered. It is of special interest that various autoantibodies are increased in older experimental animals and humans which may relate to the observed decrease in suppressor cells. The increase in incidence of monoclonal Ig bands is also a striking feature. Many efforts are currently underway to reverse the immune defects; a few of these have met with some success.

We are indebted to the authors for their special efforts in preparing these reviews and to the publishers for their fine cooperation.

HENRY G. KUNKEL
FRANK J. DIXON

This Page Intentionally Left Blank

ADVANCES IN IMMUNOLOGY, VOL. 29

Molecular Biology and Chemistry of the Alternative Pathway of Complement[1-3]

HANS J. MÜLLER-EBERHARD[4] AND ROBERT D. SCHREIBER[5]

Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, California

I. Introduction 2
II. The Proteins 2
A. C3 4
B. Factor B: C3 Proactivator 9
C. Factor D: C3 Proactivator Convertase 12
D. β1H 13
E. C3b Inactivator 13
F. Properdin 14
III. The C3b-Dependent Feedback and Amplification 15
IV. Regulation 17
V. The Metastable Binding Site 17
VI. Initiation 19
A. The Initial Enzyme 19
B. The Stable Five-Protein System 20
C. Concentration Dependence 21
D. Restriction of Control by Activators 22
E. Discrimination between Activators and Nonactivators by Bound C3b 24
VII. The Alternative Pathway: Molecular Events 27
VIII. The Cytolytic Alternative Pathway 30
IX. Generation of Bactericidal Activity and Bacteriolysis 32
X. Lysis of Nucleated Cells 35
XI. Inactivation of Viruses 37
XII. Effects on Host–Parasite Relationship 38
XIII. Reaction Products Influencing Cellular Functions 40
XIV. Nephritic Factor: Autoantibody to C3 Convertase 42
XV. Conclusion 45
References 46

[1] This is publication No. 2003 from the Research Institute of Scripps Clinic.

[2] The work reported from this laboratory was supported by United States Public Health Service Grants AI 07007, AI 13010, and HL 16411.

[3] Dedicated to Fritz Hartmann, Professor of Medicine, Hannover Medical School, Germany, on the occasion of his sixtieth birthday.

[4] Cecil H. and Ida M. Green Investigator in Medical Research, Research Institute of Scripps Clinic.

[5] Recipient of Established Investigatorship No. 77-202 of the American Heart Association.

ISBN 0-12-022429-1

I. Introduction

The alternative pathway of complement activation may be closely linked to natural resistance to infections. It has become clear that the pathway may be activated and may operate in total absence of specific antibody or immunoglobulins. This insight was gained when it became possible to assemble the pathway *in vitro* from six isolated plasma proteins and show that the isolated component mixture functioned qualitatively and quantitatively like the alternative pathway in whole serum or plasma.

Since 1976, when the subject was last reviewed in this series (Götze and Müller-Eberhard, 1976), considerable advances have been made. The initiation of the pathway is better understood than before: we have learned that target-bound C3b apparently determines whether the pathway is set in motion or not. The complex interactions between C3b and target surface constituents and between C3b and the regulatory proteins of the pathway have received much attention and may provide a clue to a biochemical definition of alternative pathway initiation. To date, we do not know the common denominator of the chemical structures that are capable of initiating the pathway. This area requires much further work.

After discussion of the proteins of the pathway, initiation, amplification and regulation will be described with emphasis on those aspects that were insufficiently understood in 1976. The next portion deals with biological manifestations of alternative pathway activation. This area of research has been facilitated by the introduction of the isolated cytolytic alternative pathway, which includes the five proteins of the membrane attack pathway. Thus, antibody-independent killing by the cytolytic pathway of bacterial and animal cells, including virus-infected human cells, will be illuminated. Finally, the story of nephritic factor, an autoantibody to the alternative C3 convertase, will be summarized.

II. The Proteins

The six proteins of the alternative pathway of complement activation isolated from human serum are depicted in Fig. 1 (left panel) after electrophoresis in sodium dodecyl sulfate (SDS)–polyacrylamide gels under nonreducing conditions. Some of their properties are listed in Table I. Representative amino acid compositions of the six human proteins are compiled in Table II. The proteins are C3, factor B, factor D, β1H, C3b inactivator (C3bINA), and properdin. Factor B, factor D, and C3bINA are enzymes, whereas C3, β1H, and properdin lack enzy-

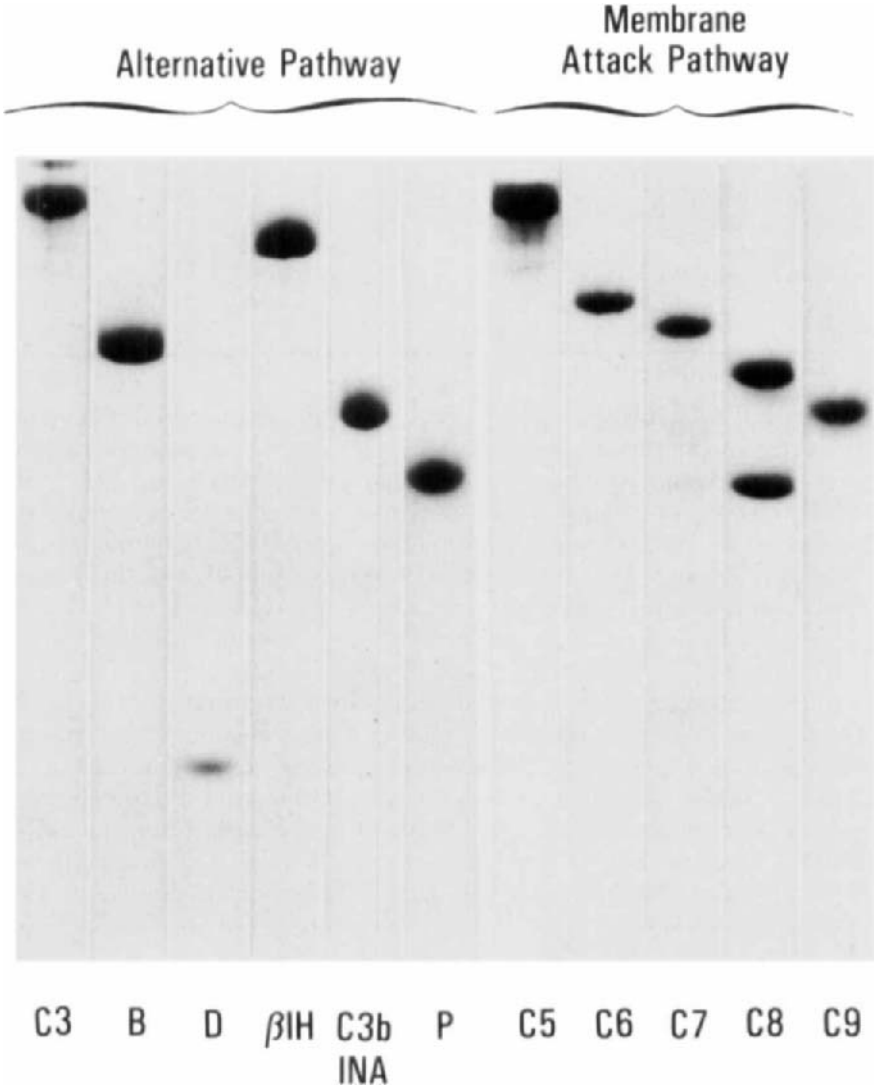


FIG. 1. The proteins of the cytolytic alternative pathway shown upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 7% gels under nonreducing conditions. Gels were stained with Coomassie Blue dye R250 (Schreiber and Müller-Eberhard, 1978a).

TABLE I
PROTEINS OF THE ALTERNATIVE PATHWAY OF COMPLEMENT ACTIVATION

Protein	Symbol	Molecular weight	No. of chains	Electrophoretic mobility	Serum conc. (μg/ml)
C3	C3	180,000	2	β	1200
C3 Proactivator	B	93,000	1	β	200
C3 Proactivator convertase	D	24,000	1	β − γ	1
C3b Inactivator	C3bINA	88,000	2	β	34
β1H	β1H	150,000	1	β	500
Properdin	P	224,000	4	γ	20

mic activity. C3, factor B, and factor D may be considered components proper, since their interactions directly lead to the formation of the C3 and C5 cleaving enzymes of the pathway. Properdin, β1H, and C3bINA may be regarded as regulators. The general molecular arrangement of the pathway may be viewed as centering around one key protein (C3) and its interactions with other molecules of the system and with biological particles.

A. C3

C3 is the precursor of several biologically active fragments and in its uncleaved form is postulated to be a subunit of the initial C3 convertase of the alternative pathway. Of all complement components, C3 was the first to be isolated and to be defined as a distinct protein (Müller-Eberhard and Nilsson, 1960; Müller-Eberhard *et al.*, 1960). Human C3 is a 9.5 S β-globulin containing 2.7% carbohydrate, i.e., 1.8% hexose, 0.2% fucose, 0.5% hexosamine, and 0.5% sialic acid. The amino acid composition has been determined in several laboratories (Budzko *et al.*, 1971; Molenaar *et al.*, 1974; Tack *et al.*, 1979) (Table II). It has a molecular weight of 180,000 and consists of two nonidentical polypeptide chains held together by disulfide bonds and noncovalent forces. The partial specific volume is 0.736 ml gm^{-1} (Tack and Prahl, 1976). The size of the α chain is 110,000 daltons, that of the β chain 70,000 daltons (Bokisch *et al.*, 1975; Nilsson *et al.*, 1975; Tack *et al.*, 1979). Serine is the N-terminal amino acid residue of both chains, and the C-terminal residue of the α chain. The C terminus of the β chain is occupied by alanine (Tack *et al.*, 1979). The sequence of the N-terminal 91 residues of the α chain (Hugli, 1975; Tack *et al.*, 1979) and of the N-terminal 10 residues of the β chain (Tack *et al.*, 1979) has been established (Fig. 2). There exists a certain degree of homology in primary structure between C3 and C4 (Gorski *et al.*, 1979b) and C3

TABLE II
AMINO ACID COMPOSITION OF THE PROTEINS OF THE ALTERNATIVE PATHWAY (RESIDUES PER 1000 RESIDUES)

Amino acid	C3[a]	B[b]	D[c]	β1H[d]	C3bINA[e]	P[f]
Lysine	71	72	36	67	75	46
Histidine	17	25	41	24	21	27
Arginine	49	52	81	44	39	87
Aspartic acid	93	100	91	94	99	46
Threonine	61	56	41	64	58	56
Serine	65	76	71	67	78	71
Glutamic acid	134	115	91	120	111	150
Proline	48	60	71	77	41	114
Glycine	60	85	107	85	94	125
Alanine	60	46	107	33	57	55
Half-cystine	13[g]	28[h]	NR[i]	54[h]	54[h]	49[h]
Valine	89	71	81	50	65	45
Methionine	19	17[h]	15	13[h]	14[h]	14[h]
Isoleucine	48	46	25	53	45	13
Leucine	93	70	117	49	55	50
Tyrosine	34	41	20	59	40	14
Phenylalanine	37	26	5	26	36	19
Tryptophan	7[j]	16[j]	NR	21[j]	18[j]	20[k]
Total residues	998	1002	1000	1000	1000	1001

[a] Tack *et al.* (1979).
[b] Lesavre *et al.* (1979).
[c] Davis *et al.* (1979).
[d] Pangburn *et al.* (1979).
[e] Pangburn *et al.* (1977).
[f] Minta and Lepow (1974).
[g] Determined as S-carboxymethyl cysteine; determined as cysteic acid, a value of 23 was obtained.
[h] Determined after performic acid oxidation.
[i] NR, not reported.
[j] Determined after base hydrolysis.
[k] Assumed to be 2%.

and C5 (Fernandez and Hugli, 1977). C3 is synthesized in the liver (Alper *et al.*, 1969; Colten, 1972; McClelland and van Furth, 1976; Brade *et al.*, 1977a) and also by macrophages and monocytes (McClelland and van Furth, 1976; Einstein *et al.*, 1977; Brade *et al.*, 1978). It is synthesized as a single polypeptide chain which, after translation, is processed in the cytoplasm to the two-chain molecule that occurs in serum (Brade *et al.*, 1977a; Patel and Minta, 1979). C3 exhibits genetic polymorphism that is demonstrable by gel electrophoresis Wieme and Demeulenaere, 1967; Alper and Propp, 1968; Azen and Smithies, 1968). Unlike C2, C4, and factor B, C3 is not linked genetically to his-

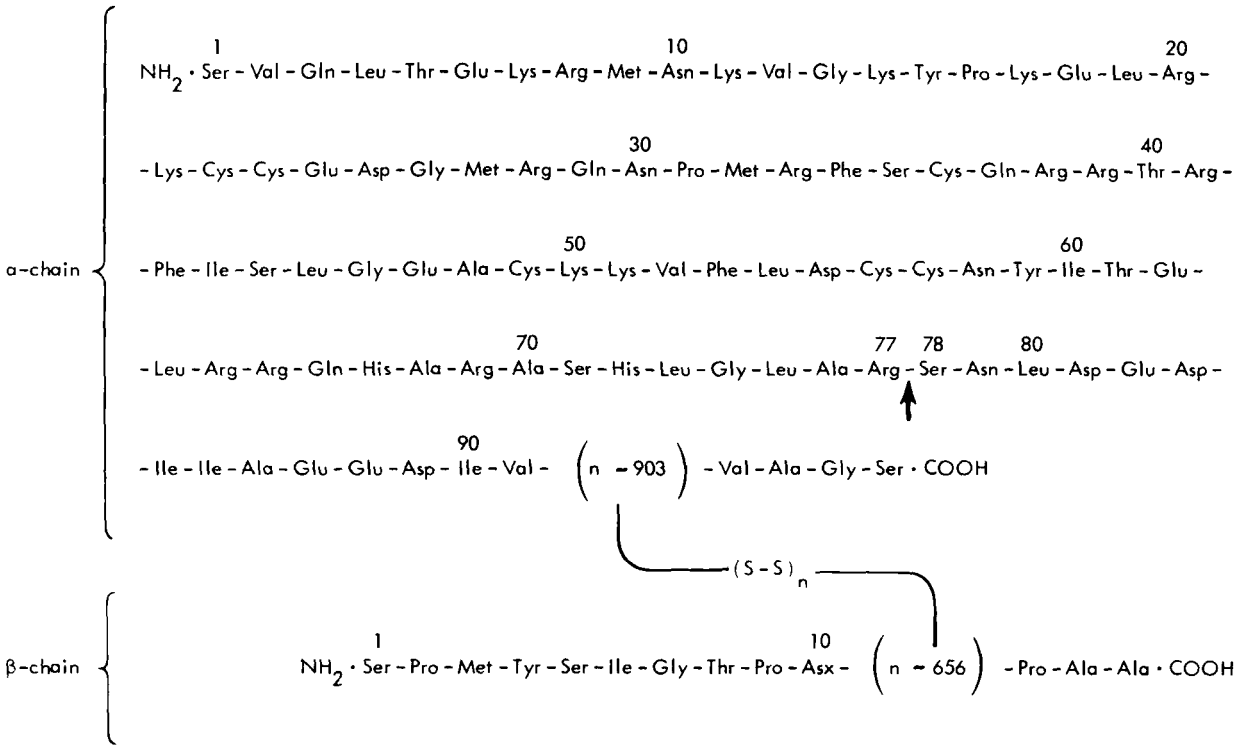


FIG. 2. Partial primary structure of human C3. The arrow denotes the bond of the α chain that is cleaved by the C3 convertase of the classical or alternative pathway upon activation of C3. The N-terminal sequence 1–77 constitutes the anaphylatoxin C3a. C3b is composed of the α chain, minus C3a, plus the β chain (Hugli, 1975; Tack *et al.*, 1979).

TABLE III
C3 FRAGMENTS AND THEIR BIOLOGICAL ACTIVITIES

Fragment	Molecular weight	Polypeptide chains	Biological activity[a]
C3a	9,000	1	Anaphylatoxin; histamine release; cellular enzyme release; smooth muscle contraction
C3b	170,000	2	Recognition (AP); binding to biological particles; subunit C3/C5 convertase (AP); subunit C5 convertase (CP); receptor for activated properdin; ligand for cellular C3b receptor
$C3b_i$	170,000	3	Ligand for cellular $C3b_i$ receptor
C3c	140,000	3	Precursor of C3e
C3d	25,000	1	Ligand for cellular C3d receptor
C3e	12,000	1	Induction of leukocytosis

[a] AP, alternative pathway; CP, classical pathway.

tocompatibility antigens in man. In the mouse, however, serum C3 levels have been shown to be linked to the *H-2* locus and there is evidence that the C3 gene is located in proximity to *H-2* on chromosome 17 (Ferreira and Nussenzweig, 1975, 1976; Natsuume-Sakai *et al.*, 1978; Penalva Da Silva *et al.*, 1978). Several individuals have been described with a homozygous C3 deficiency, and these individuals suffer from severe recurrent infections (Lachmann and Rosen, 1978).

Table III lists the physiological fragments of C3 that arise during the processes of activation and control of the molecule. Figure 3 describes the proposed topology of the fragments in relation to the intact C3 molecule. Activation of C3 occurs upon hydrolysis of peptide bond 77 (Arg-Ser) of the α chain by C3 convertase (Hugli and Müller-Eberhard, 1978; Tack *et al.*, 1979). This cleavage leads to the formation of C3a (molecular weight 9000), which is one of the three complement-derived anaphylatoxins (Dias Da Silva *et al.*, 1967; Cochrane and Müller-Eberhard, 1968; Hugli and Müller-Eberhard, 1978) and of the major fragment C3b (MW 171,000). C3a is a very basic peptide, having an electrophoretic mobility at pH 8.6 of $+2.1 \times 10^{-5}$ cm^2 V^{-1} sec^{-1} (Budzko *et al.*, 1971). Its primary sequence has been determined (Fig. 2) (Hugli, 1975). Its structure is homologous to the structure of C4a

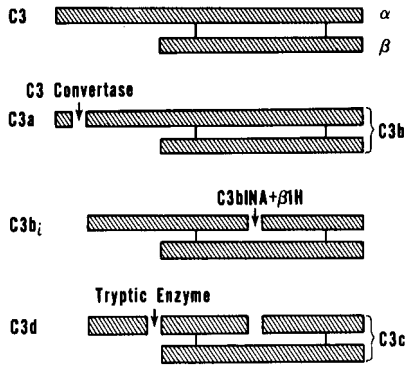


FIG. 3. Schematic description of the physiological activation and control of C3. In succession, three enzymes cause liberation of C3a anaphylatoxin, generation of C3b and its transient binding site, inactivation of C3b and fragmentation of $C3b_i$ into two immunochemically distinct pieces (Müller-Eberhard, 1978).

(Gorski *et al.*, 1979a,b) and C5a (Fernandez and Hugli, 1977, 1978). Circular dichroism analysis showed the fragment to contain 45% α-helical conformation (Hugli *et al.*, 1975), which is similar to that of C4a (Gorski *et al.*, 1979b) and C5a (Morgan *et al.*, 1974; Fernandez and Hugli, 1978). Concomitant with the C3a-C3b cleavage, a metastable binding site (Müller-Eberhard *et al.*, 1966; Müller-Eberhard, 1975) is expressed on C3b through which C3b can firmly attach to a large variety of biological particles, including immune complexes, yeast cell walls, bacteria, parasites, and animal cells (see later) (Müller-Eberhard, 1975; Law and Levine, 1977; Capel *et al.*, 1978).

Particle-bound C3b has the following functions:

1. It is an opsonin; i.e., it marks target particles for ingestion by phagocytic cells and serves as a ligand to C3b specific cell surface receptors (Gigli and Nelson, 1968; Huber *et al.*, 1968).
2. It is the acceptor of activated factor B and thus a subunit of the target bound alternative C3 convertase (Daha *et al.*, 1976c; Götze and Müller-Eberhard, 1976; Medicus *et al.*, 1976a,c; Vogt *et al.*, 1977).
3. It is the substrate modulator of the target bound C5 convertase (Vogt *et al.*, 1978).
4. It serves as receptor for activated properdin (Schreiber *et al.*, 1975).
5. It binds β1H, which results in blocking of the factor B binding site or in dissociation of the b fragment of factor B from the C3/C5 convertase (Weiler *et al.*, 1976; Whaley and Ruddy, 1976a; Conrad

et al., 1978; Nagaki *et al.*, 1978; Pangburn and Müller-Eberhard, 1978a) and in conformational adaptation of C3b to the action of C3b inactivator (Whaley and Ruddy, 1976a; Pangburn *et al.*, 1977).
6. It appears to be capable of distinguishing between an activator and a nonactivator of the alternative pathway (Pangburn and Müller-Eberhard, 1978a; Schreiber *et al.*, 1978; Pangburn *et al.*, 1980), which is reflected in a differential accessibility of bound C3b to the regulator β1H (Fearon and Austen, 1977a,b; Pangburn and Müller-Eberhard, 1978a; Kazatchkine *et al.*, 1979a).

Control of the two primary fragments of C3 is exerted such that C3a is inactivated by the serum carboxypeptidase B, which removes the essential C-terminal arginine residue (Bokisch and Müller-Eberhard, 1970; Budzko *et al.*, 1971), and C3b is inactivated by the C3b inactivator (Tamura and Nelson, 1967; Lachmann and Müller-Eberhard, 1968; Ruddy and Austen, 1971), which cleaves the α chain of C3b into a 67,000 and a 40,000 dalton fragment (Pangburn *et al.*, 1977). Since both α fragments are disulfide bonded to the β chain, the cleaved molecule ($C3b_i$) has the same molecular size as intact C3b. For the inactivation of fluid phase C3b by C3b inactivator, β1H is absolutely required (Pangburn *et al.*, 1977). Inactivation of bound C3b proceeds without β1H but is 30-fold enhanced by it. Apparently free C3b and bound C3b have different conformations.

$C3b_i$ is highly susceptible to attack by tryptic enzymes (Lachmann and Müller-Eberhard, 1968; Pangburn *et al.*, 1977; Natsuume-Sakai *et al.*, 1978), which cleave the α chain in the N-terminal region and thereby produce the two fragments C3c (MW 140,000) and C3d (MW 30,000). The C3d portion contains the particle binding site of C3b and therefore remains bound when C3c is cleaved off (Ruddy and Austen, 1971; Bokisch *et al.*, 1975; Law *et al.*, 1979a). Further tryptic degradation of C3c produces the fragment C3e (MW 10,000), which is probably derived from the α chain and has leukocytosis inducing activity (Ghebrehiwet and Müller-Eberhard, 1979). The fragments C3b and $C3b_i$ have specific receptors on polymorphonuclear leukocytes and monocytes (Ross *et al.*, 1973; Theofilopoulos *et al.*, 1974; Ross and Rabellino, 1979). The various binding sites of C3b and their functions are enumerated in Table IV.

B. Factor B: C3 Proactivator

This protein is the precursor of the key enzyme of the alternative pathway. It was first recognized through its ability to form a C3 cleaving enzyme together with cobra venom factor (Müller-Eberhard *et al.*, 1966b; Müller-Eberhard and Fjellström, 1971; Cooper, 1973). It is a

TABLE IV
BINDING SITES ON C3b AND ITS PRODUCTS

Binding site	Function
Metastable	Binding of C3 to biological particles
Discriminatory	Differentiation between alternative pathway activators and nonactivators
β1H	Disassembly of C3/C5 convertase, conformational adaptation of C3b to cleavage by C3bINA
C3bINA	Control of C3b
Factor B	Formation of C3/C5 convertase
Properdin	Stabilization of C3/C5 convertase
C5	Modulation of C5 for cleavage by C3/C5 convertase
C3b receptor	Phagocytosis, cell–cell interaction
$C3b_i$ receptor	Phagocytosis, cell–cell interaction
C3d receptor	Cell–cell interaction

thermolabile, single-chain 5.9 S β-globulin having a molecular weight of 93,000 (Götze, 1975; Curman *et al.*, 1977; Kerr and Porter, 1978) and a carbohydrate content of 7.3%, i.e., 1% galactose, 1.4% mannose, 0.1% fucose, 2.3% glucosamine, and 2.3% sialic acid (Curman *et al.*, 1977). The diffusion coefficient is 5.4×10^{-7} cm^2 sec^{-1}, the partial specific volume 0.721 ml gm^{-1} and the frictional ratio 1.28 (Curman *et al.*, 1977). The amino acid composition of factor B has been determined in several laboratories (Boenisch and Alper, 1970; Curman *et al.*, 1977; Kerr and Porter, 1978; Lesavre *et al.*, 1979) (Table II). Its concentration in human serum is 200 μg/ml. Factor B exhibits molecular polymorphism that is genetically linked to C2 (Alper *et al.*, 1972; Bitter-Suermann *et al.*, 1977) and to the major histocompatibility locus in man (Allen, 1974) and monkey (Ziegler *et al.*, 1975a,b). The genes for factor B and C2 are located side by side on chromosome 6 of man, approximately 5 centimorgans (cM) from the HLA-D locus (Raum *et al.*, 1976). The protein is synthesized by macrophages (Brade *et al.*, 1978) and by lymphocytes (Halbwachs and Lachmann, 1976). Factor B is activated by enzymic cleavage into two fragments, the MW 33,000 B*a*, which behaves as an α-globulin, and the MW 60,000 Bb, which has the electrophoretic mobility of γ-globulin (Boenisch and Alper, 1970; Götze and Müller-Eberhard, 1971; Götze, 1975). Physiological activation is accomplished by factor D, which can act on factor B only when it is in Mg^{2+}-dependent complex with C3b (Medicus *et al.*, 1976a; Vogt *et al.*, 1977; Lesavre and Müller-Eberhard, 1978). The activation fragment Ba is released during the reaction, and Bb, the active site carrying fragment, remains in complex with C3b, forming the alternative pathway C3 convertase.

Partial structural analysis has shown that Ba is derived from the N-terminal, and Bb from the C-terminal, region of factor B. The N terminus of Ba and B is blocked. The C terminus of Ba is occupied by Arg, and that of Bb and of B is Leu. Apparently, the C terminus of Ba is linked to the N terminus of Bb in the intact B molecule, and activation by factor D occurs by a trypsin-like cleavage of an Arg-Lys peptide bond at a site containing three basic residues, Lys-Arg-Lys (Fig. 4) (Lesavre *et al.*, 1979). Definite proof must await the amino acid sequence of the petide that overlaps the site of scission in factor B. Although the N terminus was found blocked in the above investigations, others reported proline (Curman *et al.*, 1977) or threomine (Kerr and Porter, 1978) to be in N-terminal position of factor B.

Whereas Bb can act on its natural substrates only when in complex with C3b, it hydrolyzes the synthetic ester acetyl-glycyl-lysine methyl ester also in its unassociated form (Cooper, 1971). The enzyme is inhibitable by DFP and binds 1 mol of the inhibitor per mole of Bb (Medicus *et al.*, 1976b). By this criterion, Bb is a serine protease. Circular dichroism spectra showed that 17% of the factor B polypeptide chain is folded in a α-helical arrangement, 22% of Bb, and 0% of Ba (Lesavre *et al.*, 1979). By comparison, C3a, C4a, and C5a contain 40–50% α-helical structure (Hugli and Müller-Eberhard, 1978; Gorski *et al.*, 1979b). Using [^{14}C]*p*-CMB, both Ba and Bb were found to contain each one reactive sulfhydryl group. Factor B bound 2 mol of *p*-CMB which reduced its cytolytic activity by 50% (Lesavre *et al.*, 1979). Upon dissociation of *p*-CMB by treatment with cysteine, full activity was recovered. Guinea pig Ba has been reported to have chemotactic activity for neutrophils (Hading *et al.*, 1978), which could not be shown for human Ba (Lesavre *et al.*, 1979). Human Bb has marked cell spreading activity with respect to monocytes and macrophages

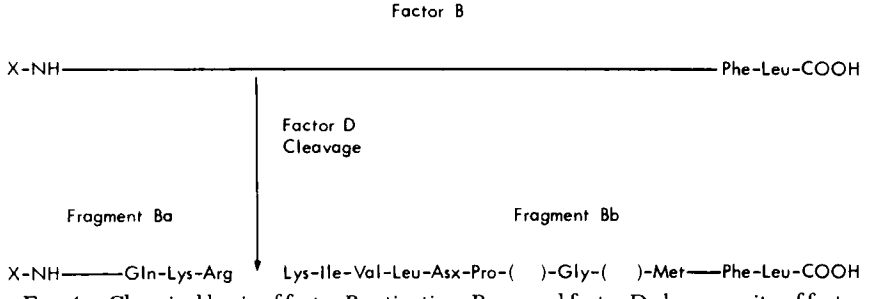


FIG. 4. Chemical basis of factor B activation. Proposed factor D cleavage site of factor B and partial N- and C-terminal sequences of factor B and its fragments (Lesavre *et al.*, 1979).

(Bianco *et al.*, 1979; Götze *et al.*, 1979a,b; Sundsmo and Götze, 1979).

Factor B and C2 are strikingly similar. Like factor B, C2 is a serine protease that is activated by cleavage into a smaller (MW 35,000) and a larger (MW 80,000) fragment (Polley and Müller-Eberhard, 1968; Nagasawa and Stroud, 1977b). The larger fragment bears the DFP inhibitable active site (Medicus *et al.*, 1976b), which is capable of hydrolyzing acetyl-glycyl-lysine methyl ester (Cooper, 1975b) and acts on the same natural substrates that factor B acts on, but only when in complex with its modulator, C4b (Müller-Eberhard *et al.*, 1967). C2 has two reactive sulfhydryl groups, which, upon oxidation with iodine, form an intramolecular disulfide bond, and this modification increases the cytolytic activity of C2 15- to 20-fold (Polley and Müller-Eberhard, 1967). Unlike C2, factor B is totally inert to treatment with iodine (Mak *et al.*, 1977).

C. Factor D: C3 Proactivator Convertase

Factor D is the activating enzyme of precursor C3/C5 convertase, C3b,B (Müller-Eberhard and Götze, 1972; Götze, 1975). It converts this bimolecular complex to the active enzyme C3b,Bb by cleavage of an arginyl–lysine bond in the B subunit (Lesavre *et al.*, 1979). As such, it is absolutely necessary for alternative pathway initiation and amplification (Schreiber *et al.*, 1975, 1976b, 1978; Brade *et al.*, 1977b; Lesavre and Müller-Eberhard, 1978) Expression of its enzymic activity depends entirely on modulation of its substrate, factor B, by C3b. Factor D requires no activation, it occurs in serum or plasma in active form (Lesavre and Müller-Eberhard, 1978). It is a single-chain glycoprotein that has an *s* rate of 2.5 S and a molecular weight of 24,000 (Dierich *et al.*, 1974; Götze, 1975; Volanakis *et al.*, 1977; Lesavre *et al.*, 1979). It has the electrophoretic mobility of a fast γ-globulin, but in serum it migrates as an α-globulin (Müller-Eberhard and Götze, 1972; Lesavre and Müller-Eberhard, 1978; Davis *et al.*, 1979). It is a true trace protein of serum, its concentration being 1 μg/ml (Lesavre *et al.*, 1979).

Factor D binds and is inhibited by DFP (Fearon *et al.*, 1974). It does not hydrolyze synthetic ester substrates, nor does it cleave the B chain of insulin (Lesavre and Müller-Eberhard, 1978). However, it does hydrolyze the factor Xa substrate *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide HCl, although at very low initial rate. The amino acid composition has been reported (Volanakis *et al.*, 1977; Davis *et al.*, 1979) (Table II). The amino-terminal acid sequence was shown to be Ile-Leu-Gly-Gly-Arg-Glx-Ala-Glx-Ala- (Davis *et al.*, 1979). This sequence indicates that factor D is distinct from, but homologous with,

other plasma serine proteases, including $C1\bar{r}$, $C1\bar{s}$, factor Xa, factor XIa, plasmin, and thrombin.

It has been suggested that factor D is antigenically and functionally related to thrombin such that it might be a thrombin fragment (Davis *et al.*, 1978). It is now clear that factor D differs from α-thrombin in that it has neither esterolytic activity nor common antigenic determinants and that α-thrombin, which has esterolytic activity, has no factor D-like activity (Lesavre and Müller-Eberhard, 1978)

D. β1H

β1H is a 6 S β-globulin that controls the function of the alternative pathway through its affinity for a strategic site on C3b (Weiler *et al.*, 1976; Whaley and Ruddy, 1967a; Conrad *et al.*, 1978; Nagaki *et al.*, 1978; Pangburn and Müller-Eberhard, 1978a). It blocks access of factor B to C3b, dislodges Bb from $\overline{C3b,Bb}$ in active form, and modulates C3b for attack by the C3b inactivator. It is absolutely required for inactivation of fluid phase C3b by the C3b inactivator, and it enhances the rate of bound C3b inactivation 30-fold (Pangburn *et al.*, 1977). It contains approximately 16% carbohydrate, and its amino acid composition (Pangburn *et al.*, 1979) is listed in Table II. Although β1H was first described in 1965 (Nilsson and Müller-Eberhard, 1965), its participation in the alternative pathway was not recognized until 1976 (Weiler *et al.*, 1976; Whaley and Ruddy, 1976a,b).

E. C3b Inactivator

The C3bINA is a 5.5 S endopeptidase with β-globulin mobility (Tamura and Nelson, 1967; Lachmann and Müller-Eberhard, 1968) and a molecular weight of 88,000 (Lachmann and Müller-Eberhard, 1968; Fearon, 1977; Pangburn *et al.*, 1977). The carbohydrate content of the protein, not including sialic acid, is 10.7%, i.e., 7.5% neutral hexose and 3.2% glucosamine (Pangburn *et al.*, 1977). The amino acid composition (Pangburn *et al.*, 1977) is shown in Table II. The protein is composed of two nonidentical polypeptide chains with molecular weights of 50,000 and 38,000 bound together by disulfide bonds and noncovalent forces (Fearon, 1977; Pangburn *et al.*, 1977). C3bINA activity is not inactivatable by heating at 56°C for 60 minutes, soybean trypsin inhibitor, or DFP (Lachmann and Müller-Eberhard, 1968). C3bINA action on C3b results in production of $C3b_i$ (Tamura and Nelson, 1967; Lachmann and Müller-Eberhard, 1968; Ruddy and Austen, 1969) by effecting cleavage of the α chain of the molecule (Bokisch *et al.*, 1975; Gitlin *et al.*, 1975) into two fragments of molecular weights 67,000 and 40,000 (Pangburn *et al.*, 1977). It also cleaves the α chain of

C4b, which leads to the generation of C4c and C4d fragments (Cooper, 1975a; Pangburn *et al.*, 1977; Natsuume-Sakai *et al.*, 1978). This reaction requires the C4 binding protein as a cofactor (Shiraishi and Stroud, 1975; Nagasawa and Stroud, 1977a; Fujita *et al.*, 1978; Fujita and Nussenzweig, 1979; Gigli *et al.*, 1980). Several individuals have been described with a homozygous C3bINA deficiency, and these individuals are highly susceptible to recurrent infections (Abramson *et al.*, 1971; Lachmann and Rosen, 1978).

F. PROPERDIN

Although properdin is the protein through which the alternative pathway was recognized (Pillemer *et al.*, 1954,1956), it is not an essential component of the pathway (Medicus *et al.*, 1976c; Schreiber and Müller-Eberhard, 1978a; Schreiber *et al.*, 1978, 1979, 1980). It fulfills the role of an enchancing regulator of the C3/C5 convertase, and as such it is recruited last in the assembly of the pathway (Medicus *et al.*, 1976b,c). Properdin is a 5.4 S γ_2-glycoprotein, first isolated in 1968 (Pensky *et al.*, 1968), which consists of four apparently identical polypeptide chains held together by noncovalent forces (Minta and Lepow, 1974; Götze *et al.*, 1977). Its partial specific volume is 0.7 ml/gm, and the molecular weight was reported to be 186,000 (Minta and Lepow, 1974) or 224,000 (Pensky *et al.*, 1968; Götze *et al.*, 1977). The carbohydrate content of the protein is 9.8%, i.e., 3.8% hexose, 0.7% fucose, 1.5% hexosamine, and 3.8% sialic acid (Minta and Lepow, 1974). The amino acid composition of the protein (Minta and Lepow, 1974) which is listed in Table II reveals that glutamic acid, proline, and glycine constitute 40% of the amino acid residues. At least two functional forms have been described, native properdin (nP) and activated properdin ($\overline{P}$) (Götze *et al.*, 1977; Medicus *et al.*, 1980). $\overline{P}$ is capable of (*a*) assembling the alternative, soluble C3 convertase in serum in absence of activators of the pathway; (*b*) binding to C3b-carrying particles in absence of factors B and D; and (*c*) stabilizing the assembled, labile C3/C5 convertase on the surface of particles. Native properdin can interact with the assembled, labile C3/C5 convertase in a binding-activation reaction and thereby stabilize the enzyme, but it cannot induce formation of C3 convertase in serum, nor can it bind to C3b-carrying particles. On the basis of their differential properties, assays were developed that allow quantitation of nP and P (Medicus *et al.*, 1980). P may be removed from a mixture of nP and P by passing the mixture over a Sepharose-C3b column (Medicus *et al.*, 1980).

The two forms of properdin have been reported to differ in electrophoretic mobility, subunit structure, and immunochemical properties

TABLE V
AMINO- AND CARBOXY-TERMINAL AMINO ACID SEQUENCES OF NATIVE (nP) AND ACTIVATED PROPERDIN ($\overline{P}$)

nP	NH_2-Asx-Pro-Val-Leu-(X)-Phe-Thr-Glx-(Tyr)-Glx-Glx. . . .
$\overline{P}$	NH_2-Asx-Pro-Val-Leu-(X)-Phe-Thr-Glx-(Tyr)-Glx-Glx. . . .
nP	. . . .Asp-Gly-Pro-Ser-Leu-COOH
$\overline{P}$	. . . .Asp-Gly-Pro-Ser-Leu-COOH

(Minta, 1976). However, a more detailed analysis of the two forms could not verify such differences. Under appropriate conditions, nP and $\overline{P}$ were found to be identical with regard to immunoelectrophoretic behavior, antigenic properties, subunit size, and terminal amino acid sequences (Table V) (Götze *et al.*, 1977; Medicus *et al.*, 1980). It was proposed, therefore, that nP and $\overline{P}$ differ only in conformation. The interconvertibility of the two forms seems to support this view. Native properdin at 1 mg/ml can be completely converted to $\overline{P}$ by freezing at − 80°C and thawing. On the other hand, $\overline{P}$ may be converted to nP by exposure to 0.8–1 *M* guanidine HCl for 1 hour at room temperature (Medicus *et al.*, 1980).

When evidence was sought for the existence of different conformational forms of properdin, circular dichroism analysis indeed detected considerable variations in tertiary structure when several properdin preparations were compared. It became evident that the protein can change from random coil to almost complete β structure, but that this alteration was not accompanied by an apparent change in activity (Medicus *et al.*, 1980). Although nP always showed random coil structure, $\overline{P}$ showed either random coil or β structure. Apparently, the conformation change that determines whether the molecule has nP or $\overline{P}$ activity is too subtle to be detectable by CD analysis.

The very strong positive ellipticity at 231 nm that was invariably exhibited by all properdin preparations examined is unusual and unexplained.

III. The C3b-Dependent Feedback and Amplification

Basic to the understanding of the alternative pathway is the C3b-dependent positive feedback mechanism (Müller-Eberhard and Götze, 1972). As illustrated in Fig. 5, a molecule of C3b and a molecule of factor B form, in the presence of Mg^{2+} the stable bimolecular complex C3b,B, which has no enzymic activity (Nicholson *et al.*, 1975; Medicus, *et al.*, 1976a; Vogt, *et al.*, 1977; Lesavre and Müller-Eberhard, 1978). In complex with C3b, factor B becomes susceptible to cleavage

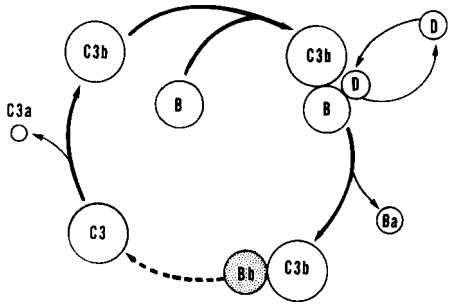


FIG. 5. The C3b-dependent positive feedback. One molecule of C3b can create many molecules of C3 convertase in the presence of an unlimited supply of factor B (B). Since factor D, the activating enzyme of factor B, is not consumed in the reaction, a chain reaction is created (Müller-Eberhard, 1978).

by factor D, its activating enzyme, which results in formation of the C3 convertase of the alternative pathway, $\overline{C3b,Bb}$. The Ba fragment of factor B is dissociated in the process (Vogt *et al.*, 1974). In acting upon C3, the enzyme supplies in a short period of time many molecules of C3b, each of which is capable of initiating the formation of a molecule of C3 convertase provided the supply of factor B is not limiting. Factor D, which has no precursor but is always available in active form, is not incorporated into the enzyme complex and can therefore activate many C3b,B complexes (Lesavre and Müller-Eberhard, 1978). In its uncontrolled form, the process resembles a chain reaction. Whereas this mechanism was first demonstrated in cell-free solution (Müller-Eberhard and Götze, 1972), it was subsequently shown also to operate on the surface of cells (Fearon *et al.*, 1973).

The alternative pathway feedback is relatively unique in that the product (C3b) of the substrate (C3) of the C3 activating enzyme ($\overline{C3b,Bb}$) becomes a subunit of that enzyme (Müller-Eberhard and Götze, 1972). Such feedback does not exist in the classical pathway of complement activation. Here, the C3 cleaving enzyme contains C4b instead of C3b as the nonenzymic subunit, and although C3 and C4 are homologous in primary structure (Gorski *et al.*, 1979b), C3b cannot substitute for C4b as a subunit of the classical C3 convertase.

Theoretically, the positive feedback or alternative pathway amplification must be of considerable biological importance. A very small event, insignificant by itself, such as deposition of one or a few molecules of C3b on a biological particle may eventuate, given the appropriate microenvironment, in the formation of a molecule of C3 convertase, subsequent deposition of many more C3b molecules, and thereby formation of many C3/C5 convertase molecules. The feed-

back thus constitutes the driving force of the alternative pathway. In addition to enzyme formation, amplification leads to opsonization of a particle and, of course, to production of C3a anaphylatoxin.

IV. Regulation

Regulation of the positive feedback, and thereby of the entire alternative pathway, is provided by four known mechanisms: spontaneous decay of the metastable binding site of activated C3b, degradation of target-bound C3b, spontaneous decay of the C3/C5 convertase and active disassembly of this enzyme. β1H binds to C3b, and this binding is competitive with that of B or Bb. As a result, β1H either blocks the formation of $\overline{C3b,Bb}$ or disassembles the enzyme, dissociating Bb in inactive form (Conrad *et al.*, 1978; Pangburn and Müller-Eberhard, 1978a: Kazatchkine *et al.*, 1979a). In complex with β1H, C3b is readily cleaved and inactivated by C3bINA (Whaley and Ruddy, 1976b; Pangburn *et al.*, 1977). With the formation of inactive C3b ($C3b_i$), β1H is released and both control proteins are free to attack the next molecule of enzyme or of C3b. Control of formation and function of the alternative C3 convertase may be formulated as follows:

$$C3b + \beta1H \rightleftharpoons C3b,\beta1H \quad (1)$$

$$\overline{C3bBb} + \beta1H \rightarrow C3b,\beta1H + Bb_i \quad (2)$$

$$C3b,\beta1H + C3bINA \rightarrow C3b_i + \beta1H + C3bINA \quad (3)$$

The equilibrium constant for the binding of β1H to C3b on sheep erythrocytes was reported to be 10^9 l/M (Conrad *et al.*, 1978). Others found an affinity constant at equilibrium of $1 \times 10^7\ M^{-1}$ (Kazatchkine *et al.*, 1979b). The affinity of B for C3b in the presence of Mg^{2+} was only $^1/_5$ that of β1H. Binding of β1H to C3b was approximately stoichiometric. Binding of C3bINA could also be demonstrated by stabilizing the enzyme substrate complex EC3b, β1H, C3bINA at 0°C. Binding of C3bINA to EC3b in the presence of β1H was 30-fold greater than in its absence, it was stoichiometric with respect to bound β1H, and the binding constant at 0°C was $10^8\ M^{-1}$ (Pangburn and Müller-Eberhard, 1978b).

V. The Metastable Binding Site

Although complement can function in cell-free solution, it has the unusual ability to transfer itself from solution to the surface of biological particles and to function as a solid phase enzyme system and membrane attack mechanism. The capacity of complement to mark and

prepare particles for ingestion by phagocytic cells and to attack and kill cells is based on this ability of complement molecules to transfer from the fluid phase to a solid phase. Transfer is accomplished through activation of metastable binding sites which are transiently revealed by the respective activating enzymes. Cleavage of a critical peptide bond leads to dissociation or dislocation of the activation fragment of a given component and to exposure of structures that are concealed in the native molecule. Owing to the metastable binding site, a molecule can bind to a suitable acceptor and establish firm association with it. Failing collision with the acceptor within a finite time period after activation, the site decays and the molecule remains unbound in the fluid phase. The rapid decay of the metastable binding sites imposes rigorous spatial constraints on the activated complement system and prevents spreading of the effects of activation beyond the microenvironment of the activation site.

The concept of the metastable binding site was originally developed to interpret the binding of C4 to unsensitized erythrocytes through the catalytic action of fluid phase $C1\bar{s}$ (Müller-Eberhard and Lepow, 1965) and was further elaborated upon in order to explain the binding reaction of C3 (Müller-Eberhard *et al.*, 1966a). As to the alternative pathway, the expression of its biological activity is entirely dependent on the binding of C3b to targets through its metastable binding site. Unlike the classical pathway, which establishes direct contact with the target through antibody, C4b and C3b, the alternative pathway establishes contact exclusively through C3b.

Present evidence indicates that C3b may be covalently linked to target particles through a hydroxylamine-labile bond and through hydrophobic interactions (Law and Levine, 1977). In these experiments C3b was bound to zymosan by enzymic activation of C3. While the bound C3b could not be eluted by treatment of the particles with detergent or acid, it was released by 1 *M* hydroxylamine (Law *et al.*, 1979b). That the hydroxylamine sensitive bond is an ester bond was suggested by the association of hydroxamate with the released protein. The bond formed between the metastable binding site and zymosan (Z) was thus proposed to be Z-O-CO-C3b (Law *et al.*, 1979b). By exposure of the bound C3b to β1H and C3bINA it was shown that binding of C3b occurs via the MW 67,000 portion of the C3b α chain and that this fragment is the precursor of the immunochemically defined C3d piece (Law *et al.*, 1979a). The covalent nature of the bond was further demonstrated by experiments in which serum containing radiolabeled C3b was incubated with [^{3}H]glucose-S-S-Sepharose. After removal of extraneous protein, the Sepharose beads were treated

with a disulfide cleaving reagent, this resulted in elution of C3b. Upon SDS–gel electrophoresis of the reduced protein, ^{3}H was found to be associated with the α chain of C3b, strongly suggesting the formation of a covalent bond between the α chain and the carbohydrate (Mann *et al.*, 1980).

There is another pertinent observation regarding the metastable binding site. Cleavage of C3 into C3a and C3b by trypsin or inactivation of C3 by hydroxylamine or hydrazine frees a sulfhydryl group which is located in the C3d portion of the molecule (Tack *et al.*, 1980). Following up these observations, it was possible to show that [^{14}C]methylamine binds to C3 within the C3d domain thereby preventing C3b uptake by cells upon enzymatic activation of C3. Binding of methylamine and inactivation of C3 was associated with the appearance of a reactive sulfhydryl group, also located in the C3d portion of C3 (Pangburn, 1980). In another serum protein, α_2-macroglobulin, methylamine was shown to bind to the γ-carboxyl group of a glutamic acid residue (Swenson and Howard, 1979). It may be postulated therefore that C3 contains within its C3d domain a thioester bond which is protected from spontaneous hydrolysis by the conformation of native C3. Upon cleavage of peptide bond 77 of the α-chain, the putative thioester bond of C3b becomes exposed such that its active carbonyl group either reacts with water, which results in decay of the metastable site, or forms a C–O bond with carboydrate.

VI. Initiation

Initiation is the first of two phases of activation of the pathway, the second being amplification. Initiation itself is a two-step process, consisting of random deposition of C3b on the surface of biological particles and then of a discriminatory interaction of bound C3b with surface structures. It is the latter reaction that determines whether the pathway is propagated or abrogated. Thus, according to present information, particle-bound C3b appears to fulfill the recognition function of the alternative pathway (Schreiber *et al.*, 1978; Pangburn *et al.*, 1980).

A. The Initial Enzyme

The enzyme responsible for setting in motion the feedback mechanism, and thus for initiating the pathway, is generated when native C3, factors B and D, and Mg^{2+} interact in free solution at physiological concentration (Fearon and Austen, 1975b; Schreiber *et al.*, 1978). Although not known with certainty, the initial enzyme appears to be the

fluid phase complex $\overline{C3,Bb}$, where native C3 rather than C3b serves as a subunit (Schreiber *et al.*, 1978). The first molecule of C3b produced by this hypothetical enzyme will then set in motion the positive feedback reaction. That no extraneous enzyme is involved is indicated by the total dependence of C3 activation on factor B and factor D (Table VI). Because factor D occurs in serum or plasma in active form (Lesavre and Müller-Eberhard, 1978) always ready to act on its substrate, provided it is suitably presented, there is probably a continuous low grade formation of $\overline{C3,Bb}$. The ability of uncleaved C3 to function as a modulator of factor B may be due to the occurrence of reversible C3 conformers or to the existence of a weak affinity between C3 and factor B. That native C3 may indeed be a part of the initial enzyme is supported by our knowledge of the mechanism of action of nephritic factor and of activated properdin (Schreiber *et al.*, 1975). These molecules appear to function by arresting native C3 and activated factor B in an enzymically active complex.

Another possibility has to be considered which is related to the hydroxylamine-labile bond of C3. It is probable that this bond which may be postulated to be a thioester bond (see above) undergoes spontaneous hydrolysis at a very low rate. It also is conceivable that certain chemical groups on the surface of cells facilitate breaking of this bond and that as a result, uncleaved C3 becomes bound to cell surface carbohydrates via the active carbonyl group preserved in the putative thioester. C3 thus bound to the surface of an activator may functionally behave like bound C3b and serve as subunit of the initial C3 convertase. According to this hypothesis, the initial enzyme would be a surface bound complex of uncleaved C3 and Bb rather than a fluid phase enzyme.

B. The Stable Five-Protein System

The control of the reaction mixture containing native C3, factors B and D, and Mg^{2+} is provided by β1H and C3bINA and may concern only the product of the initial enzyme, C3b, and amplification. Examination of the controlled five-protein system at 37°C (Table VI) revealed a remarkable stability in that neither C3 nor factor B consumption could be detected during several hours of incubation. However, it must be assumed that a very small number of C3b molecules are continually produced by the initial enzyme.

For initiation of the pathway by activators, five proteins are sufficient: C3, Factor B, factor D, β1H, and C3bINA (Schreiber *et al.*, 1978). Properdin and immunoglobulins are not required. It is thought that upon introduction of biological particles into the stable fluid

TABLE VI
INITIAL ENZYME AND CONTROL OF FLUID PHASE C3 CONSUMPTION

Reaction mixture[a]	Percent C3 remaining (at 37°C)				
	2 Min	1 Hr	2 Hr	4 Hr	8 Hr
C3	100	100	99	98	95
C3, B, D	0	0	0		
C3, B, D, β1H, C3bINA	100	100	100	96	93

[a] Physiological concentrations.

phase system, the initial enzyme ($C3,\overline{Bb}$) deposits a small number of C3b molecules on the surface of these particles. C3 deposition from the fluid phase is a random process involving activation of the metastable binding site of C3 (Müller-Eberhard *et al.*, 1966a) and does not distinguish between activators and nonactivators of the pathway. The concept that initial deposition of C3b is a random event was advanced by Lachmann and associates (Lachmann and Nicol, 1973; Nicol and Lachmann, 1973; Lachmann and Halbwachs, 1975) in the form of the "tick-over" hypothesis. It predicts that low level C3b deposition occurs on host cells and foreign particles alike. Suggestive evidence to support this prediction is the finding of C3d antigenic determinants on freshly obtained human erythrocytes and of their increase upon storage of blood (Szymanski and Ogden, 1979).

C. CONCENTRATION DEPENDENCE

The alternative pathway has been known to be highly concentration dependent (Leon, 1956; Sandberg and Osler, 1971). In serum, a dilution of 1:10 to 1:20 abrogates the function of the pathway. In isolated form the pathway proteins lost their capacity of being activated by gram-negative bacteria at $^1/_{16}$ of their physiological concentration (Schreiber *et al.*, 1979). It is likely that formation of the initial enzyme is the most concentration-dependent reaction of the pathway. Although reversible interaction between C3b and factor B have clearly been demonstrated (Nicholson *et al.*, 1975; Medicus *et al.*, 1976c; Vogt *et al.*, 1977; Lesavre and Müller-Eberhard, 1978), an interaction of factor B with native C3 remains to be shown.

The concentration dependency of β1H and C3bINA function may be demonstrated by varying their concentration over a wide range in the purified five-protein system (Schreiber *et al.*, 1978). At low concentration of the two regulators, C3 and factor B consumption was rapid and complete within minutes. As the concentration was raised toward physiological concentration of β1H and C3bINA, neither C3

nor factor B consumption could be detected for 8 hours at 37°C (Schreiber *et al.*, 1978). When the concentration of β1H alone was varied, the results were similar (Fearon and Austen, 1977b). This observation is explicable on the basis of the absolute dependence of C3bINA function on the presence of β1H for fluid phase cleavage of C3b (Pangburn *et al.*, 1977). However, when the concentration of C3bINA alone was decreased from physiological to very low values, there was predominant inactivation of factor B (Fearon and Austen, 1977b). This observation is explicable by the ability of β1H to dissociate the Bb fragment from its complex with C3b and to cause rapid decay of the C3 convertase without inactivation of C3b (Conrad *et al.*, 1978; Nagaki *et al.*, 1978; Pangburn and Müller-Eberhard, 1978a; Kazatchkine *et al.*, 1979a). Increasing the factor B concentration causes enhancement of the rate of activation (Mak *et al.*, 1977; Nydegger *et al.*, 1978a), as did increasing factor D (Lesavre and Müller-Eberhard, 1978) or native properdin concentrations.

D. Restriction of Control by Activators

Introduction of known activators of the pathway into the five-protein system leads to deposition of C3b and formation of C3/C5 convertase on the surface of the activating particle. No other components are required for these initiating events to occur (Schreiber *et al.*, 1978) (Fig. 6). The reason for successful attack of activating particles by the alternative pathway is that on their surface both C3b and the C3/C5 convertase are relatively protected from destruction by the regulatory proteins (Fearon and Austen, 1977a,b). C3b is not readily cleaved by the C3b inactivator, and Bb is not readily dissociated from $\overline{C3b,Bb}$ by β1H.

It has become clear that initiation is determined by whether or not β1H can interact with surface-bound C3b (Pangburn and Müller-Eberhard, 1978a). If C3b is placed in a microenvironment that reduces its capacity to interact with β1H, it has a finite half-life to participate in the formation of the alternative pathway C3/C5 convertase. It thus escapes immediate control and effects amplification of C3b formation through the feedback mechanism.

There are a large number of activators of the alternative pathway, which include polysaccharides, lipopolysaccharides, certain immunoglobulins, viruses, fungi, bacteria, some animal cells and parasites (reviewed in Götze and Müller-Eberhard, 1976). The common denominator of these activators is still unknown, although in general carbohydrate is a constituent of most of them. All activators tested to date show reduced β1H–C3b interaction on their surface (Pangburn

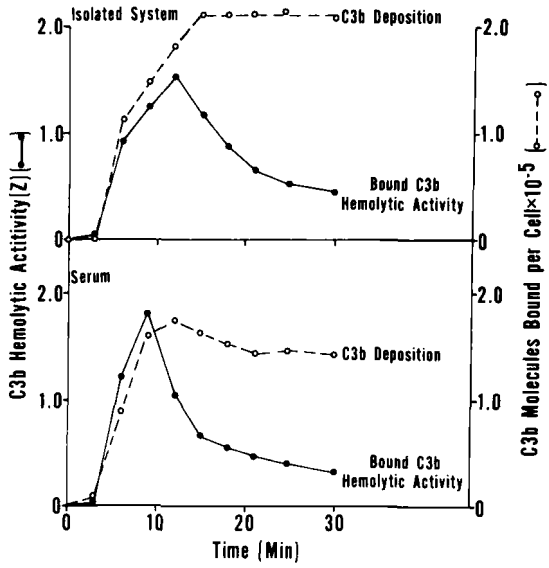


FIG. 6. Six isolated proteins (C3, factor B, factor D, β1H, C3bINA, and properdin) exhibit alternative pathway function. Reaction mixtures contained 4×10^8 rabbit erythrocytes in 400 μl of either serum depleted in C4, C5, and C6 (lower) or a mixture of proteins containing C3 (1200 μg/ml), factor B (200 μg/ml), factor D (2 μg/ml), C3bINA (34 μg/ml), β1H (470 μg/ml), and properdin (20 μg/ml) (upper). Both reaction mixtures contained ^{125}I-labeled C3 (5 μg/ml) and 1.0 m*M* Mg^{2+}. Incubation was performed at 23°C, and 20-μl samples were removed at designated times to determine C3b deposition and hemolytic activity of the bound C3b (Schreiber *et al.*, 1978).

et al., 1979). In order to express the activating potential quantitatively, the restriction index of β1H control was introduced (Pangburn *et al.*, 1980). By using radiolabeled β1H and radiolabeled, bound C3b, the ratio of β1H to C3b on a particle bearing C3b can be determined. Arbitrarily, this ratio was defined as 1.0 for sheep erythrocytes, which do not activate the pathway. Potent activators such as rabbit erythrocytes or zymosan have a restriction index of 0.1, which indicates that β1H–C3b interaction on their surface is only one-tenth that characteristic for sheep erythrocytes.

It was possible to convert nonactivators to activators by defined chemical modification (Fearon, 1978; Pangburn and Müller-Eberhard, 1978a; Pangburn et al., 1980). Two approaches were used. By removing 80% of cell-bound sialic acid from sheep erythrocytes, their restriction index was reduced from 1.0 to 0.3 and the cells became activators of the pathway (Fearon, 1978; Pangburn and Müller-Eberhard, 1978a; Kazatchkine *et al.*, 1979a). By and large, sialic acid in gly-

coproteins is linked to galactose residues (Tooze, 1973). Removal of sialic acid would therefore reveal galactose and other neutral and amino sugars.

The second approach involved the incorporation of an isolated, chemically defined lipopolysaccharide (LPS) of *Escherichia coli* 04 into the membrane of sheep erythrocytes (Pangburn *et al.*, 1980). Incorporation of approximately 25,000 monomeric and base-hydrolyzed LPS structures (MW 10,000) per cell were needed to generate alternative pathway activating activity to the extent where 50% cytolysis occurred upon exposure to C4-depleted human serum. The restriction index declined from 1.0 to 0.4.

E. Discrimination between Activators and Nonactivators by Bound C3b

Because the susceptibility of C3b to β1H control is reduced on the surface of activators, it was postulated that the C3b molecule possesses a discriminatory site that is capable of distinguishing between activators and nonactivators (Schreiber *et al.*, 1978; Pangburn *et al.*, 1980). This concept is based on the following experimental evidence. First, human C3b was bound to rabbit erythrocytes and the cells were then exposed at 23°C to autologous rabbit β1H and C3bINA. The functional half-life of the C3b was 18 minutes. The same human C3b when bound to sheep erythrocytes and exposed to the same rabbit β1H and C3bINA had a functional half-life of only 1.6 minutes. A similar difference in half-life was observed when the control proteins were of human rather than rabbit origin (Schreiber *et al.*, 1978). It was this experiment that suggested a recognition function for C3b but provided no information on whether or not the metastable binding site of C3b was responsible for this function. The results apparently ruled out β1H and C3bINA as recognition proteins because they failed to protect autologous cells from attack. Second, C3b bound to sheep erythrocytes, which is susceptible to control, became resistant to control upon removal of cell surface sialic acid (Fearon, 1978; Pangburn and Müller-Eberhard, 1978a). This experiment ruled out that the proposed discrimination by C3b was a function of the metastable binding site, but it shed no light on the nature of the uncovered chemical structures made available to C3b by the neuraminidase treatment. Third, C3b bound to sheep erythrocytes was rendered resistant to β1H and C3b inactivator control upon introduction of a bacterial lipopolysaccharide into the microenvironment of the C3b molecules (Pangburn *et al.*, 1980). This experiment shows that the functional state of C3b on a nonactivator may be changed to one that supports

alternative pathway activation upon introduction of defined molecular structures into the microenvironment of the C3b without alteration of membrane sialic acid content.

Earlier, the function of discriminating between activators and nonactivators was postulated to reside in a separate initiating factor (Medicus *et al.*, 1976c; Schreiber *et al.*, 1976a). Experiments that gave rise to this notion used serum reagents depleted of immunoglobulins. In retrospect, it is probable that these reagents were also depleted of C3bINA. Although this enzyme was appreciated as an important ingredient of the alternative pathway, its involvement in initiation together with β1H was not clear at that time. The factor therefore is considered identical with C3bINA and not a separate entity.

The observations that nonactivating sheep erythrocytes may be converted to activators by removal of cell surface sialic acid, and that an inverse relationship exists between sialic acid content and the capacity of mouse erythrocytes to activate the human alternative pathway (Nydegger *et al.*, 1978b), have led to the formulation of two hypotheses as to the manner in which bound C3b performs its discriminatory function. The "β1H-antagonist" hypothesis (Pangburn and Müller-Eberhard, 1978a; Schreiber *et al.*, 1978; Pangburn *et al.*, 1980) proposes that a distinct site on bound C3b interacts with cell surface markers and that this interaction is antagonistic toward binding of fluid phase β1H to bound C3b. Engagement of the marker site on C3b may allosterically inactivate the β1H binding site on C3b. The "β1H-protagonist" hypothesis proposes that C3b on nonactivators interacts with surface structures, such as sialic acid, in such a manner that β1H binding to C3b is enhanced (Kazatchkine *et al.*, 1978a). It seems possible that surface bound C3b can be modulated through both types of interactions. The data presented in Table VII (Pangburn *et al.*, 1980) are consistent with the view that sialic acid can, in some instances, function to regulate alternative pathway activation and in others does not. Whereas the removal of 80% of the sialic acid from sheep erythrocytes generates an alternative pathway activating surface with a restriction index of 0.3, removal of sialic acid from human erythrocytes resulted in neither a low restriction index nor a surface that activated the alternative pathway. Neuraminidase-treated human erythrocytes possessed a sialic acid surface density only 33% higher than did similarly treated E_S. The introduction of base-hydrolyzed LPS into the unmodified sheep erythrocyte membrane generates an activating surface with a restriction index of 0.4 without changing membrane sialic acid content. Perhaps the most illuminating fact is that unbound C3b in solution free of sialic acid is effectively controlled by

TABLE VII
COMPARISON OF THE RESTRICTION INDEX (RI) WITH MEMBRANE SIALIC ACID CONTENT

Cell[a]	Sialic acid/10^9E (nmol)	Sialic acid removed	Sialic acid/surface area[b]	RI
E_S	11	—	1.6	1.0
E_S (neuraminidase)	2.2	80%	0.3	0.3
E_S-BH-LPS	11	—	1.6	0.4
E_H	32	—	2.3	1.0
E_H (neuraminidase)	5.6	82%	0.4	1.2
E_H-BH-LPS	32	—	2.3	0.6
E_R	2.0	—	0.2	0.1

[a] E_S, sheep erythrocyte; E_H, human erythrocyte; BH-LPS, base-hydrolyzed lipopolysaccharide.
[b] 10^{-9} μmol/μm^2; (Pangburn *et al.*, 1980).

β1H with respect to its interaction with factor B (see above). These observations suggest that a low sialic acid density on the surface of a particle is not required for alternative pathway activation and lend support to the "β1H-antagonist" hypothesis.

It has been shown that an activator could be changed into a nonactivator by defined chemical modification. Zymosan particles, which are excellent activators and have a restriction index of 0.1, became nonactivators upon attachment by cyanogen bromide of heparin glucosaminoglycan (Kazatchkine *et al.*, 1979b). Approximately 12 million substitutions were required to render a zymosan particle a nonactivator, on whose surface C3b molecules are totally susceptible to the control proteins. In these experiments C3b was deposited after heparin attachment to the zymosan particles; i.e., the possible recognition function of the metastable binding site was not ruled out.

There are other cell surface constituents with an effect on regulation of the alternative pathway. Human erythrocyte membranes contain a 1.2×10^6 dalton protein that causes decay dissociation of cell-bound $\overline{C3b,Bb}$. It also modulates C3b for cleavage inactivation by C3bINA. While the protein resembles β1H in those respects, its function on C3b and $\overline{C3b,Bb}$ is not influenced by sialic acid residues in the microenviroment (Fearon, 1979). In the case of human erythrocytes, the protein may fulfill a "self" protecting function.

Figure 7 is a pictorial representation of the discriminatory site concept as it relates to the proposed recognition function of C3b and to activation of the alternative pathway: A C3b molecule bound to the

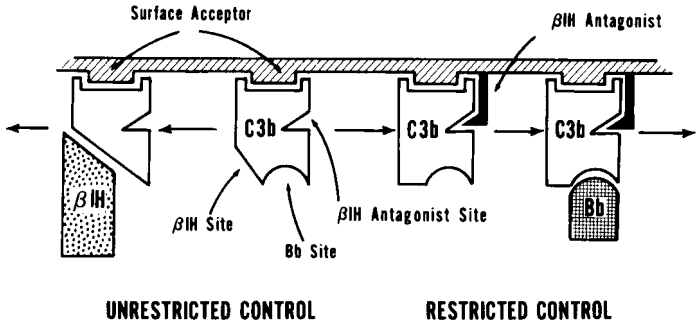


FIG. 7. A diagrammatic representation of the proposed mechanism by which the human alternative pathway discriminates between activating (restricted control) and nonactivating (unrestricted control) surfaces. The hypothesis suggests that activators possess surface molecules (β1H antagonists) that are recognized by bound C3b. Interaction of C3b with these surface markers inactivates the β1H binding site of C3b. As a result, formation of C3 convertase may proceed (Pangburn *et al.*, 1980).

surface of a biological particle via its metastable binding site possesses potential binding sites for β1H, factor B, and surface structures that may be defined functionally as "β1H-antagonists." If the surface on which the C3b resides lacks "β1H-antagonists," the discriminatory site of C3b remains unengaged, and consequently unrestricted β1H binding occurs. C3bINA rapidly cleaves C3b to which β1H is bound, and the alternative pathway is not activated. On surfaces that possess structures that function as "β1H-antagonists," binding of β1H to C3b is reduced and C3b is free to bind factor B. These conditions allow formation of the C3 convertase, amplification via C3b-dependent feedback, generation of the C5 convertase, and activation of the membrane attack pathway. The specificity of the proposed discriminatory site of C3b remains to be defined, and a direct physical interaction between C3b and isolated cell surface markers remains to be demonstrated.

It should be stressed that C3b is not the only conceivable candidate for alternative pathway recognition function. It is possible that β1H in some way participates in the discriminatory process.

VII. The Alternative Pathway: Molecular Events

The sequence of events constituting the alternative pathway is schematically represented in Fig. 8 (Medicus *et al.*, 1976c; Schreiber *et al.*, 1978). Activation consists of initiation and amplification. Initiation is nonspecific, as it does not require immunoglobulins. Initiation

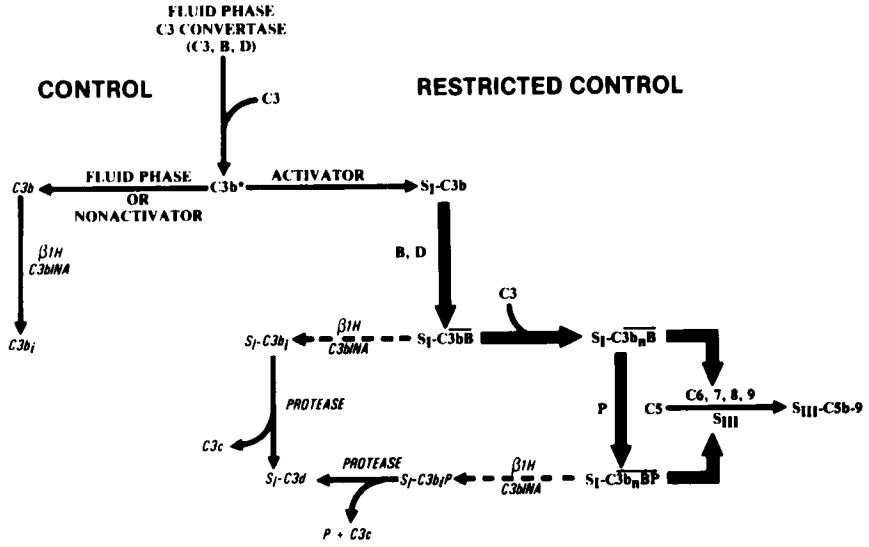


FIG. 8. Molecular concept of the alternative pathway. "Control" represents the situation that would exist in the fluid phase or upon introduction of a nonactivator particle. "Restricted control" would occur upon introduction of a pathway activator. C3b* denotes nascently formed C3b before decay of its metastable binding site. For further explanation, see text (Schreiber *et al.*, 1978).

is at least a two-step process involving random deposition of C3b on the activator particle through its metastable binding site and discriminatory interaction of bound C3b with surface markers. The random event is a function of the initial C3 convertase, which is a fluid phase enzyme of the probable composition $C\overline{3},\overline{Bb}$. The magnitude of the resultant C3b deposition is low owing to the small amount of initial enzyme available and the low efficiency of C3b binding. When bound to a nonactivator, C3b binds β1H and becomes inactivated through the combined action of C3bINA and β1H. When bound to an activator, the ability of C3b to bind β1H is diminished and, consequently, formation of C3 convertase ($\overline{C3b},\overline{Bb}$) and amplification commence.

As the multiplicity of bound C3b molecules increases around each enzyme complex, the enzyme C5 convertase ($\overline{C3b},\overline{Bb},\overline{3b}$) is formed from C3 convertase (Daha *et al.*, 1976c; Medicus *et al.*, 1976a,c). The additional C3b molecule is needed for modulation of C5 for enzymic attack (Hammer *et al.*, 1976; Vogt *et al.*, 1978). The active site responsible for C3 and C5 cleavage resides in the Bb subunit of the enzyme.

The C3/C5 convertase is innately labile and decays at 37°C with a half-life of 1.5 minutes (Fearon and Austen, 1975a; Medicus *et al.*,

1976a). Decay is due to spontaneous dissociation of the Bb subunit in inactive form (Medicus *et al.*, 1976a,c). Native properdin upon collision with the labile C3/C5 convertase becomes physically associated with it and is "activated" in the process (Medicus *et al.*, 1976a, 1980; Götze *et al.*, 1977) Binding-activation of properdin results in stabilization of the enzyme such that its half-life at 37°C increases to 10 minutes. Upon eventual decay of the enzyme and destruction of C3b by the control proteins, properdin is released partially in "activated form." This form is able to bind to C3b directly without the aid of factor B, but it retains the ability to stabilize the preformed enzyme.

As amplification results in the accumulation of many bound C3b molecules, biological particles become opsonized (Gigli and Nelson, 1968; Huber *et al.*, 1968). Such opsonized particles are recognized by cells that are endowed with C3b-specific cell surface receptors, e.g., polymorphonuclear leukocytes, monocytes, macrophages, and B lymphocytes. Bound C3b that was degraded to $C3b_i$ or C3d also can interact with specific cell surface receptors and thereby can promote contact of particles with certain cells (Ross *et al.*, 1973; Ross and Rabellino, 1979).

The cobra venom factor (CVF)-dependent enzyme ($\overline{CVF,Bb}$) is a close relative of the alternative C3/C5 convertase (Müller-Eberhard *et al.*, 1966b; Müller-Eberhard and Fjellström, 1971; Vogt *et al.*, 1974). CVF forms with mammalian factor B a C3 and C5 activating enzyme. It differs from the physiological C3/C5 convertase in that it is unusually stable and can act on C5 in the absence of C3 (Medicus, 1977). CVF has been used extensively for experimental depletion of animals of circulating C3.

There are marked similarities between the alternative and the classical pathways of complement activation. The initial enzymes of both pathways catalyze the formation of target-bound C3 convertase. Instead of C3b, the classical enzyme utilizes the homologous protein C4b as nonenzymic subunit. And instead of Bb, the classical enzyme contains the genetically related C2a as catalytic subunit. Both enzymes are serine proteases (which cleave the same peptide bond in their substrates), and both require C3b as a cofactor for C5 activation (Vogt *et al.*, 1978). The difference between the pathways are these: The antibody-dependent classical pathway has in C1q a unique recognition protein, whereas the antibody-independent alternative pathway utilizes for recognition bound C3b that has many other functions. Second, whereas the alternative pathway is endowed with a unique amplification mechanism, the classical pathway is devoid of such a mechanism.

VIII. The Cytolytic Alternative Pathway

That the alternative pathway is potentially cytolytic has been known for a number of years. Rabbit erythrocytes, for instance, are readily lysed by human serum in which the classical pathway was inhibited (Platts-Mills and Ishizaka, 1974). Although not absolutely required in this system, immunoglobulins were assigned an enhancing role in alternative pathway activation (Polhill *et al.*, 1978; Nelson and Ruddy, 1979).

Recently, it has been possible to assemble the cytolytic alternative pathway entirely from 11 isolated proteins of human serum (Schreiber and Müller-Eberhard, 1978a). The mixture consists of the 6 proteins of the alternative pathway of activation which are described above, and the 5 proteins of the membrane attack pathway, C5, C6, C7, C8, and C9. The membrane attack proteins are demonstrated in Fig. 1 (right panel) as they appear upon SDS–polyacrylamide gel electrophoresis under nonreducing conditions. Some of their properties are summarized in Table VIII.

A detailed description of the five proteins and their interactions is beyond the scope of this review and will appear in a review on the membrane attack mechanism of complement (Podack and Müller-Eberhard, 1980). Suffice it to say that, upon cleavage of peptide bond 74 of the α chain of C5 by either the classical or the alternative C5 convertase (Hugli and Müller-Eberhard, 1978), the 5 proteins fuse into a firm tetradecamolecular complex, known as the membrane attack complex (MAC) (Kolb and Müller-Eberhard, 1973; Podack *et al.*, 1978). The MAC is the dimer of C5b-9 (Biesecker *et al.*, 1979). It has a characteristic ultrastructure that is responsible for the electron microscopic image of the typical membrane lesions caused by complement (Bhakdi and Tranum-Jensen, 1978; Biesecker *et al.*, 1979). The membranolytic activity of the MAC has been attributed to MAC insertion and transmembrane channel formation (Mayer *et al.*, 1979) or to the

TABLE VIII
PROTEINS OF THE MEMBRANE ATTACK PATHWAY OF COMPLEMENT

Protein	Molecular weight	No. of chains	Electrophoretic mobility	Serum conc. (μg/ml)
C5	180,000	2	β_1	70
C6	128,000	1	β_2	60
C7	121,000	1	β_2	55
C8	154,000	3	γ_1	55
C9	72,000	1	α	60

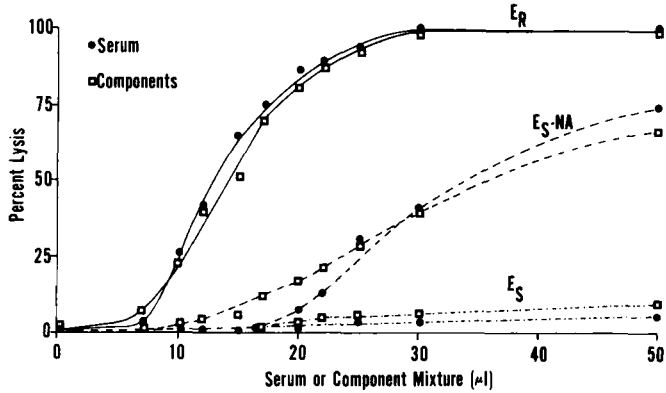


FIG. 9. Assembly of an intact cytolytic alternative pathway from eleven purified proteins of human serum. Reaction mixtures (each 160 μl) containing 10^7 erythrocytes, Mg-GVB, and various amounts of either C4-depleted serum or the isolated component mixture were incubated for 10 minutes at 37°C. Reactions were stopped by addition of 1 ml of cold Mg-GVB and centrifugation. Shown are the dose-response curves for lysis of rabbit erythrocytes (E_R), neuraminidase-treated sheep erythrocytes (E_S-NA), and untreated sheep erythrocytes (E_S) (Schreiber and Müller-Eberhard, 1978a).

appearance of high affinity phospholipid binding sites during MAC assembly (Podack *et al.*, 1979). It has been shown that the interaction of the MAC with membranes results in binding of phospholipid molecules to the MAC and in local reorganization of the lipid bilayer (Esser *et al.*, 1979). Thus, MAC action eventuates in lysis of erythrocytes, killing of nucleated cells and bacteria, and destruction of enveloped viruses.

The 11-protein mixture of the cytolytic alternative pathway was stable during incubation at 37°C; i.e., no spontaneous activation occurred. When rabbit erythrocytes were introduced into the reaction mixture, the cells were lysed to the same extent as in C4-depleted human serum (Fig. 9). Immunoglobulins were not present in the isolated component mixture. The presence of properdin was not essential for lysis, although it enhanced pathway activity two- to threefold (Schreiber and Müller-Eberhard, 1978a)

Sheep erythrocytes did not activate the purified cytolytic alternative pathway (nor C4-depleted human serum) and were not lysed. However, neuraminidase-treated sheep erythrocytes (Pangburn and Müller-Eberhard, 1978a) activated the pathway and were lysed, although not to the same extent as were rabbit erythrocytes (Fig. 9).

It thus became clear that the 11 isolated proteins constitute an intact cytolytic alternative pathway that is capable of coupling the initiation and amplification sequence with the cytolytic membrane attack se-

quence. The system functions independent of immunoglobulin, is not dependent on properdin, and is quantitatively and qualitatively comparable to the alternative pathway in human serum.

IX. Generation of Bactericidal Activity and Bacteriolysis

The alternative pathway has been shown to be activated by a number of bacterial organisms (Götze and Müller-Eberhard, 1971; Fine *et al.*, 1972; Jasin, 1972; Forsgren and Quie, 1974; Fine, 1975; Reed, 1975; Traub and Kleber, 1976; Stephens *et al.*, 1977), lipopolysaccharides from gram-negative bacteria (Gewurz *et al.*, 1968; Götze and Müller-Eberhard, 1971; Marcus *et al.*, 1971; Dierich *et al.*, 1973; Galanos and Lüderitz, 1976; Morrison and Kline, 1977), and cell walls or isolated teichoic acid from gram-positive bacteria (Winkelstein *et al.*, 1976; Winkelstein and Tomsz, 1978). Information regarding the ability of the alternative pathway to kill bacteria has been scarce. That the pathway may be bactericidal was suggested earlier by experiments with serum depleted of factor B (Götze and Müller-Eberhard, 1971) or deficient in C2 (Reed and Albright, 1974) or C4 (Root *et al.*, 1972). There are also reports that antibody is required for bacterial killing by the pathway (Osawa and Muschel, 1960; Michael *et al.*, 1962; Reed and Albright, 1974) and reports showing that bacterial lipopolysaccharide induced selective C3-C9 consumption in agammaglobulinemic sera. Consumption proceeded even in precolosteral piglet sera, which contain less than 2.5×10^{-6} mg of immunoglobulin per milliliter (Gewurz *et al.*, 1970). Although, on the basis of the latter work, it appeared conceivable that lipopolysaccharide can activate C3-C9 without antibody, the hypothesis was favored that antibody is involved in lipopolysaccharide-complement interaction (Webb and Muschel, 1968; Gewurz *et al.*, 1970). Thus the question of antibody involvement has not been clearly answered. That, in addition to complement, lysozyme is needed for the lysis of bacteria has been established (Inoue *et al.*, 1959; Muschel *et al.*, 1959; Spitznagel, 1966).

Utilizing the purified cytolytic alternative pathway, it was shown that certain bacteria do activate the alternative pathway in the absence of antibody or immunoglobulin and are killed in the process. *Escherichia coli* K12 W1485 introduced into the isolated system activated the pathway and subsequently lost viability (Schreiber *et al.*, 1979). Lysis of the bacteria required, in addition to the 11 isolated complement proteins, also lysozyme. Dose response and kinetics of lysis indicated that the purified cytolytic alternative pathway, supplemented with lysozyme, and C4-depleted human serum had equivalent bacteriolytic activity despite the complete lack of immunoglobulins in the purified

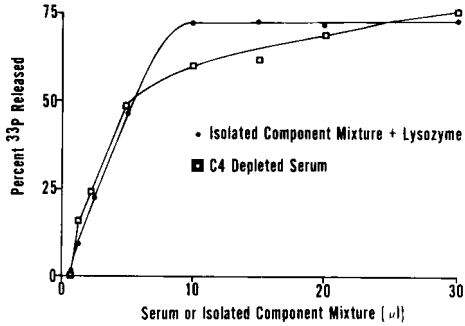


FIG. 10. Lysis of *Escherichia coli* K12 W1485 by the 11 isolated proteins of the cytolytic alternative pathway and lysozyme. Incubations at 37°C for 60 minutes were performed using the isolated component mixture or C4-depleted serum. Lysozyme concentrations used were 10 μg of egg white lysozyme per milliliter of isolated component mixture (Schreiber *et al.*, 1979).

protein mixture (Fig. 10). Lysis diminished with dilution of the protein mixture and became undetectable at 1/16 the physiological concentration of the proteins. Figure. 11 shows scanning electron micrographs of bacteria that were killed by treatment with the purified cytolytic alternative pathway or lysed when lysozyme was also present. As can be seen, the killed bacteria are approximately twice as large as the untreated controls, and irregular surface protrusions and indentations are evident. The increase in size could be verified by light-scattering analysis. Lysed bacteria had lost any resemblance to their controls and had assumed a random polymorphic appearance.

Deletion of properdin reduced the killing or lytic activity of the system by 50%, indicating that properdin was not essential but enhanced the extent of the reaction. All other proteins, including C9, were needed for bacterial killing and lysis. It has become evident that C9 functions by inducing C5b-9 dimer formation (Podack and Müller-Eberhard, 1980). It appears therefore that maximal aggregation of the membrane attack complex is necessary to achieve the degree of reorganization of the outer lipid bilayer of the bacteria that effects killing. Similar conditions appear to be required for the passage of lysozyme through the lipid membrane to gain access to the peptidoglycan layer: Lysis could not be effected by pretreatment of the bacteria with lysozyme and subsequent exposure to the pathway proteins.

Thus, this study constitutes a clear example for the ability of the cytolytic alternative pathway to express bactericidal activity and to do so without antibody. It should be stressed that it is not known today to what extent the large variety of gram-negative and gram-positive bacteria can be recognized and attacked by the alternative pathway.

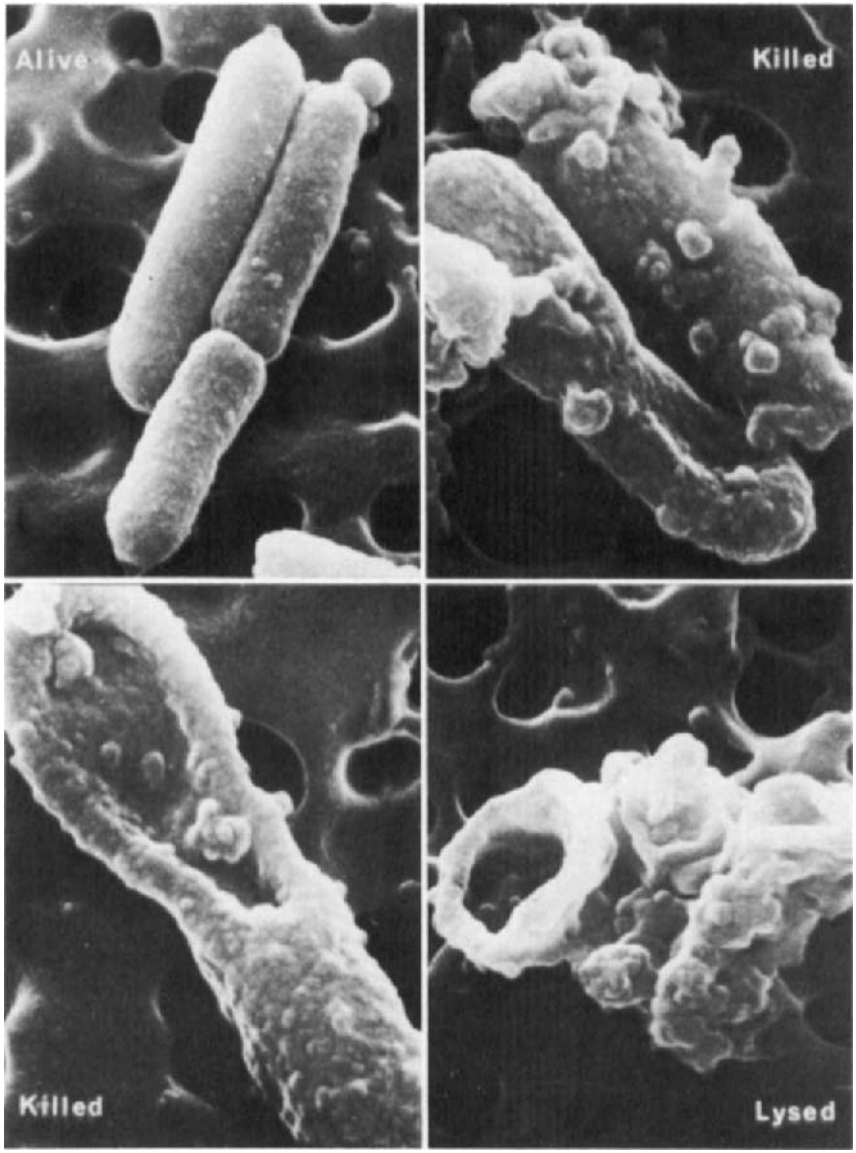


FIG. 11. Scanning electron micrographs of *Escherichia coli* K12 W1485 exposed to the purified cytolytic alternative pathway. Shown are *E. coli* treated at 37°C for 60 minutes with buffer or the heat-inactivated (56°C, 30 minutes) isolated component mixture (upper left panel), the isolated component mixture (upper right, lower left), or the isolated component mixture containing 10 μg of egg white lysozyme per milliliter (lower right). $\times$30,000.

X. Lysis of Nucleated Cells

Several kinds of nucleated cells have been shown to activate the alternative pathway, including those of heterologous and homologous origin (Theofilopoulos *et al.*, 1974; Joseph *et al.*, 1975; Perrin *et al.*, 1976; Theofilopoulos and Perrin, 1976, 1977 Eidinger *et al.*, 1977; Kierszenbaum and Budzko, 1977; Yefenof *et al.*, 1977; Ehrnst, 1978; Hamuro *et al.*, 1978; McConnell *et al.*, 1978; Sissons *et al.*, 1979; Wilson *et al.*, 1979; Schreiber *et al.*, 1980). Pathway-mediated killing of one group of cells requires antibody (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Ehrnst, 1978; Sissons *et al.*, 1979), whereas killing of another group does not (Kierszenbaum and Budzko, 1977; Theofilopoulos and Perrin, 1977; Schreiber *et al.*, 1980). Certain cells are activators of the pathway by virtue of their expressing viral antigen on their surface. Other activating cells do not express detectable surface antigens of viral origin. Only limited information is available at present on the extent to which nucleated mammalian cells including tumor cells are susceptible to this mechanism of eradication.

In 1974 the suggestion was made that a human lymphoblastoid cell line, designated Raji, activated the alternative pathway in human serum (Theofilopoulos *et al.*, 1974). Verification came in 1976 when it was shown that incubation of Raji cells with human serum blocked in the classical pathway resulted in specific deposition of C3 and properdin onto the cell surface and consumption of C3 and factor B in the fluid phase (Budzko *et al.*, 1976; Theofilopoulos and Perrin, 1976). Activation of the pathway resulted in Raji cell lysis, although up to 24 hours of incubation was required for lysis to reach completion (Theofilopoulos and Perrin, 1977). Lysis was much accelerated when the cells were pretreated with puromycin (Baker *et al.*, 1977). Activation and lysis were shown to be independent of antibody by use of either preabsorbed serum or hypogammaglobulinemic serum. The property of activating the alternative pathway appears to reside in Raji cell surface structures which are Epstein–Barr (EB) virus induced. Lymphoma cell lines which were EB virus negative and apparently unable to activate the pathway acquired this ability after infection with EB virus (Yefenof *et al.*, 1977; McConnell *et al.*, 1978).

Lysis of Raji cells could be effected by the 11 proteins of the purified cytolytic alternative pathway, not including immunoglobulins (Schreiber *et al.*, 1980). The precise mechanism of Raji cell killing was investigated by correlating cellular binding of radiolabeled proteins with the release from the cytoplasm of radioactive markers and by measuring the effect of inhibition of protein synthesis on both events. A kinetic analysis of the measured events is shown in Fig. 12. Pathway

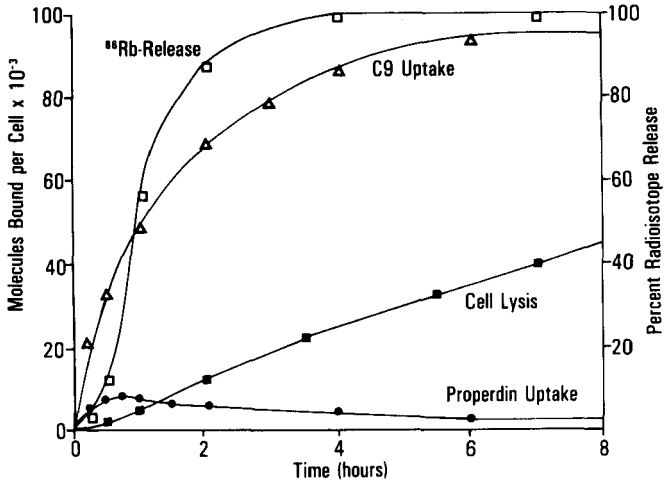


FIG. 12. Injury and death of Raji cells caused by the purified cytolytic alternative pathway. Comparison of the kinetics of pathway activation (properdin uptake), membrane attack complex formation (C9 uptake), production of initial membrane lesion (^{89}Rb release), and Raji cell lysis (^{51}Cr release) (Schreiber *et al.*, 1980).

activation was relatively rapid as evidenced by the fact that properdin uptake was maximal at 1 hour (8000 molecules per cell). Membrane attack complex formation was maximal at 4 hours as indicated by C9 uptake (90,000 molecules per cell). Development of the initial membrane lesion (^{86}Rb release) closely paralleled C9 uptake. However, cell death as measured by ^{51}Cr release occurred slowly and reached completion only after 10–20 hours. The rate of cell lysis was about seven times lower than that of formation of the initial lesion. When cell metabolism was inhibited by puromycin, the kinetics of cell lysis paralleled that of C9 uptake and ^{86}Rb release.

These studies show that Raji cells appear to be weak activators of the alternative pathway. Deposition of properdin and C9 was approximately ten times slower than their deposition on strong pathway activators. Control of cell bound C3b by β1H was reduced (restriction index 0.6; see above) and intermediate to strong activators (rabbit erythrocytes 0.1) and nonactivators (sheep erythrocytes 1.0). The large lag between occurrence of the initial membrane lesion and cell lysis may be due to cellular defense against complement attack. This assumption is supported by disappearance of the lag following inhibition of cell metabolism. Nevertheless, it is remarkable that 90,000 C9 molecules per cell corresponding to 15,000 MAC are not capable of effecting rapid cell death. Temporal resistance to lysis may be due not only to cellular defense, but also to the rate of MAC accumulation on

the target cell. If this rate is high, then Raji cells lyse within 1 hour (Podack and Müller-Eberhard, 1980).

While evidence is accumulating showing that the alternative pathway operates without antibody, there are clear examples for antibody requirement in pathway-mediated cell lysis. Virus-infected cells in the presence of antiviral IgG can be lysed by the alternative pathway. Measles virus-infected cells are the most extensively studied in this regard (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Ehrnst, 1978; Sissons *et al.*, 1979, 1980). Cell lysis was dependent on cell surface expression of viral antigen. Cells that produced nonclustered viral antigen on their surface required bivalent antibody for induction of lysis (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Sissons *et al.*, 1979, 1980). Cells that expressed viral antigen in clusters could be lysed with the Fab′ fragment of the antibody on their surface (Ehrnst, 1978).

The virus-infected cell model appeared to become an exception in alternative pathway biology. The role of antibody in this system was unclear and is still puzzling, especially since very large numbers of molecules are involved, 5×10^7 antiviral IgG molecules per cell being required for 50% cell lysis (Sissons *et al.*, 1979). It has now become apparent that in the virus-infected cell model activation of the alternative pathway and cell lysis have different requirements. Utilizing the six isolated proteins of the alternative pathway, it was shown that the pathway can be initiated on the surface of measles virus-infected cells independent of antibody (Sissons *et al.*, 1980). Activation was manifested by progressive specific uptake of radiolabeled C3 onto the cell surface. The rate of C3 uptake was the same in the presence and the absence of properdin. However, properdin did increase the rate of C3 uptake when antiviral IgG was bound to the cell surface. Lysis of the virus-infected cells exposed to the purified cytolytic alternative pathway did require antiviral IgG and did require properdin (Sissons *et al.*, 1979).

It appears likely that antibody and properdin, which increase the rate of C3 uptake by the virus-infected cells, also increase the rate of MAC accumulation on the cells. Thus, as in the case of Raji cells (see above), fast accumulation of MAC on a nucleated target cell may facilitate lysis.

XI. Inactivation of Viruses

An impressive literature is developing describing the classical pathway's capacity to inactivate viruses with or without antiviral antibody (Cooper, 1979). In contrast, little is known about the role of the alternative pathway in virus neutralization. Thirty years ago it was ob-

served that New Castle disease virus (NDV), mumps virus, and influenza virus of type A or B could be inactivated by nonimmune human serum (Ginsberg and Horsfall, 1949). That inactivation of NDV may proceed through the alternative pathway was suggested by a requirement for properdin and magnesium ions (Wedgwood *et al.*, 1956). Reinvestigation of NDV neutralization using human serum immunochemically depleted of factor B or C4 indicated that either the alternative or the classical pathway can effect viral inactivation in the absence of antibody (Welsh *et al.*, 1976; Welsh, 1977). The mechanism of neutralization of NDV has yet to be defined. Only one other virus has been shown to interact with the alternative pathway, Moloney leukemia virus. This retrovirus is an extremely weak activator of the pathway, and pathway-mediated lysis of the virion has not been demonstrated (Bartholomew *et al.*, 1978).

XII. Effects on Host–Parasite Relationship

A number of different parasites have been shown to activate the alternative pathway. These include members of the helminth and protozoan families (reviewed in Santoro *et al.*, 1979a). Pathway activation can have three biological consequences that affect the relationship between parasite and host: two result in host defense; the third facilitates parasitemia. These consequences are (*a*) the direct antibody-independent lysis of the parasite; (*b*) the induction of cell-mediated killing of the parasite; and (*c*) mediation of red cell parasitization.

Susceptibility to killing via the alternative pathway may be dependent on the stage of differentiation of the parasite. The cercariae of *Schistosoma mansoni* are killed in normal serum deficient in C4 (Machado *et al.*, 1975). Alternatively, they are not killed but lose their tails and become transformed to schistosomula, which are infective upon injection into mice (Greenblatt *et al.*, 1980). The conversion of *S. mansoni* cercariae to schistosomula by normal serum is a process that depends upon an intact alternative pathway and membranolytic activity (Greenblatt *et al.*, 1980). Schistosomula were thought to be resistant to normal serum despite causing alternative pathway activation (Ramalho-Pinto *et al.*, 1978) and despite the fact that they are lysed by IgG class antibody and the classical pathway (Santoro *et al.*, 1979a). It is now clear that under appropriate conditions schistosomula are killed by normal chicken, guinea pig, monkey, or human serum (Santoro *et al.*, 1979b). This action was clearly shown to proceed via the alternative pathway and not to require antibody. As the schistosomula ma-

ture, the schistosomulicidal activity of serum is less apparent, probably because the organisms lose the surface constituents that activate the alternative pathway.

Trypanosoma were thought to be killed in serum via the alternative pathway, but only in presence of specific antibody (Flemmings and Diggs, 1978). In contrast, it has been demonstrated that *Trypanosama cruzi* is killed by chicken serum (Kierszenbaum *et al.*, 1976) and *Trypanosoma cyclops* by human serum (Kierszenbaum and Weinman, 1977) in total absence of antibody via the alternative pathway. *Toxoplasma gondi* was originally believed to be killed in serum via the alternative pathway, but has recently been shown to be killed only via the classical pathway in the presence of antibody (Schreiber and Feldman, 1980).

The cellular mode of complement-dependent parasite killing operates through C3b. Owing to activation of the alternative pathway at the schistosomular surface, C3b becomes bound to the parasite. Eosinophils (Ottesen *et al.*, 1977; Ramalho-Pinto *et al.*, 1978) and mast cells (Sher, 1976; Sher and McIntyre, 1977), which possess C3b receptors, have been shown *in vitro* to adhere to the C3b-coated organism. In the case of eosinophils, C3b-mediated adherence induced killing of the parasite within 18 hours (Ramalho-Pinto *et al.*, 1978). Killing has not been demonstrated as yet for mast cells. However, mast cells are capable of phagocytizing zymosan particles or sheep erythrocytes coated with C3b (Vranian *et al.*, 1980). Phagocytosis is not accompanied by secretion of intracellular mediators or release of lysosomal enzymes. It is considerably enhanced by treatment of the bound C3b with β1H and C3bINA. Thus it appears that mast cells are endowed with functional cell surface receptors for C3b and $C3b_i$ in addition to IgG receptors (Vranian *et al.*, 1980).

An unusual adaptation of a parasite to the effects of complement is exemplified by *Babesia rodhaini* (Chapman and Ward, 1977). This protozoan uses the alternative pathway to effect penetration into the erythrocytes of its host, the rat. That the pathway mediates parasitization was shown by its abrogation when sera depleted of C3 or factor B were used in the test system. It is thought that bound C3b interacts with C3b receptors on the rat erythrocytes, thereby bringing the parasite into proper contact for penetration. Although this parasite can be killed by complement, it appears that in this particular case the alternative pathway facilitates parasite propagation.

Considering the very high incidence of parasitic disease in man on several continents, in depth studies of the interactions of molecular and cellular host systems with parasites are of utmost biomedical im-

portance. The available information constitutes a stimulus and an initial base from which to launch such investigations.

XIII. Reaction Products Influencing Cellular Functions

Activation of the alternative pathway produces protein fragments that function as ligands between cells or as activators of cellular functions. C3b molecules on the surface of a target react with specific receptors on phagocytic cells and thus promote the adherence phase of phagocytosis (Gigli and Nelson, 1968; Huber *et al.*, 1968; Ross *et al.*, 1973; Ross and Rabellino, 1979). C3a and C5a are hormone-like messengers (reviewed in Hugli and Müller-Eberhard, 1978) that release histamine from mast cells and from basophils and hydrolytic enzymes from polymorphonuclear leukocytes (Ward and Newman, 1969; Goldstein and Weissmann, 1974) and contract smooth muscle (Cochrane and Müller-Eberhard, 1968). Both peptides induce increased vascular permeability. Injected into the human skin, minute amounts of C3a (2×10^{-12} mol) or of C5a (1×10^{-15} mol) produce an immediate erythema and edema (Vallota and Müller-Eberhard, 1973). C3a is said to have tumorolytic activity (Ferluga *et al.*, 1976, 1978), and C5a is a powerful chemotactic agent for polymorphonuclear leukocytes, monocytes, and macrophages. The physical and chemical properties of human C3a were described above in context with those of C3. The molecular properties of human C5a are as follows: the molecular weight is 11,200, the electrophoretic mobility at pH 8.6 is -1.7×10^{-5} cm^2 V^{-1} sec^{-1}; the α-helical content is approximately 40% (Morgan *et al.*, 1974). The molecule consists of 74 amino acid residues, has a known primary structure (Fernandez and Hugli, 1978) with threonine in N-terminal and arginine in C-terminal position. The C-terminal arginine is essential for the anaphylatoxin, but not the chemotactic function of the molecule. C5a has a sizable carbohydrate moiety. Liberation of C3a, C3b, and C5a is not unique to the alternative pathway, since it is also effected by the classical pathway. In contrast, liberation of Ba and Bb is a function of the alternative pathway. Ba (from guinea pig serum) has been reported to have chemotactic activity for guinea pig polymorphonuclear leukocytes (Hadding *et al.*, 1978). Similarly, a mixture of factors B and D and C3b of human origin, which generates Ba, was found to contain chemotactic activity (Ruddy *et al.*, 1975). Bb derived from human factor B has cell spreading activity for human monocytes and mouse macrophages (Bianco *et al.*, 1979; Götze *et al.*, 1979a,b; Sundsmo and Götze, 1979).

Cells capable of phagocytosis, such as polymorphonuclear leuko-

cytes, monocytes, macrophages, and mast cells, have cell surface receptors for C3b. B lymphocytes also have receptors that are specific for C3d (Ross *et al.*, 1973). These receptors were not found on peripheral granulocytes or mast cells (Vranian *et al.*, 1980). Recently, a cell surface receptor for $C3b_i$ has been described to be present on polymorphonuclear leukocytes, monocytes (Ross and Rabellino, 1979), and mast cells (Vranian *et al.*, 1980). This receptor is distinct from the C3b and the C3d receptors. Earlier reports on the occurrence of the C3d receptor on phagocytic cells will have to be reevaluated in the light of the known mechanism of $C3b_i$ and C3d formation (Pangburn *et al.*, 1977). The function of C3b in phagocytosis has not been completely elucidated. It is clear that C3b enhances IgG-mediated phagocytosis (Huber *et al.*, 1968). It has also been reported that C3b alone can mediate ingestion of particles by polymorphs (Gigli and Nelson, 1968), monocytes (Huber *et al.*, 1968), activated macrophages (Bianco *et al.*, 1975), and mast cells (Vranian *et al.*, 1980). However, nonactivated freshly explanted macrophages do not ingest C3b-coated particles, although they bind such particles through their surface C3b receptors (Vranian *et al.*, 1980). Peritoneal macrophages could be converted *in vitro* from cells mediating only attachment to cells promoting both attachment and ingestion of C3b-coated particles. Conversion required treatment of the cells with supernatants of cultures containing T lymphocytes and triggered macrophages (Griffin and Griffin, 1979). The role of $C3b_i$–$C3b_i$ receptor interaction in phagocytosis remains to be investigated.

Engagement of particle-bound C3b with C3b receptors (or of IgG with Fc receptors) on phagocytic cells results in increased oxidative metabolic activity (Babior *et al.*, 1973; Curnutte and Babior, 1974; Goldstein *et al.*, 1975). The products of this activity, superoxide anions, hydrogen peroxide, hydroxyl radicals and singlet oxygen, have been postulated to be involved in the actual bactericidal process (Klebanoff, 1974; Johnston *et al.*, 1975). Soluble C3b has been observed to enhance intracellular killing of bacteria by monocytes (Leijh *et al.*, 1979).

Macrophages spread under the influence of alternative pathway activation, and this reaction is regarded as an expression of cell activation. The protein fragment Bb, which is the catalytic site carrying subunit of C3 convertase, was shown to be responsible for the cell spreading effect (Götze *et al.*, 1979a). Recently, it was found that Bb imparts the same effect on peripheral monocytes (Sundsmo and Götze, 1979). In both instances, Bb activity is a function of its enzymic site; DFP treatment abolished spreading activity (Götze *et al.*, 1979a;

Sundsmo and Götze, 1979). Preliminary evidence suggests that Bb acts on membrane-associated C5 in eliciting the spreading response (Götze *et al.*, 1979b). In inducing spreading Bb inhibits cell migration, and this effect is countered by the action of C5a on the cells (Bianco *et al.*, 1979). Thus, motility of macrophages may be manipulated *in vitro* and may indeed be regulated *in vivo* by two functionally antagonistic complement reaction products.

The influence of complement reaction products on cell behavior and function is an area of research that is still in its infancy. There are a large number of products that have not been explored for biological functions. And there are complement-specific cell surface receptors whose functional potential has remained unknown. Cell membrane-associated complement proteins and their possible role as receptors or mediators of messages deserve full exploration.

XIV. Nephritic Factor: Autoantibody to C3 Convertase

Although nephritic factor is neither a component nor an accessory factor of the alternative pathway, its discussion is included in this review because of its unusual and highly specific effects on the pathway.

Nephritic factor (C3NeF or NF) is an autoantibody with specificity for conformations or neoantigen(s) characteristic for the bimolecular complex of C3 and activated factor B (Davis *et al.*, 1977a,b; Daha *et al.*, 1978; Schreiber and Müller-Eberhard, 1978b; Scott *et al.*, 1978; Schreiber and Müller-Eberhard, 1980). As such, it physically combines with the complex and augments the association of its subunits and thereby allows expression of C3 convertase activity (Schreiber *et al.*, 1976b; Daha *et al.*, 1977). NF is an unusual serum constituent that occurs in patients with hypocomplementemic chronic glomerulonephritis (Pickering *et al.*, 1968; Spitzer *et al.*, 1969) and partial lipodystrophy (Williams *et al.*, 1972). While it is probably responsible for the marked hypocomplementemia in respective patients, a pathogenetic role of NF has not been documented to date.

The history of description and elucidation of NF stretches from 1965 to 1978, when a clear definition emerged. After the description of the disease "persistent hypocomplementemic glomerulonephritis" (West *et al.*, 1965) or "progressive glomerulonephritis" (Gotoff *et al.*, 1965), a serum factor was found in afflicted patients that consumed complement when added to normal serum (Pickering *et al.*, 1968). This activity (NF) was shown to inactivate specifically C3 and to be

dependent on normal serum factors. The magnesium requirement of NF function and its independence of C4 (Spitzer *et al.*, 1969; Vallota *et al.*, 1970) suggested a relationship of the normal serum factors to the alternative pathway of complement activation. Subsequent investigations identified the serum cofactors as factor B (Ruley *et al.*, 1973), C3 (Vallota *et al.*, 1974), and factor D (Vallota *et al.*, 1974). Notable was that C3 not only served as substrate in the NF system, but also as cofactor of the NF-dependent C3 convertase (Schreiber *et al.*, 1975) and that properdin was not required (Williams *et al.*, 1973; Schreiber *et al.*, 1975). With increasing understanding of the alternative pathway it became possible to delineate the mechanism of action of NF as binding to (Schreiber *et al.*, 1976b) and stabilizing (Schreiber *et al.*, 1975; Daha *et al.*, 1976a) the alternative pathway C3 convertase.

While studies on NF mechanism were underway, parallel investigations led to the isolation of the protein and its identification as an immunoglobulin (Vallota *et al.*, 1974; Schreiber *et al.*, 1976b; Daha *et al.*, 1977). An early claim that NF is an immunoglobulin (Thompson, 1971) was eventually confirmed (Amos *et al.*, 1977; Davis *et al.*, 1977a,b; Daha *et al.*, 1978; Schreiber and Müller-Eberhard, 1978b; Scott *et al.*, 1978; Schreiber and Müller-Eberhard, 1980).

Some of the results on NF reported in the literature have required reinterpretation. In brief, a relationship of NF to immunoglobulins was temporarily refuted (Vallota *et al.*, 1974; Daha *et al.*, 1975; Schreiber *et al.*, 1976b) until it was fully verified. One of the reasons for failing to recognize NF isolated from patient T.A. (Schreiber *et al.*, 1976b) as immunoglobulin was due to its unusual monoclonality, which hampered immunochemical and structural identification. Neither an immunochemical relationship of NF to properdin (Daha *et al.*, 1975) nor a functional requirement for properdin by NF (Vallota *et al.*, 1974) could subsequently be verified. That factor D was found to be unessential for NF activity (Daha *et al.*, 1976b; Sissons *et al.*, 1976) could be explained on the basis of trace contamination of NF preparations with DFP-sensitive enzyme activity (Schreiber *et al.*, 1976b). That physical binding of NF to the preformed C3 convertase initially escaped detection (Daha *et al.*, 1976a) remains unexplained. That the full elucidation of NF was difficult is undoubtedly due to the fact that the protein occurs in very small amounts and that its separation from the bulk of IgG had to await the elaboration of methods based on specific affinity (Schreiber *et al.*, 1976b; Daha *et al.*, 1977).

The first specific isolation of NF was accomplished by using binding to and release from its specific antigen, $\overline{C3b,Bb}$ (Schreiber *et al.*,

1976b). NF_{TA} is a heat-stable 7 S γ-globulin (Schreiber and Müller-Eberhard, 1980). Examination of its chain structure by SDS–polyacrylamide gel electrophoresis under reducing conditions revealed the presence of two types of chains having molecular weights of 62,000 and 25,000. For the detection of the MW 25,000 species, it was necessary to use a lysine-specific labeling reagent (Bolton-Hunter) because the more commonly used tyrosine-specific reagent (chloramine T) failed to effect radioiodination. This differential susceptibility to labeling is unusual and distinguishes NF_{TA} from other IgG proteins. The larger than usual size of the heavy chain also is relatively rare. A light and heavy chain structure typical of IgG has been described for other NF preparations (Davis *et al.*, 1977b; Daha *et al.*, 1978; Scott *et al.*, 1978). Thus, on the basis of chain structure, electrophoretic mobility, and size, NF is indistinguishable from IgG. By and large, NF contains antigenic determinants of light chains, γ chains, and the Fab and Fc fragments (Amos *et al.*, 1977; Davis *et al.*, 1977b; Daha *et al.*, 1978; Schreiber and Müller-Eberhard, 1978b, 1980; Scott *et al.*, 1978). On the basis of antigenic expression and electrophoretic distribution, most NF are oligoclonal. A notable exception is NF_{TA}, which contains only λ chain determinants, is antigenically deficient in the C_H3 domain, and possesses unique antigenic determinants to which anti-iodiotypic antibody could be elicited (Schreiber and Müller-Eberhard, 1978b, 1980). The IgG nature of NF is also supported by the observation that NF has the capacity to pass the placenta (Davis *et al.*, 1977b) and that, in association with cell-bound C3 convertase, it binds C1q and activates the isolated C1 complex (Sobel *et al.*, 1979).

The mechanism of action of NF is based on its specificity for the alternative pathway C3 convertase. Unlike most other autoantibodies that inhibit biological function, NF enhances the function of the C3 convertase. This enhancement is expressed in a 30-fold increase in half-life of the enzyme at 37°C. The NF_{TA}-C3 convertase has an affinity of 10^9 liters/mol, which is indicative of strong association (Schreiber and Müller-Eberhard, 1980). Because the complex is stable, it is possible to demostrate the fluid phase NF-C3 convertase complex by ultracentrifugation (Daha *et al.*, 1976a; Schreiber *et al.*, 1976b). The question regarding the possible contribution of NF to disease mechanisms remains open. In patients with glomerulonephritis and circulating NF, kidney biopsies have often revealed deposition of C3 and properdin in the glomerular capillaries. In those instances, IgG often is not detectable or occurs only in small amounts (Westberg *et al.*, 1971; Habib *et al.*, 1973). No attempts are known to elute IgG with NF activity from affected kidneys.

XV. Conclusion

It has been the aim of this review to impart a representative image of the molecular organization and dynamics of the alternative complement pathway and to point out unanswered questions pertaining thereto. No attempt was made to render an encyclopedic account of the subject. Emphasis was placed on the newest insights into relevant protein–protein interactions, chemical structure, biochemical events, and biological manifestations of pathway activation. Because of the approach chosen, the bibliography may not be complete. Preference was given to the recent literature; a large percentage of the references listed refer to work published from 1976 on.

The biomedical relevance of the alternative pathway as part of the complement system is unquestionalbe. It is now abundantly clear that the pathway can operate without antibody. It is capable of distinguishing between targets and nontargets. Once activated, it can mark particles for phagocytosis, induce killing of nucleated cells or of bacteria, and cause lysis of enveloped viruses or virus-infected cells. It generates biologically active protein fragments, such as Bb, C3a, C3b, and C5a, that influence function and behavior of inflammatory cells and cells of the immune system. Observation of individuals with homozygous deficiencies of one or the other protein of the alternative pathway, once they are found, will greatly illuminate the physiogenic role of this pathway, especially the extent to which it contributes to natural resistance phenomena. That the pathway can function independent of specific antibody suggests that its ancestral precursor may have served as a humoral defense mechanism already early in phylogeny.

From the group of the six proteins, C3 stands out because it is a protein of versatility and multiple functions. C3 is the precursor of several physiological protein fragments, the largest of which, C3b, is endowed with a metastable target binding site, a stable site for specific cell surface receptors, a binding site for the catalytic enzyme subunit Bb, and sites for the regulatory proteins properdin, β1H, and C3b inactivator. It has now come to light that, in all probability, target-bound C3b can recognize constituents on the surface of biological particles.

C3 has two evolutionary relatives among the complement proteins, C4 and C5, all three proteins being homologous in primary structure. Like C3, C4 and C5 are each the precursor of a peptide with anaphylatoxin activity and of a b fragment that expresses a metastable binding site. At least in part, C4 is the functional counterpart of C3 in the classical pathway and C5 in the membrane attack pathway, inasmuch as C5 constitutes the nucleus of the membrane attack complex assembly.

For all these reasons, it appears desirable and justified to tackle elucidation of the entire structure of the C3 molecule.

Knowledge of the structure of the C3 molecule would be particularly important and illuminating if the present perception is correct that C3b fulfills the critical recognition function within the alternative pathway.

REFERENCES

Abramson, N., Alper, C. A., Lachmann, P. J., Rosen, F. S., and Jandl, J. H. (1971). *J. Immunol.* **107,** 19.

Allen, F. H., Jr. (1974). *Vox Sang.* **27,** 382.

Alper, C. A., and Balavitch, D. (1976). *Science* **191,** 1275.

Alper, C. A., and Propp, R. P. (1968). *J. Clin. Invest.* **47,** 2181.

Alper, C. A., Johnson, A. M., Birtch, A. G., and Moore, F. D. (1969). *Science* **16,** 286.

Alper, C. A., Boenisch, T., and Watson, L. (1972). *J. Exp. Med.* **135,** 68.

Amos, N., Sissons, J. G. P., and Peters, D. K. (1977). *Pathol. Biol.* **25,** 390.

Azen, E. A., and Smithies, O. (1968). *Science* **162,** 905.

Babior, B. M., Kipnes, R. S., and Curnutte, J. T. (1973). *J. Clin. Invest.* **52,** 741.

Baker, P. J., Lint, T. F., Mortensen, R. F., and Gewurz, H. (1977). *J. Immunol.* **118,** 198.

Bartholomew, R. M., Esser, A. F., and Müller-Eberhard, H. J. (1978). *J. Exp. Med.* **147,** 844.

Bhakdi, S., and Tranum-Jensen, J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5655.

Bianco, C., Patrick, R., and Nussenzweig, V. (1970). *J. Exp. Med.* **132,** 702.

Bianco, C., Griffin, F. M., and Silverstein, S. C. (1975). *J. Exp. Med.* **141,** 1278.

Bianco, C., Götze, O., and Cohn, Z. A. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 888.

Biesecker, G., Podack, E. R., Halverson, C. A., and Müller-Eberhard, H. J. (1979). *J. Exp. Med.* **149,** 448.

Bitter-Suermann, D., Krönke, M., Brade, V., and Hadding, U. (1977). *J. Immunol.* **118,** 1822.

Boenisch, T., and Alper, C. A. (1970). *Biochim. Biophys. Acta* **221,** 529.

Bokisch, V. A., and Müller-Eberhard, H. J. (1970). *J. Clin. Invest.* **49,** 2427.

Bokisch, V. A., Dierich, M. P., and Müller-Eberhard, H. J. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1989.

Brade, V., Hall, R. E., and Colten, H. R. (1977a). *J. Exp. Med.* **146,** 759.

Brade, V., Bentley, C., Bitter-Suermann, D., and Hadding, U. (1977b). *Z. Immuntoetsforsch.* **152,** 402.

Brade, V., Fries, W., and Bentley, C. (1978). *J. Immunol.* **120,** 1766.

Budzko, D. B., Bokisch, V. A., and Müller-Eberhard, H. J. (1971). *Biochemistry* **10,** 1166.

Budzko, D. B., Lachmann, P. J., and McConnell, I. (1976). *Cell. Immunol.* **22,** 98.

Capel, P. J. A., Groeneboer, O., Grosveld, G., and Pondman, K. W. (1978). *J. Immunol.* **121,** 2566.

Chapman, W. E., and Ward, P. A. (1977). *Science* **196,** 67.

Cochrane, C. G., and Müller-Eberhard, H. J. (1968). *J. Exp. Med.* **127,** 371.

Colten, H. R. (1972). *J. Clin. Invest.* **51,** 725.

Conrad, D. H., Carlo, J. R., and Ruddy, S. (1978). *J. Exp. Med.* **147,** 1792.

Cooper, N. R. (1971). *Prog. Immunol., Int. Congr. Immunol., 1st,* p. 567.

Cooper, N. R. (1973). *J. Exp. Med.* **137,** 451.

Cooper, N. R. (1975a). *J. Exp. Med.* **141,** 890.
Cooper, N. R. (1975b). *Biochemistry* **14,** 4245.
Cooper, N. R. (1979). *Compr. Virol.* **15,** 123.
Curman, B., Sandberg-Trägårdh, L., and Peterson, P. A. (1977). *Biochemistry* **16,** 5369.
Curnutte, J. T., and Babior, B. M. (1974). *J. Clin. Invest.* **53,** 1662.
Daha, M. R., Fearon, D. T., and Austen, K. F. (1975). *Clin. Res.* **23,** 401A.
Daha, M. R., Fearon, D. T., and Austen, K. F. (1976a). *J. Immunol.* **116,** 1.
Daha, M. R., Fearon, D. T., and Austen, K. F. (1976b). *J. Immunol.* **116,** 568.
Daha, M. R., Fearon, D. T., and Austen, K. F. (1976c). *J. Immunol.* **117,** 630.
Daha, M. R., Austen, K. F., and Fearon, D. T. (1977). *J. Immunol.* **119,** 812
Daha, M. R., Austen, K. F., and Fearon, D. T. (1978). *J. Immunol.* **120,** 1389.
Davis, A. E., Arnaout, M. A., Alper, C. A., and Rosen, F. S. (1977a). *N. Engl. J. Med.* **297,** 144.
Davis, A. E., Ziegler, J. B., Gelfand, E. W., Rosen, F. S., and Alper, C. A. (1977b). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3980.
Davis, A. E., Rosenberg, R. D., Fenton, J. W., Bing, D. H., Rosen, F. S., and Alper, C. A. (1978). *J. Immunol.* **120,** 1771.
Davis, A. E., Zalut, C., Rosen, F. S., and Alper, C. A. (1979). *Biochemistry* **18,** 5082.
Dias Da Silva, W., Eisele, J. W., and Lepow, I. H. (1967). *J. Exp. Med.* **126,** 1027.
Dierich, M. P., Bitter-Suermann, D., König, W., Hadding, U., Galanos, C., and Rietschel, E. T. (1973). *Immunology* **24.** 721.
Dierich, M. P., Hadding, U., König, W., Limbert, M., Schorlemmer, H. U., and Bitter-Suermann, D. (1974). *Immunochemistry* **11,** 527.
Ehrnst, A. (1978). *J. Immunol.* **121,** 1206.
Eidinger, D., Bello, E., and Mates, A. (1977). *Cell. Immunol.* **29,** 174.
Einstein, L. P., Hansen, P. J., Ballow, M., Davis, A. E., Davis, J. S., Alper, C. A., Rosen, F. S., and Colten, H. R. (1977). *J. Clin. Invest.* **60,** 963.
Esser, A. F., Kolb, W. P., Podack, E. R., and Müller-Eberhard, H. J. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1410.
Fearon, D. T. (1977). *J. Immunol.* **119,** 1248.
Fearon, D. T. (1978). *Proc. Natl. Acad. Sci. U. S. A.* **75,** 1971.
Fearon, D. T. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5867.
Fearon, D. T., and Austen, K. F. (1975a). *J. Exp. Med.* **142,** 856.
Fearon, D. T., and Austen, K. F. (1975b). *J. Immunol.* **115,** 1357.
Fearon, D. T., and Austen, K. F. (1977a). *J. Exp. Med.* **146,** 22.
Fearon, D. T., and Austen, K. F. (1977b). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 1683.
Fearon, D. T., Austen, K. F., and Ruddy, S. (1973). *J. Exp. Med.* **138,** 1305.
Fearon, D. T., Austen, K. F., and Ruddy, S. (1974). *J. Exp. Med.* **139,** 355.
Ferluga, J., Schorlemmer, H. U., Baptista, L. C., and Allison, A. C. (1976). *Br. J. Cancer* **34,** 626.
Ferluga, J., Schorlemmer, H. U., Baptista, L. C., and Allison, A. C. (1978). *Clin. Exp. Immunol.* **31,** 512.
Fernandez, H. N., and Hugli, T. E. (1977). *J. Biol. Chem.* **252,** 1826.
Fernandez, H. N., and Hugli, T. E. (1978). *J. Biol. Chem.* **253,** 6955.
Ferreira, A., and Nussenzweig, V. (1975). *J. Exp. Med.* **141,** 513.
Ferreira, A., and Nussenzweig, V. (1976). *Nature (London)* **260,** 613.
Fine, D. P. (1975). *Infect. Immun.* **12,** 772.
Fine, D. P., Marney, S. R., Jr., Calley, D. G., Sergent, J. S., and Dep Prez, R. M. (1972). *J. Immunol.* **109,** 807.
Flemmings, B., and Diggs, C. (1978). *Infect. Immun.* **19,** 928.

Forsgren, A., and Quie, P. G. (1974). *Infect. Immun.* **10,** 402.
Fujita, T., and Nussenzweig, V. (1979). *J. Exp. Med.* **150,** 267.
Fujita, T., Gigli, I., and Nussenzweig, V. (1978). *J. Exp. Med.* **148,** 1044.
Galanos, C., and Lüderitz, O. (1976). *Eur. J. Biochem.* **65,** 403.
Gewurz, H., Shin, H. S., and Mergenhagen, S. E. (1968). *J. Exp. Med.* **128,** 1049.
Gewurz, H., Pickering, R. J., Snyderman, R., Lichtenstein, L. M., Good, R. A., and Mergenhagen, S. E. (1970). *J. Exp. Med.* **131,** 817.
Ghebrehiwet, B., and Müller-Eberhard, H. J. (1979). *J. Immunol.* **123,** 616.
Gigli, I., and Nelson, R. A., Jr. (1968). *Exp. Cell Res.* **51,** 45.
Gigli, I., Fujita, T., and Nussenzweig, V. (1980). *J. Immunol.* **124,** 1521.
Ginsberg, H. S., and Horsfall, F. L. (1949). *J. Exp. Med.* **90,** 475.
Gitlin, J. D., Rosen, F. S., and Lachmann, P. J. (1975). *J. Exp. Med.* **141,** 1221.
Goldstein, I., and Weissmann, G. (1974). *J. Immunol.* **113,** 1583.
Goldstein, I., Roos, D., Kaplan, H. B., and Weissmann, G. (1975). *J. Clin. Invest.* **56,** 1155.
Gorski, J. P., Hugli, T. E., and Müller-Eberhard, H. J. (1979a). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 1010.
Gorski, J. P., Hugli, T. E., and Müller-Eberhard, H. J. (1979b). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5299.
Gotoff, S. P., Fellers, F. X., Vawter, G. F., Janeway, C. A., and Rosen, F. S. (1965). *N. Engl. J. Med.* **273,** 524.
Götze, O. (1975). *In* "Proteases and Biological Control" (E. Reich, D. B. Rifkin, and E. Shaw eds.), p. 255. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Götze, O., and Müller-Eberhard, H. J. (1971). *J. Exp. Med.* **134,** 90s.
Götze, O., and Müller-Eberhard, H. J. (1976). *Adv. Immunol.* **24,** 1.
Götze, O., Medicus, R. G., and Müller-Eberhard, H. J. (1977). *J. Immunol.* **118,** 525.
Götze, O., Bianco, C., and Cohn, Z. A. (1979a). *J. Exp. Med.* **149,** 372.
Götze, O., Bianco, C., Sundsmo, J. S., and Cohn, Z. A. (1979b). *In* "Mononuclear Phagocytes—Functional Aspects" (R. van Furth, ed.). Martinus Nijhoff Medical Division, The Hague.
Greenblatt, H. C., Eveland, L. K., and Morse, S. I. (1980). *J. Immunol.* **124,** 1523.
Griffin, J. A., and Griffin, F. M. (1979). *J. Exp. Med.* **150,** 653.
Habib, R., Kleinknecht, C., Gubler, M. C., and Levy, M. (1973). Clin. Nephrol. **1,** 194.
Hadding, U., Hamuro, J., and Bitter-Suermann, D. (1978). *J. Immunol.* **120,** 438.
Halbwachs, L., and Lachmann, P. J. (1976). *Scand. J. Immunol.* **5,** 697.
Hammer, C. H., Abramovitz, A. S., and Mayer, M. M. (1976). *J. Immunol.* **117,** 830.
Hamuro, J., Hadding, U., and Bitter-Suermann, D. (1978). *Immunology* **34,** 695.
Huber, H., Polley, M. J., Linscott, W. D., Fudenberg, H. H., and Müller-Eberhard, H. J. (1968). *Science* **162,** 1281.
Hugli, T. E. (1975). *J. Biol. Chem.* **250,** 8293.
Hugli, T. E., and Müller-Eberhard, H. J. (1978). *Adv. Immunol.* **26,** 1.
Hugli, T. E., Morgan, W. T., and Müller-Eberhard, H. J. (1975). *J. Biol. Chem.* **250,** 1479.
Inoue, K., Tanigawa, Y., Takubo, M., Santani, J., and Amano, T. (1959). *Biken J.* **2,** 1.
Jasin, H. E. (1972). *J. Immunol.* **109,** 26.
Johnston, R. B., Jr., Keele, B. B., Jr., Mirsa, H. P., Lehmeyer, J. E., Webb, L. S., Bachner, R. L., and Rajagopalan, K. V. (1975). *J. Clin. Invest.* **55,** 1357.
Joseph, B. S., Cooper, N. R., and Oldstone, M. B. A. (1975). *J. Exp. Med.* **141,** 761.
Kazatchkine, M. D., Fearon, D. T., and Austen, K. F. (1979a). *J. Immunol.* **122,** 75.

Kazatchkine, M. D., Fearon, D. T., Silbert, J. E., and Austen, K. F. (1979b). *J. Exp. Med.* **150,** 1202.
Kerr, M. A., and Porter, R. R. (1978). *Biochem. J.* **171,** 99.
Kierszenbaum, F., and Budzko, D. B. (1977). *Cell. Immunol.* **29,** 137.
Kierszenbaum, F., and Weinman, D. (1977). *Immunology* **32,** 245.
Kierszenbaum, F., Ivanyi, J., and Budzko, D. B. (1976). *Immunology* **30,** 1.
Klebanoff, S. J. (1974). *J. Biol. Chem.* **249,** 3724.
Kolb, W.P., and Müller-Eberhard, H. J. (1973). *J. Exp. Med.* **138,** 438.
Lachmann, P. J., and Halbwachs, L. (1975). *Clin. Exp. Immunol.* **21,** 109.
Lachmann, P. J., and Müller-Eberhard, H. J. (1968). *J. Immunol.* **100,** 691.
Lachmann, P. J., and Nicol, P. A. E. (1973). *Lancet* **1,** 465.
Lachmann, P. J., and Rosen, F. S. (1978). *Springer Semin. Immunopathol.* **1,** 339.
Law, S.K., and Levine, R. P. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 2701.
Law, S. K., Fearon, D. T., and Levine, R. P. (1979a). *J. Immunol.* **122,** 759.
Law, S. K., Lichtenberg, N. A., and Levine, R. P. (1979b). *J. Immunol.* **123,** 1388.
Leijh, P. C. J., van den Barselaar, M. T., van Zwet, T. L., Daha, M. R., and van Furth, R. (1979). *J. Clin. Invest.* **63,** 772.
Leon, M. A. (1956). *J. Exp. Med.* **102,** 285.
Lesavre, P., and Müller-Eberhard, H. J. (1978). *J. Exp. Med.* **148,** 1498.
Lesavre, P., Hugli, T. E., Esser, A. F., and Müller-Eberhard, H. J. (1979). *J. Immunol.* **123,** 529.
McClelland, D. B. L., and van Furth, R. (1976). *Immunology* **31,** 855.
McConnell, I., Klein, G., and Lint, T. F. (1978). *Eur. J. Immunol.* **8,** 453.
Machado, A. J., Gazzinelli, G., Pellegrino, J., and Dias Da Silva, W. (1975). *Exp. Parasitol.* **38,** 20.
Mak, L. W., Majewski, J., and Lachmann, P. J. (1977). *Clin. Exp. Immunol.* **30,** 211.
Mann, J., O'Brien, R., Alper, C. A., Rosen, F. S., and Babior, B. M. (1980). *J. Immunol.* **124,** 1531.
Marcus, R. L., Shin, H. S., and Mayer, M. M. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 1351.
Mayer, M. M., Hammer, C. H., Michaels, D. W., and Shin, M. L. (1979). *Mol. Immunol.* **15,** 813.
Medicus, R. G. (1977). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36,** 1244.
Medicus, R. G., Götze, O., and Müller-Eberhard, H. J. (1976a). *J. Exp. Med.* **144,** 1076.
Medicus, R. G., Götze, O., and Müller-Eberhard, H. J. (1976b). *Scand. J. Immunol.* **5,** 1049.
Medicus, R. G., Schreiber, R. D., Götze, O., and Müller-Eberhard, H. J. (1976c). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 612.
Medicus, R. G., Esser, A. F., Fernandez, H. N., and Müller-Eberhard, H. J. (1980). J. Immunol. *124* (in press).
Michael, S. G., Whitby, J. C., and Landy, M. (1962). *J. Exp. Med.* **115,** 131.
Minta, J. O. (1976). *J. Immunol.* **117,** 405.
Minta, J. O., and Lepow, I. H. (1974). *Immunochemistry* **11,** 361.
Molenaar, J. L., Helder, A. W., Müller, M. A. C., Goris-Mulder, M., Jonker, L. S., Bronwer, M., and Pondman, K. W. (1974). *In* "The Third Human Complement Component" (J. L. Molenaar, ed.), p. 72. Graduate Press, Amsterdam.
Morgan, W. T., Vallota, E. H., and Müller-Eberhard, H. J. (1974). *Biochem. Biophys. Res. Commun.* **57,** 572.
Morrison, D. C., and Kline, L. F. (1977). *J. Immunol.* **118,** 362.

Müller-Eberhard, H. J. (1975). *Annu. Rev. Biochem.* **44,** 697.
Müller-Eberhard, H. J. (1978). *In* "Molecular Basis of Biological Degradative Processes" (R. Berlin, H. Hermann, I. H. Lepow, and J. Tanzer, eds.), p. 65. Academic Press, New York.
Müller-Eberhard, H. J., and Fjellström, K.-E. (1971). *J. Immunol.* **107,** 1666.
Müller-Eberhard, H. J., and Götze, O. (1972). *J. Exp. Med.* **135,** 1003.
Müller-Eberhard, H. J., and Lepow, I. H. (1965). *J. Exp. Med.* **121,** 819.
Müller-Eberhard, H. J., and Nilsson, U. R. (1960). *J. Exp. Med.* **111,** 217.
Müller-Eberhard, H. J., Nilsson, U. R., and Aronsson, T. (1960). *J. Exp. Med.* **111,** 201.
Müller-Eberhard, H. J., Dalmasso, A. P., and Calcott, M. A. (1966a). *J. Exp. Med.* **123,** 33.
Müller-Eberhard, H. J., Nilsson, U. R., Dalmasso, A. P., Polley, M. J., and Calcott, M. A. (1966b). *Arch. Pathol.* **82,** 205.
Müller-Eberhard, H. J., Polley, M. J., and Calcott, M. A. (1967). *J. Exp. Med.* **125,** 359.
Muschel, L. H., Carey, W. F., and Baron, L. S. (1959). *J. Immunol.* **82,** 38.
Nagaki, K., Iida, K., Okubo, M., and Inai, S. (1978). *Int. Arch. Allergy Appl. Immunol.* **57,** 221.
Nagasawa, S., and Stroud, R. M. (1977a). *Immunochemistry* **14,** 749.
Nagasawa, S., and Stroud, R. M. (1977b). *Proc. Natl. Acad. Sci. U. S. A.* **74,** 2998.
Natsuume-Sakai, S., Hayakawa, J. I., and Takahashi, M. (1978). *J. Immunol.* **121,** 491.
Nelson, B., and Ruddy, S. (1979). *J. Immunol.* **122,** 1994.
Nicholson, A., Brade, V., Schorlemmer, H. U., Burger, R., Bitter-Suermann, D., and Hadding, U. (1975). *J. Immunol.* **115,** 1108.
Nicol, P. A. E., and Lachmann, P. J. (1973). *Immunology* **24,** 259.
Nilsson, U. R., and Müller-Eberhard, H. J. (1965). *J. Exp. Med.* **122,** 277.
Nilsson, U. R., Mandle, R. J., Jr., and McConnell-Mapes, J. A. (1975). *J. Immunol.* **114,** 815.
Nydegger, U. E., Fearon, D. T., and Austen, K. F. (1978a). *J. Immunol.* **120,** 1404.
Nydegger, U. E., Fearon, D. T., and Austen, K. F. (1978b). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 6078.
Osawa, E., and Muschel, L. H. (1960). *J. Immunol.* **84,** 203.
Ottesen, E. A., Stanley, A. M., Gelfand, J. A., Gadek, J. E., Frank, M. M., Nash, T. E., and Cheever, A. W. (1977). *Am. J. Trop. Med. Hyg.* **26,** 134.
Pangburn, M. K. (1980). *Fed. Proc.* In press.
Pangburn, M. K., and Müller-Eberhard, H. J. (1978a). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2416.
Pangburn, M. K., and Müller-Eberhard, H. J. (1978b). *J. Immunol.* **120,** 1791.
Pangburn, M. K., Schreiber, R. D., and Müller-Eberhard, H. J. (1977). *J. Exp. Med.* **146,** 257.
Pangburn, M. K., Schreiber, R. D., and Müller-Eberhard, H. J. (1979). Unpublished observations.
Pangburn, M. K., Morrison, D. C., Schreiber, R. D., and Müller-Eberhard, H. J. (1980). *J. Immunol.* **124,** 977.
Patel, F., and Minta, J. O. (1979). *J. Immunol.* **122,** 1582.
Penalva Da Silva, F., Hoecker, G. F., Day, N. K., Vienne, K., and Rubinstein, P. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 963.
Pensky, J., Hinz, C. F., Jr., Todd, E. W., Wedgwood, R. J., Boyer, J. T., and Lepow, I. H. (1968). *J. Immunol.* **100,** 142.
Perrin, L. H., Joseph, B. S., Cooper, N. R., and Oldstone, M. B. A. (1976). *J. Exp. Med.* **143,** 1027.

Pickering, R. J., Gewurz, H., and Good, R. A. (1968). *J. Lab. Clin. Med.* **72,** 298.
Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C. (1954). *Science* **120,** 279.
Pillemer, L., Blum, L., Lepow, I. H., Wurz, L., and Todd, E. W. (1956). *J. Exp. Med.* **103,** 1.
Platts-Mills, T. A. E., and Ishizaka, K. (1974). *J. Immunol.* **113,** 348.
Podack, E. R., and Müller-Eberhard, H. J. (1980). *Adv. Immunol.* (in press).
Podack, E. R., Biesecker, G., Kolb, W. P., and Müller-Eberhard, H. J. (1978). *J. Immunol.* **121,** 484.
Podack, E. R., Biesecker, G., and Müller-Eberhard, H. J. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 897.
Polhill, R. B., Jr., Newman, S. L., Pruitt, K. M., and Johnston, R. B., Jr. (1978). *J. Immunol.* **121,** 371.
Polley, M. J., and Müller-Eberhard, H. J. (1967). *J. Exp. Med.* **126,** 1013.
Polley, M. J., and Müller-Eberhard, H. J. (1968). *J. Exp. Med.* **128,** 533.
Ramalho-Pinto, F. J., McLaren, D. J., and Smithers, S. R. (1978). *J. Exp. Med.* **147,** 147.
Raum, D., Glass, D., Carpenter, C., Alper, C. A., and Schur, P. H. (1976). *J. Clin. Invest.* **58,** 1240.
Reed, W. P. (1975). *Immunology* **28,** 1051.
Reed, W. P., and Albright, E. L. (1974). *Immunology* **26,** 205.
Root, R. K., Ellman, L., and Frank, M. M. (1972). *J. Immunol.* **109,** 477.
Ross, G. D., and Rabellino, E. M. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 1467.
Ross, G. D., Polley, M. J., Rabellino, E. M., and Grey, H. M. (1973). *J. Exp. Med.* **138,** 798.
Ruddy, S., and Austen, K. F. (1969). *J. Immunol.* **102,** 533.
Ruddy, S., and Austen, K. F. (1971). *J. Immunol.* **107,** 742.
Ruddy, S., Austen, K. F., and Goetzl, E. J. (1975). *J. Clin. Invest.* **55,** 587.
Ruley, E. J., Forristal, J., Davis, N. C., Andres, C., and West, C. D. (1973). *J. Clin. Invest.* **52,** 896.
Sandberg, A. L., and Osler, A. G. (1971). *J. Immunol.* **107,** 1268.
Santoro, F., Bernal, J., and Capron, A. (1979a). *Acta Trop.* **36,** 5.
Santoro, F., Lachmann, P. J., Capron, A., and Capron, M. (1979b). *J. Immunol.* **123,** 1551.
Schreiber, R. D., and Feldman, H. A. (1980). *J. Infect. Dis.* (in press).
Schreiber, R. D., and Müller-Eberhard, H. J. (1978a). *J. Exp. Med.* **148,** 1722.
Schreiber, R. D., and Müller-Eberhard, H. J. (1978b). *J. Immunol.* **120,** 1796.
Schreiber, R. D., and Müller-Eberhard, H. J. (1980). *Scand. J. Immunol.* (in press).
Schreiber, R. D., Medicus, R. G., Götze, O., and Müller-Eberhard, H. J. (1975). *J. Exp. Med.* **143,** 760.
Schreiber, R. D., Götze, O., and Müller-Eberhard, H. J. (1976a). *J. Exp. Med.* **144,** 1062.
Schreiber, R. D., Götze, O., and Müller-Eberhard, H. J. (1976b). *Scand. J. Immunol.* **5,** 705.
Schreiber, R. D., Pangburn, M. K., Lesavre, P., and Müller-Eberhard, H. J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3948.
Schreiber, R. D., Morrison, D. C., Podack, E. R., and Müller-Eberhard, H. J. (1979). *J. Exp. Med.* **149,** 870.
Schreiber, R. D., Pangburn, M. K., Medicus, R. G., and Müller-Eberhard, H. J. (1980). *Clin. Immunol. Immunopathol.* **15,** 384.
Scott, D. M., Amos, N., Sissons, J. G. P., Lachmann, P. J., and Peters, D. K. (1978). *Clin. Exp. Immunol.* **32,** 12.

Sher, A. (1976). *Nature (London)* **263,** 334.
Sher, A., and McIntyre, S. L. (1977). *J. Immunol.* **119,** 722.
Shiraishi, S., and Stroud, R. M. (1975). *Immunochemistry* **12,** 935.
Sissons, J. G. P., West, R. J., Fallows, J., Williams, D. G., Boucher, B. J., Amos, N., and Peters, D. K. (1976). *N. Engl. J. Med.* **294,** 461.
Sissons, J. G. P., Schreiber, R. D., Perrin, L. H., Cooper, N. R., Müller-Eberhard, H. J., and Oldstone, M. B. A. (1979). *J. Exp. Med.* **150,** 445.
Sissons, J. G. P., Oldstone, M. B. A., and Schreiber, R. D. (1980). *Proc. Natl. Acad. Sci. U. S. A.* **77,** 559.
Sobel, A. T., Cooper, N. R., and Schreiber, R. D. (1979). *J. Immunol.* **122,** 34.
Spitzer, R. E., Vallota, E. H., Forristal, J., Sudora, E., Stitzel, A., Davis, N. C., and West, C. D. (1969). *Science* **164,** 436.
Spitznagel, J. K. (1966). *J. Bacteriol.* **91,** 401.
Stephens, C. G., Williams, R. C., Jr., and Reed, W. P. (1977). *Infect. Immun.* **17,** 296.
Sundsmo, J. S., and Götze, O. (1979). *Cell. Immunol.* (in press).
Swenson, R. P., and Howard, J. B. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4313.
Szymanski, I. O., and Ogden, P. R. (1979). *Vox Sang.* **36,** 213.
Tack, B. F., and Prahl, J. W. (1976). *Biochemistry* **15,** 4513.
Tack, B. F., Morris, S. C., and Prahl, J. W. (1979). *Biochemistry* **18,** 1497.
Tack B. F., Janatova, J., Lorenz, P. E., Schechter, A. N., and Prahl, J. W. (1980). *J. Immunol.* **124.** In Press.
Tamura, N., and Nelson, R. A., Jr. (1967). *J. Immunol.* **99,** 582.
Theofilopoulos, A. N., and Perrin, L. H. (1976). *J. Exp. Med.* **143,** 271.
Theofilopoulos, A. N., and Perrin, L. H. (1977). *Science* **195,** 878.
Theofilipoulos, A. N., Bokisch, V. A., and Dixon, F. J. (1974). *J. Exp. Med.* **139,** 696.
Thompson, R. A. (1971). *Immunology* **22,** 147.
Tooze, J. (1973). "The Molecular Biology of Tumor Viruses," p. 173. Cold Spring Harbor Monograph Series, Cold Spring Harbor, New York.
Traub, W. H., and Kleber, I. (1976). *Infect. Immun.* **13,** 1343.
Vallota, E. H., and Müller-Eberhard, H. J. (1973). *J. Exp. Med.* **137,** 1109.
Vallota, E. H., Forristal, J., Spitzer, R. E., Davis, N. C., and West, C. D. (1970). *J. Exp. Med.* **131,** 1306.
Vallota, E. H., Götze, O., Spiegelberg, H. L., Forristal, J., West, C. D., and Müller-Eberhard, H. J. (1974). *J. Exp. Med.* **139,** 1249.
Vogt, W., Dieminger, L., Lynen, R., and Schmidt, G. (1974). *Hoppe-Seyler's Z. Physiol. Chem.* **355,** 171.
Vogt, W., Dames, W., Schmidt, G., and Dieminger, L. (1977). *Immunochemistry* **14,** 201.
Vogt, W., Schmidt, G., von Buttlar, B., and Dieminger, L. (1978). *Immunology* **34,** 29.
Volanakis, J. E., Schrohenloher, R. E., and Stroud, R. M. (1977). *J. Immunol.* **119,** 337.
Vranian, G., Conrad, D., and Ruddy, S. (1980). *J. Immunol.* **124,** 1544.
Ward, P. A., and Newman, L. J. (1969). *J. Immunol.* **102,** 93.
Webb, P. M., and Muschel, L. H. (1968). *Can. J. Microbiol.* **14,** 545.
Wedgwood, R. J., Ginsberg, H. S., and Pillemer, L. (1956). *J. Exp. Med.* **104,** 107.
Weiler, J. M., Daha, M. R., Austen, K. F., and Fearon, D. T. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 3268.
Welsh, R. M., Jr. (1977). *J. Immunol.* **118,** 348.
Welsh, R. M., Jr., Cooper, N. R., Jensen, F. C., and Oldstone, M. B. A. (1976). *Virology* **74,** 432.
West, C. D., McAdams, A. J., McConville, J. M., Davis, N. C., and Holland, N. H. (1965). *J. Pediat.* **67,** 1089.

Westberg, N. G., Naff, G. B., Boyer, J. T., and Michael, A. F. (1971). *J. Clin. Invest.* **50,** 642.
Whaley, K., and Ruddy, S. (1976a). *J. Exp. Med.* **144,** 1147.
Whaley, K., and Ruddy, S. (1976b). *Science* **193,** 1011.
Wieme, R. J., and Demeulenaere, L. (1967). *Nature (London)* **214,** 1042.
Williams, D. G., Scopes, J. W., and Peters, D. K. (1972). *Proc. R. Soc. Med.* **65,** 591.
Williams, D. G., Charlesworth, J. A., Lachmann, P. J., and Peters, D. K. (1973). *Lancet* **1,** 447.
Wilson, A. B., Lachmann, P. J., and Coombs, R. R. A. (1979). *Immunology* **37,** 25.
Winkelstein, J. A., and Tomsz, A. (1978). *J. Immunol.* **120,** 174.
Wiklenstein, J. A., Bocchini, J. A., Jr., and Schiffman, G. (1976). *J. Immunol.* **116,** 367.
Yefenof, E., Klein, G., and Kuarnung, K. (1977). *Cell. Immunol.* **31,** 225.
Ziegler, J. B., Alper, C. A., and Balner, H. (1975a). *Nature (London)* **254,** 609.
Ziegler, J. B., Watson, L., and Alper, C. A. (1975b). *J. Immunol.* **114,** 1649.

This Page Intentionally Left Blank

ADVANCES IN IMMUNOLOGY, VOL. 29

Mediators of Immunity: Lymphokines and Monokines

ROSS E. ROCKLIN, KLAUS BENDTZEN,[1] AND DIRK GREINEDER

Department of Medicine, Tufts University School of Medicine and New England Medical Center Hospital, Boston, Massachusetts; and Harvard Medical School and Robert B. Brigham Hospital, Boston, Massachusetts

I. Introduction 56
II. Lymphokines 58
A. Migration Inhibitory Factors 58
B. Macrophage Activating Factor 70
C. Chemotactic Factors 73
D. Cytotoxic and Cytostatic Factors 76
E. Lymphocyte Mitogenic Factor 82
F. Lymphocyte Factors Regulating Antibody Responses 85
G. Interferon 91
H. Immunoglobulin Binding Factor 95
I. Tissue Factor: Procoagulant Activity 96
J. Colony-Stimulating Activity 98
K. Osteoclast Activating Factor 99
III. Cell Types Involved in Lymphokine Synthesis 101
IV. Regulation and Pharmacologic Modulation of Lymphokine Production and Action 103
A. Modulation of Lymphokine Production 103
B. Modulation of Lymphokine Action 107
V. Antibodies to Lymphokines 109
A. Preparation of Antilymphokine Antibodies 110
B. Characterization of Antisera 110
VI. *In Vivo* Significance of Lymphokines 112
VII. Clinical Relevance of the Measurement of Lymphokines 114
VIII. Monokines 115
A. Lymphocyte Activating Factor 115
B. B Cell Differentiating Factor or PFC Helper Factor 118
C. B Cell Activating Factor 119
D. Monocyte Pyrogen 120
E. Thymic Differentiation Factor 121
F. Colony-Stimulating Activity 121
G. Mononuclear Cell Factor 121
H. Neutral Proteinases 122
I. Complement Components 123
IX. Biologic Significance of Monokines 124
X. Conclusion 125
References 127

[1] On leave from Laboratory of Clinical Immunology, Department of Medicine TA, Rigshospitalet University Hospital, Copenhagen, Denmark.

ISBN 0-12-022429-1

I. Introduction

Cellular immune reactions are mediated by T lymphocytes, and the expression of these phenomena include cutaneous delayed-type hypersensitivity, contact allergy, resistance to infection by facultative intracellular microorganisms, graft rejection, and tumor surveillance. These reactions result from complex interactions between T cells and B cells, T cells and other T cells, T cells and macrophages. The basis for these interactions on a molecular level is the subject of this review.

The large body of evidence at hand indicates that over 95% of the mononuclear cells comprising the infiltrate of delayed cutaneous hypersensitivity reactions represent cells that have been nonspecifically recruited to the site of reaction. That is, only a small number of infiltrating cells are specifically sensitized to the antigen that initiates the reaction. These findings have led to the supposition that the few sensitized cells initiating the reaction are responsible for producing substances that recruit the other cells and amplify the inflammatory response. The nonimmunoglobulin factors presumed to play a role in the expression of these reactions have been collectively termed "mediators of cellular immunity" and include substances produced by lymphocytes (lymphocyte mediators, products of activated lymphocytes, or lymphokines) and monocytes (monokines). These factors are produced in minute quantities but have marked biologic effects on a variety of cell types including T and B lymphocytes, macrophages, eosinophils, basophils, and neutrophils. Although they were originally described as *in vitro* phenomena, increasing evidence suggests that they play an important role in *in vivo* cellular immune reactions as well.

Because lymphokines and monokines are produced in minute quantities, and are therefore difficult to purify, there has been a tendency to ascribe each biologic activity to the presence of a distinct molecule. This has generated a profusion of lymphokine–monokine activities without regard to whether a biologic effect is actually due to a separate substance or to an unrelated biologic activity of the same molecule. With this in mind, we have so organized this review that the lymphokines are discussed in relation to their effects on certain target cells, as shown in Table I.

Lymphokines can also be classified functionally according to their effects: inhibitory, stimulatory, or inflammatory (Pick, 1977b). The inhibitory lymphokines include materials that lyse their target cells (lymphotoxin) or inhibit their proliferation (proliferation inhibitory factor, clonal inhibitory factor, inhibitor of DNA synthesis, or immune

TABLE I
LYMPHOCYTE MEDIATORS (LYMPHOKINES)

A. Mediators affecting macrophages
1. Migration inhibitory factor (MIF)
2. Macrophage activating factor (MAF) (indistinguishable from MIF)
3. Chemotactic factors for macrophages
4. Antigen-dependent MIF
B. Mediators affecting polymorphonuclear (PMN) leukocytes
1. Chemotactic factors
2. Leukocyte inhibitory factor (LIF)
3. Eosinophil stimulation promoter (ESP)
C. Mediators affecting lymphocytes
1. Mitogenic factors
2. Factors enhancing antibody formation (antigen-dependent and antigen-independent)
3. Factors suppressing antibody formation (antigen-dependent and antigen-independent)
D. Mediators affecting other cells
1. Cytotoxic factors, lymphotoxin (LT)
2. Growth inhibitory factors (? same as LT)
3. Osteoclastic factor (OAF)
4. Collagen-producing factor
5. Colony-stimulating factor
6. Interferon
E. Immunoglobulin-binding factor (IBF)
F. Procoagulant (tissue factor)

interferon). The stimulatory lymphokines include mitogenic factors that act upon lymphocytes or macrophages, hematopoietic colony-stimulating factors, and lymphokines that mediate T–B and T–T cell cooperation. The inflammatory lymphokines include those factors related to the expression of cellular immunity including MIF, MAF, chemotactic factors as well as factors that influence vascular permeability and the clotting system.

In this review, we attempt to describe the physicochemical characteristics and known mechanisms of action of some of the better studied lymphokines and monokines. The literature citations proceed from the last review in this series by Bloom (1971). This subject has also been reviewed previously by David and David (1972), Pick and Turk (1972), and David and Rocklin (1978). The methodology involved with each *in vitro* assay system is not described in this review. The interested reader is referred to Bloom and David (1976) and Rose and Friedman (1976).

II. Lymphokines

A. Migration Inhibitory Factors

The demonstration of cell-mediated immunity *in vivo* was originally based upon the appearance of a delayed-type skin reaction (24–48 hours) after the intracutaneous injection of antigen. The first step toward the development of assay systems that represented *in vitro* correlates of delayed hypersensitivity was taken by Holst in 1922. He observed that human blood leukocytes were sometimes inhibited in their migration in vertically placed capillary tubes when exposed to tuberculin. These experiments were later elaborated upon in a different system by Rich and Lewis (1932), who demonstrated tuberculin-induced migration inhibition of spleen cells or leukocytes from immunized animals. Since then, a variety of assay systems using the principle of macrophage and leukocyte migration inhibition have been developed (reviewed by Pekarek and Krejci, 1974).

Two of these migration techniques have gained widespread use—the capillary tube method and the agarose method. The capillary tube technique has been reviewed by Bloom (1971) and David and David (1972) and will not be detailed here. It is sufficient to mention that peritoneal exudate cells (consisting predominantly of macrophages and some lymphocytes) or blood leukocytes (consisting predominantly of granulocytes and lymphocytes as well as a small number of monocytes) are packed into small glass capillary tubes. The cell portions of the tubes are then placed horizontally in chambers containing tissue culture medium with or without antigen. During the incubation period of 20–24 hours, the cells migrate out of the tubes and spread out onto the glass surface at the bottom of the chambers. A modification of this technique has been described by Clausen (1971). Blood leukocyte suspensions are placed in wells cut in agarose and incubated at 37°C for 18–24 hours. During this period, the polymorphonuclear (PMN) cells migrate out of the wells between the agarose and the supporting dish. The presence of specific antigen to which the lymphoid cells are sensitive causes the release of certain lymphokines—macrophage-migration inhibitory factor (MIF) and leukocyte inhibitory factor (LIF)—that inhibit the migration of the indicator cells (macrophages or PMN leukocytes).

The one-step direct assays of migration inhibition have also been modified to two-step, indirect methods. First, sensitized lymphocytes are stimulated by antigen; than the supernatant is transferred to a target cell culture, usually buffy coat or PMN leukocytes or macrophages. Both the direct and indirect methods have shown excellent

correlation with skin test reactivity (Astor *et al.*, 1973; Clausen, 1974; Hoffman *et al.*, 1976; Bloom, 1971; David and David, 1972).

A recent modification of the agarose technique has been described by Harrington and Stastny (1973). They determined the migration of cells out of macrophage-containing agar droplets submerged in medium. This method requires fewer migrating cells than the previously described techniques. However, in this technique, the immunologically committed cells are embedded in agarose and not immediately accessible to antigen added to the medium; this method may, therefore, not be optimal for direct migration inhibition studies.

Two of the effector molecules released during the interaction between lymphocytes and antigen (termed MIF and LIF) are capable of reacting with the actively migrating cells of the leukocyte suspensions. Thus, MIF inhibits the migration of macrophages and monocytes, and LIF selectively inhibits the random movement of PMN leukocytes (Rocklin, 1974b). Both mediators appear to act without apparent genetic restriction, since MIF and LIF from one species will be active on allogeneic and even on xenogeneic target cells. However, some species specificity does exist, because MIF activity assayed on xenogeneic cells requires higher concentrations of mediator than that required for migration inhibition of homologous target cells (see Bloom, 1971).

1. Macrophage-Migration Inhibitory Factor (MIF)

In 1963, Svejcar and Johanovsky postulated that a soluble mediator was responsible for the inhibition of macrophage migration observed in double spleen explants cultured in the same chamber. One of the explants was taken from a specifically sensitized animal, the other from a nonsensitive one. Svejcar and Johanovsky added antigen to the chamber and observed inhibition of cell migration also from the spleen fragment of the nonsensitive animal. Later, release of the soluble mediator from sensitized lymphocytes reacting with the corresponding antigen was unequivocally demonstrated (see Bloom, 1971; David and David,1972).

In adition to the specific antigen-induced MIF production by lymphocytes from blood, lymph nodes, spleen, peritoneal exudate, and thymus, lymphocytes from several animal species, including man, can be triggered in a nonspecific manner to produce MIF. Thus, stimulation with plant lectins, such as phytohemagglutinin (PHA) and concanavalin A (Con A), and with antigen–antibody complexes result in production of MIFs with apparently similar physicochemical and immunochemical properties (Remold *et al.*, 1972; Kotkes and Pick,

1975b; Geczy *et al.*, 1975). The presence of MIF in supernatants of allogeneic cell cultures has been reported by some investigators (Bartfeld and Atoynatan, 1971a), but not by others (Geczy *et al.*, 1976b). Even particulate or insolubilized stimulants, such as Bacillus Calmette-Guérin (BCG) and agarose-immobilized Con A, are effective (Pick, 1972; Friedrich *et al.*, 1975). MIF-like activities have also been found in culture supernatants of established lymphoid cell lines, in nonlymphoid cell lines such as fibroblasts, and in a range of human tumor cell lines (Papageorgiou *et al.*, 1972; Tubergen *et al.*, 1972; Poste, 1975; Yoshida *et al.*, 1976). The biochemical relationship between these mediators and lymphocyte-derived MIF remains to be determined.

Migration inhibition factor is not found in appreciable amounts in extracts of nonstimulated lymphocytes, and MIF production by stimulated cells is blocked if the cells are treated with inhibitors of protein synthesis (see Bloom, 1971). Several investigators have shown that the production of MIF is independent of lymphocyte proliferation. Thus, inhibition of lymphocyte replication by X-irradiation and by treatment of the cells with vinblastine, cytosine arabinoside, or mitomycin C, or with 5-bromo-2-deoxyuridine and light, do not interfere with MIF production (Bloom *et al.*, 1972; Mizoguchi *et al.*, 1973; Rocklin, 1973; Visakorpi,1974). MIF activity can be detected as early as 2–6 hours after antigen stimulation, i.e., in the early G_1 phase of the cell cycle, and the production of the mediator continues until the S phase. MIF activity in the early G_1 phase has been detected in association with broken-cell microsomal preparations (Prystkowsky *et al.*, 1975).

A complex regulation of MIF action and MIF production has been suggested. Thus, Ward and Rocklin (1975) described a substance in normal human serum that blocked the effect of human MIF on guinea pig macrophages. Cohen and Yoshida (1977) obtained a soluble factor, termed MIF inhibitory factor (MIFIF), from nonsensitized T cells stimulated by Tuberculin purified protein derivative (PPD), which when added to B cells, interfered with their MIF production. Preliminary findings indicated that MIFIF did not act directly on MIF, nor did it compete with an MIF receptor on the indicator cell. The suppressor factor was found to be nondialyzable and heat labile.

The production of MIF by lymphocyte subpopulations is discussed in Section III as is the pharmacologic modulation of MIF production, (Section IV).

a. Biochemical Properties of MIF. The properties of MIF from guinea pig, mouse, and man are listed in Table II. Generally, MIFs from all species appear to be proteins or glycoproteins with molecular

TABLE II
PHYSICOCHEMICAL PROPERTIES OF MACROPHAGE-MIGRATION INHIBITORY FACTOR (MIF)[a]

Property	Guinea pig	Mouse	Human
Molecular weight (Sephadex chromatography)	25,000–43,000 (pH 5 MIF) 65,000 (pH 3 MIF)	48,000–67,000	23,000–55,000
Chymotrypsin treatment	Sensitive	Sensitive	Sensitive
Neuraminidase treatment	Sensitive	—	Resistant
Heat stability: 80°C	—	Sensitive	—
56°C	Stable	Stable	Stable
Isoelectric point	3–4.5 (pH 3 MIF)	—	4–6
	5–5.5 (pH 5 MIF)	—	—
Polyacrylamide gel electrophoresis	Prealbumin	β-Globulin	Albumin
Buoyant density (CsCl)	Denser than protein	Denser than protein	Protein
Salt precipitation, $(NH_4)_2SO_4$	50–80% saturation		

[a] Data from Dumonde *et al.*, 1972; Remold *et al.*, 1972; Rocklin *et al.*, 1972; Kuhner and David, 1976; Remold and Mednis, 1977; Weiser *et al.*, 1979.

weights less than those immunoglobulins. However, on the basis of known physicochemical properties significant interspecies differences in the molecular structure of MIF seem to exist. This is further evidenced by the fact that MIF from one animal may act as an immunogen when injected into animals of other species.

No complete purification of MIF has been achieved, and it may well be that migration inhibitory activity of whole supernatants of unseparated lymphoid cells may be attributed to more than one molecule. The heterogeneity of antigen-induced, particularly of mitogen-induced, guinea pig MIF has long been suspected, since MIF activity obtained after molecular sieve chromatography in some instances are associated with a relatively high molecular weight fraction (approximately 56,000–82,000) as well as with a low molecular weight fraction (approximately 12,000–56,000) (Yoshida and Reisfeld, 1970; Dumonde *et al.*, 1972; Remold *et al.*, 1972; Sorg and Bloom, 1973). Recently, Remold and Mednis (1977) provided further evidence that MIF activity obtained from guinea pig lymphocytes can be separated into at least two distinct molecular species. Using isoelectrofocusing

of partially purified MIF, they demonstrated the presence of a high moledular weight (55,000) MIF with an isoelectric point of 3–4.5, and a low molecular weight (25,000) MIF having an isoelectric point of 5–5.5. Preliminary experiments with polyacrylamide pore gradient gels supported the view that two sizes of MIF exist.

Very little is known about the molecular structure of MIF, except that human MIF may contain disulfide bonds, judged by the irreversible blocking of MIF activity by the reducing agent L-cysteine (Bartfeld and Atoynatan, 1971b). Guinea pig MIF, on the other hand, is dissociated into fragments of lower molecular weight by treatment with agents such as 1 *M* mercaptoethanol and 6 *M* guanidine HCl, but the fragments reassociate to biologically active MIF molecules upon removal of the agents (Kotkes and Pick, 1977).

Some controversy previously existed as to the possible protease nature of MIF. Thus, in a study by Havemann *et al.* (1972), the activity of human MIF was irreversibly blocked by $2.5 \times 10^{-4} M$ diisopropylfluorophosphate (DFP), and MIF activity was significantly reduced by treatment with antiproteases, such as pancreas-, soybean-, and lima bean trypsin inhibitors, and aprotinin. These findings could not be reproduced by David and Becker (1974) using guinea pig MIF or by Rocklin and Rosenthal (1977) using human MIF. On the other hand, Poste (1975) reported that MIF-like activity in supernatants of simian virus 40-transformed mouse 3T3 cells was significantly reduced by $5 \times 10^{-4} M$ DFP, by animal and plant trypsin inhibitors, and by high concentrations ($5 \times 10^{-2} M$) of ϵ-aminocaproic acid (EACA). That there might not be any correlation between protease inhibition and MIF inhibition by EACA was suggested by Houck and Chang (1975), studying the effects of EACA and its analogs on an MIF-like activity obtained from thymus extracts. EACA, at concentrations below $10^{-3} M$, was ineffective in blocking MIF activity, whereas analogs of EACA had strong inhibitory potency. However, the most active anti-MIF compounds possessed very little antitryptic activity, and the most active antitrypsin agent, EACA itself, had little or no anti-MIF activity. To solve the problem of whether or not MIF in one of its molecular forms has a proteolytic potency, highly purified and well defined MIF should be tested, and the ability of synthetic substrates to interfere with the blocking activity of an irreversible serine protease inhibitor, such as DFP, should be demonstrated.

b. Mode of Action of MIF. The MIF appears to interact with macrophages at the cell surface, and receptor models for MIF–macrophage interaction have been proposed (Leu *et al.*, 1972; Remold, 1973). Thus, MIF activity is removed from supernatants after incubation

with macrophages, whereas lymphocytes and PMN leukocytes have no absorbing capacities. Furthermore, trypsin-treated macrophages do not respond to MIF, but this effect is reversible after removal of the enzyme and further culture for 24 hours, presumably due to regeneration of an MIF receptor (Leu *et al*, 1972; Manheimer and Pick, 1973). It has also been shown that the monosaccharide α-L-fucose specifically blocks the biologic activity of MIF-rich supernatants obtained from guinea pig lymphocyte cultures. Furthermore, guinea pig macrophages treated with fucosidase, an enzyme that cleaves terminal fucose residues from oligosaccharides, no longer respond to MIF (Remold, 1973). These findings suggest that α-L-fucose constitutes part of an MIF receptor that may be susceptible to the action of trypsin.

Recent studies using the same enzyme suggest that α-L-fucose is also the terminal sugar on a receptor for human MIF on human blood monocytes (Rocklin, 1976). The evidence for an MIF receptor is further strengthened by the fact that inhibition of macrophage migration does not require contact between the migrating cells and MIF for the entire migration period. Exposure of macrophages to MIF for 2 hours at 37°C, followed by washing of the cells still results in marked migration inhibition (Manheimer and Pick, 1973). Recently Higgins *et al.* (1978) and Liu *et al.* (1978) have suggested that fucogangliosides serve as MIF recognition units on macrophages. They showed that aqueous glycolipids obtained from guinea pig macrophages enhanced the responsiveness of other macrophages to MIF. Furthermore, this effect was abolished after either fucosidase or neuraminidase treatment of the active glycolipids, indicating that fucose and sialic acid may be important parts of the putative MIF receptor.

The study of the interaction of MIF with macrophage receptors is complicated by the ability of one or several macrophage surface esterase(s) to inactivate MIF (Remold, 1974). Thus, guinea pig macrophages pretreated with DFP or with soybean trypsin inhibitor demonstrate enhanced inhibition of migration when exposed to MIF. Serum pretreated with DFP and then freed of the inhibitor does not enhance MIF activity. The most likely explanation for these findings is that there is an active esterase(s) on the macrophage surface that destroys MIF activity. Moreover, physiological concentrations of the plasma esterase and protease inhibitors α_2-macroglobulin, α_1-antitrypsin, C1-inhibitor and antithrombin–heparin cofactor also enhance the response *in vitro* or macrophages to MIF (Remold and Rosenberg, 1975). This suggests a mechanism by which esterase inhibitors of normal plasma may play a regulatory role in cell-mediated immune reactions *in vivo*.

The mechanism by which the inhibitory signal reaches the interior of the macrophage is largely unknown, although altered membrane calcium transport and changes in the cellular levels of cAMP and cGMP seem to be involved. Furthermore, MIF appears to increase microtubular density in macrophages, and this may in fact be the primary event, alterations in cyclic nucleotide levels being a consequence rather than the cause of the changes in cellular microtubular function (Pick and Grunspan-Swirsky, 1977). These effects will be further discussed in the section on pharmacologic modulation of the production and function of lymphokines.

c. Antigen-Dependent MIF. The question of whether or not the activity of some MIF preparations is dependent on or potentiated by the presence of the antigen used to elicit the mediator was first raised by Svejcar *et al.* (1967). They found that MIF obtained by treatment of sensitive lymphocytes with low concentrations of antigen was inactive unless additional specific antigen was added to the supernatants. Since then, the necessity for the presence of specific antigen for the activity of MIF has been stressed by several investigators (reviewed by David and David, 1972). In one instance, specific antigen was insolubilized by conjugation to polyaminostyrene (Amos and Lachmann, 1970). Supernatants from lymphocytes cultured with insoluble antigen and from which antigen had been removed by centrifugation were largely devoid of MIF activity. However, MIF activity could be restored by adding the specific antigen, indicating that a lymphokine (a receptor for antigen?) capable of recognizing the antigen was produced, and that the lymphokine (receptor?)–antigen complex possessed MIF-like properties. This MIF was not a cytophilic antibody, since it was smaller than immunoglobulins. Why the MIF was not absorbed from the supernatants by the insoluble antigen is not known.

In several studies, the continued presence of antigen has not been necessary to express MIF activity. In one such study by Yoshida *et al.* (1972), MIF was generated by guinea pig lymphocytes sensitized to various DNP–protein conjugates. MIF was still active after removal of the antigens by anti-DNP agarose bead columns, and addition of antigen to the antigen-depleted supernatants did not increase MIF activity. Moreover, MIF activity obtained from cultures of lymphocytes stimulated with DNP-protein coupled to agarose beads, an experimental system closely resembling that described by Amos and Lachmann above, did not depend on the presence of antigen.

Little is known about the molecular structure of antigen-dependent MIF. A factor obtained from mouse lymoh node lymphocytes (probably T cells) has a molecular weight between 50,000 and 100,000 and

an isoelectric point of approximately 6.5 (Krejci *et al.*, 1976). It is species nonspecific, and it appears to be a glycoprotein, since it binds to Con A–agarose bead adsorbents. A similar finding has been reported by Lowe and Lachmann (1974), who found an antigen-dependent MIF activity in sheep lymph draining a tuberculin reaction.

2. *Leukocyte-Migration Inhibitory Factor (LIF)*

The distinction between MIF and LIF was first clarified by Rocklin (1974b). He demonstrated, using the capillary tube technique, that human MIF-rich fractions obtained by molecular sieve chromatography (MW 23,000) selectively inhibited the migration of human monocytes and guinea pig macrophages with no detectable effect on the mobility of PMN leukocytes. The fractions responsible for migration inhibition of human buffy coat cells or purified PMN leukocytes were those that contained molecules eluting together with albumin (MW 68,000). The active principle was termed leukocyte (migration) inhibitory factor. By the use of the leukocyte migration agarose technique (LMAT), these results were later confirmed in the guinea pig system by Hoffman *et al.* (1977), who also demonstrated that LIF, like MIF and most other lymphokines, can cross species barriers, since guinea pig PMN leukocytes were susceptible to the action of human LIF (Hoffman *et al.*, 1975). In the LMAT, both mononuclear and PMN leukocytes are known to migrate. However, the migration of mononuclear cells is very poor compared to that of PMN cells (Hoffman *et al.*, 1975, 1976). Thus, it seems that LIF not only plays the dominant role in the inhibition of migration observed in the LMAT using purified neutrophils but also when a mixed population of buffy coat cells is used (Hoffman *et al.*, 1975; Bendtzen, 1975a). The LIF appears to be produced by steroid-sensitive lymphocytes. Production of LIF is dependent upon intact cellular protein synthesis, judged by the blocking effect of puromycin (Bendtzen, 1975b; Gorski *et al.*, 1976a). However, LIF synthesis is dissociable from cell proliferation (Gorski *et al.*, 1975).

The lymphocyte subpopulation(s) responsible for LIF production has not been clearly established. Antigen-stimulated, enriched T cell populations (less than 2% B cells) obtained by passing blood mononuclear cells through Sephadex antihuman Fab columns produce LIF equally as well as the enriched B cell population (98% B cells), which can be obtained by eluting the same columns with immunoglobulin (Chess *et al.*, 1975). Furthermore, in experiments using mitogen-activation of lymphocytes, neither purified T cells nor B cells produced LIF. Monocytes, or combinations of monocytes and B cells, were also

nonproductive, whereas combinations of T and B cells elaborated significant amounts of the lymphokine (Weisbart *et al.*, 1977). These findings are difficult to interpret, unless one assumes that LIF production is the result of a collaborative event between T and B cells, or that LIF production is a property of a less abundant lymphocyte subpopulation distinct from conventional T and B cells. The latter suggestion is supported by findings of Neville and Lischner (1978), who showed that a fraction of nonadherent human peripheral blood lymphocytes absent from B or T cell-enriched populations, but not separable from K cells, can be activated by IgG immune complexes to produce LIF. IgM immune complexes were not effective, and the Fc region of IgG was required for cell activation. Moreover, a protease-sensitive surface membrane receptor (the Fc receptor?) was shown to be involved in the immune complex-induced LIF production.

Specific lymphocyte stimulation by antigens as well as nonspecific stimulation by mitogens, such as Con A and PHA, result in production of LIFs with apparently similar physiocochemical and immunochemical properties (Rocklin, 1974b, 1975; Bendtzen, 1977a). Detectable amounts of LIF in these cultures appear within 3–9 hours, and maximum production is seen after 1–2 days (Bendtzen *et al.*, 1975). Whether the LIF produced in mixed lymphocyte cultures is identical with antigen- or mitogen-induced LIF is not known (Gorski *et al.*, 1976a).

a. Physicochemical and Biochemical Properties of LIF. Human LIF is a protein by virtue of its susceptibility to chymotrypsin and its buoyant density in isopycnic centrifugation studies (Rocklin, 1975). It is also resistant to treatment with neuraminidase. On disc gel electrophoresis at pH 9.1, the migration velocity of LIF is similar to that of albumin, and the molecular weight of the lymphokine as determined by Sephadex G-100 column chromatography is 68,000 (Rocklin, 1975) or slightly less (Bendtzen, 1975a). When exposed to 56°C for 1 hour, LIF is rather stable, but it is destroyed at 80'C for 30 minutes; it is stable when treated for 30 minutes at pH 4–10, but partially destroyed at pH 3 and 11 (Bendtzen, 1976). LIF appears to contain one or several disulfide linkage groups critical for its biologic activity. Very little is known about LIF obtained from animal lymphocytes. However, guinea pig LIF is a relatively heat-stable protein of MW 68,000–158,000. It is resistant to neuraminidase and sensitive to chymotrypsin treatment (Hoffman *et al.*, 1977). Human LIF appears to be a hydrolytic enzyme susceptible to the irreversible serine protease and esterase inhibitors, phenylmethylsulfonylfluoride (PMSF) and DFP (Bendtzen, 1976; Rocklin, 1975; Rocklin and Rosenthal, 1977). The LIF-neu-

tralizing effect of PMSF and DFP is irreversible and dose-, time-, pH-, and temperature-dependent (Bendtzen, 1977b; Rocklin and Urbano, 1978). Furthermore, LIF activity, blocked by PMSF, can be reestablished by the nucleophilic enzyme reactivator pralidoxime methanesulfonate (Bendtzen, 1977d). Substrate competition experiments show that arginine esters and amides selectively protect LIF against the blocking effect of PMSF, probably by preventing the inhibitor from gaining access to the active site of the lymphokine (Bendtzen, 1977a,d; Rocklin and Urbano, 1978).

By a similar experimental approach in which phosphate esters were coincubated with PMSF and LIF, a protective effect of cGMP on LIF has recently been demonstrated (Bendtzen, 1977e). The effect of cGMP was highly specific, since chemically related nucleotides, including 5′-GMP and cAMP, were ineffective; an exception was 2′,3′-cCMP, which was effective but only at high concentrations (Bendtzen, 1978). Inasmuch as the relative, rather than the absolue, concentrations of cGMP and PMSF determined the degree of protection afforded by cGMP, the nucleotide appears to compete with PMSF for the reactive site on the LIF molecule, thereby protcting the enzyme against the inhibitor. However, the complete lack of structural resemblance between PMSF and cGMP indicates that the mechanism of interaction between LIF and the two reagents might not be a simple competitive one. Therefore, although direct evidence is still lacking, there is reason to believe that cGMP acts as an allosteric effector capable of inducing conformational changes in the LIF molecule and, consequently, a decrease in the affinity for PMSF (Bendtzen, 1978).

Unfortunately, the proposed esterolytic or proteolytic activity of LIF is difficult to test directly because of the very small amounts of LIF produced by stimulated lymphocytes, and because of release of contaminating enzymes even by nonstimulated lymphocytes (Bendtzen, 1979a). However, the use of a highly sensitive radioenzymic assay has enabled the direct demonstration of arginine-specific esterase activities in rather crude (Rocklin and Rosenthal, 1977) and in highly purified LIF preparations (Bendtzen, 1979a,b). The resulting esterase concentration of the latter preparations was approximately 10^{-11} M, judged by affinity radiolabeling with tritiated DFP, and the average esterolytic activity of the purified preparations corresponded to that of 0.3 ng of human thrombin per milliliter, calculated on the basis of unconcentrated supernatants (Bendtzen, 1979a).

When radioenzymic assays were carried out on purified LIF preparations, and their control counterparts were subjected to the same purification procedure, the rate of esterolysis of purified LIF, but not

of "purified" control materials, was repeatedly reduced in the presence of cGMP at concentrations as low as 10^{-7} *M* (Bendtzen, 1979a,b). Again, the effect of cGMP was highly selective, since several structurally related cyclic and noncyclic nucleotides were ineffective; the only exception was 2′,3′-cCMP, which was effective at concentrations of 10^{-3} *M* or above. Interestingly, the assumed competitive nature of the cGMP-induced reduction in the affinity of LIF for PMSF and, possibly, for the LIF substrate could be verified in the direct esterolytic assay by kinetic experiments (Bendtzen, 1979b).

b. Mode of Action of LIF. The interaction between LIF and PMN leukocytes seems very similar to that described in the MIF-macrophage system. Exposure of neutrophils to LIF for 1 hour at 37°C is sufficient to inhibit the mobility of the cells for the next 24 hours, whereas exposure to the lymphokine for shorter periods is less effective (Lomnitzer *et al.*, 1977; K. Bendtzen, unpublished findings). The acquired inhibition of migration of neutrophils pulse-treated with LIF also depends on the temperature at which the pulse-treatment is carried out, and selective, temperature-dependent loss of LIF activity follows absorption with PMN leukocytes but not with mononuclear cells or erythrocytes (K. Bendtzen, unpublished findings). Such removal of LIF might be due either to a specific interaction between the lymphokine and the neutrophils or to the inactivation of LIF by these cells. A receptor for LIF on the neutrophil surface has been suggested, and, like that described for MIF on macrophages, it also appears to contain sugar. However, the nature of the carbohydrate is a matter of controversy, since α-L-fucose and *N*-acetyl-D-glucosamine have both been postulated to be important parts of the LIF receptor (Bendtzen, 1975a; Rocklin, 1976).

It should be pointed out that cytophilic antibodies and antigen–antibody complexes, which may influence monocyte and macrophage mobility, play little or no role as a modifier of PMN leukocyte migration, at least in the LMAT (Hoffman *et al.*, 1975).

The mode of action, in molecular terms, of LIF on the target cell is larely unknown. However, circumstantial evidence indicated that LIF, at least in the capillary tube tecnique, affects microfilament function of PMN leukocytes, possibly through changes in the cellular levels of cyclic nucleotides. Thus, cytochalasin B, which interferes with the organization of contractle microfilaments, causes marked inhibition of PMN leukocyte locomotion along with an increased adhesiveness of these cells to nylon wool; cAMP and agents known to increase intracellular concentrations of cAMP reverse both these effects (Lomnitzer *et al.*, 1976b). Similarly, LIF-treated neutrophils escape

migration inhibition if treated with cAMP-generating agents, and cell migration per se is enhanced by some of these agents, particularly the potent phosphodiesterase inhibitors papaverine and dipyridamole (Bendtzen and Palit, 1977). Direct addition of cAMP or its lipid soluble derivative, dibutyryl cAMP, has been found by Lomnitzer *et al.* (1976a) to enhance the migration of neutrophils out of capillary tubes. Others, however, have been able to confirm these findings neither in the capillary tube technique (Hill *et al.*, 1975) nor in the LMAT (Bendtzen and Palit, 1977).

The natural substrate(s) for LIF and its relationship to the putative LIF receptor on neutrophils is unknown, as is the biologic significance of the cGMP-mediated modulation of LIF activity. Indeed, the exact biologic role of LIF is uncertain, since the lymphokine may have effects on the leukocytes other than those involved strictly in cell migration. Thus, nonpurified supernatants of PHA-activated lymphocytes increase the electrophoretic mobility of PMN cells. However, in a recent study involving a laser Doppler technique of electrophoretic light scattering, results differing with the above study were obtained (Petty *et al.*, 1980). Polymorphonuclear leukocytes incubated with unfractionated supernatants obtained from antigen-stimulated lymphocytes significantly decreased the mode electrophoretic mobility of these cells. Two activities (MW 30,000–60,000 and 10,000–20,000) recovered after molecular sieve chromatography were responsible for these effects. Fractions containing LIF activity had no effect on the electrophoretic mobility, suggesting that the inhibition of PMN leukocyte migration by LIF did not involve an alteration in the leukocyte surface charge density. Neutrophils treated with unfractionated supernatants show marked stimulation of phagocytic activity, glucose oxidation through the hexose monophosphate shunt, and increased nitroblue tetrazolium reductive capacity (Lomnitzer *et al.*, 1977). Whether these effects are due to LIF needs to be clarified.

A pathway of amplification of LIF activity on PMN leukocytes has recently been described (Goetzl and Rocklin, 1978). It was found that Sephadex G-100 fractions containing human LIF inhibited the random migration and chemotaxis of human PMN leukocytes as assessed in a Boyden chamber micropore filter assay. Incubation of PMN leukocytes with LIF released additional inhibitory activity, distinct from LIF, which resembled the neutrophil-immobilizing factor (NIF) (Goetzl and Austen, 1972) by virtue of its size (MW 4000), inactivation by trypsin and preferential noncytotoxic inhibition of spontaneous migration, and chemotaxis of PMN leukocytes as compared to mononuclear leukocytes. Thus, LIF inhibits PMN leukocyte migration by a

direct action on the cells and by an amplification pathway that is mediated by low molecular weight chemotactic inhibitors similar to NIF.

Although it remains to be determined whether some or all of these effects are caused by LIF, a likely intracellular mediator of such a variety of effects could be cGMP. Thus, recent experiments indicate that a PMSF-sensitive factor in supernatants of Con A-stimulated human lymphocytes causes a transient threefold increase in PMN leukocyte cGMP levels (Bendtzen and Klysner, 1980). This substance, possibly LIF, might initiate a series of consecutive biosynthetic reactions leading to an increase in cellular levels of cGMP. This would provide an attractive mechanism by which LIF activity could be regulated. Many similar examples of end-product regulatory systems are known in biology (Stadtman, 1970). Also, since cGMP is thought to play a role in lymphocyte activation and proliferation (Hadden *et al.*, 1975a), the possibility should be considered that LIF, a product of activated lymphocytes whose biologic activity appears to be regulated by cGMP, might exercise important biologic functions in processes governing lymphocyte activation and gene expression.

c. Antigen-Dependent LIF. The expression of LIF activity in highly diluted supernatants of antigen-stimulated lymphocytes may, at least under certain experimental conditions, depend upon the presence of specific antigen, as reported by Weisbart *et al.* (1975). They showed that LIF activity uniformly disappeared when supernatants were diluted 1:50, but the inhibitory activity could be restored by adding specific antigen. Since the addition of non-cross-reacting antigen was ineffective, this LIF activity was not only antigen dependent but apparently also antigen specific. The factor was nondialyzable and stable at 37°C for 30 minutes. It migrated in the albumin region in polyacrylamide gel electrophoresis, a property that seems to exclude cytophilic antibody as responsible for this effect.

B. Macrophage Activating Factor

It has become apparent that enhancement of macrophage function during infection has an immunologic basis and that *in vivo* activation of macrophages requires interaction between antigen and sensitized lymphocytes (Mackaness, 1969).

Although direct evidence is still lacking, *in vitro* studies indicate that the specific lymphocyte–antigen interaction may be translated into macrophage activation by means of lymphokines produced by antigen-activated lymphocytes. Thus, macrophages incubated in the presence of supernatants obtained from lymphocytes stimulated with antigens or mitogens exhibit a number of morphologic and functional

changes typical of cellular activation. The mediator responsible for these effects is referred to as macrophage activating factor (MAF).

Some of the cellular changes observed after treatment for 1–3 days with MAF are shown in Table III. Macrophage plasma membrane alterations appear to be fundamental to most of these effects, but the exact mechanism of action of MAF is not understood. Surface changes may also account for the ability of macrophages activated *in vivo* (Hibbs, 1972; Meltzer *et al.*, 1975) and *in vitro* (Piessens *et al.*, 1975) to kill tumor cells preferentially to nonneoplastic cells.

1. Biochemical Properties of MAF

The physicochemical properties of MAF obtained from guinea pigs and man are indistinguishable from those of MIF. Guinea pig MAF, like MIF, elutes from Sephadex G-100 chromatographic columns with molecules having molecular weights between 35,000 and 68,000. Both MAF and MIF activities are destroyed by neuraminidase and have a buoyant density slightly greater than that of albumin as determined by isopycnic centrifugation in CsCl (Nathan *et al.*, 1973). Whether MAF, like guinea pig MIF, is heterogeneous, or whether MAF will be distinguished from one or both MIF species (previously discussed) by isoelectrofocusing experiments remains to be determined. In man, MAF and MIF cochromatograph on Sephadex G-100 gel columns (Rocklin *et al.*, 1974b).

TABLE III

SOME BIOLOGIC EFFECTS ATTRIBUTED TO MACROPHAGE ACTIVATING FACTOR

1. Increased surface adherence (Nathan *et al.*, 1971; Rocklin *et al.*, 1974b)
2. Increased ruffled membrane movement (Nathan *et al.*, 1971)
3. Increased membrane adenylate cyclase activity (Remold-O'Donnell and Remold, 1974)
4. Increased phagocytic activity (Nathan *et al.*, 1971; Schmidt *et al.*, 1973)
5. Increased pinocytic activity (Meade *et al.*, 1974)
6. Increased hexose monophosphate oxidation (Nathan *et al.*, 1971; Rocklin *et al.*, 1974b)
7. Increased protein synthesis (Pantalone and Page, 1977)
8. Collagenase secretion (Wahl *et al.*, 1975; Pantalone and Page, 1977)
9. Increased complement C2 production (Littman and Ruddy, 1977)
10. Increased activity and release of lysosomal enzymes and of plasminogen activator (Pantalone and Page, 1977; Klimetzek and Sorg, 1977; Vassalli and Reich, 1977)
11. Increased tumoricidal capacity (Piessens *et al.*, 1975)
12. Increased bacteriostatic (-cidal) ability (Godal *et al.*, 1971; Fowles *et al.*, 1973; Patterson and Youmans, 1970)

2. *Mode of Action of MAF*

Typically, the MAF-induced activation of macrophages occurs after 2–3 days, and, on the basis of kinetic experiments, it appears that this 3-day period consists of two stages. In the first, requiring 1–2 days, the macrophages are refractory to the influence of the activating factor but undergo changes that render them receptive. In the second, they respond to MAF with detectable morphologic and biochemical changes (Nathan *et al.*, 1973).

Although MIF activity is detectable within 24 hours, this does not rule out the possibility that migration inhibition and activation of macrophages are different manifestations of the same lymphokine.Thus, it seems that macrophages cultured *in vitro* respond to the lymphokine with migration inhibition before they become sensitive to its activating influence. Moreover, if MIF-rich material is allowed to remain in contact with macrophages or monocytes, the cells escape inhibition and ultimately begin to migrate. Indeed, at this later stage, they often show enhanced mobility in addition to the other signs of activation. Activation by MAF is enhanced if the macrophages are pretreated with agents that also enhance MIF activity, such as esterase inhibitors or a surface active agent, diazotized sulfanilic acid (Remold, 1977).

It is interesting that the initial latent period of MAF-induced macrophage activation may be considerably shortened if macrophages are cultured in the presence of antigen and specifically sensitized T lymphocytes (Simon and Sheagren, 1971; Krahenbuhl *et al.*, 1973). Under these conditions, macrophages show enhanced bactericidal activity as early as 24 hours after initiation of the cultures. Why lymphocytes themselves have a greater influence on macrophage activation than their mediators is not clear. Perhaps direct contact between the two cell types, which is known to occur during macrophage "processing" of antigens, or the involvement of other essential factors derived from stimulated lymphocytes may facilitate the induction of bactericidal activity. For instance, a specific macrophage arming factor (SMAF), similar to the one described by Evans *et al.* (1972) in mice immunized with tumor cells, might cooperate with nonspecific MAF in these types of experiments. SMAF was produced by educated T lymphocytes stimulated with the specific tumor cells. The mediator had a specific recognition site for the tumor cells as well as a cytophilic moiety for macrophages that rendered these cytotoxic to the target cells. Very little is known about the structure of this lymphokine except that it appears to be smaller than immunoglobulin, with a molecular weight of approximately 50,000–60,000 (Evans *et al.*, 1972). The relationship of this mediator to antigen-specific MIF (discussed

above) is completely unknown, as are the biochemical events that trigger the intracellular changes in macrophages treated with MAF or SMAF.

C. Chemotactic Factors

Adaptive transfer experiments years ago showed that the majority of cells infiltrating the site of a delayed hypersensitivity reaction are nonsensitive macrophages derived from a rapidly dividing monocyte precursor. The additional finding that only a small percentage of the lymphocytes present in the lesion are specifically sensitive to the antigen that induced the reaction, suggested the possibility that a few sensitized cells produce soluble chemotactic factors that recruit the inflammatory cells. Indeed, *in vitro* studies have demonstrated that activated lymphocytes elaborate soluble factors that are chemotactic for a variety of cell types including macrophages, neutrophils, eosinophils, basophils, and lymphocytes. The stimulants for their production include soluble antigens, mitogens, graft-versus-host (GVH) reactions, and agents that activate Fc, immunoglobulin, and C3 receptors.

1. Monocyte–Macrophage Chemotactic Factor (MCF)

Monocyte–macrophage chemotactic factor (MCF) has been described in a number of species including guninea pigs, rats, and man (Table IV). The MCF has been generated after stimulation of lymphocytes by specific antigens, such as orthochlorobenzoyl bovine globulin and tuberculin PPD, mitogens such as PHA, GVH reaction, and stimulation of the C3 recepor (Altman *et al.*, 1973; Wahl *et al.*, 1974;

TABLE IV
Physicochemical Properties of Lymphokine Chemotatic Factor[a]

Properties	Monocyte–macrophage	PMN leukocyte
Temperature (56°C, 30 min)	Stable[b]	Stable[c]
Sieve chromatography	24,000–55,000[c]; 12,000[b]	24–55,000[c]
Polyacrylamide gel electrophoresis	Albumin[c]	Prealbumin[c]
Isoelectric point	10.1 and 5.6[b]	—
Chymotrypsin	Sensitive[b]	Sensitive[c]
RNase	Resistant[b]	Resistant[c]
Neuraminidase	Resistant[b]	Resistant[c]

[a] Data from Altman *et al.*, 1973, 1975; Ward *et al.*, 1969; Snyderman *et al.*, 1977; Wahl *et al.*, 1974a; Ward and Rocklin, 1975.

[b] Human.

[c] Guinea pig.

Ward *et al.*, 1975; Ward and Volkman, 1975; Sandberg *et al.*, 1975; Koopman *et al.*, 1976; Snyderman *et al.*, 1977). The MCF, as well as other chemotactic factors, are synthesized within the first 24 hours after activation of lymphocytes *in vitro*. Guinea pig MCF has an estimated molecular weight on gel filtration of 24,000–55,000 (Ward *et al.*, 1969), and in man of 12,500 (Altman *et al.*, 1973, 1975; Snyderman *et al.*, 1977). On polyacrylamide gel electrophoresis, guinea pig MCF migrates with albumin and thus can be distinguished from MIF and the neutrophil chemotactic factor (Ward *et al.*, 1969). Both human and guinea pig MCFs are heat stable and antigenically distinct from C3 and C5. Human MCF has been found to be heterogeneous with respect to its isoelectric point, a major peak being found at pH 10.0 and a moderate peak of activity at 5.6. It should be noted that C5a, which is chemotactic for macrophages, has an isoelectric point of 8.7, thus distinguishing it from the lymphocyte-derived chemotactic factor.

That stimulation of the C3 receptor on B cells can generate MCF activity has shed some light on the requirements of cell activation (Sandberg *et al.*, 1975; Koopman *et al.*, 1976). The incubation of guinea pig B cells with a fragment of C3 (C3b) results in the liberation of MCF. T cells do not respond under these conditions. Furthermore, no lymphocyte proliferation occurs under these conditions, in spite of the fact that MCF activity can be detected. Of interest is the finding that MCF production can be blocked by the addition of anti-C3 to B cells *in vitro*, although anti-C3 will not block the activity of this factor once it is produced. When C3b is cleaved to form C3c and C3d, it is no longer able to trigger B cells to generate MCF. The fact that C3b can activate spleen cells to make MCF indicates that it does so by cross-linking of the C3b and C3d receptors. The MCF activity produced by stimulation of B cells with C3b has the same physicochemical characteristics as that derived from lipopolysaccharide (LPS), antigen, or PHA-stimulated lymphocytes.

2. *Chemotactic Factors for PMN Leukocytes*

The chemotactic factor(s) for neutrophils, basophils, and eosinophils in guinea pig and mouse has a molecular weight range of 24,000–55,000. The first chemotactic activity described for eosinophils was that of Cohen and Ward (1971). They showed that antigen-stimulated guinea pig lymphocytes liberated a factor that combined with antigen and antibody in complex to generate an eosinophil chemotactic factor. Once the factor was generated, the continued presence of the complexes was not necessary. This eosinophil chemotactic factor was anti-

gen-specific in that the antigen or antibody complex must be the same as the antigen that stimulated production of the factor. The chemotactic activity was also species specific as it did not attract rabbit eosinophils. This factor, when injected *in vivo* into skin of guinea pigs, caused an accumulation of eosinophils. Some neutrophil chemotactic activity was also generated by this process but was 4- to 6-fold less than the corresponding eosinophil chemotactic activity. It was later shown by these workers that the guinea pig eosinophil chemotactic factor eluted from Sephadex G-100 gels with molecules having molecular weights of 12,000 and 70,000 (Torisu *etal.*, 1973). Furthermore, antibody-coated Sepharose columns absorbed the precursor or inactive form of the chemotactic factor. The other eosinophil migration activity was reported in mice (Greene and Colley, 1974; Colley *et al.*, 1977). This factor has been termed eosinophil stimulation promoter (ESP). Its activity is measured by a stimulation in migration of eosinophils in agar. The promoter could be generated in mouse lymphocytes by exposing them to specific antigens. Its production was inhibited by puromycin. The factor was heat stable and was inactivated by treatment with chymotrypsin, but not by RNase or neuraminidase. The apparent molecular weight of ESP by gel filtration on Sephadex G-75 was 24,000–56,000. Preparations of ESP were subsequently shown to have chemotactic activity for eosinophils and macrophages (Colley *et al.*, 1977). A basophil chemotactic factor is elaborated by guinea pig lymphocytes exposed to DNP-BSA *in vitro* (Ward *et al.*, 1975). The basophil chemotactic factor is partly heat labile and has the same apparent molecular weight by sucrose density gradient analysis as the monocyte chemotactic factor. Of interest is the finding that the basophil chemotactic activity can be absorbed out by preincubation of this material with monocytes, whereas the converse is not true.

3. *Chemotactic Factor for Lymphocytes*

Concanavalin A, MLC, or stimulation of the IgG receptor on B cells liberates a chemotactic factor for lymphocytes (Ward *et al.*,1977). Rat lymphocytes from spleen, lymph node, and thymus respond to this chemotactic factor. It appears to be selective in its activity in that only T cells respond to the chemotactic factor derived in MLC, whereas only B cells respond to the anti-immunoglobulin generated material.

4. *Chemotactic Factor for Fibroblasts*

Human lymphocytes stimulated with tuberculin PPD or PHA produce a chemotactic factor for human dental fibroblasts (Postlethwaite *et al.*, 1976a). This factor has been shown to be different from the ma-

crophage chemotactic factor in that it is heat stable, trypsin sensitive, and neuraminidase resistant, as well as having a molecular weight of 22,000.

5. *Identity of Chemotactic Factors*

None of the laboratories investigating these factors at present have been able to test their factors in the same species on homologous target cells. Therefore, it is impossible to determine whether one factor is responsible for all the chemotactic activities described above or whether there are distinct chemotactic factors for each inflammatory cell. From some of the evidence we presented here, it would appear that the macrophage chemotactic factor can be differentiated on the basis of physicochemical characteristics from the granulocyte chemotactic factors. Whether the granulocyte chemotactic factors for eosinophils, neutrophils, or basophils are distinct from one another is not clear at present. However, it is readily apparent that all these lymphocyte derived chemotactic factors may play an important part in mobilizing different effector cells in various immune reactions leading to inflammation.

D. Cytotoxic and Cytostatic Factors

Stimulated lymphocytes produce a number of soluble substances that are cytostatic or cytotoxic to susceptible target cells *in vitro*. These include colony inhibition factor (CIF), proliferation inhibitory factor (PIF), lymphotoxins (LT), and inhibitors of DNA synthesis (IDS). Lymphocytes from a variety of tissues (blood, lymph nodes, spleen) from several animal species, including man, produce these materials when stimulated by specific antigen, mitogens, or allogeneic cells. Cytotoxic activities have also been detected in supernatants from lymphoid cell lines.

Compared with most other lymphokines, these factors occur late after stimulation of lymphoid cells, i.e. in the late G_1 and S phase of the lymphocyte activation cycle, 2–5 days after activation (Daynes and Granger, 1974). In spite of this, and with the possible exception of IDS, their secretion does not require DNA synthesis. However, blocking of cellular protein synthesis by puromycin inhibits LT as well as IDS production (see page 80).

1. *Lymphotoxins (LT)*

a. LT Production. Generally, the greatest yield of LT is obtained from PHA-activated lymphocytes, and these cells continue to produce LT even after the mitogen has been removed (Daynes and Granger, 1974). In contrast, Con A-stimulated human lymphocytes require the

continuous presence of this mitogen for LT production. Thus, LT release is rapidly reduced after removal of Con A, but the lymphokine synthesis can be reinitiated by addition of fresh Con A. Since blast transformation and lymphocyte DNA synthesis are not significantly affected by removal of the mitogen, once the process is triggered, these findings indicate that the mechanisms controlling LT release may be turned on and off irrespective of the stage of lymphocyte differentiation.

b. Physicochemical Properties of LT. The chemical properties of LTs differ between animal species. However, human, mouse, and guinea pig LT appear to be proteins having molecular weights between 35,000 and 150,000: guinea pig LT: 35,000–55,000 (Coyne *et al.*, 1973); human LT: 45,000–100,000 (Walker *et al*, 1976); mouse LT: 90,000–150,000 (Kolb and Granger, 1970); and rat LT: 90,000 (Namba and Waksman, 1976). The major component of human LT is a material of MW 75,000–100,000. This component is referred to as α-LT (Walker *et al.*, 1976; Hiserodt *et al.*, 1976a). A smaller component, termed β-LT has a MW of approximately 45,000, and this group of LTs again appears to be heterogeneous (Hiserodt *et al.*, 1976b). An even smaller and very unstable compound, termed γ-LT, has been recently described. For a comparison of human LTs, see Table V).

TABLE V
SOME PHYSICOCHEMICAL PROPERTIES OF HUMAN LYMPHOTOXINS (LTs)[a]

Property	α-LT	β-LT	γ-LT
Molecular weight (molecular sieve chromatography)	75,000–100,000	45,000–50,000	10,000–15,000
Trypsin treatment	Stable?	—	—
Pronase treatment	Sensitive	—	—
DNase, RNase, and neuraminidase treatment	Stable	—	—
Heat stability: 80°C	Sensitive	Sensitive	Sensitive
56°C	Stable	—	—
Treatment at pH 5–11	Stable	—	—
Storage at 4°C	Stable	Unstable	Unstable
Isoelectric point	6.8–8.0	—	—
Polyacrylamide gel electrophoresis	α_2,β-Globulin	Heterogeneous β-LT_1, β-LT_2	—
Neutralized by anti-α-LT antibodies	Yes	No	No
Peak production after mitogen stimulation	2–3(5) Days	8–24 Hours	<24 Hours

[a] Data from Walker *et al.*, 1976; Hiserodt *et al.*, 1976a,b.

It is not completely clear whether the effects of lysis and growth inhibition are the results of separate molecules or one mediator having apparently different activities (LT, PIF, and CIF). Thus, studies by Jeffes and Granger (1976) show that α-LT at high concentrations causes cell destruction; at intermediate concentrations, permanent inhibition of growth; and at low levels, a transitory inhibition of cell division.

c. Mechanism of Action of LT. Although lymphokines such as the LTs have been proposed to be the cytodestructive mediators in T lymphocyte-mediated cell damage, the precise mechanism of action of lymphocyte killer cells is still unknown and a subject of controversy. At least two pathways for nonantibody-dependent, cell-mediated cytotoxicity may be operative—one involving LT, and the other requiring intimate contact between the plasma membranes of killer and target cells. In fact, several mechanisms may be operative, since various subpopulations of effector lymphocytes may be involved, and the target cell destruction may be antigen specific or nonspecific. However, in most systems used to study T cell killing, only target cells in direct contact with sensitized effector lymphocytes are lysed, whereas bystander cells not carrying the sensitizing antigen are left unharmed (Cerottini and Brunner, 1974). Since LTs have not been detected in the supernatants of these cultures, it has been claimed that cytotoxic lymphokines are not involved in direct, lymphocyte-mediated cytodestructive reactions. This view is supported by the findings by M. K. Gately *et al.* (1976), who showed that neutralizing antibodies against LT failed to affect the lytic activity of alloimmunized effector cells. Furthermore, the exquisite specificity observed in these immune reactions contrasts to the total lack of specificity of LT-induced cell damage. However, the specificity of direct T cell-mediated lysis may reside in the interaction between lymphocytes and target cell antigens, not in the stage of actual cell injury. Thus, nonspecific cytotoxic lymphokines might accumulate in the contact area between the activated lymphocyte and the target cell, resulting in destruction of the latter.

The fact that cytolysis by immune lymphocytes involves a secretory process (Plaut *et al.*, 1973) and an effector-cell-independent step (Hiserodt and Granger, 1977) supports the involvement of soluble lymphocyte mediators in these reactions. Furthermore, in contrast to the findings of M. K. Gately *et al.* (1976), Hiserodt and Granger (1977) recently found that antisera against human LTs are capable of blocking lymphocyte-mediated cytolysis. This indicates that lymphocytes, at least in man, deposit LT-like molecules on the target cell surface

during the period of physical contact between the cells. These molecules, then, are responsible for the cell lysis that occurs at a later stage, irrespective of the presence of lymphocytes. In light of these findings, it appears possible that the absence of LT molecules from supernatants of cytotoxic lymphocyte cultures is due to a rapid binding of the molecules to the target cell membrane. The membrane damage induced by LT might promote the separation of the lymphocytes from the target cell and a subsequent early cessation of lymphokine secretion. Such a mechanism would inhibit indiscriminate destruction of potential target cells as well as nonspecific cell damage in the host.

A limited capacity, high affinity component on susceptible target cells capable of binding LT has been demonstrated in absorption studies and in binding studies using radiolabeled LT (Hessinger *et al.*, 1973; Tsoukas *et al.*, 1976). Furthermore, an elutable surface component obtained by trypsinization or scraping of target cells has been described, which when added to other target cells greatly enhance their susceptibility to LT-mediated cytolysis (Lies, 1975). This component, probably representing an LT receptor, may help to localize LT deposits on the surface of the target cells. Support for this hypothesis was recently presented by Friend and Rosenau (1977). Using immunoelectron microscopy with ferritin- or peroxidase-labeled antibody, they were able to demonstrate a patchy localization of LT on target cell membranes, often in areas overlying a microfilament web.

The cell lysis induced by LT has been observed *in vitro* by use of cinematography (Russell *et al.*, 1972). Two types of lysis were shown to occur. One is a slow process that is completed in 1–2 hours, during which time the target cells are observed to swell and eventually disrupt. The other occurs suddenly within 2–5 minutes with shrinkage and marked agitation of the cell prior to lysis.

Very little is known about the metabolic changes that lead to cell death. No alterations in aerobic or anaerobic glycolysis of LT-treated cells have been found, and the K^+ pump abnormalities as determined by the flux of radiolabeled rubidium have not been detected either (Rosenau *et al.*, 1973). Initial measurement of total protein synthesis of LT-treated cells also appears to be unaltered, but changes in the production of selective proteins have not been unequivocally excluded in these types of experiments (Kunitomi *et al.*, 1975). Target cells exposed to LT have been shown to increase their RNA polymerase II activity (Kunitomi *et al.*, 1975). This may be a secondary result, however, since combined treatment with LT and actinomycin D results in a marked synergistic effect on cell lysis, indicating that the increase in RNA synthesis is a compensatory reaction for the LT-in-

duced injury (Rosenau *et al.*, 1973). Finally, it should be mentioned that intracellular changes in cyclic nucleotide levels may be of importance in LT-induced cytodestructive reactions (see page 107).

2. *Inhibitor of DNA Synthesis (IDS)*

A factor capable of reducing lymphocyte DNA replication was first described by Smith *et al.* (1970) in supernatants of human lymphoblastoid cell lines. Pulse treatment of lymphocytes with these supernatants, which contained a mitogenic factor, resulted in increased thymidine incorporation into DNA, whereas suppression of DNA synthesis was observed if the supernatants were allowed to remain in contact with the cells during the final 24 hours of culture. This could be explained by the simultaneous presence of an irreversible mitogenic activity and a reversible activity capable of inhibiting DNA synthesis.

Further experiments have now clearly demonstrated the presence of a nonspecific suppressor factor in supernatants of lymphocytes stimulated with specific antigen, mitogens, or allogeneic cells. The factor, called inhibitor of DNA synthesis (IDS), is usually detected by its ability to suppress [^{3}H]thymidine incorporation into mitogen-stimulated lymphocytes.

The mediator is physicochemically distinct from α-LT, PIF, and MIF (Namba and Waksman, 1976), and IDS has no direct cytotoxic effect on lymphocytes, even at high concentrations. It has been produced by lymphoid cells from rat, mouse, pig, and man. Animal studies indicate that it is a product of a steroid-sensitive T lymphocyte subpopulation demonstrating nonspecific suppressor cell activity, and it is produced in large quantities by spleen and thymus cells from rats injected with large tolerogenic doses of antigen. This indicates that IDS may play a role in the nonspecific suppression of immune responses associated with prolonged high levels of antigenic stimulation (Namba *et al.*, 1977). The production of human IDS, as in the case of α-LT, usually occurs after a longer time interval than that of most other known lymphokines, with maximum activity detectable between 2 and 5 days after initiation of the cultures (Namba and Waksman, 1975b; Lee and Lucas, 1977). The kinetics of production, however, may depend upon the anatomic region from which the lymphocytes are obtained. Thus, Con A-stimulated mouse spleen cells produce detectable amounts of IDS within 24 hours, thymus cells within 2–3 days, and lymph node cells after 3–4 days (Namba *et al.*, 1977). IDS synthesis by Con A-stimulated lymph node cells is abrogated when the cells are treated with mitomycin C, suggesting

that DNA synthesis is necessary for IDS production (Namba *et al.*, 1977).

a. Physicochemical Properties of IDS. Rat IDS appears to be a relatively heat-stable glycoprotein, judged by its resistance to heating to 56°C and its sensitivity to trypsin and periodate treatment. On Sephadex gel filtration two peaks of activity may be recovered with estimated molecular weights of 80,000 and 160,000–200,000. Isoelectrofocusing analysis demonstrates that rat IDS is highly acidic and has an estimated p*I* of 2.7–3.0 (Namba and Waksman, 1975b). Human IDS appears to be a heat-sensitive protein of MW 40,000–80,000. It inhibits DNA synthesis in stimulated lymphocytes as well as in growing HeLa cells (Lee and Lucas, 1977).

b. Mechanism of Action of IDS. IDS is not species-specific but rather cell-specific, acting primarily but not exclusively on lymphocytes (Namba and Waksman, 1975b; Wagshal *et al.*, 1978). Kinetic experiments using rat IDS indicate that the suppressor effect follows multihit kinetics; i.e., attachment of several molecules of IDS is required in order to inhibit lymphocyte DNA synthesis (Namba and Waksman, 1975b). This suggests that, in situations in which IDS is produced in small amount, its action would be restricted to the immediate vicinity of the producing cell.

The mechanism of action of IDS in molecular terms is not clear at present. Its effect on PHA-stimulated mouse lymphocytes is restricted to the late G_1 phase of the cell cycle (16–29 hours after mitogen activation), and the suppressive effect of the factor is reversible when it is removed before this phase is reached (Namba and Waksman, 1975b). The effect becomes partially reversible by 30 hours. When lymphocytes stimulated with mitogen for 3 hours or 20 hours are tested for their ability to absorb IDS activity, only the latter cells are effective (Wagshal and Waksman, 1978). This suggests that the cell cycle specificity of IDS action may be due to appearance of cell receptors for IDS only during a limited phase of the mature lymphocyte cell cycle. Jegasothy *et al.* (1976) demonstrated that IDS obtained from Con A-stimulated rat lymph node cells induced a late two- to fourfold increase in the intracellular levels of cAMP. The IDS used in these experiments was partially purified by molecular sieve chromatography and ion exchange chromatography. Control supernatants devoid of IDS activity were subjected to the same purification procedures and had no effect on PHA-stimulated lymphocytes. The addition to lymphocytes of cAMP or dibutyryl cAMP at concentrations that doubled the intracellular levels of the nucleotide also inhibited the PHA-induced DNA

synthesis. These results suggest that increased intracellular cAMP levels may mediate (directly or indirectly) the inhibitory effect of IDS. In fact, several lines of evidence suggest that cAMP may regulate cell proliferation, high concentrations halting DNA replication and lower ones promoting it (Strom *et al.*, 1977). The possibility, however, that the IDS-induced increase in cellular cAMP levels is a secondary effect, and not by itself responsible for the suppression of DNA synthesis, should be borne in mind. Thus, recent findings by Lee and Lucas (1977) indicate that IDS may act as an inhibitor of DNA polymerase. This effect was not mediated by cAMP, since the highly purified lymphokine, obtained by PHA-stimulated human tonsil cells, inhibited the activity of calf thymus DNA polymerase in a cell-free assay. However, as in the case of other lymphokines, definitive proof of the mechanism of action of IDS must await either its isolation in a chemically pure state or at least a more precise biochemical or immunochemical characterization of the mediator.

The IDS may be closely related to or identical with a recently described factor capable of suppressing the proliferative responses in MLR (MLR-suppressor factor) (Rich and Rich, 1975). This mediator is produced by *in vivo* sensitized mouse spleen cells after challenge *in vitro* by mitomycin C-treated allogeneic spleen cells of the strain used for sensitization. Although antigen specificity of the MLR suppressor factor for stimulator cell alloantigens was absent, genetic restriction of the interaction with MLR responder cells was demonstrated. It appears that a receptor, specific for the factor and required for a suppressive interaction, is expressed only by genetically homologous cells. This receptor seems to be coded for by *I* region genes. Experiments in which MLR responder cells were tested for the ability to absorb the MLR suppressor factor further indicate that the receptor is dynamically expressed by T lymphocytes only after an antigenic or mitogenic signal has been provided. Thus, normal spleen or thymus cells did not absorb MLR suppressor factor activity, whereas Con A-activated and alloantigen-stimulated cells effectively removed the suppressor activity (Rich and Rich, 1976).

E. Lymphocyte Mitogenic Factor

Operationally, lymphocyte mitogenic factor (LMF) activity is said to be present in supernatants of lymphocytes stimulated with antigens, mitogens, or allogeneic cells when they stimulate the cellular incorporation of radiolabeled thymidine in cultures of autologous or nonrelated lymphocytes under conditions where no other stimulant has been provided. This activity will be greater than the stimulatory ef-

fects seen in control supernatants to which the same amount of antigen or mitogen has been added. Since the first reports on LMF activity in supernatants of human mixed leukocyte cultures (Bain and Lowenstein, 1964; Kasakura and Lowenstein, 1965; Gordon and MacLean, 1965), several activities have been described that nonspecifically stimulate other lymphocytes to undergo blast transformation and increased DNA synthesis. These have been called lymphocyte stimulating factor, potentiating factor, lymphocyte recruiting factor or transforming factor, and blastogenic factor.

Under conditions where unseparated lymphocytes are stimulated with specific antigen or allogeneic cells, LMF appears to be elaborated predominantly by T cells (Geha and Merler, 1974; Kasakura, 1977; Littman and David, 1978). However, in the presence of T cells, B lymphocytes may also make LMF or signal T lymphocytes to increase LMF production (Oates *et al.*, 1972; Blomgren, 1976a; Kasakura, 1977). The production of a B cell LMF functionally distinct from T cell LMF has been suggested by Kasakura (1977).

Most LMF activities described are usually produced within the first 6 hours of culture, maximum activity being detectable 1–3 days after lymphocyte activation (Mills, 1975; Littman and David, 1978; Geha and Merler, 1974). The release of LMF is inhibited by puromycin, X-irradiation, and actinomycin D (Geha and Merler, 1974).

a. Physicochemical Properties. The LMF made by guinea pig lymphocytes is a macromolecule of MW 15,000–30,000 (Mills, 1975; C. L. Gately *et al.*, 1975). It is heat stable at 56°C for 30 minutes. It is resistant to treatment with RNase and DNase, but is destroyed by treatment with proteolytic enzymes, such as trypsin. Human LMF is relatively stable at 56°C, has a molecular weight between 20,000 and 50,000, is destroyed by proteolytic enzymes but not affected by RNase, neuraminidase, or α-amylase (Rocklin *et al.*, 1974a; Geha and Merler, 1974; Littman and David, 1976) (see Table VI).

The molecular properties of guinea pig LMF released by lymphocytes stimulated with soluble antigen or mixed lymphocyte cultures (MLC) have been separated using immunochemical and physicochemical techniques (Geczy, 1977). Neutralizing antibodies raised against MLC-induced LMF did not interfere with the biologic activity of antigen-induced LMF. The two factors differed slightly with regard to molecular weight and isoelectric point (antigen-induced LMF: MW 20,000–25,000 and p*I* 7.5; MLC LMF: MW 15,000–18,000 and p*I* 6.5). The MLC-induced LMF activity was inhibited by α-L-fucose, whereas antigen-induced LMF was not. It is of considerable interest that the antibody raised against MLC-induced LMF also inhibited the

TABLE VI
PHYSICOCHEMICAL PROPERTIES OF LYMPHOCYTE MITOGENIC FACTOR[a]

Property	Guinea pig	Human
Temperature (56°C, 30 min)	Stable	Stable
Sieve chromatography	15,000–30,000	20,000–50,000
Isoelectric point	6.5–7.5	—
RNase	Resistant	Resistant
DNase	Resistant	Resistant
Trypsin	Sensitive	Sensitive
Neuraminidase	—	Resistant
α-Amylase	—	Resistant

[a] Data from Mills, 1975; C. L. Gately *et al.*, 1975; Rocklin *et al.*, 1974b; Geha and Merler, 1974; Littman and David, 1976; Geczy, 1977.

MLC reaction itself. Since immunoglobulins to lymphocyte surface antigens had been previously removed, this effect was attributed to the ability of the antibody to inhibit a soluble lymphocyte product, possibly LMF, not to a direct cellular action of the antibody.

b. Mode of Action. Both B and T lymphocytes can respond to LMF, but the B cell response is often (Littman and David, 1978), but not always (Blomgren, 1976b) quantitatively greater than the response of T cells. In experimental situations where specific stimulation of lymphocytes with antigen has been used to elicit LMF production, the expression of LMF activity is usually independent of and unaffected by the presence of the antigen (Jones *et al.*, 1973; Littman and David, 1978). An exception to this has been described by Geha and Merler (1974). Their LMF, which was produced by antigen-stimulated T lymphocytes, induced proliferation of lymphocytes from tonsils, lymph nodes, spleen, and blood. Maximal stimulation, however, was observed in lymphocytes that normally do not proliferate in response to antigen, such as thymocytes, newborn lymphocytes, and circulating B cells. Interestingly, this latter function was expressed only in the presence of antigen, but the antigen need not be the same as the one used to elaborate LMF. This antigen-dependent LMF may be identical or closely related with the "potentiating factor" described by Janis and Bach (1970).

The existence of two functionally distinct LMF species has recently been described by Kasakura (1977). In his experiments, human LMF produced in B cell-enriched cultures stimulated both B cells and T cells to proliferate, whereas LMF produced in T cell cultures stimulated only B cells.

Lymphocyte mitogenic factor appears to exert its effect through contact with the responder cell for at least 48 hours. Furthermore, LMF activity can be absorbed out by an excess of target cells, suggesting that LMF mediates its effect via a receptor or that LMF is rendered inactive as a result of LMF–cell interaction (Geha and Merler, 1974). The molecular mechanisms by which LMF accelerates DNA synthesis in unstimulated lymphocytes is completely unknown, as is the possible role of LMF *in vivo*. It seems likely, however, that mitogenic factors released from comparatively few sensitive lymphocytes may amplify the immune response by activating or recruiting nonsensitive lymphocytes to produce greater amounts of lymphokine. This has recently been demonstrated *in vitro* by C. L. Gately *et al.*, (1976). The authors showed that highly purified LMF preparations induced the production of lymphotoxin (LT), and more LT was elaborated by LMF-stimulated lymphocytes obtained from recently immunized animals.

It should be borne in mind that the fact that B lymphocytes appear to be very sensitive to the effects of LMF suggests that this group of lymphokines may play a significant role in humoral immune reactions as well.

F. Lymphocyte Factors Regulating Antibody Responses

The complex cooperative interactions between T and B lymphocytes in antibody reponses have been analyzed by many investigators, and much effort has been mounted to identify biologically active substances capable of augmenting or suppressing the antibody responses. Since the immunologic and physicochemical properties of these factors have been the subject of many recent review articles, they will only be briefly summarized in this chapter. For a more complete discussion of the molecular biology of some of these mediators, see Pierce and Kapp (1976), Tada *et al.*, (1977) and Möller (1975).

Two distinct classes of T cell helper and suppressor factors may be distinguished: antigen-specific and antigen-nonspecific (see Table VII).

The antigen-specific helper factors, released by primed T cells upon challenge with specific antigen, induce B cells and plasma cells to produce immunoglobulin that is directed exclusively against the antigen used to elicit the helper factor. In experimental systems where hapten–carrier conjugates have been used to induce a secondary antihapten antibody response, these factors have been shown to possess specificity for the carrier component. Similar, but not identical, antigen-specific factors are involved in T–T cell interaction in the generation of suppressor T cells involved in humoral (Tada *et al.*,

TABLE VII
SOLUBLE ANTIGEN-SPECIFIC AND NONSPECIFIC LMYPHOCYTE FACTORS INVOLVED IN HUMORAL IMMUNE RESPONSES

A. Antigen-specific factors
1. Helper activity
"IgT"[a]
Specific T cell helper factor[b]
Specific T cell enhancing factor[c]
2. Suppressor activity
"IgT"?[d]
Specific suppressive T cell factor[e]
Allotype suppression factor[f]
B. Antigen-nonspecific factors
1. Helper activity
T cell replacing factor[g,h] Soluble enhancing factor[i] Nonspecific mediator[j]
Allogeneic effect factor[j,k]
2. Suppressor activity
Antibody inhibitory material[l]
Soluble immune response suppressor[m,n]
Antibody initiation suppressor factor[o]
Type 1 and type 2 interferon (see page 91)

[a] Feldmann and Basten (1972).
[b] Taussig (1974).
[c] Tada *et al.* (1977).
[d] Feldmann *et al.* (1974).
[e] Tada *et al.* (1973).
[f] Jacobson (1973).
[g] Gorczynski *et al.* (1972).
[h] Schimpl and Wecker (1972).
[i] Rubin and Coons (1972).
[j] Dutton *et al.* (1971).
[k] Armerding and Katz (1974).
[l] Ambrose (1969).
[m] Rubin and Coons (1972).
[n] Rich and Pierce (1974).
[o] Douglas and Rubin (1977).

1973; Jacobson, 1973) as well as in cell-mediated (Zembala *et al.*, 1975) immune responses.

The nonspecific helper and suppressor factors, which enhance or suppress the response of B cells to a variety of antigens, are produced by T cells challenged either with alloantigens, mitogens, or, if primed T cells are used, with the relevant antigen. The T lymphocytes used to generate helper and suppressor factors differ in membrane phenotype as exemplified in the mouse, using the Ly antigens as membrane markers. Thus, whereas short-lived, Ly1,2,3$^+$ cells in many systems

appear to act as amplifiers of both types of responses, the long-lived Ly 1^+ lymphocytes function as helper cells, and the Ly2,3$^+$ cells mediate suppression (Jandinski *et al.*, 1976; Cantor *et al.*, 1976; Feldmann *et al.*, 1977). Some helper and suppressor factors have been shown to act directly on B cells, whereas others act on T cells or on macrophages (see later).

The immunobiology and biochemistry of helper and suppressor factors is described in Sections 1–4 that follow.

1. Antigen-Specific Helper Factors

The antigen-specific helper factor that is capable of replacing T cells in a cooperation-dependent antibody response is a glycoprotein of MW 35,000–60,000 (Taussig, 1974). The factor cannot be removed by an anti-mouse immunoglobulin adsorbent, but it is removed by anti-Ia adsorbents, indicating that at least part of the molecule must be coded for by genes in the *I* region (Taussig and Munro, 1974). Moreover, the putative B cell receptor for the helper factor also appears to be coded for by genes in the *I* region, since treatment of B cells with anti-Ia antiserum prevents the cells from absorbing the mediator (Munro and Taussig, 1975). Similar findings have been reported by Tada *et al.* (1977), who investigated a carrier-specific T cell factor capable of enhancing a T cell-dependent antihapten antibody response. Their factor was also found to have *I* region gene expression, and the target cell acceptor site appeared to carry *I* region determinants as well. For a number of reasons, this factor is clearly different from the one described by Tausig. First, the factor was found in T cell extracts. Second, the target for the factor appears to be the T cell rather than the B cell. And, third, the enhancing molecule is not capable of replacing T cells, since addition of the factor to T cell-depleted spleen cells does not induce an antihapten antibody response.

Another type of antigen-specific helper factor has been described by Feldmann and Basten (1972). These authors showed that a mediator released from carrier-specific T cells stimulated by hapten–carrier conjugate triggered an antihapten antibody response in hapten-primed B cells. The specific cooperative activity could be removed by anti-μ-chain and anti-κ-chain antibody absorbents. Since the mediator also contained antigen, it was concluded that the factor was a complex containing T cell-released Ig ("IgT") and antigen, and that the "IgT" had similarities to IgM. The factor was also shown to be cytophilic for macrophages. It has been hypothesized that this mediator may have different functions in cell cooperation events: (*a*) "IgT"-antigen complexes bound to macrophages may promote the processing of antigen

important in B cell triggering; (*b*) by occupying the receptors for "IgT" on macrophages these complexes may induce the phenomenon of antigenic competition; and (*c*) "IgT"–antigen complexes may interact dirctly with lymphocytes, resulting in specific T cell suppression (Feldmann *et al.*, 1974).

2. *Antigen-Nonspecific Helper Factors*

The nonspecific helper factors, T cell replacing factor (TRF), nonspecific mediator (NSM), and soluble enhancing factor (SEF), are produced by T cells stimulated by alloantigens or by Con A. They are also generated by sensitized T cells challenged with the specific antigen, but once produced these factors act nonspecifically in initiating or facilitating the response of B cells to non-cross-reacting antigens. Although not unequivocally demonstrated, all three mediators seem to act directly on B cells. It is conceivable that these factors may be structurally identical or closely related (Dutton *et al.*, 1971; Schimpl and Wecker, 1972; Harwell *et al.*, 1976; Gorczynski *et al.*, 1972; Rubin and Coons, 1972).

T cell replacing factor is produced by Ly 1^+ cells (Pickel *et al.*, 1976). The mediator can substitute for T cells in the primary IgM response against heterologous red blood cells. The secondary, predominantly IgG, response to SRBC has also been reconstituted with TRF (Schimpl and Wecker, 1975). The TRF is not involved in the initial triggering of B cells but rather acts on B cells that have already proliferated upon contact with antigen. The mediator appears to be a glycoprotein, and H-2-associated gene products are not part of the molecule (Hubner *et al.*, 1978). Soluble enhancing factor (SEF) nonspecifically enhances anti-SRBC IgG responses as well as IgG and IgE antihapten antibody responses (Rubin and Coons, 1972; Kishimoto and Ishizaka, 1975). Mouse SEF has a molecular weight of 75,000; it is heat resistant and protease sensitive (Rubin and Coons, 1972). Present evidence indicates that distinct SEFs are involved in the IgG and IgE antibody responses (Kishimoto and Ishizaka, 1975).

That nonspecific helper factors may play a role not only in B cell-mediated events has been shown recently by Plate (1976). She obtained a TRF-like activity from antigen-stimulated T cells that was capable of substituting for T helper cells during the differentiation of precursor T cells into killer cells. The relationship of this factor to the TRF involved in humoral immune immune responses or to other lymphokines, such as LMF, is unknown.

The TRF, NSM, and SEF differ significantly from another nonspecific helper factor secreted by *in vivo* alloantigen-activated mouse

T lymphocytes during short-term *in vitro* reactions with alloantigens. This mediator, allogeneic effect factor (AEF), acts directly on B cells and substitutes for T cells in primary IgM anti-SRBC antibody responses as well as in secondary IgG antihapten responses (Armerding and Katz, 1974). The T cell subpopulation responsible for AEF production in this secondary MLC system appears to be that of the Ly 1^+ phenotype, and macrophages are not required for optimal production (Eshhar *et al.*, 1977). The AEF is a glycoprotein with a subunit structure consisting of two, noncovalently associated components of MW 10,000–12,000 and 40,000, respectively. It is devoid of immunoglobulin determinants, but it comprises both Ia antigenic determinants and β_2-microglobulin (Armerding *et al.*, 1977).

3. *Antigen-Specific Suppressor Factors*

The antigen-specific factor(s) involved in mouse T–T cell interactions in the generation of humoral immune response suppression has many features in common with the antigen-specific T cell helper factor described by Taussig. The factor is a protein of MW 35,000–55,000; it has specificity and affinity for carrier and *I* region, but no immunoglobulin, determinants, and the acceptor sites on the target cells are coded for by *I* region genes (Tada *et al.*, 1977). In contrast to the helper factor, however, this factor acts on T cells, and only on T cells from syngeneic animals. The mediators also differ with respect to the ease with which they are released from activated lymphocytes.

The helper factor is easily detectable in supernatants from stimulated lymphocytes, whereas the suppressor factor usually has to be extracted from stimulated thymocytes or spleen cells. An exception to this has been reported by Jacobson (1973), who used the phenomenon of chronic allotype suppression as a model for the regulation of antibody synthesis by a soluble suppressor T cell factor. It should be noted that cellular cooperation mediated by specific suppressor factors is not limited to antibody responses. Thus, an antigen-specific, soluble T cell factor capable of suppressing passive transfer of contact hypersensitivity in mice has been described (Zembala *et al.*, 1975). This factor has a molecular weight of approximately 50,000 and appears to exert its effect through the macrophage. It is reported to be resistant to trypsin digestion.

4. *Antigen-Nonspecific Suppressor Factors*

Several nonspecific suppressor factors have been reported by Ambrose (1969), Rubin and Coons (1972), Rich and Pierce (1974), and Douglas and Rubin (1977) (Table VII) that are released by sensitized

lymph node or spleen cells after challenge with the specific antigen or by spleen cells incubated with Con A.

The factor described by Ambrose was produced by primed rabbit lymph node cells stimulated *in vitro* with the specific antigen. Cells undergoing a secondary immune response elaborated the factors only in the immunoglobulin producing phase, i.e., from 1 week up to 4 weeks after addition of antigen. The factor, termed antibody inhibitory material (AIM), inhibited antibody synthesis in an immunologically nonspecific manner, and the inhibition also occurred during the immunoglobulin producing phase (second week) of the antibody response (Ambrose, 1973). This lymphokine may participate in a normal control mechanism for the production of antibodies. Little is known about the molecular biology of AIM, except that it appears to be Pronase-resistant but susceptible to RNase treatment; the molecular weight range of AIM, estimated by ultrafiltration studies, is 10,000 –50,000 (Ambrose, 1973).

Another nonspecific suppressor factor inhibits the inductive rather than the immunoglobulin productive phase of antibody synthesis to a newly introduced antigen. It appears to be distinct from AIM. This factor, termed soluble immune response suppressor factor (SIRS) is an early product of mouse spleen cells stimulated with antigen or Con A. In appropriate dilutions, it nonspecifically suppresses 5-day plaque-forming cell (PFC) responses to SRBC as well as antihapten antibody responses. SIRS is a product of $Ly2,3^+$ T cells. Sensitivity of cells producing SIRS to X-irradiation has been reported by some investigators (Pierce and Kapp, 1976), but not by others (Thomas *et al.*, 1975). The molecular properties of mouse SIRS have been described by Thomas *et al.* (1975) and Tadakuma *et al.* (1976). The factor is a glycoprotein. It is stable at 56°C for 30 minutes, but not at 80°C or at pH 2. It does not possess an antigen binding site, nor does it contain immunoglobulin determinants. The activity of SIRS is not affected by treatment with DNase or RNase, but its activity is lost after treatment with proteolytic enzymes. The molecular weight, estimated by molecular sieve chromatography, is between 48,000 and 67,000. These characteristics distinguish SIRS from type 1 interferon, which has also been reported to suppress PFC responses to SRBC (see page 94), but not from MIF. Indeed, SIRS and MIF copurify in experiments using gel filtration, polyacrylamide gel electrophoresis, and isopycnic centrifugation (Tadakuma *et al.*, 1976). The similarities between SIRS and MIF have been further substantiated by the recent observation that the target cell of SIRS activity is also the macrophage (Tadakuma and Pierce, 1976). The SIRS-treated macrophage appears to exert its suppressive effects

via a second factor(s) (Tadakuma and Pierce, 1978). The kinetics of PFC suppression in cultures containing SIRS-treated macrophages indicate that the response develops normally for 3 days. On day 4, approximately 24 hours after a significant decrease in DNA synthesis is seen, the suppression becomes apparent. This suggests that the SIRS-induced suppression is ultimately due to limitation of proliferation of responding B cells (Tadakuma and Pierce, 1978). Whether or not this event is mediated through an elevation of the intracellular levels of B cell cAMP is not known. The nonspecific suppressor factor described by Douglas and Rubin (1977) has many biologic properties in common with SIRS. This antibody initiation suppressor factor (AISF), generated by stimulation of primed mouse T lymphocytes with specific antigen, suppresses the initiation of the *in vitro* primary and secondary PFC responses to SRBC. The kinetics of its production and suppressor function are very similar to those of SIRS, but AISF appears to be smaller (approximate MW 34,000) and more heat labile (inactivated at 56°C for 30 minutes) than SIRS (Douglas and Rubin, 1977).

G. Interferon

Operationally, interferon (IF) is defined as a cellular mediator produced in response to, and acting to prevent replication of, an infecting virus (in the infected cell). However, since the discovery of interferon by Isaacs and Lindemann in 1957 as a normal defensive response of an animal to viral infection, a number of other biologic activities, such as inhibition of tumor growth and interference with the immune response, have been ascribed to this mediator.

Although interferon has not been completely purified, evidence has accumulated to suggest that multiple substances having antiviral activity in a given animal species bear the name of interferon but differ in other ways.

Interferons are glycoproteins synthesized *in vivo* and *in vitro* by a wide variety of cells in response to a wide variety of stimuli, including viruses, double-stranded RNA, and synthetic polymers. Synthesis of interferon by lymphocytes can be induced by the same viral stimuli. However, lymphocytes also produce interferon when stimulated with such agents as bacteria, plant mitogens, and antilymphocyte serum. Interferons are also found in the supernatants from mixed lymphocyte cultures and lymphocytes stimulated with specific antigens. This latter group of interferons, referred to as "immune" or type 2 interferon, should be distinguished from the "classical" or type 1 interferon produced by a variety of cell types as a result of viral infection. These two types of interferon are antigenically distinct, since antibod-

ies raised against type 1 interferon do not inhibit the activity of type 2 interferon. Furthermore, type 1 interferon is stable to pH 2.0, whereas type 2 interferon is acid labile (Youngner and Salvin, 1973). Only type 2 interferon may be regarded as a lymphokine and a mediator of cellular immunity. The properties of type 1 interferon, reviewed by Friedman (1977), will not be discussed in detail here.

1. Production of Type 2 Interferon

Induction of type 2 interferon by mitogen-stimulated lymphocytes usually takes several hours to occur, reaching a peak at 1–3 days, whereas interferon production by antigen-stimulated lymphocytes is characterized by an unusual duration (7 days) of culture (Green *et al.*, 1969). The cell type(s) producing type 2 interferon has not been unequivocally established. In fact, it is not clear whether this mediator is elaborated by lymphocytes or macrophages, or both. Epstein *et al.* (1971b) reported that a threefold increase in production of type 2 interferon was observed when macrophages were added to lymphocyte cultures. However, macrophages by themselves did not produce the mediator. The possibility that T lymphocytes and macrophages collaborate in the production of type 2 interferon was further investigated by Neumann and Sorg (1977). They found that macrophages were responsible for the antigen- and mitogen-induced type 2 interferon production by unfractionated murine spleen cells. When the cells were depleted of macrophages by passage through glass bead columns and, subsequently, nylon wool columns, type 2 interferon was no longer produced. On the other hand, purified macrophages from spleens of immunized animals elaborated type 2 interferon without further stimulation, and macrophages from unimmunized animals could be induced to produce the mediator by supernatants of activated T cells.

2. Biochemical Properties of Type 2 Interferon

Very little is known about the physicochemical properties of type 2 interferon. The molecular weight of mouse type 2 interferon is 45,000–80,000; it is rather stable at 56°C but unstable at pH 2.0 (Youngner and Salvin, 1973). Interferon is sensitive to treatment with trypsin but insensitive to DNase and RNase. Of considerable interest is the observation that the physicochemical properties of mouse MIF (isolated from serum) are very similar to those of type 2 interferon (Youngner and Salvin, 1973), and it possible that these mediators are identical or closely related molecules being defined by different functional assays.

3. *Mode of Action of Type 2 Interferon*

The present knowledge regarding the molecular mechanisms of interferon action has been obtained almost entirely by examining the interaction between type 1 interferon and virus-infected susceptible cells. Since the "immune interferon" belongs to a different interferon species, the molecular mechanisms by which this group of mediators exerts its effect may not necessarily be the same as those for type 1 interferon. The antiviral action of type 1 interferon has been the subject of extensive review (see Friedman, 1977) and will be discussed only briefly here (Table VIII).

The antiviral activity of type 1 interferon appears to be relatively species specific because, with few exceptions, it confers the best protection to cells of the homologous species. Interferon does not inactivate viruses directly, nor does it interfere with viral absorption to cells. There is no apparent need for interferon to enter cells in order to exert its effect. Moreover, the antiviral state does not develop in enucleated cells or in cells treated with actinomycin D or cycloheximide, indicating that transcription and translation of the cellular genome are required for antiviral activity to occur. The mechanism by which interferon transmits its signal to the interior of the target cell is not known. However, it is believed that interferon interacts with a glycoli-

TABLE VIII
BIOLOGICAL EFFECTS ASCRIBED TO INTERFERON[a,b]

Effects on cell surfaces
Increased expression of surface histocompatibility antigens
Increased net negative charge
Altered binding of Con A, TSH, and cholera toxin to cell surfaces
Effect on cell division
Inhibits multiplication of tumor cells and normal cells *in vitro* and *in vivo*
Effects on specialized cell functions
Increases phagocytosis by macrophages
Enhances synthesis of prostaglandins
Effects on the immune system
Enhances cytotoxicity of sensitized lymphocytes against tumor cell
Increases IgE-mediated histamine release from basophils
Inhibits antibody response to T cell-dependent and independent antigens *in vitro* and *in vivo*
Inhibits certain delay-type hypersensitivity reactions
Inhibits DNA synthesis in lymphocytes stimulated with phytohemagglutinin and allogeneic cells

[a] Modified from Gresser (1977).

[b] Most of these effects have been observed using type 1 interferon (see text).

pid-like receptor on the target cell surface, which in man may be coded for by chromosome 21 (see Pitha, 1977). How the result of the interaction between type 1 interferon and its receptor is transmitted to the interior of the cell is poorly understood, but alterations in the intracellular levels of cAMP or cGMP or the induction of an intracellular antiviral protein have both been proposed as possible effector mechanisms. The question of how the interferon-induced changes in target cell metabolism actually inhibit the multiplication of viruses is also largely unanswered. However, there is reason to believe that the interferon-treated cells are rendered incapable of synthesizing viral nucleic acid, viral proteins, or both.

It has become increasingly clear that the antiviral effect of interferon may be but one of many manifestations of the cellular effects of interferon. Thus, a variety of biologic effects other than the antiviral ones have been reported for interferon, some of which are listed in Table VIII. Most of these effects have been attributed to type 1 interferon. However, little work has been done to clarify biologic effects other than the antiviral ones of type 2 interferon, and some or all of the effects ascribed to type 1 interferon may also be found in type 2 interferon preparations. In fact, the suppression of plaque-forming cell responses has been shown to be a property of both types of interferon (Johnson *et al.*, 1977), and the kinetic patterns of the responses appear to be very similar to those induced by the lymphokine soluble immune response suppressor (SIRS). This has lead to the proposal that SIRS and type 2 interferon may represent different biologic functions of the same molecule (Pierce and Kapp, 1976).

A serious problem in these experiments, in which rather crude materials are often used, is the extent to which the various biologic effects ascribed to interferon are in fact due to other contaminating lymphokines and cytokines. Thus, when considering the possible varied effects of "immune interferon," the inhibition of DNA synthetic responses of lymphocytes stimulated with mitogens or allogeneic cells might be due to IDS (see page 80); the enhancement of lymphocyte cytotoxicity might be attributed to LT (see page 76); the stimulation of macrophage phagocytosis might be due to MAF (see page 70); and, as already mentioned, the suppression of antibody synthesis might be caused by SIRS (see page 90).

However, whether or not "immune interferon" has a multitude of effects on immune reactions, the mere antiviral effect of type 2 interferon produced by lymphocytes or macrophages after a specific immunologic reaction appears to be important to the host. This is substantiated by the observation that persons who are unable to mount an adequate cellular immune response are plagued by viral infections.

H. Immunoglobulin Binding Factor

Lymph node lymphocytes from rats immunized with guinea pig encephalitogenic protein produce a factor that combines with IgG complexed to antigen, inducing hemagglutination of IgG-sensitized erythrocytes and protecting them from complement-mediated hemolysis (Fridman *et al.*, 1974). This factor, termed immunoglobulin binding factor (IBF), prevents the fixation of CIq, thus protecting IgG-sensitized sheep erythrocytes from lysis. The IBF does not bind to IgM or protect IgM-coated red blood cells from hemolysis.

Production of IBF was initially shown to be induced by an allogeneic stimulus (Fridman *et al.*, 1974). More recently, IBF activity was detected in the culture fluid of a mouse thymoma cell line L-5178-Y that is theta and Fc receptor positive (Fridman *et al.*, 1977). In this latter study, IBF was shown to have another biologic activity: suppression of plaque-forming cell generation *in vitro* in response to sheep red blood cells. The authors also reported that IBF was made by T cells and bound to immunoglobulin-coated Sepharose columns. The latter property has enabled physicochemical characterization and purification of IBF using the immunoglobulin binding and plaque-forming cell generation assays to follow the purification steps. The initial characterization of IBF by antigen or mitogen-stimulated cells included the observation that the molecular weight of IBF was approximately 150,000 and that it focused with an isoelectric point of 6.30 (Fridman *et al.*, 1974) (Table IX). Subsequent characterization using the cell line material with internal labeling revealed that the immunoglobulin binding and the plaque-forming suppressing activities

TABLE IX
Physicochemical Properties of Immunoglobulin Binding Factor[a] in Mouse

Temperature (56°C, 30 min)	Labile
Sieve chromatography	140,000, 300,000
Isoelectric point	6.30
SDS PAGE[b]	80,000
2-Mercaptoethanol	40,000 and 20,000
Trypsin	Sensitive
Pronase	Sensitive
Neuraminidase	Sensitive

[a] Data from Fridman *et al.*, 1974, 1977; Neuport-Sautes *et al.*, 1977; Joskowicz *et al.*, 1977.

[b] Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

eluted in two peaks, one approximately MW 140,000 and the other greater than 300,000 (Neuport-Sautes *et al.*, 1977). On polyacrylamide gel electrophoresis under reducing conditions with sodium dodecyl sulfate, IBF activity was recovered in a single peak at MW 80,000. In the presence of 2-mercaptoethanol, this material dissociated into a major unit of MW 40,000 and a smaller 20,000 unit suggesting that the two chains of 40,000 and 20,000 were linked by disulfide bridges. Under normal culture conditions, however, the materials existed in polymeric forms of MW 140,000 and greater than 300,000. In a subsequent study, IBF activity produced by activated T cells was purified by affinity chromatography on insolubilized IgG columns. The eluted material was iodinated, treated with 2-mercaptoethanol, and run on SDS gels (Joskowicz *et al.*, 1977). These studies demonstrated that radioactivity was present in two peaks at MW 38,000 and 18,000. In other studies, IBF was shown to be heat labile (inactivated at 56°C for 30 minutes), and its activity was destroyed by treatment with neuraminidase, trypsin, or Pronase, indicating a glycoprotein nature. It was also known that the MW 38,000 chain was necessary for the binding to IgG. Furthermore, it was reported that IBF bears or is associated with Ia determinants.

These studies suggest that IBF could be the soluble form of the Fc receptor on T cells (Neuport-Sautes *et al.*, 1977). This conclusion was inferred on the basis of observations that IBF was specific for the Fc portion of IgG, it was produced by Fc positive T cells or an Fc-positive lymphoid cell line, and the kinetics of production of IBF parallel the kinetics of Fc receptor shedding by activated T cells. Thus, IBF functionally may have biologic activity detected in one system as an ability to interfere with the fixation of complement to antigen–antibody complexes and therefore is potentially able to diminish an inflammatory response. In another system, its activity is detected by inhibiton of plaque formation, and a regulatory role in antibody production is suggested.

I. Tissue Factor: Procoagulant Activity

Niemetz (1972) first showed that peritoneal leukocytes spontaneously released a procoagulant activity *in vitro*. The amount of this material was increased 10-fold in the culture fluids when endotoxin was injected intraperitoneally in the animals prior to lavage. Rickles *et al.* (1973) subsequently showed that mononuclear cells from normal subjects and patients with hemophilia elaborated a procoagulant-like activity *in vitro* following stimulation by specific antigens, such as tuberculin PPD, and nonspecifically by PHA. The authors showed that

this procoagulant activity could correct the clotting time in factor 8-deficient plasma despite the absence of detectable antihemophilia factor antigen in the serum. It was also shown that the material was bound to cell membranes, since activity could be detected after destruction of lymphocytes as well as being present in the supernatant. Treatment of cells with drugs such as actinomycin D, puromycin, or cycloheximide inhibited the generation of the lymphocyte coagulant activity in response to endotoxin stimulation. It was reasoned that the drugs either inhibited the synthesis of new procoagulant activity or directly inhibited active material.

In another study, tissue factor was generated *in vitro* in an allogeneic system (Rothberger *et al.*, 1978). In these studies, 8- to 240-fold more procoagulant activity was generated in mixed lymphocyte reactions than by each individual's cells cultured alone. Combinations of lymphocytes from syngeneic twins did not result in increased tissue factor generation.

The initial assumption made regarding the cell type producing tissue factor was that it was a lymphokine. However, subsequent studies have revealed that, in fact, tissue factor may be a monokine (Rickles and Bobrave, 1975; Edwards and Rickles, 1978). It was shown in these studies that T cells separated by an E rosette technique in the absence of monocytes did not make tissue factor in response to PHA. Furthermore, monocytes alone did not elaborate tissue factor when stimulated with PHA. The latter implies that T cells are necessary in order to initiate production of the material, but this occurs by cell–cell interaction, the T cell being activated to produce some principle that subsequently stimulates the monocytes to elaborate the factor. In support of this interpretation, LPS appeared to activate monocytes directly in the absence of T cells to generate tissue factor. It is also of interest that immune complexes can stimulate the generation of tissue factor activity (Rothberger *et al.*, 1977).

This procoagulant activity is antigenically distinct from factor 8 and has been identified as being tissue factor using purified antisera to this material. It is very labile and thus far, has not been well characterized.

Both a physiologic and pathophysiologic role of tissue factor generation by mononuclear leukocytes has been proposed. It has been suggested that tissue factor production and the clotting system in general, may be involved in the expression of delayed cutaneous hypersensitivity (Edwards and Rickles, 1978). Delayed hypersensitivity skin test reactivity, the *in vitro* proliferative response, and tissue factor production to environmental antigens, such as tuberculin PPD, SK-SD, mumps, and monilia, was compared in 24 normal subjects before and

during anticoagulant treatment with coumadin. It was found that anticoagulant therapy in these subjects decreased their ability to express delayed hypersensitivity skin reactions and produce tissue factor, although proliferative responses to these antigens were intact. These results, and others in which it has been demonstrated that fibrin deposition is present at sites of delayed cutaneous hypersensitivity reactions (Colvin *et al.*, 1973), imply that the participation of the clotting system is necessary for the demonstration (i.e., induration) of skin reactivity. In the latter studies, anticoagulant therapy obliterated induration and fibrin deposition at biopsied skin sites, but did not interfere with the mononuclear cell infiltration. This suggests that the induration is due to fibrin deposition, not to the cellular infiltrate. Furthermore, it is known that patients with systemic lupus erythematosus, rheumatoid arthritis, and renal homograft rejection have inflammatory reactions that histologically resemble a Schwartzman phenomenon, as the tissue damage is characterized by thrombosis and fibrin deposition. The question has been raised whether these phenomena could be due in part to participation of tissue factor.

J. Colony-Stimulating Activity

Bone marrow precursors of granulocytes and monocytes, will grow to form colonies of fully differentiated cells of the granulocytic and monocytic variety *in vitro* provided a stimulating factor is added to the culture medium. Many cell types have previously been shown to elaborate a colony-stimulating activity (CSA), including blood monocytes and tissue macrophages, and it now appears that lymphocytes also have this capacity. Mouse lymphocytes from thymus and spleen spontaneously release a CSA activity that is increased two- to fivefold after stimulation of the cells with mitogens, such as PHA, Con A, or pokeweed mitogen. Spleen cells appear to be better able to produce this factor than thymocytes (Ruscetti and Chervenick, 1975a).

CSA activity is slowly released into the culture medium of cells stimulated with mitogens and reaches maximum by day 7. Although this finding correlates with uptake of tritiated thymidine, it has recently been shown that the two phenomena can be separated. For example, actinomycin D, vinblastine, irradiation, and mitomycin all significantly reduce DNA synthesis but have no effect on the release of CSA (Ruscetti and Chervenick, 1975b).

Production and release of CSA depend upon the continuous binding of the mitogen to the cell. α-Methylmannoside added to Con A-stimulated cultures at any time during the culture period blocks the release of CSA. In contrast, α-methylmannoside had no effect on the

production of CSA or thymidine incorporation of cells stimulated with PHA. Restimulation of α-methylmannoside inhibited cultures with Con A and allowed normal CSA release (Ruscetti and Chervenick, 1975b). The latter implies that an actual control step may be occurring for the release of CSA at the lymphocyte membrane. That CSA production depends upon active cell protein synthesis has been shown in studies in which puromycin and cycloheximide have been used.

It appears that T cells are required for the production of CSA by experiments in which anti-T cell sera, but not anti-B cell sera, abolish CSA release (Ruscetti and Chervenick, 1975a). Furthermore, T cell-deficient nude mice do not make CSA to pokeweed mitogen stimulation. These findings suggest that T cell participation in the production of CSA is required but does not establish whether this cell is actually making the factor or merely required for its production by monocytes–macrophages.

At present CSA has not been well characterized, but it has been described to be a heat-stable glycoprotein having an apparent molecular weight between 40,000 and 60,000 after gel filtration on Sephadex G-100 (Ruscetti and Chervenick, 1975a).

Another factor has been described that affects macrophage growth but is measured by its effect on DNA synthesis rather than by colony formation. This factor, termed macrophage mitogenic factor, induces proliferation of nonimmune oil-induced, monocyte-derived peritoneal and noninduced alveolar macrophages (Hadden *et al.*, 1973, 1975b). The factor was made by sensitized guinea pig lymph node lymphocytes stimulated by specific antigen. The factor has molecular weight range of 35,000–70,000 by gel filtration (Sephadex G-100) and appears to be distinct from MIF.

K. Osteoclast Activating Factor

There are a number of substances capable of causing resorption of bone. These include parathyroid hormone, prostaglandin E_1, vitamin D metabolites, and other sterols. In addition to the above, lymphocytes also produce a bone resorbing activity (Horton *et al.*, 1972). This mediator has been termed osteoclast activating factor (OAF). The production of OAF has been triggered by lymphocytes from patients stimulated specifically with dental plaque antigens, in a mixed lymphocyte reaction, or nonspecifically by mitogens such as PHA (Horton *et al.*, 1972, 1979). This factor has been shown to form and activate osteoclasts in bone explants.

The technique for measuring bone resorption in an organ culture utilizes ^{45}Ca-labeled shafts of radius or ulna from a 19-day-old rat

fetus. The culture supernatants obtained from unstimulated and activated lymphocytes are then incubated with the organ culture for 4–6 days. The ratio of ^{45}Ca released into the medium from control and treated bone cultures is a measure of the bone resorption.

Physicochemical characterization of OAF revealed that it is heterogeneous. In early studies, OAF was reported to have an apparent molecular weight between 13,000 and 25,000 by gel filtration on Sephadex G-100 (Mundy *et al.*, 1974a). In more recent studies, two OAF activities were detected (Mundy and Raisz, 1977). One molecular species was found to have an apparent molecular weight of 18,000. However, it was found that, when this material was placed in 1 *M* NaCl or 2 *M* urea, it dissociated into a low molecular weight material of 140. This activity is referred to as "little" OAF. The low molecular weight material was then chromatographed on BioGel P6 and found to have an apparent molecular weight of 1330–3500. Furthermore, when this low molecular weight OAF was placed in low ionic strength buffers, it associated to the high molecular weight (18,000) material. One explanation for the heterogeneity in size of OAF following these chromatographic procedures is the fact that it may bind to protein. For example, when OAF is prepared in serum-free medium, one peak of activity is recovered (Lubin *et al.*, 1974). In contrast, when OAF is prepared from cells incubated initially in plasma, multiple peaks of activity are recovered. The OAF has been shown to be sensitive to trypsin and Pronase in one study, indicating that it has a protein nature (Lubin *et al.*, 1974). Furthermore, Mundy and Raisz (1977) have shown that the low molecular weight OAF is sensitive to digestion by trypsin and papain, but the MW 18,000 material is not.

Osteoclast activating factor has been shown to be distinct from other agents that cause bone resorption. For example, it is heat labile relative to parathyroid hormone, vitamin D, or prostaglandin E_2 (Mundy *et al.*, 1974a). It also exhibits a different dose response in terms of calcium release than do these other agents, it is not lipid soluble, and it does not react to antibodies against the other materials. Furthermore, indomethacin, which blocks prostaglandin synthetase activity, does not inhibit the production of OAF by human lymphocytes (Horton *et al.*, 1979).

Osteoclast activating factor may be important in causing the hypercalcemia seen in neoplastic disease or in the pathogenesis of bone resorption in dental caries. It was shown in one study (Mundy *et al.*, 1974b) that a bone-resorbing and osteoclast-activating factor was produced in bone marrow cultures of 6 or 7 patients with multiple myeloma. This material was found to have the same physicochemical char-

acteristics as OAF, not those of other calcium releasers from bone. Furthermore, peripheral blood lymphocytes from patients with multiple myeloma produced normal amounts of OAF *in vitro*. It is of interest that various cell lines from patients with multiple myeloma, Burkett's lymphoma, and other malignant myelomas liberate an OAF-like activity having the physicochemical characteristics of OAF made by peripheral blood lymphocytes (Mundy *et al.*, 1974a).

III. Cell Types Involved in Lymphokine Synthesis

That T lymphocytes mediate cellular immunity is undisputed. However, whether or not B lymphocytes also play a role in the expression of this reaction is far from being clear. Through the development of methods to purify subpopulations of T cells or B cells, it has been possible to evaluate which cell types produce lymphokines. Studies from a number of laboratories indicate that both T and B lymphocytes have the capacity to make mediators. For example, MIF, chemotactic factor for macrophages, LIF, type 2 interferon, mitogenic factor, and lymphotoxin are made by activated T or B cells (Yoshida *et al.*, 1973; Rocklin *et al.*, 1974a; Mackler *et al.*, 1974; Wahl *et al.*, 1974b). The trigger may be antigen or mitogen for T cells; antigen, mitogen, or stimulation of the C3 or immunoglobulin receptor may activate B cells. It becomes apparent that, although the production of lymphocyte mediators correlates with the *in vivo* state of cellular immunity of the host, one is not necessarily measuring the function of a particular cell type. Several lymphocyte mediators including MIF, LIF, and the macrophage chemotactic factor, from both T and B cells have an identical chromatographic pattern (Rocklin *et al.*, 1974b; Chess *et al.*, 1975; Altman *et al.*, 1975).

That B lymphocytes make lymphokines, in the apparent absence of T lymphocytes, is of particular interest. The latter implies that B cells could participate in, amplify, or perhaps even initiate a cellular immune reaction. However, it should be pointed out that, although B cells produce lymphokines, they do so only if T cell function in that individual is intact. For example, in a T cell-deficient patient (DiGeorge syndrome) who has normal numbers of B cells, no MIF is made (Rocklin *et al.*, 1972).

T cells and B cells can elaborate lymphokines, but they do so with the cooperation of another cell type, the macrophage. Lymphocyte activation in general requires the presence of a macrophage-like cell. Thus, the *in vitro* proliferative response to mitogens and antigens, as well as the production of lymphocyte mediators, requires the pres-

ence of macrophages or monocytes in the culture (Cline and Swett, 1968; Twomey *et al.*, 1970, 1978; Epstein *et al.*, 1971a; Wahl *et al.*, 1974b; Nelson and Leu, 1975; Ohishi and Onoue, 1975; Rosenstreich *et al.*, 1976). In particular, it has been shown that the production of MIF, type 2 interferon, and the macrophage chemotactic factor are macrophage-dependent reactions. Furthermore, histocompatibility between the macrophage and the lymphocyte appears to be essential (Shevach *et al.*, 1975). The B cell requirement for macrophages is less stringent than that required for T cells (Twomey *et al.*, 1978; Wahl *et al.*, 1974a). This appears to be possible because B cells may be activated by their C3 or immunoglobulin receptors. Therefore, B cells, in the absence of macrophages, could be capable of initiating a cellular immune reaction.

In addition to being produced by antigen- or mitogen-stimulated lymphocytes, lymphokine-like activities have been detected in the culture fluids of lymphoid cell lines and nonlymphoid cell types. Thus, culture fluids from spontaneously growing established cell lines contain MIF-like, lymphotoxin-like, interferon-like, and skin reactive factor activities (Florentin *et al.*, 1975; Granger *et al.*, 1970; Papageorgiou *et al.*, 1972; Haase *et al.*, 1970; Calebaugh and Paque, 1974). It is of interest that both T cell-derived and B cell-derived cell lines have the capacity to liberate lymphokines. In some instances, the activities derived from the cell lines and those from antigen- or mitogen-stimulated lymphocytes have similar physicochemical characteristics. It remains to be shown, however, whether these activities are in fact, identical with the lymphokines derived from antigen-stimulated lymphocytes. It should also be noted that material with MIF-like activity has been extracted from thymus and has also been found in the culture fluids of some dividing mouse fibroblasts (Houck *et al.*, 1973; Tubergen *et al.*, 1972).

Under other nonimmunologic circumstances, it has been shown that lymphokine activities can be recovered from culture fluids derived from cells infected with viruses. For example, MIF activity has been detected in supernatants derived from mouse fibroblasts infected with SV40, and monkey kidney cells infected with Newcastle disease virus, mumps virus, or SV40 (Hammond *et al.*, 1974; Flanagan *et al.*, 1973; Cohen *et al.*, 1975). In addition, chemotactic factors for neutrophils and macrophages have also been detected in these supernatants (Ward *et al.*, 1972). Further studies are necessary to determine whether or not the mediators produced by the virus-infected cells are identical to those produced by lymphocytes. Preliminary evidence suggests that there is some cross-reactivity, because anti-MIF anti-

serum prepared against lymphocyte-derived MIF is capable of adsorbing MIF obtained from SV40-infected African green monkey cells (Yoshida *et al.*, 1975b). It is possible that virus-induced mediators play an important role in host defense against viral infection. One could hypothesize that this nonimmunologic triggering of lymphokine production is similar to the nonimmunologic triggering of other host defense mechanisms, such as complement via the alternative pathway. Mouse fibroblasts that have been transformed by SV40 produce an MIF-like substance and also exhibit a loss of dense-staining cell coat material similar to that observed in macrophages that have been treated with MIF (Hammond *et al.*, 1974). Possibly the MIF-like substance accounts for the alterations in cell contact behavior that accompanied transformation by oncogenic viruses.

IV. Regulation and Pharmacologic Modulation of Lymphokine Production and Action

Pharmacologic modulation *in vitro* of lymphokine production and activity can occur at the afferent, central, and efferent levels of the immune response. In the afferent stage, pharmacologic agents may interact directly with antigen (or mitogen) or they may interfere with macrophage binding and processing of the antigen. Drugs may influence the synthesis and release of the mediators in the central stage of the response or may react with the mediators themselves or interfere with their action on target cells in the efferent stage. Finally, pharmacologic agents may modify directly the target cells or biologically active factors released from target cells exposed to lymphokines.

In this section, we discuss primarily the effects of agents that influence the metabolism of a variety of eukaryotic cell types on lymphokine production and activity. Modulation of lymphokine function by antilymphokine antibodies is discussed in Section V.

A. Modulation of Lymphokine Production

Evidence has emerged that there may be a natural regulation of mediator production by lymphocytes (see Table X). Thus, antigen-stimulated T lymphocytes, or more likely a subpopulation of T cells, release a mediator that, when incubated with B cells stimulated with the same antigen, prevents them from making MIF (Cohen and Yoshida, 1977). Although many explanations are possible, there is reason to believe that the MIF inhibitory factor (MIFIF) acts directly on the B cells to prevent synthesis or release of MIF. The presence of a natural regulator mechanism that prevents excessive mediator production may also account for the previous observation that more MIF, LIF, and LT are

TABLE X

PHARMACOLOGIC MODULATION OF THE PRODUCTION OF MIGRATION INHIBITORY FACTOR (MIF), LEUKOCYTE INHIBITORY FACTOR (LIF), AND LYMPHOTOXIN (LT)[a]

Agents and their effects	Effects on the production of		
	MIF	LIF	LT
Blocking of DNA synthesis and/or DNA replication			
Mitomycin C	Reduction (14), none (13)	None (3)	—
Cytosine arabinoside	None (4)	—	—
X-Irradiation	Reduction (18), none (17)	None (8)	None (21)
5-Bromo-2-deoxyuridine and light	None (16)	—	—
Blocking of DNA-dependent RNA synthesis			
Actinomycin D	Reduction (6, 13, 14)	Reduction (3)	—
Blocking of protein synthesis			
Puromycin	Reduction (6, 13, 14)	Reduction (2, 8)	Reduction (21)
Pactamycin, emetine	Reduction (10)	—	Reduction (10)
Cycloheximide	—	Reduction (3)	Reduction (21)
Blocking of microfilaments			
Cytochalasin B	Reduction (15)	Reduction (3)	Reduction (22)
Blocking of microtubules			
Colchicine	None (10,15)	None (3)	None (10)
Vinblastine	None (4, 10, 15)	—	None (10)
Elevated cAMP levels			
cAMP, dibutyryl cAMP	Reduction (15)	Reduction (3, 12)	Reduction (11, 15a, 19)
β-Adrenergic stimulators	None (15)	—	Reduction (15a)
PGE_1	Reduction (7)	Reduction (12)	Reduction (19)
Cholera toxin	None (10)	—	None (10)
Phosphodiesterase inhibitors	Reduction (15)	Reduction (2, 5, 12)	Reduction (11)
Elevated cGMP levels			
Cholinergic drugs	Enhancement (9)	Reduction (3)	Reduction (15a)
Miscellaneous			
Glucocorticoids	None (1, 14), reduction (20)	Reduction (2)	Reduction (21)

[a] Numbers in parentheses indicate references: (1) Balow and Rosenthal, 1973; (2) Bendtzen, 1975b; (3) Bendtzen and Rocklin, unpublished findings; (4) Bloom *et al.*, 1972; (5) Coeugniet *et al.*, 1977; (6) David and David, 1972; (7) Gorden *et al.*, 1976; (8) Gorski *et al.*, 1976a; (9) Hadden *et al.*, 1973; (10) Henney *et al.*, 1974; (11) Lies and Peter, 1973; (12) Lomnitzer *et al.*, 1976a; (13) Mizoguchi *et al.*, 1973; (14) Pekarek *et al.*, 1976; (15) Pick, 1974; (15a) Prieur and Granger, 1975; (16) Rocklin, 1973; (17) Salvin and Nishio, 1972; (18) Visakorpi, 1974; (19) Wagshal and Waksman, 1978; (20) Wahl *et al.*, 1975; (21) Williams and Granger, 1969; (22) Yoshinaga *et al.*, 1972.

produced if the culture medium is changed during lymphokine production than if no change is made. Previously, these findings have usually been attributed to exhaustion of nutritional ingredients in the medium or to destruction of the lymphokines during culture.

The effects of various metabolic inhibitors on the synthesis and release of MIF, LIF, and LT as well as the effects of agents that influence intracellular levels of cyclic nucleotides in lymphocytes are summarized in Table X.

There is general agreement that the *in vitro* production of at least some lymphokines is dissociated from lymphocyte blastogenesis. This is in accordance with the notion that many effector functions in cell-mediated immunity studied *in vitro* are performed by nondividing cells. Also, proliferation is not a prerequisite for production of most of the known mediators of T–B cell interactions. Thus, X-irradiated spleen cells still produce SIRS (Pierce and Kapp, 1976) and treatment with mitomycin C has no effect on TRF (Schimpl and Wecker, 1976) and AEF production (Eshhar *et al.*, 1977). On the other hand, the elaboration of IDS appears to require cell division because mitomycin C and bromodeoxyuridine suppress its production (Namba *et al.*, 1977). This may not be surprising, considering that the production of this mediator occurs at a very late stage of the cell cycle (late G_1 to S phase). In contrast, the production of MIF, LIF, and LT ocurs in the early G_1 phase.

With no known exception, the production of lymphokines appears to be dependent upon an intact protein synthesis apparatus. It is not known whether protein synthesis is needed for the actual synthesis of the lymphokine or its secretion from the cell. Double radiolabeling studies indicate, however, that MIF and other lymphokines in the guinea pig are synthesized de novo or in increased amounts in response to antigen and mitogens (Sorg and Bloom, 1973).

The mechanism of lymphocyte triggering is not fully understood, but measurable RNA, protein, and DNA synthesis are preceded by one or more steps initiated by the interaction of antigen or mitogen with the lymphocyte membrane. Cap formation and pinocytosis of the antigen- or mitogen-receptor complex are recognized to be essential steps leading to lymphocyte activation. These steps depend upon intact microfilament function, which may be inhibited by the mold metabolite cytochalasin B. As shown in Table X, cytochalasin B also blocks early events of lymphocyte triggering as measured by the production of lymphokines. The effects of cytochalasin B have been found to be reversible and therefore not due to generalized cell damage. On the other hand, agents that inhibit the formation of microtu-

bules and interfere with the formation of mitotic spindles, such as the alkaloid colchicine and the mold product vinblastine, do not influence early mediator production by stimulated lymphocytes.

The production of many if not all lymphokines appears to be completely blocked by the addition of cAMP and by agents that increase the endogeneous levels of cAMP. Thus, agents that activate the cAMP-generating enzyme adenylate cyclase (e.g., PGE_1) and agents that inhibit the cAMP-degrading enzyme cAMP-phosphodiesterase (e.g., theophylline, dipyridamole, and papaverine) all reduce or abolish the production of lymphokines (see Table X). The inability of cholera toxin, a potent and sustained activator of adenylate cyclase, to block the release of MIF and LT in one study (Henney *et al.*, 1974), and the lack of effect of isoproterenol on MIF production in another study (Pick, 1974) is difficult to explain. However, it may be related to the kinetics of reaction of these drugs. Cholera toxin elevates cAMP levels in most tissues (including lymphocytes) after a latent period of up to several hours whereas the stimulating effect of isoproterenol is very rapid (minutes). The signal mediated by the increased levels of cAMP induced by isoproterenol may therefore be of insufficient intensity or of too short a duration to prevent the lymphocytes from entering the G_1 phase, in which most mediators are produced. On the other hand, the elevation of cAMP concentrations induced by cholera toxin may appear too late to block lymphokine production, since the G_1 phase has already been entered.

The explanation agrees with the observation that the production of LT by mitogen-stimulated lymphocytes during the early G_1 phase is inhibited by PGE_1 and dibutyryl cAMP. The fact that cholera toxin inhibits the release of type 2 interferon from mitogen-activated lymphocytes, a process that occurs in the late G_1 and the S phase of the cell cycle, may also be seen in this context (Johnson, 1977). It is interesting that elevated cGMP levels may enhance MIF production (Hadden *et al.*, 1973). Increases in cGMP levels or in the ratio between cGMP and cAMP concentrations may be one of several triggering events after mitogen stimulation that enable lymphocytes to leave the resting stage and enter the cell cycle.

The mechanism by which glucocorticoids suppress immunologic functions is on the whole unknown despite many years of empirical use of these agents in clinical immunology. In the opinion of some, but not all, investigators, glucocorticoids suppress the production of lymphokines (see Table X). In interpreting these results one must bear in mind that significant species differences exist. Also, some lymphokines may be produced primarily by steroid sensitive T_1 cells,

found in the cortex of the thymus and in the spleen. In contrast, other lymphokines may be produced by steroid resistant, recirculating T_2 cells.

B. Modulation of Lymphokine Action

For obvious reasons, it is not possible to generalize when discussing the pharmacologic modulation of the function of various lymphokines. For instance, some lymphokines seem to enhance cellular proceses (e.g., MAF), whereas others suppress the function of the target cells (e.g., IDS). Moreover, lymphocytic mediators may act directly on the target cells (e.g., LT), or they may exert their functions more indirectly through activation of other inflammatory cells (e.g., SIRS). We attempt, in Table XI, to summarize the effects on the target cell response to MIF, LIF, and LT of agents that are known to interfere with essential metabolic pathways.

The influence on MIF and LIF action by agents that interfere with macrophage DNA replication, and macrophage and PMN leukocyte transcription and translation, appears to be limited. However, LT activity is significantly enhanced when target cells, usually mouse L cell fibroblasts, are treated with these agents. Cells treated with LT have been shown to increase their RNA polymerase II activity, but the total protein synthesis of these cells is initially unaltered (Kunitomi *et al.*, 1975). The marked synergistic effect on cell lysis by drugs that interfere with RNA and protein synthesis indicate that the increased RNA polymerase II activity is not directly caused by LT, but rather by compensatory reaction in response to the LT-induced injury. Although the total protein synthesis of LT-treated cells is unaltered, it cannot be excluded that the production of selective proteins is temporarily enhanced in an effort to counteract the effects of LT. If this is prevented by drugs that block protein synthesis, enhanced and/or early cell injury might result.

From the effects of agents that raise intracellular cAMP levels (including cAMP itself), it appears the cAMP counteracts events occurring after exposure of macrophages to MIF and of PMN leukocytes to LIF. In order to prevent the action of these lymphokines, the agents must be present during the initial stage of the interaction between mediators and migrating cells (Kotkes and Pick, 1975a; Lomnitzer *et al.*, 1976a; Bendtzen and Palit, 1977).

Macrophages treated with MIF-containing supernatants show a prolonged (24 hour) but minimal decrease in cAMP concentrations compared with cells treated with medium alone. However, the discrete cAMP-lowering effect is similar to (Higgins *et al.*, 1976) or only

TABLE XI

PHARMACOLOGIC MODULATION OF THE ACTION OF MIGRATION INHIBITORY FACTOR (MIF), LEUKOCYTE INHIBITORY FACTOR (LIF), AND LYMPHOTOXIN (LT)[a]

Agents and their effects	Effects on the action of		
	MIF	LIF	LT
Blocking of DNA synthesis and/or DNA replication			
Mitomycin C	Reduction (10)	—	None (12)
X-Irradiation	—	—	Increase (12)
Blocking of DNA-dependent RNA synthesis			
Actinomycin D	None (10, 14)	None (3)	Increase (17, 19)
Blocking of protein synthesis			
Puromycin	Reduction (14)	Reduction (2)	Increase (19)
Cycloheximide	Reduction (14)	None (3)	Increase (19)
Blocking of microfilaments			
Cytochalasin B	—	None (3)	—
Blocking of microtubules			
Colchicine	None (6), reduction (13)	None (9)	None (6, 12)
Vinblastine	None (6), reduction (13)	None (9)	None (6)
Elevated cAMP levels			
cAMP, dibutyryl cAMP	Reduction (7)	Reduction (4, 9)	Reduction (16)
β-Adrenergic stimulators	Reduction (7, 8)	Reduction (4)	Reduction (16)
PGE_1	Reduction (7), none (5, 8)	Reduction (9)	—
Cholera toxin	Reduction (6)	—	—
Phosphodiesterase inhibitors	Reduction (15), blocking (7)	Reduction (4, 9)	Reduction (16)
Elevated cGMP levels			
cGMP, dibutyryl cGMP	None (15)	Reduction (4)	—
Cholinergic drugs	None (8)	None (4)	Reduction (16)
Imidazole	—	Reduction (4)	Reduction (16)
Miscellaneous			
Glucocorticoids	Reduction (1, 10, 18)	None (2)	Reduction (11)

[a] Numbers in parentheses indicate references: (1) Balow and Rosenthal, 1973; (2) Bendtzen, 1975b; (3) Bendtzen, unpublished findings; (4) Bendtzen and Palit, 1977; (5) Gordon *et al.*, 1976; (6) Henney *et al.*, 1974; (7) Koopman *et al.*, 1973; (8) Kotkes and Pick, 1975a; (9) Lomnitzer *et al.*, 1976a; (10) Pekarek *et al.*, 1976; (11) Peter, 1971; (12) Peter *et al.*, 1973; (13) Pick and Abrahamer, 1973; (14) Pick and Manheimer, 1973; (15) Pick and Manheimer, 1974; (16) Prieur and Granger, 1975; (17) Rosenau *et al.*, 1973; (18) Wahl *et al.*, 1975; (19) Williams and Granger, 1969.

slightly more pronounced than (Pick, 1977b) the decrease seen when macrophages are treated with supernatants from unstimulated lymophocytes. The reduction in cAMP concentrations has been attributed to diminished cAMP synthesis rather than to increased cAMP degradation (Pick and Grunspan-Swirsky, 1977). Thus, direct inhibition of macrophage adenylate cyclase has not been demonstrated (Pick and Grunspan-Swirsky, 1977). In fact, a late (24–48 hour) serum-dependent increase in adenylate cyclase activity of intact macrophages exposed to MIF-rich lymphocyte supernatants has been reported (Remold-O'Donnell and Remold, 1974). It is therefore probable that MIF, if at all active in this system, interferes with the early signal transfer from membrane receptors to the enzyme. The possible effect of MIF on the intracellular level of cGMP is not known.

Although the effects of LIF on PMN leukocyte cAMP levels are grossly similar to those observed in MIF-treated macrophages (Bendtzen *et al.*, 1977), LIF-containing supernatants cause a threefold or higher increase in the cGMP levels of PMN leukocytes (Bendtzen and Klysner 1980). This effect is detectable within 3 minutes of exposure to LIF, and it subsides within 3 hours. The cGMP-generating factor, which seems to interfere with the degradation of cGMP rather than the synthesis of the nucleotide, is susceptible to the serine protease inhibitor PMSF and may therefore prove to be identical with LIF.

In discussing the possible influence of lymphokines on target cell cyclic nucleotides or on any other biochemically measurable parameter, one should always bear in mind that the mere demonstration of such an effect, whether synergistic or antagonistic, does not justify any conclusion regarding the mechanism of action of the lymphokine under study. Thus, in the case of LIF, the apparent cGMP-increasing effect of the lymphokine may be totally unrelated to its effect on PMN cell mobility, since it is impossible to mimic LIF activity by exogeneous cGMP (Bendtzen and Palit, 1977). This does not rule out a possible role of cGMP as a second mediator of hitherto unrecognized effects of LIF on PMN leukocytes.

V. Antibodies to Lymphokines

The availability of antisera to lymphokines is of obvious importance for the analysis of their mechanism of action *in vitro* and for the investigation of the role played by each of these mediators *in vivo*. Until recently, progress in this area was very slow mainly because of difficulties in obtaining sufficient quantities of highly purified lymphokine preparations. A number of investigators have now reported the

development of antisera to animal and human lymphokines. Antibodies capable of binding guinea pig MIF (Yoshida *et al.*, 1975a; Geczy *et al.*, 1975), guinea pig LT (M. K. Gately *et al.*, 1975), guinea pig mixed lymphocyte culture LMF (Geczy, 1977), and rat IDS (Namba and Waksman, 1977) have been produced, as have immunoglobulins to the following human lymphokines: α-LT (Walker and Lucas,1974; Boulos *et al.*, 1974), β-LT (Walker *et al.*, 1976; Lewis *et al.*, 1977), LIF (Bendtzen, 1977a), and MIF (McLeod *et al.*, 1977; Block *et al.*, 1978). None of these antibody preparations appear to be monospecific, i.e., directed against only one well defined lymphokine. However, absorption with serum proteins and with mononuclear cells may lead to immunoglobulins selectively directed against only a few products of activated mononuclear cells.

A. Preparation of Antilymphokine Antibodies

With few exceptions, conventional biochemical techniques, such as molecular sieve chromatography, ion exchange chromatography, preparative gel electrophoresis, and isoelectrofocusing, have been used for purification of the lymphokines prior to immunization. Another method of purification was reported by Yoshida *et al.* (1975a). They first treated Sephadex G-100 fractions rich in guinea pig MIF with an antibody directed against the corresponding fractions of control supernatant in order to reduce the level of contaminants common to each. This partially purified lymphokine preparation was then used as an immunogen to obtain the final antiserum. A modification of this rapid method, in which the lymphokine-containing supernatants themselves are used to elicit antibodies against contaminants, has proved to be well suited for producing antibodies even to very labile lymphokines, such as human LIF (Bendtzen, 1977a). Indeed, the critical features of the process of antibody induction to short-lived lymphokines, in addition to repeated exposure of the animals, appears to be rapid handling of the lymphokine preparations to preserve maximum antigenicity of the mediator (Lewis *et al.*, 1977).

B. Characterization of Antisera

The preparations used to raise antisera have contained several mediators, leading to the production of antibodies with multiple specificitis. For example, one antiserum raised against partially purified guinea pig MIF will remove MIF, macrophage CF, and "skin reactive factor" activities from supernatants of stimulated homologous lymphoid cells. It will not, however, remove LT, LMF, or neutrophil CF activities, indicating that at least some of these mediators are immuno-

chemically different (Kuratsuji *et al.*, 1976). Interestingly, this antiserum also binds to neutrophil CF, but not to MIF produced by lymphoid cells from man (Yoshida *et al.*, 1976). This suggests that a partial phylogenetic similarity in the molecular structure of lymphokines may exist. Another antiserum is capable of removing LT activity, but not LMF or MIF activities (M. K. Gately *et al.*, 1975), and yet another antibody preparation reacts with MIF, but not with LMF or "skin reactive factor" activities (Geczy *et al.*, 1975). Thus, by the use of antisera raised against guinea pig MIF and LT, it can now be concluded that MIF, LT, and LMF in this species differ immunochemically from each other. It is of interest that both anti-MIF antisera mentioned above also depress delayed hypersensitivity skin reactions when injected along with antigen in appropriately immunized guinea pigs. Most reported antilymphokine antisera differ significantly from classical antilymphocyte sera by virtue of their noncytotoxicity to lymphoid cells, the inability to prevent T rosette formation, and the inability of lymphocytes and lymphoblasts to absorb out the antilymphokine activities. In addition, antibodies raised against lymphocytes do not remove lymphokine activities (Geczy *et al.*, 1975; Bendtzen, 1977a).This indicates that the antilymphokine preparations contain antibodies against newly released products of activated lymphocytes, not merely antibodies to surface antigens.

Characterization in biochemical terms of antilymphokine antisera has been very difficult to achieve primarily because commonly used immunochemical techniques such as immunodiffusion and immunoelectrophoresis and conventional staining techniques cannot be used. Although highly concentrated lymphokine preparations will produce precipitates in these techniques, these precipitates have never been proved to be attributed to the lymphokine under study and its corresponding antibody. In most, if not all instances, they appear to be caused by contaminating proteins and their corresponding antibodies.

The hitherto most extensive characterization of an antilymphokine antiserum, in biochemical as well as in biologic terms, has been performed by Geczy and collaborators (Geczy *et al.*, 1975, 1976a,b; Sorg and Geczy, 1976; Hentges *et al.*, 1977). Their antiserum, raised against partially purified MIF produced by Con A-stimulated guinea pig lymphocytes, has been analyzed for specificity by means of a sensitive radioactive double labeling technique that distinguishes mediators produced in increased amounts or synthesized *de novo* by activated lymphocytes (Sorg and Bloom, 1973). The antiserum recognized primarily three products of stimulated lymphocytes with molecular weights of approximately 60,000, 45,000, and 30,000. One of these, the

45,000 MW product, appears to be guinea pig MIF (Sorg and Geczy, 1976). Another of these, probably the 30,000 MW product, may be a blastogenic factor produced during a mixed lymphocyte culture reaction *in vitro,* since the antibody suppressed the proliferation of responder cells in these cultures. Its inhibitory activity was not influenced by absorption with lymphoid cells, and it appeared to inhibit the ability of a soluble factor to stimulate unsensitized cells rather than interfering with the synthesis of this lymphocyte mediator (Geczy *et al.,* 1976b). Recent findings by Hentges *et al.* (1977) suggest that the antiserum also inhibits a product(s) of allogeneic cell interactions *in vivo,* since it inhibited normal and immune lymphocyte transfer in guinea pigs.

As mentioned previously, the antiserum of Geczy and collaborators has also been used to elicit a "secondary" antibody against MLC LMF, and this immunoglobulin has been used to demonstrate the difference between LMF produced by antigen-stimulated lymphocytes and LMF elaborated from lymphocytes during MLC reactions (Geczy, 1977) (see page 82).

Although radioimmunoassays of individual lymphokines have not yet been developed, antilymphokine antibodies as previously described, have already proved to be highly valuable tools for the investigation of component parts of cellular immune reactions. Moreover, the inherent nonspecificity of most biologic lymphokine assays may become less of a problem in future laboratory work, since antilymphokine antibodies may be used for neuralization tests to ensure that the result of a bioassay of a particular lymphokine actually reflects the appropriate biologic activity. The availability of antilymphokine antibodies also opens up the possibility of detecting lymphokines in situations where it is difficult or impossible to obtain appropriate control materials, such as detemination of lymphokines in body fluids and in supernatants of *in vivo* activated lymphocytes (Palit *et al.,* 1978).

VI. *In Vivo* Significance of Lymphokines

Although most of the effector molecules have been produced and described *in vitro,* several reports on the detection of lymphokine-like activities *in vivo* have accumulated (see Table XII). Lymphokine-like activities have been recovered in extracts of delayed hypersensitivity skin reactions (CF for lymphocytes and macrophages) (Cohen *et al.,* 1973), in lymph draining these sites (MIF and MF) (Hay *et al.,* 1973), in arthritic joint fluids (MIF, LT, and MF) (Stastny *et al.,* 1975; Peter *et al.,* 1971), in peritoneal fluid and in serum from sensitized animals

TABLE XII
In Vivo EVIDENCE OF LYMPHOKINE PARTICIPATION IN IMMUNOLOGIC REACTIONS[a]

Injection of Lymphokine In Vivo	
Lymphokine	Observed biologic effect
1. Skin reactive factor	Accelerated delayed hypersensitivity (DHS) skin reaction
2. Migration inhibitory factor	Drop in blood monocyte level
3. Migration inhibitory factor	Decreased peritoneal macrophage content
4. Lymphocyte mitogenic factor	Germinal center proliferation
Recovery of Lymphokine Activity during Immunologic Reaction	
Tissue or reaction	Lymphokine(s) detected
1. Lymph fluid	MIF, LMF
2. Serum	MCF, IF, MIF, LT
3. Joint fluid	MIF, LMF, LT
4. DHS skin extract	MCF

[a] Data from Cohen *et al.*, 1973, 1974; Hay *et al.*, 1973; Stastny *et al.*, 1975; Salvin *et al.*, 1975; Sonozaki *et al.*, 1975; Postlethwaite *et al.*, 1976b; Yoshida *et al.*, 1975b; Savel and Moehring, 1971; Palit *et al.*, 1978; Kataria *et al.*, 1976; Kelley *et al.*, 1972; Kelley and Wolstencroft, 1974; Yoshida and Cohen, 1974.

after intraperitoneal or intravenous challenge with specific antigen (CF for macrophages, interferon, and MIF) (Salvin *et al.*, 1975; Sonozaki *et al.*, 1975; Postlethwaite *et al.*, 1976b), and in serum of patients with various lymphoproliferative disorders (MIF) (Cohen *et al.*, 1974;Yoshida *et al.*, 1975b). Also spontaneous release of LT-, MIF-, and LIF-like activities by mononuclear cells isolated from blood of patients suffering from cancer (Savel and Moehring, 1971), infectious mononucleosis (Palit *et al.*, 1978), and sarcoidosis (Kataria *et al.*, 1976) has been detected. Although the findings strongly suggest that lymphokines play a role in *in vivo* immune events, they generally do not offer definite proof that this is so and that the mediators are actually produced by lymphocytes *in vivo*. Thus, the effects observed in the biologic assays used to detect conventional lymphokines may be caused by substances other than lymphokines. To verify this, and to demonstrate that lymphokines produced *in vivo* are identical to those produced *in vitro*, more sophisticated characterization of these molecules is needed. This has recently been accomplished by the use of antibodies raised against highly purified human LIF. Thus, mononuclear blood cells from patients with infectious mononucleosis spontaneously release a mediator immunochemically similar to LIF ob-

tained from Con A-stimulated lymphocytes *in vitro* (Palit *et al.*, 1978).

Lymphokines generate *in vitro* have also been demonstrated to exhibit biologic activities *in vivo* when administered to animals. Skin inflammation resembling typical delayed-type reactions [previously considered to be due to a separate lymphokine, the skin reactive factor (see Bloom, 1971)], chronic synovitis (Andreis *et al.*, 1974), alteration of lymph node architecture and germinal center proliferation (Kelley *et al.*, 1972; Kelley and Wolstencroft, 1974), and disappearance of macrophages from the peritoneal cavity (Sonozaki and Cohen, 1971) have been shown to follow local injections of lymphokines. By analogy to the latter reaction, intravenous injection of antigen into previously immunized animals results in a reduction in the number of circulating monocytes (Yoshida and Cohen, 1974). This reaction has been shown to be a function of the state of delayed hypersensitivity of the animals and may, therefore, be a manifestation of lymphokines released during cell-mediated immune reactions *in vivo*. Other lymphokines will enhance antibody production when injected (Krejci *et al.* 1973), and local injection of lymphokine-rich materials into cutaneous tumors in man has been shown to cause their temporary regression (Papermaster *et al.*, 1976).

In a discussion of the *in vivo* significance of lymphokines, it should be emphasized that some of the biologic assays discussed in this paper have already proved to be clinically valuable, since the antigen-induced production *in vitro* of MIF, LIF, and other mediators is closely associated with the presence of cell-mediated immunity *in vivo* (David and David, 1972). Moreover, the mitogen-induced production of LIF and the production of LIF in mixed lymphocyte culture reactions have proved to be useful for assessing immunocompetence in man (Gorski *et al.*, 1976b,1977).

VII. Clinical Relevance of the Measurement of Lymphokines

The detection of lymphokine production has been used to evaluate cellular immunity in a wide variety of clinical situations (Rocklin *et al.*, 1974a). The presence of sensitized lymphocytes has been detected to tissue and tumor antigens, substances that should ordinarily not be injected *in vivo* to elicit delayed cutaneous hypersensitivity reactions. In addition, measurement of lymphokine production has been used to detect drug sensitivity. It should be noted that the presence of antigen-sensitized cells does not necessarily imply a pathogenic role in a disease, since it must be recalled that both cellular immunity and antibody production may arise secondary to tissue damage rather than be the cause of it. It would appear at present that the usefulness of these

assays would be to assess cellular immunity per se in patients suspected of having a defect in this system, and to monitor the effects of therapy on the immune system.

Abnormal production of lymphokines may occur for a variety of reasons. These would include a defect in lymphocyte function, a defect in macrophage function related to lymphocyte activation, a lack of antigen-specific lymphocytes that can produce the lymphokine(s), their blocking or destruction by regulatory substances or drugs, and intrinsic deficiencies or external factors that prevent the target cells from responding. Further studies leading to the purification of lymphokines, including the generation of antisera to individual lymphokines, classification of the lymphocyte subpopulations producing them, understanding their action and their control, and the development of more quantitative assays should clarify the basic function of each and greatly add to their clinical usefulness.

VIII. Monokines

Mononuclear phagocytes have a central role in host resistance and inflammation. They are actively involved in phagocytosis and digestion of microorganisms, cells, and debris. In addition, they have evolved a close functional association with lymphocytes that participate in the induction of specific immunity to antigens. The means by which mononucler phagocytes carry out their many functions remain unclear. In this part of the review, we discuss the possible biologic role of secretory products from monocytes–macrophages in mediating immunologic reactions.

Mononuclear phagocytes secrete a vast array of molecules with a large number of activities. Some of these products are summarized in Table XIII. In this review on chemical mediators of immunity, we have arbitrarily chosen to focus only on macromolecular products of mononuclear phagocytes, as only these are generally considered to be "monokines." Some monokines may have dual functions. For example, it has been demonstrated that complement and interferon have regulatory functions as well as being important in host defense mechanisms. A paucity of information is present concerning the biologic significance of the proteinases secreted by macrophages, although important regulatory as well as catabolic functions have been postulated.

A. Lymphocyte Activating Factor

The activity called lymphocyte activating factor (LAF) was first described by Gery and co-workers (Gery *et al.*, 1971; Gery and Waksman, 1972), who demonstrated that murine thymocytes cultured in the

TABLE XIII
MONOKINES: REGULATORY PROTEINS SECRETED BY MONONUCLEAR PHAGOCYTES

Lymphocyte activating factor (LAF)
B cell differentiation factor (PFC helper factor)
B cell activating factor (BAF)
Monocyte pyrogen (EP)
Thymic differentiation factor (TDF)
Colony-stimulating activity (CSA)
Mononuclear cell factor (MCF)
Neutral proteinases
Plasminogen activator
Elastase
Collagenase
Complement proteins

presence of supernatants derived from human or murine cells stimulated with bacterial LPS or PHA exhibited enhanced DNA synthesis. Subsequent studies showed that this activity was derived from human- and murine-adherent cells (Gery and Waksman, 1972), and recent work has confirmed that macrophages are the cellular source of LAF in experiments using murine peritoneal cells, human peripheral blood monocytes, and murine macrophage cell lines (Calderon *et al.*, 1975; Unanue *et al.*, 1976b; Blyden and Handschumacher, 1978; Lachman *et al.*, 1977a,b). These studies also established that a variety of stimuli in addition to LPS and PHA could be used to induce LAF production by monocytes–macrophages. Latex particles, antibody-coated red cells, viable *Listeria* organisms, antigen–antibody complexes, barium and beryllium salts, dimethyl sulfoxide, and phorbol myristate acetate (PMA) were found to increase LAF production by murine and human mononuclear phagocytes (Unanue *et al.*, 1976a; Blyden and Handschumacher, 1978; Mizel *et al.*, 1978a). Production of LAF by murine peritoneal macrophages or the macrophage tumor line $P388D_1$ can also be enhanced by coculture with activated T lymphocytes via a cell contact-dependent mechanism (Unanue *et al.*, 1976a; Mizel *et al.*, 1978b). Meltzer and Oppenheim (1977) have also reported enhanced LAF production following treatment of murine peritoneal cells with lymphokine-rich supernatants.

Lymphocyte activating factor from several sources has been partially purified. Some of the physical characteristics are summarized in Table XIV. Human LAF has an apparent molecular weight of 12,000–14,000 and a p*I* of 6.8–7.0. Additional peaks of higher apparent molecu-

TABLE XIV

PHYSICAL CHARACTERISTICS OF LOW MOLECULAR WEIGHT MONOKINES

Mediator	Source: species cell	Usual stimulant	Molecular weight		Isoelectric point	References[a]
			Monomer	Polymers		
LAF	Human monocyte	LPS	12,000–14,000	85,000	6.8–7.0[b]	(1–3)
	Human leukocyte	Allogeneic cells	14,000–15,000	—	—	(4)
	Mouse P388D$_1$	LPS, PMA	16,000	75,000–85,000[c]	5.0–5.4	(5)
	Mouse PEC	None	13,000–21,000	—	—	(6)
BAF	Human monocyte	LPS	18,000	—	—	(7)
EP	Human monocyte	Staphlococcus, LPS	15,000	38,000–45,000[d]	6.9	(8)
MCF	Human monocyte	Con A	14,000	—	—	(9)
PFC-helper	Human leukocyte	Allogeneic cells	14,000–15,000	—	—	(4)

[a] Key to references: (1) Koopman *et al.*, 1978; (2) Blyden and Handschumacher, 1978; (3) Lachman *et al.*, 1977b; (4) Koopman *et al.*, 1977; (5) Mizel *et al.*, 1978a; (6) Calderon *et al.*, 1975; (7) Wood and Cameron, 1978; (8) Dinarello, 1979; (9) Dayer *et al.*, (in press).

[b] p*I* of polymers probably 5.2–5.9 (Lachman *et al.*, 1977b).

[c] Only form isolated by Lachman *et al.* (1977a). Possibly a polymer or else a complex with albumin. p*I* = 4.8–5.0.

[d] Trimer with a p*I* of 5.1 (Dinarello *et al.*, 1974).

lar size have been reported but not further characterized (Gery and Handschumacher, 1974; Wood *et al.*, 1976; Biller and Unanue, 1977; Blyden and Handschumacher, 1978). At present, it is unclear whether these represent aggregates of low molecular weight LAF or whether LAF can exist as a complex with a carrier protein such as albumin. Murine LAF has a similarly low molecular size with the exception of one report for LPS-induced LAF from the cell line $P388D_1$ (Lachman and Metzgar, 1979). Both human and murine LAF exhibit charge heterogeneity when further purified on DEAE (Koopman *et al.*, 1977, 1978; Mizel *et al.*, 1978c). Other properties of human and murine LAF include resistance to treatment with periodate, iodacetate, protease inhibitors (PMSF or DFP), trypsin, papain, and neuraminidase, whereas chymotrypsin and Pronase readily degrade LAF (Calderon *et al.*, 1975; Blyden and Handschumacher, 1978; Mizel, 1979).

Expression of LAF activity occurs readily across allogeneic and xenogeneic barriers (Gery and Waksman, 1972; Calderon *et al.*, 1975; Blyden and Handschumacher, 1978). In addition to its direct thymocyte mitogenic effect, LAF is also capable of augmenting PHA- or concanavalin A-induced thymocyte proliferation (comitogenic effect).

It is also likely that LAF is the same molecule as that stimulating plaque-forming cell responses, as discussed in the next two sections of this review.

B. B Cell Differentiating Factor or PFC Helper Factor

Murine macrophages and human adherent peripheral blood leukocytes spontaneously elaborate an activity with an apparent molecular weight of 13,000–15,000 that augments *in vitro* plaque-forming cell (PFC) response of T-deficient mouse spleen cells or nude mouse spleen cells (Calderon *et al.*, 1975; Koopman *et al.*, 1977, 1978). The production of this activity can be enhanced by conditions exactly analogous to those discussed above for LAF, i.e., the addition of macrophage activators such as latex, LPS, or activated T cells (Unanue *et al.*, 1976a,b); Koopman *et al.*, 1977). As we have mentioned in the preceding section, it is likely that LAF and PFC helper activities are properties of a single molecule. Neither group of investigators studying these activities has been able to resolve one from another. In particular, Koopman *et al.* (1977, 1978) have systematically but unsuccessfully attempted to do this. It is important to point out that Koopman *et al.* (1977) have identified two PFC-helper factors derived from human mixed leukocyte cultures, which they designated HP-1 (MW 14,000) and HP-2 (MW 40,000–52,000). HP-1 and HP-2 act synergistically to augment PFC responses. Although HP-1 is probably identical to LAF,

HP-2 does *not* appear to be high molecular weight LAF. HP-2 is most likely derived from lymphocytes and is probably lymphocyte mitogenic factor (LMF), which is generated in the mixed allogeneic cell cultures.

During their attempts to resolve LAF from PFC-helper factor, Koopman *et al.* (1977, 1978) have partially purified HP-1 using Sephadex gel filtration, DEAE-cellulose chromatography, CM-cellulose chromatography, and polyacrylamide gel electrophoresis, and they have obtained results equivalent to those reported by others for LAF (Blyden and Handschumacher, 1978; Calderon *et al.*, 1975; Mizel *et al.*, 1978c; Mizel, 1979).

C. B Cell Activating Factor

Wood and co-workers have described a monokine derived from human monocytes that enhances *in vitro* PFC responses of T-depleted murine spleen cells, which they have named B cell activating factor (BAF) (Wood and Cameron, 1978). Although this activity is essentially the same as that described in the preceding section, it has been argued that BAF is a distinct monokine based on the apparent separation of BAF from LAF (Wood *et al.*, 1976).

Human monocyte supernatants containing BAF activity were concentrated by ultrafiltration through XM-50 membranes, and the retentate was chromatographed on Sephadex G-75. Columns were assayed for LAF, CSA, and BAF activities. The BAF eluted with an apparent molecular weight of 18,000. This is surprising since XM-50 membranes generally do not retain molecules much less then 50,000 MW. This suggested that BAF was associated with a carrier molecule during ultrafiltration and dissociated during gel filtration. Both CSA and LAF activities were eluted with the void volume. No low molecular weight LAF was found in these experiments, suggesting that this activity may have been lost during XM-50 concentration. Further purification data will be required to determine more definitively whether BAF and LAF-PFC helper factor are indeed discrete molecules (with identical activities and sizes).

Production of BAF, like that of LAF, can apparently be enhanced by a variety of monocyte stimulants including LPS, PHA, and mycostatin (Wood and Cameron, 1978). However, a recent report has established that all the stimuli used, as well as the commercial tissue culture medium in which the cells are grown, is usually contaminated with very small amounts of LPS and that this is sufficient to induce BAF production. Dose response curves of LPS-induced BAF production have shown that 10^{-9} to 10^{-12} gm of LPS per milliliter is sufficient to trigger near maximal BAF (Wood and Cameron, 1978). This report takes on

added significance when viewed in the context of the observation by Lachman and Metzgar(1979) that LAF production by human monocytes can also be triggered by similar concentrations of LPS. The amounts of LPS needed for maximal BAF or LAF induction are so small that, unless extraordinary care is taken, almost all manipulations will lead to monocyte activation by the contaminating LPS. Because of these observations, it now becomes necessary to reexamine for LPS content, by means of the *Limulus* lysate assay, all stimuli that are applied to human monocytes in order to rule out this artifact.

D. Monocyte Pyrogen

Phagocytic leukocytes can be stimulated to release endogenous pyrogen by a wide variety of exogenous substances, such as viruses, bacteria, bacterial products, LPS, pyrogenic steroids, and adjuvants (Bodel, 1974; Dinarello, 1979).

Mononuclear phagocytes can also be stimulated to release endogenous pyrogen if cocultured with activated T cells or lymphokine-rich supernatants derived from sensitized lymphocytes activated by specific antigen (Atkins and Francis,1978; Atkins *et al.*, 1978). This lymphokine appears to act selectively on monocytes. It is clear that conditions favorable for release of monocyte pyrogen resemble those already discussed for LAF.

Human monocyte pyrogen has been purified and shown to have a molecular weight of 15,000 and a p*I* of 6.9 (Dinarello, 1979). This subunit readily aggregates to a trimer (MW 45,000) with a p*I* of 5.1 (Dinarello, 1979; Dinarello *et al.*, 1974). Lachman *et al.* (1977b) have shown that human LAF has essentially identical physical characteristics, raising the possibility that these two activities could reside within a single molecule. Support for such a possibility was provided by the studies of Rosenwasser *et al.* (1979) who demonstrated that affinity purified human monocyte derived pyrogen could subserve a LAF-like function in antigen-specific T cell activation. Additional similarities are that neither molecule contains neuraminic acid and both are active across allogeneic and xenogeneic barriers. A possible difference is that pyrogen appears to be somewhat more labile in storage than LAF and that pyrogen is degraded by trypsin whereas LAF is not [although LAF has the properties of a protein as shown by its degradation by chymotrypsin (Blyden and Handschumacher, 1978; Dinarello *et al.*, 1974)].

More recent data have established that a number of mouse histiocytic and myelomonocytic tumor cell lines spontaneously release both endogenous pyrogen and LAF (Bodel, 1978), and preliminary observations suggest that both activities may be associated with high mo-

lecular weight molecules that could represent aggregates of the MW 15,000 subunit (Lachman *et al.*, 1977a; Bodel, 1978). By contrast, the human histiocytic tumor cell line U-937 does not produce LAF (Lachman and Metzgar, 1979) although it appears to release pyrogen (Bodel, 1978). If these observations are confirmed, this may be a strong argument against the possible molecular identity of human LAF and monocyte pyrogen.

E. Thymic Differentiation Factor

Murine peritoneal exudate cells can be stimulated by activated T cells or opsonized sheep erythrocytes (either *in vivo* or *in vitro*) to produce a thymic differentiation factor (TDF), also called thymic maturation factor (Unanue *et al.*, 1976b; Beller and Unanue, 1977). The TDF appears to be secreted by macrophages and eluted from Sephadex G-75 with an apparent molecular weight of 35,000–40,000. Immature thymocytes (low in H-2 antigen and high in TL antigen content) cultured in TDF for 1–2 days are converted into cells with high levels of H-2 antigen and low levels of TL antigen, which is characteristic of the cortisone-resistant mature thymocyte. Simultaneously, these mature thymocytes become better responder cells in an MLR- or PHA-induced proliferation assay. Maturation precedes thymocyte replication and is not inhibited by blocking thymocyte proliferation with mitomycin C. TDF activity cannot be replaced either with 2-mercaptoethanol or standard preparations of mouse interferon; TDF appears to be functioning in a manner analogous to thymic hormone. Since the thymus is known to contain macrophages *in vivo* and even when cultured *in vitro*, thymic epithelial cultures are contaminated with macrophages. The cellular source of thymic hormone therefore needs to be reevaluated.

F. Colony-Stimulating Activity

Colony-stimulating activity (CSA) has already been discussed in the section on lymphokines because many cell types, including lymphocytes, can be induced to produce CSA. Normal blood monocytes, peritoneal exudate macrophages, and monocyte tumor cell lines can be stimulatd to secrete CSA in response to a variety of stimuli, including LPS, synthetic adjuvants, opsonized zymosan, and phorbol myristate acetate (Ralph *et al.*, 1977; Staber *et al.*, 1978; Unanue, 1976). Little is known about the physical characteristics of the macrophage CSA.

G. Mononuclear Cell Factor

Human peripheral blood mononuclear phagocytes produce a factor, mononuclear cell factor (MCF), that stimulates collagenase and pros-

taglandin E_2 (PGE_2) release by cultured rheumatoid synovial cells several hundredfold (Dayer *et al.*, 1979, in press, submitted). The rheumatoid synovial cells can be distinguished from mononuclear phagocytes because they lack conventional macrophage markers. Mononuclear cell factor is made by purified monocytes, but cannot be detected in supernatants from purified T or B lymphocytes. There is no detectable collagenase activity in MCF (monocyte derived) or in the supernatants from purified T or B lymphocytes. The MCF production by monocytes is stimulated by addition of Con A, Fc fragments, latex, LPS, and activated T cells. Although some of these agents stimulate PGE_2 synthesis and secretion by human monocytes, the effect on MCF production is not mediated by PGE_2 because addition of indomethacin does not alter MCF production.

The apparent molecular weight of MCF based on gel filtration data is 14,000. The relationship of MCF to other monokines is not known at present. It is likely that MCF differs from LAF because the degree of stimulation of MCF obtained with LPS is small (about 2-fold), whereas Con A and Fc fragments produce a 10-fold enhancement.

H. Neutral Proteinases

Considerable attention has been given in recent years to the production and secretion of the neutral proteinases, plasminogen activator, elastase, and collagenase by monocytes and macrophages. Of these, plasminogen activator has been the most extensively studied. Monocytes–macrophages from mouse, human, rabbit, and guinea pig produce plasminogen activator (Unkeles *et al.*, 1974; Gordon *et al.*, 1974a; Werb, 1978), and the amount generated is modulated by inflammatory and immunologic stimuli. Resident peritoneal macrophages from untreated mice do not produce the enzyme whereas peritoneal macrophages obtained after intraperitoneal injection of inflammatory agents secrete plasminogen activator (Unkeles *et al.*, 1974). Phagocytosis of latex particles enhances plasminogen activator production of appropriately primed macrophages (e.g., endotoxin-induced peritoneal exudate cells) (Gordon *et al.*, 1974b). Other agents that increase macrophage plasminogen activator production include Con A, phorbol myristate acetate, asbestos, activated T lymphocytes, and lymphokine-rich supernatants. (Vassalli *et al.*, 1977; Greineder *et al.*, 1977; Klimetzek and Sorg, 1977). By contrast, plasminogen activator production is inhibited by glucocorticosteroids, colchicine, vinblastine, cholera toxin, and compounds increasing macrophage AMP (Vassalli *et al.*, 1976; Werb, 1978).

Similar results have been obtained in studies of macrophage elas-

tase and collagenase production, although the scope of these experiments has been more limited. Unstimulated mouse peritoneal macrophages produce little elastase, whereas alveolar macrophages or thioglycolate-induced peritoneal macrophages secrete easily detectable levels (White *et al.*, 1977; Werb and Gordon, 1975a). Phagocytic stimuli enhance elastase secretion whereas glucocorticosteroids inhibit elastase production (Werb and Gordon, 1975a; Werb, 1978). Similarly, unstimulated macrophages secrete little collagenase, whereas thioglycolate-induced or phagocytically active macrophages demonstrate markedly enhanced enzyme production (Werb and Gordon, 1975b). Treatment of guinea pig macrophages with lymphokine-rich supernatants enhances collagenase secretion (Wahl *et al.*, 1975). This contrasts with the data obtained with human monocytes where no collagenase activity is detected using mononuclear cell factor obtained from stimulated monocyte cultures (Dayer *et al.*, submitted). Glucocorticosteroids inhibit macrophage collagenase production (Werb, 1978).

I. Complement Components

Human monocytes and animal peritoneal macrophages have been demonstrated to secrete a number of complement components. Production of at least some of these components is augmented when macrophages are activated. C2, C4, and factor B have clearly been shown to be synthesized *de novo* by animal macrophages (Dolten, 1974; Hadding *et al.*, 1976). Production of C2 and C4 is markedly enhanced when macrophages are allowed to phagocytose heat-killed pneumococci (Colten, 1974). Human monocytes have also been shown to secrete C2, and they do so earlier and to an augmented degree when cocultured with activated T lymphocytes or lymphokine-rich supernatants (Einstein *et al.*, 1976; Littman and Ruddy, 1977). In these studies, the secreted complement components have been synthesized *de novo* by the macrophages as determined by incorporation of radioactive precursor amino acids into the complement molecule or by inhibition of complement secretion when macrophages are treated with cycloheximide (Colten, 1974; Littman and Ruddy, 1977; Einstein *et al.*, 1976; Hadding *et al.*, 1976).

Other Products Secreted by Mononuclear Phagocytes

In addition to the monokines described above, mononuclear phagocytes secrete other compounds that do not appear to qualify as monokines. Some of these include low molecular weight products, such as hydrogen peroxide, superoxide anion, prostaglandins, and thymidine.

The production of these agents is actively modulated by the monocytes–macrophages in response to a variety of stimuli, and the agents themselves have important regulatory functions. We have not included them in our list of monokines largely because of their low molecular weight.

Monocytes and macrophages also secrete lysozyme. Because lysozyme is considered to be a constitutive enzyme that is secreted at a relatively fixed rate and independent of macrophage activation, it is not likely to have a regulatory role such as that of the other monokines. For this reason, we have not discussed lysozyme in greater detail.

The lysosomal hydrolases appear to represent another group of monocyte products whose primary role is more likely to involve intracellular metabolism than extracellular mediator activity. However, recent studies make that categorization more problematic because macrophages may regulate secretion and synthesis of these enzymes in response to a variety of immunologically relevant stimuli (Schnyder and Baggliolini, 1978; Pantalone and Page, 1977).

IX. Biologic Significance of Monokines

Monocytes and macrophages have a central role in host defenses and cellular immunity. The mechanisms by which they perform their many functions remain to be clarified, and so it is not surprising that the *in vivo* significance of the monokines also remains unclear. Since the early experiments in cellular immunology, macrophage culture fluid or "conditioned medium" has frequently been found to replace some or all of the requirement for macrophages in *in vitro* systems. It seems likely therefore that at least some monocyte–macrophage functions are mediated through soluble factors. At present, the only monokine with a well established *in vivo* function is monocyte endogenous pyrogen, and even in this case, it is likely that the fever is only one manifestation of the full biologic spectrum of pyrogen activity. This concept is illustrated by the expanding range of biologic functions currently being ascribed to one monokine, LAF. Initially, LAF was identified as a factor mitogenic for thymocytes. At present, it appears likely that LAF may be identical to pyrogen, PFC helper factor and B-cell activating factor. The *in vivo* range of LAF activity may well be even broader and include activities that have as yet no *in vitro* correlates. The idea of one factor with multiple activities has already been established for other mediators, such as interferon. Similarly, the activity of the neutral proteases produced by monocytes will most likely

not be limited to a single substrate or even a single biologic unit. This is illustrated by the multiplicity of functions already ascribed to plasminogen activator, which is currently implicated in ovulation, insulin secretion, and B cell activation, in addition to its role in fibrinolysis. As a consequence, we suggest that the most appropriate question to ask about the biologic significance of monokines is not whether the mediators have an *in vivo* function, but rather which of the several functions that each mediator is likely to have will be of greatest importance to the maintenance of homeostasis.

X. Conclusion

We have described in this review a variety of soluble factors that are prime candidates for mediating various immunologic reations, particularly those relating to cellular immunity. As more and more information is uncovered relating to their *in vivo* effects, the exact extent of their role in these reactions will be more clearly defined. One of the more interesting observations made is that these factors can be produced by a variety of nonlymphoid sources. This implies a more general biologic role for lymphokines and monokines in host defense and other homeostatic mechanisms. What still remains unique about this process, however, is the fact that the lymphocyte has evolved a special triggering mechanism, which the other cells capable of producing these factors do not have. The latter can be viewed as another example of the redundancy present in biologic systems. The ability of cell types other than lymphocytes to produce lymphokine- and monokine-like factors provides a safeguard for the organism. Furthermore, the biologic effects of lymphokines and monokines can be duplicated to varying degrees by other immunologic systems, such as antigen–antibody complex effects on target cells and various components of the complement system.

By virtue of the nature of their biologic activity, a role for lymphokines and monokines in the expression of cellular immunity can be suggested. After activation of T or B lymphocytes, substances would be produced to mobilize cells both by way of vessel permeability effects (skin reactive factor or vascular permeability factor) and by direct chemotactic activity on macrophages, neutrophils, basophils, eosinophils, and other lymphocytes. In the classical delayed-type hypersensitivity reaction, the macrophage infiltration predominates; in the cutaneous basophil type of hypersensitivity, basophils predominate; in some types of tumor rejection, lymphocytes are present in large num-

bers; and in chronic reactions, eosinophils may be present to a greater degree than other inflammatory cells. Once the inflammatory cells, e.g., macrophages and PMN leukocytes, are recruited to the site, their random movement can be retarded by MIF and LIF so that they are retained there. Furthermore, as suggested previously, this may be followed by an activated state. The latter would lead to enhanced phagocytosis and suppression of intracellular replication of a variety of bacterial, viral, or other pathogens and, in some instances, an ability to kill them. The extracellular release by activated macrophages of lysosomal hydrolases as well as other monokines, could result in both target cell damage (e.g., tumors) and damage to normal tissue. Release of lymphotoxin or growth inhibitory factors would have corresponding effects on the target cell, as would interferon on viral replication.

This reaction could be amplified by the lymphocyte mitogenic factors and by the monokine: lymphocyte activating factor. The latter would have the effect of nonspecifically activating lymphocytes so that they could produce more lymphokines, this resulting in the recruitment and activation of other cells. The elaboration of a procoagulant activity initiates the clotting system and subsequent deposition of fibrin, in the tissues and intravascularly. Lymphokines and monokines could also be involved in a healing phase of this reaction by the production of factors that stimulate collagen synthesis. It may turn out that the nonimmunologic function of lymphokines and monokines may be as important as the immunologic ones.

Lymphokines and monokines are also involved in the regulation of antibody synthesis. It appears that the interaction of specific antigen with the appropriate immunoglobulin receptor on B cells provides an initial signal for cell activation but is not sufficient to trigger immunoglobulin synthesis unless a T helper lymphokine (second signal) is also provided. Monokines, such as LAF, could be involved in activating T helper cells to facilitate production of helper factors and directly activate B cells as well (B cell differentiation factor and BAF). The antigen nonspecific helper lymphokines could also help amplify the amount of antibody made. On the other hand, antigen-specific and nonspecific suppressor factors could decrease the amount of antibody synthesized, thus regulating excess production. Furthermore, the suppressor factors could also terminate antibody responses altogether via effects on the B cell itself or by suppressing the function of the T helper cell. Depending upon the relative amounts of helper and suppressor factors, the net result would be a given level of specific antibody. With further advances in the biochemical isolation of these factors, more insight will be provided for their role in these processes.

REFERENCES

Altman, L. C., Snyderman, R., Oppenheim, J. J., and Mergerhagen, S. E. (1973). *J. Immunol.* **110,** 801.

Altman, L. C., Chassy, B., and Mackler, B. F. (1975). *J. Immunol.* **115,** 18.

Ambrose, C. T. (1969). *J. Exp. Med.* **130,** 1003.

Ambrose, C. T. (1973). *J. Exp. Med.* **137,** 1369.

Amos, H. E., and Lachmann, P. J. (1970). *Immunology* **18,** 269.

Andreis, M., Stastny, P., and Ziff, M. (1974). *Arthritis Rheum.* **17,** 537.

Armerding, D., and Katz, D. H. (1974). *J. Exp. Med.* **140,** 19.

Armerding, D., Eshhar, Z., and Katz, D. H. (1977). *J. Immunol.* **119,** 1468.

Astor, S. H., Spitler, L. E., Frick, O. L., and Fudenberg, H. H. (1973). *J. Immunol.* **110,** 1174.

Atkins, E., and Francis, L. (1978). *Infect. Immun.* **21,** 806.

Atkins, E., Francis, L., and Bernheim, H. (1978). *Infect. Immun.* **21,** 813.

Bain, B., and Lowenstein, L. (1964). *Science* **145,** 1315.

Balow, J. E., and Rosenthal, A. S. (1973). *J. Exp. Med.* **137,** 1031.

Bartfeld, H., and Atoynatan, T. (1971a). *Nature (London), New Biol.* **230,** 246.

Bartfeld, H., and Atoynatan, T. (1971b). *Nature (London), New Biol.* **231,** 157.

Bendtzen, K. (1975a). *Acta Allergol.* **30,** 327.

Bendtzen, K. (1975b). *Acta Pathol. Microbiol. Scand. Sect. C* **83,** 447.

Bendtzen, K. (1976). *Acta Pathol. Microbiol. Scand. Sect. C* **84,** 471.

Bendtzen, K. (1977a). *Cell. Immunol.* **29,** 382.

Bendtzen, K. (1977b). *Scand. J. Immunol.* **6,** 125.

Bendtzen, K. (1977c). *Scand. J. Immunol.* **6,** 133.

Bendtzen, K. (1977d). *Scand. J. Immunol.* **6,** 1055.

Bendtzen, K. (1977e). *Scand. J. Immunol.* **6,** 1357.

Bendtzen, K. (1978). *Scand. J. Immunol.* **8,** 53.

Bendtzen, K. (1979a). *J.Clin. Lab. Immunol.* **2,** 37.

Bendtzen, K. (1979b). *Biochim. Biophys. Acta* **566,** 183.

Bendtzen, K., and Klysner, R. (1980). *Immunopharmacology* (in press).

Bendtzen, K., and Palit, J. (1977). *Acta Pathol. Microbiol. Scand. Sect. C* **85,** 317.

Bendtzen, K., Anderson, V., andBendixen, G. (1975). *Acta Allergol.* **30,** 133.

Bendtzen, K., Thode, J., and Madsen, S. N. (1977). *Acta Pathol. Microbiol. Scand. Sect. C* **85,** 473.

Block, L. H., Jaksche, H., Bamberger, S., and Ruhenstroth-Bauer, G. (1978). *J. Exp. Med.* **147,** 541.

Blomgren, H. (1976a). *Scand. J. Immunol.* **5,** 1173.

Blomgren, H. (1976b). *Scand. J. Immunol.* **5,** 1179.

Bloom, B. R. (1971). *Adv. Immunol.* **13,** 101.

Bloom, B. R., and David, J. R. eds. (1976). "*In Vitro* Methods of Cell-Mediated and Tumor Immunity." Academic Press, New York.

Bloom, B. R., Gaffney, J., and Jimenez, L. (1972). *J. Immunol.* **109,** 1395.

Blyden G., and Handschumacher, R. E. (1978). *J. Immunol.* **118,** 1631.

Bodel, P. (1974). *J. Exp. Med.* **140,** 954.

Bodel, P. (1978). *J. Exp. Med.* **148,** 1503.

Bodel, P., and Miller, H. (1976). *Proc. Soc. Exp. Biol. Med.* **151,** 93.

Boulos, G. N., Rosenau, W., and Goldberg, M. L. (1974). *J. Immunol.* **112,** 1347.

Calderon, J., Kiely, J. M., Lefke, J. L., and Unanue, E. R. (1975). *J. Exp. Med.* **142,** 151.

Calebaugh, D. L., and Paque, R. E. (1974). *Cell. Immunol.* **11,** 286.

Cantor, H., Shen, F. W., and Boyse, E. A. (1976). *J. Exp. Med.* **143,** 1391.

Cerottini, J. C., and Brunner, K. T. (1974). *Adv. Immunol.* **18,** 67.
Chess, L., Rocklin, R. E., MacDermott, R. P., David, J. R., and Schlossman, S. F. (1975). *J. Immunol.* **115,** 315.
Clausen, J. E. (1971). *Acta Allergol.* **26,** 56.
Clausen, J. E. (1974). "The Agarose Migration Migration Inhibition Technique for in Vitro Demonstration of Cell-Mediated Immunity in Man. A Review." N. O. Møllers Bog-Frykkeu, Copenhagen.
Cline, M. J., and Swett, V. C. (1968). *J. Exp. Med.* **128,** 1309.
Coeugniet, E., Bendtzen, K., Søeberg, B., and Bendixen, G. (1977). *Acta Med. Scand.* **201,** 197.
Cohen, M. C., Zeschke, R., Bigazzi, P. E., Yoshida, T., and Cohen, S. (1975). *J. Immunol.* **114,** 1641.
Cohen, S., and Ward, P. A. (1971). *J. Exp. Med.* **133,** 133.
Cohen, S., and Yoshida, T. (1977). *J. Immunol.* **119,** 719.
Cohen, S., Ward, P. A., Yoshida, T., and Burek, C. L. (1973). *Cell. Immunol.* **9,** 363.
Cohen, S., Fisher, B., Yoshida, T., and Bettigole, R. E. (1974). *N. Engl. J. Med.* **290,** 882.
Colten, H. R. (1974). *Prog. Immunol.* **1,** 183.
Colvin, R. B., Johnson, R. A., Mihm, M. C., and Dvorak, H. F. (1973). *J. Exp. Med.* **138,** 686.
Coyne, J. A., Remold, H. G.,Rosenberg, S. A., and David, J. R. (1973). *J. Immunol.* **110,** 1630.
David, J. R., and Becker, E. L., (1974). *Eur. J. Immunol.* **4,** 287.
David, J. R., and David, R. A. (1972). *Prog. Allergy* **16,** 300.
David, J. R., and Rocklin, R. E. (1978). *In* "Immunologic Diseases" (M. Samter, ed.), p. 307. Little, Brown, Boston, Massachusetts.
Dayer, J. M., Breard, J., Chess, L., and Krane, S. M. (submitted for publication).
Dayer, J. M., Goldring, S. R., Robinson, D. R., and Krane, S. M. (1979). *Biochim Biophys. Acta* (in press).
Daynes, R. A., and Granger, G. A. (1974). *Cell. Immunol.* **12,** 252.
Dinarello, C. A. (1979). *Fed. Proc., Fed. Am. Soc. Exp.Biol.* **38,** 52.
Dinarello, C. A., Goldin, N. P., and Wolff, S. M. (1974). *J. Exp. Med.* **139,** 1369.
Douglas, G. N., and Rubin, A. S. (1977). *Immunology* **33,** 745.
Dumonde, D. C., Page, D. A., Matthew, M., and Wolstencroft, R. A. (1972). *Clin. Exp. Immunol.* **10,** 25.
Dutton, R. W., Falkoff, R., Hirst, J. A., Hoffmann, M., Kappler, J. W., Kettman, J. R., Lesley, J. F., and Vann, D. (1971). *Prog. Immunol.* **1,** 355.
Dvorak, H. F., Orenstein, N. S., Rypysc, J., Colvin, R. B., and Dvorak, A. M. (1978). *J. Immunol.* **120,** 766.
Economon, J. W., and Shin, H. S. (1978). *J. Immunol.* **121,** 1446.
Edwards, R. L., and Rickles, F.R. (1978). *Science* **200,** 541.
Einstein, L. P., Scheeberger, E. E., and Colten, H. R. (1976). *J. Exp. Med.* **143,** 114.
Epstein, L. B., Cline, M. J., and Merigan, T. C. (1971a) *Cell. Immunol.* **2,** 602.
Epstein, L. B., Cline, M. J., and Merigan, T. C. (1971b). *J. Clin. Invest.* **50,** 744.
Eshhar, Z., Armerding, D., Waks, T., and Katz, D. (1977). *J. Immunol.* **119,** 1457.
Evans,R., Grant, C. K., Cox, H., Steele, K., and Alexander, P. (1972). *J. Exp. Med.* **136,** 1318.
Farrar, J. J., and Koopman, W. J. (1978). *In* "Biology of the Lymphokines" (S. Cohen, E. Pick, and J. J. Oppenheim, eds.). Academic Press, New York.
Feldmann, M., and Basten, A. (1972). *J. Exp. Med.* **136,** 49.
Feldmann, M., Basten, A., Boylston, A., Erb, P., Gorczynski, R., Greaves, M., Hogg, N.,

Kilburn, D., Kontiainen, S., Parker, D., Pepys, M., and Schrader, J. (1974). *Prog. Immunol. II* **3,** 65.
Feldmann, M., Beverley, P. C. L., Woody, J., and McKenzie, I. F. C. (1977). *J. Exp. Med.* **145,** 793.
Ferrier, M., Birrien, J. L., Leclere, C., Chedid, L., and Liacopoulos, P. (1978). *Eur. J. Immunol.* **8,** 558.
Flanagan, T. D., Yoshida, T., and Cohen, S. (1973). *Infect. Immun.* **8,** 145.
Florentin, I., Bruley, M., and Belpomme, D. (1975). *Cell. Immunol.* **17,** 285.
Fowles, R. E., Fajardo, I. M., Leibowitch, J. L., and David, J. R. (1973). *J. Exp. Med.* **138,** 952.
Fridman, W. H., Nelson, R. A., and Liabeuf, A. (1974). *J. Immunol.* **113,** 1008.
Fridman, W. H., Guimezanes, A., and Gisler, R. H. (1977). *J. Immunol.* **119,** 1266.
Friedman, R. M. (1977). *Bacteriol. Rev.* **41,** 543.
Friedman, S. A., Remold-O'Donnell, E., Piessens, W. F. (submitted for publication)
Friedrich, W., Lazary, S., Geczy, C., and deWeck, A. L. (1975). *Int. Arch. Allergy* **49,** 504.
Friend, D. S., and Rosenau, W. (1977). *Am. J. Pathol.* **86,** 149.
Gately, C. L., Gately, M. K., and Mayer, M. M. (1975). *J. Immunol.* **114,** 10.
Gately, C. L., Gately, M. K., and Mayer, M. M. (1976). *J. Immunol.* **116,** 669.
Gately, M. K., Gately, C. L., Henney, C. S., and Mayer, M. M. (1975). *J. Immunol.* **115,** 817.
Gately, M. K., Mayer, M. M., and Henney, C. S. (1976). *Cell. Immunol.* **27,** 82.
Geczy, C. L. (1977). *J. Immunol.* **119,** 2107.
Geczy, C. L., Friedrich, W., and deWeck, A. L. (1975). *Cell Immunol.* **19,** 65.
Geczy, C. L., Geczy, A. F., and deWeck, A. L. (1976a). *J. Immunol.* **117,** 66.
Geczy, C. L., Geczy, A. F., and deWeck, A. L. (1976b). *J. Immunol.* **117,** 1824.
Geha, R. S., and Merler, E. (1974). *Cell. Immunol.* **10,** 86.
Gery, I., and Waksman, B. H. (1972). *J. Exp. Med.* **136,** 143.
Gery, I., Gershon, R. K., and Waksman, B. H. (1971). *J. Immunol.* **107,** 1778.
Godal, T., Rees, R. J. W., and Lamvik, J. O. (1971). *Clin. Exp. Immunol.* **8,** 625.
Goetzl, E. J., and Austen, K. F. (1972). *J. Exp. Med.* **136,** 1564.
Goetzl, E. J., and Rocklin, R. E. (1978). *J. Immunol.* **121,** 891.
Gorczynski, R. M., Miller, R. G., and Phillips, R. A. (1972). *J. Immunol.* 108, 547.
Gordon, D., Bray, M. A., and Morley, J. (1976). *Nature (London)* **262,** 401.
Gordon, J., and MacLean, L. D. (1965). *Nature (London)* **208,** 795.
Gordon, S. (1976). *Ann. N. Y. Acad. Sci.* **278,** 176.
Gordon, S., Todd, J., and Cohn, Z. A. (1974a). *J. Exp. Med.* **139,** 1228.
Gordon, S., Unkeles, J. C., and Cohn, Z. A. (1974b). *J. Exp. Med.* **140,** 995.
Gorski, A. J., Dupont, B., Hansen, J. A., and Good, R. A. (1975). *Proc. Natl. Acad. Sci.* **72,** 3197.
Gorski, A. J., Dupont, B., Hansen, J. A., and Good, R. A. (1976a). *J. Immunol.* **117,** 865.
Gorski, A. J., Dupont, B., Hansen, J. A., O'Reilly, R., Smithwick, E., Gorski, R., and Good, R. A. (1976b). *Clin. Exp. Immunol.* **26,** 505.
Gorski, A. J., Dupont, B., Hansen, J. A., Safai, B., Pahwa, S., and Good, R. A. (1977). *J. Immunol.* **118,** 858.
Granger, G. A., Moore, G. E., White, J. G., Matzinger, P., Sundsmo, J. S., Shupe, S., Kolb, W. P., Kramer, W. P., and Glade, P. R. (1970). *J. Immunol.* **104,** 1476.
Green, J. A., Cooperband, S. R., and Kibrick, S. (1969). *Science* **164,** 1415.
Greene, B. M., and Colley, D. G. (1974). *J. Immunol.* **113,** 910.
Greineder, D. K., Connorton, K. J., and David, J. R. (1977). *Clin. Res.* **25,** 483A.

Gresser, I. (1977). *Cell Immunol.* **34,** 406.
Haase, A. T., Johnson, J. S., Kasel, J. A., Margolis, S., and Levy, H. B. (1970). *Proc. Soc. Exp. Biol. Med.* **133,** 1076.
Hadden, J. W., Hadden, E. M., Meetz, G., Good, R. A., Haddox, M. K., and Goldberg, N. D. (1973). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **32,** 1022. (Abstr.)
Hadden, J. W., Johnson, E. M., Coffey, R. G., and Johnson, L. D. (1975a) *In* "Immune Recognition" (A. S. Rosenthal, ed.), p. 359. Academic Press, New York.
Hadden, J. W., Sadlik, J. R., and Hadden, E. M. (1975b). *Nature (London)* **257,** 483.
Hadden, J. W., Sadlik, J. R., and Hadden, E. M. (1978). *J. Immunol.* **121,** 231.
Hadding, U., Bentley, C., Bitter-Suermann, D., and Brede, V.(1976). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **35,** 654.
Hamilton, J., Vassali, J. D., and Reich, E. (1976). *J. Exp. Med.* **144,** 1689.
Hammond, M. E., Roblin, R. O., Dvorak, A. M., Selvaggio, S. S., Black, P. H., and Dvorak, H. F. (1974). *Science* **185,** 956.
Harrington, J. T., and Stastny, P. (1973). *J. Immunol.* **110,** 752.
Harwell, L., Kappler, J. W.,and Marrack, P. (1976). *J. Immunol.* **116,** 1379.
Havemann, K., Horvat, M., Sodomann, C. P., Havermann, K., and Burger, S. (1972). *Eur. J. Immunol.* **2,** 97.
Hay, J. B., Lachmann, P. J., and Truka, Z. (1973). *Eur. J. Immunol.* **3,** 127.
Henney, C. S., Gaffney, J., and Bloom, B. R. (1974). *J. Exp. Med.* **140,** 837.
Hentges, F., Geczy, C. L., Geczy, A. F., and deWeck, A. L. (1977). *Immunology* **32,** 905.
Hessinger, D. A., Daynes, R. A., and Granger, G. A. (1973). *Proc. Natl. Acad. Sci. U. S. A.***70,** 3082.
Hibbs, J. B. (1972). *Science* **180,** 868.
Higgins, T. J., Winston, C. T., and David, J. R. (1976). *Cell. Immunol.* **27,** 11.
Higgins, T. J., Sabatino, A. P., Remold, H. G., and David, J. R. (1978). *J. Immunol.* **121,** 880.
Hill, H. R., Estensen, R. D., Quie, P. G., Hogan, N. A., and Goldberg, N. D. (1975). *Metabolism* **24,** 447.
Hiserodt, J. C., and Granger, G. A. (1977). *J,. Immunol.* **119,** 374.
Hiserodt, J. C., Prieur, A. M., and Granger, G. A. (1976a). *Cell. Immunol.* **24,** 277.
Hiserodt, J. C., Fair, D. S., and Granger, G. A. (1976b). *J. Immunol.* **117,** 1503.
Hoffman, P. M., Spitler, L. E., Hsu, M., and Fudenberg, H. H. (1975). *Cell. Immunol.* **18,** 21.
Hoffman, P. M., Spitler, L. E. and Hsu, M. (1976). *Cell. Immunol.* **21,** 358.
Hoffman, P. M., Hanes, D. M., Horsmanheimo, M. and Fudenberg, H. H. (1977). *Cell. Immunol.* **30,** 92.
Holst, P. M. (1922) *Tubercle (Edinburgh)* **3,** 249.
Horton, J. E., Raisz, L. G., Simmons, H. A., Oppenheim, J. J., and Mergerhagen, S. E. (1972). *Science* **177,** 793.
Horton, J. E., Koopman, W. J., Farrar, J. J., Fuller-Bonar, J., and Mergerhagen, S. E. (1979). *Cell. Immunol.* **43,** 1.
Houck, J. C., and Chang, C. M. (1975). *Proc. Soc. Exp. Biol. Med.* **148,** 104.
Houck, J. C., Chang, C. M., and Platt, M. (1973). *Proc. Soc. Exp. Biol. Med.* **143,** 858.
Hubner, L., Muller, G., Schimpl, A., and Wecker, E. (1978). *Immunochemistry* **15,** 33.
Humes, J. L., Bonney, P. J., Pelus, L., Dahlgren, M. E., Sadowski, S. J., Kuehl, F. A., and Davies, P. (1977). *Nature (London)* **269,** 149.
Isaacs, A., and Lindemann, J. (1957). *Proc. R. Soc. London Ser B* **147,** 258.
Jacobson, E. B. (1973). *Eur. J. Immunol.* **3,** 619.
Jandinski, J., Cantor, H., Tadakuma, T., Peavy, D. L., and Pierce, C. W. (1976). *J. Exp. Med.* **143,** 1382.

Janis, M., and Bach, F. H: (1970). *Nature (London)* **225,** 238.
Jeffes, E. W. B., and Granger, G. A. (1976). *J. Immunol.* **117,** 174.
Jegasothy, B. V., Pachner, A. R., and Waksman, B. H. (1976). *Science* **193,** 1260.
Johnson, H. M. (1977). *Nature (London)* **265,** 154.
Johnson, H. M., Blalock, J. E., and Baron, S. (1977). *Cell. Immunol.* **33,** 170.
Jones, J. M., Hart, D. A., and Nisonoff, A. (1973). *Cell. Immunol.* **9,** 186.
Joskowicz, M., Raboardin-Combe, C., Neauport-Sautes, C., and Fridman, W. H. (1977). *J. Immunol.* **121,** 777.
Kataria, Y. P., LoBuglio, A. F., and Bromberg, P. A. (1976). *Am. Rev. Respir. Dis.* **113,** 315.
Kasakura, S. (1977). *Immunology* **33,** 23.
Kasakura, S., and Lowenstein, L. (1965). *Nature (London)* **208,** 794.
Kelley, R. H., and Wolstencroft, R. A. (1974). *Clin. Exp. Immunol.* **18,** 321.
Kelley, R. H., Wolstencroft, R. A., Dumonde, D. C., and Balfour, B. M. (1972). *Clin. Exp. Immunol.* **10,** 49.
Kishimoto, T., and Ishizaka, K. (1975). *J. Immunol.* **114,** 1177.
Klimetzek, V., and Sorg, C. (1977). *Eur. J. Immunol.* **7,** 185.
Kolb, W. P., and Granger, G. A. (1970). *Cell. Immunol.* **1,** 122.
Koopman, W. J., Gillis, M. H., and David, J. R. (1973). *J. Immunol.* **110,** 1609.
Koopman, W. J., Sandberg, A. L., Wahl, S. M., and Mergerhagen, S. E. (1976). *J. Immunol.* **117,** 331.
Koopman, W. J., Farrar, J. J., Oppenheim, J. J., Fuller-Bonar, J., and Dougherty, S. (1977). *J. Immunol.* **119,** 55.
Koopman, W. J., Farrar, J. J., and Fuller-Bonar, J. (1978). *Cell. Immunol.* **35,** 92.
Kotkes, P., and Pick, E. (1975a). *In* "The Immunological Basis of Corrective Tissue Disorders" (L. G. Silvestri, ed.), pp. 141–153. North-Holland Publ., Amsterdam.
Kotkes, P., and Pick, E. (1975b). *Clin. Exp. Immunol.* **19,** 105.
Kotkes, P. and Pick, E. (1977). *Isr. J. Med. Sci.* **13,** 1045. *(Abstr.)*
Krahenbuhl, J. L., Rosenberg, L. T., and Remington, J. S. (1973). *J. Immunol.* **111,** 992.
Krejci, J., Pekarek, J., Svejcar, J., and Johanovsky, J. (1973). *Cell. Immunol.* **7,** 322.
Krejci, J., Pekarek, J., Rozprimova, L., Svejcar, J., and Johanovsky, J. (1976). *Immunology* **31,** 283.
Kuhner, A. L., and David, J. R. (1976). *J. Immunol.* **116,** 140.
Kunitomi, G., Rosenau, W., Burke, G. C., and Goldberg, M. L. (1975). *Am. J. Pathol.* **80,** 249.
Kuratsuji, T., Yoshida, T., and Cohen, S. (1976). *J. Immunol.* **117,** 1985.
Kurland, J., and Bockman, R. (1978). *J. Exp. Med.* **147,** 952.
Lachman, L. B., and Metzgar, R. S. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 932.
Lachman, L. B., Hacker, M. P., Blyden, G. T., and Handschumacher, R. E. (1977a) *Cell. Immunol.* **34,** 416.
Lachman, L. B., Hacker, M. P., and Handschumacher, R. E. (1977b). *J. Immunol.* **119,** 2019.
Lee, S. C., and Lucas, Z. J. (1977). *J. Immunol.* **118,** 88.
Leu, R. W., Eddleston, A. L. W. F., Hadden, J. W., and Good, R. A. (1972). *J. Exp. Med.* **136,** 589.
Lewis, F. A., Carter, C. E., and Colley, D. G. (1977). *Cell. Immunol.* **32,** 86.
Lewis, J. E., Carmack, C. E., Yamamoto, R., and Granger, G. A. (1977). *J. Immunol. Methods* **14,** 163.
Lies, R. B. (1975). *Cell. Immunol.* **17,** 96.
Lies, R. B., and Peter, J. B. (1973). *Cell. Immunol.* **8,** 332.
Littman, B. H., and David, J. R. (1976). *Cell. Immunol.* **27,** 334. (Abstr.)

Littman, B. H., and David, J. R. (1978). *Cell. Immunol.* **39,** 374.
Littman, B. H., and Ruddy, S. (1977) *J. Exp. Med.* **145,** 1344.
Liu, D. Y., Higgins, T. J., Petschek, K. D., Remold, H. G., and David, J. R. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **37,** 1400. (Abstr.)
Lomnitzer, R., Rabson, A. R., and Koornhof, H. J. (1976a). *Clin. Exp. Immunol.* **24,** 42.
Lomnitzer, R., Rabson, A. R., and Koornhof, H. J. (1976b). *Clin. Exp. Immunol.* **25,** 303.
Lomnitzer, R.,Glover, A., and Rabson, A. R. (1977). *Clin. Exp. Immunol.* **29,** 501.
Loskutoff, D. J., and Edgington, T. S. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3903.
Lowe, D. M., and Lachmann, P. J. (1974). *Scand. J. Immunol.* **3,** 423.
Luben, R. A., Mundy, G. R., Trummel, C. L., and Raisz, L. G. (1974). *J. Clin. Invest.* **53,** 1473.
Mackaness, G. B. (1969). *J. Exp. Med.* **129,** 973.
Mackler, B. F., Altman, L. C., Rosenstreich, D. L., and Oppenheim, J. J. (1974). *Nature (London)* **249,** 834.
McLeod, T. F., Baskin, B. L., Meltz, S. K., Glade, C. S., and Glade, P. R, (1977). *Cell. Immunol.* **32,** 370.
Manheimer, S., and Pick, E. (1973). *Immunology* **24,** 1027.
Meade, C. J., Lachmann, P. J., and Brenner, S. (1974). *Immunology* **27,** 227.
Meltzer, M. S., and Oppenheim, J. J. (1977). *J. Immunol.* **118,** 77.
Meltzer, M. S., Tucker, R. W., Sanford, K. K., and Leonard, E. J. (1975). *J. Natl. Cancer Inst.* **54,** 1177.
Mills, J. A. (1975). *J. Immunol.* **114,** 45.
Mizel, S. B. (1979). *J. Immunol.* **122,** 2167.
Mizel, S.B., Oppenheim, J. J., and Rosenstreich, D. L. (1978a). *J. Immunol.* **120,** 1497.
Mizel, S. B., Oppenheim, J. J., and Rosenstreich, D. L. (1978b). *J. Immunol.* **120,**1504.
Mizel, S. B., Rosenstreich, D. L., and Oppenheim, J. J. (1978c). *Cell. Immunol.* **40,** 230.
Mizoguchi, Y., Yamamoto, S., and Morisawa, S. (1973). *J. Biochem.* **73,** 467.
Möller, G. (1975). *Transplant. Rev.* **23,** 136.
Morris, K. M., Colten, H. R., and Bing, D. H. (1978). *J. Exp. Med.* **148,** 1007.
Mundy, G. R., and Raisz, L. G. (1977). *J. Clin. Invest.* **60,** 122.
Mundy, G. R., Luben, R. A., Raisz, L. G., Oppenheim, J. J., and Buell, D. N. (1974a). *N. Engl. J. Med.* **290,** 867.
Mundy, G. R., Raisz, L. G., Cooper, R. A., Schechter, G. P., and Salmon, S. E. (1974b). *N. Engl. J. Med.* **291,** 1041.
Munro, A. J., and Taussig, M. J. (1975). *Nature (London)* **256,** 103.
Namba, Y., and Waksman, B. H. (1975a). *J. Immunol.* **115,** 1018.
Namba, Y., and Waksman, B. H. (1975b). *Inflammation* **I,** 5.
Namba, Y., and Waksman, B. H. (1976). *J. Immunol.* **116,** 1140.
Namba, Y., and Waksman, B. H. (1977). *Immunochemistry* **14,** 143.
Namba, Y., Jegasothy, B. V., and Waksman, B. H. (1977). *J. Immunol.* **118,** 1379.
Nathan, C. F., Karnovsky, M. L., and David, J. R. (1971). *J. Exp. Med.* **133,** 1356.
Nathan, C. F., Remold, H. G., and David, J. R. (1973). *J. Exp. Med.* **137,** 275.
Nelson, R. D., and Leu, R. W. (1975). *J. Immunol.* **114,** 606.
Neumann, C., and Sorg, C. (1977). *Eur. J. Immunol.* **7,** 719.
Neumann, C., and Sorg, C. (1978). *Eur. J. Immunol.* **8,** 582.
Neuport-Sautes, C., and Fridman, W. H. (1977). *J. Immunol.* **119,** 1269.
Neville, M. E., and Lischner, H. W. (1978). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **37,**1362. (Abst.)
Niemetz, J. (1972). *J. Clin. Invest.* **51,** 307.
Oates, C. M., Bissenden, J. F., Maini, R. N., Payne, L. N., and Dumonde, D. C. (1972). *Nature (London) New Biol.* **239,** 137.

Ohishi, M., and Onoue, K. (1975). *Cell. Immunol.* **18,** 220.
Oppenheim, J. J., Mizel, S. B., and Meltzer, M. S. (1978). *In* "Biology of the Lymphokines" (S. Cohen, E. Pick, and J. J. Oppenheim, eds.). Academic Press, New York.
Palit, J., Bendtzen, K., and Andersen, V. (1978). *Clin. Exp. Immunol.* **31,** 66.
Pantalone, R., and Page, R. C. (1977). *J. Reticubendothel. Soc.* **21,** 343.
Papageorgiou, P. S., Henley, W. L., and Glade, P. R. (1972). *J. Immunol.* **108,** 494.
Papermaster, B. W., Holtermann, O. A., Klein, E., Djerassi, I., Rosher, D., Dao, T., and Costanzi, J. J. (1976). *Clin. Immunol. Immunopathol.* **5,** 31.
Patterson, R. J., and Youmans, G. P. (1970). *Infect. Immun.* **1,** 600.
Pekarek, J., and Krejci, J. (1974). *J. Immunol. Methods* **6,** 1.
Pekarek, J., Svejcar, J., Nonza, K., and Johanovsky, J. (1976). *Immunology* **31,** 773.
Peter, J. B. (1971). *Cell. Immunol.* **2,** 199.
Peter, J. R., Konen, T., Stempel, K., and Cardin, C. (1971). *Arthritis Rheum.* **14,** 179.
Peter, J. B., Stratton, J. A., Stempel, K. E., Yu, D., and Cardin, C. (1973) *J. Immunol.* **111,** 770.
Petty, H. R., Smith, B. A., Ware, B. R., and Rocklin, R. E. (1980). *Cell. Immunol.*, in press.
Pick, E. (1972). *Isr. J. Med. Sci.* **8,** 654. (Abst.)
Pick, E. (1974). *Immunology* **26,** 649.
Pick, E. (1977a). *Cell. Immunol.* **32,** 329.
Pick, E. (1977b). *In* "Immunopharmacology" (J. W. Hadden and R. G. Coffey, eds.), p. 163. Plenum, New York.
Pick, E., and Abrahamer, H. (1973). *Int. Arch. Allergy* **44,** 215.
Pick, E., and Grunspan-Swirsky, A. (1977). *Cell. Immunol.* **32,** 340.
Pick, E., and Manheimer, S. (1973). *Int. Arch. Allergy* **45,** 295.
Pick, E., and Manheimer, S. (1974). *Cell. Immunol.* **11,** 30.
Pick, E., and Turk, J. L. (1972). *Clin. Exp. Immunol.* **10,** 1.
Pickel, K., Hammerling, V., and Hoffmann, M. K.(1976). *Nature (London)* **264,** 72.
Pierce, C. W., and Kapp, J. A. (1976). *Contemp. Top. Immunobiol.* **5,** 91.
Piessens, W. F., Churchill, W. H., and David, J. R. (1975). *J. Immunol.* **114,** 293.
Pitha, P. M. (1977). *Nature (London)* **269,** 374.
Plate, J. M. D. (1976). *Nature (London)* **260,** 329.
Plaut, M., Lichtenstein, L. M., and Henney, C. S. (1973). *J. Immunol.* **110,** 771.
Poste, G. (1975). *Cancer Res.* **35,**2558.
Postlethwaite, A. E., Snyderman, R., and Kang, A. H. (1976a). *J. Exp. Med.* **144,** 1188.
Postlethwaite, A. E., Townes, A. S., and Kang, A. H. (1976b). *J. Immunol.* **117,** 1716.
Prieur, A., and Granger, G. A. (1975). *Transplantation* **20,** 331.
Prystowsky, M. B., Sorokin, C. F., Ceglowski, W. S., Hirschhorn, K., and Glade, P. (1975). *Int. Arch. Allergy Appl. Immunol.* **48,** 225.
Ralph, P., Broxmeyer, H. E., and Nakoinz, I. (1977). *J. Exp. Med.* **146,** 611.
Remold, H. G. (1973). *J. Exp. Med.* **138,** 1065.
Remold, H. G. (1974). *J. Immunol.* **112,** 1571.
Remold, H. G. (1977). *J. Immunol.* **118,** 1.
Remold, H. G., and Mednis, A. D. (1977). *J. Immunol.* **118,** 2015.
Remold, H. G., and Rosenberg, R. G. (1975). *J. Biol. Chem.* **250,** 6608.
Remold, H. G., David, R. A., and David, J. R. (1972). *J. Immunol.* **109,** 578.
Remold-O'Donnell, E., and Remold, H. G. (1974). *J. Biol. Chem.* **249,** 3622.
Rich, A. R., and Lewis, M. R. (1932). *Bull. Johns Hopkins Hosp.* **50,** 115.
Rich, R. R., and Pierce, C. W. (1974). *J. Immunol.* **112,** 1360.
Rich, S. S., and Rich, R. R. (1975). *J. Exp. Med.* **142,** 1391.
Rich, S. S., and Rich, R. R. (1976). *J. Exp. Med.* **144,** 1214.

Rickles, F. R., and Bobrave, A. M. (1975). *Blood* **46,** 1046A.
Rickles, F. R., Hardin, J. A., Pitlick, F. A., Hager, L. W., and Conrad, M. E. (1973). *J. Clin. Invest.* **52,** 1427.
Rocklin, R. E. (1973). *J. Immunol.* **110,** 674.
Rocklin, R. E. (1974a). *Prog. Clin. Immunol.* **2,** 21.
Rocklin, R. E. (1974b). *J. Immunol.* **112,** 1461.
Rocklin, R. E. (1975). *J. Immunol.* **114,** 1161.
Rocklin, R. E. (1976). *J. Immunol.* **116,** 816.
Rocklin, R. E., and Rosenthal, A. S. (1977). *J. Immunol.* **119,** 249.
Rocklin, R. E., and Urbano, A. M. (1978). *J. Immunol.* **120,** 1409.
Rocklin, R. E., Rosen, F., and David, J. R. (1970). *N. Engl. J. Med.* **282,** 1340.
Rocklin, R. E., Remold, H. G., and David, J. R. (1972). *Cell Immunol.* **5,** 436.
Rocklin, R. E., MacDermott, R. P., Chess, L., Schlossman, S. F., and David, J. R. (1974a). *J. Exp. Med.* **140,** 1303.
Rocklin, R. E., Winston, C. T. and David, J. R. (1974b). *J. Clin. Invest.* **53,** 559.
Rose, N., and Friedman, H. (1976). "Manual of Clinical Immunology." American Society for Microbiology, Washington, D. C.
Rosenau, W., Goldberg, M. L., and Burke, G. C. (1973). *J. Immunol.* **111,** 1128.
Rosenstreich, D. L., Farrar, J., and Dougherty, S. (1976). *J. Immunol.* **116,** 131.
Rosenwasser, L. J., Dinarello, C. A., and Rosenthal, A. S. (1979). *J. Exp. Med.* **150,** 709.
Rothberger, H., Zimmerman, T. S., and Spregelberg, H. Z. (1977). *J. Clin. Invest.* **59,** 529.
Rothberger, H., Zimmerman, T. S., and Vaughn, J. H. (1978). *J. Clin. Invest.* **62,** 649.
Rubin, A. S., and Coons, A. H. (1972). *J. Immunol.* **108,** 1597.
Ruscetti, F. W., and Chervenick, P. A. (1975a). *J. Immunol.* **114,** 1513.
Ruscetti, F. W., and Chervenick, P. A. (1975b). *J. Clin. Invest.* **55,** 520.
Russell, S. W., Rosenau, W., Goldberg, M. L., and Kunitomi, G. (1972). *J. Immunol.* **109,** 784.
Salvin, S. B., and Nishio, J. (1972). *J. Exp. Med.* **135,** 985.
Salvin, S. B., Ribi, E., Granger, D. L., and Youngner, J. S. (1975). *J. Immunol.* **114,** 354.
Sandberg, A. L., Wahl, S. M., and Mergerhagen, S. G. (1975). *J. Immunol.* **115,** 139.
Savel, H., and Moehring, T. (1971). *Proc. Soc. Exp. Biol. Med.* **137,** 374.
Schimpl, A., and Wecker, E. (1972). *Nature (London) New Biol.* **237,** 15.
Schimpl, A., and Wecker, E. (1975). *Transplant. Rev.* **23,** 176.
Schmidt, M. E., Douglas, S. D., and Rubin, A. (1973). *Cell. Immunol.* **9,** 45.
Schnyder, J., and Baggliolini, M. (1978). *J. Exp. Med.* **148,** 435.
Shevach, E. M., Lee, L., and Ben-Sasson, S. Z. (1975). *In* "Immune Recognition" (A. Rosenthal, ed.), p. 627. Academic Press, New York.
Simon, H. B., and Sheagren, J. N. (1971). *J. Exp. Med.* **133,** 1377.
Smith, R. T., Bausher, J. A. C., and Adler, W. H. (1970). *Am. J. Pathol.* **60,** 495.
Snyderman, R., Meadows, L., and Amos, D. B. (1977). *Cell. Immunol.* **30,** 225.
Sonozaki, H., and Cohen, S. (1971). *Cell. Immunol.* **2,** 341.
Sonozaki, H., Papermaster, V., Yoshida, T., and Cohen, S. (1975). *J. Immunol.* **115,** 1657.
Sorg, C., and Bloom, B. R. (1973). *J. Exp. Med.* **137,** 148.
Sorg, C., and Geczy, C. L. (1976). *Eur. J. Immunol.* **6,** 688.
Stadtman, E. R. (1970). *In "The Enzymes"* (P. Boyer, ed.), 3rd ed., Vol. 1, p. 397. Academic Press, New York.
Stastny, P., Rosenthal, M., Andreis, M., and Ziff, M. (1975). *Arthritis Rheum.* **18,** 237.
Strom, T. B., Lundin, A. P., and Carpenter, C. B. (1977). *Prog. Clin. Immunol.* **3,** 115.
Svejcar, J., and Johanovsky, J. (1963). *In "Proceedings of the Fifth European Congress on Allergy,"* p. 375. Schwabe, Basel.

Svejcar, J., Johanovsky, J., and Pekarek, J. (1967). Z. *Immuntaetsforsch.* **133,** 259.
Tada, T., Okumura, K., and Taniguchi, M. (1973). *J. Immunol.* **111,** 952.
Tada, T., Taniguchi, M., and David, C. S. (1977). *Cold Spring Harbor Symp. Quant. Biol.* **41,** 119.
Tadakuma, T., and Pierce, C. W. (1976). *J. Immunol.* **117,** 967.
Tadakuma, T., and Pierce, C. W. (1978). *J. Immunol.* **120,** 481.
Tadakuma, T., Kuhner, A. L., Rich, R. E., David, J. R., and Pierce, C. W. (1976). *J. Immunol.* **117,** 323.
Taussig, M. J. (1974). *Nature (London)* **248,** 234.
Taussig, M. J., and Munro, A. J. (1974). *Nature (London)* **251,** 63.
Thomas, D. W., Roberts, W. K., and Talmage, D. W. (1975). *J. Immunol.* **114,** 1616.
Togewa, A., Oppenheim, J. J., and Mizel, S. B. (1979). *J. Immunol.* **122,** 2112.
Torisu, M., Yoshida, T., Ward, P. A., and Cohen, S. (1973). *J. Immunol.* **111,** 1450.
Trinchieri, G., Santoli, D., Dee, R. R., and Knowles, B. B. (1978). *J. Exp. Med.* **148,** 1299.
Tsoukas, C. D., Rosenau, W., and Baxter, J. D. (1976). *J. Immunol.* **116,** 184.
Tubergen, D. G., Feldman, J. D., Pollock, E. M., and Lerner, R. A. (1972). *J. Exp. Med.* **135,** 255.
Twomey, J. J., Sharkey, O., Brown, J. A., Laughter, A. H., and Jordan, P. H. (1970). *J. Immunol.* **104,** 845.
Twomey, J. J., Lazar, S., and Rocklin, R. E. (1978). *Clin. Immunol. Immunopathol.* **8,** 396.
Unanue, E. R., and Kiely, J. M. (1978). *J. Immunol.* **119,** 925.
Unanue, E. R., Beller, D. J., Calderon, J., Kiely, J. M., and Stadecker, M. J. (1976a). *Am. J. Pathol.* **85,** 465.
Unanue, E. R., Kiely, J. M., and Calderon, J. (1976b). *J. Exp. Med.* **144,** 155.
Unkeles, J. C., Gordon, S., and Reich, E. (1974). *J. Exp. Med.* **139,** 834.
Vassalli, J. D., and Reich, E. (1977). *J. Exp. Med.* **145,** 429.
Vassalli, J. D., Hamilton, J., and Reich, E. (1976). *Cell* **8,** 271.
Vassalli, J. D., Hamilton, J., and Reich, E. (1977). *Cell* **11,** 695.
Visakorpi, R. (1974). *Immunology* **27,** 145.
Wagshal, A. B., and Waksman, B. H. (1978). *J. Immunol.* **121,** 966.
Wagshal, A. B., Jegasothy, B. V., and Waksman, B. H. (1978). *J. Exp. Med.* **147,** 171.
Wahl, L. M., Wahl, S. M., Mergerhagen, S. E., and Martin, G. R. (1975). *Science* **187,** 261.
Wahl, S. M., Iverson, G. M., and Oppenheim, J. J. (1974a). *J. Exp. Med.* **140,** 1631.
Wahl, S. M., Wilton, J. M., Rosenstreich, D. L., and Oppenheim, J. J. (1974b). *J. Immunol.* **114,** 1296.
Wahl, S. M., Altman, L. C., and Rosenstreich, D. L. (1975). *J. Immunol.* **115,** 476.
Walker, S. M., and Lucas, Z. J. (1974). *J. Immunol.* **113,** 813.
Walker, S. M., Lee, S. C., and Lucas, Z. J. (1976). *J. Immunol.* **116,** 807.
Ward, P. A., and Rocklin, R. E. (1975). *J. Immunol.* **115,** 309.
Ward, P. A., and Volkman, A. (1975). *J. Immunol.* **115,** 1394.
Ward, P. A., Remold, H. G., and David, J. R. (1969). *Science* **163,** 1079.
Ward, P. A., Cohen, S., and Flanagan, T. D. (1972). *J. Exp. Med.* **135,** 1095.
Ward, P. A., Dvorak, H. G., Cohen, S., Yoshida, T., Data, R., and Selvaggio, S. S. (1975). *J. Immunol.* **114,** 1523.
Ward, P. A., Unanue, E. R., Goralnick, S. J., and Schreiner, G. F. (1977). *J. Immunol.* **119,** 416.
Weisbart, R. H., Isaacson, J., Bluestone, R., and Goldberg, L. S. (1975). *Immunology* **29,** 223.

Weisbart, R. H., Billing, R. J., Paulus, H. E., Yu, D. T., Fau, P. T., and Clements, P. J. (1977). *Clin. Res.* **25,** 370A.
Werb, Z. (1978). *J. Exp. Med.* **148,** 1695.
Werb, Z., and Gordon, S. (1975a). *J. Exp. Med.* **142,** 346.
Werb, Z., and Gordon, S. (1975b). *J. Exp. Med.* **142,** 361.
White, R., Liu, H. S., and Kuhn, C. (1977). *J. Exp. Med.* **146,** 802.
Williams, T. W., and Granger, G. A. (1969). *J. Immunol.* **103,** 170.
Wood, D. D., and Cameron, P. M. (1978). *J. Immunol.* **121,** 53.
Wood, D. D., Cameron, P. M., Poe, M. T., and Morris, C. A. (1976). *Cell. Immunol.* **21,** 88.
Yoshida, T., and Cohen, S. (1974). *J. Immunol.* **112,** 1540.
Yoshida, T., and Reisfeld, R. A. (1970). *Nature (London)* **226, 856.**
Yoshida, T., Janeway, C. A. and Paul, W. E. (1972). *J. Immunol.* **109,** 201.
Yoshida, T., Sonozaki, H., and Cohen, S. (1973). *J. Exp. Med.* **138,** 784.
Yoshida, T., Bigazzi, P. E., and Cohen, S. (1975a). *J. Immunol.* **114,** 688.
Yoshida, T., Edelson, R., Cohen, S., and Green, I. (1975b). *J. Immunol.* **114,** 915.
Yoshida, T., Kuratsuji, T., Takada, A., Takada, Y., Minowada, J., and Cohen, S. (1976). *J. Immunol.* **117,** 548.
Yoshinaga, M., Waksman, B. H., and Malawista, S. E. (1972). *Science* **176,** 1147.
Youngner, J. S., and Salvin, S. B. (1973). *J. Immunol.* **111,**1914.
Zembala, M., Asherson, G. L., Mayhew, B., and Krejci, J. (1975). *Nature (London)* **253,** 72.

ADVANCES IN IMMUNOLOGY, VOL. 29

Adaptive Differentiation of Lymphocytes: Theoretical Implications for Mechanisms of Cell–Cell Recognition and Regulation of Immune Responses

DAVID H. KATZ

Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, La Jolla, California

I. Introduction 138
A. Cell–Cell Interactions in Immune Responses 138
B. Immune Response (*Ir*) Genes 139
C. Involvement of Major Histocompatibility Complex Genes in Cell–Cell Interactions 140
II. Basis for Genetic Restrictions on Cell Interactions 142
III. The Concept of Adaptive Differentiation 144
IV. Recent Experiments with Bone Marrow Chimeras 145
V. Theoretical Considerations on the Mechanisms of Adaptive Differentiation 147
A. Explanation of Statements 1–7 149
B. Mechanisms of Adaptive Differentiation in Normal Circumstances 151
C. Mechanisms of Adaptive Differentiation in Bone Marrow Chimeras 154
VI. Extrathymic versus Intrathymic Influences on Adaptive Differentiation 160
VII. The Concept of Environmental Restraint and Possible Explanatory Mechanisms 167
A. The Extrathymic Environment Exerts the Major Influence on the Restriction Phenotype of Helper T Lymphocytes 167
B. (Responder × Nonresponder) F_1 T Cells Can Be Taught Preferentially to Help Nonresponder, Rather than Responder, B Cells 178
C. Antiself Receptor Responses Could Explain Environmental Restraint 181
VIII. The Case for B Cell Adaptive Differentiation and Pertinence to the Concept of Interacting "Partner cell Sets" 184
IX. Relevance of the Proposed Mechanisms of Adaptive Differentiation to Certain Unresolved Immunologic Puzzles 192
A. Recognition Mechanisms in the Immune System 192
B. The Nature and Function of *Ir* Genes 196
X. Conclusions 201
References 203

ISBN 0-12-022429-1

I. Introduction

The major histocompatibility complex (MHC) is a family of genes encoding a variety of cell surface marcomolecules first identified and studied for their role in transplantation rejection reactions (1–3). The transplantation antigens encoded by MHC genes in several different species have been thoroughly analyzed serologically and, in more recent years, by modern biochemical techniques (4–7). Two features of the MHC have been particularly provocative in stimulating numerous speculations and posing questions, many of which are still not definitively answered. These are (*a*) the high degree of polymorphism within the family of MHC genes in any given species; and (b) the high frequency of immunocompetent lymphocytes existing in any one individual that appear to be specific for MHC products (i.e., alloantigens) displayed on cells of other individual members of the species. Since it is fair to assume that there should be no evolutionary purpose in preserving a recognition capability, particularly in high frequency, within the native cells of one individual to "protect" it from invading cells of any other member of the species, the reason such cells exist in high frequency has been indeed a great mystery. Various speculations have been made in attempts to address these two provocative aspects of the MHC and recognition processes associated with antigens encoded by MHC genes (8–18), but little definitive evidence is available on these points.

During the past decade, three fundamental observations have been made in the field of immunology that bear directly on the issues raised in the preceding paragraph and furthermore have revolutionized our conceptual understanding of cellular and molecular mechanisms involved in the functions of the immune system. These will be briefly summarized in the following sections.

A. Cell–Cell Interactions in Immune Responses

This era was ushered in by the observations of several investigators (19–21) that first demonstrated that the development of antibody responses in certain instances required cooperative interactions between two distinct classes of lymphocytes, those derived from the thymus (T cells) and those derived from the bone marrow (B cells). Subsequent studies documented that B cells were the actual precursors of antibody-forming cells, and T cells provided an important auxiliary function necessary for B cell precursors to fully develop into mature antibody-secreting cells. By the early 1970s, it was clear that T lymphocytes exerted sophisticated regulatory effects on other lymphocytes of both classes and in this way dictated the quality and

magnitude of most immune responses (22, 23). It is now quite clear that T cell regulation of the immune system spans the entire spectrum from enhancement to suppression and involves complex cellular interactions between T cells and macrophages, T cells and T cells, and T cells and B cells; these cellular interactions reflect what we now understand to be a complex network of communication processes that are at the same time finely tuned and (usually) unmistakable. Moreover, T cells participating in different regulatory functions or exerting different effector roles in cell-mediated immunity are now known to belong to distinct subclasses identifiable by their phenotypic expressions of distinct cell surface antigenic markers (24–26).

B. Immune Response (*Ir*) Genes

Another revolutionary series of concepts that have evolved during the past decade have been those concerned with the role of the major histocompatibility gene complex in the immune system. As previously stated, extensive analyses have been made of the MHC in several species, particularly in the *HLA* system of humans (reviewed in reference 3) and the *H-2* system of mice (reviewed in reference 13). In the mid-1960s fundamental discoveries were made by Benacerraf and McDevitt and their respective co-workers that demonstrated for the first time a function of MHC genes in the control of immune responses to antigens other than those related in any obvious way to transplantation antigens or reactions or both (reviewed in references 11 and 12). Responder strains and nonresponder strains were distinguished by their ability or inability to manifest both cell-mediated (delayed hypersensitivity) and humoral (antibody production) immune responses to the revelant antigens.

Studies utilizing congenic-resistant strains of mice established the linkage of responder or nonresponder status to genes in the MHC, and the MHC genes controlling such responses were termed immune response (*Ir*) genes (27, 28). Such genes were shown to be inherited in a Mendelian fashion as autosomal dominant traits in most instances, although more recently the responder phenotype has been shown in selected cases to result from gene complementation, thus implicating the involvement of at least two *Ir* genes in the control of such responses (reviewed in reference 29). Analyses with intra-*H-2* recombinant strains of mice established the fact that *Ir* genes were not linked to the terminal *K* (left) or *D* (right) regions of the complex, but rather were located in a new region, adjacent and to the right of the *K* region, which was appropriately denoted as the *I* region (30); the corresponding portion of the human *HLA* complex appears to be the *HLA-D* region (31–33).

In view of the facts that (*a*) all responses controlled by *Ir* genes involved the participation of T lymphocytes; (*b*) the specificity of such control appeared to be of a high order; and (*c*) the nature of the molecules functioning as receptors on T cells was for many years uncertain and appeared to be different from the immunoglobulin molecules serving as receptors on B lymphocytes, it was proposed that *Ir* genes encoded the molecular products functioning as T cell receptors (28). However, the bulk of both direct and indirect evidence presently available contradicts the validity of this possibility. Thus, it has been established by studies performed initially by Ramseier and Lindemann (34), and more recently by Binz and Wigzell (35, 36) and Rajewsky and colleagues (37, 38) that T cell receptors possess idiotypic determinants identical to those found on immunoglobulin receptors on B cells (39–44). Since idiotypic determinants are characteristic of the region comprising the specific antigen-combining site, these findings strongly indicate that the variable (*V*) region genes encoding antigen specificity in immunoglobulins are also responsible for dictating specificity of T cell receptors. Additional information indicates that these T cell receptor molecules do not possess conventional immunoglobulin markers, are not linked in any obvious way to the MHC, and are linked in inheritance to immunoglobulin heavy chain genes.

C. Involvement of Major Histocompatibility Complex Genes in Cell–Cell Interactions

Another major conceptual advance during recent years has been that concerned with the role of MHC genes in governing cell–cell interactions and communication in the development of immune responses. These ideas arose during the course of studies designed to ascertain the nature of the mechanism(s) by which different populations and subpopulations of lymphocytes interact with one another as well as with macrophages. The basic concept that histocompatibility gene products are integrally involved in the mechanism of regulatory cell interactions initially arose from certain unexpected observations made in experiments conducted 9–10 years ago. In these experiments, it was found that transfer of histo*in*compatible T cells to previously immunized recipient animals resulted in circumvention of the normal requirement for antigen-specific helper T cells in secondary antibody responses. This phenomenon was termed the "allogeneic effect" and was shown to reflect the development of an active graft-versus-host reaction in recipient lymphoid organs (reviewed in reference 45). Extensive analyses of this phenomenon revealed that the allogeneic effect bore remarkable parallels to normal regulatory T cell–B

cell interactions in isogeneic or syngeneic combinations and hence was postulated to be an analogous model for regulatory cell interactions in the immune response (45).

The fact that the final pathway in the allogeneic effect involved interactions at the histocompatibility molecules on the cell surface of the target T or B cells employed in the system led to the consideration that perhaps precisely the same pathway was involved in syngeneic interactions, occurring perhaps by similar molecular mechanisms. In order to ascertain the validity of this possibility, experiments were conducted to investigate whether physiologic T cell–B cell interactions in the mouse involved genetic restrictions associated with MHC genes. The basic observation from such studies was that antigen-specific T cells were capable of providing specific helper function for primed B cells of histocompatible, but *not* of histoincompatible, donor origin in secondary antibody responses of the IgG class (46, 47). It was further established that cooperative interactions could be obtained between reciprocal mixtures of F_1 hybrid and parental T and B cells (46), and, moreover, that the presence of primed histoincompatible T cells did not appreciably alter cooperative interactions between histocompatible T and B cells (48, 49) thus arguing against the possibility of nonspecific or specific suppresive effects contributing to the observed genetic restrictions in the systems. At about the same time, Kindred and Shreffler (50) demonstrated a requirement of *H-2* identity for successful thymus reconstitution of athymic nude mice, (50) and Shevach and Rosenthal made the fundamental discovery of MHC-linked genetic restrictions in macrophage–T cell interactions in guinea pigs (51, 52), thereby closing the loop of regulatory cell interactions in immune responses and associating control of such interactions with MHC genes.

Studies utilizing congenic-resistant mouse strains established that the genetic restriction in cooperative T cell–B cell interactions is linked to the *H-2* gene complex (47). Further studies documented that the critical genetic locus or loci involved in controlling such interactions map in the *I* region of the histocompatibility complex (53), a remarkable association with the same region containing *Ir* genes and also the genes encoding cell surface macromolecules known to be the most potent alloantigens in terms of stimulating one type of transplantation reaction, namely, the mixed lymphocyte reaction (MLR) (54). The original interpretation of these observations was that these genetic restrictions reflected the existence of cell surface molecules, distinct from antigen-specific receptors, that play a crucial role in mediating effective cell–cell interactions between T and B lymphocytes (46, 47). It was further postulated that genetic identity between the T cell

and the B cell was necessary for the relevant T cell surface molecules to bind to the corresponding B cell molecules, which were termed "acceptor" sites, for effective interactions between primed T and B lymphocytes in development of antibody responses. Subsequently, the respective molecules were defined as cell interactions (CI) molecules and the *I* region genes coding them as *CI* genes (55).

Later, the involvement of histocompatibility gene products in controlling the ability of cytotoxic T lymphocytes (CTL) effectively to lyse virus-infected, chemically modified or minor H antigen-bearing target cells was found (56–60). In such circumstances, it has been shown that CTL are most efficient in lysing target cells derived from a similar histocompatibility genotype (reviewed in references 61 and 62). The critical genetic locus or loci involved in controlling interactions between CTL and target cells map in the *K* and *D* regions of the histocompatibility complex (63, 64), thus differing from those involved in T cell–B cell interactions which are located in the *I* region. Postulated mechanisms concerning these genetic restrictions will be discussed below.

Thus, it is clear from the foregoing paragraphs that a remarkable transition has taken place in the science of immunology in recent years, both from our understanding of how the immune system functions in its complex ways and in the level of sophistication with which we are now able to approach even more fundamental questions concerning regulatory phenomena as they pertain to the immune system per se and, in the broad sense, to normal and abnormal developmental processes in general as viewed through the very accessible windows of the lymphoid system. It is nevertheless worth noting that despite the very extensive experimentation and energy devoted to such questions, the precise mechanism of cell–cell communication in the immune system, namely, whether this involves direct cell–cell contact, activity of secreted (or released) molecules, or a combination of cell contact and mediator release, has still not been definitively established. This merely emphasizes the considerable magnitude of the stumbling blocks that yet must be successfully hurtled if we are to further advance our grasp of how this intricate and delicately balanced system works.

II. Basis for Genetic Restrictions on Cell Interactions

Essentially two major concepts have evolved to explain the MHC-linked genetic restrictions on cell interactions. The first hypothesis, which stemmed from analysis of such restrictions in T cell–B cell in-

teractions, considered that interactions among various cell types in the immune system are mediated by cell interaction (CI) molecules located on the cell surface, at least some of which are encoded by MHC genes (i.e., *I* region genes in this case), and are quite distinct from the lymphocyte receptors specific for conventional antigens (46, 47, 55). The CI molecule concept therefore emphasizes a dual recognition mechanism that involves at least two distinct molecular interactions in lymphocyte activation, one utilizing antigen-specific receptors and the second consisting of reactions between the relevant CI structures and their corresponding receptors. The second major concept, derived primarily from studies in the CTL system, considered that T lymphocytes have receptors that do not recognize antigen alone, but antigen in some form of association with MHC gene products on cell surface membranes; this concept of "altered-self" (65) recognition by T lymphocytes has subsequently been modified in various ways, but all versions still differ substantially from the CI molecule concept in predicting the existence of a *single* receptor on T cells simultaneously recognizing modified determinants on the cell surface. As yet, no definitive proof has been obtained to establish which of these two models is correct.

These two very distinct hypotheses concerning the basis of MHC restrictions on cellular interactions among components of the immune system place such genetic restrictions in very different biologic perspectives. Single recognition mechanisms, such as the altered-self model, limit the biologic connotations of these genetic restrictions to the immune system. The CI molecule concept of dual recognition, on the other hand, suggests the existence of a mechanism for highly specific *self-recognition* that—aside from its obvious importance for proper cell communication in the immune system, where it has first been discovered and analyzed—provides a general mechanism for control of effective cell interactions in nonimmunologic organ systems as well. Moreover, delineation of the mechanisms responsible for conserving self-recognition and, alternatively, understanding processes that potentially disturb it (and the consequences of such disturbances to the individual) could open an important avenue toward elucidating crucial events in normal and abnormal cell differentiation in eukaryotic organisms.

In many ways, therefore, what we have been studying as a curious phenomenon in the immune system may have substantial biological implications. Thus, in this review the issue of genetic restrictions in cellular interactions is discussed in terms of its broadest potential biologic context. A theory is proposed to explain the mechanisms governing self-recognition among interacting cell types in the immune

system. This theory extends eariler interpretations (55, 66) of experimental observations in our own laboratory and certain others and is written to address the broad issue of the selective processes that are postulated to be concerned with cell communication and differentiation not only within, but also outside of, the immune system.

III. The Concept of Adaptive Differentiation

The validity of the CI molecule concept as an appropriate interpretation of the basis for genetic restrictions in T cell–B cell interactions came under question amid the reports of other investigators who failed to find similar restrictions in different systems in which T cell–B cell cooperative responses were analyzed. Particularly important were those studies performed with cells obtained from bone marrow chimeras. Bone marrow chimeras are generally prepared by reconstituting lethally irradiated recipient mice with bone marrow stem cells from one or more donor mouse strains. When prepared properly, the chimeric state is manifested by full reconstitution of the irradiated host with hematopoietic cells that are entirely of donor origin; this can be proved by analyzing the lymphoid cells of such chimeras for their *H-2* type using appropriate anti-*H-2* alloantibodies. In immunologic terms, the lymphocytes in a bone marrow chimera display mutual and specific immunologic tolerance (67, 68). Studies were made with lymphocytes obtained from bone marrow chimeric mice that had been prepared by reconstituting lethally irradiated $(A \times B)F_1$ recipients with a mixture of bone marrow cells derived from each of the respective parents, *A* and *B* (69). In such circumstances, T lymphocytes originally derived from donors of different *H-2* haplotypes (i.e., parent *A* and *B*), but which had differentiated within a mutual host F_1 environment, were found to be independently capable of interacting effectively with B cells derived from conventional donors of the opposite parential type (69).

Since the parental *A* and *B* lymphoid populations of such chimeras were mutually tolerant of one another (i.e., unable to exert reciprocal alloreactivity), the question arose whether the *failure* of partner cells derived from nontolerant histoincompatible donors to interact effectively might be due to some type of inhibitory influences resulting from subtle alloreactivity between such cells. However, this seemed untenable for a number of reasons discussed more fully elsewhere (26, 66, 70, 71). Furthermore, cell mixture experiments designed to test directly this possibility failed to detect any suppressive or interference mechanisms in the initial studies (48, 49).

It became necessary, therefore, to address the paradox that consisted of (*a*) the striking degree of MHC-linked genetic restrictions imposed upon effective T cell–B cell interactions; (*b*) the absence of demonstrable suppressive influences to explain such genetic restrictions; and (*c*) the seemingly contradictory data obtained with T and B lymphocyte populations derived from bone marrow chimeras. These paradoxical observations seemed to be most logically explained by a concept of *adaptive differentiation* of lymphoid cell precursors (49, 66, 70, 71). We proposed that the process of stem cell differentiation is critically regulated by histocompatibility molecules on cell surfaces, and that such differentiation is "adaptive" to the environment in which it takes place. This concept, in brief, predicted that (*a*) during early differentiation lymphoid cell precursors "learn" the relevant compatibilities required of them for *effective* cell–cell interactions, and, moreover, (*b*) this learning process is dictated by the MHC phenotype of the environment in which such differentiation takes place.

The first experimental approach employed to address this possibility focused attention on the capacity of B lymphocytes to undergo adaptive differentiation. Indeed, the results of such studies (66, 70) were consistent with the hypothesis. Thus, B lymphocytes obtained from unprimed $(A \times B)F_1$ hybrid donors were sensitized to antigen in adoptive antigen-primed irradiated recipients of each parental type, *A* and *B*, or in completely unrelated recipients (strain *C*). When tested in subsequent secondary antibody responses, such B lymphocytes displayed a clear preference for interacting with T lymphocytes derived from the same strain as that used as the initial priming host. These results clearly suggested the existence of an undefined mechanism that dictated the optimal partner cell interaction preference ultimately manifested by B cells primed under such circumstances.

IV. Recent Experiments with Bone Marrow Chimeras

More recently, analysis of the cooperative activities of lymphocytes that have differentiated in the environments of bone marrow chimeras has been undertaken. Initially, such studies focused on the ability of chimeric T lymphocytes to interact effectively with B lymphocytes obtained from coventional donors of the same or opposite parental type; indeed, interactions that appeared to cross *H-2* barriers were demonstrated in certain circumstances using chimeric lymphocytes (69, 72–75). Bone marrow chimeras were also used to analyze genetic restrictions in the CTL–target cell systems. The first of such studies (76), subsequently confirmed by others (77, 78), demonstrated that sen-

sitization of single parent $A \rightarrow (A \times B)F_1$ chimeras[1] by either virus infection or inoculation of hapten-modified parent *B* cells resulted in development of CTL (of parent *A* type) capable of lysing virus-infected or hapten-modified target cells of parent *B* type, although with considerably less efficiency than that observed on target cells of isologous parent *A* type (76–78).

Quite recently, the issue of genetic restrictions in T cell–B cell interactions has been extensively reinvestigated in several laboratories using bone marrow chimeras prepared in various combinations between two parental strains *A* and *B*, and their corresponding $(A \times B)F_1$ hybrid (79–84). In our own studies (79), the five types of chimeras in such studies can be represented as follows: (*a*) $F_1 \rightarrow F_1$; (*b*) $A \rightarrow F_1$; (*c*) $B \rightarrow F_1$; (*d*) $F_1 \rightarrow A$; and (*e*) $F_1 \rightarrow B$. In these studies, attention was paid not only to the cooperative helper activities of T cells derived from such chimeras, but also to the partner cell interaction preference of B lymphocytes which had been primed in these chimeric environments.

To summarize our observations briefly (79), parallel results were obtained with *both* lymphocyte types and demonstrated the following points quite clearly:

1. $F_1 \rightarrow F_1$chimeric lymphocytes displayed no restrictions in their abilities to cooperate with all of the various partner cell combinations, results that parallel precisely the cooperative capabilities of conventional F_1 T cells.
2. Parent $A \rightarrow F_1$ and parent $B \rightarrow F_1$ chimeric cells displayed phenotypic preferences that were indistinguishable from conventional parental cells—i.e., in manifesting cooperative preference for partner cells only from F_1 donors or from parental donors corresponding to the *H-2* haplotype of the original bone marrow donor.
3. $F_1 \rightarrow$ parent *A* and $F_1 \rightarrow$ parent *B* chimeric T and B cells displayed restricted preference in cooperating only with partner lymphocytes sharing the *H-2* haplotype (either entirely or codominantly) of the parental chimeric host. In other words, cells originally of F_1 donor origin no longer behaved as typical F_1 cells, but rather displayed restricted cooperative activity similar to activity that would be observed in interactions employing conventional parent T or B cells. Similar findings have been made in the CTL–target cell system (85–87), although these were restricted to T cells.

[1] The notations for compositions of bone marrow chimeras are as follows: $A \rightarrow (A \times B)F_1$ indicates *A* type bone marrow cells were used to reconstitute lethally irradiated $(A \times B)F_1$ recipients.

Three points are worth emphasizing about these observations. First, the failure of parent → F_1 or F_1 → parent chimeric T or B cells to cooperate with partner lymphocytes of the opposite parental haplotype cannot be explained by the existence of a conventional suppressive mechanism, whether subtle or otherwise. This possibility was ruled out by documenting the capability of such cells to cooperate effectively with partner lymphocytes from either conventional F_1 or F_1 → F_1 chimeric donors; furthermore, in a direct test of this possibility by cell mixture experiments, no inhibitory effects of the chimeric cells employed could be detected (79).

Second, the finding that lymphocytes from semiallogeneic parent → F_1 chimeras were unquestionably incapable of interacting with partner cells of the opposite parental haplotype (79) is consistent with certain studies (75, 88), but not all (72), on cooperative T cell–B cell interactions with chimeric lymphocytes. Moreover, the failure of T lymphocytes from *single* parent → F_1 chimeras to interact effectively with B cells of the opposite parental type is a significant contrast with the ability of T lymphocytes from *double* parent →F_1 chimeras to reciprocally interact with B cells of opposite parental type (69, 75). As discussed later, the basis for these differences appears to be an important clue to the mechanism(s) underlying adaptive differentiation.

Third, the significance of the findings made with B lymphocytes that have differentiated in F_1 → parent chimeras to the whole issue pertaining to single vs dual recognition models cannot be overemphasized. Thus, quite unlike the situation with T cells, were the argument can be (and has been) made that T lymphocytes manifesting restrictions of the one type or another could be reflecting their receptor specificities for antigen-plus-"self" (65), this argument does not easily explain the findings on B cell adaptation; indeed, B cell adaptive differentiation is almost exclusively explainable by a dual recognition, i.e., CI molecule, model (as will be explained later).

V. Theoretical Considerations on the Mechanisms of Adaptive Differentiation

The evidence described above not only supports the likelihood that adaptive differentiation normally dictates the phenotypic expression of preferential cell–cell interactions among components of the immune system, but also provides some interesting insights into how this process may occur during normal development. The model proposed here as an explanation for adaptive differentiation of lymphocytes can be summarized by the following seven statements; more detailed explanation on the various points highlighted in these

statements will follow thereafter. This basic model was initially summarized by Katz *et al.* (79).

1. All cell interactions within an individual member of the species are interactions of *SELF-RECOGNITION*. These self-recognition processes are mediated through cell surface cell interaction (CI) molecules (defined in statement 4).
2. Each individual member of a species possesses the genotypic library for *all* CI molecule specificities expressed in the species. This library spans not only many different specificities, but a whole spectrum (i.e., from low to high) of binding affinities between the two interacting molecules.
3. One of the earliest and most important decisions in morphogenesis is which CI phenotype will be worn by the background environment. Once this decision has been made, by whatever mechanism, the phenotype of self-reactivity characteristic of that individual is firmly established.
4. CI molecules are defined as follows: In any interaction between two partner cells, at least one of the two CI molecules is a product of MHC gene(s); the second molecule may either be an MHC gene product or a product of non-MHC gene; i.e., it could be, in part, encoded by a *V* gene. Thus, in any set of two interacting CI mulecules, one molecule can be considered to be the ligand (hereafter termed $CI_{A,B,\text{ or }C}$) whereas the second molecule may be considered to be the specific receptor for that ligand (hereafter termed $\alpha CI_{A,B,\text{ or }C}$).
5. Determined by the CI phenotype of the environment, differentiating lymphocytes undergo a process of *selection* that involves deletion or abortion of cells with high affinity receptors for native (i.e., self) CI molecules; this deletion process is accompanied by a corollary process in which cells with low-to-moderate affinity receptors for native CI molecules predominantly emerge. These cells then constitute the functional interacting populations involved in self-regulating further differentiation and responsiveness.
6. A concomitant selection process occurs among the cells bearing receptors for CI molecules expressed predominantly in other individual members of the species (i.e., nonself). In these cases, cells with high affinity receptors predominate, whereas low-to-moderate affinity nonself cells are eliminated. The later cells can serve no useful function in the inappropriate environment and without this selection mechanism might proliferate uncontrollably.
7. Based on the aforementioned points, all cell interactions within a species, even those occurring between cells from *different* individual

members of the species, are interactions of self-recognition. They differ only in the binding affinities of interactions—i.e., those occurring among partner cells involved in physiologic responses within the same individual are of low-to-moderate affinity, whereas those occurring between cells from different individuals are of high affinity; these high affinity reactions are most probably those that we classically have termed *alloreactions*.

A. Explanation of Statements 1–7

The first basic assumption of this model is that normal cell–cell interactions take place by a process of self-recognition. For example, in individual *A*, self-recognition is mediated by αCI_A receptors (one of an interacting pair of CI molecules) with the corresponding CI_A molecules. This terminology holds for all self-recognition interactions among partner cells of *A*-type irrespective of the affinities of the αCI_A molecules involved. However, the affinities of the αCI molecules involved will determine the functional consequences of such reactions.

Another important point of this model is that every progenitor stem cell in any individual member of the species possesses the genotypic *potential* for encoding the entire repertoire of CI and αCI molecules of the species. The mechanism by which the decision is made as to which CI phenotype will be expressed in a given individual is not immediately apparent. It is clear, however, that it is governed by heritable genetic messages and certainly must occur very early, perhaps even at the time of fertilization.

In terms of the CI molecules involved in such interactions, it is clear that at least *one* of the two partner CI molecules is a product of the MHC; in those cases of interactions involving regulatory (i.e., helper or suppressor) T lymphocytes, macrophages, and B lymphocytes (in any combination), the relevant MHC-linked CI molecules are probably *I* region products, whereas in interactions between CTL and target cells, the MHC-linked CI molecules are either *K* or *D* region products. As stated above, the αCI molecules involved in any interacting pair could be either an MHC gene product, a product of non-MHC genes (i.e., *V* genes), or composed of products encoded by both MHC and non-MHC genes.

It is implicit in the assumption that at least one of the CI molecules in any interacting pair displays a range of affinities from low-to-high that a receptor–ligand relationship exists between any two interacting CI molecules. The existence of a spectrum of differing affinities is an important aspect of the model because of the functional flexibility that is afforded to the system in this way. For example, in functional cell

interactions that occur normally among interacting cells during the development of a physiologic immune response (i.e., T–B, T–T, T–macrophage interactions), the ultimate consequence of such interactions may reflect the relative affinities of CI molecules involved. Thus, when the desired result is the further differentiation and/or multiplication of the target cells, such interactions may be of low affinity. When the desired end result is to abort further differentiation and/or proliferation of target partner cells, such interactions may be of moderate-to-high affinity.

Another important aspect of this model concerns the consequences of high affinity binding events between two partner cells. The point, quite simply, is that such high affinity interactions could result in functional abortion of one or perhaps both of the interacting partner cells. One could envisage, for example, that whenever sufficiently high affinity binding occurs, a process of *modulation* results in loss of one or both of the interacting CI molecules either by shedding or endocytosis (or a combination of both). Such modulated cells would be unable thereafter to reexpress their membrane-bound CI molecules, a situation analogous to be inability of very immature B lymphocytes to reexpress surface-bound immunoglobulin receptor molecules following modulation by anti-immunoglobulin antibodies (89, 90). This would provide a convenient mechanism for deleting the majority of undesirable cells with high affinity αCI receptors specific for the self CI phenotype of a given individual, since such cells would be exposed to an excess of self CI molecules present throughout the native environment. Second, such a process would provide a mechanism that would limit the frequency of functionally irrelevant cells with low affinity αCI receptors for nonself, since high affinity cells of corresponding specificity would react with and abort these cells.

It is worth emphasizing that this provides an important selective pressure for maintaining a certain frequency of high affinity cells capable of reacting with nonself CI molecules. In this respect, the considerations presented here differ fundamentally from the hypothesis proposed recently by Janeway *et al.* (91). Central to their model is the notion that the low affinity receptor for self (which they termed receptor two) on a given cell is capable of binding with high affinity to certain nonself MHC antigens (i.e., CI ligand molecules). The model presented herein is in general agreement with that of Janeway *et al.* (91) with respect to the important aspect of affinity differences being a crucial point of distinction between interactions with self (low) vs nonself (high) CI specificities. However, we believe that these are *separate*

receptors encoded independently in the genome and expressed on separate cells altogether; the reasons for this are discussed later in greater detail.

Additionally, it is important to emphasize the perpetual nature of these selection porcesses within a given individual, since cells of all potential CI and αCI molecule specificities and affinities would be constantly reemerging from the stem cell pool. In these terms, therefore, *adaptive differentiation* reflects the net result of these concomitant *selection* processes by which the functional interacting cell sets corresponding to the CI phenotype of the environment are favored, whereas those corresponding to CI specificities of the remainder of the species are not.

In the sections that follow we will examine how one can envisage the process of adaptive differentiation of murine lymphocytes as it may occur in normal and experimentally contrived circumstances. To minimize the complexity of our examples, we have limited the entire species repertoire of CI molecules in these schemes to a total of three, termed *A*, *B*, and *C*.

B. Mechanisms of Adaptive Differentiation in Normal Circumstances

Following the reasoning and definitions discussed above, we can now examine how adaptive differentiation may occur under normal circumstances. It should be noted that in the schemes that follow we deliberately have not defined the cell types discussed as being either B or T lymphocytes, since we consider the mechanisms proposed here to be equally valid for lylmphocytes of either class. Indeed, there is no a priori reason to restrict the capabilities for self-recognition to cells of the T lymphocyte class. Moreover, the critical involvement of *macrophages* in the selection processes that take place cannot be overstated. It is abundantly clear that these important cells play a central role in selecting T lymphocytes during antigen-driven responses (51, 52, 92). We believe that they perform similar functions in antigen-driven selection of B cells and perhaps also during *antigen-independent* selection processes occurring in normal differentiation. Finally, it should be stated that the concepts outlined here for adaptive differentiation of lymphocytes might be equally applicable to cell differentiation outside the immune system, although the CI molecules involved in other cell systems need not be the same as those used in the lymphoid system.

1. Adaptive Differentiation in Homozygous Individuals

The sequence of events involved in adaptive differentiation in a homozygous individual, parent *A*, is diagrammatically illustrated in Fig. 1. As shown, the progenitor stem cell gives rise to three types of self-recognizing cells, *A*, *B*, and *C*; at the outset, the full affinity spectra of αCI_A, αCI_B, and αCI_C receptors are expressed. However, when differentiation occurs in the environment of individual *A*, the process of selection, by modulation or otherwise (see above), results in deletion of the high affinity αCI_A cells. This permits predominant emergence of low-to-moderate affinity αCI_A cells that will functionally interact with CI_A molecule-bearing partner cells. A concomitant selection process operates on cells with αCI_B and αCI_C receptor specificities emerging from the stem cell pool. These cells, which differentiate in the absence of environmental selection, develop a closed system of internal self-regulation whereby high affinity αCI_B and αCI_C cells limit the growth potential of CI_B and CI_C molecule-bearing cells, which are, after all, the only available target cells with which they can react in that environment. Thus, as suggested previously, a mechanism is required for limiting the quantities of such nonself cells since they would (*a*) serve no useful functional purpose in the environment of individual *A*; and (*b*) not be susceptible to usual self-limiting control mechanisms built into the regulatory cell interactions of normal immune responses in that environment. The overall net result of this process would be (*a*) minimal numbers of low-to-moderate affinity αCI_B- and αCI_C-type cells; and (*b*) a relative excess of high affinity αCI_B-and αCI_C-type cells.

In functional terms, the small quantities of low-to-moderate affinity αCI_B and αCI_C cells would explain why in most instances effective cell interactions are restricted to partner cells derived from individuals of genetically identical MHC type; the fact that such cells may exist in very small numbers would explain why Pierce and Klinman, using a very sensitive assay system, were able to detect effective interactions between gentically dissimilar partner cells (93). Also, in functional terms, the existence of high affinity αCI_B and αCI_C-type cells would explain typical patterns of alloaggressive behavior observed following comixture of lymphocyte populations derived from different individual members of the species.

It is important to reiterate the point that since adaptive differentiation is a continuous process, high affinity αCI_A clones would constantly reemerge from the progenitor cell pool. It is not necessary to assume that *all* such high affinity cells are deleted before migrating to

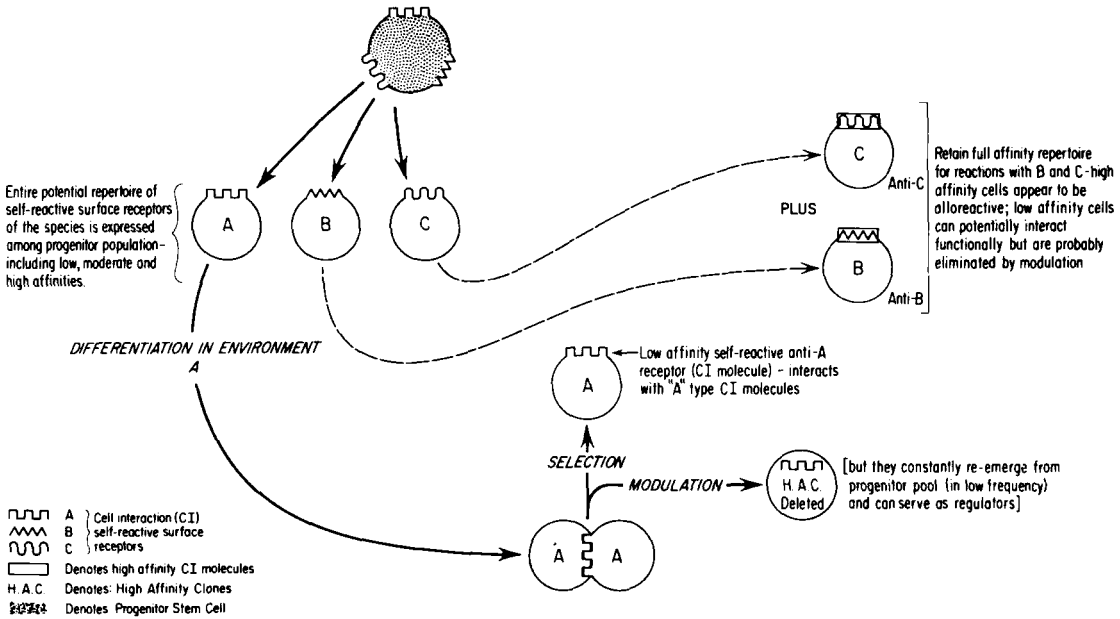


FIG. 1. Adaptive differentiation in the physiologic host: parent A. See text for explanation of Figs. 1–5.

peripheral lymphoid organs, and perhaps such cells may even perform an important regulatory role in the process of becoming deleted.

2. *Adaptive Differentiation in Heterozygous Individuals* [$(A \times B)F_1$]

Following the same principles outlined in the preceding section, Fig. 2 illustrates adaptive differentiation in the heterozygous $(A \times B)F_1$ individual. Here the differentiating stem cells undergo bipartisan selection, since the environment presents both CI_A- and CI_B-type molecules. Therefore, both high affinity αCI_A and αCI_B cells are deleted, leaving cells with low-to-moderate affinity αCI_A and αCI_B specificity as the predominant interacting populations. Population C, for reasons stated above, would consist predominantly of high affinity αCI_C molecules. It follows that cells from this individual would be unreactive (tolerant) when confronted with cells of parent A or parent B origin, and, conversely, they would display a typical MLR when confronted with third party cells from individual C.

It is important to note that this scheme illustrates the resulting cell populations of A and B specificities, respectively, as independent cell populations. Thus, in terms of functional cell interactions, distinct clones of self-recognizing lymphocytes interact with cells of corresponding CI molecule specificity. This contrasts with serologic and biochemical data that have illustrated quite clearly that the detectable MHC antigens are *codominantly* expressed on most cells of an F_1 hybrid individual (94), and further indicates our limited knowledge of the precise nature of the CI molecules. It is obvious, however, that only one of the two CI molecules need be independently expressed to fulfill this prediction, and its seems likely that this would be the αCI receptor entity (non-MHC gene product?) of the two.

C. Mechanisms of Adaptive Differentiation in Bone Marrow Chimeras

Having established the ground rules for physiologic adaptive differentiation in normal homozygous or heterozygous individuals, we can now consider how such differentiation events might occur during reconstitution of hematopoietic cells in bone marrow chimeras.

1. *Adaptive Differentiation of Parent A Bone Marrow in* $A \rightarrow (A \times B)F_1$ *Chimeras*

A scheme for adaptive differentiation of parent A bone marrow in $A \rightarrow (A \times B)F_1$ chimeric host can be *theoretically* constructed along the lines illustrated in Fig. 3. Bone marrow from parent A donors con-

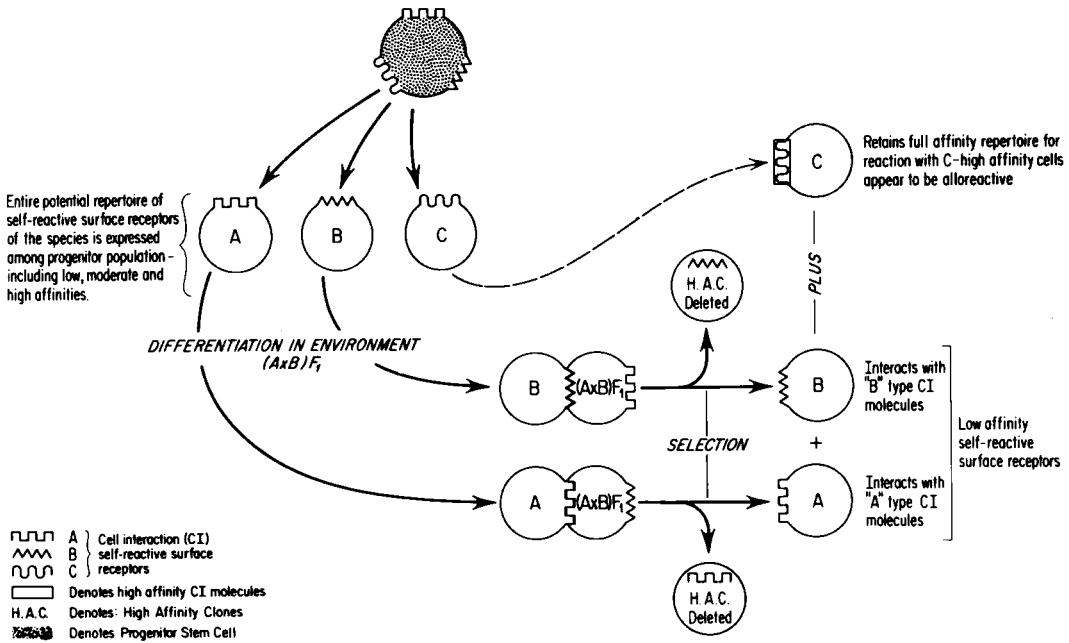


FIG. 2. Adaptive differentiation in the physiologic host: $(A \times B)F_1$.

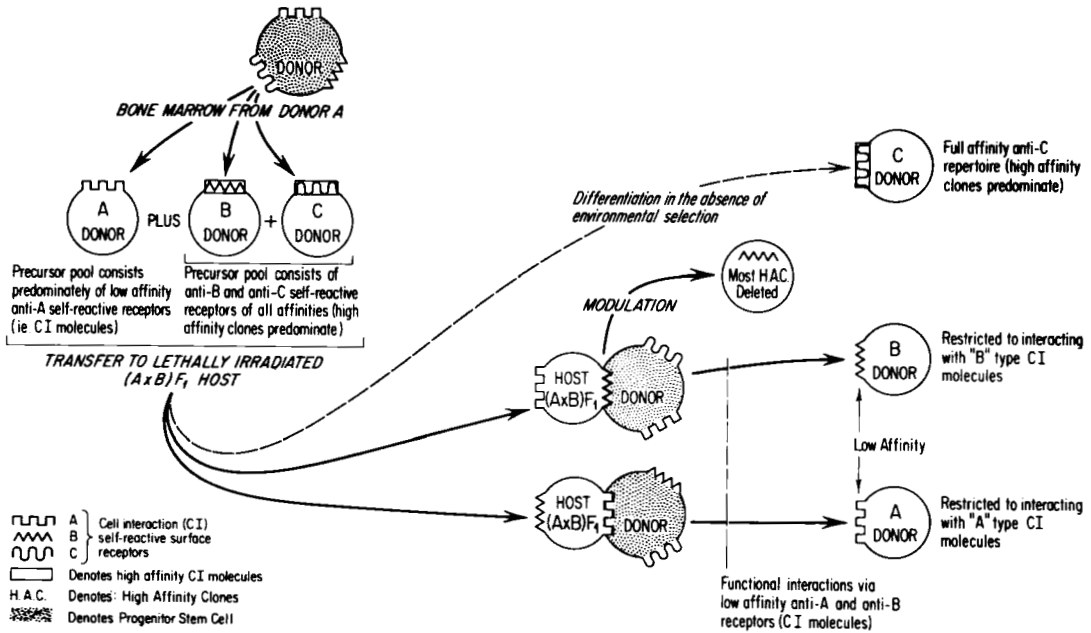


FIG. 3. Theoretical adaptive differentiation of parent A bone marrow in $A \rightarrow (A \times B)F_1$ chimeras.

tains (*a*) progenitor stem cells; (*b*) precursor cells with predominantly low affinity self-reactive αCI_A receptors; and (*c*) precursor cells of predominantly high affinity αCI_B and αCI_C specificities. When such cells are transferred to lethally irradiated $(A \times B)F_1$ hosts, the selection process would presumably favor differentiation of cells with predominantly low affinity αCI_A and αCI_B specificities. Since most of the high affinity αCI_B clones would presumably be deleted by the selection process, the result would be a state of operational immunologic tolerance (inability to react against target cells from individual *B*); no selection process of this type would exist to delete high affinity αCI_C clones in an $(A \times B)F_1$ environment, thereby maintaining normal reactivity against third party type *C* targets. This is, indeed, consistent with experimental findings (67, 68).

In terms of functional cell interaction capabilities, the scheme illustrated in Fig. 3 suggests that the resulting lymphoid cells should interact effectively with partner cells of both *A* and *B* type. However, as pointed out above, this has not been consistently borne out by experimental observations. Thus, attempts to demonstrate successful T-cell –B cell cooperative interactions in these circumstances reveal that such cells generally retain their haplotype preference for interacting with partner cells identical with the original donor (i.e., parent *A*), particularly when *in situ*-primed chimeric cells are analyzed in this respect (79).

The failure of parent $A \rightarrow (A \times B)F_1$ chimeric lymphocytes effectively to interact with partner cells of parent *B* type in the T-cell–B cell cooperative systems is most likely explained by the restricted macrophage–T cell axis present in such reconstituted mice. Thus, the bulk of the macrophages existing in the lymphoid organs of such chimeras would be of donor (i.e., parent *A*) type. In view of the importance of genetic identity in macrophage–T cell interactions, as discussed above (51, 52), it seems plausible that the major selection process at that level would favor predominant emergence of *A*-type preference among interacting lymphoid cells when primed in the chimeric environment. Very recent experiments (95), described below (Section V,B), validate this interpretation.

2. *Adaptive Differentiation in* $F_1 \rightarrow$ *Parent Bone Marrow Chimeras*

The scheme depicted in Fig. 4 illustrates adaptive differentiation of $(A \times B)F_1$ bone marrow in parent *A* chimeric hosts. The bone marrow consists of progenitor stem cells, predominantly low affinity αCI_A and αCI_B precursors and predominantly high affinity αCI_C. In the parent *A*

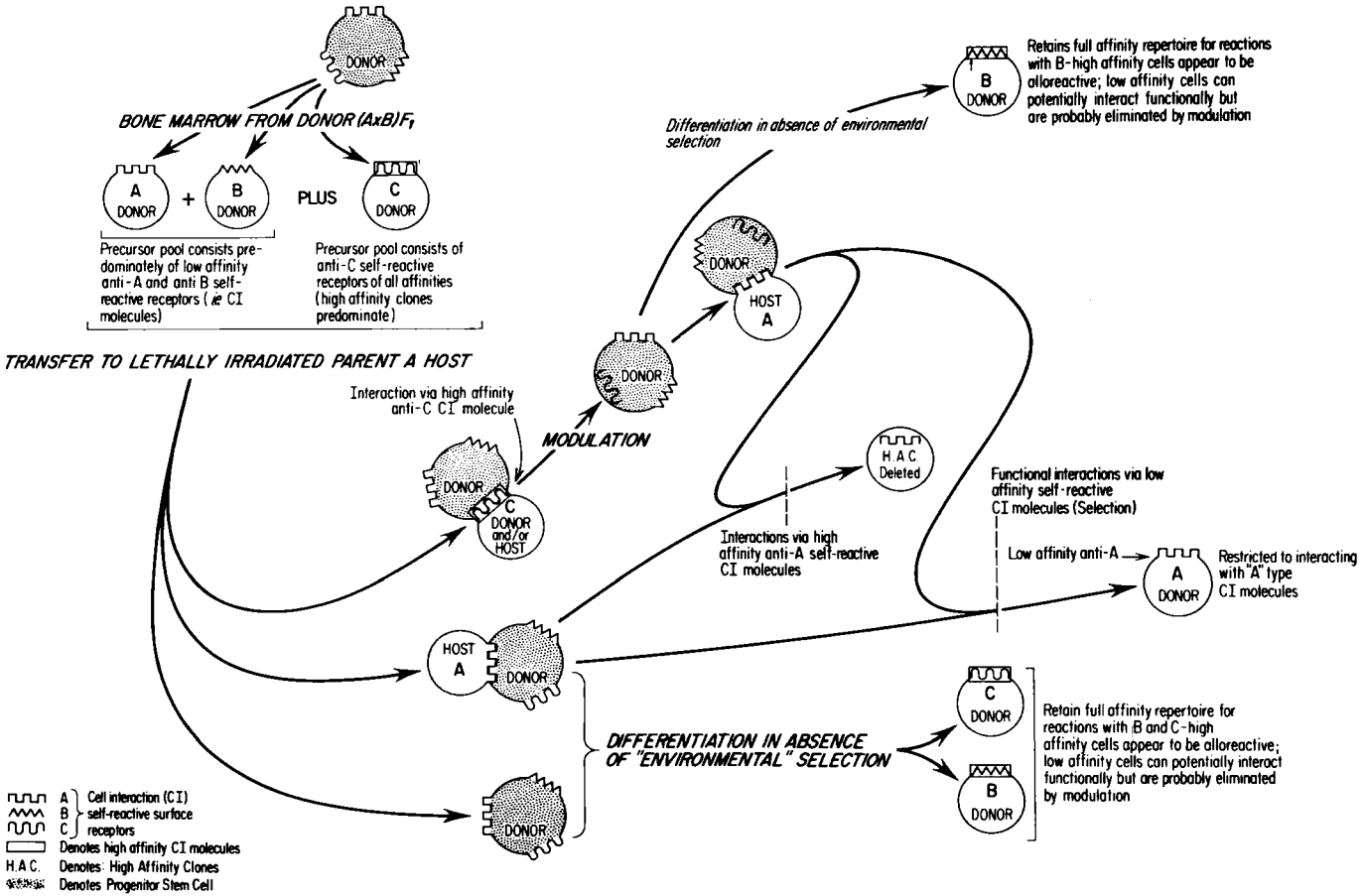


FIG. 4. Adaptive differentiation of $(A \times B)F_1$ bone marrow in semiallogeneic $(A \times B)F_1 \rightarrow A$ parent chimeras.

environment, predominantly CI_A-type molecules are presented to these donor cells. Accordingly, selection would preferentially eliminate emerging high affinity αCI_A cells, while allowing predominant emergence of low affinity αCI_A cells. In the absence of environmental selection, high affinity αCI_C populations would be maintained as would have been the case in the native $(A \times B)F_1$ environment.

The important difference imposed upon these differentiating cells by the parent *A* environment from that which existed in the native F_1 environment is that some high affinity αCI_B cells might be allowed to emerge from the progenitor stem cells as illustrated (far left and far right) near bottom of Fig. 4. Such high affinity cells could then exert their normal regulatory function of aborting clones of low-to-moderate affinity αCI_B specificity, which, in this environment, would have little or no useful purpose. The consequence of all this would be a shift in the phenotypic expression of functional cell interaction capabilities to one that is relatively restricted to partner cells of parent *A* type (since selection in this environment has favored mainly low affinity cells of αCI_A specificity). However, recent evidence suggests that another mechanism might exist in these circumstances to create the operational restriction observed in $F_1 \rightarrow$ parent chimeras (see Section V,B).

3. *Adaptive Differentiation in Double Parent* $A + B \rightarrow (A \times B)F_1$ *Chimeras*

The last experimental model to explain is the selection process by which lymphoid cells of two distinct orgins, parent *A* and parent *B*, differentiate in the environment of a neutral $(A \times B)F_1$ host to emerge with the capacities to interact functionally with partner cells of each reciprocal haplotype. As pointed out above, this has been observed in previous studies using T cell–B cell cooperative interactions as the assay system (69). A diagrammatic illustration of the postulated mechanisms involved in such circumstances is presented in Fig. 5.

These respective bone marrow populations differentiate in the environment of $(A \times B)F_1$ hosts as follows: Cells from donor *A* (*a*) maintain predominantly low affinity αCI_A self-reactive cells for obvious reasons (bottom, right); and (*b*) undergo environmental selection that deletes the high affinity αCI_B cells; this results in emergence of a functional interacting population of low affinity αCI_B reactivity. Concomitant in time, the donor *B* bone marrow cells (top right) undergo the corollary selection process whereby (*a*) the low affinity αCI_B reactive cells are maintained; and (*b*) modulation and deletion of high affinity αCI_A cells permits emergence of predominantly low affinity cells with αCI_A specificity. In both populations, the absence of environmental selection maintains cells with predominantly high affin-

ity αCI_C reactivity (as they would have been in each respective native environment). The overall consequence of this coadaptive differentiation is mutual and specific immunologic tolerance with maintenance of normal reactivity toward a third party, parent *C*, target.

This scheme raises a new explanation for results obtained with lymphoid cells from such double-parent bone marrow chimeras. Previously, it was concluded that cooperative interactions can occur across an MHC barrier when tolerant chimeric T cells are mixed with B cells of the opposite parental MHC type to which they have been rendered immunologically tolerant (69). However, Fig. 5 illustrates that what actually may occur in such situations is that there are effective cell–cell interactions between cells of parent *A* type and cells of parent *B* type, *but the interacting cells themselves are reciprocally self-specific.* Thus, cells with low affinity αCI_A reactivity from the parent *A* population may actually be interacting with cells of corresponding *A* type specificity that are present in the parent *B* population as a result of the selection process described above. Likewise, cells in the parent *B* population with low affinity αCI_B reactivity find willing partners among the cells of the parent *A* population that possess CI_B-type molecules (which have been similarly selected by adaptive differentiation). This implies that the interacting capabilities between partner lymphocytes are, indeed, quite restricted and specific.

VI. Extrathymic versus Intrathymic Influences on Adaptive Differentiation

A significant point that has arisen from certain of the adaptive differentiation studies concerns the possible role played by the thymic microenvironment in directing the self-recognition capabilties of T lymphocytes. Thus, in the CTL system Zinkernagel *et al.* (86), and subsequently Fink and Bevan (96), reported that $(A \times B)F_1$ precursors of CTL that have matured in an F_1 mouse whose thymus was replaced by a homozygous parent *A* thymus graft generate CTL (following immunization) that display a certain degree of restriction in their lytic activity for target cells possessing the same *H-2K* and/or *H-2D* genetic regions as parent *A*. A similar interpretation was ascribed by Waldmann *et al.* (97) and Bevan and Fink (98) to results of their studies concerning the effects of thymus grafts on development on *H-2* restrictions in helper T cell functions involved in antibody responses.

Studies in our own laboratory, however, have consistently failed to substantiate this interpretation concerning the role of the thymic microevironment in dictating self-recognition restrictions insofar as T cell–B cell cooperative interactions are concerned. Our studies, like

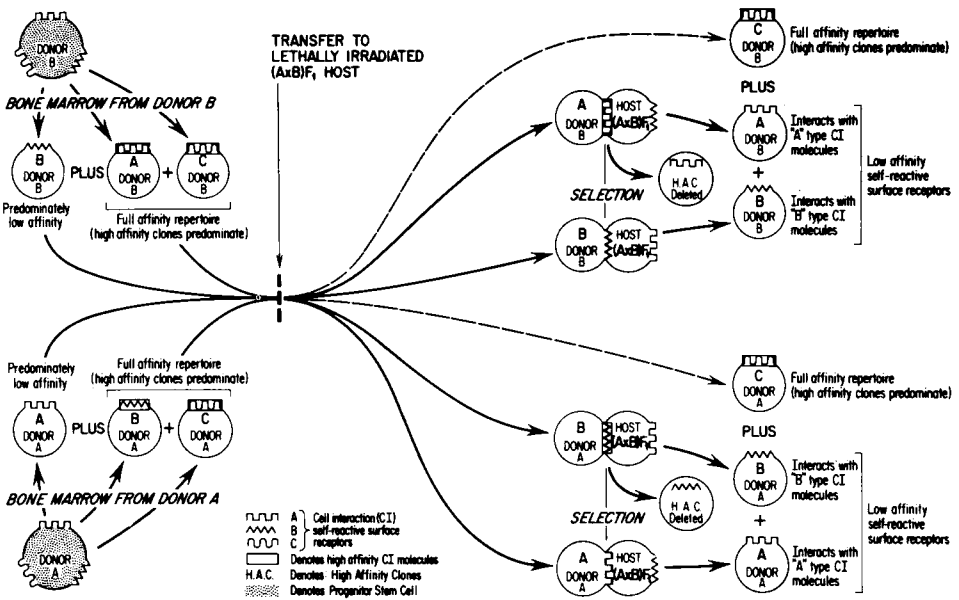


FIG. 5. Adaptive differentiation of parent A and parent B bone marrow in $A + B \rightarrow (A \times B)F_1$ chimeras.

the others mentioned above (86, 96–98), made use of artificially constructed bone marrow chimeras prepared by reconstituting adult-thymectomized, lethally irradiated F_1 mice with syngeneic F_1 bone marrow together with transplanted thymuses from either F_1 or parental donors. Reconstituted "thymic" chimeras of these types were then immunized with keyhole limpet hemocyanin (KLH), and their KLH-specific helper T cells so induced were tested for the cooperative helper activity they could provide to 2,4-dinitrophenyl (DNP)-primed B lymphocytes derived from conventional F_1 or parental donors in developing secondary anti-DNP antibody responses to DNP-KLH. A representative experiment of this type is summarized in Fig. 6, where it can be clearly seen that the thymus certainly does not restrict the partner cell preference displayed by helper T cells differentiating in such "thymic" chimera environments (99). Moreover, even where some slight thymic influence might exist in terms of helper T cell interactions with B cells for developing antibody responses of the IgG class, the same did not hold true for antibody responses of the IgE class (cf. groups XIV and XV, Fig. 6).

This absence of restricted partner cell preference of helper T cells from parental thymus-grafted F_1 chimeras in our hands (99) represents a significant difference from the results in the CTL systems (86, 96) and in certain other T cell–B cell cooperative interaction systems (97, 98) that indicated a somewhat greater restriction in self-specificity of CTL imposed by the thymic microenvironment in which precursors of such cells had differentiated. It is unlikely that any obvious technical points, such as contaminating parental T cells leaking out of the parental thymus graft or the existence of some type of suppressive mechanism(s), could explain these differences in outcome, since these possibilities were explored in our own studies (99) as well as certain of the others (86, 96) and were found to be absent. Indeed, our own concern about the technique of thymus grafting prompted us to use two different methods of preparing donor thymuses before transplantation; the results obtained were comparable in either case (99).

Another set of experiments that has been cited often as proving a crucial role of the thymus in dictating self-specificity restrictions was reported by von Boehmer *et al.* (100). These studies utilized the *H-2*-restricted CTL response of female murine T cells to the H-Y antigen of male cells. In this system, certain murine strains are high responders to H-Y antigens, while others are low responders; moreover, low responsiveness is, in certain instances, presumably a manifestation of lack of Y antigen-specific CTL precursors, whereas in other in-

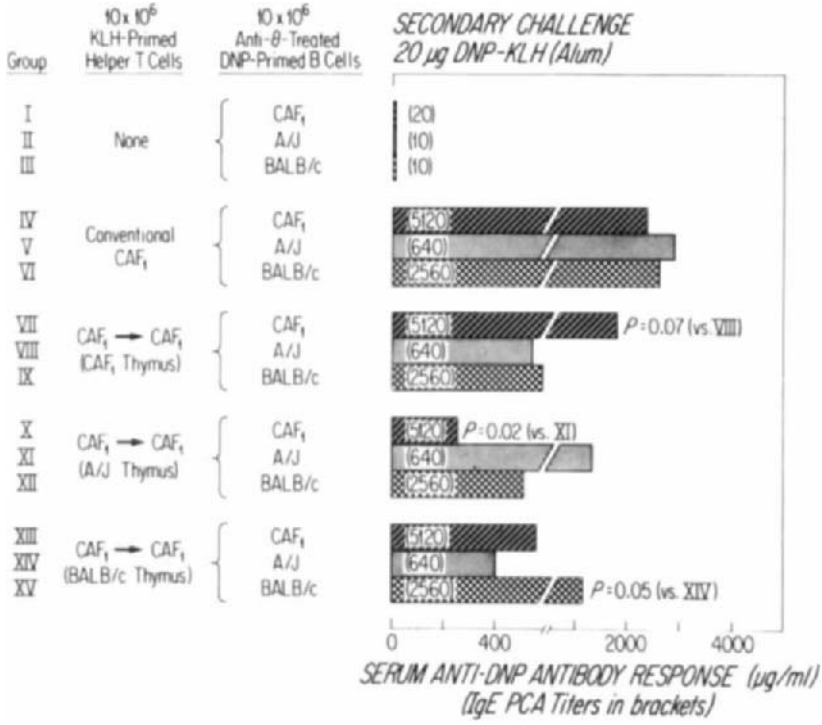


FIG. 6. *In vivo* helper activity of $CAF_1 \rightarrow CAF_1$ thymic chimera T cells for F_1 and parental *B* cells. Irradiated (650 rads) CAF_1 recipients (5 per group) were injected intravenously with 10×10^6 helper T cells from keyhole limpet hemocyanin (KLH)-primed conventional CAF_1 or CAF_1 thymic chimera donor mice together with 10×10^6 anti-θ serum-treated spleen cells from DNP-ASC-primed CAF_1, A/J, or BALB/c donor mice as indicated on the left of the figure. All recipients were secondarily challenged with 20 μg of DNP-KLH in alum shortly after cell transfer on day 0. Seven days later, all mice were bled for analysis of serum antibody levels and also killed for analysis of quantities of splenic DNP-specific PFC. On the right of the figure, the horizontal bars represent the geometric mean levels of serum anti-DNP antibodies in groups of four mice each. IgE anti-DNP antibody responses, as detected by PCA analysis, are indicated within parentheses enclosed in each corresponding horizontal data bar. With one exception, only those p values representing significant differences between relevant groups are indicated; although we do not consider a p value of 0.07 to be within significant limits, one such p value is indicated merely to illustrate the differences between groups VII and VIII. [Taken from Katz *et al.* (99), with permission.]

stances there is a presumed absence of *I* region restricted helper T cells for Y antigen-specific CTL precursors. When stem cells from low responders (due to deficiencies in *I* region-restricted helper cells) were found to differentiate into cells capable of displaying the high

responder phenotype in the environment of (low responder × high responder) F_1 recipients, this was interpreted as indicating intrathymic "learning" of the high responder phenotype under such circumstances (100). However, careful scrutiny of the data, and the system used to derive it, clearly indicates that the results can readily be interpreted in terms of the classical "schlepper" effect described many years ago in *Ir* gene-controlled antibody responses to hapten–protein conjugates (101). Such studies, first reported by Green *et al.* (101), demonstrated that an animal unable to respond to an antigen because it lacks the required *Ir* gene may nevertheless be stimulated to form antibodies against determinants on that antigen, when immunized with the antigen bound to an immunogenic carrier (or schlepper) to which its helper T cells are able to respond. By analogy, the study of von Boehmer *et al.* (100), described above, can be similarly interpreted when one realizes that the only differences between the two studies are that the most recent studies (*a*) deal with helper activity for precursors of CTL rather than precursors of antibody-forming B cells; and (*b*) utilize cooperating antigenic determinants that are native constituents of cell surface membranes rather than soluble hapten-carrier conjugates.

It is pertinent to point out that, subsequent to our own studies discussed above (99), studies from other laboratories similarly cast substantial doubts on the validity of the notions concerning the thymus in dictating *H-2* restrictions. Thus, Lattime *et al.* (102) have constructed triparental chimeras of the type $(A \times B)F_1 \rightarrow (A \times C)F_1$ and then analyzed the nature of *H-2*-restricted CTL responsiveness displayed by lymphocytes obtained from such chimeras. If the thymus were, indeed, solely (or even significantly) responsible for dictating self-specificity, then cells from a full reconstituted $(A \times B)F_1 \rightarrow (A \times C)F_1$ chimera would be expected to be restricted to target cells of haplotypes corresponding to those displayed by the host thymus (i.e., types *A* and *C*). Lattime *et al.* (102) found, however, that CTL derived from such triparental chimeras displayed *H-2*-restricted lytic activity against target cells of all three parental types (i.e., *A*, *B*, and *C*), although they were unable to lyse target cells derived from donors of unrelated haplotypes. Similarly, Good and his colleagues (103) have recently been successful in creating fully allogeneic murine bone marrow chimeras that manifested intact humoral as well as cell-mediated immune responsiveness. Although there was some transient deficiency in primary antibody response capacities of such chimeras, they displayed perfectly intact abilities to develop vigorous antibody responses upon

secondary antigenic stimulation; this clearly is not compatible with concepts that place the thymus in a central role for dictating self-specificity restrictions.

Unfortunately, there is no definitive or adequate explanation for these rather substantial differences in results concerning the influence of the thymus on self-specificity of differentiating T lymphocytes. It is difficult to escape the possibility that these substantial differences in results may be signaling to us a need to examine more critically, and to interpret more cautiously, the data obtained with the increasingly popular bone marrow chimera systems. Recently, bone marrow chimeras of various types have been employed in immunologic studies conducted in any number of different laboratories. When all the different findings are carefully examined, one is struck by the obvious diversity in experimental results obtained, not only from one laboratory to another but also within the same laboratory from one year to the next (cf. 74, 75) This is not altogether surprising when one considers that many variables, some readily apparent and some not, determine success and extent of reconstitution, health of the chimeras, and, hence, ultimate immunologic function manifested by chimeric cells. Moreover, differences in genetic restriction patterns of chimeric cell function have been observed depending on whether such functions were analyzed using *in vivo* or *in vitro* systems, a point emphasized very nicely by studies of Erb *et al.* (104).

One point of importance in this regard is that the nature of results obtained could reflect the choice of thymic chimeras utilized in any such analyses. In our own studies, for example, we took great care to screen all thymic chimeras for functional reconstitution according to their abilities to develop essentially normal anti-KLH antibody responses, an excellent criterion for establishing whether KLH-specific helper T cell activity had been restored to normal levels; only those thymic chimeras manifesting evidence of full reconstitution were employed as donors of helper T cells to be tested for their cooperating phenotypes (99). Since we discarded those thymic chimeras that developed lower-than-normal anti-KLH responses, it is possible that we may have selected for those helper T cells that had passed through all stages of the normal T cell differentiation pathway and hence been influenced by both intra- and extrathymic events. In none of the other studies performed in these systems (86, 87, 96–98, 100), as discussed above, were comparable functional criteria employed as guidelines for selecting the thymic chimeras actually utilized, and this might account for some of the differences in results obtained.

A second point worth emphasizing concerns the degrees of restriction observed in those studies in which some thymic influence on the restriction phenotypes of T cells seemed evident. Not infrequently, careful scrutiny of the data reveals variable "leakiness" in the restriction patterns observed. It is worth reiterating that *H-2* restriction in T cell–B cell cooperation as we originally defined it in conventional mouse systems is a *true* restriction, i.e., incompatible cell mixtures fail to respond altogether. In contrast, the results obtained in certain of the thymic chimera studies represent examples of *preference* in the sense that higher responses were obtained with certain cell mixtures than with others. The significant qualitative difference between true restriction, on the one hand, and preference, as seen in certain cases of studies with thymic chimeras, on the other, underscores the importance of extrathymic influences on T cell adaptive differentiation.

It may in fact be the case that bone marrow chimeras simply pose to many variables that cannot be adequately controlled, and which make such experimental animals different enough from a conventional intact mouse that we should perhaps reconsider the extent to which the chimera models are employed as the primary approach to answer many of the questions for which they are now used. We know, for example, that a sublethally irradiated parental mouse exerts a very definite allogeneic effect on transferred antigen-primed F_1 donor cells (105). We were somewhat surprised to find in recent studies that exposure of parental mice to lethal and supralenthal doses of irradiation (1100 rads and above) resulted in allogeneic effects exerted on transferred F_1 donor cells that were as strong or stronger than those observed at lower sublethal doses (99). This raises an obvious question as to what extent, if any, the parental environment may exert or influence or both, selection processes by virtue of such allogeneic effects, particularly during the early phases of immunologic reconstitution.

In any case, it should be emphasized that the discrepancies that have been reported among the various investigations conducted with chimeras do not argue against the ability of lymphocytes to learn their preferential cooperating phenotype, but rather it emphasizes the caution we must use in ascribing the major source of the environmental influence imposed. On the basis of the data described above, and additional data to be presented below (see Section VII), it is now quite clear that the extrathymic environment plays the most critical role in determining the ultimate cooperating phenotype of T lymphocytes that have differentiated under these circumstances. Consistent with this interpretation are studies of Kindred, who has reproducibly failed

to find evidence of intrathymic "learning" in nude mice grafted with allogeneic thymuses (106, 107).

VII. The Concept of Environmental Restraint and Possible Explanatory Mechanisms

In Section VII, we present certain recent experimental studies that may shed additional insights both on the mechanisms involved in adaptive differentiation and on certain plausible explanations for the seemingly contradictory findings obtained in different laboratories utilizing bone marrow chimera models.

A. The Extrathymic Environment Exerts the Major Influence on the Restriction Phenotype of Helper T Lymphocytes

The experiments described in this section were designed to analyze further the sites of major influence in determining the ultimate preferential cooperating phenotype(s) of regulatory helper T lymphocytes (95). Such experiments were conducted to extend our observations (see Section VI) demonstrating that the thymic microenvironment exerts relatively little influence on the cooperative phenotype of helper T cells (99). Since those findings represented a significant departure from other studies, which indicated that adaptive differentiation of precursors of CTL (86, 96) and helper T cells (97, 98) is predominantly influenced by the genotypes of the thymic microenvironment available to them during differentiation, it became essential to delineate more precisely the location(s) of dominant influence on helper lymphocyte maturation with regard to the self-recognition capabilities normally displayed by such cells.

Thus, we conducted analyses utilizing lymphocytes that had differentiated in several types of environments and then were removed from those environments and primed with antigen in the adoptive environment of sublethally irradiated, thymectomized F_1 recipients. Helper T cells induced in such adoptive priming circumstances were then analyzed for their partner cell preferences when mixed with primed B lymphocytes of either parental or F_1 origin in a standard hapten-carrier adoptive secondary assay system. The types of environments provided for initial differentiation consisted of either (*a*) bone marrow chimeras constructed by reconstituting lethally irradiated F_1 or parental mice, with intact thymuses, with either F_1 or parental bone marrow cells in various conbinations; (*b*) intact parental mice rendered tolerant as neonates to the MHC determinants of a second

parental strain; and (*c*) bone marrow chimeras prepared by reconstituting adult-thymetomized, lethally irradiated F_1 mice with either single-parent bone marrow cells or mixtures of bone marrow cells from both parents together with transplanted thymuses obtained from donors of one or the other parental type (95).

1. Parent → F_1 and F_1 → Parent Chimeric T Cells Display Unrestricted Helper Activity for Parental B Cells after Passage and Repeated Priming in Irradiated F_1 Recipients

In the first experiment of this type (Fig. 7), we investigated the extent to which restriction in parent → F_1 and F_1 → parent chimeric T cells was absolute (or not). To do this, helper T cells were primed to KLH in the chimeric environments of F_1 → F_1, parent → F_1 and F_1 → parent combinations. After a suitable period of time the splenic T cells from such chimeras were removed and transferred to irradiated F_1recipients for further adoptive priming to KLH. The cooperating phenotypes of such adoptively primed chimeric T cells were compared to that of conventional CAF_1 helper T cells in terms of providing helper activity for conventional F_1, A/J, and BALB/c B cells. In this way, we were able to ask two central questions about such chimeric T cells, namely, whether (*a*) the restriction observed when cells are obtained directly from the chimera are indeed absolute; and (*b*) the extent to which any restrictions that may be observed is conditioned by antigen-driven events in the chimeric environment. As shown in Fig. 7, it is quite obvious that (*a*) the levels of helper activities were substantial in all cases; and (*b*) such helper activities were totally unrestricted; i.e., irrespective of the nature of the initial chimeric environment, such cells were capable of helping either parental B cell type quite effectively.

2. Analysis of the Cooperating Phenotype of T Cells from Neonatally Tolerant BALB/c Mice (BALB[Tol.$_A$])

Spleen cells from BALB[Tol.$_A$] mice (BALB/c mice rendered tolerant as neonates to A/J), were adoptively primed to KLH in irradiated, thymectomized recipients of either parental BALB/c or CAF_1 type. Such KLH-primed activated T cells were then analyzed for their cooperating phenotypes in secondary adoptive transfer recipients. As shown in Fig. 8, BALB[Tol.$_A$] cells primed in BALB/c recipients displayed the restricted cooperation pattern normally encountered with conventional BALB/c T cells, namely, inability to provide help for B cells of A/J type (group IV), while providing excellent helper activity for BALB/c or CAF_1 B cells (groups V and VI, respectively). In con-

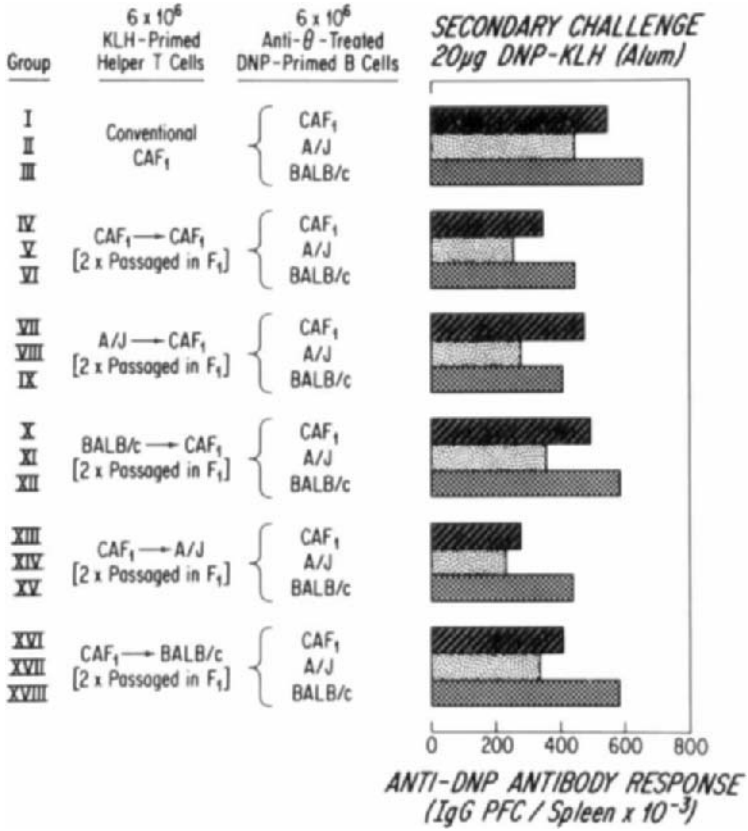


FIG. 7. Parent → F_1 and F_1 → parent chimeric T cells display unrestricted helper activity for parental B cells after passage and repeated priming in irradiated F_1 recipients. Conventional CAF_1 mice and chimeras of the types indicated on the left of the figure were primed with 20 μg of keyhole limpet hemocyanin (KLH) in CFA. Six weeks later, spleen cells from such KLH-primed conventional or chimeric donors were transferred intravenously (35×10^6 donor cells per recipient) to thymectomized, irradiated (650 rads) CAF_1 recipients; all recipients were immunized with 25 μg of KLH in CFA. Seven days later, these first recipients were killed, and their spleen cells were transferred intravenously to a fresh set of thymectomized, irradiated F_1 recipients that were adoptively primed with KLH in an identical fashion for additional 7 days. At the end of the second adoptive-priming period, the KLH-primed helper T cells were transferred together with anti-θ serum plus C-treated spleen cells from DNP-ASC-primed conventional CAF_1, A/J, or BALB/c donors into irradiated (but nonthymectomized) CAF_1 recipients (five per group). Adoptive secondary responses in all the various recipient groups were elicited by challenge with 20 μg of DNP-KLH in alum, and the DNP-specific IgG PFC responses were enumerated 7 days later. On the right of the figure, the horizontal bars represent the geometric mean levels of splenic PFC in groups of five mice each. There were no significant differences among the relevant comparative groups. [Taken from Katz *et al.* (95) with permission.]

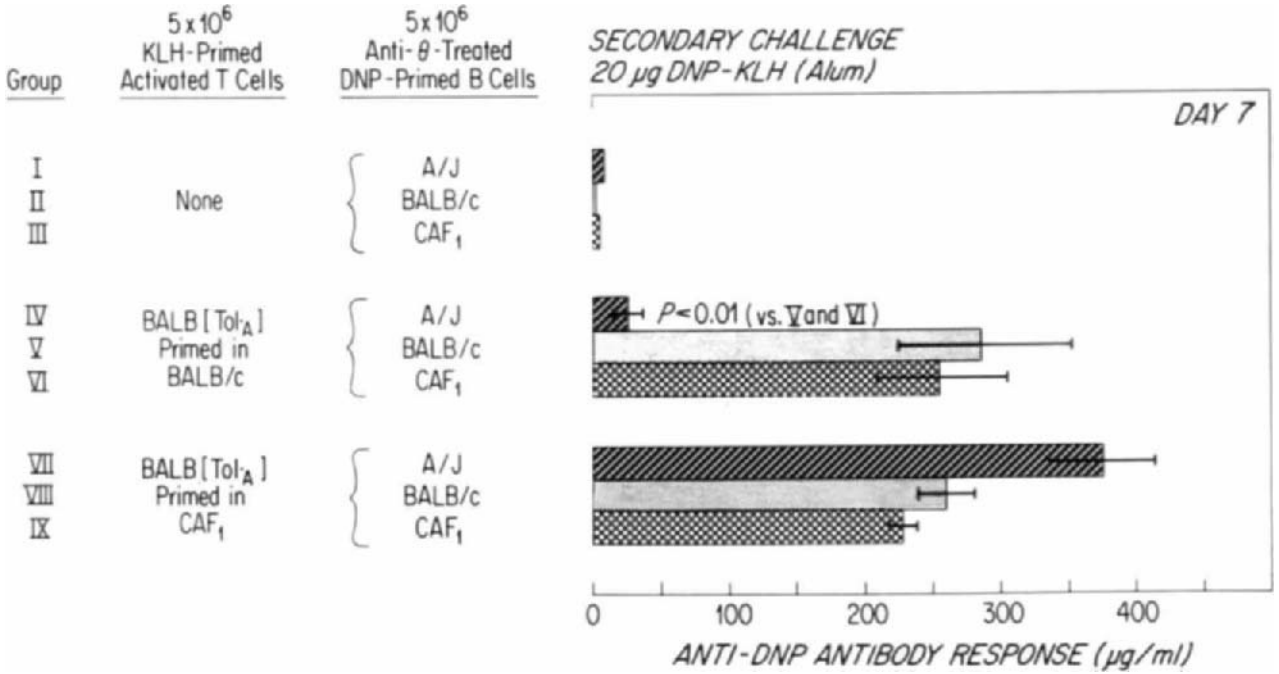


FIG. 8. T cells from neonatally tolerant BALB/c mice (BALB[Tol.$_A$]) can be activated to KLH in adoptive F_1 recipients as helper cells capable of cooperating with A/J partner B cells. Spleen cells from BALB[Tol.$_A$] mice were adoptively primed to KLH in thymectomized, irradiated recipients of either BALB/c or CAF_1 type. Seven days later, such adoptively primed helper cells were transferred (5×10^6 per recipient) together with an equal number of DNP-primed B cells derived from A/J, BALB/c, or CAF_1 donors into irradiated CAF_1 recipients; as controls for these KLH-primed activated cells, irradiated CAF_1 mice that did not receive any source of potential helper T cells were comparably primed with KLH for 7 days and cells from such control donors were transferred together with the different types of B cells into irradiated CAF_1 recipients (groups I–III). All recipient mice (5 per group) were secondarily challenged with 20 μg of DNP-KLH in alum and bled 7 days later for titration of IgE anti-DNP antibody levels. The data presented on the right of the figure are geometric mean levels and standard errors of serum anti-DNP antibodies in groups of four mice each. The one pertinent p value is illustrated. [Taken from Katz *et al.* (95), with permission.]

trast, BALB[Tol.$_A$] helper T cells adoptively primed in CAF_1 recipients were totally unrestricted in their capacity to provide help for either parental B cell type as well as those of F_1 type (groups VII–IX).

3. *Analysis of the Cooperating Phenotypes of Thymic Chimera T Cells Adoptively Primed in F_1 Recipients*

a. Parent → F_1 Thymic Chimera T Cells Display Unrestricted Helper Activity for Parental B Cells after Adoptive Priming in F_1 Recipients. Spleen cells from thymic chimeras of the types A/J → CAF_1 [A Thy] and BALB/c → CAF_1 [BALB Thy] as well as spleen cells from conventional CAF_1 donor mice were adoptively primed to KLH in irradiated, thymectomized CAF_1 recipients. As shown in Fig. 9, all three types of adoptively primed helper T cells were unrestricted in their capacities to provide help for either conventional parental A/J or BALB/c B cells or conventional CAF_1 B cells. Although not shown, helper cells from these single-parent → F_1 chimeras, primed *in situ*, displayed the expected restricted cooperating phenotype (79) when tested concomitantly with the adoptively primed chimeric helper cells (not shown).

b. T Cells of One Parental Type Derived from Double-Parent Thymic Chimeras Display Unrestricted Helper Activity for Parental B Cells after Adoptive Priming in F_1 Recipients. "Chim BALB/c" spleen cells were obtained from double-parent thymic chimeras of the types A/J + BALB/c → CAF_1 [A Thy] and A/J + BALB/c → CAF_1 [BALB Thy] by treatment of spleen cells from such chimeras with BALB/c anti-A/J serum + C *in vitro*. These two respective populations, i.e., "Chim. BALB/c" [A/J Thy] and "Chim. BALB/c" [BALB Thy], as well as conventional CAF_1 spleen cells were adoptively primed to KLH in irradiated, thymectomized CAF_1recipients and then tested for helper activity in secondary adoptive responses together with conventional parental A/J or BALB/c or CAF_1 DNP-primed B cells. It is obvious from the data presented in Fig. 10 that all three types of adoptively primed activated T cells were quite effective in providing unrestricted helper activity for all the partner B cell types.

4. *Interpretation*

Two important conclusions can be reached from the data presented in Figs. 7–10. First, MHC restrictions on the cooperating phenotype displayed by helper T lymphocytes are not dictated by the genotype of the thymic microenvironment in which such cells undergo certain of their presumably early phases of differentiation. Second, the ulti-

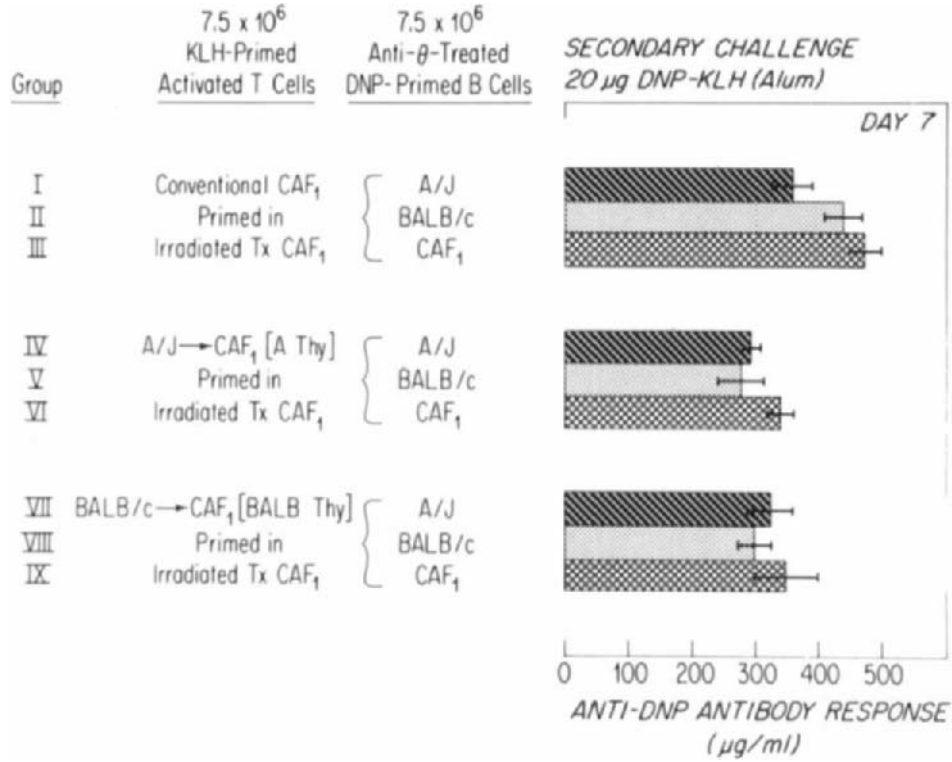


FIG. 9. Parent → F_1 thymic chimera T cells display unrestricted helper activity for parental B cells following adoptive priming in thymectomized, irradiated F_1 recipients. Spleen cells from unprimed conventional CAF_1 or single-parent → F_1 thymic chimeras of the types indicated on the left were adoptively primed for 7 days in thymectomized, irradiated CAF_1 recipients in the manner described in Fig. 8. (A/J → CAF_1 [A Thy] denotes chimeras constructed by reconstituting thymectomized, lethally irradiated CAF_1 mice with A/J bone marrow cells and thymus grafts from A/J donors.) Seven days later, these keyhole limpet hemocyanin (KLH)-primed activated T cells were transferred together with equal numbers (7.5×10^6 of each per recipient) of DNP-primed B cells obtained from conventional A/J, BALB/c, or CAF_1 donors into irradiated CAF_1 recipients (five per group). All recipients were secondarily challenged with 20 μg of DNP-KLH in alum and bled 7 days later for titration of IgG anti-DNP antibody levels. The data presented are geometric mean levels and standard errors of serum anti-DNP antibodies in groups of five mice each. There were no statistically significant differences among the relevant comparative groups. [Taken from Katz *et al.* (95) with permission.]

mate interaction restriction patterns displayed by fully mature helper T cells are conditioned by the extrathymic environment in which peripheral differentiation takes place, including elements introduced into the environment, although not native to it, to which such cells can become exposed during their differentiation processes.

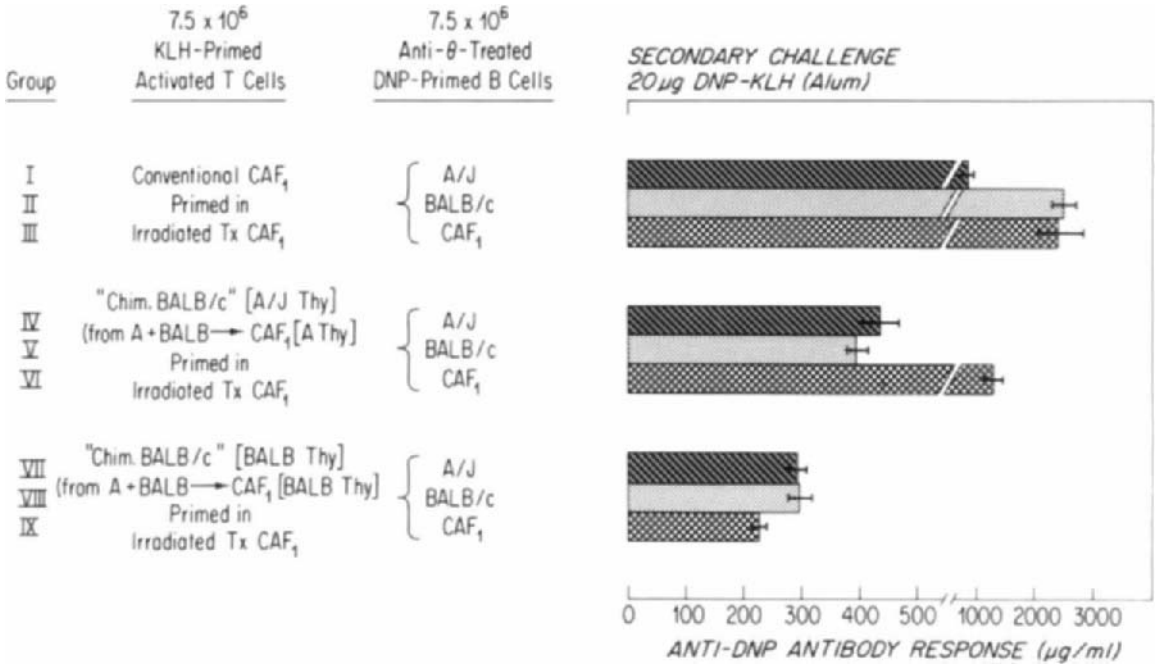


FIG. 10. T cells of one parental type derived from double parent $A + B \rightarrow F_1$ thymic chimeras display unrestricted helper activity for parental B cells following adoptive priming in thymectomized, irradiated F_1 recipients. Spleen cells from either conventional CAF_1 donor mice or spleen cells of the type designated "Chim. BALB/c" (double-parent chimera cells treated *in vitro* with BALB/c anti-A/J serum + C) obtained from thymectomized double-parent $\rightarrow F_1$ chimeras reconstituted with either parental A/J or BALB/c thymuses were adoptively primed in a manner identical to that described in Fig. 8. Likewise, analysis of the cooperating phenotypes of such adoptively primed helper T cells was conducted in secondary adoptive transfer recipients exactly as in Fig. 9. The data presented are geometric mean levels and standard errors of serum anti-DNP antibodies in groups of five mice each. There were no statistically significant differences among the relevant comparative groups. [Taken from Katz *et al.* (95), with permission.]

These conclusions are clear from the fact that, irrespective of the genotype of the thymus epithelium available to them, T cells differentiating under four different types of circumstances in which specific transplantation tolerance existed to all partner cell types employed in the analyses could be adoptively primed in F_1 recipients to develop helper T cell populations capable of interacting indiscriminately with all the relevant parental and F_1 partner B cell types. Since adoptive priming was performed in heavily irradiated, *thymectomized* F_1 recipients, any possible contribution of either host F_1 lymphocytes or F_1 thymic epithelium to the unrestricted cooperating phenotypes of the helper cells induced in this way was thereby eliminated.

We have no clear explanation for previous contrasting data obtained with adoptively primed $F_1 \rightarrow$ parent chimera cells in the CTL system (87) or neonatally tolerant or chimeric cells in another T cell–B cell collaboration system (81, 83). It should be noted, however, that the unrestricted cooperating phenotypes demonstrated in the above studies (Figs. 7–10) are not likely to be explained by some heretofore undetected sharing of a relevant *H-2* specificity between BALB/c and A/J. These particular murine strains have consistently manifested extraordinarily tight *H-2* restriction phenotypes when cells from conventional mice of these strains are analyzed. Moreover, if such specificity sharing existed between BALB/c and A/J, one should have observed evidence of this with $F_1 \rightarrow$ parent and/or parent $\rightarrow F_1$ helper cells primed *in situ*, but such has not been the case with chimeras prepared in these strain combinations (79).

An important point that emerges somewhat more subtly from these experiments is that the potential for unrestricted cooperative function was inherent in all the various cell populations tested, even in their initial differentiating environments. In other words, the unrestricted cooperative phenotype was not something learned for the first time during the adoptive priming process. This reasoning follows from the fact that, as mentioned above, adoptive priming was performed in thymectomized recipients and, more important in regard to this point, over a rather short period of time; if T cells capable of interacting effectively with all the tested partner cell types had not preexisted in their original environment, it seems unlikely that all the cooperating cell types would have emerged in quantitatively indistinguishable proportions in such rapid fashion. Moreover, the studies conducted with cells from nonthymectomized bone marrow chimeras (Fig. 7) make the additional point that the cooperating phenotype is not "locked in" irrevocably by antigen encounter itself. This follows from the fact that such cell populations were initially primed in their native

chimeric environment and then removed for further adoptive priming in F_1 recipients; had the population been restricted by their initial encounter with antigen in the chimeric environment, one might have expected to find at least quantitative differences in the levels of helper cell activities generated for the "inappropriate" partner cell type in the adoptive priming circumstance. Since this was not the case, It is quite unlikely that antigen encounter per se created any irrevocable restriction on the T cell populations generated during priming in the chimeric environment.

This brings us to consider two interrelated issues raised by these findings. First, how can one satisfactorily explain certain of the *apparent* restrictions displayed by T cells differentiating in some of the chimeric situations when such cells are tested for their interaction phenotypes after being taken directly from the chimeric environment? This is particularly true for T cells derived from single-parent $\rightarrow F_1$ and $F_1 \rightarrow$ single-parent chimeras. The first case is less problematic since one can easily dismiss apparent restrictions in single-parent $\rightarrow F_1$ chimeric T cells by placing the blame on absence of suitable macrophage antigen-presenting cells displaying the second parental cell interaction (CI) type; this explanation is based on the assumption that most, if not all, of the macrophage component in such chimeras would be derived from the reconstituting parental bone marrow population. Indeed, it has been shown by others that the T cells from single-parent $\rightarrow F_1$ chimeras could be induced to develop interaction capabilities with partner cells of the second parental type following exposure to antigen-presenting F_1 macrophages (81–83).

Greater difficulties arise in making similar considerations about T lymphocytes derived from chimeras of the $F_1 \rightarrow$ single parental type. Using the same reasoning mentioned above, it would be expected that the macrophage component in such chimeras would be of F_1 type if most or all of such cells were derived from the reconstituting bone marrow inoculum. However, as discussed in Section IV, we (79) and others (80–84) demonstrated that $F_1 \rightarrow$ single-parent chimeric T cells primed *in situ* displayed restricted helper activity for partner B cells identical to the parental host type. The data presented in Fig. 7 clearly document, however, that $F_1 \rightarrow$ parent T cells, while displaying a restricted phenotype when tested directly from their native environment, are not at all restricted once removed from the parental environment and adoptively primed in F_1 recipients. This means that the restriction observed with cells taken directly from the chimeric environment in these situations is actually a *pseudorestriction*. In reference to MHC-linked self-recognition, the term restriction connotes

certain definitions about the self-receptor repertoire, presence or absence of which determines the inherent interaction capabilities of cells from a given individual. Since the previously described studies document unequivocally that self-recognition receptors for all the potential partner cells in the various circumstances tested are in fact present, it seems more reasonable to consider such pseudorestriction phenomena as manifestations of *environmental restraint.*

Environmental restraint describes the process by which the environmental milieu can exert nonpermissive influences on the development of functional interacting partner cells corresponding to one of the possible (and actually existing) CI phenotypes inherent in a given lymphoid cell population. In other words, despite the fact that the F_1 lymphoid cells residing in an $F_1 \rightarrow$ parent chimera consist of self-recognizing subpopulations corresponding to each of the two inherited parental CI types, the parental host environment is permissive for expression (in that environment) of only that subpopulation corresponding to the CI phenotype of the parental host; that same environment is nonpermissive for emergence of the second parental type subpopulation for reasons that have yet to be delineated and may be explained by the mechanism postulated in Section VII C. Finally in this regard, the demonstration that $F_1 \rightarrow$ single-parent chimera T cells are truly unrestricted (i.e., analogous to conventional F_1 lymphocytes) must be borne in mind in interpreting any studies utilizing such chimera T cells as representative of a restricted (i.e., parent-like) population, as in the case of the recent studies of Singer *et al.* (108).

Irrespective of what the actual mechanism of environmental restraint may be, these experiments emphasize the importance of extrathymic events in determining the cooperating phenotypes of cells obtained from various situations. Although true in all four situations presented in Figs. 7–10, this fact is particularly underscored by the observations with cells derived from neonatally tolerant BALB/c mice and by those obtained with isolated parental cells from double-parent $\rightarrow F_1$ chimeras reconstituted with thymus of only one parental type. Central to the extrathymic influences on the self-recognition restriction phenotypes of the cells analyzed was the existence of specific immunologic tolerance to transplantation antigens of the opposite parental type. In the neonatally tolerant situation, tolerance was induced at an obviously very early stage of lymphoid cell differentiation in such mice. Since the tolerance state itself was sufficient to determine the ultimate cooperating phenotypes of cells differentiating in such circumstances, this indicates that the extrathymic environment must contribute to these processes by providing the appropriate mi-

lieu for relevant selection processes that will dictate the interacting phenotypes of cells developing in that environment.

To reiterate what we have postulated above (Section V), we believe that adaptive differentiation reflects the occurrence of two concomitant selection processes (79). First, cells with high affinity self-receptors (i.e., for CI molecules of the native environment or, in the case of neonatal tolerance, for CI determinants of the inducing cell population) are deleted, thereby allowing emergence of low-to-moderate affinity self-recognizing cells that will become the functional interacting components of the system. Second, and concomitantly, cells with high affinity receptors for CI determinants of other members of the species, which are not confronted by large numbers of foreign CI determinants in the environment in which they find themselves, react with the only target molecules of such type that they find, i.e., those present on other cells of similar CI type; this would eliminate the low-to-moderate affinity cells of corresponding CI type, which would have no functional purpose in the wrong environment in which they exist and might replicate uncontrollably. This would explain the selective pressures that exist to maintain a reasonable frequency of such high affinity nonself, or alloreactive, cells. When neonatal tolerance is induced, the high affinity cells capable of recognizing the cell type used for the induction of tolerance would be deleted, thereby allowing emergence of the low affinity component.

The data presented in Fig. 8 with adoptively primed neonatally tolerant BALB/c spleen cells, particularly the fact that it was necessary to remove them from their native environment in order for them to manifest their unrestricted potential, provides strong support for the existence of this postulated mechanism. Furthermore, it should be noted that, since inherent in the adaptive differentiation concept is the fact that cells from all individuals are genetically capable of displaying functional self-recognition for partner cells of all members of the species, it is logical that a certain proportion of cells exist at any given time, albeit in low frequency, and can be called upon to display such interaction capabilties under the appropriate circumstances. Normally, perhaps owing to mechanisms of environmental restraint, we do not appreciate their existence. However, under certain circumstances, notably with highly sensitive assay systems such as those described by Pierce and Klinman (93), their presence can be demonstrated.

Studies presented in Section VII,B provide data that seem to be illuminating with respect to the mechanism underlying environmental restraint.

B. (Responder × Nonresponder) F_1 T Cells Can Be Taught to Preferentially Help Nonresponder, Rather Than Responder, B Cells

Several years ago, we reported that T cells from (responder × nonresponder) F_1 hybrids primed to the synthetic terpolymer, L-glutamic acid, L-lysine, L-tyrosine (GLT), to which responses are governed by *H*-2-linked *Ir-GLT* genes, were restricted in their ability to provide GLT-specific help for DNP-primed B cells from the respective parental mice in response to DNP-GLT (109). Thus, such F_1 T cells were able to provide normal helper activity for DNP-specific B cells from responder, but not from nonresponder, donor mice. This finding contrasted sharply with the indiscriminant ability of F_1 T cells to interact effectively with partner B cells from *either* parent when the carrier antigen employed was not one to which responses were governed by a known *Ir* gene. This observation has subsequently been confirmed by others in studies conducted in mice (110, 111) and guinea pigs (112).

These observations were interpreted as an indication that in heterozygous individuals independent subpopulations of interacting T lymphocytes existed, one each corresponding to the respective parental type (71, 109, 113). Hence, we envisaged that stimulation of a (responder × nonresponder) F_1 T cell population by GLT would sensitize only the population of T cells able to recognize and react with the functional cell interaction (CI) phenotype of the responder parent; F_1 T cells corresponding to the nonresponder parent CI phenotype would not be stimulated by GLT. This situation would therefore be manifested as defective ability of F_1 T cells to interact with nonresponder B cells irrespective of the antigen specificity of the latter. This original interpretation (71, 109, 113) has been reinforced by the subsequent demonstrations of the existence of independent F_1 T cell subpopulations reactive with each respective parental CI phenotype (114–119).

Recently, we have reported results of experiments designed to determine whether the normally restricted cooperating phenotype of (responder × nonresponder) F_1 T cells specific for GLT could be experimentally manipulated to express a different cooperating phenotype (120). Specifically, we were interested in generating GLT-specific F_1 T cells that might now express cooperative helper activity for nonresponder B cells. We have found that this can, indeed, be done by inducing a transient allogeneic effect during the period of priming of F_1 mice to GLT. Moreover, the conditions in which the allogeneic effect is induced determines, in a critical fashion, the ultimate cooperating

phenotype observed with the resulting F_1 GLT-primed helper T cells. This is illustrated by the experimental data presented below.

CAF_1 T cells obtained from mice primed to GLT, either in the absence of any allogeneic effect or in the presence of an allogeneic effect induced by one or the other parental cell type, display the pattern of cooperative helper activity for DNP-primed B cells from either A/J, BALB/c, or CAF_1 donor mice that is depicted in Fig. 11. Note that CAF_1 helper T cells primed to GLT in the absence of an allogeneic effect provide no help for B cells from A/J mice (group II), while providing help for B cells from BALB/c (group VI) or CAF_1 (group X) donor mice, results identical to the original findings (109).

The remarkable findings are those pertaining to the patterns of F_1 helper activity, for the various B cells, that were generated under the influence of allogeneic effects induced by one or the other parental cell type. First, note that F_1 helper T cells generated under the influence of an allogeneic effect induced by either A/J or BALB/c parental cells were clearly and significantly enhanced in their levels of cooperative activity for B cells derived from F_1 donors (groups XI and XII). This pattern of indiscriminately enhanced F_1 helper T cell activity as manifested with F_1 B cells did not hold true when such F_1 T cells were assayed for their ability to help nonresponder A/J or responder BALB/c parental B cells. Thus, F_1 T cells generated under the influence of an allogeneic effect induced by parental BALB/c cells were clearly capable of providing GLT-specific help for nonresponder A/J B cells (group IV), but were unable to provide detectable help for responder BALB/c cells (group VIII). Conversely, F_1 T cells generated during an allogeneic effect induced by parental A/J cells did not display effective helper activity for nonresponder A/J B cells (group III), while exhibiting significantly enhanced helper activity of responder BALB/c cells (group VII).

These studies make two important points. First, the usually restricted phenotype of (responder × nonresponder) F_1 T cells that typically is permissive only for providing cooperative helper activity for B cells of responder, but not of nonresponder, type *can* be changed by inducing an allogeneic effect during the priming of such F_1 mice. This is true *provided the allogeneic effect is induced by cells derived from the opposite*, i.e., responder, parental type. Second, actually, the ultimate cooperating phenotype of GLT-primed F_1 helper T cells is differentially and reciprocally directed toward B cells of one parental type or the other depending on which parental donor cells are used for inducing the allogeneic effect that takes place during the priming regimen. Thus, F_1 T cells primed to GLT under influence of an allogen-

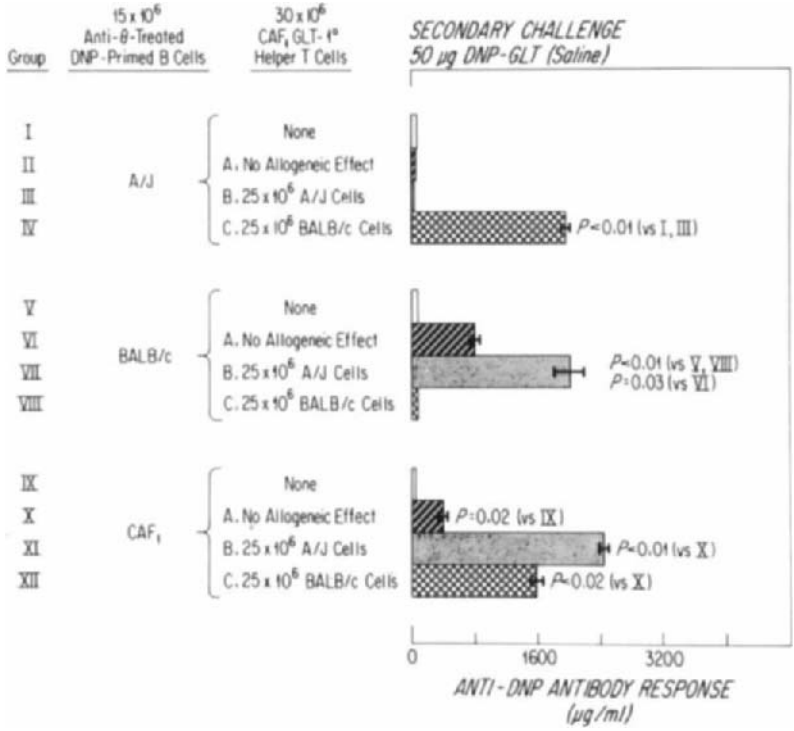


FIG. 11. Helper T cell activity of (responder × nonresponder) F_1 spleen cells primed to GLT under the influence of an allogeneic effect. Irradiated (650 rads) CAF_1 recipient mice were injected with intravenously with 15×10^6 DNP-primed B cells from either A/J, BALB/c, or CAF_1 donors, either in the absence of helper cells or together with 30×10^6 spleen cells taken from F_1 mice primed to L-glutamic acid–L-lysine–L-tyrosine terpolymer (GLT) as follows: (a) helper T cell donors, primed in the absence of an allogeneic effect, were primed with 50 μg of GLT in CFA followed 10 days later by a second injection of 50 μg in saline; (b) helper T cell donors were similarly primed to GLT under the influence of an allogeneic effect induced by iv injection (on day 10 after initial immunization with GLT) of 25×10^6 spleen cells from either parental A/J or BALB/c donors just prior to the second injection of 50 μg of GLT in saline. Spleens were removed from such donor mice 7 days after the second injection to be used as helper cells. All adoptive recipients were secondarily challenged with 50 μg of DNP-GLT in saline. The data are presented as geometric mean levels of serum anti-DNP antibodies of individual mice in groups of 5 mice each bled on day 7 after cell transfer and secondary challenge. Horizontal lines represent the range of standard errors, and relevant p values of statistically significant differences are indicated. [Taken from Katz *et al.* (120), with permission.]

eic effect induced by parental BALB/c cells now provide effective help for nonresponder A/J B cells, but do not do so for responder BALB/c B cells, and vice versa. On the other hand, F_1 T cells primed to GLT under the influence of an allogeneic effect induced by either parental cell type display significantly enhanced levels of helper activity for B cells derived from F_1 donors.

The fact that the allogeneic effect induces such exquisite discriminatory helper activities when F_1 T cells are assayed on parental B cells, but loses this discriminatory aspect when F_1 B cells serve as the partner B cells in the assay, is perhaps the most pertinent aspect of these findings with respect to understanding the regulatory events that these data reflect. Parenthetically, the results with F_1 B cells provide additional arguments against any significant contribution made by contaminating parental B cells that may be carried over in the final assay system; but this possibility has been more directly circumvented as described elsewhere (120).

Since the interpretation of these findings may bear directly on the mechanism of *environmental restraint* defined above (Section VII,A,4), these issues will be discussed together in the next section VII,C.

C. Antiself Receptor Responses Could Explain Environmental Restraint

We believe that the results presented in Fig. 11 reflect the consequences of two interdependent events that are schematically illustrated in Fig. 12. The first event, is that the allogeneic effect induced by one parental cell type exerts powerful stimulatory signals that substantially augment the normal differentiaton signals induced by immunization with antigen alone; this has been amply documented to occur in many previous studies [see Chapter XI of Katz (26)]. The consequence of such stimulatory signals is reflected in the significant enhancement of GLT-specific helper T cell activity provided to B cells from F_1, BALB/c and A/J donors, as shown in Fig. 11. Of particular note is the capacity of the allogeneic effect, induced by BALB/c cells, to draw out permissiveness of F_1 cells in providing GLT-specific help to the nonresponder A/J B cells. One point that should be clarified is that this finding is by no means directly analogous to either (*a*) our own earlier studies demonstrating that an appropriately timed allogeneic effect would convert the normally tolerogenic effects of DNP-derivatized D-glutamic acid, D-lysine (D-GL) to an immunogenic signal for DNP-primed B cells (121, 122); or (*b*) that of Ordal and Grumet (123), who demonstrated the capacity of an allogeneic effect to permit

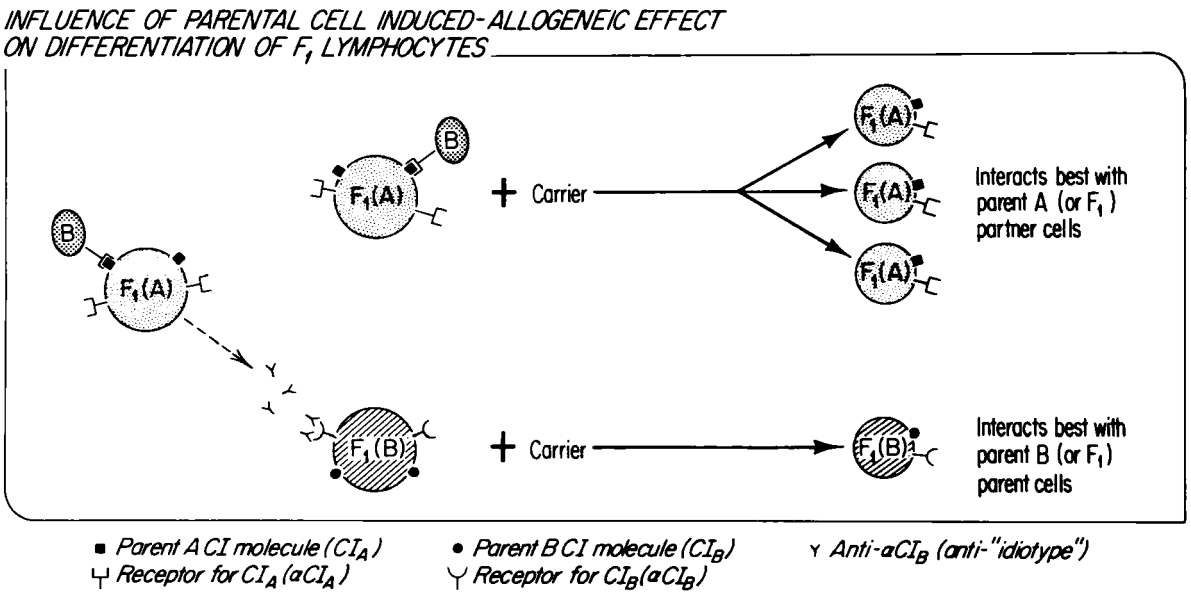


FIG 12. Events illustrating results presented in Fig. 11. See text for explanation.

nonresponder mice to make IgG (as well as IgM) antibody responses to (T,G)-A—L. In both the aforementioned instances, the allogeneic effect provided a necessary stimulus to target *B* cells, thereby replacing a normal interaction step missing in both circumstances. In our present studies, the allogeneic effect has obviously provided the necessary stimulus, in addition to antigen, to encourage the differentiation of the subset of "A/J-type" T cells capable of interacting with DNP-primed A/J B cells. The reasons for the failure of such cells to differentiate through the helper cell pathway in response to GLT under normal circumstances is a central question to the whole issue of what role is actually served by *Ir* genes and their products. This issue will be discussed in greater detail below in Section IX,B.

The second of the two interdependent events, and by far the more difficult to address, pertains to the explanation for the very striking discriminatory aspects of helper activities when F_1 T cells are assayed on *parental* B cells. Obviously, no direct evidence is presently available to permit conclusions concerning this aspect of our data. Nevertheless, we would like to suggest that, aside from the stimulatory consequences of the allogeneic effect in inducing helper T cell function as discussed above, a second consequence may be the stimulation of an F_1 response against certain of its own receptors that self-recognize native CI determinants (αCI). Thus, as illustrated in Fig. 12, when parental *B*-type cells induce an allogeneic effect in an $(A \times B)F_1$, the response within the F_1 would be directed against self-receptors for *B*-type CI molecules (anti-αCI_B), and vice versa. These types of anti- "idiotype" responses against self-recognizing receptors would be capable of preventing development of helper T cells belonging to the corresponding parental-type T cell subpopulation without adversely affecting the development of helper cells corresponding to the opposite parental-type T cell subpopulation.

It is rather simple to envisage how such a mechanism of postulated anti-α CI responses could explain phenotypic environmental restraint. Thus, in the case of an $(A \times B)F_1 \rightarrow$ parent *A* chimera, it is possible that, for reasons that have yet to be defined, the induction of an immune response within that environment involves the concomitant induction of an anti-αCI_B response that would blunt the emergence of operational cells of the αCI_B subpopulation; on the other hand, the absence of an anti-αCI_A response would allow emergence of the αCI_A interacting subpopulations. The operational consequences of this sequence of responses would be that the parent *A* environment would be permissive for cells of the CI_A phenotype and nonpermis-

sive for cells of the CI_B phenotype in terms of their participating in responses taking place within that environment. Once removed from such conditions of environmental restraint, however, as in the situations involving adoptive priming in conventional F_1 enviornments (Section VII, A), both subpopulations (i.e., CI_B as well as CI_A) would emerge and display their functional interacting capabilities. This is precisely what the data presented above demonstrate.

If studies currently in progress demonstrate anti-αCI reactions to be the mechanism responsible for the data presented in Fig. 11, and for the circumstances of environmental restraint observed in certain of the chimeric situations, then one wonders (*a*) if responses of this type occur normally in the pathways of immune regulation; (*b*) whether virgin cells and primed cells are similarly susceptible to the effects of such reactions, or not; and (*c*) what relationship such anti-αCI responses might have to the whole picture portrayed by *Ir* genes and the specificity they appear to display for the antigens under their control. This latter point is discussed in greater detail in Section IX,B.

VIII. The Case for B Cell Adaptive Differentiation and Pertinence to the Concept of Interacting "Partner Cell Sets"

Most thinking on the matter of self-recognition and MHC-linked genetic restrictions on cell–cell interactions is oriented predominantly around T lymphocytes and macrophages. It is presently easier to cope with the notion that T lymphocytes are endowed with self-recognition capabilities, by whatever molecular mechanism, and that they do utilize this capability largely at the macrophage surface membrane level. There is a no a priori reason, however, to consider that B lymphocytes are not similarly subject to comparable selective events based on self-recognition of MHC-encoded CI molecules. Indeed, we have previously obtained experimental data, in both conventional (66) and bone marrow chimera animals (79), which strongly suggest that this is the case.

[Recently, Sprent and Bruce (123a) have claimed that they were unable to confirm our results on B cell adaptive differentiation as reported by us with B cells differentiating in $F_1 \rightarrow$ parent chimeras (79). It is pertinent to point out, however, a substantial difference in experimental design which readily explains their failure to observe B cell adaptive differentiation, in contrast to our findings. In their study (123a), they used as sources of helper T cells lymphocytes obtained from $F_1 \rightarrow$ parent chimeras, assuming that such helper cells were equivalent in their "restricted" phenotype to helper T cells derived

from conventional parental donors (it was the latter type of T cells that we employed in our studies to demonstrate restricted cooperating phenotypes of $F_1 \rightarrow$ parent chimeric B cells). Since we had specifically reported the importance of using conventional parent T cells in order to observe adaptive differentation of $F_1 \rightarrow$ parent chimeric B cells (79), and since it is quite obvious that T cells from $F_1 \rightarrow$ parent chimeras are *pseudorestricted* only in their native environment due to environmental restraint (see Section VII,A), we do not consider the data reported by Sprent and Bruce (123a) to be a valid refutation of the existence of mechanisms of B cell adaptive differentiation.]

Since, as discussed in Section VI, it is becoming increasingly evident that studies in bone marrow chimera systems must be interpreted cautiously owing to certain uncontrollable potential artifacts, we have sought to address this question under conditions in which conventional animals could be employed. The results of such experiments (124) are presented and discussed in this section.

The question addressed by the following studies is as follows: In the development of a given antibody response, do interacting T and B lymphocytes pair off into partner cell sets in accordance with inherent and *reciprocal* self-recognition capabilities? In other words, is it correct to view any given lymphocyte population as consisting of many subsets of self-recognizing partner cells, any pair of which interact optimally when there is a "best-fit" reciprocal recognition between them? If this were, indeed, true then the case for adaptive differentiation of B lymphocytes would be considerably strengthened.

In order to ask this question, we made use of the system of *Ir* gene-restricted responses to the synthetic terpolymer, GLΦ. This terpolymer was the one studied extensively by Dorf and Benacerraf in defining the complementing α and β *Ir* genes in the mouse (125). Thus, mice of the parental B10.A and B10 strains are phenotypic nonresponders to GLΦ; the recombinant 5R strain is a phenotypic responder, since it has received the α gene from the B10.A and the β gene from the B10 portion of its recombinant *H-2* genome. The (B6A)F_1 hybrid is a phenotypic responder, since it inherited the α gene from the A parent and the β gene from the B6 parent, albeit on separate chromosomes.

In earlier studies (126), we addressed the question of whether complementation in this sytem reflected the presence of one gene in T cells and the other in B cells. Our results clearly demonstrated that this was not the case, and that functional expression of responsiveness required the presence of both genes in each cell type, or at least in T cells. In the course of performing such studies, however, we made an

unexpected and unexplained observation. Thus, GLΦ-primed F_1 T cells, which were fully capable of providing excellent help to DNP-FγG-primed B cells from responder 5R mice, were quite inefficient in providing help for similarly primed DNP-specific B cells from identical F_1 donors in a typical adoptive transfer system (126). It is to the explanation of this paradoxical result that the present study has been addressed.

There is nothing overtly defective in F_1 responder mice in terms of their ability to generate cooperative hapten-carrier responses to DNP-GLΦ, at least among interacting cells within an intact mouse. Thus, immunization of an intact (B6A)F_1 mouse with DNP-GLΦ generates reasonable anti-DNP antibody responses. Moreover, spleen cells from such DNP-GLΦ-primed F_1 mice can readily develop adoptive secondary anti-DNP responses when challenged with DNP-GLΦ in irradiated F_1 recipient mice (124).

Why, then, did we previously observe such an obvious defect in the ability to elicit cooperative interactions between mixtures of GLΦ-primed F_1 T cells and DNP-primed F_1 B cells (126)? Perhaps the fact that the cooperative mixtures of T and B cells in our previous adoptive transfer responses utilized cell populations that had been independently primed in separate donor environment contributed to this puzzling finding. To address this possibility, the experiment summarized in Fig. 13 was performed (124).

In this study, B cells were primed in 5R donor mice with DNP-FγG. DNP-specific (B6A)F_1 B cells were, on the other hand, primed in two different ways: one population was primed with DNP-FγG while the second population was primed with DNP-GLΦ. All B cell populations were thoroughly depleted of T cells by double anti-θ serum plus complement treatments and then tested for cooperative activity with either KLH-primed (B6A)F_1 helper cells in response to DNP-KLH or GLΦ-primed F_1 helper cells in response to DNP-GLΦ. The experimental system employed for this type of adoptive transfer experiment was the same as that used in all the *in vivo* genetic restriction studies reported in the past (see reference 46 for full description).

As shown by groups I–III at the top of Fig. 13, excellent and comparable cooperative responses to DNP-KLH were obtained in all cases. Responses to DNP-GLΦ were not comparable among the three B cell types as shown by groups IV–VI at the bottom. Thus, while the DNP-FγG-primed 5R B cells received optimal help from GLΦ-primed (B6A)F_1 T cells, little or no cooperation occurred between such T cells and DNP-FγG-primed F_1 B cells. This is consistent with the results we reported previously, as already described (126). In striking con-

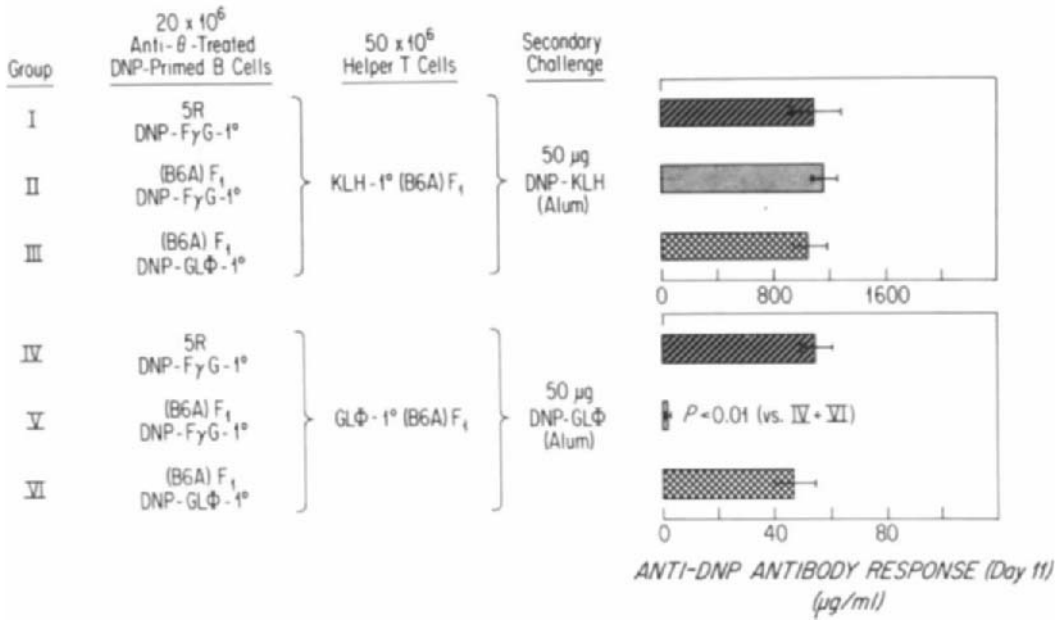


FIG. 13. Restricted partner cell preference of F_1 B lymphocytes induced during responses controlled by complementing *Ir-GLΦ* genes. Spleen cells from either keyhole limpet hemocyanin (KLH)-primed (groups I–III) or GLΦ-primed (group IV–VI) (B6A)F_1 donors were injected intravenously into unirradiated (B6A)F_1 hybrid recipients. Twenty-four hours later, all recipients were irradiated and shortly thereafter injected with DNP-primed B cells from either 5R or (B6A)F_1 donor mice which had been primed with either DNP-FγG or DNP-GLΦ as indicated. All B cells were obtained from spleen cell preparations treated *in vitro* with anti-θ serum plus complement *twice* consecutively. After transfer of the B cell populations, recipient mice were challenged with either 50 μg of DNP-KLH in alum or 50 μg of DNP-GLΦ in alum as indicated. Mean serum levels of anti-DNP antibody of groups of 5 mice on day 11 after secondary challenge are illustrated. Horizontal bars represent ranges of standard errors, and statistically significant differences are indicated on the figure. [Taken from Katz. (124) with permission.]

trast, when DNP-specific F_1 B cells were obtained from donors primed with DNP-GLΦ, this cooperative defect was overcome.

This study demonstrates that in the complementing *Ir-GLΦ* gene system a high degree of restriction is placed upon which DNP-specific B cells can accept effective help from GLΦ-specific T cells. Thus, F_1 mice derived from two GLΦ-nonresponder parents are phenotypic responders and can develop anti-DNP (as well as anti-GLΦ) antibody responses when immunized with DNP-GLΦ. Spleen cells taken from F_1 mice primed in this way can effectively transfer adoptive secondary responses to DNP-GLΦ in irradiated F_1 recipients (124). This clearly illustrates that the respective DNP-specific B cells and GLΦ-specific helper T cells are able to communicate fully with one another in the circumstances just mentioned. Likewise, DNP-specific F_1 B cells primed in one animal with either DNP-GLΦ or DNP-FγG can accept help from KLH-primed T cells generated in another F_1 donor when these two cell populations are transferred together to an irradiated F_1 recipient and challenged with DNP-KLH (Fig. 13).

However, when these same two populations of DNP-primed F_1 cells are transferred together with GLΦ-primed F_1 T cells, *only* those primed with DNP-GLΦ are able effectively to accept help from the GLΦ-specific helper T cells that function in this system (Fig. 13). This clear restriction in effective partner cell cooperation appears to reflect the fact that, after exposure to irradiation, the remaining GLΦ-primed T cells whose helper function we see in this assay system represent a select subpopulation of the total GLΦ-specific helper cells generated by immunization of F_1 mice with GLΦ. It must be remembered that in our genetic restriction protocol helper T cells are exposed to irradiation *in situ* to eliminate both enhancing and suppressive allogeneic effects (46). While substantial amounts (but not all) of helper activity are retained after irradiation of helper cell populations primed with strong carriers such as KLH, this is not true with helper cells primed with some of the weaker antigens, such as certain synthetic polypeptides. In our first studies with GLΦ (126), we noted this to be a particular problem with GLΦ-primed helper cells that routinely display marked diminution in helper activity after exposure to irradiation. It is with this select population of *radioresistant* GLΦ-primed F_1 T cells that a portion of the total DNP-specific B cells from the DNP-GLΦ-primed F_1 population is able to effectively cooperate in developing the response to DNP-GLΦ observed; obviously, other DNP-specific B cells in the DNP-GLΦ-primed population are able to interact effectively with KLH-primed helper T cells in response to DNP-KLH. The

pertinent point is that priming with an unrelated DNP-carrier, such as DNP-FγG, generates F_1 DNP-specific B cells that are clearly *lacking* a corresponding subset capable of accepting help from this select subpopulation of GLΦ-primed F_1 T cells.

The fact that DNP-FγG-primed 5R B cells were *not* lacking a subset of DNP-specific B cells capable of interacting with this select subpopulation of GLΦ-primed F_1 T cells is both interesting and pertinent. As discussed previously (126), this substantial difference observed between $F_1 \leftrightarrow$ 5R and $F_1 \leftrightarrow F_1$ T and B cell interactions probably reflects the preferential efficiency of a cis chromosomal relationship of the $\alpha(+)$ and $\beta(+)$ alleles in the respective interacting lymphocyte classes. The basis for the cis–trans effect in complementing *Ir* gene systems may be the result of gene dosage or other mechanisms, as discussed elsewhere (127).

The possible meaningful explanations for this difference in communication ability in this system are actually quite limited. Certain possible explanations can be readily dismissed. First, the response obtained when DNP-specific B cells originated from F_1 donors primed to DNP-GLΦ did not reflect contaminating residual T cells in this donor B cell population, since secondary challenge of such cells with DNP-GLΦ in irradiated recipients which did not receive GLΦ-primed F_1 T cells failed to elicit any detectable antibody even as long as 21 days after transfer and secondary challenge (124). Second, the absence of a cooperative response between F_1 DNP-FγG-primed B cells and radioresistant GLΦ-primed F_1 T cells is not a manifestation of some strange suppression phenomenon. For example, GLΦ-primed F_1 T cells do not disturb cooperative interactions between GLΦ-primed 5R T cells and DNP-primed 5R B cells; nor do they interfere with cooperative interactions between F_1 DNP-FγG-primed B cells and KLH-primed F_1 T cells. Finally, F_1 DNP-FγG-primed B cells do not disturb interactions between F_1 DNP-GLΦ-primed B cells and GLΦ-primed F_1 T cells (124).

Third, it is not readily obvious how a macrophage–T cell interaction defect could explain the observations reported here. For one thing, the priming of the respective B and T cell populations took place in all instances within F_1 animals, which should provide the relevant macrophage component. Moreover, all adoptive transfers were made to irradiated F_1 recipients, which again should provide the appropriate macrophages for antigen presentation. In this regard, it is pertinent to point out data of Schwartz *et al.* (128) that demonstrated that in this very same complementing *Ir-GLΦ* system effective anti-

gen presentation for stimulation of T cell proliferation *in vitro* could be accomplished only with F_1 macrophages. But, as just stated, this requirement was clearly fulfilled in the system reported here.

This brings us to the two possible explanations that have substance to them, if correct. One of these is based on considerations of *idiotypy* as used in the conventional sense nowadays. To explain these results in terms of idiotypy, the argument would be that a response such as that elicited by DNP-GLΦ, itself a relatively restricted antigen, might tend to be one characterized by a dominant antibody idiotype and as such might require the participation of idiotype-specific helper T cells in order for such clones to be generated. Indeed, recent data reported by others have been interpreted as indirect evidence in favor of such operational mechanisms (129, 130). Moreover, Kipps *et al.* (131) have shown the anti-DNP response to DNP-GLΦ to be of limited heterogeneity. A DNP-FγG-primed F_1 B-cell population may lack dominance of this particular idiotype and hence be unable to cooperate effectively with GLΦ-primed F_1 helper T cells. Since the converse would be the case with DNP-GLΦ-primed F_1 B cells, the results observed would thereby be explained. There is no evidence against this possibility, nor for it. We do not find it very attractive for a number of reasons, principal among which is that we cannot find a logical explanation of why the dominance of this idiotype would be restricted to (B6A)F_1 mice and not also be characteristic of the response generated by B10.A (5R) recombinant mice. Since this phenomenon of restricted cooperating preference was observed only in $F_1 \leftrightarrow F_1$, but not in $F_1 \leftrightarrow$ 5R, combinations, the idiotype possibility diminishes in likelihood.

The second possibility, and the interpretation we favor for these results, is based on the following reasoning. B lymphocytes, like T lymphocytes, are selected by at least two independent events. One of these selective events occurs through the surface membrane-bound immunoglobulin receptors for the antigen involved, i.e., in this case, the DNP haptenic determinant. A second process of selection occurs in which subsets of antigen-specific T cells, as well as macrophages, direct the further differentiation of subsets of B lymphocytes each corresponding in appropriate specificity of their surface membrane-bound MHC-encoded CI molecules. Put in other terms, one might view the lymphoid system as being comprised of many subsets of self-recognizing partner cell matches, and the ultimate processes of differentiation with the population at large to reflect the sum total of events taking place within each matched set of such partner cells. Normally, we do not see evidence of restrictions in terms of partner

cell matches, particularly when we immunize with complex antigens, which are obviously under heterogeneous genetic control. When a highly restricted antigen is used, however, such as GLΦ in this case, and especially in a further restricted circumstance where first, the responder phenotype is a consequence of complementation between two genes inherited on separate chromosomes, and, second, cooperative function is further limited (in this case by irradiation) to a selected subpopulation of helper cells, such restrictions become more easily detected. In this respect, the bone marrow chimera experiments merely provide another type of window through which to view these selection processes irrespective of whether we are examining this among T lymphocytes or B lymphocytes.

The questions that now need exploration are the molecular mechanisms by which such selection processes as are necessary for adaptive differentiation take place. In particular, we need to know the nature of both the self-recognizing receptors and the target CI determinants to which such receptors are directed, and we need to know whether idiotype specificity is involved. It should be noted that if one considers idiotypy as it pertains to the self-recognizing receptors (rather than the antigen-specific receptors), then idiotype phenomena of this variety could very well pertain to the mechanism(s) explaining the results described here.

It is pertinent to note that investigators other than ourselves have recently corroborated the point that B lymphocytes exhibit self-recognizing capabilities and undergo adaptive differentiation. Thus, Marusic and Perkins (132) have demonstrated quite clearly the reconstitution of antibody responses in thymectomized, irradiated, bone marrow-reconstituted heterozygous mice occurs only when the recipient B cells have differentiated in a environment compatible with the H-2 phenotype of the reconstituting T cells. For example, triparental chimeric T cells of the type $(A \times B)F_1 \rightarrow (A \times C)F_1$ are effective in reconstitution B cells in thymectomized chimeras of the type $(A \times C)F_1 \rightarrow (A \times B)F_1$, but not of the type $(A \times C)F_1 \rightarrow (A \times C)F_1$. These data, like the DNP-GLΦ studies described above (Fig. 13), illustrate a requirement for reciprocal self-recognition by B lymphocytes of its cooperating partner T lymphocyte in order for effective interactions to take place. Additionally, it is worth noting that the adaptive differentiation of self-recognition by B lymphocytes in the study of Marusic and Perkins (132) represents selection processes occurring totally under the influence of the extrathymic environment since thymectomized recipients were employed as the chimeric host in which B cell differentiation occurred.

Thus, it is becoming increasingly clear that we should be flexible toward the idea that effective interaction between any two partner lymphocytes, regardless of their classification, may involve a reciprocal or two-way molecular interaction—each cell expressing a CI target determinant and self-recognition receptors for such determinants expressed by the proper target cell, and only when an appropriate match is satisfied in both directions will optimal effective communication take place.

IX. Relevance of the Proposed Mechanisms of Adaptive Differentiation to Certain Unresolved Immunologic Puzzles

Any theoretical framework costructed to explain differentiation events in the immune system must take into account at least certain of the major unresolved questions. Foremost among many of our field are the interrelated issues of recognition mechanisms in general, T cell recognition in particular, and the nature and function of MHC-linked *Ir* genes. It is on these three indisputably intertwined points that the remainder of this discourse will focus. These issues have been addressed in recent hypothetical papers by several other investigators who have been directly working in the pertinent areas (91, 133–135), and naturally there are some areas of overlap as well as clear differences among the various opinions (as will be pointed out).

A. Recognition Mechanisms in the Immune System

The only undebatable point concerning questions of immunologic recognition mechanisms is that B lymphocytes have Ig molecules on their surface membranes that are specifically capable of binding antigenic determinants. As stated above, the exact molecular nature of corresponding antigen-binding receptors on T cells has not yet been delineated, although substantial evidence supports the likelihood that at least certain of these receptors on T cells have antigen-combining sites encoded by the same heavy chain *V* genes used by B cells for synthesis of their Ig receptors (35–38).

The controversial points are as follows: Are recognition events in the immune system singular or dualistic in nature? Are T cells unique in their clear dependency on reacting with MHC gene products, in addition to nonMHC antigens (by whatever mechanism), in order to be properly activated, or is this a more general requirement including B cells as well? Why are so many cells within a given individual capable of reacting to nonself alloantigens?

To briefly summarize our own thinking on these points:

1. We favor the possibility that there are two recognition systems that operate in concert with one another for purposes of cell triggering —one receptor for MHC (i.e., CI) molecules on partner cells, and a second receptor for non-MHC (i.e., conventional) antigens; these receptors coexist, *but as separate entities*, on each individual lymphocyte. The alternative possibility, namely, that a single receptor binds to a "complex antigenic determinant" comprised of antigenic fragments associated with self-MHC determinants, seems unlikely to us for reasons given in detail elsewhere (70, 71). Nevertheless, formal proof for one or the other of these two possibilties is still lacking.

2. Although the greatest experimental emphasis thus far has been placed on T cell functions and the genetic restrictions imposed thereupon, this alone does not provide a very strong argument against a more general requirement for other cells, notably B lymphocytes (but not to exclude macrophages), to also recognize and react with CI molecules on interacting partner cells in order to consummate the purpose of the interaction. Indeed, as described in Section VIII we (66, 79, 124) and others (132) have obtained data that speak strongly in favor of this possibility.

As already stated, we believe, therefore, that B lymphocytes have two independent recognition mechanisms (like T cells), one of which is directed toward self CI molecules. Hence, when partner cells (i.e., T and B lymphocytes) interact with one another, we envisage a two-way bridge being created by the binding of each cell to the other via their respective αCI receptors and the corresponding CI target molecules recognized by such receptors; this is *in addition* to the binding of antigenic determinants by antigen-specific receptors on each cell. Likewise, we believe that, just as macrophages exert selective pressures on T cells during antigen-induced responses, they exert comparable selective pressures on B cells via similar MHC-linked recognition events (i.e., recognition of macrophage CI molecules by αCI receptors on B cells). T cells can likewise exert such selective pressures on one another and on B cells and vice versa.

3. As stated at the outset, the existence of relatively large numbers of alloreactive cells has been one of the most perplexing issues in transplantation biology. As previously discussed, the adaptive differentiation model proposed here (see Section V) provides an explanation for the high frequency of cells that appear to be alloreactive when appropriately manipulated experimentally and also suggests an im-

portant function that they may have as regulatory cells within their native environment, i.e., to provide an important "brake" to minimize replication of cells bearing the corresponding nonself CI molecules. The proposed importance of this regulatory surveillance mechanism provokes the logical question of what provides an analogous brake for growth of low-to-moderate affinity αCI_A-type cells in individual *A*. This brake is inherent in the sophisticated regulatory feedback control mechanisms that constitute the normal operation of the immune system of individual *A*; indeed, it is conceivable that *A*-type cells performing negative regulatory functions that limit or ablate any given response may do so by binding to target CI molecules with higher than usual affinity combining sites.

In this regard, it should be noted that among the most significant possibilities that stem from the adaptive differentiation mechanism are those that concern the way we view those events involved in development of leukemia and surveillance mechanisms against neoplastically transformed cells. As already stated, low affinity αCI_B-type cells would be not only useless (in the functional sense) in the wrong environment of individual *A* but, more important, insensitive to normal regulatory control mechanisms inherent in the process of functional cell interactions. Leukemia may quite simply be a manifestation of the replication of such cells in the absence of any regulatory control. Hence, any condition that renders ineffective the normal surveillance mechanism (i.e., that mediated by high affinity αCI molecule-binding cells of corresponding type) for controlling the growth of such cells could result in uncontrolled proliferation of low-to-moderate affinity cells of inappropriate CI type. This could result either from a defect in the capacity of high affinity cells to perform their normal surveillance role or, alternatively, the development of insensitivity of cells of low-to-moderate affinity to the normal modulation mechanism by which high affinity cells might abort such cells. Likewise, cells of the native CI phenotype could, for similar reasons, escape normal self-recognition regulatory control and proliferate indiscriminately.

This raises some interesting speculations as to how the inappropriate expression of either endogenous or exogenous viral genes could play an important and determining role in such processes. Thus, it is now clear the major envelope glycoproteins of endogenous C-type viruses constitute what has recently become recognized as a highly polymorphic family of genes and their products (136, 137). As a consequence of recombination events, the capacity for generation of a large number of new virus entities has been shown to exist within the DNA compartment of the mammalian genome. Indeed, the association of

viral recombinants with the onset or occurrence of thymic leukemias in the mouse has been virtually established during the past 2–3 years (138–140). The major envelope glycoproteins (gp70) are present on the surface membranes of normal lymphoid cells (among other) in mice carrying the endogenous viral genes. These macromolecular products of parental viral genes do not, however, interfere detectably with normal processes of regulatory cell–cell communication (i.e., self-recognition) as shown by recent experiments in our laboratory (141). However, the glycoprotein products of viral gene recombination events could markedly disturb such self-recognition processes. For example, recombinant gp70 present on the cell surface could (*a*) mask or alter the CI molecule or the αCI receptors for such molecules (or both); or (*b*) perturb normal membrane fluidity in some way that leaves affected cells insensitive to modulation. This disturbance in normal self-recognition would hinder normal surveillance or regulatory control mechanisms and consequently permit uncontrollable proliferation, i.e., leukemia. In a sense, therefore, leukemic cells may be not truly malignant at all, but rather normal cell variants that, in effect, find themselves either in an inappropriate environment or unrecognizable in the appropriate environment.

As stated earlier, the aspect of our thinking with respect to differences in affinity being a crucial point of distinction between interactions with self (low) vs nonself (high) CI specificities is in agreement with the model proposed recently by Janeway *et al.* (91). Where our reasoning differs fundamentally from theirs is that we believe that low affinity receptors for self CI molecules and high affinity receptors capable of reacting with nonself CI molecules exist on *separate* cells altogether. As mentioned previously, Janeway and colleagues have proposed that the same receptor that binds to self with low affinity is capable of binding with high affinity to certain nonself MHC antigens (91). If this were so, it becomes difficult (though not impossible) to account for the fact that there are B lymphocytes capable of producing alloantibodies specific for nonself MHC antigens (including Ia antigens); such alloantibodies, which would have high affinity for nonself, would likewise have the capacity to bind to self with low affinity, a situation of potential danger to the individual because of the likely interference with normal physiologic cell interactions that would result from the presence of such antibodies.

Our thinking also agrees with that of Janeway *et al.* in considering that the anti-idiotype antibodies directed against alloantigen receptors in the rat that have been analyzed so extensively and well by Binz and Wigzell (35, 36) are actually recognizing idiotypes on the second

of two receptors on T cells; in our terminology the Binz–Wigzell anti-idiotypic antibodies are reacting with high affinity αCI receptors. If the model proposed here is, in principle, correct, we would further state that it should be possible to induce antibodies capable of reacting with low-affinity αCI receptors for self CI molecules. Such anti-αCI antibodies would be capable of blocking physiologic cell–cell interactions. By extension of the Binz–Wigzell findings, one could draw the tentative conclusion that the CI molecule receptor involved in any partner cell interaction has a combining site encoded by immunoglobulin *V* region genes.

One attractive aspect of this conclusion, if correct, is that it provides an understandable link between processes of adaptive differentiation, which are presumed to be essential components of normal cell differentiation, and selective pressures that could initiate somatic events crucial for generating diversity of antigen recognition in the immune system. Somewhat different from Jerne's earlier proposal that diversity was generated as a consequence of selective pressures *against* recognition of self (142), we envisage the selective pressure *for* self-recognition (of the effective functional type) as being a possible provocation for somatic events capable of generating such enormous diversity. Indeed, if Jerne's more recent network theory (143), which postulates that recognition of idiotypes by anti-idiotypes, etc., itself generates diversity for all the antigens in the universe (since they correspond to the inherent diversity of idiotypes expressed on immunocompetent cells) is correct, then at the furthest extreme one merely needs to provoke the first somatic event as a consequence of one or more events in adaptive differentiation; that one event will itself become the initiator for a cascade of subsequent somatic events.

In either case, all this reasoning is consistent with the thinking first addressed by Wilson (12, 18) that on alloreactive cells antigen-specific receptors of varying specificities most likely coexist with receptors for MHC alloantigens. This is precisely analogous to the situation for self-reactive cells, which would have receptors for various non-MHC antigens coexisting on the same cells that express receptors for self CI molecules, as proposed by us previously (26, 70, 71, 79, 144, 145) and elaborated upon here.

B. The Nature and Function of *Ir* Genes

1. Some Current Thoughts of Others on Ir Genes

Before discussing our own thinking on this issue, it is pertinent to analyze other recent speculations concerning *Ir* gene function. Al-

though several examples could be cited, we believe that two of these, proposed by investigators who have devoted considerable energy to this problem, are of particular interest and attractiveness. Thus, Benacerraf (134) has recently made a strong statement of the case in favor of *Ir* gene function being located at the level of macrophage antigen presentation, a notion originally proposed by Rosenthal, Shevach, Paul, and their colleagues (51, 115, 128, 133). Benacerraf (134) formalized such notions further by stating that the products of *Ir* genes, present on the surface membrane of the macrophage, could have combining site activity capable of reacting with defined sequences of a limited number of amino acids (i.e., three or four) within the structure of a larger macromolecule; the consequence of this reaction between *Ir* gene product and a given amino acid sequence would be highly specific display of the resulting complex formed between the exogenous antigen and the macrophage-bound *Ir* gene product. This complex would then be recognized specifically by T cells with receptors consisting of combining sites of corresponding specificity for a given complex. This model therefore postulates that the T cell sees antigen oriented in a unique structural way by virtue of its reaction with the *Ir* gene products; although the implied suggestion is that such recognition occurs via a *single* receptor entity, a dual recognition model is not altogether ruled out.

The attractiveness of this model is that it accounts for the exquisite specificity of *Ir* gene function without demanding an extraordinarily large number of distinct *Ir* genes. Thus, Benacerraf (134) has explained the possibility that specificity differences could be accounted for by imagining that a limited number of *Ir* genes, each capable of reacting with a defined sequence of three or four amino acids, could display a highly diverse antigenic universe depending on differences in the way that each complex macromolecule would subsequently become oriented on the macrophage surface membrane after reacting with the *Ir* gene product(s). Uniqueness, therefore, would be more a property of the inherent structural attributes of the exogenous antigen than of any enormous number of individual *Ir* genes. This model also accounts for most of the work demonstrating the level of control by *Ir* genes in terms of macrophage–T cell interactions and the like.

The second model, recently proposed by Schwartz (135), also considers that T cells see antigen in a form of a complex developed by associations with *Ir* gene products on the macrophage surface via a single receptor for this "complex antigenic determinant" (CAD). Schwartz goes further in proposing that self antigens associate with *Ir* gene products and potentially reactive T cells for such self CADs are

deleted during the process of tolerance induction early in the course of ontogeny. Non-self antigens also must interact with *Ir* gene products to form CADs, which are then necessarily recognized by T cells with corresponding receptor specificity. *Ir* gene defects are thereby explained as reflecting those situations in which a CAD formed between a nonself antigen and *Ir* gene product exhibits sufficient mimicry with the self CAD; since T cells capable of recognizing self CAD have been deleted, any CAD of sufficient mimicry will naturally fail to elicit a response. This model likewise accounts for many observations made recently, particularly those that focused on the relationship of *Ir* gene function to macrophage antigen presentation.

However, neither of these models adequately account for several observations that appear to be central to all these issues.

Let us first consider the allogeneic effect, the descriptive term given to the biological consequences of a transient transplantation reaction whereby one cell population provides differentiation signals to a target cell population as a result of reacting with such target cells via specific allorecognition (see Chapter XI of reference). It is pertinent that the allogeneic effect represents precisely the circumstance in whch a reaction with MHC-encoded target molecules (i.e., *I* region molecules), concomitant in time with the exposure of the target cell to its relevant antigenic determinants, results in the delivery of stimulatory signals to such target cells that drive them onward along the pathway of differentation. Since the alloreactive cells that mediate this effect need not exhibit any relationship to the non-MHC antigen employed, it is clear that the pertinent recognition necessary for the triggering events is that concerned with the MHC-encoded cell surface target molecules. This exemplifies, therefore, the occurrence of two independent recognition events, i.e., one directed to CI molecule determinants and the other directed to non-MHC antigens, capable of triggering lymphocytes. Clearly, there is no obvious relationship between such triggering events and the need for complex antigenic determinants, of the type described above, in such circumstances.

Perhaps more important is the fact that several experimental situations have been used to demonstrate the ability of an appropriately timed allogeneic effect to overcome *Ir* gene defects. On the one hand, it has been possible to demonstrate the capacity of an allogeneic effect to permit nonresponder mice to make IgG (as well as IgM) antibody responses to a synthetic antigen under *Ir* gene control (123; see above). In this instance, the allogeneic effect provided a necessary stimulus to target B cells, thereby replacing a normal interaction step missing in such circumstances. As summarized in Section VII,B, we

have recently used the allogeneic effect as a mechanism for stimulating the induction of (responder × nonresponder) F_1 helper T cells capable of effectively interacting with nonresponder B cells.

It should be pointed out, moreover, that the fact that the allogeneic effect was effective in inducing GLT-specific helper T cells, presumably of the nonresponder "A/J-type" subset, must be viewed with serious consideration in the context of any speculation that *Ir* gene products react specifically with antigenic determinants at the level of the macrophage to orient and display the relevant determinants for recognition by T cells of corresponding specificity (133–135). It is difficult to envisage, for example, how an allogeneic effect could circumvent an *absolute* requirement for *Ir* gene-controlled antigen display by macrophages. Rather, these data (Fig. 11) seem to argue against any such absolute requirement for macrophage presentation and tend to favor the possibility that the allogeneic effect permits the development of GLT-specific F_1 helper cells capable of interacting with nonresponder B cells by either direct stimulation of the "A/J-type" subset of F_1 GLT-specific T cells to differentiate into effective helper cells, or by elimination of some type of inherent suppressive mechanism that normally blocks development of GLT-specific helper T cells capable of cooperating with nonresponder B cells.

2. *Our Own Current Thoughts on Ir Gene Mechanisms*

If *Ir* genes do not encode the combining site(s) of antigen-specific T cell receptors, what role is served by these genes? We believe that an important clue to the answer to this question is the concomitant location in the I region of the *Ir* genes and the genes responsible for effective cell–cell communication among different lymphoid cells, and for encoding determinants that are strong stimulants of alloreactivity in MLR. We further believe that the answer is, in fact, quite simple: *Ir genes encode CI molecules.*

If *Ir* genes are actually *CI* genes and if CI molecules are distinct entities from antigen-specific receptors. how then do *Ir* genes exert such apparent specificity for antigen in responses over which they display control? In the context of the adaptive differentiation model being proposed here, there are three possible answers to this question depending on whether *Ir* genes encode molecules serving as (*a*) αCI receptors alone (at least in part); (*b*) target CI molecules themselves; or (*c*) both αCI receptors and target CI molecules. These possibilities will be considered separately.

If *Ir* genes encode αCI receptor molecules alone, either in part or whole, then it must be assumed that the affiliation of specific antigen-

binding receptors on a given lymphocyte and the αCI receptor expressed by that same cell is somehow linked, either genetically or epigenetically, within a given individual of the species. Nevertheless, such affiliations could be initially random with the species at large. As already discussed, during the process of adaptive differentiation within a given individual high affinity αCI cells that are reactive with self CI molecules of that individual's environment are deleted. It follows, then, that among the populations of deleted high affinity cells would exist a certain fraction of the *V* gene repertoire for non-MHC antigens; this fraction of the repertoire would therefore be functionally silent. Thus, if the antigen receptor for *X* happened to be affiliated consistently with a high affinity αCI_A receptor molecule within the environment of individual *A*, then functional deletion of such high affinity αCI_A cells would concomitantly be phenotypically displayed as an inability to develop responses to antigen *X*. Since abortion of high affinity αCI_A cells merely implies *functional* deletion of their CI molecule-binding capability, such cells could still be present in the population. In this case, they would display their antigen-specific receptors capable of binding *X* but be impotent with respect to cooperating with partner cells to make an appropriate response; this is consistent with experimental observations (146–149).

If *Ir* genes encode target CI molecules, rather than αCI receptors, how can their apparent specificity for antigen be envisaged? Here again, the assumptions mentioned above with regard to the affiliation between antigen-specific receptors and αCI receptors on the same cell still pertain. However, an additional mechanism for *Ir* gene-linked unresponsiveness now enters the equation. Let us assume that in individual *A* there is heterogeneity among CI_A that we can denote $CI_{A1,A2,A3,}$; for each CI_A specificity, there will be corresponding αCI_A receptors i.e., αCI_{A1}, αCI_{A2}, αCI_{A3}, and so on, each of which will be affiliated with certain antigen-specific receptors. Let us further assume that the *Ir-GLT* gene encodes, in nonresponder individual *A*, CI_{A1} molecules; specificity of *Ir* gene function in this case reflects an affiliation on the same cells of anti-GLT receptors with αCI_{A1} receptors. Anything that prevents the reaction $\alpha CI_{A1} \rightarrow CI_{A1}$ could be manifested as specific unresponsiveness to GLT; for example, something analogous to an anti-αCI_{A1} idiotype reaction, as suggested in Section VII,C. Perhaps a nonresponder individual displays that phenotype because, for some reason, αCI_{A1} is particularly effective in eliciting a strong (and early) anti-αCI_{A1} reaction, which, in turn, blunts any possible response to GLT from developing.

These two possible mechanisms by which *Ir* genes may function are by no means mutually exclusive. It is pertinent to emphasize the fact that although in both circumstances the phenotypic effect is displayed as poor responsiveness or none, there is no defect in *Ir* gene expression per se, but rather the consequence of the expression of the relevant *Ir* gene. Moreover, if both mechanisms are valid, one can easily understand how *Ir* gene complementation can occur in certain circumstances when responder F_1 hybrids are derived from two nonresponder parental strains; in such cases, one nonresponder parent would be so as a result of one of these mechanisms, and vice versa. In those situations where nonresponsiveness may reflect the third possibility mentioned above, namely that *Ir* genes encode both αCI receptors and target CI molecules, and where both mechanisms just discussed operate simultaneously to result in nonresponsiveness, one would not except to find a suitable complementing nonresponder partner strain to give rise to a responding F_1 hybrid.

This definition of *Ir* genes also explains why it is easier to find *Ir* gene defects in highly inbred individuals and more difficult to detect such defects as population heterozygosity increases. It seems likely that as heterozygosity increases, so would heterogeneity of CI molecule specificities possessed by any one individual. This, in turn, would increase the likelihood that loss of a given αCI molecule-binding cell (either by deletion because of high affinity or because of anti-αCI reactions), with concomitant loss of responsiveness to the antigen for which specific receptors were affiliated on the same cell, might be compensated by the association of receptors for the same antigen on cells possessing a different αCI specificity. Finally, consistent with this reasoning is the experimental finding that tetraparental bone marrow chimeras constructed from bone marrow populations of nonresponder and responder parent origins fail to manifest cooperative activity between the responder T cell and nonresponder B cell (111).

X. Conclusions

The process of adaptive differentiation is one in which highly effective selective mechanisms are initiated by contact between developing cells and the normal cell interaction (CI) molecules predominantly expressed in the surrounding environment. These selective mechanisms involve self-recognition and result in functional deletion of clones of cells that possess high affinity receptors for CI molecules that are predominantly expressed in the environment. The con-

sequence of this process is to shift the affinity spectrum of self-recognition toward the lower end, where cells possessing such low-to-moderate affinity αCI molecules and their corresponding receptors are then capable of engaging in functional interactions necessary for development and maintenance of the system.

A second consequence is that cells with self-recognition capabilities for all the other CI molecules in the species express such CI molecule receptors but, in the absence of environmental selection, are shifted toward the higher end of the affinity spectrum of CI binding capabilities. These cells may play a very important functional role in a surveillance mechanism that operates to eliminate the majority of low-to-moderate affinity cells of the same CI specificity that have no useful function in the inappropriate environment in which they find themselves. Because of their high affinity binding capacity for CI molecules of other individual members of the species, certain of these cells mainifest alloaggressive responses in mixed lymphocyte reactions and other transplantation reactions.

Although the precise nature of the molecular interactions involved in these processes have yet to be defined, it seems clear that they must occur to a very great extent by direct cell contact. For several years, we have stressed the fact that self-recognition appears to be a fundamental biologic process concerned with control of many types of developmental and differentiation events. In this article, we have presented some new thoughts on possible mechanisms of self-recognition and regulatory interactions in the immune system. In particular, new speculations concerning *Ir* genes and the manner in which they function have been discussed in light of recent experimental observations as reviewed here. Clearly, only time and sophisticated molecular approaches to these issues will ultimately sort out the correct answers.

ACKNOWLEDGMENTS

I am grateful to all my colleagues for their participation in the studies in our laboratory that have contributed to the notions presented here. Cherie Dichter and Keith Dunn provided excellent assistance in the preparation of the manuscript. I am furthermore grateful to Drs. Frank Dixon, Sam Waksal, Richard Gershon, and Charles Janeway, Jr. For carefully reviewing and offering helpful comments on early versions of the manuscript and to Dr. Richard Lerner for doing the same and, moreover, providing valuable insights from a different biologic perspective. Dr. Norman Klinman patiently read and commented constructively on several versions of the treatise.

This is publication No. 135 from the Department of Cellular and Developmental Immunology and No. 1937 from the Immunology Departments, Scripps Clinic and Research Foundation. The work performed in our laboratory was supported by NIH Grants AI-13781, AI-13874, and RR-05514 and National Foundation Grant 1-540.

REFERENCES

1. Shreffler, D. C. (1977). *In* "HLA and Disease" (J. Dausset and A. Svejggaard, eds.), p. 28. Williams & Wilkins, Baltimore, Maryland.
2. Klein, J. (1975). "Biology of the Mouse Histocompatibility-2 Complex." Springer-Verlag, Berlin and New York.
3. Bach, F. H., and van Rood, J. J. (1976). *N. Engl. J. Med.* **295,** 806, 872, 927.
4. Nathenson, S. G., and Cullen, S. E. (1974). *Biochim. Biophys. Acta* **334,** 1.
5. Silver, J., and Hood, L. (1976). *Contemp. Top. Mol. Immunol* **5,** 35.
6. Strominger, J. L., Chess, L., Humphreys, R. E., Mann, D., Parham, P., Robb, R., Schlossman, S., Springer, T., and Terhorst, C. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 621. Academic Press, New York.
7. Schwartz, B. D., and Cullen, S. E. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 691. Academic Press, New York.
8. Bodmer, W. F. (1972). *Nature (London)* **237,** 139.
9. Bodmer, W. F. (1973). *Transplant. Proc.* **5,** 1471.
10. Burnet, F. M. (1973). *Nature (London)* **245,** 359.
11. Simonsen, M. (1974). *Allergology, Proc. 8th Congr. Int. Assoc. Allergology,* p. 146. Excerpta Med. Found., Amsterdam.
12. Wilson, D. B. (1974). *In* "Progress in Immunology II" (L. Brent and J. Holborow, eds.), Vol. 2, p. 145. North Holland/American Elsevier, Amsterdam/New York.
13. Klein, J. (1976). *Contemp. Top. Immunobiol.* **5,** 297.
14. Snell, G. D. (1976). *Transplant. Proc.* **8,** 147.
15. Janeway, Jr., C. A., Wigzell, H., and Binz, H. (1976). *Scand. J. Immunol.* **5,** 993.
16. Doherty, P. C., Blanden, R. V., and Zinkernagel, R. M. (1976). *Transplant. Rev.* **29,** 89.
17. Miller, J. F. A. P., and Vadas, M. A. (1977). *Scand. J. Immunol.* **6,** 771.
18. Wilson, D. B., and Herber-Katz, E. (1977). *Cold Spring Harbor Symp. Quant. Biol.* **41,** 559.
19. Claman, H. N., Chaperon, E. A., and Triplett, R. F. (1966). *Proc. Soc. Exp. Biol. Med.* **122,** 1167.
20. Davies, A. J. A., Leuchars, E., Wallis, V., Marchant, R., and Elliott, E. V. (1976). *Transplantation* **5,** 222.
21. Mitchell, G. F., and Miller, J. F. A. P. (1968). *J. Exp. Med.* **128,** 821.
22. Katz, D. H., and Benacerraf, B. (1972). *Adv. Immunol.* **15,** 1.
23. Gershon, R. K. (1973). *Contemp. Top. Immunol.* **3,** 1.
24. Cantor, H. and Weissman, I. (1976). *Prog. Allergy* **20,** 1.
25. Cantor, H. and Boyse, E. A. (1977). *Cold Spring Harbor Symp. Quant. Biol.* **41,** 23.
26. Katz, D. H. (1977). "Lymphocyte Differentiation, Recognition, and Regulation." Academic Press, New York.
27. McDevitt, H. O., and Benacerraf, B. (1969). *Adv. Immunol.* **11,** 31.
28. Benacerraf, B., and McDevitt, H. O. (1972). *Science* **175,** 273.
29. Benacerraf, B., and Katz, D. H. (1975). *In* "Immunogenetics and Immunodeficiency" (B. Benacerraf, ed.), p. 117. Medical and Technical Publ., London.
30. McDevitt, H. O., Deak, B. D., Shreffler, D. C., Klein, J. Stimpling, J. H., and Snell, G. D. (1972). *J. Exp. Med.* **135,** 1259.
31. van Rood, J. J., van Leeuwen, A., Termijtelen, A., and Keunign, J. J. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 31. Academic Press, New York.

32. Kunkel, H. G., Winchester, R. J., Fu, S. M., and Dupont, B. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 71. Academic Press, New York.
33. Bach, F. H., Jarrett-Toth, E., Benike, C. J., Sheehy, M. J., Sondel, P. M., and Bach, M. L. (1976). *J. Exp. Med.* **144,** 549.
34. Ramseier, H., and Lindenmann, J. (1972). *Transplant. Rev.* **10,** 57.
35. Binz, H., and Wigzell. H. (1977). *Cold Spring Harbor Symp. Quant. Biol.* **41,** 275.
36. Binz, H., and Wigzell, H. (1977). *Contemp. Top. Immunobiol.* **7,** 113.
37. Rajewsky, K., Hammerling, G. J., Black, S. J., Berek, C., and Eichmann, K. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 445. Academic Press, New York.
38. Krawinkel, U., Cramer, M., Berek, C., Hammerling, G., Black, S. J., Rajewsky, K., and Eichmann, K. (1977). *Cold Spring Harbor Symp. Quant. Biol.* **41,** 285.
39. Davie, J. M., and Paul, W. E. (1971). *J. Exp. Med.* **134,** 495.
40. Unanue, E. R. (1971). *J. Immunol.* **107,** 1168.
41. Nossal, G. J. V., and Lewis, H. (1972). *J. Exp. Med.* **135,** 1416.
42. Raff, M. C., Feldmann, M., and De Petris, S. (1973). *J. Exp. Med.* **137,** 1024.
43. Vitetta, E. S., and Uhr, J. W. (1975). *Science* **189,** 964.
44. Marchalonis, J. J. (1975). *Science* **190,** 20.
45. Katz, D. H. (1972). *Transplant. Rev.* **12,** 141.
46. Katz, D. H., Hamaoka, T., and Benacerraf, B. (1973). *J. Exp. Med.* **137,** 1405.
47. Katz, D. H., Hamaoka, T., Dorf, M. E., and Benacerraf, B. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2626.
48. Katz, D. H., Hamaoka, T., Dorf, M. E., and Benacerraf, B. (1974). *J. Immunol.* **112,** 855.
49. Katz, D. H., Chiorazzi, N., McDonald, J., and Katz, L. R. (1976). *J. Immunol.* **117,** 1853.
50. Kindred, B., and Shreffler, D. C. (1972). *J. Immunol.* **109,** 940.
51. Rosenthal, A. S., and Shevach, E. M. (1973). *J. Exp. Med.* **138,** 1194.
52. Shevach, E. M., and Rosenthal, A. S. (1973). *J. Exp. Med.* **138,** 1213.
53. Katz, D. H., Graves, M., Dorf, M. E., DiMuzio, H., and Benacerraf, B. (1975). *J. Exp. Med.* **141,** 263.
54. Bach, F. H., Widmer, M. B., Bach, M. L., and Klein, J. (1972). *J. Exp. Med.* **136,** 1430.
55. Katz, D. H., and Benacerraf, B. (1975). *Transplant. Rev.* **22,** 175.
56. Shearer, G. M. (1974). *Eur. J. Immunol.* **4,** 527.
57. Zinkernagel, R. M., and Doherty, P. C. (1974). *Nature (London)* **248,** 701.
58. Koszinowski, U., and Ertl, H. (1975). *Nature (London)* **255,** 552.
59. Bevan, M. J., (1975). *J. Exp. Med.* **142,** 1349.
60. Gordon, R. D., Simpson, E., and Samelson, L. E. (1975). *J. Exp. Med.* **142,** 1108.
61. Shearer, G. M., Schmitt-Verhulst, A.-M., and Rehn, T. G. (1976). *In* "The Role of the Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 133. Academic Press, New York.
62. Zinkernagel, R. M., and Doherty, P. C. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 203. Academic Press, New York.
63. Schmitt-Verhulst, A.-M., and Shearer, G. M. (1975). *J. Exp. Med.* **142,** 914.
64. Blanden, R. V., Doherty, P. C., Dunlop, M. B. C., Gardner, I. D., and Zinkernagel, R. M. (1975). *Nature (London)* **254,** 269.
65. Zinkernagel, R. M., and Doherty, P. C. (1974). *Nature (London)* **251,** 574.

66. Katz, D. H., and Benacerraf, B. (1976). *In* "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D. H. Katz and B. Benacerraf, eds.), p. 355. Academic Press, New York.
67. von Boehmer, H., Sprent, J., and Nabholz, M. (1975). *J. Exp. Med.* **141,** 322.
68. Sprent, J., von Boehmer, H., and Nabholz, M. (1975). *J. Exp. Med.* **142,** 321.
69. von Boehmer, H., Hudson, L., and Sprent, J. (1975). *J. Exp. Med.* **142,** 989.
70. Katz, D. H. (1976). *Transplant. Proc.* **8,** 405.
71. Katz, D. H. (1977). *Cold Spring Harbor Symp. Quant. Biol.* **41,** 611.
72. Sado, T., and Kamisaku, H. (1975). *J. Immunol.* **115,** 1607.
73. Waldmann, H., Pope, H., and Munro, A. J. (1975). *Nature (London)* **258,** 728.
74. Waldmann, H., Pope, H., and Munro, A. J. (1976). *J. Exp. Med.* **144,** 707.
75. Waldmann, H. (1977). *Immunol. Rev.* **35,** 121.
76. Pfizenmaier, K., Starzinski-Powitz, A., Rodt, H., Rollinghoff, M., and Wagner, H. (1976). *J. Exp. Med.* **143,** 999.
77. Zinkernagel, R. M. (1976). *Nature (London)* **261,** 139.
78. von Boehmer, H., and Haas, W. (1976). *Nature (London)* **261,** 141.
79. Katz, D. H., Skidmore, B. J., Katz, L. R., and Bogowitz, C. A. (1978). *J. Exp. Med.* **148,** 727.
80. Sprent, J. (1978). *J. Exp. Med.* **147,** 1838.
81. Waldmann, H., Pope, H., Brent, L., and Bighouse, K. (1978). *Nature (London)* **274,** 166.
82. Sprent, J. (1978). *Immunol. Rev.* **42,** 108.
83. Waldmann, H. (1978). *Immunol. Rev.* **42,** 202.
84. Kappler, J. W., and Marrack, P. (1978). *J. Exp. Med.* **148,** 1510.
85. Bevan, M. J. (1977). *Nature (London)* **269,** 417.
86. Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P., and Klein, J. (1978). *J. Exp. Med.* **147,** 882.
87. Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Streilein, J. W., and Klein, J. (1978). *J. Exp. Med.* **147,** 897.
88. Marusic, M., Goodman, J. W., and Shinpock, S. G. (1977). *Cell. Immunol.* **33,** 72.
89. Sidman, C. L., and Unanue, E. R. (1975). *Nature (London)* **257,** 149.
90. Raff, M. C., Owen, J. J. T., Cooper, M. D., Lawton, III, A. R., Megson, M., and Gathings, W. E. (1975). *J. Exp. Med.* **142,** 1052.
91. Janeway, Jr., C. A., Wigzell, H., and Binz, H. (1976). *Scand. J. Immunol.* **5,** 993.
92. Unanue, E. R. (1976). *Am. J. Pathol.* **83,** 396.
93. Pierce, S. K., and Klinman, N. R. (1976). *J. Exp. Med.* **144,** 1254.
94. Cullen, S. E., Schwartz, B. D., Nathenson, S. G., and Cherry, M. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 1394.
95. Katz, D. H., Katz, L. R., Bogowitz, C. A., and Bargatze, R. F. (1980). *J. Immunol.* **124,** 1750.
96. Fink, P. J., and Bevan, M. J. (1978). *J. Exp. Med.* **148,** 766.
97. Waldmann, H., Pope, H., Bettles, C., and Davies, A. J. S. (1979). *Nature (London)* **277,** 137.
98. Bevan, M. J., and Fink, P. J. (1978). *Immunol. Rev.* **42,** 3.
99. Katz, D. H., Katz, L. R., Bogowitz, C. A., and Skidmore, B. J. (1979). *J. Exp. Med.* **149,** 1360.
100. von Boehmer, H., Haas, W., and Jerne, N. K. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2439.
101. Green, I., Paul, W. E., and Benacerraf, B. (1969). *Proc. Natl. Acad. Sci. U.S.A.* **64,** 1095.

102. Lattime, E. C., Gershon, H. E., and Stutman, O. (1980). *J. Immunol.* **124,** 274.
103. Onóe, K., Fernandes, G., Nair, M., and Good, R. A. (1980). *J. Exp. Med.* **151,** 115.
104. Erb, P., Meier, B., Matsunaga, T., and Feldmann, M. (1979). *J. Exp. Med.* **149,** 686.
105. Hamaoka, T., Osborne, D. P., Jr., and Katz, D. H. (1973). *J. Exp. Med.* **137,** 1393.
106. Kindred, B. (1975). *Scand. J. Immunol.* **4,** 653.
107. Kindred, B. (1978). *Immunol. Rev.* **42,** 60.
108. Singer, A., Hathcock, K. S., and Hodes, R. J. (1979). *J. Exp. Med.* **149,** 1208.
109. Katz, D. H., Hamaoka, T., Dorf, M. E., Maurer, P. H., and Benacerraf, B. (1973). *J. Exp. Med.* **138,** 734.
110. Pierce, S. K. (1977). *In* "Immune System: Genetics and Regulation" (E. E. Sercarz, L. A. Herzenberg, and C. F. Fox, eds.), p. 447. Academic Press, New York.
111. Press, J. L., and McDevitt, H. O. (1977). *J. Exp. Med.* **146,** 1815.
112. Yamashita, U., and Shevach, E. M. (1978). *J. Exp. Med.* **148,** 1171.
113. Katz, D. H., and Benacerraf, B. (1974). *In* "The Immune System: Genes, Receptors, Signals" (E. E. Sercarz, A. R. Williams, and C. F. Fox, eds.), p. 569. Academic Press, New York.
114. Skidmore, B. J., and Katz, D. H., (1977). *J. Immunol.* **119,** 694.
115. Paul, W. E., Shevach, E. M., Thomas, D. W., Pickeral, S. F., and Rosenthal, A. S. (1977). *Cold Spring Harbor Symp. Quant. Biol.* **41,** 571.
116. Miller, J. F. A. P., and Vadas, M. A. (1977). *Scand. J. Immunol.* **6,** 771.
117. Thomas, D. W., and Shevach, E. M. (1978). *J. Immunol.* **120,** 638.
118. McDougal, J. S., and Court, S. P. (1978). *J. Immunol.* **120,** 445.
119. Swierkosz, J. E., Rock, K., Marrack, P., and Kappler, J. W. (1978). *J. Exp. Med.* **147,** 554.
120. Katz, D. H., Katz, L. R., Bogowitz, C. A., and Maurer, P. H. (1979). *J. Exp. Med.* **150,** 20.
121. Katz, D. H., Davie, J. M., Paul, W. E., and Benacerraf, B. (1971). *J. Exp. Med.* **134,** 201.
122. Osborne, D. P., Jr., and Katz, D. H. (1973). *J. Exp. Med.* **137,** 991.
123. Ordal, J. C., and Grumet, F. C. (1972). *J. Exp. Med.* **136,** 1195.
123a. Sprent, J., and Bruce, J. (1979). *J. Exp. Med.* **150,** 715.
124. Katz, D. H. (1979). *J. Immunol.* **122,** 1937.
125. Dorf, M. E., and Benacerraf, B. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 3671.
126. Katz, D. H., Dorf, M. E., and Benacerraf, B. (1976). *J. Exp. Med.* **143,** 906.
127. Dorf, M. E., Maurer, P. H., Merryman, C. F., and Benacerraf, B. (1976). *J. Exp. Med.* **143,** 889.
128. Schwartz, R. H., Yano, A., and Paul, W. E. (1978). *Immunol. Rev.* **40,** 153.
129. Bottomly, K., Mathieson, B. J., and Mosier, D. E. (1978). *J. Exp. Med.* **148,** 1216.
130. Woodland, R., and Cantor, H. (1978). *Eur. J. Immunol.* **8,** 600.
131. Kipps, T. J., Benacerraf, B., and Dorf, M. E. (1978). *J. Immunol.* **120,** 46.
132. Marusic, M., and Perkins, E. H. (1979). *J. Immunol.* **122,** 1116.
133. Rosenthal, A. S. (1978). *Immunol. Rev.* **40,** 136.
134. Benacerraf, B. (1978). *J. Immunol.* **120,** 1809.
135. Schwartz, R. H. (1978). *Scand. J. Immunol.* **7,** 3.
136. Stockert, E., Old, L. J., and Boyse, E. A. (1971). *J. Exp. Med.* **133,** 1334.
137. Elder, J. H., Jensen, F. C., Bryant, M. L., and Lerner, R. A. (1977). *Nature (London)* **267,** 23.
138. Kawashima, K., Ikeda, H., Hartley, J. W., Stockert, E., Rowe, W. P., and Old, L. J. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4680.
139. Hartley, J. W., Wolford, N. K., Old, L. J., and Rowe, W. P. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 789.

140. Elder, J. H., Gautsch, J. W., Jensen, F. C., Lerner, R. A., Hartley, J. W., and Rowe, W. P. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4676.
141. Yamamoto, H., and Katz, D. H. (1978). *Cell. Immunol.* **41,** 414.
142. Jerne, N. K. (1971). *Eur. J. Immunol.* **1,** 1.
143. Jerne, N. K. (1974). *Ann. Immunol. (Paris)* **125C,** 373.
144. Katz, D. H., and Skidmore, B. J. (1978). *In* "The Molecular Basis of Cell–Cell Interaction" (R. A. Lerner and O. Bergsma, eds.), p. 327. Alan R. Liss, Inc., New York.
145. Katz, D. H. (1978). *In* "Cell–Cell Recognition" (A. S. G. Curtis, ed.), p. 411. Cambridge Univ. Press, London and New York.
146. Dunham, E. K., Unanue, E. R., and Benacerraf, B. (1972). *J. Exp. Med.* **136,** 403.
147. Hammerling, G. J., Masuda, T., and McDevitt, H. O. (1973). *J. Exp. Med.* **137,** 1180.
148. Hammerling, G. J., and McDevitt, H. O. (1974). *J. Exp. Med.* **140,** 1180.
149. Kennedy, L. J., Jr., Dorf, M. E., Unanue, E. R., and Benacerraf, B. (1975). *J. Immunol.* **114,** 1670.

This Page Intentionally Left Blank

ADVANCES IN IMMUNOLOGY, VOL. 29

Antibody-Mediated Destruction of Virus-Infected Cells

J. G. PATRICK SISSONS AND MICHAEL B. A. OLDSTONE

Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California

I. Introduction 209
II. Viral Antigen Expression and the Antibody Response 211
A. Viral Antigen Expression on the Surfaces of Infected Cells 211
B. The Antibody Response to Viral Infection 213
III. Effect of Antibody Alone on Virus-Infected Cells 215
A. Antibody-Induced Redistribution of Surface Viral Antigens 215
B. Antibody-Induced Antigenic Modulation of Virus-Infected Cells 221
C. Effect of Antibody Binding to Infected Cells on Synthesis and Expression of Viral Proteins 224
D. Interaction of IgG and the Herpes Simplex Virus-Induced Fc Receptor 226
E. Summary and Conclusions 227
IV. Antibody- and Complement-Mediated Lysis of Virus-Infected Cells 229
A. Complement 229
B. Lysis of Virus-Infected Cells by Antibody and Heterologous Complement 230
C. Lysis of Virus-Infected Human Cells by Human Serum 232
D. Analysis of the Requirement for Antibody in Complement-Mediated Lysis 235
E. Analysis of the Requirement for the Alternative Complement Pathway 237
F. Aspects of Target Cell Structure Affecting Antibody- and Complement-Mediated Lysis 241
G. Summary and Conclusions 242
V. Antibody-Dependent Cell-Mediated Cytotoxicity of Virus-Infected Cells 245
A. Introduction 245
B. ADCC of Virus-Infected Cells 247
C. Summary and Conclusions 249
VI. Conclusions 251
A. Role of Viral Antigenic Modulation *in Vivo* 252
B. Role of Antibody-Dependent Killing of Virus-Infected Cells *in Vivo* 253
C. Conclusion 254
References 255

I. Introduction

The nature of immune responses elicited by viral infections in animal hosts and the role such responses play in eliminating infection,

ISBN 0-12-022429-1

producing tissue injury, or encouraging virus persistence have attracted extensive interest in recent years. These interactions are inevitably intricate. Added to the fundamental complexity of the immune response itself is the complicated nature of viruses as antigens, with their intracellular site, ability to replicate, multiple separate antigenic molecules, and, in some cases, ability to infect and replicate in lymphocytes, macrophages, and their precursor cells.

A number of immunologic mechanisms have been defined by which virus-infected cells can be killed *in vitro*. Antibody-independent cell-mediated cytotoxicity against virus infected target cells has received great attention. This is especially so in the last 5 years, since the demonstration that killing of virus-infected cells by specifically sensitized cytotoxic T cells occurs and is H-2 restricted in the mouse. This subject has recently been reviewed in this series by Zinkernagel and Doherty (1979). Even more recently the "spontaneous" cytotoxicity of murine and human lymphocytes for virus-infected targets (and for uninfected targets and tumor cell lines) has become the subject of intensive investigation, with the demonstration that the activity of natural killer cells can apparently be augmented by interferon. This topic, as it pertains to virus-infected cells, has again been reviewed for the murine (Welsh, 1978) and human (Santoli and Koprowski, 1979) systems.

Serum antibody, produced in response to virus infections, is of major importance in preventing the spread of infection by virtue of neutralizing free virus in extracellular fluids (reviewed by Daniels, 1975; Burns and Allison, 1975). Virus neutralization by antibody is enhanced by complement; antibody and complement can lyse enveloped virions, and complement may lyse or neutralize some viruses in the absence of antibody (reviewed by Oldstone, 1975; Cooper, 1979; Cooper and Welsh, 1979).

Antibody can also act in a number of ways on virus-infected cells, and this review is concerned with those effects of the immune response on virus-infected cells that are mediated by antibody. We deal with the actions of antibody alone, of antibody and complement, and of antibody interacting with cytotoxic cells, on virus-infected target cells. It should become apparent that, despite the foregoing emphasis on the role of antibody in neutralizing free virus and of cytotoxic T cells in killing virus-infected cells, antibody can profoundly affect virus-infected cells *in vitro;* there is little reason for thinking these actions may be less important *in vivo* than other cytotoxic mechanisms demonstrated *in vitro*. Furthermore, as with cytotoxic T cells, antibody-mediated effects on virus-infected cells can occur prior to re-

lease of progeny virus, eliminating a source of continuing virus production.

In surveying this subject, emphasis is given to the effect of antibody on virus-infected cells in homologous human systems and, particularly in Sections III and IV, to work from this laboratory.

II. Viral Antigen Expression and the Antibody Response

A. Viral Antigen Expression on the Surfaces of Infected Cells

Some aspects of the mechanism by which viral proteins are expressed on the surfaces of infected cells merit discussion, as these are the target antigens whose recognition initiates immune lysis. All enveloped RNA viruses, which acquire an outer lipid envelope by budding from the host cell plasma membrane, express viral glycoproteins on the cell surface. This group comprises, in part, togaviruses, negative strand RNA viruses (arena-, rhabdo-, orthomyxo-, and paramyxoviruses) and retroviruses. DNA viruses, such as herpes viruses, which bud from the nuclear membrane, and poxviruses, which manufacture their own envelope, also express structural viral glycoproteins on the cell surface. In addition, virus infection may cause expression of nonstructural viral proteins (e.g., tumor-specific transplantation antigens) or alter expression of host membrane proteins.

It is worthwhile briefly to summarize the mechanism for assembly of enveloped viruses. After viral entry into a cell and uncoating, transcription of viral mRNA and its translation into viral proteins on host cell ribosomes follow. Enveloped viruses have three main classes of structural proteins: nucleocapsid proteins, matrix (M) proteins, and envelope glycoproteins. The nucleocapsid protein is associated with the viral nucleic acid, the M protein (not present in togaviruses) is a peripheral membrane protein associated with the cytoplasmic surface of the cell membrane (see Choppin and Compans, 1975). The glycoproteins are inserted into the lipid bilayer of the plasma membrane, probably as transmembrane proteins, with a hydrophobic region in the bilayer and a hydrophilic glycosylated portion projecting on the outer surface (reviewed in Compans and Kemp, 1978).

The evidence suggests that viral glycoproteins are synthesized, glycosylated, and transported to the plasma membrane by the same mechanism as host cell integral membrane and secretory glycoproteins. The work of Lodish and colleagues on vesicular stomatitis virus

(VSV) glycoprotein provides a model for this process (Rothman and Lodish, 1977). Briefly, the nascent polypeptide chain synthesized on membrane-associated polyribosomes, is inserted through the membrane of the endoplasmic reticulum, insertion apparently being mediated by a specific "signal" peptide sequence in the chain in accordance with the "signal hypothesis"(Blobel and Dobberstein, 1975). Glycosylation then occurs on the noncytoplasmic side of the membrane utilizing the host cell glycosylating enzymes, and the protein is transported to the plasma membrane in a vesicle that fuses with the membrane. Viral glycoproteins appear to be mobile in the lipid bilayer, as are host cell integral membrane proteins (see Section III), and become distributed at random over the surface of the cell. Prior to the process of budding, glycoproteins locate in the area of budding; M protein aligns under the glycoproteins on the cytoplasmic side of the membrane, the nucleocapsids also align under the M protein, and the whole complex is then enveloped in a bud of plasma membrane. In the virion, as in the cell, the glycoproteins are thus the exposed viral proteins. The lipid envelope reflects the composition of the cell plasma membrane from which it is derived (Lenard and Compans, 1974). Most evidence indicates that host cell membrane proteins are almost totally excluded from the viral envelopes during budding (see Holland and Kiehn, 1970). There is some circumstantial evidence that H-2 antigens can be incorporated into Friend leukemia virus (FLV) (Bubbers and Lilly, 1977). This was based on adsorption of antisera to H-2 by FLV purified from viremic mice. However, virus had to be disrupted to demonstrate this adsorption, and there is as yet no firm confirmatory biochemical evidence demonstrating incorporation of gene products of the major histocompatibility complex (MHC) into enveloped viruses. It does seem definite that cellular actin, a peripheral membrane protein, can be incorporated into some enveloped viruses, e.g., measles virus, VSV, and influenza virus, as demonstrated by its presence in purified virions. However, this is probably a passive association, as exposure of infected cells to cytochalasin B does not prevent incorporation of actin into VSV or influenza virions, or inhibit budding (Griffin and Compans, 1979). Hence, contractile protein function is apparently not required for the budding process.

Viruses may in some circumstances bud from particular domains of the plasma membrane. Studying two different cultured lines of epithelial cells grown as monolayers, Boulan and Sabatini (1978) found that influenza virus, simian virus 5, and Sendai virus budded exclusively from the apical (free) surface whereas VSV budded only from the basolateral plasma membrane. This implies that certain viruses

might be less susceptible to immune recognition at the cell surface because they bud from areas of the cell removed from direct contact with blood.

A point worth making is that these viruses must express virus-specific proteins on the cell membrane before release of free virions occurs. It is also apparent that lysis of a cell infected with a budding virus would be unlikely to result in release of infectious virus, as the nucleocapsids would be devoid of the envelope glycoproteins that mediate virion attachment to the cell membrane prior to entry.

The herpes simplex viruses (HSV), which have been widely used in *in vitro* cytotoxicity studies, are structurally much more complex, coding for some 50 viral polypeptides (Roizman and Furlong, 1974). Viral proteins are inserted in cytoplasmic and plasma membranes, and the viral cores can gain envelopes by budding from any of these membranes. An intriguing feature of HSV is their ability to induce the appearance of an Fc receptor for human or rabbit IgG on the membrane of infected cells (Watkins, 1964; Westmoreland and Watkins, 1974). Although the structural relationship of this receptor to the "endogenous" Fc receptors on lymphocytes and phagocytic cells is unknown, it seems possible that this is a virus-encoded gene product, and it can reportedly be blocked by a $F(ab')_2$ antibody to HSV (McTaggart *et al.*, 1978; Adler *et al.*, 1978). It has also been reported that cytomegalovirus can induce Fc receptors on infected cells (Rahman *et al.*, 1976; Westmoreland *et al.*, 1976). It remains to be seen whether this novel surface structure participates in any definite way in immunologic reactions. Some preliminary evidence indicating that this may be so is summarized in Section III.

Unlike other DNA viruses, poxviruses replicate in the cytoplasm rather than the nucleus and, unlike budding viruses, synthesize their own lipid membranes, but nevertheless express viral antigens on the cell surface, which can appear as early as 1 hour after infection (Ada *et al.*, 1976).

B. The Antibody Response to Viral Infection

Studies with poliovirus in rabbits (Svehag and Mandel, 1964) and humans (Ogra *et al.*, 1968) show a similar sequence in the development of antibody responses to that described for other viral and nonviral antigens. After immunization the level of neutralizing IgM antibody attains maximal titers in 3–4 weeks and is undetectable by 3 months. IgG titers rise in parallel but continue to increase for several months and persist at lower levels for years. Serum IgA antibody is first detectable several weeks after immunization, and titers rise for

several months. Secretory IgA responses occur at local sites of infection of mucosal surfaces with live virus. Subsequent challenge with virus elicits a further transient IgM, in addition to a continuing IgG, response. Studies with a number of other viruses show a similar pattern of response. Many viral antigens appear to be thymus dependent, as indicated by their eliciting no antibody or only small amounts of IgM antibody in nude mice (Burns *et al.*, 1975; reviewed in Oldstone, 1979). Although in all these studies of the immune response antibody is measured as neutralizing antibody, it is very likely that the same antibodies also recognize antigens on infected cells; neutralizing antibodies are directed against external proteins on the virion, which are likely to be those expressed also on infected cell membranes.

Much of the work on the relative importance of responses to individual viral antigens has been done with influenza virus. This is largely because the biochemistry of influenza virus, including detailed knowledge of the structure of the glycoproteins, is better understood than for other viruses. Passive transfer of antibody to the M or nucleoprotein (both internal) polypeptides did not protect infected mice, whereas transfer of antibody to the hemagglutinin (HA) or, to a lesser extent, the neuraminidase, was protective (see Virelizier *et al.*, 1979). Direct visualization of the bromelain-extracted influenza HA and its reaction with IgG antibody, by electron microscopy, shows that antibody reacts with the tip of the molecule—the end of the spike farthest from the membrane of the virion or cell membrane (Wrigley *et al.*, 1977), and the only site on the HA likely to be sterically accessible to antibody on the virion.

In paramyxoviruses the relative response to the two envelope proteins may be important in effective immunity. Norrby and associates (Norrby *et al.*, 1975; Norrby and Penttinen, 1979) showed that formalin-inactivated mumps vaccine or Tween 80–ether-inactivated measles vaccine induce antibodies only to the viral HA, not to the fusion (F) protein (or hemolysin), compared to immunization with live attenuated viruses, which induces antibodies against the F protein. In the case of measles virus, exposure to wild-type virus after immunization with the inactivated virus sometimes produces the clinical syndrome of "atypical measles" with prominent pulmonary infiltrates and severe rash, and the inactivated vaccines generally produce a poor level of immunity.

The extensive literature on the subject of humoral immune responses and viral antigens is reviewed by Ogra *et al.* (1975) and Burns and Allison (1975).

III. Effect of Antibody Alone on Virus-Infected Cells

In the absence of any effector system, such as complement or antibody-dependent cytotoxic K cells, antibody itself can act on virus-infected cells to affect the release of progeny virus and expression of viral antigens. These actions appear to be at least partially reversible and, in some circumstances, capable of protecting the infected cell from subsequent immunologic attack.

A. Antibody-Induced Redistribution of Surface Viral Antigens

The phenomenon of capping of integral membrane proteins by antibodies or lectins is well described. Binding of antibody initially causes patching of the antigen, followed by polar redistribution of the cross-linked membrane proteins as a cap. The initial patching is energy independent, whereas capping is temperature and energy dependent and involves the participation of the cell contractile proteins. Divalent antibody is required to produce capping. Binding of antibody appears to induce transmembrane linkages of integral membrane proteins to intracellular actomyosin-containing filaments at the patching stage for cells in suspension (Bourgignon and Singer, 1977) and in monolayers (Ash *et al.*, 1977). Most, if not all, integral membrane proteins can behave in this way. The subject as it applies to B lymphocytes has been reviewed in this series by Schreiner and Unanue (1976).

Viral glycoproteins expressed on cell membranes can also be capped by antibody as shown independently for measles (Joseph and Oldstone, 1974; Lampert *et al.*, 1975; Ehrnst and Sundquist, 1975) and influenza (Rutter and Mannweiler, 1976) and subsequently for other viruses. Capping of measles viral glycoproteins required active cell metabolism, a functioning cytoskeleton, membrane ATP, and divalent antibody (Joseph and Oldstone, 1974) (see Table I). These glycoproteins are thus capable of lateral diffusion in the membrane and behave in this respect as do host cell integral membrane proteins (Fig. 1). Electron microscopy studies of measles virus-infected cells showed that the nucleocapsids move in concert with the glycoproteins on the cytoplasmic side of the membrane as the latter cap (Lampert *et al.*, 1975), indicating that a transmembrane connection exists between the surface and internal polypeptides. In studies using fluoresceinated monospecific antisera, low concentrations of cytochalasin B are reported to prevent this associated movement of nucleocapsids (Tyrell and Ehrnst, 1979). More-detailed studies are needed to deter-

TABLE I
INHIBITION OF CAP FORMATION IN HELA CELLS INFECTED WITH MEASLES VIRUS[a]

Reagent	Final concentration of sample	Percentage Inhibition of capping
Sodium azide	10^{-2} *M*	57
Sodium azide	10^{-3} *M*	42
2,4-Dinitrophenol	10^{-5} *M*	50
2,4-Dinitrophenol	10^{-6} *M*	30
Oligomycin D	10^{-7} *M*	70
Oligomycin D	10^{-8} *M*	20
N,N'-Dicyclohexyl carbodiimide	10^{-4} *M*	0
N,N'-Dicyclohexyl carbodiimide	10^{-5} *M*	0
1-Ethyl-3(3-dimethylaminopropyl) carbodiimide	10^{-2} *M*	0
1-Ethyl-3(3-dimethylaminopropyl) carbodiimide	10^{-3} *M*	0
Colchicine	10^{-2} *M*	84
Colchicine	10^{-3} *M*	31
Cytochalasin B	10 μg/ml	92
Cytochalasin B	1 μg/ml	38
Vinblastine sulfate	10^{-5} *M*	69
Vinblastine sulfate	10^{-6} *M*	31
Ethylenedinitrile tetraacetate	10^{-1} *M*	0
Ethylenedinitrile tetraacetate	10^{-2} *M*	0

[a] Effect of various inhibitors on antibody-induced cap formation on measles virus-infected HeLa cells (from Joseph and Oldstone, 1974). Cells were preincubated with these inhibitors for 15 minutes at 37°C and then with FITC conjugated antibody to measles for 1 hour at 37°C.

mine whether separate viral envelope glycoprotein species expressed on the membrane cocap, or whether they can behave as independent molecules.

The evidence on whether host cell membrane proteins, particularly products of the MHC genes, cocap with viral proteins is somewhat contradictory, partly owing to studies with different systems and antisera. Rauscher virus gp70 was initially reported to cocap with H-2 on tumor cells (Schrader *et al.*, 1975). Later, using well defined antisera, it was found that retroviral gp69/71 cocapped with H-2 and with TL on a mouse thymoma line (Bourgignon *et al.*, 1978). However, in this same study, capping of T200, which is a host cell surface glycoprotein, also induced cocapping of H-2, and of the Thy 1 and TL antigens. This suggested that H-2 antigens may cocap with a variety of independent cell surface molecules, possibly, as postulated in this report, because they are all linked to the actomyosin filaments (Bourgignon *et al.*,

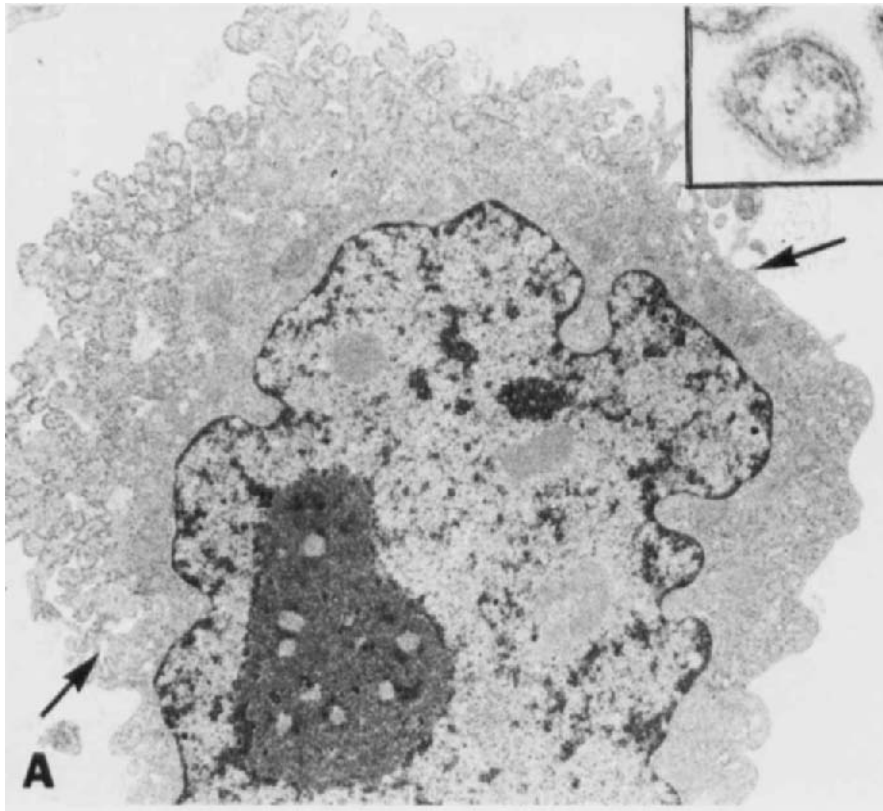
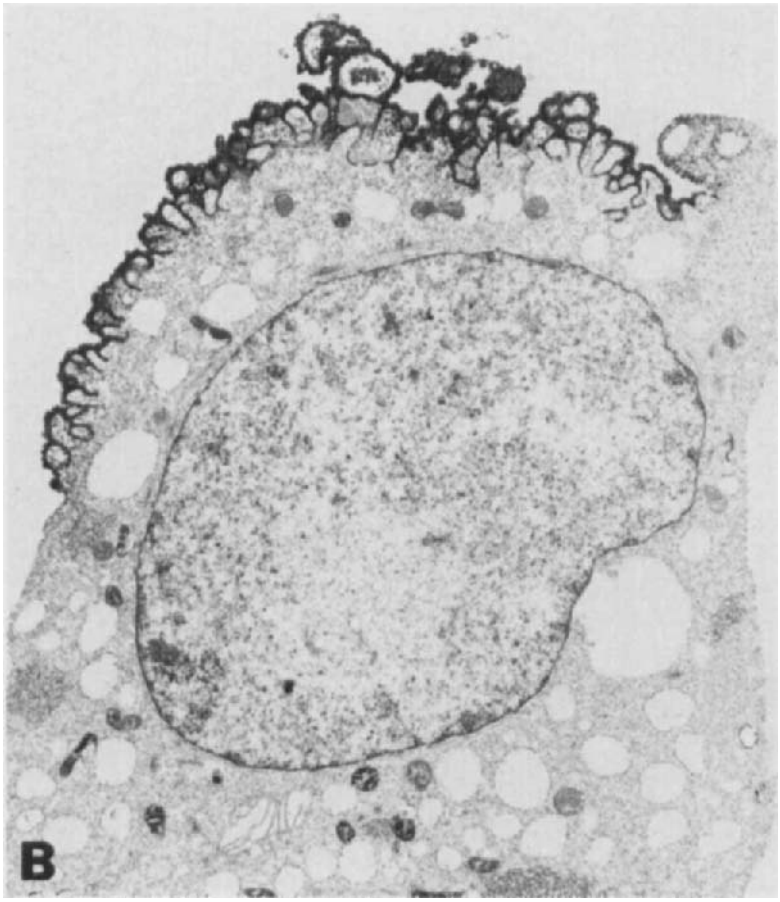
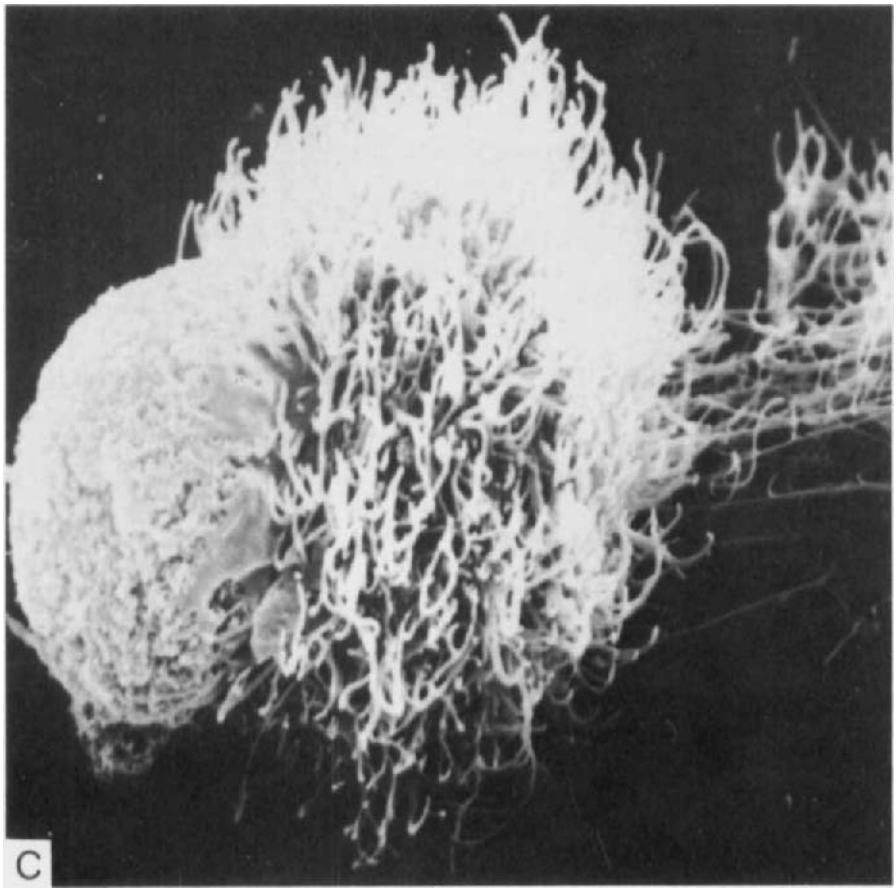


FIG. 1. (A) Electron micrograph of a measles virus-infected HeLa cell after incubation with IgG antibody to measles virus for 1 hour at 37°C. The budding virions have moved to one pole (between arrows) of the cell. Inset shows higher magnification of measles virion. (B) Measles virus-infected HeLa cell exposed to antimeasles virus antibody and peroxidase-labeled antihuman IgG antibody. Capping of the viral antigen –antibody complex has occurred. Minimal endocytosis of the peroxidase label was observed. (C) Scanning electron microscopy of measles virus-infected HeLa cell after capping with antibody to measles, showing movement of the microvilli into a polar position (Lampert *et al.*, 1975).

1978). In contrast to those reports with retroviruses, measles viral antigens expressed on the plasma membrane do not cocap with HLA antigens (Haspel *et al.*, 1977). Lymphocytic choriomeningitis virus (LCMV) expresses one surface glycoprotein on infected cells, and this viral polypeptide does not cocap with either H-2K or H-2D determinants (M. B. A. Oldstone, unpublished observations). Furthermore, in a recent careful biochemical study using immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-



PAGE), no evidence for molecular association of MHC molecules and Moloney virus gp70 could be found on a Moloney virus lymphoma line (Fox and Weissman, 1979). One major interest of these observations is their relevance to the debate on whether cytotoxic T cells recognize MHC products and viral antigen as a complex, or as two independent molecules (see Zinkernagel and Doherty, 1979). In this context additional evidence arguing against a direct molecular association of H-2 antigens and viral glycoproteins, at least occurring during transcription or translation, is provided by experiments mixing independently synthesized proteins. Thus, secondary *in vitro* cytotoxic T cell responses can be generated by liposomes containing isolated



H-2 antigens and Sendai viral glycoproteins (Finberg *et al.*, 1977); and heterokaryons formed by fusing cells bearing SV40 antigens, but inappropriate H-2 antigens, with uninfected cells of appropriate H-2 haplotype, are reported to act as targets for H-2 restricted cytotoxic T cells (Watt and Gooding, 1980).

The actual binding of antibody to determinants at the cell surface could be affected by a number of local factors. As an example, Sissons *et al.* (1979b) investigated the binding site of measles virus antibodies to the surface of radioiodinated measles virus-infected HeLa cells. After exposure of intact cells to IgG containing measles antibody at 4°C to inhibit subsequent capping, the cells were washed and deter-

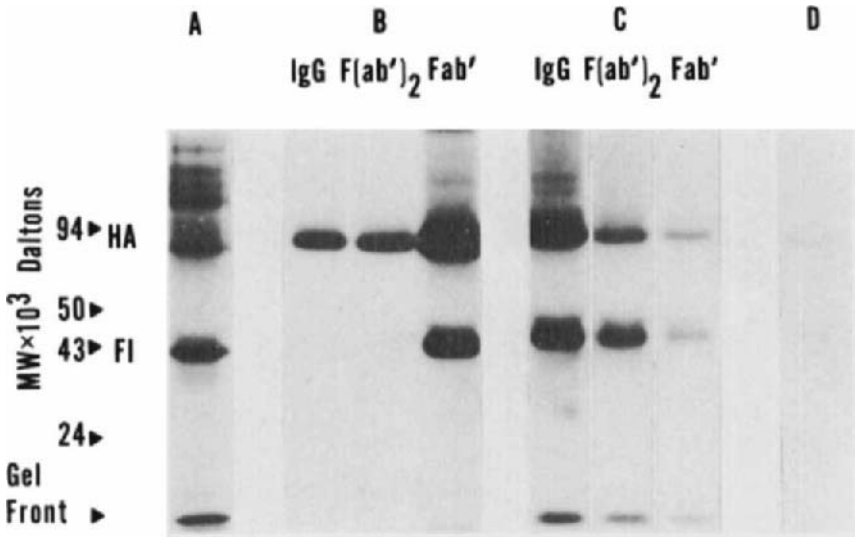


FIG. 2. Binding sites of IgG antibody and its fragments on surface of measles virus-infected HeLa cells. Autoradiogram depicts reduced SDS-PAGE analysis of immunoprecipitates from surface radioiodinated HeLa cells acutely infected with measles virus. (A) Detergent-solubilized cells, not immunoprecipitated. (B) IgG, $F(ab')_2$, or Fab' added in equal amounts to intact cells, followed by washing, detergent solubilization, and addition of protein A. (C) IgG, $F(ab')_2$, or Fab' added after detergent solubilization of cells. (D) Control. HA, measles virus hemagglutinin; F1, the major reduction fragment of the measles virus fusion (F) protein. Note that IgG and $F(ab')_2$ added to intact cells bind only to HA, whereas the univalent Fab' binds to both HA and F1 (panel B). This could be explained by divalent antibody binding to HA sterically hindering the access of antibody to F. (From Sissons *et al.*, 1979b.)

gent solubilized, after which the material binding to staphylococcal protein A was analyzed by SDS-PAGE. IgG antibody bound to both viral glycoproteins on cells infected 24 hours, but at later stages of infection antiviral IgG or $F(ab')_2$ bound only to the HA glycoprotein whereas the same amount of Fab' bound to both HA and F glycoproteins (Fig. 2). These findings were interpreted as showing that, at high densities of antigen, divalent antibody to HA could sterically block the access of antibody to F at the cell surface. Studies of actual binding sites of antibody at the infected cell surface would be of interest in regard to other viruses.

B. Antibody-Induced Antigenic Modulation of Virus-Infected Cells

Antigenic modulation is a term originally introduced by Boyse *et al.* (1963, 1967) to describe the reversible loss of the TL^+ (thymic leukemia antigen) phenotype from TL^+ leukemia cells passaged in immunized mice. These cells lost their sensitivity to lysis when a new source of anti-TL antibody and guinea pig complement was added, but the TL^+ phenotype and susceptibility to lysis returned after culture in the absence of antibody or passage in unimmunized mice. Antigenic modulation of TL, and in other tumor systems, is reviewed in detail by Stackpole and Jacobson (1978). It now seems that a similar phenomenon can occur when virus-infected cells are exposed to antibody, although the mechanism may not necessarily be precisely identical to the TL system.

1. Antigenic Modulation of Measles-Infected Cells

Joseph and Oldstone (1975) found that culture of measles virus-infected HeLa cells in the presence of antibody to measles virus rendered them resistant to lysis when exposed to a fresh source of human antibody and human complement. The authors termed this "antibody-induced viral antigenic modulation." Antibody-induced measles virus antigenic modulation has subsequently been studied in detail. HeLa cells persistently or acutely infected with measles virus were incubated in heat-inactivated human serum containing antibody to measles virus and then maintained in suspension culture with 20% of the same heat-inactivated human serum. At timed points cells were removed from culture and washed. The infected cells' susceptibility to lysis by human antiviral antibody and complement underwent a progressive decline, which was maximal by 12 hours. This correlated with loss of accessible viral antigens from the cell surface as detected by surface staining with fluoresceinated anti-measles IgG or by binding of radiolabeled antibody (see Fig. 3). After the initial few hours of culture in the presence of antibody, only minimal degrees of cap formation were evident on the measles-infected cells. Acutely infected cells maintained in culture regained their susceptibility to lysis by 48 hours, whereas persistently infected HeLa cells regained susceptibility only if washed and recultured in the absence of antibody. This difference probably reflects the differing rate and density of viral glycoprotein synthesis between the two cell types, the acutely infected cell synthesizing enough viral antigen to eventually bind all the antibody in the experimental system used and escape modulation. Under these

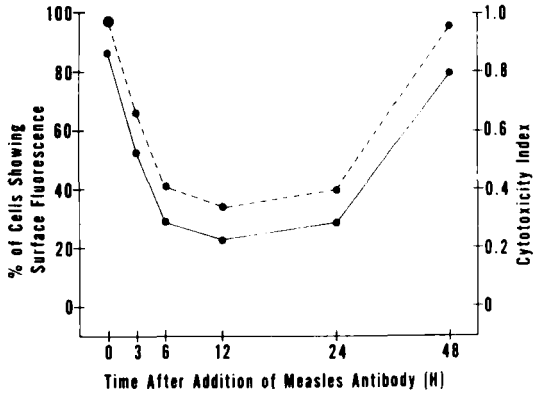


FIG. 3. Antigenic modulation of measles viral antigens expressed on the surface of acutely infected HeLa cells. Alteration of surface expression of measles viral antigens (●----●) and antibody-mediated complement (C)-dependent lysis (●——●) after incubation of acutely infected HeLa cells with measles virus antibodies. HeLa cells were infected with measles virus at a multiplicity of infection of 0.2, and on day 4 of infection they were cultured in suspension with antiviral antibody. Surface expression of measles virus antigens and susceptibility to antibody-mediated C-dependent lysis parallel each other. (From Joseph and Oldstone, 1975.)

same conditions, cells persistently infected with measles virus completely lost their susceptibility to lysis by peripheral blood lymphocytes from immune subjects, within 3 days in culture (Oldstone and Tishon, 1978).

Culture of cells infected with measles virus for longer periods in the presence of antibody can result in the selection of attenuated virus (Rustigian, 1966; Gould and Almeida, 1977), although the biochemical structure of such selected viruses has not been examined carefully.

2. *Fate of Virus Antigen–Antibody Complex on Infected Cells*

The fate of the virus antigen–antibody complex on the infected cell is important to any understanding of the mechanism of antigenic modulation. In general, the approach used to study ligand interactions with cell surface molecules has been to label one or the other component of the complex and follow its fate. When ^{125}I-labeled IgG antibody was bound to the surface of measles virus-infected cells at the start of the culture period, under the conditions described above for antigenic modulation, about 40% of the radioactivity was still cell associated at 12 hours and nearly all was protein bound (Joseph and Oldstone, 1975; Perrin and Oldstone, 1977). The fate of the antibody

was followed in detail by Perrin and Oldstone (1977). After 3 hours in culture 30% of the [^{125}I]IgG was present in the supernatant, and analysis on linear sucrose density gradients showed that a third of this labeled IgG sedimented in a peak heavier than 7 S IgG. This peak of heavier IgG also contained measles virus HA but not nucleocapsid, as detected by immunoprecipitation with specific antibodies. Material from this peak bound to rheumatoid factor and C1q, and to Raji cells, indicating the presence of immune complexes. Thus, these findings suggested that a significant amount of complexed antibody and viral glycoprotein was shed from the cell surface, and the absence of nucleocapsids indicated that the antibody was unlikely to be complexed with whole virus. In contrast, only a small amount of antibody appeared to be endocytosed. This was shown by studying cell lysates in SDS in which most of the [^{125}I]IgG was 7 S, but some non-TCA precipitable material was present, suggesting intracellular degradation in lysosomes (Perrin and Oldstone, 1977).

Immunoelectron microscopy studies of measles-infected cells capped with IgG antibody to measles and then labeled with peroxidase conjugated anti-IgG rarely showed any internalized peroxidase (Lampert *et al.*, 1975). Hence, endocytosis of the viral antigen–antibody complex appears to be unusual in the 80-minute duration of these latter experiments and accounts for only a minority of the complex during the longer interval of modulating conditions studied by Perrin and Oldstone (1977). Some endocytosis of peroxidase label was seen in electron microscopy studies of antibody-treated measles-infected syncytial cells (P. W. Lampert and M. B. A. Oldstone, unpublished observations; Hooghe-Peters *et al.*, 1979) and influenza-infected cells (Rutter and Mannweiler, 1976) after several hours of incubation. However, the use of the second peroxidase-coupled antibody has been shown to favor endocytosis of membrane proteins, at least in B lymphocytes (Schreiner and Unanue, 1976) and makes the system more artificial compared to the situation *in vivo*.

In recent experiments the fate of viral cell surface antigens was followed by surface radioiodination of measles virus-infected cells and maintenance of the cells in culture with or without antibody. The rate of disappearance of ^{125}I-labeled viral glycoproteins from the cell was then determined by running cell lysates on SDS–PAGE and excising and counting the viral glycoprotein bands. After an initial acceleration, the turnover rate of the labeled viral glycoprotein in cells cultured with antiviral antibody was no greater than in cells cultured in the absence of antibody (Fujinami and Oldstone, 1980; Fujinami *et al.*, 1980). This suggests that, after initial shedding, antibody does not

subsequently enhance the net rate of viral glycoprotein loss from the cell under the culture conditions used.

In contrast to the fate of viral antigen–antibody complexes on the plasma membrane, ligand interaction with surface Ig or Fc receptors on B lymphocytes results primarily in their endocytosis (reviewed Schreiner and Unanue, 1976). The fate of other cell surface molecules complexed with antibody is less defined, although it appears that MHC molecules on B cells are less likely to be endocytosed and may be shed (Unanue *et al.*, 1972; reviewed by Schreiner and Unanue, 1976).

3. *Antigenic Modulation in Other Viral Systems*

The only other viral systems in which antigenic modulation has been studied involve oncogenic murine retroviruses. Gross virus leukemia antigens can be modulated *in vivo* in immunized mice (Aoki and Johnson, 1972) and *in vitro* (Ioachim and Sabbath, 1979), as shown by resistance to antibody and complement-dependent lysis. There is also evidence for antigenic modulation in FLV-induced leukemia. This was demonstrated *in vitro* with FLV-infected cells by Genovesi *et al.* (1977) and is likely to play a role *in vivo* (Doig and Cheseboro, 1979). Presence of antiviral antibody, loss of FLV antigens from the leukemic cell surface, and recovery from viremia were all associated with a single genetic locus, named *RFV-3*, which is not linked to the MHC. It was suggested that *RFV-3* acts as an immunoregulatory gene influencing production of cytotoxic anti-FLV antibodies. Nevertheless, despite this evidence for antigenic modulation, progressive fatal leukemia occurred unless appropriate separate *H-2*-linked genes, influencing recovery from leukemia, were associated with the *RFV-3* locus (Doig and Cheseboro, 1979).

Mouse mammary tumor virus antigens can also be specifically modulated *in vitro* (Calafat *et al.*, 1976). Shedding of viral antigen–antibody complexes was also prominent in this study, as assessed by immunoelectron microscopy. These studies are further reviewed by Oldstone *et al.* (1980) and Stackpole and Jacobson (1978).

C. Effect of Antibody Binding to Infected Cells on Synthesis and Expression of Viral Proteins

Antibody in the medium of infected cultured cells results in a decrease of detectable virus in the medium. The cause is in part complexing of antibody with free virus released from the cell, but also cross-linking of viral glycoproteins on the cell surface by antibody, thereby inhibiting release of virus, probably by interfering with the

budding process. The release of influenza virus was inhibited by antibody to the HA and, less effectively, by antibody to neuraminidase (Dowdle *et al.*, 1974); Fab fragments of antiviral IgG had earlier been reported not to inhibit release of virus (Becht *et al.*, 1971).

In addition to these effects of antibody on the release of virus, there is evidence to suggest that antibody may also affect the synthesis and/or expression of viral proteins inside the cell. Fujinami and Oldstone (1979) have recently studied the effect of specific antiviral antibody [under the conditions used by Joseph and Oldstone (1975)] on the expression of individual measles virus polypeptides in acutely infected HeLa cells. After culture in the presence of antiviral antibody in the medium for 6–12 hours, the cells were washed and their incorporation of various labeled precursors into viral polypeptides was studied and compared to controls. It was found that expression of the F protein was diminished as detected by decreased incorporation of [^{35}S]methionine. Since turnover of F protein at the cell surface was not appreciably accelerated (Fujinami *et al.*, 1980), this observation most likely represents diminished synthesis of F protein. Interestingly, expression of a cytoplasmic viral polypeptide, the measles virus phosphoprotein (P) was also diminished (as assessed by [^{35}S]methionine and ^{32}P incorporation) (see Fig. 4). P protein is associated with the transcriptive complex and is probably necessary for replication of the viral genome (Mountcastle and Choppin, 1977). The effects were specific for antiviral antibody, as they could be produced exclusively by purified antiviral IgG, but not by incubation with antibody to the HeLa cell surface or nonimmune IgG. Hence, regulation of an internal viral polypeptide by antibody initially present outside the cell can occur. Whether a transmembrane signal is produced by antibody binding to the plasma membrane or whether the effect is mediated by pinocytosed antibody acting inside the cell is not known.

Yagi *et al.* (1978) observed changes in the polypeptide profile of mouse mammary tumor virus when tumor cells were cultured in the presence of antibody *in vitro,* although these changes were not analyzed in detail with regard to individual proteins.

There is evidence that antibodies can inhibit viral transcription and translation in cell-free systems. Thus, antibody to the VSV viral transcriptase immediately inhibited RNA transcription by VSV nucleocapsids (Imblum and Wagner, 1975), and antibody to Newcastle disease virus nucleocapsid had the same effect (Miller and Stone, 1979). These results, presumably produced by direct binding of antibody to viral enzymes, may be useful tools in molecular virology. However, the observation that antibody can affect viral polypeptide expression

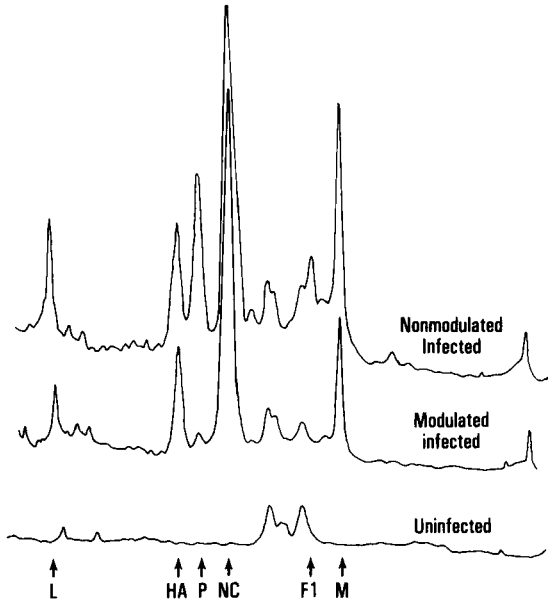


FIG. 4. Inhibition of expression of measles virus phosphoprotein (P) and fusion (F) protein in infected cells by antibody to measles virus. Polypeptide profile of modulated and nonmodulated infected HeLa cells, represented by a densitometer scan of reduced SDS-PAGE of cells pulse labeled with [^{35}S]methionine for 2 hours. Prior to labeling, cells were incubated with or without antibody for 6 hours. P protein and F (seen here as its major reduction fragment F1) are reduced in cells cultured with antibody. Peak found in uninfected cells migrating just before F1 probably represents cellular actin (P is found inside the cell; F is expressed on the cell's surface). (From Fujinami and Oldstone, 1979, 1980.)

in intact cultured cells suggests that the synthesis, transport, or assembly of viral polypeptides could be significantly affected by antibody *in vivo*.

D. Interaction of IgG and the Herpes Simplex Virus-Induced Fc Recptor

Both HSV and cytomegalovirus can induce formation of an Fc receptor for IgG on the surface of infected cells. Costa *et al.* (1977) cultured Vero cells infected with HSV-2 in the presence of nonimmune rabbit IgG or its Fc fragment at concentrations of 5–10 mg/ml and found a marked reduction in virus production. Since the IgG had no neutralizing effect on free virus or cytotoxic effect on infected cells, it was suggested that binding to the Fc receptor inhibited the growth of

HSV. Costa *et al.* did not record the effect of $F(ab')_2$ fragments or of the same IgG preparation on other virus-infected cells. Adler *et al.* (1978) reported that preincubation of HSV-1 infected rat neuroma cells with aggregated nonimmune IgG resulted in loss of susceptibility to lysis by antibody and complement. The effect of native nonaggregated IgG was not reported; although this report mentions that no capping was observed when FITC-aggregated IgG was used, there have been no systematic studies of the fate of HSV Fc receptors binding IgG. In contrast, Rager-Zisman *et al.* (1976) reported that aggregated nonimmune IgG could mediate cytotoxicity of HSV-infected cells by normal mouse peritoneal macrophages. The authors postulated that the cause was cross-linking of Fc receptors on the HSV-infected targets and the effector cytotoxic cells by the aggregated IgG. These reports thus suggest that the presence of the HSV Fc receptor may inhibit viral replication on the one hand, and might interfere with or enhance immune cytolysis on the other. Clearly, more detailed studies in this system are warranted, and the presence of the HSV Fc receptor must be taken into account in any assessment of immune cytolysis of herpes virus-infected cells.

E. Summary and Conclusions

Antibody binding to the surface of virus-infected cells can affect virus production and release in the absence of an effector system. A complex and dynamic interaction exists between antibody and the viral polypeptides expressed on the plasma membrane. Clearly, antibody can induce redistribution and capping of viral proteins on the cell surface. Most of the measles viral antigen and antibody complexed on the plasma membrane is subsequently shed rather than endocytosed; however, it is difficult to know whether shedding of complexes is more likely to occur with viral membrane proteins on the cell surface than with other membrane proteins. Although IgG–anti-IgG complexes on the B lymphocyte membrane are predominantly endocytosed, this may be peculiar to signaling in the B cell. While other host membrane proteins complexed with ligands may not be as rapidly endocytosed, endocytosis, with subsequent degradation in lysosomes, is believed to be the normal route of metabolism for host cell membrane proteins as indicated by the studies of surface radioiodinated mouse L cells by Hubbard and Cohn (1976). Hormone receptors complexed with their specific ligands probably are endocytosed and degraded in the same way (Catt *et al.*, 1979). Viral glycoproteins, which are destined for export from the membrane, may be more sus-

ceptible to shedding; when they are actually in segments of the membrane where virus budding has commenced, this might be even more likely. Electron microscopic studies on measles virus-infected cells undergoing capping show that budding virus is visible in the microvilli forming the cap (Lampert *et al.*, 1975).

Antibody-induced viral antigenic modulation as described for measles virus-infected cells has some similarities to the original TL system of Boyse *et al.* (1963, 1967). Hence, capping is not essential and some antibody and viral antigen is still present on the cell at the time of maximal resistance to antibody- and complement-mediated lysis. However, Yu and Cohen (1974) could not detect any change in TL synthesis in TL^+ cells undergoing modulation, but showed by surface radioiodination that there was accelerated removal of TL from the cell surface, at least during the early time course of modulation. In contrast, diminished synthesis may contribute to the loss of surface viral antigen in the presence of antibody in measles virus-infected cells. There is no suggestion of any requirement for C3 to produce modulation of measles virus-infected cells, whereas there is one in the TL system (Stackpole *et al.*, 1978). It may be concluded that the loss of surface viral antigen induced by antibody and leading to antigenic modulation could result from a combination of (*a*) shedding of antigen–antibody complexes; (*b*) blocking of antigenic sites by IgG remaining on the membrane; and (*c*) diminution of synthesis of surface viral polypeptides. Because cells can generally be rendered resistant to lysis by antibody and complement via relatively modest reduction of surface antigens, it is important to note that, in addition to being resistant to antibody-dependent complement-mediated lysis, modulated measles virus-infected cells are also resistant to antibody-dependent cell-mediated cytotoxicity (Oldstone and Tishon, 1978).

Antibody binding to intact cells can affect the intracellular expression of viral polypeptides as shown in measles virus-infected HeLa cells (Fujinami and Oldstone, 1979). This area has just begun to be studied at a biochemical level, and it will be interesting to see the parallels and contrasts that arise with other membrane receptor–ligand interactions, such as those between hormones and their receptors. The major interest in these observations lies in the possibility that such effects of antibody may represent mechanisms whereby virus persistence could be established *in vivo*. The consequence would be a cell rendered resistant to immune injury because of lost surface viral markers, but still retaining the potential to shed virus once antibody is removed or to infect adjacent cells by cell-to-cell spread. This possibility is further discussed in Section VI.

IV. Antibody- and Complement-Mediated Lysis of Virus-Infected Cells

Virus-infected cells can be lysed by specific antibody and complement. The mechanism of this reaction is discussed in detail in this section. Emphasis is given to work with homologous human systems, and with measles virus-infected cells in particular. Enveloped viruses, as free virions, can also serve as targets for antibody- and complement-mediated lysis; this topic has been reviewed by Oldstone (1975) and by Cooper and Welsh (1979).

A. Complement

The human complement system is composed of some 20 plasma proteins, including its intrinsic components and the proteins that regulate activation of the pathways. The mechanisms by which the system is activated, produces membrane lesions and mediates some of its biological effects are now relatively well understood. The mechanism of activation of the classical pathway (Müller-Eberhard, 1975) and the mechanism by which the membrane attack pathway (C5-9) produces membrane lesions (Podack and Müller-Eberhard, 1980) have been reviewed recently. The alternative pathway of complement activation is the subject of a review in this volume by Schreiber and Müller-Eberhard. Only points that are relevant to the present discussion are mentioned here.

Activation of the classical pathway is usually dependent on the interaction of the Fc portion of IgG or IgM complexed to antigen with the C1q subunit of C1. C1 is activated and enzymically catalyzes assembly of the classical pathway C3 convertase $\overline{C4b2a}$ by sequential proteolytic cleavage of C4 and C2. After cleavage of C3, association of C3b with $\overline{C4b2a}$ gives the classical pathway C5 convertase $\overline{C4b2a3b}$.

The alternative pathway of complement activation consists of six proteins: C3, factors B and D, β1H, C3b inactivator, and properdin. Current evidence indicates that initiation of the alternative pathway on the surface of known activators, such as rabbit erythrocytes (Fearon and Austen, 1977a; Pangburn and Müller-Eberhard, 1978), zymosan (Fearon and Austen, 1977b), and certain strains of *Escherichia coli* (Schreiber *et al.*, 1979), occurs because C3b from the fluid phase is bound there and relatively protected from its serum inactivators. There is probably a low level of background C3b generated from the interaction of native C3, factor B, and factor D. In the fluid phase or on the surface of nonactivators, β1H and C3b inactivator rapidly inactivate this C3b, whereas the access of β1H to C3b bound to the surface of an activator is restricted: factor B can then bind to C3b and is

cleaved by factor D generating the alternative pathway C3 convertase $\overline{C3bBb}$, the active site being on the Bb fragment of factor B. Amplification of C3 turnover with further surface deposition of C3b and acquisition of C5 cleaving activity by $\overline{C3bBb}$ then occur. Native properdin binds to C3b and retards the otherwise rapid decay of enzymic activity of $\overline{C3bBb}$. Properdin is thus a regulatory protein and is not required for initiation of the pathway [see Schreiber and Müller-Eberhard (this volume) for further details].

Consequent upon activation of either pathway, C5 is cleaved and the membrane attack complex is assembled from C5-9. This complex is inserted into the lipid bilayer of cell membranes as a dimer of C5b-9 (Biesecker *et al.*, 1979), giving the typical electron microscopic complement membrane lesion. Osmotic lysis of the cell then results, either because a hydrophilic protein channel is created by insertion of the membrane attack complex, or because its insertion results in rearrangement of the phospholipids and creation of a lipid channel in the bilayer (Podack and Müller-Eberhard, 1980).

B. Lysis of Virus-Infected Cells by Antibody and Heterologous Complement

Many reports of antibody- and complement-dependent lysis of virus-infected cells involved heterologous sources of antibody and complement. A number of studies from 1963 onward showed that cells infected with togaviruses, ortho- and paramyxoviruses, rhabdoviruses, arenaviruses, retroviruses, coronaviruses, and enteroviruses (all enveloped RNA viruses) and with the DNA HSV and poxviruses, could all be lysed by antibody and heterologous complement. These studies are summarized in Table II, and by Rawls and Tompkins (1975). These reports demonstrate convincingly that cells infected with a variety of RNA and DNA viruses are susceptible to lysis by antibody and complement. However, heterologous sera, particularly rabbit serum, which is frequently used as a complement source, may contain natural antibodies reacting with heterologous cell surface or viral antigens. For example, Rawls and Tompkins (1975) found that rabbit serum, as a complement source, lysed certain HSV-infected cells in the presence of antibody, whereas guinea pig or human serum did not. A prozone phenomenon is often observed with rabbit or guinea pig serum as a complement source (Ehrnst, 1977; Hicks *et al.*, 1976). These are not particular problems if the principal purpose is to assay potentially cytotoxic antiviral antibodies, as it was in a number of the studies in Table II. For instance, in cytotoxicity testing for H-2 or HLA typ-

TABLE II
SUDIES OF ANTIBODY- AND COMPLEMENT-MEDIATED LYSIS OF VIRUS-INFECTED CELLS USING HETEROLOGOUS COMPLEMENT SOURCES[a]

Virus	Antibody source	Complement source	References
DNA			
Herpes simplex	Rabbit	Guinea pig	Roane and Roizman (1964)
Herpes simplex	Human	Guinea pig	Smith *et al.* (1972)
Herpes simplex	Rabbit	Rabbit	Brier *et al.* (1971)
Shope papilloma	Rabbit	Guinea pig	Wahren (1963)
Vaccinia	Rabbit	Rabbit	Brier *et al.* (1971)
RNA			
Friend leukemia	Mouse	Guinea pig	Wahren (1963)
Influenza	Rabbit	Rabbit	Brier *et al.* (1971)
Lymphocytic choriomenigitis virus	Mouse	Guinea pig	Oldstone and Dixon (1971)
Measles (persistently infected cells)	Human (normal and SSPE[b] sera)	Guinea pig	Kibler and ter Meulen (1975)
Measles (persistently infected cells)	Human	Rabbit/guinea pig	Ehrnst (1977)
Measles (persistently infected cells)	Rabbit	Rabbit	Minagawa and Yamada (1971)
Mumps	Human/guinea pig	Guinea pig	Oldstone and Dixon (1971)
Rabies	Human and mouse	Guinea pig	Wiktor *et al.* (1968)
Sendai/Newcastle disease virus	Rabbit/mouse horse	Guinea pig/rabbit	Eaton and Scala (1970), Brier *et al.* (1971)
Simian virus 5	Rabbit	Guinea pig	Holmes *et al.* (1969)

[a] Summary of earlier studies of antibody- and complement-dependent lysis of virus-infected cells, in which heterologous sources of antibody and complement were used. Most of these studies were designed primarily to detect cell surface viral antigens or antibody to them, rather than to analyze the mechanism of cell lysis.

[b] SSPE, subacute sclerosing panencephalitis.

ing, the presence of natural antibodies in the complement source is often used to "rig" the system to produce maximum lysis (Ferrone *et al.*, 1974). However, the presence of such natural antibodies makes it difficult to analyze the mechanism of antibody- and complement-mediated lysis of virus-infected cells. Thus, use of heterologous systems provides results that are of doubtful relevance to the situation *in vivo*.

C. Lysis of Virus-Infected Human Cells by Human Serum

The lysis of virus-infected human cells by human serum has been studied in detail in a homologous system by Oldstone, Cooper, and colleagues (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Sissons *et al.*, 1979a). In addition to having obvious direct relevance to human infection, the use of a homologous system avoids the problems inherent in the use of heterologous sera as a complement source referred to above.

Lysis by human serum of cells infected with HSV types 1 and 2, influenza A, parainfluenza 1, 2, 3, and 4, mumps, and measles viruses was dependent on the presence in serum of IgG antibody specific for the relevant virus and of complement (reviewed by Oldstone and Lampert, 1979). In addition to infected human epithelioid cell lines (HeLa, HEP 2), virus-infected lymphoblastoid, neural and glial cell lines of human origin were also lysed. No lysis occurred in serum from agammaglobulinemic children, despite an intact complement system. Serum lacking antibody to a particular virus would not lyse cells infected with that virus, but would do so after addition of IgG containing specific antibody to that virus (Perrin *et al.*, 1976). Acquisition by serum of the ability to lyse virus-infected cells has been demonstrated after immunization with mumps virus and measles virus in individuals without preexisting antibody. Sera obtained before immunization and 3 days after it would not lyse infected cells, but became able to produce specific lysis of mumps virus or measles virus-infected cells 6 days afterward, maximum lytic ability being attained 10 days after immunization (Perrin *et al.*, 1976; and unpublished observations).

A consistent finding in these studies was the requirement for an intact alternative pathway of complement activation in human serum for lysis to occur (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Sissons *et al.*, 1979a). Thus the ability of serum to lyse virus-infected cells was extremely sensitive to dilution, 90% of the cytolytic activity being lost at a 1:6 dilution, and was abrogated by heating serum at 50°C (which inactivates factor B). In contrast, lysis still occurred in serum with 0.01 *M* EGTA and 1 m*M* Mg^{2+}, which selectively chelates Ca^{2+} and thus inactivates the classical pathway (Joseph *et al.*, 1975). This dependence on an intact alternative pathway was confirmed by using human sera that were immunochemically depleted of specific complement components by affinity column chromatography with monospecific antisera coupled to Sepharose 4B (Perrin *et al.*, 1976). Lysis of cells infected with mumps, herpes simplex, influenza, or measles viruses

TABLE III
ROLE OF ALTERNATIVE COMPLEMENT PATHWAY IN ANTIBODY- AND COMPLEMENT-DEPENDENT LYSIS OF VIRUS-INFECTED HELA CELLS BY SERUM[a]

Depletion experiments	Reconstitution experiments	
Component depleted	Percent lysis in depleted serum	Percent lysis on addition of depleted component
None (normal serum)	95	—
Factor B	5	95
Factor D	7	95
Properdin	20	95
C4	95	—
C2	95	—

[a] Human sera containing IgG antibody to measles virus were immunochemically depleted of various complement components by affinity chromatography, except for C2, which was obtained from a patient with genetic deficiency of C2. Depleted sera were reconstituted with a physiologic concentration of the purified depleted component. Lysis was assessed by the eosin microcytotoxicity assay (1). Data represent the results of three experiments. Spontaneous lysis of infected cells in MEM was 5 ± 10%; lysis of uninfected HeLa cells in reagents was 5 ± 5% (mean ± 2 SD). In similar additional experiments cells infected with mumps, influenza, parainfluenza, and HSV types 1 and 2 were lysed by antibody and C4-depleted, but not factor B-depleted, serum. Reconstitution with factor B restored lysis (see Perrin *et al.*, 1976; Sissons *et al.*, 1979a).

was completely abrogated in serum thus depleted of factor B or of factor D and diminished by 80% in properdin-depleted serum. Reconstitution of the depleted component with physiologic concentrations of the purified protein restored the lytic ability in each case (see Table III) (Perrin *et al.*, 1976; Sissons *et al.*, 1979a).

In contrast, serum immunochemically depleted of C4 or genetically deficient in C2, both components necessary for formation of the classical pathway C3 convertase $C\overline{42}$, was as efficient as whole serum in lysing measles virus-infected cells (Perrin *et al.*, 1976). Furthermore, dose related lysis curves showed that C4 depleted serum with no measles virus antibody was quantitatively equal to the original whole serum in its ability to lyse antibody coated measles virus-infected cells (Fig. 5). The kinetics of lysis in whole and C4-depleted serum were also identical (Fig. 6) (Sissons *et al.*, 1979b).

These observations thus showed that the lysis of virus-infected cells by human serum depends on antibody and the alternative comple-

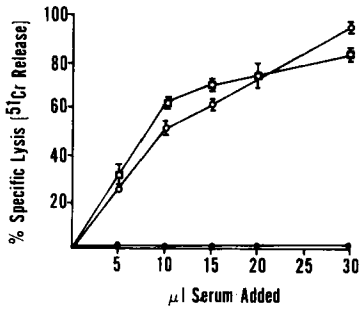


FIG. 5. Equivalent dose-related immune lysis of measles virus-infected HeLa cells coated with antibody by whole and C4-depleted serum. Lysis of HeLa cells acutely infected with measles virus and coated with antiviral IgG by whole serum lacking antibody to measles (○); by the same serum immunochemically depleted of C4 (●); or by the serum immunochemically depleted of factor B (□). Uninfected cells or infected cells without antibody were not lysed. (From Sissons *et al.*, 1979b.)

ment pathway. The lack of any requirement for C4 or C2 showed that activation of the classical pathway is not necessary for lysis, and that the alternative pathway must therefore be initiated by a mechanism other than recruitment in consequence of classical pathway activation.

There are very few other reported studies of lysis of virus-infected cells by human serum. Hicks *et al.* (1976) reported lysis of measles virus-infected monkey (Vero) cells by antibody and human serum. Specific lysis was observed with both C2-deficient and Mg^{2+} EGTA-treated serum and in serum heated at 50°C or adsorbed with zymosan, although none of these was as effective as whole serum. These authors therefore concluded that lysis could be mediated by alternative and classical pathways, but that neither pathway alone was as effective as

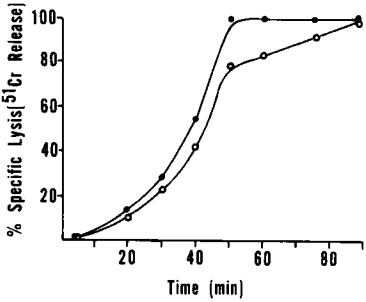


FIG. 6. Kinetics of immune lysis of measles virus-infected HeLa cells. Lysis of HeLa cells acutely infected with measles virus and coated with antiviral IgG by whole (○) and C4 depleted (●) serum; 20 μl of serum was used for each time point. (From Sissons *et al.*, 1979b.)

both combined. Again, human serum produced no lysis in the absence of antibody in these studies. The finding of some classical pathway mediated lysis, in contrast to the findings discussed above, is probably related to the use of a different cell line and a heterologous system.

D. Analysis of the Requirement for Antibody in Complement-Mediated Lysis

The absolute requirement for antibody in complement-mediated lysis of virus-infected cells by serum is documented above. The amount of cell bound antibody required to induce lysis was determined by using measles virus-infected HeLa cells as targets and employing a binding assay with radiolabeled IgG. No lysis occurred until a mean of greater than 5×10^6 to 1×10^7 molecules of IgG had bound per infected cell (Joseph *et al.*, 1975; Perrin *et al.*, 1976; Sissons *et al.*, 1979a) (Fig. 5). This is a large amount of IgG, although the measles virus infected HeLa cell has a large surface area.

The $F(ab')_2$ fragment of IgG can also induce lysis of mumps virus- and measles virus-infected cells (Perrin *et al.*, 1976), and the dose related lysis curves for whole IgG and $F(ab')_2$ in whole and C4 depleted serum are near-identical (Sissons *et al.*, 1979b). However, Fab' fragments are ineffective in inducing lysis. At least 10 times more Fab' than $F(ab')_2$ or IgG molecules bound per infected cell were required to produce 50% lysis, which was the maximum attainable with Fab' in these experiments. At this high density it is difficult to exclude the possibility that Fab' fragments reassociated despite being initially alkylated (see Fig. 7) (Sissons *et al.*, 1979b). These experiments thus

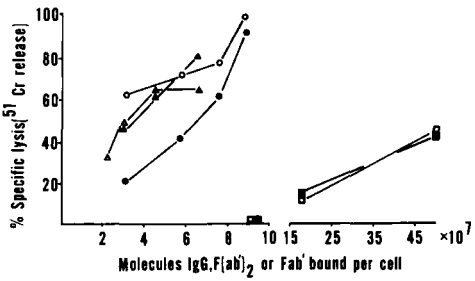


FIG. 7. Comparison of the dose related immune lysis of measles virus-infected HeLa cells produced by IgG and its $F(ab')_2$ or Fab' fragments. Lysis induced by IgG in whole (○) and C4 depleted (●) serum; by $F(ab')_2$ in whole (△) and C4-depleted (▲) serum; and by Fab' in whole (□) and C4-depleted (■) serum. Production of 50% lysis required binding of at least 10 times more Fab' than $F(ab')_2$ or IgG molecules.

show a requirement for divalent antibody, but no requirement for the Fc portion, for IgG to induce lysis of virus-infected cells by the alternative pathway. The fact that $F(ab')_2$ (which cannot bind C1q) is as effective as whole IgG is further proof of the lack of requirement for classical pathway activation for lysis.

Fab' and Fab in addition to $F(ab')_2$ fragments were reported as able to induce lysis of measles virus-infected cells in one other study, but rabbit or guinea pig sera were used as complement sources (Ehrnst, 1978). These findings are difficult to interpret because a heterologous complement source was used, and no determination was made of the amount of Fab bound to the cell. In contrast, Fab' fragments blocked the lysis of HSV-infected cells by IgG antibody and rabbit complement in a study by Brier *et al.* (1971).

This requirement for divalency in the homologous human system suggests that antibody-induced redistribution of viral antigens on the cell membrane may be required for production of lysis. Prevention of capping with cytochalasin D or sodium azide does not abrogate complement-mediated lysis (Perrin *et al.*, 1976). However, these inhibitors of actin polymerization do not prevent local patching of viral antigen on the cell membrane by antibody.

Antibody directed against different viral polypeptides expressed on the cell surface can mediate lysis of measles virus-infected cells. Measles virus has two surface glycoproteins, HA and F. Using a persistently infected cell line and a heterologous complement source, Ehrnst (1977) reported that antibodies to HA induced lysis by the alternative pathway, whereas antibody to F induced lysis by the classical pathway. Again, the amount of antibody bound per cell was not determined in these experiments; by immunoprecipitation and SDS-PAGE analysis of infected cells, the antisera used by Ehrnst were not monospecific (J. G. P. Sissons and M. B. A. Oldstone, unpublished observations), making these results at best difficult to interpret. In contrast, using cell surface labeling and immunoprecipitation to assess the site of antibody binding on acutely infected cells, Sissons *et al.* (1979b) found that human IgG antibodies that bound specifically only to HA or only to F protein were equally effective in inducing lysis by the alternative pathway (in C4-depleted serum) and that neither antibody would induce lysis by the classical pathway alone (in factor B-depleted serum). An equivalent amount of antibody bound to either viral polypeptide induced an equal degree of lysis.

The subclass of IgG seems unlikely to be of direct importance in the induction of lysis, especially since the Fc region is not required. Although some experiments indicate that IgG1 is the major subclass in-

volved in lysis of measles virus-infected cells (Ehrnst, 1978; L. H. Perrin and M. B. A. Oldstone, unpublished observations), this probably reflects the fact that 70% of all serum IgG is of the IgG1 subclass.

There is virtually no information on whether other human immunoglobulin classes can induce complement-dependent lysis of virus-infected cells. This would be of interest insofar as most viruses induce an early IgM response on primary infection or immunization and may also produce a serum IgA response (Ogra *et al.*, 1975). Joseph *et al.* (1975) reported that, at amounts equivalent to or greater than cytolytic IgG, IgA, and IgM isolated from convalescent sera of patients with measles or after vaccination (unpublished observations) did not induce lysis of measles virus-infected cells. However, as might be expected, measles virus antibody titers in the IgM and IgA preparations were very low compared to titers in the IgG fraction. Rabbit IgM antibody appears to be relatively ineffective in inducing lysis of virus-infected cells when compared to IgG (Brier *et al.*, 1971; Ehrnst, 1975).

The precise molecular mechanism by which antibody produces lysis of virus-infected cells in conjunction with complement remains to be determined. However, the findings described in the next section show that IgG is not required for initiation of the alternative pathway by measles virus-infected cells, although it is required for cell lysis.

E. Analysis of the Requirement for the Alternative Pathway

Recent work has further confirmed the role of the alternative pathway in lysing virus-infected cells. These experiments have again used measles virus-infected HeLa cells as a representative model.

The alternative pathway of complement activation has recently been assembled *in vitro* from its isolated constituent proteins in highly purified form. A mixture composed of C3, factors B and D, native properdin, and the two control proteins, β1H and C3b INA, all at physiologic concentrations, can mediate deposition of C3b onto activators of the alternative pathway, such as rabbit erythrocytes, with an efficiency equal to serum (Schreiber *et al.*, 1978). Addition to this mixture of the five purified proteins of the membrane attack pathway, C5-9, creates a system with cytolytic capacity. This purified cytolytic alternative pathway can lyse rabbit erythrocytes and (with lysosyme) *E. coli* as efficiently as C4-depleted serum (Schreiber and Müller-Eberhard, 1978; Schreiber *et al.*, 1979). In recent experiments it was shown that the purified cytolytic alternative pathway could lyse antibody-coated measles virus-infected cells with an efficiency comparable to that of C4-depleted or whole human serum. As in serum, no lysis

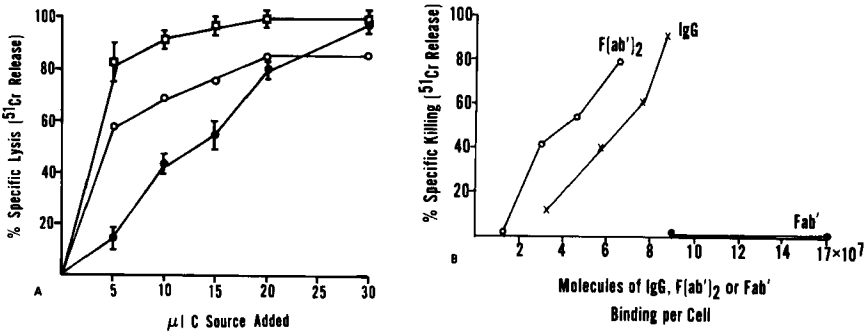


FIG. 8. (A) Dose-related immune lysis of measles virus-infected HeLa cells coated with IgG antibody to measles virus by the following sources of complement: whole human serum lacking antibody to measles (□); human serum immunochemically depleted of C4 (●); and the purified cytolytic alternative pathway (○). There was no lysis of uninfected HeLa cells or infected cells without antibody. (B) Requirements for IgG, $F(ab')_2$, and Fab' for immune lysis of measles virus-infected cells via the purified cytolytic alternative pathway. Measles virus-infected HeLa cells (2×10^5) were preincubated with increasing amounts of IgG (×) or its $F(ab')_2$ (○) and Fab' (●) fragments. Then 5×10^3 cells were incubated with 20 μl of the purified complement components. Data for lysis are plotted against the number of molecules of IgG, $F(ab')_2$, or Fab' binding per measles virus-infected cell, as determined in a parallel binding assay using ^{125}I-labeled antibody or fragments. (A and B reproduced from Sissons *et al.*, 1979a.)

occurred in the absence of antibody, and $F(ab')_2$ and IgG gave near identical dose related lysis curves, but Fab' produced no lysis. Approximately 5×10^7 molecules of IgG or $F(ab')_2$ bound per infected cell were required to induce 50% lysis (Sissons *et al.*, 1979a) (see Fig. 8).

Omission of properdin from the purified cytolytic alternative pathway totally abrogated lysis of measles virus-infected cells. This is noteworthy insofar as properdin was not essential for the lysis of rabbit erythrocytes or *E. coli* by the purified cytolytic alternative pathway (Schreiber and Müller-Eberhard, 1978; Schreiber *et al.*, 1979). This difference may well reflect the fact that nucleated cells require more complement-mediated membrane damage to produce lysis. The use of this system, composed of 11 highly purified complement proteins, provided conclusive evidence that the known proteins of the alternative and membrane attack pathways with IgG antibody are sufficient for lysis of the measles virus-infected cell, without other serum factors. These findings and the efficacy of $F(ab')_2$ also excluded any possibility that alternative pathway activation by antibody-coated virus-infected cells could be occurring by the "C1 bypass activation pathway" described by May and Frank (1973), in which sheep erythrocytes bearing rabbit antibody apparently initiate the alterna-

tive pathway by a mechanism involving C1. Hence, a dominant role is established for the alternative pathway in the complement-dependent lysis of virus-infected cells by human serum.

The requirement for both specific antibody and an intact alternative pathway for lysis of virus-infected cells at first suggested that antibody might be required for activation of the alternative pathway in this system. This would be distinctly unusual, as activation of the alternative pathway by known particulate activators (rabbit erythrocytes, zymosan, *E. coli* 04) is independent of immunoglobulin. The question therefore arose whether, despite being required for lysis, IgG was actually necessary for activation of the pathway on the virus-infected cell. Recent experiments show that measles virus-infected cells activate the alternative pathway independently of antibody. Sissons *et al.* (1980) found that measles virus-infected HeLa cells incubated in a mixture of highly purified C3, factors B and D, native properdin, β1H, and C3b INA (the purified alternative pathway of complement activation, without C5-9) showed progressive linear uptake of [^{125}I]C3b onto the cell surface (Fig. 9). This C3b uptake, which averaged 1 to 1.5×10^6 C3b molecules per cell after 90 minutes, was specific in that it was not shown by uninfected HeLa cells and was blocked by 0.01 *M* EDTA. Measles virus-infected HeLa cells also showed progressive uptake of [^{125}I]C3b from human serum immunochemically depleted of C4 and IgG. In addition to being independent of IgG, [^{125}I]C3b uptake from the purified alternative pathway onto measles-infected cells in the absence of antibody was also independent of properdin, the rate of uptake being the same in the absence and in the presence of properdin, and similar to the rate of [^{125}I]C3 uptake by infected cells in the presence of IgG but the absence of properdin. These experiments are summarized in Fig. 9. Thus, the rate of [^{125}I]C3b uptake by infected cells was increased only in the presence of IgG and properdin together. Because both IgG and properdin are also required for lysis, this suggests that some interaction between the two, possibly affecting the rate of C3b uptake, is important in the mediation of lysis.

In these experiments, significant [^{125}I]C3 uptake by measles virus-infected cells was first demonstrable 12–18 hours after infection, by this time all cells were expressing viral polypeptides on their surface, indicating that viral replication must occur before alternative pathway activation becomes detectable. In all the above experiments the absolute amount of C3b ultimately bound by infected cells alone was similar or identical to the amount bound by infected cells coated with IgG, only the rate of uptake being influenced by IgG.

Further work is needed to determine whether this ability to activate

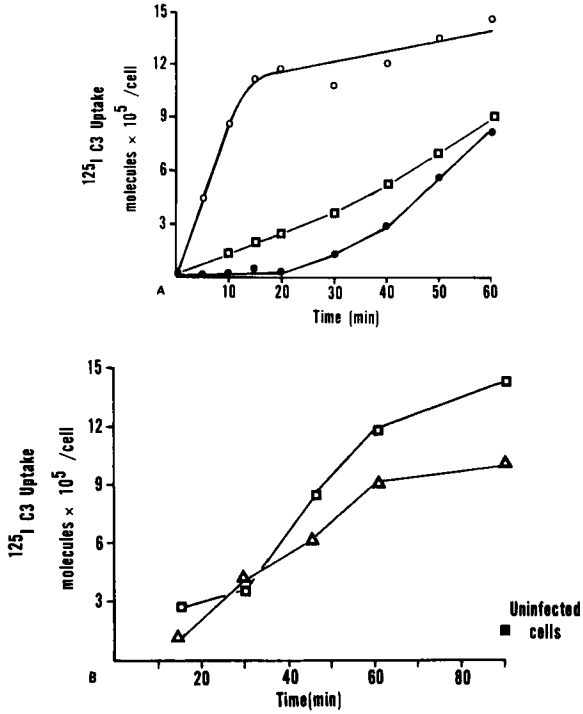


FIG. 9. (A) Uptake of ^{125}I-labeled C3 on to measles virus-infected HeLa cells coated with IgG from the purified alternative pathway (○) and from the purified alternative pathway without properdin (●). □: Uptake of [^{125}I]C3 onto measles virus-infected HeLa cells, without IgG, from the purified alternative pathway. [^{125}I]C3 uptake in the presence of 0.01 *M* EDTA has been subtracted at each time point. (B) Uptake of [^{125}I]C3 on measles virus-infected HeLa cells via the purified alternative pathway in the presence (□) or the absence (△) of properdin. (■)[^{125}I]C3 uptake on uninfected HeLa cells via the purified alternative pathway. (A and B from Sissons *et al.*, 1980.)

the alternative pathway independent of antibody is a general property of cells infected with viruses. However, it should be noted that a number of human lymphoblastoid cell lines can activate the alternative pathway in human serum (Budzko *et al.*, 1976; Theofilopoulos and Perrin, 1976; McConnell and Lachmann, 1976; McConnell *et al.*, 1978). McConnell *et al.* (1978) have reported that this ability correlates with transformation by Epstein–Barr (EB) virus. They found that cell lines positive for EB virus-determined nuclear antigen (EBNA) activated the pathway, as shown by C3 conversion in Mg-EGTA serum and deposition of C3 on cells detected by immunofluorescence. EBNA negative lines did not deposit C3 on their membranes but acquired the ability to do so after EB virus transformation and ac-

quisition of EBNA. EB virus-transformed cells are mostly nonproducing and do not express detectable structural viral antigens on their surfaces. There presumably must be changes in the membrane as shown by the presence of the uncharacterized lymphocyte-determined membrane antigen (Svedmyr and Jondal, 1975). Despite the ability of Raji cells, one of these EBNA-positive lymphoblastoid lines, to activate the alternative pathway, lysis of the cells occurred only after prolonged incubation in serum for 7–8 hours and was independent of antibody (Theofilopoulos and Perrin, 1977). In contrast, measles virus-infected HeLa cells did not lyse (as measured by specific ^{51}Cr release) even after 18 hours of incubation in serum without antibody to the virus (J. G. P. Sissons, unpublished observations).

There is supportive but less direct evidence suggesting that measles virus-infected cells (Hicks *et al.*, 1976) and Sendai virus-infected cells (Okada *et al.*, 1979) can activate the alternative pathway. This is implied by their lysis in C4-deficient guinea pig serum independent of antibody. It was also suggested that retrovirus-infected cells can activate the alternative pathway, as evidenced by using Mg-EGTA serum and a rosetting assay to detect cell-bound C3 (Okada and Baba, 1974). However, it seems equally likely that retrovirus-infected cells might activate the classical pathway, since it is already known that free retrovirus activates the classical pathway independent of antibody by binding C1q via its surface glycoproteins (Welsh *et al.*, 1975; Cooper *et al.*, 1976; Bartholomew *et al.*, 1978).

F. Aspects of Target Cell Structure Affecting Antibody- and Complement-Mediated Lysis

The principal aspects of target cells affecting lysis are the density and presentation of viral antigen expression on the cell surface. Any factor decreasing surface antigen expression tends to decrease susceptibility to lysis. Thus, events that cause the virus-infected cell to produce mutants defective for surface glycoproteins or defective interfering virus, which suppresses replication of the standard virus, result in diminished viral antigen expression on the cell surface. Welsh and Oldstone (1977) found that the susceptibility of cultured neuroblastoma cells infected with LCMV to lysis by antiviral antibody and heterologous complement declined with increasing time in culture. This was related to diminished LCMV surface antigen expression, which resulted from generation of defective interfering LCMV. The resistance to lysis was specific for LCMV in that both acutely and persistently infected cells were still susceptible to lysis by anti-H-2 and complement.

Antigenic modulation of measles virus-infected cells by antibody,

as described in Section III, also renders cells resistant to human antibody- and complement-mediated lysis. Joseph and Oldstone (1975) found that after 12 hours of culture in the presence of antibody, cells both acutely and persistently infected with measles virus lost their susceptibility to antibody- and complement-dependent lysis.

At certain doses addition of interferon to cultured cells expressing LCMV antigens is followed by a reduction in the expression of viral antigens on the cell surface and susceptibility to immune lysis (M. B. A. Oldstone and T. Merigan, unpublished observations).

A further mechanism whereby infected target cells could escape lysis by antibody and complement is the expression of new membrane proteins that block access of antibody to surface antigens. Celis *et al.* (1979) showed that murine myeloma LPC-1 cells grown as an ascites tumor express increased amounts of a surface glycoprotein (gp100) that seemed able to block binding of cytotoxic antibodies to H-2 antigens. Removal of this protein with proteases restored susceptibility to immune lysis by anti-H-2 and complement or cytotoxic lymphocytes. Whether similar events can occur with viral antigens is unknown.

The cell cycle can also influence susceptibility to lysis. Cikes and Friberg (1971) found that Moloney virus-transformed lymphocytes were susceptible to lysis by antibody and complement only in the G1 phase, not during S, G2, or M. Yet viral antigen was present and accessible to antibody, and complement was activated throughout the cell cycle (Lerner *et al.*, 1971). Complement membrane lesions were also present on the cells at different phases of the cell cycle, although immune lysis was again restricted to G1 (Cooper *et al.*, 1974). Consequently, the plasma membrane may vary in its ability to withstand complement-mediated damage at various phases of the cell cycle, presumably because of factors affecting membrane repair.

Susceptibility of nucleated cells in general to complement-mediated lysis is probably related in part to their ability to effect membrane repair. For instance, complex changes in the synthesis and release of several lipid classes have been reported in tumor cell lines upon their exposure to antibody and complement (Schlager *et al.*, 1978). However, there is no direct information on how virus infection can affect the cell's ability to repair its membrane.

G. Summary and Conclusions

In summary, cells infected with a number of different RNA and DNA budding viruses are lysed by human serum, and this lysis is dependent on IgG antibody and the alternative pathway of complement. More detailed studies using measles virus have shown that $F(ab')_2$ is

as effective as whole IgG in inducing lysis but that Fab′ is ineffective. Furthermore, IgG is not required for activation of the alternative pathway, which occurs on the surface of measles virus-infected cells independently of antibody. Properdin is not required for activation of the alternative pathway, but is required with IgG for lysis of infected cells. The major remaining questions concerning the mechanism of this reaction are as follows: Do cells infected with other viruses activate the alternative pathway? How do virus-infected cells activate the alternative pathway? What is the mechanism by which IgG induces lysis?

The mechanism by which measles virus-infected cells activate the alternative pathway may be similar to that described for other activators, activator-bound C3b being protected from β1H. It has been proposed that specific surface molecules on activating particles might act as "β1H antagonists," restricting access of β1H to C3b (Pangburn and Müller-Eberhard, 1978; Pangburn *et al.*, 1979). It is conceivable that the viral glycoproteins, which are inserted in the membrane as integral membrane proteins, could function in this way. Alternatively, some other virus-induced change in the membrane may be responsible. For example, virus infection could result in expression or release of membrane proteases that might cleave C3. Immunoelectron microscopy studies in which measles virus-infected cells were incubated in human serum containing antibody to measles, and then stained with ferritin-conjugated antisera to C3, factor B, or properdin, showed that the antisera bound to the viral antigen–antibody complex on the cell membrane. Adjacent sites on the membrane, where viral spikes were not apparent, showed no labeled antibody binding. This suggests that membrane deposition of C3 occurs at the sites of insertion of viral glycoproteins (Oldstone and Lampert, 1979).

The fact that properdin is not required for activation of the pathway by measles virus-infected cells accords with its known function, which is to delay intrinsic decay of $\overline{C3bBb}$. Properdin can be recruited only when multiple C3b molecules are deposited on a surface in close spatial association, and activation of the pathway must therefore precede its recruitment (Fearon and Austen, 1975; Medicus *et al.*, 1976). The requirement for properdin in complement-dependent lysis of measles virus-infected cells, in contrast to its nonessential role in lysing other activators, may reflect the fact that a nucleated cell with the capacity for membrane repair requires more extensive complement-mediated membrane damage before lysis results. Properdin would be expected to enhance C3 and C5 turnover and membrane deposition.

The fact that virus-infected cells are not lysed by antibody and the

classical pathway alone in human serum requires explanation. The classical pathway is *activated* by IgG antibody bound to virus-infected cells, as shown by uptake of C4 and C3 (assessed by immunofluorescence) from factor B-depleted serum (Perrin *et al.*, 1976) and binding of [^{125}I]C3 following assembly of $C\overline{42}$ on the surface of IgG bearing measles-infected cells (M. K. Pangburn and J. G. P. Sissons, unpublished observations); however, it is not known whether membrane lesions result. It is possible that, despite activation of the classical pathway, insufficient membrane damage occurs to achieve lysis in the absence of the amplification provided by the alternative pathway C3b-dependent feedback mechanism.

The mechanism whereby IgG induces lysis by the alternative pathway remains to be determined. The requirement for divalent antibody suggests that patching of viral polypeptides on the membrane may be important. The fact that the rate of C3 uptake onto measles-infected cells is enhanced only when IgG and properdin are both present suggests that IgG may facilitate the uptake of properdin. Further experiments are needed to test these possibilities.

There are a few other instances in which antibody and the alternative pathway are both required to lyse cells. Human lymphocytes coated with human alloantisera can be lysed by the alternative pathway in human serum, although not all the alloantisera used in this study had this ability and some were effective in inducing lysis only by the classical pathway (Ferrone *et al.*, 1973). *Trypanosoma cruzii* are also lysed by IgG antibody and the alternative pathway (Krettli and Nussenzweig, 1977). Additionally, there is some evidence to suggest that a serum factor, probably IgG, enhances the alternative pathway-dependent lysis of rabbit erythrocytes by human serum (Polhill *et al.*, 1978; Nelson and Ruddy, 1979), although IgG is not essential for this reaction. It is apparent that considerably more surface-bound IgG is required to induce complement-dependent lysis of virus-infected cells than is required for antigenic modulation or antibody-dependent cell-mediated cytotoxicity. The possible implications of this are discussed in Section V.

Further work showing that cells infected with other viruses can activate the alternative pathway independent of antibody would strengthen the evidence for viewing the alternative pathway as a means of nonspecific host defense against microorganisms. It is also important to determine whether free viruses can activate the pathway. The observations of Wedgewood *et al.* (1956) and of Welsh (1977) suggest that Newcastle disease virus may be inactivated by the alternative pathway independent of antibody in human serum (reviewed by Cooper and Welsh, 1979).

V. Antibody-Dependent Cell-Mediated Cytotoxicity of Virus-Infected Cells

A. Introduction

The main emphasis in this review is directed toward the effect of antibody alone or antibody and complement on virus-infected cells. Now we discuss antibody-dependent cell-mediated cytotoxicity (ADCC) briefly, both to illustrate the range of actions that antibody may have on virus-infected cells and to place all these actions of antibody in a comparative context. This section deals only with ADCC as applied to virus-infected cells, with emphasis on human systems and the role of antibody, rather than on detailed discussion of the effector cells involved.

1. General Aspects of ADCC

ADCC has been studied in a number of different systems (reviewed by McLennan, 1972; Perlmann *et al.*, 1972; Lovchik and Hong, 1977; Perlmann and Cerottini, 1979). Although the lysis of antibody-coated erythrocytes can be mediated by phagocytic cells—polymorphonuclear leukocytes (PML) and macrophages—the lysis of nucleated cells sensitized with antibody by nonimmune, human peripheral blood lymphocytes (PBL) is mediated predominantly by lymphocytes that have Fc receptors. These cells do not have conventional B or T lymphocyte markers (surface Ig and sheep erythrocyte (E) rosette negative) and thus fall within the "null cell" population. However, there is evidence that some PBL able to mediate ADCC can be found in the subpopulation of T cells with Fc receptors for IgG (see Perlmann and Cerottini, 1979) as has also been reported for virus-infected targets (Santoli and Koprowski, 1979). This T cell subset also contains cells able to suppress antibody production by B cells (Moretta *et al.*, 1977). Although cells that can mediate ADCC are often referred to as K cells, this is a functional definition and is not based on the presence of unique surface markers. ADCC is usually rapid; membrane damage can be detected 10 minutes after the reaction's inception, and cell lysis is usually completed by 4–8 hours. There is evidence to suggest that the effector cell is inactivated by interaction with its targets (Ziegler and Henney, 1975), which may result from modulation of the Fc receptors on the effector cell surface (Perlmann and Cerottini, 1979).

In assaying for ADCC, exogenous antibody is conventionally added to the cytotoxicity assay in the presence of PBL. However, PBL can exhibit cytotoxicity for a range of target cells in the absence of added antibody, and this activity is mediated, in part, by a class of cells func-

tionally designated as natural killer (NK) cells. There has been some uncertainty over whether or not this spontaneous or natural cytotoxicity could be dependent on small amounts of antibody present in the assay system. For this reason, recent observations concerning human NK cells are summarized below.

2. *Lysis of Virus-Infected Cells by Natural Killer Cells*

The spontaneous cytotoxicity exhibited by unsensitized human PBL for various cultured cell lines and virus-infected cells *in vitro* has received increasing attention. This lysis, mediated by NK cells, has been recently reviewed in human (Herberman *et al.*, 1979; Saksela *et al.*, 1979; Santoli and Koprowski, 1979; Perlmann and Cerottini, 1979) and murine (Herberman *et al.*, 1979; Kiessling *et al.*, 1979; Welsh, 1978) systems.

The bulk of evidence indicates that human NK cells cannot be easily separated from cells mediating ADCC, both being chiefly null cells with Fc receptors. Depletion of NK cells by target binding and cross competition experiments suggests that the same cell population mediates both activities. However, the mechanism of target recognition differs, and a functional distinction between cells mediating ADCC and NK activity can be made on this basis. The available evidence (summarized in the reviews above) suggests that NK cells do not recognize and bind to target cells via their Fc receptors or depend on antibody for cytotoxicity. Furthermore, NK cells do not exhibit conventional immunologic specificity but can lyse a wide range of infected or uninfected target cells. In contrast, cells mediating ADCC recognize and bind to target cells sensitized with antibody via their Fc receptors and require antibody for cytotoxicity, which thus confers immunologic specificity on the reaction. An obvious and important question is whether small amounts of IgG antibody secreted during the course of a cytotoxicity assay, or bound to cytotoxic cells via their Fc receptors, could account for NK cell cytotoxicity. The case against a role for IgG in NK cell cytotoxicity is supported by experiments showing a failure to block NK cell activity by F(ab')2 or Fab anti-IgG, anti-Fab or protein A; all these reagents can block the IgG-Fc receptor interaction and effectively inhibit ADCC by exogenous antibody (Kay *et al.*, 1978; Trinchieri *et al.*, 1978). However, Pape *et al.*, (1979) could inhibit a significant part, but not all, of NK cell activity against K-562 cells by these methods. The question of whether ADCC can account for some NK cell cytotoxicity is thus not resolved.

Interferon enhances the activity of human NK cells *in vitro* (Trinchieri and Santoli, 1978; Herberman *et al.*, 1979) and *in vivo* (Hudd-

lestone *et al.*, 1979). However, it is not clear whether interferon can also enhance the ability of PBL to mediate ADCC (Trinchieri and Santoli, 1978; Herberman *et al.*, 1979). Virus-infected cells (and tumor- and virus-transformed cell lines) can induce interferon release from PBL. Interferon can then enhance the activity of NK cells within the PBL population and contribute to lysis of target cells in the course of a cytotoxicity assay (Santoli *et al.*, 1978). Thus, distinguishing NK cell activity from ADCC presents difficulties. Unless carefully defined reagents are used to block IgG-Fc receptor interactions with uninfected and infected cell targets, it is difficult to interpret their relative importance during lysis of virus-infected cells by PBL.

When NK activity is induced *in vitro* by interferon released during a cytotoxicity assay, cytotoxicity is maximal at 16–18 hours, whereas ADCC in the presence of exogeneously added antibody is usually maximal by 4–8 hours. However, killing by NK cells already induced by preexposure to interferon *in vivo* or *in vitro* is maximal at 6–8 hours, whereas ADCC dependent on antibody secreted during an *in vitro* assay might be expected to have slower kinetics. Hence, clearly distinguishing between NK cytotoxicity and ADCC on the basis of kinetics is also difficult. It is possible that if the same cell is involved in both NK cell killing and ADCC, as current evidence suggests, then attachment to target cells sensitized by antibody via its Fc receptor may induce a more efficient cytotoxic mechanism than binding via the putative NK cell receptor.

B. ADCC of Virus-Infected Cells

The mechanism of ADCC appears to be the same for different cells infected with a variety of viruses and is unlikely to differ from that involved in ADCC of other nucleated cells. Although there are numerous descriptions of virus-infected cells killed by human PBL, only those reports in which ADCC activity was deliberately assayed by addition of exogenous antibody are discussed below.

The first examples concerned ADCC of HSV-infected cells by human PBL (Shore *et al.*, 1974, 1976; Rager-Zisman and Bloom, 1974) as well as mouse peritoneal exudate cells (Rager-Zisman and Bloom, 1974). Both reports emphasized that ADCC could be produced by antibody concentrations several hundredfold less than those needed to produce antibody and complement dependent lysis of the same virus-infected cells. Extending these findings, Shore *et al.* (1978) and Kohl *et al.* (1977) noted that the effector cell in the human PBL population could be either an adherent cell or a nonadherent Fc receptor-positive lymphocyte. The adherent cell required higher antibody concentra-

tion and displayed slower kinetics of killing than the nonadherent cell. This is the only report of an adherent mononuclear cell mediating ADCC of virus-infected cells in a human system; others have found that eliminating adherent cells did not diminish or might enhance ADCC. Other studies with HSV (Möller-Larssen *et al.*, 1977) suggested that trace amounts of antibody present in the medium used for washing PBL could mediate ADCC by PBL in the absence of added IgG. The added complication of the HSV-induced Fc receptor (discussed in Section III) should be recalled. Lysis of HSV-infected cells may occur in part by interaction involving this receptor. Rager-Zisman *et al.* (1976) suggested that even traces of aggregated IgG in fetal bovine serum could cross-link target and mouse peritoneal effector cell Fc receptors to produce apparent ADCC of HSV infected cells, although the possibility of NK cell killing was not considered.

In studies with mumps virus-infected cells (Andersson *et al.*, 1975; Härfast *et al.*, 1977), high concentrations of rabbit antibody to mumps virus inhibited, but lower concentrations enhanced, PBL-mediated cytotoxicity. Antibody independent but virus specific cytotoxicity mediated by Fc receptor bearing PBL was also noted in these studies (Härfast *et al.*, 1978).

Several laboratories have independently assayed PBL-mediated killing of measles virus-infected targets. Antibody to measles virus enhanced the killing of measles virus-infected cells by PBL harvested from normal immune subjects (Perrin *et al.*, 1977a; Kreth and ter Meulen, 1977; Ewan and Lachmann, 1977). Antibody from normal immune subjects or patients with subacute sclerosing panencephalitis (SSPE) also enhanced the cytotoxicity of PBL obtained from patients with SSPE (Kreth and ter Meulen, 1977; Perrin *et al.*, 1977a; A. Tishon, J. H. Huddlestone, and M. B. A. Oldstone, unpublished observations). Since patients' sera enhanced cytotoxicity, these observations are at variance with the earlier report of Ahmed *et al.* (1974) of a "blocking factor" in the sera of SSPE patients that inhibited lymphocyte-mediated activity (migration inhibition, proliferation) against measles virus-infected cells, although Ahmed *et al.* did not assay cytotoxicity. Perrin *et al.* (1977a) calculated that an average of 4 to 5 $\times$ 10^5 antibody molecules bound per measles virus-infected target (HeLa) cell was required to induce ADCC. This contrasts with the much greater (at least 10-fold) amount of cell-bound antibody required to induce antibody- and complement-dependent lysis in the same system (see Section IV).

Respiratory syncytial virus-infected cells were lysed by ADCC (Scott *et al.*, 1977) using colostrum as well as serum as sources of IgG

antibody. ADCC has also been used to detect IgG antibodies to EB virus (Jondal, 1976), but only lytically infected cells that express virus-specific surface antigens, not transformed cells, can be used as targets.

C. Summary and Conclusions

1. Role of Antibody in ADCC of Virus-Infected Cells

It is clear that addition of specific antibody can enhance the cytotoxicity of PBL for virus-infected cells. Minimal amounts of antibody are effective for ADCC activity. Because the kinetics of ADCC are usually rapid, increased ionic efflux from target cells being observable at 10 minutes in some systems (Ziegler and Henney, 1975), it seems unlikely that antibody would produce significant modulation during the course of a cytotoxicity assay. Nevertheless, it is possible that the inhibitory effects of high doses of antibody on ADCC might be related to capping or redistribution of viral antigens by antibody; inhibition might also be produced by modulation and loss of Fc receptors on the effector cells by aggregated IgG (Perlmann and Cerottini, 1979). IgG antibody has been responsible for ADCC of virus-infected cells in all the human studies cited. At present there is no published work on the relative efficacy of antibodies directed against different viral antigenic determinants on the cell surface in mediating ADCC. However, unpublished work from this laboratory (L. H. Perrin and M. B. A. Oldstone) suggests that there may be differences in the ability of antibody to measles virus HA and antibody to measles virus F protein to produce ADCC.

If spontaneous cytotoxicity by NK cells does involve recognition of target antigens by a receptor distinct from the Fc receptor on cells within the null cell population, it is conceivable that antiviral antibody on the target cell surface might sterically hinder binding of such cells to their target antigen, whatever that antigen is, on the infected cell. Antibody might thus inhibit NK cell binding as it simultaneously facilitated K cell binding.

2. Role of Antibody in Spontaneous PBL-Mediated Cytotoxicity of Virus-Infected Cells

There are a number of reports that evaluate the spontaneous cytotoxicity of human PBL for virus-infected targets without added antibody. Some selected reports are discussed that indicate a possible role for antibody. In some cases there is evidence that cytotoxicity is dependent on antibody adsorbed to effector cells as suggested by studies of Greenberg *et al.* (1977) with influenza and Möller-Larssen *et al.*

(1977) with HSV. An alternative explanation would be that antibody is produced by plasma cells in the course of the cytotoxicity assay. After vaccination of human subjects with measles virus or vaccinia virus, Perrin *et al.* (1977b, 1978) found that cytotoxic PBL were generated. Enhanced lysis of virus-infected targets by PBL began at day 5, peaked by day 7, and was rapidly dissipated by day 15 after immunization. These induced cytotoxic PBL showed immune specificity. PBL generated after vaccination with measles virus killed measles virus-infected target cells preferentially, but not vaccinia virus-infected targets. Similarly, cytotoxic PBL generated after vaccinia vaccination showed enhanced killing of vaccinia-infected targets, but not of measles virus-infected targets. The target cells used were autologous fibroblasts and homologous and xenogeneic cultured cell lines. PBL cytotoxicity induced by measles virus or vaccinia virus vaccines was not mediated by T cells (E-rosetting cells), but by Fc receptor positive, E rosette negative cells. The specific cytotoxicity for virus-infected target cells was inhibited 95% by $F(ab')_2$ antihuman IgG. Further, experiments using different subsets of PBL from immune and nonimmune subjects showed that Fc receptor bearing lymphocytes from either immune or nonimmune persons lysed virus-infected targets in the presence of B cells from immune donors. Hence, two populations within PBL participated in the killing of virus-infected targets. In addition to the Fc receptor positive effector lymphocytes, it is likely that B lymphocytes producing antibody were present. The evidence for antibody playing a role in these circumstances is compelling, but synthesized antibody has not been identified directly. However, PBL from subjects 7 days after immunization or natural infection with influenza virus have recently been shown to secrete specific antibody to the viral hemagglutinin *in vitro*, coinciding with a peak in cytotoxicity by these PBL against influenza infected target cells (Greenberg *et al.*, 1979). However, in other instances the enhanced cytotoxicity against virus-infected targets shown by human PBL in the absence of added antibody is likely to result from interferon-induced NK cell activity (Santoli and Koprowski, 1979).

3. *Effector Cell in ADCC of Virus-Infected Cells*

The abundance of evidence indicates that the effector cells mediating ADCC of virus infected targets are the same as those mediating ADCC of other nucleated cell targets by human PBL, namely, nonadherent cells with Fc receptors that do not rosette with sheep E. The report of an adherent cell mediating ADCC against an HSV-infected target cell (Kohl *et al.*, 1977) could relate to special circumstances in the HSV system. However, in most other studies the cytotoxicity of

adherent cells had not been studied directly, their ineffectiveness being indirectly deduced from the fact that their depletion from PBL does not diminish cytotoxicity by the remaining PBL. Human adherent mononuclear cells can reportedly mediate ADCC against uninfected sensitized nucleated targets (K562 and CLA-4 cells) (Horwitz *et al.*, 1979).

Rouse *et al.* (1976) noted that neutrophils were the predominant effector cell in a bovine system, killing sensitized targets infected with a bovine herpes virus. They also reported that complement enhanced ADCC mediated by neutrophils in this system (Rouse *et al.*, 1977). However, no clear distinction was made between an effect of complement enhancing ADCC, or causing independent membrane damage that summated with that produced by ADCC. There are no reports of neutrophils mediating ADCC of virus-infected targets in a human system. Enhanced ADCC activity against measles virus-infected targets by PBL (without neutrophils) in the presence of complement has been the subject of extensive experiments in this laboratory, but has not been demonstrated (J. Huddlestone, unpublished observations). Although some K cells probably have low affinity C3 receptors, no convincing role for C3 has been shown in ADCC of other nucleated cell targets (Perlmann and Cerottini, 1979).

4. Conclusion

IgG antibody is highly efficient in its ability to sensitize virus-infected cells for lysis by human PBL. The amount of cell-bound specific antiviral antibody required for ADCC is at least 10- to 100-fold less than that required for antibody-dependent complement-mediated cytotoxicity and the kinetics of ADCC are faster than those of modulation. At present, in virus-infected human target models there is no evidence that complement potentiates ADCC activity. Although precise assessment of the role of ADCC in cytotoxicity assays is complicated by the presence of NK cell activity in the same lymphocyte population, ADCC appears to be a more efficient cytotoxic mechanism *in vitro* than NK cell cytotoxicity. The relative role of ADCC *in vivo* in man during acute viral infections, as well as that of NK cells induced by interferon and that of cytotoxic T cells, remains to be determined.

VI. Conclusions

There is ample evidence from *in vitro* studies that, in the absence of any cytotoxic effector system, antibody can inhibit virus production and can prevent infected cells from subsequent immunologic attack.

In the presence of complement- or antibody-dependent cytotoxic lymphocytes, antibody can mediate the destruction of virus-infected cells. As with other types of cytotoxicity demonstrated *in vitro*, it is difficult to be certain about the relative importance of these actions *in vivo*. Complement alone, NK cells, cytotoxic T cells, macrophages and interferon are all potentially able to contribute to the control of virus infection *in vivo*, in addition to any effects dependent on antibody.

A. Role of Viral Antigenic Modulation *in Vivo*

There is evidence that antibody-induced viral antigenic modulation occurs *in vivo*. This is well documented for the tumor virus models alluded to in Section III, namely for Gross leukemia virus (Aoki and Johnson, 1972) and FLV (Doig and Cheseboro, 1979), as it is for the TL system (Boyse *et al.*, 1963, 1967). The evidence is the loss of surface viral antigens *in vivo* despite the presence of cytoplasmic viral antigen and the reexpression of surface antigen when leukemic spleen cells from affected animals are transferred to an antibody-free environment. The reason why antigenic modulation, rather than immune lysis, of infected cells should occur is not clear. However, both complement-mediated lysis and ADCC are relatively inefficient in the mouse compared to human systems; for instance, the DBA/2 mice with dormant FLV erythroleukemia [studied by Wheelock *et al.* (1972) and Genovesi *et al.* (1977)] are C5 deficient. In addition, antibody might block T cell cytotoxicity (Welsh and Oldstone, 1977; Effros *et al.*, 1979), or the infection could adversely affect the function of cytotoxic effector cells.

It is not known whether antigenic modulation of virus-infected cells occurs *in vivo* in man. However, one situation with possible relevance to the *in vitro* antigenic modulation of measles-infected cells is the disease subacute sclerosing panencephalitis (SSPE). SSPE is a rare disease characterized by chronic progressive brain damage afflicting children or young adults. Measles virus has been isolated predominantly from the brain and lymphoid tissue by cocultivation techniques, and electron microscopy shows accumulations of measles viral nucleocapsids in brain cells (ter Meulen *et al.*, 1972). Thus, SSPE is a persistent measles virus infection in man, but the reasons for the latent period of several years between typical measles virus infection and the insidious onset of SSPE as well as for the localization in the brain are unknown. Patients with SSPE have high titers of antibody to measles in their sera and cerebrospinal fluids (CSF), but no evidence of impaired antibody and complement killing (Perrin *et al.*, 1976; Joseph *et al.*, 1975) or ADCC (Kreth and ter Meulen, 1977; Per-

rin *et al.*, 1977a) of measles virus-infected target cells. CSF has no detectable hemolytic complement, either in normal subjects or in patients with SSPE. We have postulated that in the presence of antibody, but absence of complement or paucity of cytotoxic lymphocytes in local areas of the central nervous system, antibody could modulate viral antigens expressed on infected cell surfaces. This could prevent cell fusion and death and initiate a state of viral persistence. These conditions would provide selective pressure favoring the emergence of mutant viruses, such as temperature-sensitive or defective interfering virus (Oldstone, 1981). There is experimental evidence suggesting that antibody can be associated with modification of measles infection and facilitation of a persistent infection. Albrecht *et al.* (1977) found that an SSPE isolate produced a chronic encephalitis in animals with preexisting antibody to measles, compared to an acute fatal disease in animals without antibody. Similarly, Wear and Rapp (1971) found that maternal antibody was required to produce latent brain infection upon inoculation of newborn suckling hamsters with an adapted strain of human measles virus.

HSV is commonly maintained in a latent state within nerve ganglia in the central nervous system. It has been suggested that antibody to HSV could maintain HSV latency in infected ganglia, as evidenced by passively transferred antibody preventing reactivation in latently infected ganglia implanted into nonimmune mice (Stevens and Cook, 1974). This has been interpreted as a possible example of antibody-induced antigenic modulation (Stevens and Cook, 1974; Joseph and Oldstone, 1975). Others have suggested that an additional mechanism may maintain latency, because in their experiments reactivation of HSV occurred despite high titers of HSV antibody both *in vitro* and in immunosuppressed mice (Openshaw *et al.*, 1979).

B. Role of Antibody-Dependent Killing of Virus-Infected Cells *in Vivo*

In murine models of virus infection, there are a number of reports of protection from acute virus infection by passive transfer of antibody (reviewed in part by Allison, 1974), although the protection is not invariable. However, the protective effect of antibody could result from neutralization of free virus rather than any cytotoxic effect on infected cells. Hicks *et al.* (1978) showed that decomplementation with cobra venom factor increased the mortality and severity of pneumonia from influenza in mice (disease was also more severe in C5-deficient animals) despite serum neutralizing antibody responses equivalent to controls. The mechanism, whether by direct lysis of infected cells or

chemotaxis of inflammatory cells, or an effect on free infectious virus, is unknown.

There is evidence that tissue injury at sites of virus persistence in chronic LCMV infection in mice or Aleutian disease of mink can be mediated by passively transferred antibody (Oldstone and Dixon, 1970; Porter *et al.*, 1972), and that decomplementation with cobra venom factor protects from death adult mice acutely infected with LCMV (Oldstone and Dixon, 1971). IgG and C3 have been demonstrated by immunofluorescence on neuronal cells infected with measles, in perivascular areas of the brains of patients with SSPE (Vandvik, 1973; Jenis *et al.*, 1973). This type of evidence, suggesting that antibody and complement can induce tissue damage by reacting with virus-infected cells, provides the strongest evidence for antibody-mediated destruction of virus infected cells *in vivo*.

The evidence provided by human immunodeficiency syndromes suggests that patients with T cell deficiencies are more likely to develop severe infections from common viral agents (e.g., measles virus, herpes virus, vaccinia virus infections). Many of the immunodeficiencies characterized by T cell defects are also accompanied by deficiency in antibody production, and many viral antigens are T dependent. Hence, antibody deficiency cannot be totally excluded from contributing to the severity of virus infections in these circumstances. Patients with hypogammaglobulinemia generally recover normally from virus infections, although whether their small amount of IgG effectively mediates ADCC is not known. Patients with deficiencies of C3 itself, or of C5-8, do not seem to be unduly predisposed to virus infections, although C3-deficient patients are prone to recurrent pyogenic bacterial infections (Lachmann and Rosen, 1979). Nevertheless, passively administered antibody can clearly exert a protective or therapeutic effect in a number of human virus infections. This applies also to immunodeficient patients who contract vaccinia virus or varicella-zoster virus infections (Pahwa *et al.*, 1979).

C. Conclusion

In this review we have documented, particularly by reference to work in human systems, that antibody exerts a number of significant effects on virus-infected cells. IgG antibody can mediate the destruction of virus-infected cells in conjunction with complement or cytotoxic lymphocytes. In addition, at a conceptual level there is evidence to suggest that antibody may enhance and confer specificity on basic nonspecific humoral and cell-mediated defense mechanisms. Thus, virus-infected cells can activate the alternative complement pathway

independent of IgG, but antibody is required for subsequent lysis of the cell. Similarly, NK cells can lyse virus-infected target cells independent of antibody and without immunologic specificity, but antibody enhances, and confers specificity on, cytotoxicity by what is probably the same class of effector cells. The importance of these reactions *in vivo*, particularly in comparison with cytotoxic T cells, remains to be uncovered, as does the possibility that antibody can block cytotoxic T cell function *in vivo* as it can *in vitro* (Welsh and Oldstone, 1977; Effros *et al.*, 1979; Zinkernagel and Doherty, 1979). Because there is limited information on the relative importance of these cytotoxic mechanisms in acute virus infections in humans, future collection and analysis of data in this area are necessary.

Finally, the ability of antibody to affect the synthesis and intracellular and surface expression of viral proteins has only recently been investigated on a molecular basis. There is already evidence that antibody can act at this level, and such mechanisms may play an important role in the establishment and maintenance of viral persistence.

ACKNOWLEDGMENTS

This is publication No. 1930 from the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California. This research was supported by U.S. Public Health Service grants NS-12428, AI-09484, AI-07007 and National Foundation March of Dimes Grant 1-364. J. G. P. S. is the recipient of U.S.P.H.S. International Research Fellowship No. 1 F05 TW-2518.

Parts of the work described were done in collaboration with many colleagues at the Research Institute of Scripps Clinic. The collaboration of Neil Cooper and Robert Schreiber in the work described in Section IV, the contributions of Luc Perrin, Peter Lampert, Barry Joseph, and Toni Tishon, and permission of Robert Fujinami to quote unpublished work are specially acknowledged. The authors thank Ms. Booz and Ms. Taxel for preparation of the manuscript.

REFERENCES

Ada, G. L., Jackson, D. C., Blanden, R. V., Tha Hla, R., and Bowern, N. A. (1976). *Scand. J. Immunol.* **5,** 23.

Adler, R., Glorioso, J. C., Cossman, J., and Levine, M. (1978). *Infect. Immun.* **21,** 442.

Ahmed, A., Strong, D. M., Sell, K. W., Thurman, G. B., Knudsen, R. C., Wistar, R., and Grace, W. R. (1974). *J. Exp. Med.* **139,** 902.

Albrecht, P., Burnstein, T., Klutch, M. J., Hicks, J. T., and Ennis, F. A. (1977). *Science* **195,** 64.

Allison, A. C. (1974). *Transplant. Rev.* **19,** 3.

Andersson, T., Stejskal, V., and Härfast, B. (1975). *J. Immunol.* **114,** 237.

Aoki, T., and Johnson, P. A. (1972). *J. Natl. Cancer Inst.* **49,** 183.

Ash, J. F., Louvard, D., and Singer, S. J. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 5584.

Bartholomew, R. M., Esser, A. F., and Müller-Eberhard, H. J. (1978). *J. Exp. Med.* **147,** 844.

Becht, H., Hämmerling, U., and Rott, R. (1971). *Virology* **46,** 337.

Biesecker, G., Podack, E. R., Halverson, C. A., and Müller-Eberhard, H. J. (1979). *J. Exp. Med.* **149,** 448.
Blobel, G., and Dobberstein, B. (1975). *J. Cell. Biol.* **67,** 835.
Boulan, E. R., and Sabatini, D. B. (1978). *Proc. Natl. Acad. Sci. U.S.A.***75,** 5071.
Bourgignon, L. Y. W., and Singer, S. J. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 5031.
Bourgignon, L. Y. W., Hyman, R., Trowbridge, I., and Singer, S. J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2406.
Boyse, E. A., Old, L. J., and Luell, S. (1963). *J. Natl. Cancer Inst.* **31,** 987.
Boyse, E. A., Stockert, E., and Old, L. J. (1967). *Proc. Natl. Acad. Sci. U.S.A.* **58,** 954.
Brier, A. M., Wohlenberg, C., Rosenthal, J., Mage, M., and Notkins, A. L. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 3073.
Bubbers, J. E., and Lilly, F. (1977). *Nature (London)* **266,** 458.
Budzko, D. B., Lachmann, P. J., and McConnell, I. (1976). *Cell. Immunol.* **22,** 98.
Burns, W. H., and Allison, A. C. (1975). *In* "The Antigens" (M. Sela, ed.), Vol. 3, pp. 480–559. Academic Press, New York.
Burns, W. H., Billups, L. C., and Notkins, A. L. (1975). *Nature (London)* **256,** 654.
Calafat, J., Hilgers, J., Van Blitterswijk, W. J., Verbeet, M., and Hageman, P. C. (1976). *J. Natl. Cancer Inst.* **56,** 1019.
Catt, K. J., Harwood, J. P., Aguilera, G., and Dufau, M. L. (1979). *Nature (London)* **280,** 109.
Celis, E., Chang, T. W., and Eisen, H. N. (1979). *J. Immunol.* **122,** 2245.
Chess, L., and Schlossman, S. F. (1977). *Adv. Immunol.* **25,** 213.
Choppin, P. W., and Compans, R. W. (1975). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. Wagner, eds.), Vol. 4, p. 95. Plenum, New York.
Cikes, M., and Friberg, S. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 566.
Compans, R. W., and Kemp, M. C. (1978). *Curr. Top. Membr. Transp.* **11,** 233.
Cooper, N. R. (1979). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 123–170. Plenum, New York.
Cooper, N. R., and Welsh, R. M. (1979). *In* "Springer Seminars in Immunopathology" (P. A. Miescher and H. J. Müller-Eberhard, eds.), Vol. 2, pp. 285–310. Springer-Verlag, Berlin and New York.
Cooper, N. R., Polley, M. J., and Oldstone, M. B. A. (1974). *J. Immunol.* **112,** 866.
Cooper, N. R., Jensen, F. C., Welsh, R. M., and Oldstone, M. B. A. (1976). *J. Exp. Med.* **144,** 970.
Costa, J., Rabson, A. S., Yee, C., and Tralka, T. S. (1977). *Nature (London)* **269,** 251.
Daniels, C. A. (1975). *In* "Viral Immunology and Immunopathology" (A. L. Notkins, ed.), p. 79. Academic Press, New York.
Doig, D., and Cheseboro, B. (1979). *J. Exp. Med.* **150,** 10.
Dowdle, W. R., Downie, J. C., and Laver, W. G. (1974). *J. Virol.* **13,** 269.
Eaton, M. D., and Scala, A. R. (1970). *Proc. Soc. Exp. Biol. Med.* **133,** 615.
Effros, R. B., Frankel, M. E., Gerhard, W., and Doherty, P. C. (1979). *J. Immunol.* **123,** 1343.
Ehrnst, A. (1975). *J. Immunol.* **114,** 1077.
Ehrnst, A. (1977). *J. Immunol.* **118,** 533.
Ehrnst, A. (1978). *J. Immunol.* **121,** 1206.
Ehrnst, A., and Sundquist, K. (1975). *Cell* **5,** 351.
Ewan, P., and Lachmann, P. J. (1977). *Clin. Exp. Immunol.* **30,** 22.
Fearon, D. T., and Austen, K. F. (1975). *J. Exp. Med.* **142,** 856.
Fearon, D. T., and Austen, K. F. (1977a). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 1683.
Fearon, D. T., and Austen, K. F. (1977b). *J. Exp. Med.* **146,** 22.

Ferrone, S., Cooper, N. R., Pellegrino, M. A., and Reisfeld, R. A. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 3665.
Ferrone, S., Cooper, N. R., Pellegrino, M. A., and Reisfeld, R. A. (1974). *Transplant. Proc.* **6,** 13.
Finberg, R., Mescher, M., and Burakoff, S. J. (1978). *J. Exp. Med.* **148,** 1620.
Fox, R. L., and Weissman, I. L. (1979). *J. Immunol.* **122,** 1697.
Fujinami, R. S., and Oldstone, M. B. A. (1979). *Nature (London)* **279,** 529.
Fujinami, R. S., and Oldstone, M. B. A. (1980). *J. Immunol.* **127,** (in press).
Fujinami, R. S., Sissons, J. G. P., and Oldstone, M. B. A. (1980). *J. Virol.* (submitted for publication).
Genovesi, E. V., Marx, P. A., and Wheelock, E. F. (1977). *J. Exp. Med.* **146,** 520.
Gould, J. J., and Almeida, J. D. (1977). *J. Med. Virol.* **1,** 111.
Greenberg, S. B., Criswell, B. S., Six, H. R., and Couch, R. B. (1977). *J. Immunol.* **119,** 2100.
Greenberg, S. B., Six, H. R., Drake, S., and Couch, R. B. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4622.
Griffin, J. A., and Compans, R. W. (1979). *J. Exp. Med.* **150,** 379.
Härfast, B., Andersson, T., Stejskal, V., and Perlmann, P. (1977). *J. Immunol.* **118,** 1132.
Härfast, B., Andersson, T., and Perlmann, P. (1978). *J. Immunol.* **121,** 755.
Haspel, M. V., Pellegrino, M. A., Lampert, P. W., and Oldstone, M. B. A. (1977). *J. Exp. Med.* **146,** 146.
Herberman, R. B., Djeu, J. Y., Kay, H. D., Ortaldo, J. R., Riccardi, C., Bonnard, G. D., Holden, H. T., Fagnani, R., Santoni, A., and Puccetti, P. (1979). *Immunol. Rev.* **44,** 43.
Hicks, J. T., Klutch, M. J., Albrecht, P., and Frank, M. M. (1976). *J. Immunol.* **117,** 208.
Hicks, J. T., Ennis, F. A., Kim, E., and Verbonitz, M. (1978). *J. Immunol.* **121,** 1437.
Holland, J. E., and Kiehn, E. D. (1970). *Science* **167,** 202.
Holmes, K. V., Klenk, H. D., and Choppin, P. (1969). *Proc. Soc. Exp. Biol. Med.* **131,** 651.
Hooghe-Peters, E. L., Rentier, B., and Dubois-Dalcq, M. (1979). *J. Virol.* **29,** 666.
Horwitz, D. A., Knight, N., Temple, A., and Allison, A. C. (1979). *Immunology* **36,** 221.
Hubbard, A. L., and Cohn, Z. A. (1976). *J. Cell. Biol.* **64,** 461.
Huddlestone, J. H., Merigan, T. C., and Oldstone, M. B. A. (1979). *Nature (London)* **282,** 487.
Imblum, R. L., and Wagner, R. R. (1975). *J. Virol.* **15,** 1357.
Ioachim, H. L., and Sabbath, M. (1979). *J. Natl. Cancer Inst.* **62,** 169.
Jenis, E. H., Knieser, M. R., Rothouse, P. A., Jensen, G. E., and Scott, R. M. (1973). *Arch. Pathol.* **95,** 81.
Jondal, M. (1976). *Clin. Exp. Immunol.* **25,** 1.
Joseph, B. S., and Oldstone, M. B. A. (1974). *J. Immunol.* **113,** 1205.
Joseph, B. S., and Oldstone, M. B. A. (1975). *J. Exp. Med.* **142,** 864.
Joseph, B. S., Cooper, N. R., and Oldstone, M. B. A. (1975). *J. Exp. Med.* **141,** 761.
Kay, H. D., Bonnard, G. D., and Herberman, R. B. (1978). *J. Immunol.* **122,** 675.
Kibler, R., and ter Meulen, V. (1975). *J. Immunol.* **114,** 93.
Kiessling, R., and Wigzell, H. (1979). *Immunol. Rev.* **44,** 165.
Kohl, S., Starr, S. E., Oleske, J. M., Shore, S. L., Ashman, R. B., and Nahmias, A. J. (1977). *J. Immunol.* **118,** 729.
Kreth, H. W., and ter Meulen, V. (1977). *J. Immunol.* **118,** 291.
Krettli, A., and Nussenzweig, R. V. (1977). *In* "Symposium on Chagas Disease." Pan-American Health Organization, New York.

Lachmann, P. J., and Rosen, F. J. (1979). *In* "Springer Seminars in Immunopathology" (P. Miescher and H. J. Müller-Eberhard, eds.), Vol. 1, No. 4. Springer-Verlag, Berlin and New York.

Lampert, P. W., Joseph, B. S., and Oldstone, M. B. A. (1975). *J. Virol.* **15,** 1248.

Lenard, J., and Compans, R. W. (1974). *Biochim. Biophys. Acta.* **344,** 51.

Lerner, R., Oldstone, M. B. A., and Cooper, N. R. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 2584.

Lovchik, J. C., and Hong, R. (1977). *Prog. Allerg.* **22,** 1.

McConnell, I., and Lachmann, P. J. (1976). *Transplant. Rev.* **32,** 72.

McConnell, I., Klein, G., Lint, T. F., and Lachmann, P. J. (1978). *Eur. J. Immunol.* **8,** 453.

McLennan, I. C. M. (1972). *Transplant. Rev.* **13,** 67.

McTaggart, S. P., Burns, W. H., White, D. O., and Jackson, D. C. (1978). *J. Immunol.* **121,** 726.

May, J. E., and Frank, M. M. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 649.

Medicus, R. G., Schreiber, R. D., Götze, O., and Müller-Eberhard, H. J. (1976). *J. Exp. Med.* **144,** 1076.

Miller, T., and Stone, H. (1979). Personal communication.

Minagawa, T., and Yamada, M. (1971). *Jpn. J. Microbiol.* **15,** 341.

Möller-Larssen, A., Heron, I., and Haahr, S. (1977). *Infect. Immun.* **16,** 43.

Moretta, L., Webb, S. R., Grossi, C. E., Lydyard, P., and Cooper, M. D. (1977). *J. Exp. Med.* **146,** 184.

Mountcastle, W. E., and Choppin, P. W. (1977). *Virology* **78,** 463.

Müller-Eberhard, H. J. (1975). *Annu. Rev. Biochem.* **44,** 697.

Nelson, B., and Ruddy, S. (1979). *J. Immunol.* **122,** 1994.

Norrby, E., and Pentinnen, K. (1978). *J. Infect. Dis.* **138,** 672.

Norrby, E., Enders-Ruckle, G., and ter Meulen, V. (1975). *J. Infect. Dis.* **132,** 262.

Ogra, P. L., Karzon, D. T., Righthand, F., and MacGillivray, M. (1968). *New Eng. J. Med.* **279,** 893.

Ogra, P. L., Morag, A., and Tiku, M. L. (1975). *In* "Viral Immunology and Immunopathology" (A. L. Notkins, ed.), p. 57. Academic Press, New York.

Okada, H., and Baba, T. (1974). *Nature* (London) **248,** 521.

Okada, H., Okada, N., and Shibata, H. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 1217 (Abstr.).

Oldstone, M. B. A. (1975). *In* "Progress in Medical Virology" (J. L. Melnick, ed.). Vol. 19, pp. 84–119. Karger, Basel.

Oldstone, M. B. A. (1979) *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), Vol. 15, pp. 1–36. Plenum, New York.

Oldstone, M. B. A. (1981). *In* "Clinical Aspects of Immunology" (P. Lachmann and D. K. Peters, eds.), 4th ed. Blackwell, Oxford. In press.

Oldstone, M. B.A., and Dixon, F. J. (1970). *J. Exp. Med.* **131,** 1.

Oldstone, M. B. A., and Dixon, F. J.(1971). *J. Immunol.* **107,** 1274.

Oldstone, M. B. A., Fujinami, R. S., and Lampert, P. W. (1980). *In* "Progress in Medical Virology" (J. L. Melnick, ed.), Vol. 26. Karger, Basel. In press.

Oldstone, M. B. A., and Lampert, P. W. (1979). *In* "Springer Seminars in Immunopathology" (P. A. Miescher and H. J. Müller-Eberhard, eds.), Vol. 2, pp. 261–283. Springer-Verlag, Berlin and New York.

Oldstone, M. B. A., and Tishon, A. (1978). *Clin. Immunol. Immunopathol.* **9,** 55.

Openshaw, H., Asher, L. V. S., Wohlenberg, C., Sekizawa, T., and Notkins, A. L. (1979). *J. Gen. Virol.* **44,** 205.

Pahwa, R., Pahwa, S., O'Reilly, R., and Good, R. A. (1979). *In* "Springer Seminars in Immunopathology" (P. Miescher and H. J. Müller-Eberhard, eds.), Vol. 1, No. 4. Springer-Verlag, Berlin and New York.

Pangburn, M. K., and Müller-Eberhard, H. J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2416.

Pangburn, M. K., Morrison, D. C., Schreiber, R. D., and Müller-Eberhard, H. J. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 1417 (Abstr.).

Pape, G. R., Troye, M., Axelsson, B., and Perlmann, P. (1979). *J. Immunol.* **122,** 2251.

Perlmann, P., and Cerottini, J. C. (1979). *In* "The Antigens" (M. Sela, ed.), Vol. 5, pp. 173–281. Academic Press, New York.

Perlmann, P., Perlmann, H., and Wigzell, H. (1972). *Transplant. Rev.* **13,** 91.

Perrin, L. H., and Oldstone, M. B. A. (1977). *J. Immunol.* **118,** 316.

Perrin, L. H., Joseph, B. S., Cooper, N. R., and Oldstone, M. B. A. (1976). *J. Exp. Med.* **143,** 1027.

Perrin, L. H., Tishon, A., and Oldstone, M. B. A. (1977a). *J. Immunol.* **118,** 282.

Perrin, L. H., Zinkernagel, R. M., and Oldstone, M. B. A. (1977b). *J. Exp. Med.* **146,** 949.

Perrin, L. H., Reynolds, D., Zinkernagel, R., and Oldstone, M. B. A. (1978). *Med. Microbiol. Immunol.* **166,** 71.

Podack, E. R., and Müller-Eberhard, H. J. (1980). *Adv. Immunol.* (in press).

Polhill, R. B., Newman, S. L., Pruitt, K. M., and Johnston, R. B. (1978). *J. Immunol.* **121,** 371.

Porter, D. D., Larsen, A. E., and Porter, H. G. (1972). *J. Immunol.* **109,** 1.

Rager-Zisman, B., and Bloom, B. R. (1974). *Nature (London)* **251,** 542.

Rager-Zisman, B., Grose, C., and Bloom, B. R. (1976). *Nature (London)* **260,** 369.

Rahman, A. A., Teschner, M., Sethi, K. K., and Brandis, H. (1976). *J. Immunol.* **117,** 253.

Rawls, W., and Tompkins, W. (1975). *In* "Viral Immunology and Immunopathology" (A. L. Notkins, ed.), p. 99. Academic Press, New York.

Roane, P. R., and Roizman, B. (1964). *Virology* **22,** 1.

Roizman, B., and Furlong, D. (1974). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), Vol. 3, pp. 229–403. Plenum, New York.

Rothman, J. E., and Lodish, H. F. (1977). *Nature (London)* **269,** 775.

Rouse, B. T., Wardley, R. C., and Babiuk, L. A. (1976). *Infect. Immun.* **13,** 1433.

Rouse, B. T., Grewal, A. J., and Babiuk, L. A. (1977). *Nature (London)* **266,** 456.

Rustigian, R. (1966). *J. Bacteriol.* **92,** 1805.

Rutter, G., and Mannweiler, K. (1976). *J. Gen. Virol.* **33,** 321.

Saksela, E., Timanen, T., Ranki, A., and Häyri, P. (1979). *Immunol. Rev.* **44,** 71.

Santoli, D., and Koprowski, H. (1979). *Immunol. Rev.* **44,** 125.

Santoli, D., Trinchieri, G., and Koprowski, H. (1978). *J. Immunol.* **121,** 532.

Schlager, S. I., Ohanian, S. H., and Borsos, T. (1978). *J. Immunol.* **120,** 1644.

Schrader, J. W., Cunningham, B. A., and Edelman, G. M. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 5066.

Schreiber, R. D., and Müller-Eberhard, H. J. (1978). *J. Exp. Med.* **148,** 1722.

Schreiber, R. D., Pangburn, M. K., Lesavre, P. H., and Müller-Eberhard, H. J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3948.

Schreiber, R. D., Morrison, D. C., Podack, E. R., and Müller-Eberhard, H. J. (1979). *J. Exp. Med.* **149,** 870.

Schreiner, G. F., and Unanue, E. R. (1976). *Adv. Immunol.* **24,** 37.

Scott, R., DeLandazuri, M. O., Gardner, P. S., and Owen, J. J. T. (1977). *Clin. Exp. Immunol.* **28,** 19.

Shore, S. L., Nahmias, A. J., Starr, S. E., Wood, P. E., and McFarlin, D. E. (1974). *Nature (London)* **251,** 350.
Shore, S. L., Black, C. M., Melewicz, F. M., Wood, R. A., and Nahmias, A. J. (1976). *J. Immunol.* **116,** 194.
Shore, S. L., Melewicz, F. M., and Gordon, D. S. (1978). *J. Immunol.* **118,** 558.
Singh, S. B., Smith, J. W., Rawls, W. E., and Tevethia, S. S. (1972). *Infect. Immun.* **5,** 352.
Sissons, J. G. P., Schreiber, R. D., Perrin, L. H., Cooper, N. R., Müller-Eberhard, H. J., and Oldstone, M. B. A. (1979a). *J. Exp. Med.* **150,** 455.
Sissons, J. G. P., Cooper, N. R., and Oldstone, M. B. A. (1979b). *J. Immunol.* **123,** 2144.
Sissons, J. G. P., Oldstone, M. B. A., and Schreiber, R. D. (1980). *Proc. Natl. Acad. Sci. U. S. A.* **77,** 559.
Smith, J. W., Adam, E., Melnick, J. L., and Rawls, W. E. (1972). *J. Immunol.* **109,** 554.
Stackpole, C., and Jacobson, J. (1978). *In* "The Handbook of Cancer Immunology" (H. Waters, ed.), pp. 55–159. Garland STPM Press, New York.
Stackpole, C. W., Jacobson, J. B., and Galuska, S. (1978). *J. Immunol.* **120,** 188.
Stevens, J. G., and Cook, M. L. (1974). *J. Immunol.* **113,** 1685.
Svedmyr, E., and Jondal, M. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1622.
Svehag, S. E., and Mandel, B. (1964). *J. Exp. Med.* **119,** 21.
ter Meulen, V., Katz, M., and Muller, D. (1972). *Curr. Top. Microbiol.* **57,** 1.
Theofilopoulos, A. N., and Perrin, L. H. (1976). *J. Exp. Med.* **143,** 271.
Theofilopoulos, A. N., and Perrin, L. H. (1977). *Science* **195,** 878.
Trinchieri, G., and Santoli, D. (1978). *J. Exp. Med.* **147,** 1314.
Trinchieri, G., Santoli, D., and Koprowski, H. (1978). *J. Immunol.* **120,** 1849.
Tyrell, D. L. J., and Ehrnst, A. (1979). *J. Cell. Biol.* **81,** 396.
Unanue, E. R., Perkins, W. D., and Karnovsky, M. J. (1972). *J. Exp. Med.* **136,** 885.
Vandvik, B. (1973). *Ann. Clin. Res.* **5,** 308.
Virelizier, J. L., Allison, A. C., and Schild, G. C. (1979). *Br. Med. Bull.* **35,** 65.
Wahren, B. (1963). *J. Natl. Cancer Inst.* **31,** 411.
Watkins, J. F. (1964). *Nature (London)* **202,** 1364.
Watts, T. S., and Gooding, L. R. (1980). *Nature (London)* **283,** 74.
Wear, D. J., and Rapp, F. (1971). *J. Immunol.* **107,** 1593.
Wedgewood, R. J., Ginsberg, H. S., and Pillemer, L. (1956). *J. Exp. Med.* **104,** 107.
Welsh, R. M. (1977). *J. Immunol.* **118,** 348.
Welsh, R. M. (1978). *J. Immunol.* **121,** 1631.
Welsh, R. M., and Oldstone, M. B. A. (1977). *J. Exp. Med.* **145,** 1449.
Welsh, R. M., Cooper, N. R., Jensen, F. C., and Oldstone, M. B. A. (1975). *Nature (London)* **257,** 612.
Westmoreland, D., and Watkins, J. F. (1974). *J. Gen. Virol.* **24,** 167.
Westmoreland, D., St. Jeor, S., and Rapp, F. (1976). *J. Immunol.* **116,** 1566.
Wheelock, E. F., Toy, S. T., Caroline, N. L., Sibal, L. R., Fink, M. A., Beverly, P. L. L., and Allison, A. C. (1972). *J. Natl. Cancer Inst.* **48,** 665.
Wiktor, T. J., Kuwert, D., and Koprowski, H. (1968). *J. Immunol.* **101,** 1271.
Wrigley, N. G., Laver, W. G., and Downie, J. C. (1977). *J. Mol. Biol.* **109,** 405.
Yagi, M. J., Blair, P. B., and Lane, M. A. (1978). *J. Virol.* **28,** 611.
Yu, A., and Cohen, E. P. (1974). *J. Immunol.* **112,** 1296.
Ziegler, H. K., and Henney, C. (1975). *J. Immunol.* **115,** 1500.
Zinkernagel, R. M., and Doherty, P. C. (1979). *Adv. Immunol.* **27,** 52.

ADVANCES IN IMMUNOLOGY, VOL. 29

Aleutian Disease of Mink[1]

DAVID D. PORTER, AUSTIN E. LARSEN, AND HELEN G. PORTER

Department of Pathology, UCLA School of Medicine, Los Angeles, California and Department of Cellular, Viral, and Molecular Biology, University of Utah College of Medicine, Salt Lake City, Utah

I. Introduction 261
II. Biology and Immunobiology of Mink 262
III. Aleutian Disease Virus 263
A. *In Vivo* Growth 263
B. *In Vitro* Growth 265
C. Properties 266
D. Infection and Host Range 266
IV. The Host Immune Response to Infection 267
A. Hypergammaglobulinemia 267
B. Specific Immune Responses 270
C. Immune Complexes 272
D. Unrelated Immune Responses 272
V. The Pathogenesis of Lesions 273
A. General Considerations and Genetic Effects 273
B. Glomerulonephritis and Arteritis 275
C. Manipulation of Lesion Production 278
D. Other Lesions 280
VI. Disease Control 281
VII. Discussion 282
References 284

I. Introduction

Aleutian disease (AD) is a common and economically important chronic or persistent virus infection of mink. The disease was first recognized in ranch-raised mink of the Aleutian genotype (Hartsough and Gorham, 1956), but it was subsequently found that all genetic types of mink could develop AD (Porter and Larsen, 1964). The immunologic and immunopathologic consequences are the most severe known in any persistent viral infection. The responsible virus (ADV) is a parvovirus that is naturally temperature-sensitive in its replication when isolated from affected mink and appears to replicate in macrophages *in vivo*. Although the virus replicates relatively rapidly *in vivo*, no lesions attributable to the infection are noted for about a month, by which time there is an antiviral antibody response of con-

[1] The authors' work was supported by grant AI-09476 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by the Mink Farmers' Research Foundation.

ISBN 0-12-022429-1

siderable magnitude and hypergammaglobulinemia develops. The γ-globulin may reach levels as high as 11 gm/100 ml late in the disease, and in some animals the heterogeneity of this increased amount of IgG becomes restricted. Aleutian disease virus circulates as infectious antigen–antibody complexes in persistently infected mink. Smaller complexes are deposited in the glomeruli and arteries and cause severe and frequently fatal inflammatory lesions. Both the host genotype and the viral genotype influence the severity of AD. Whereas immunosuppressive therapy can block the development of lesions, immunization with killed virus vaccine prior to live virus challenge increases the severity of disease.

This review will emphasize the immunologic and immunopathologic aspects of AD.

II. Biology and Immunobiology of Mink

Mink (*Mustela vison*) are members of the order Carnivora and family Mustelidae (Ewer, 1973). Several studies of the immunologic relatedness of the carnivores are available (Sarich, 1969; Seal *et al.*, 1970; Ledoux and Kenyon, 1975). Other generally familiar Mustelidae include the wolverine, marten, fisher, otter, ferret, weasel, and skunk. Because mink are so highly valued for their fur, approximately 2 million mink pelts are produced on commercial ranches in the United States and 10–12 million pelts worldwide per year. The animals mate once a year in the early spring, after which an average of four young are born to impregnated females afer a 50-day gestation period (Bowness, 1968). Most mink are killed for their pelts at 7 months of age; only the 20% used for breeding stock are retained. The reproductive physiology of mink is well described (Hansson, 1947; Shackelford, 1952). Mink on commercial ranches are generally bred twice, and most offspring result from the second mating (Shackelford, 1952; Johansson and Venge, 1951). Brother–sister and other close matings are usually avoided by ranchers, thus mink are genetically heterogeneous except for coat color genes.

Major disease problems of mink include AD, distemper, viral enteritis, and bacterial diarrhea. Other of their less common diseases have been described elsewhere (Padgett *et al.*, 1968). Distemper is readily controlled by an effective live virus vaccine. Viral enteritis, which is caused by a virus either closely related to or identical to feline panleukopenia virus, can be controlled by a killed virus vaccine. Bacterial diarrhea affects infant and young mink, but can be controlled to some degree with antibiotics. With reasonable control of the other

severe infectious diseases of mink achieved, AD has emerged as the principal health problem of commercial mink ranching.

Mink have been highly selected for coat color, and their coat color genetics have been described (Shackelford, 1950). In 1941, mink bred on an Oregon ranch developed a blue-gray coat similar in color to Aleutian blue foxes, so were similarly named. Mink of the Aleutian genotype have a lysosomal abnormality similar to that of humans with the Chediak–Higashi syndrome (Davis *et al.*, 1971). This abnormality is apparently responsible for the diluted coat color of the Aleutian genotype mink, and it is thought that the responsible gene may increase susceptibility of such mink to infectious diseases, including AD (Padgett *et al.*, 1968).

Mink are born with low or undetectable levels of IgG and no IgM or IgA (Porter, 1965; Coe and Race, 1978). Their colostrum contains 33–59% of the maternal serum levels of IgG, and milk 12–38% of the serum level of IgG. The kits achieve serum IgG levels similar to adult levels by 8 days after birth. Although small amounts of IgA were detected in milk, no evidence of transfer by nursing was obtained. The lymphoid tissues of mink were shown to synthesize IgG and IgM at birth, but IgA was not synthesized until 75 days of age. Similar observations have been made in the closely related ferret, *Mustela furo* (Suffin *et al.*, 1979).

Mink serum proteins have an overall similarity to those of other mammals (Porter and Dixon, 1966). Of note, mink have a pregnancy-specific serum protein that is more basic than immunoglobulin (Larsen *et al.*, 1971). Mink IgG appears to have only lambda light chains (Hood *et al.*, 1967), on which at least one antigenic marker is present (Coe, 1972). A basic description of mink immunoglobulins is available (Coe and Hadlow, 1972). Mink make the expected humoral immune response to a range of antigens as is discussed later in relation to AD. The only study of cellular immunity in mink showed that cells respond to nonspecific mitogens (Perryman *et al.*, 1975).

III. Aleutian Disease Virus

A. *In Vivo* Growth

Transmission of AD from mink to mink by means of diseased tissue or cell-free filtrates of such tissue was reported by Henson *et al.* (1962), Karstad and Pridham (1962), and Trautwein and Helmboldt (1962). Infectivity was shown to be readily recoverable from whole blood, serum, urine, feces, saliva, and tissues of mink with typical AD

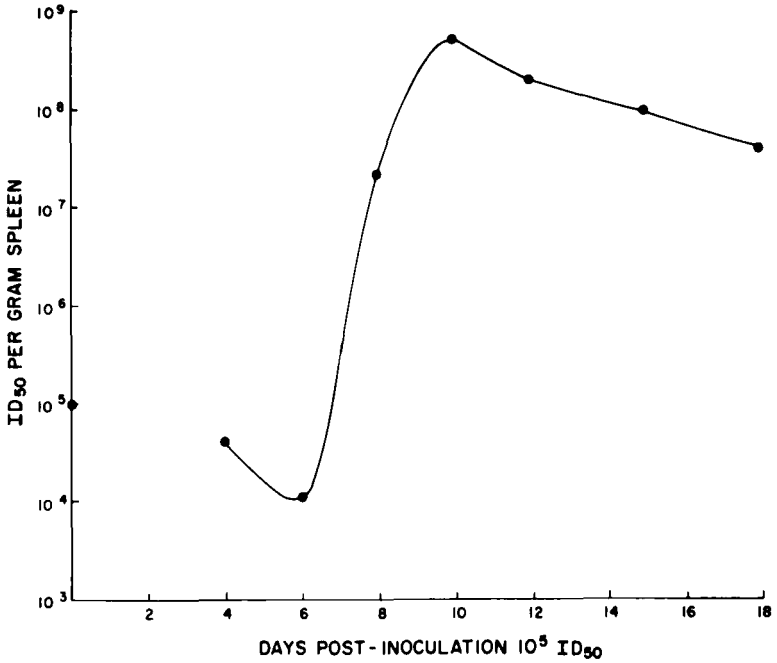


FIG. 1. A growth curve of the Utah-1 strain of Aleutian disease virus in the spleen of mink of the Aleutian genotype is shown. Each point represents the amount of infectivity recovered from a pool of the spleens of two mink. The virus was titered in normal mink. Reprinted from Porter *et al.* (1969) by permission of the *Journal of Experimental Medicine.*

(Kenyon *et al.*, 1963; Gorham *et al.*, 1964; Eklund *et al.*, 1968). When the highly virulent Utah-1 isolate of ADV was used to infect mink, maximal viral titers of nearly 10^9 ID_{50} per gram of spleen were recovered 10 days after infection (Fig. 1). Similar titers were obtained also from the liver and lymph nodes of mink infected for 10 days. Immunofluorescence studies showed ADV antigen in macrophages of spleens and lymph nodes (Fig. 2) and in Kupffer cells of the liver (Porter *et al.*, 1969). After initial observations that the antigen appeared in cytoplasm, nuclear ADV antigen also was found (D. D. Porter, unpublished observations). Virus-like particles with a diameter of 20–22 nm have been seen by electron microscopy in macrophages and Kupffer cells, mainly in cytoplasmic vacuoles but occasionally in nuclei. Areas containing the particles reacted with ADV antibody conjugated with ferritin (Shahrabadi and Cho, 1977). Peak viral titers were observed 10 days after infection then slowly fell so that 2 or more months after infection spleen titers of 10^5 ID_{50}/gm and serum titers of 10^4 ID_{50}/ml were common.

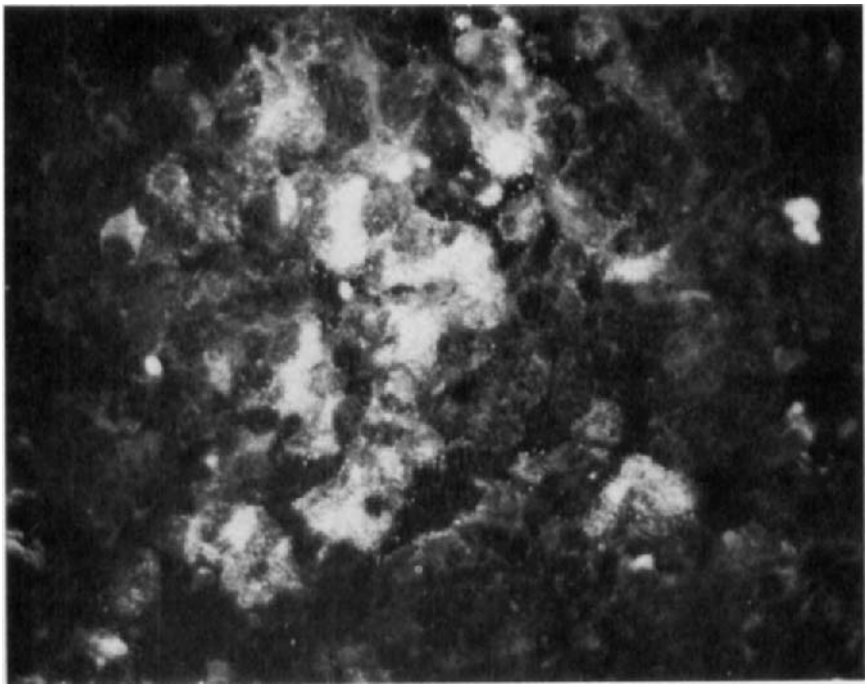


FIG. 2. The medullary area of a lymph node of an Aleutian mink infected with Aleutian disease virus (ADV) 10 days previously is shown stained with fluorescein-labeled mink ADV antibody. A group of macrophages shows powdery cytoplasmic localization of ADV antigen. Nuclear antigen, which is also present, is not apparent in the photograph. Fluorescence micrograph. ×525.

B. *In Vitro* GROWTH

The Utah-1 strain of ADV produced viral antigen and infectious virus in a continuous line of feline renal cells or in primary feline renal cells (Porter *et al.*, 1977a), but only at a temperature of 31.8°C, not at 37°C or 39°C (the body temperature of mink is 39°C). After serial passage in culture, the viruses' optimum temperature for growth became 37°C. The cultured virus had equal infectivity for cell cultures and for mink, as quantitated by an immunofluorescence focus assay used because ADV does not produce plaques in culture. In common with other parvoviruses, ADV requires rapidly dividing cells for replication and is markedly cell associated. The viral yield has been relatively low, with an average of 10^5 to 10^6 focus-forming units/ml. We suspect that defective-interfering particles are at least partially responsible for the low yields, since titers vary in a cyclic fashion. Although viral antigen is produced in mink cells and in some cells from other species, little or no infectious virus has been produced.

Persistently infected feline cell cultures are easily developed when the Utah-1 strain of ADV is used and the cultures are passaged at 31.8°C. However, the cultures permanently lose the virus after one or two passages at 37°C. Less virulent ADV strains, such as the Pullman isolate, are difficult to passage serially at 31.8°C but are successfully passaged at 28°C (unpublished observations). The Connecticut strain of ADV produces viral antigen but not infectious virus in feline cells (Hahn *et al.*, 1977).

C. Properties

ADV purified from infected mink tissue or cell cultures and negatively stained is an icosahedral structure with a diameter of 23–25 nm. The virion probably has 32 capsomers, which are hollow tubes 4.5 nm in diameter with a 1–2 nm central hole (Cho and Ingram, 1973a; Chesebro *et al.*, 1975; Porter *et al.*, 1977a). In common with other parvoviruses (reviewed by Siegl, 1976), ADV is resistant to 56°C, lipid solvents, and other chemicals. These properties have been tabulated and discussed in detail (Porter and Cho, 1980). Most of the infectivity of purified ADV bands in CsCl at a density of 1.405 to 1.43 (Porter *et al.*, 1977a; Cho, 1977). Lighter particles, which are apparent in the virus purified from infected mink organs, have a much lower specific infectivity and may represent defective interfering virus. ADV was shown to contain single-stranded DNA of three sizes, 1.2, 0.5, and 0.23 $\times$ 10^6 daltons, but it is not known whether each size class of DNA is present in all the particles. Electrophoresis of disrupted ADV on SDS–polyacrylamide gels showed four polypeptides with molecular weights of 30,000, 27,000, 20,500, and 14,000 in a ratio of 10:3:10:1. It seems possible that the 27,000 polypeptide is a dimer of the 14,000 polypeptide (Shahrabadi *et al.*, 1977). Most of the properties of ADV are similar to those of parvoviruses. However, the molecular weights of the polypeptides are only a third those of other parvoviruses, and it may be that the purification fragmented the virions' polypeptides. No immunologic relationship of ADV to 13 other parvoviruses could be shown (Porter *et al.*, 1977a).

D. Infection and Host Range

It has been shown that both horizontal and vertical infection of mink by ADV occur on commercial mink ranches (Gorham *et al.*, 1964, 1976; Padgett *et al.*, 1967). There is also experimental evidence that ADV can be transmitted by mosquitoes (Shen *et al.*, 1973), but this does not appear to be an important route of infection. Since the virus is present in urine, saliva, and feces (Kenyon *et al.*, 1963; Gor-

ham *et al.*, 1964), it is reasonable that AD can be transmitted to normal mink by biting, excreta, or contaminated handling gloves (Larsen, 1969). However, natural horizontal transmission probably occurs more often via the respiratory route than from ingestion; for example, aerosol exposure of mink to ADV regularly produces infection, but feeding them infected tissue does not always cause infection (Gorham *et al.*, 1965; unpublished data). Vertical transmission of ADV probably plays an important role in perpetuating AD in both ranch and feral mink, particularly since feral mink are quite territorial and do not have close or continuing contact with other mink after weaning (Gerell, 1970). The transplacental infection is much milder than is horizontal infection (Porter *et al.*, 1977b). In fact, asymptomatic and even nonpersistent infections by ADV are common in wild mink and strains other than the Aleutian variety (Larsen and Porter, 1975; An and Ingram, 1977, 1978: An *et al.*, 1978; Cho and Greenfield, 1978).

Although the host range of ADV has not been studied extensively, feral skunks, raccoons, and foxes may have ADV antibody (Ingram and Cho, 1974). Kenyon *et al.* (1978) inoculated ADV into 14 species of Mustelidae and found ADV antibody in the mink, ferret, weasel, fisher, marten, and striped skunk. Definite lesions of AD were observed in mink and ferrets, and possible lesions were found in striped skunks and martens. We have found (unpublished observations) a 40% incidence of ADV antibody in commercially obtained ferrets, and some of the antibody-positive ferrets have moderate hyperglobulinemia. Others have noted both disease and hyperglobulinemia in ferrets (Kenyon *et al.*, 1966, 1967; Ohshima *et al.*, 1978). Furthermore, the mink-virulent Utah-1 strain of ADV produces antibody in ferrets, although frank disease is uncommon. Finally, ferret ADV isolates may be serially passaged in this species.

IV. The Host Immune Response to Infection

A. Hypergammaglobulinemia

There is enormous expansion of the B lymphoid cell system during AD, and lymphocytes and plasma cells infiltrate many organs. The early morphologic descriptions of AD (Helmboldt and Jungherr, 1958; Obel, 1959; Leader *et al.*, 1963) emphasize the plasmacytosis. Figure 3 shows the kidney of a mink infected with ADV for 83 days, at which time approximately half the cells are lymphocytes and plasma cells that have infiltrated the interstitial areas. In mink with AD spleen and lymph nodes are enlarged and active, and plasma cells are evident in

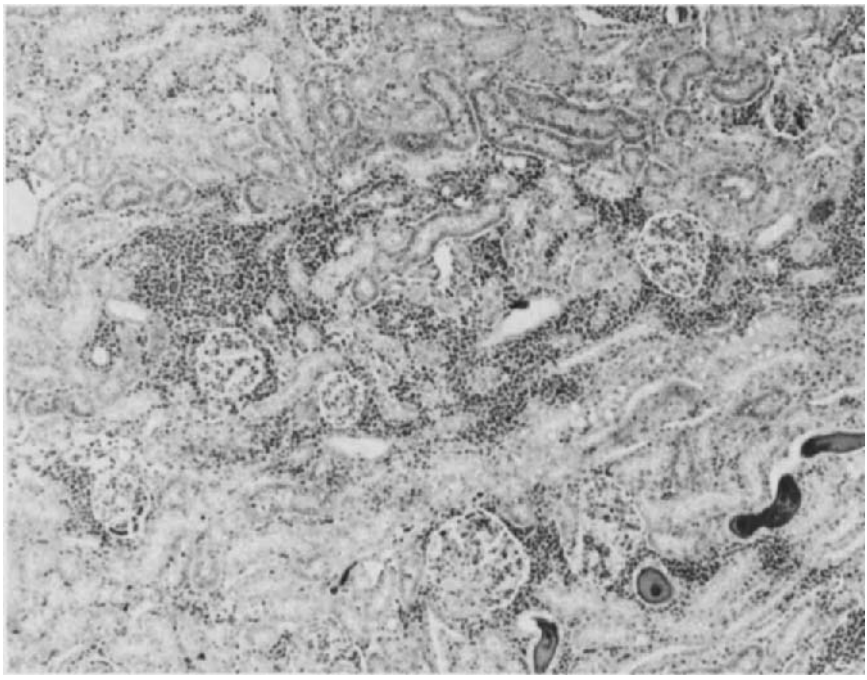


FIG. 3. The kidney of a mink of the Aleutian genotype infected with Aleutian disease virus (ADV) 83 days previously is illustrated. Large numbers of lymphocytes and plasma cells are infiltrating the interstitial areas. Hematoxylin and eosin; ×80.

the bone marrow and liver, although the thymus is morphologically normal. The number of peripheral blood lymphoid cells with surface immunoglobulin increases in some mink by 10 weeks after ADV infection, a change that is quite marked in all animals by 32 weeks after infection (Perryman *et al.*, 1975).

Accompanying the plasmacytosis, there is a marked hypergammaglobulinemia. We found that the electrophoretic γ-globulin levels of 570 morphologically normal mink were 0.74 gm/100 ml, and those of 683 naturally infected mink with lesions were 3.5 gm/100 ml (Porter and Larsen, 1964). The maximal levels of γ-globulin observed to date are about 11 gm/100 ml. Both the percentage of γ-globulin and the total serum protein are elevated during AD, which we have interpreted as a physiologic response to maintain a constant colloid osmotic pressure of plasma. The increased γ-globulin is nearly all 6.4 S IgG. The elevated IgG levels are clearly due to overproduction of the protein, since the IgG half-life is reduced from 3.45 ± 0.28 days in

normal mink to 2.19 ± 0.10 days in mink with severe AD ($p < 0.001$). No effect of AD on albumin half-life was noted ($p > 0.10$) (Porter *et al.*, 1965a). No immunologic difference has been found between the IgG of normal and AD mink in the absence of a monoclonal gammopathy. However, transient elevations of IgM and continuing elevation of IgA do occur in mink with AD (Porter *et al.*, 1977c; J. E. Coe, personal communication). Serum IgG levels are usually elevated 3–4 weeks after administration of a large ADV inoculum, or by 6–8 weeks when the dose of inoculum is small (Eklund *et al.*, 1968; Porter *et al.*, 1969; Trautwein, 1970; Bloom *et al.*, 1975). Maximal elevations of IgG are reached 3–24 months after infection begins, but generally somewhat sooner in mink of the Aleutian genotype.

The elevated IgG may show some restriction of mobility on cellulose acetate electrophoresis or immunoelectrophoresis as early as 40 days after ADV infection (Tabel and Ingram, 1970). Of mink with AD followed for a year or more, about 10% developed a monoclonal or, rarely, biclonal gammopathy as shown in Fig. 4 (Porter *et al.*, 1965b).

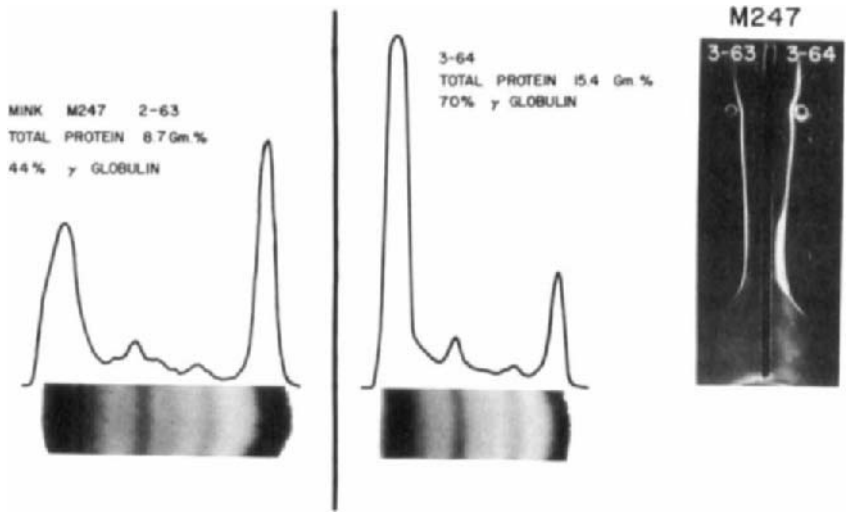


FIG. 4. The paper electrophoresis pattern on the left is that of the serum of a mink with early Aleutian disease (AD). The γ-globulin is heterogeneous. The center frame shows the serum electrophoresis of the same mink 13 months later, and the γ-globulin has become much more homogeneous. On the right, immunoelectrophoresis of the serum of this mink developed with rabbit anti-mink IgG shows a smooth and heterogeneous IgG precipitin arc early in AD. Late in the disease a localized dip and doubling of the IgG indicates the development of a monoclonal IgG. Reprinted from Porter and Larsen (1968) by permission of Academic Press.

Such mink may have Bence-Jones proteinuria, but they never have osteolytic bone lesions of the type seen in human and mouse myeloma. We have studied a small number of such mink and found that the monoclonal gammopathy may revert to a polyclonal gammopathy. In several of these mink, the γ-globulin levels fell to nearly normal and the lymphoid tissue showed fibrosis and few plasma cells. Limited studies of the monoclonal IgG proteins have shown that they carry ADV antibody activity, and individual and group-specific antigens are present that react with cat antiserums.

B. Specific Immune Responses

Antibody to ADV antigens can be shown by immunofluorescence, complement fixation, counterelectrophoresis, an ELISA assay, and viral agglutination tests, but not by viral neutralization tests (Porter *et al.*, 1969; McGuire *et al.*, 1971; Cho and Ingram, 1972; Porter *et al.*, 1977a; Burger *et al.*, 1978). The first three techniques have been compared for sensitivity and specificity (Crawford *et al.*, 1977) and seem to have the same specificity. However, the counterelectrophoresis test has only one-fourth to one-eighth the overall sensitivity of the immunofluorescence and complement fixation tests, but is more practical for routine use and better for the examination of undiluted serums. It is known that the counterelectrophoresis and complement fixation antigens are the whole virion, and that the virions can be coated and agglutinated by mink antibody. It is not certain, however, whether there are nonvirion antigens associated with ADV or which of the three or four virion antigens are reactive with mink antibody.

The time course of the ADV antibody titer measured by immunofluorescence of mink inoculated with the Utah-1 strain of ADV is shown in Fig. 5. Some mink made detectable antibody 9 days after infection, and all did so by 10 days. By day 60, the extraordinarily high mean antibody titer of 100,000 was observed (Porter *et al.*, 1969). Similar extremely high antibody titers can be shown by other techniques; complement-fixing antibody titers of 8,000 to 260,000 were present 6 weeks after infection, and titers of 5,000 to 80,000 were found 2 or more months after infection by using counterelectrophoresis (McGuire *et al.*, 1971; Cho and Ingram, 1973b), which detects antibody by 7 days after infection (Cho and Ingram, 1973b). Once the virus-specific antibody titers reach very high levels, they generally plateau, except in non-Aleutian mink that are not persistently infected (Larsen and Porter, 1975); in these, antibody titers decrease. Studies of antibody responses to the much less virulent Pullman strain of ADV (Bloom *et al.*, 1975) showed that Aleutian mink made somewhat greater responses than non-Aleutian mink, and that maximal titers of

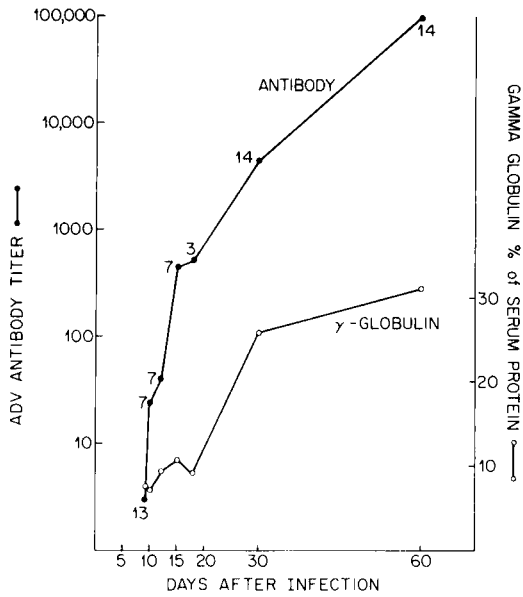


FIG. 5. Mink were inoculated with the Utah-1 strain of Aleutian disease virus (ADV). Antibody was measured by immunofluorescence. The geometric mean antibody titers are shown on a logarithmic scale, and the percentage of serum γ-globulin is shown on a linear scale. The number of mink tested at each time is indicated. The data are from Porter *et al.* (1969).

about 1024 were present by 6 weeks after infection according to the counterelectrophoresis test. In addition, lymphoid cells from the peripheral blood, spleen, and lymph nodes of infected mink synthesized immunoglobulin and ADV antibody in culture (Bloom, 1976; Perryman *et al.*, 1976).

Neutralization of ADV by virion-specific antibody has not been shown *in vivo* or in the cell culture system (Porter *et al.*, 1969, 1977a; An *et al.*, 1978), even when serums from mink or ferrets that did not develop persistent infection were used. Antiserum prepared in other species has not been tested for neutralizing antibody.

Virus present in the serum of persistently infected mink is complexed with antibody (Porter and Larsen, 1967), but, it is not known how much of the increased IgG is virus-specific antibody. In favor of a major amount of IgG being virus-specific antibody are the very high ADV antibody titers, a good correlation between the amounts of serum IgG and antibody, antibody activity of isolated monoclonal IgG, and the demonstration that 1 μg/ml of IgG from persistently infected mink gives a positive immunofluorescence test. The study of Bloom *et al.* (1975), which showed that peak ADV antibody titers were

reached long before the maximal increase in serum IgG was present, is the major evidence against most of the IgG being specific antibody. The experiments necessary to prove this point are obvious, but it has not been possible to obtain a sufficient amount of viral antigen to carry out the necessary tests.

C. Immune Complexes

ADV infectivity in the serums of persistently infected mink can be precipitated by antiserum to IgG, but not by antiserum to albumin (Porter and Larsen, 1967). Immune complexes of 9–14 S and 22–30 S can be demonstrated in the serums of many mink with severe AD by ultracentrifugation (Porter *et al.*, 1965a). These complexes are much smaller than the intact virion (125 S) and can be dissociated by urea or acid pH. The complexes contain IgG, but it is not known which, if any, of the viral antigens participate in complex formation. The presence of complexes could not be associated with specific lesions because the mink tested had such severe disease.

D. Unrelated Immune Responses

At one time it was thought that AD might be an unrestrained proliferation of all clones of B cells, and a number of studies were carried out to assess the immune responsiveness of normal and ADV-infected mink by using a variety of antigens. Although goat and sheep erythrocytes, keyhole limpet hemocyanin, peroxidase, *Shigella flexneri, Brucella abortus,* and bacteriophage T2 are good antigens in mink, bovine serum albumin, bovine insulin, bovine IgG, egg albumin, and poliovirus induce little if any antibody in mink. In testing immunologic adequacy, it has been found that non-Aleutian uninfected mink are slightly more responsive to hemocyanin and moderately more responsive to goat erythrocytes than uninfected mink of the Aleutian genotype. The difference is larger for secondary than for primary immune responses (Lodmell *et al.*, 1970, 1971), but not great enough to suggest that mink of the Aleutian genotype are immunologically defective. When mink with AD and hypergammaglobulinemia are immunized with various antigens, about one-quarter of the normal amount of antibody is produced and the suppression begins at about the time of the onset of hypergammaglobulinemia (Porter *et al.*, 1965a; Kenyon, 1966; Lodmell *et al.*, 1970, 1973; Trautwein *et al.*, 1974). If mink are immunized and then challenged with ADV, the antibody titer to the non-ADV antigens falls during the development of hyperglobulinemia, but the mink may be able to mount a normal secondary response to the nonviral antigen (Porter *et al.*, 1965a; Porter and Larsen, 1968; Lodmell *et al.*, 1970). The levels of "natural" antibodies may fall or

increase moderately in mink with AD (Tabel *et al.*, 1970). It was noted that mink surviving AD for 6 months or more (Kenyon, 1966) had a nearly normal primary response to *Brucella abortus,* but this has not been the experience with other antigens. The results clearly indicate that ADV does not indiscriminately stimulate all clones of B cells. We believe that the most likely explanation for the reduced humoral immune responsiveness of infected mink is antigenic competition, but a number of other explanations are possible (Lodmell *et al.*, 1970).

The nonspecific T cell mitogen phytohemagglutinin P produces less than normal stimulation of peripheral blood lymphocytes in infected mink (Perryman *et al.*, 1975). Furthermore, delayed skin sensitivity to purified protein derivative (PPD) was reduced in mink with AD that had been immunized with Bacillus Calmette-Guérin (BCG) (Munoz *et al.*, 1974).

Nuclear antigens and antinuclear antibodies have been noted in the serum of mink with AD (Barnett *et al.*, 1969). Although we have only occasionally found antinuclear antibody in mink and have been unable to associate it with AD, this problem should be studied further with the more sensitive and specific tests now available. We have shown blocked rheumatoid factor-like activity in some AD mink serums, but this result is difficult to evaluate (unpublished observations). Saison *et al.* (1966) reported that erythrocytes from mink with AD could be agglutinated by antiglobulin, and Cho and Ingram (1973c) have shown that the erythrocytes are coated with more C3 than IgG and that ADV antibody may be eluted from such erythrocytes McGuire *et al.* (1979) found that most mink with AD have IgG present on erythrocytes, but that the amounts are usually too small to be detected by a direct Coombs' test. However, erythrocyte half-life was reduced in mink with AD. Although hemagglutination by ADV has not been shown (Porter *et al.*, 1977a), possibly owing to low viral titers, hemagglutination is a general property of parvoviruses, and this may explain the presence of immune reactants on erythrocytes.

V. The Pathogenesis of Lesions

A. General Considerations and Genetic Effects

Highly virulent ADV strains such as Utah-1 and Guelph produce disease and death in all mink of the Aleutian genotype and about 75% of non-Aleutian mink, whereas the Pullman strain of ADV, which is of low virulence, causes progressive disease and death only in Aleutian mink. No immunologic or physical differences have been noted between the ADV strains of high and low virulence. Eklund *et al.* (1968)

noted that 50% of Aleutian mink are dead 120 days after inoculation of the Pullman strain of ADV. This strain does replicate to some extent in non-Aleutian mink, but they develop lower ADV antibody titers than do Aleutian mink (Bloom *et al.*, 1975). In the non-Aleutian mink, virus may persist in lymphoid tissue for many months in the absence of lesions (Eklune *et al.*, 1968). No difference in the mean time until death in Aleutian mink is found when the Utah-1 strain of virus is used instead of the Pullman strain, but the Utah-1 strain produces progressive infection in 75% of pastel (non-Aleutian) mink. One group of resistant pastel mink had no viremia, but manifested a transient increase in serum γ-globulin and a relatively low ADV antibody response, which in time fell to very low levels. Breeding experiments with these mink showed that no single gene was responsible for host resistance (Larsen and Porter, 1975). Another group of pastel mink without disease was found to have viremia (An and Ingram, 1977), in addition to which virus was isolated and ADV antibody was demonstrated. Pastel mink that do develop progressive AD rarely die sooner than 5 months after

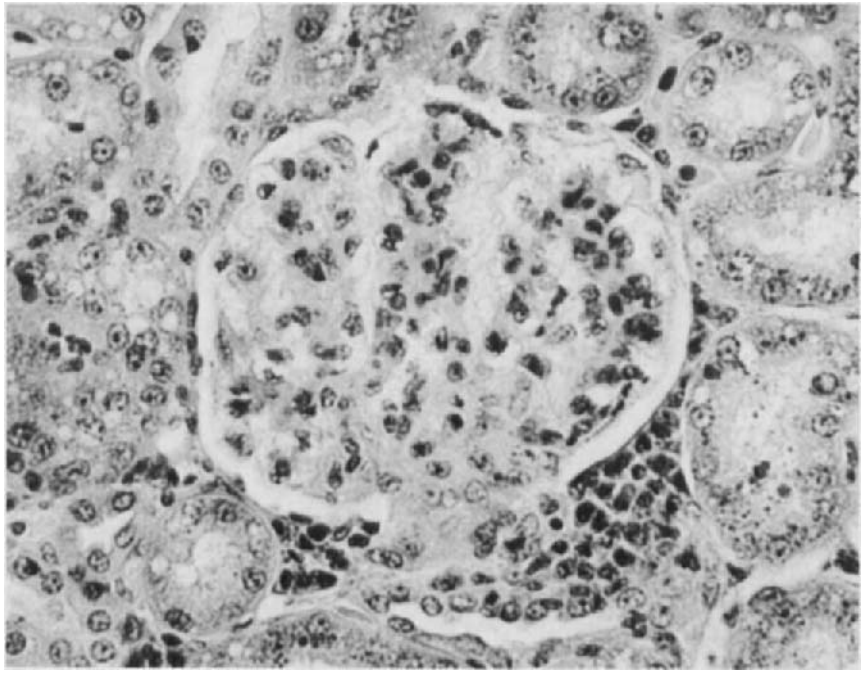


FIG. 6. A glomerulus from an Aleutian mink inoculated with the Utah-1 strain of Aleutian disease virus (ADV) 83 days earlier is shown. There is severe glomerulonephritis with obliteration of the glomerular capillaries. Hematoxylin and eosin; ×700.

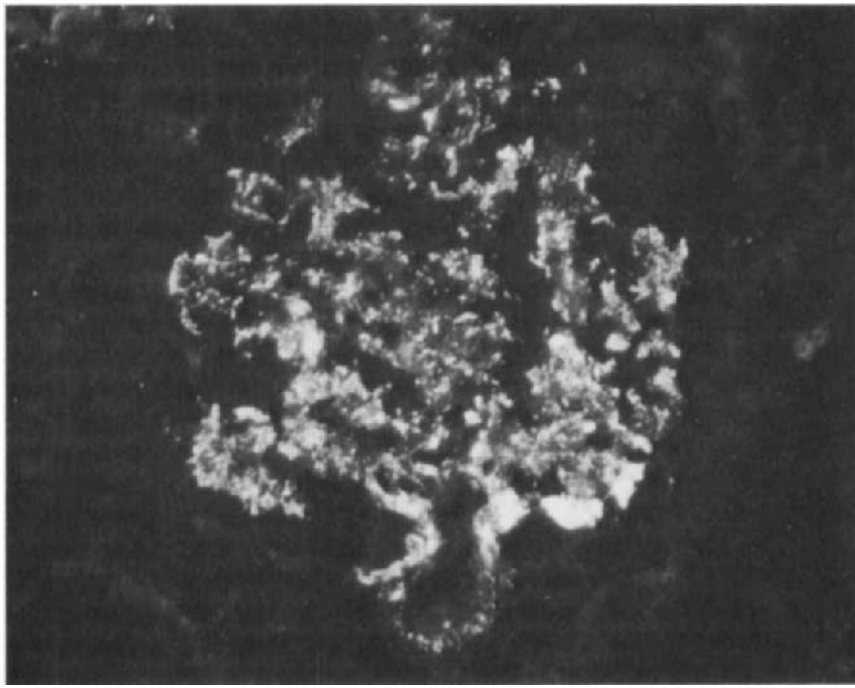


FIG. 7. A glomerulus from the same mink as that of Fig. 6, 83 days after Aleutian disease virus (ADV) infection is shown stained for the presence of mink C3. Mink complement is deposited in a finely granular pattern along the glomerular capillary walls. Fluorescence micrograph; ×600.

infection, and 50% are dead after 1 year. A few of these mink may live for their full life-span of 8 years.

Mink with progressive AD usually show few clinical signs of illness until a few days prior to death. About 85% of deaths are due to renal failure, and the remainder are caused by rupture of inflamed arteries or enlarged spleens, or intercurrent bacterial infections. Death is often associated with periods of temperature stress.

B. GLOMERULONEPHRITIS AND ARTERITIS

Persistently infected mink develop severe immune complex glomerulonephritis (Fig. 6) that progresses much faster in Aleutian than in non-Aleutian mink (Johnson *et al.*, 1975). A number of morphologic studies of this lesion have been reported (Leader *et al.*, 1963; Kindig *et al.*, 1967; Henson *et al.*, 1966, 1967, 1968; Trautwein, 1970). Immunofluorescence studies have shown IgG and C3 in a finely to coarsely granular pattern along the glomerular capillary walls, as shown in Fig. 7 (Henson *et al.*, 1969; Porter *et al.*, 1969; Pan *et al.*,

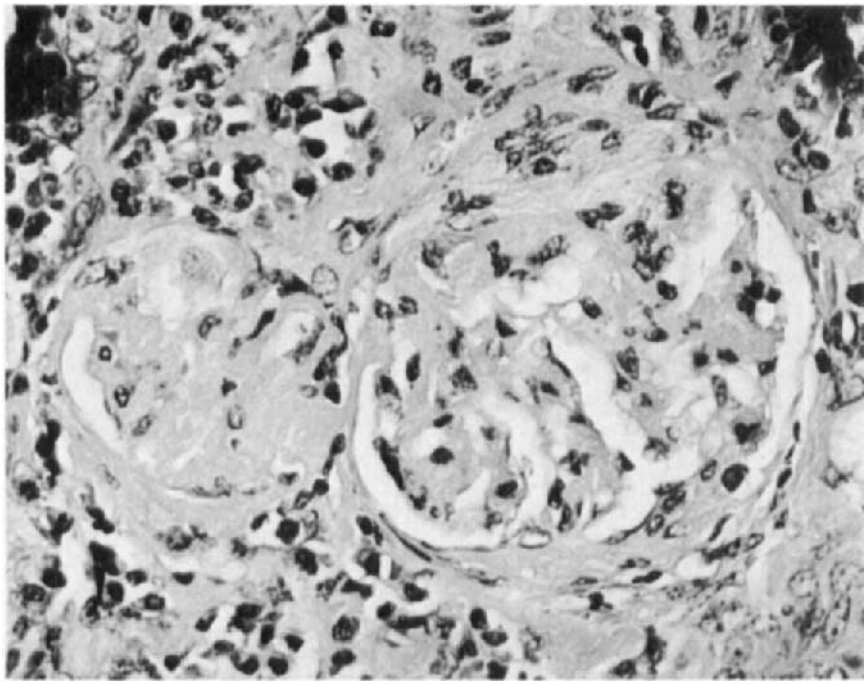


FIG. 8. Two sclerotic glomeruli from a non-Aleutian mink infected with the Utah-1 strain of Aleutian disease virus (ADV) 7 years previously are shown. This mink was in advanced renal failure and still had infectious virus in the serum and organs. Hematoxylin and eosin; ×525.

1970; Johnson *et al.*, 1975). Antivirion antibody can be eluted from the kidneys of animals with chronic glomerulonephritis (Porter *et al.*, 1969; Cho and Ingram, 1973c). By immunofluorescence ADV antigens can be found without elution in the glomerular capillary walls of mink infected transplacentally, or in other mink if the Ig and C3 are eluted with acid buffer (Porter *et al.*, 1977b). We have noted less glomerular staining by antiserum specific for mink γ-chain than by antiserum to mink C3, and we agree with Johnson *et al.* (1975) that IgM is present in affected glomeruli. Portis and Coe (1979) found a predominant deposition of IgA in diseased glomeruli. The class and specificity of the Ig deposited in the glomeruli of mink with AD deserve further study. In addition to developing glomerulonephritis, the glomeruli of mink that survive AD for a period of years may be extremely sclerotic, as shown in Fig. 8.

Acute and chronic arteritis involving small and medium-sized mus-

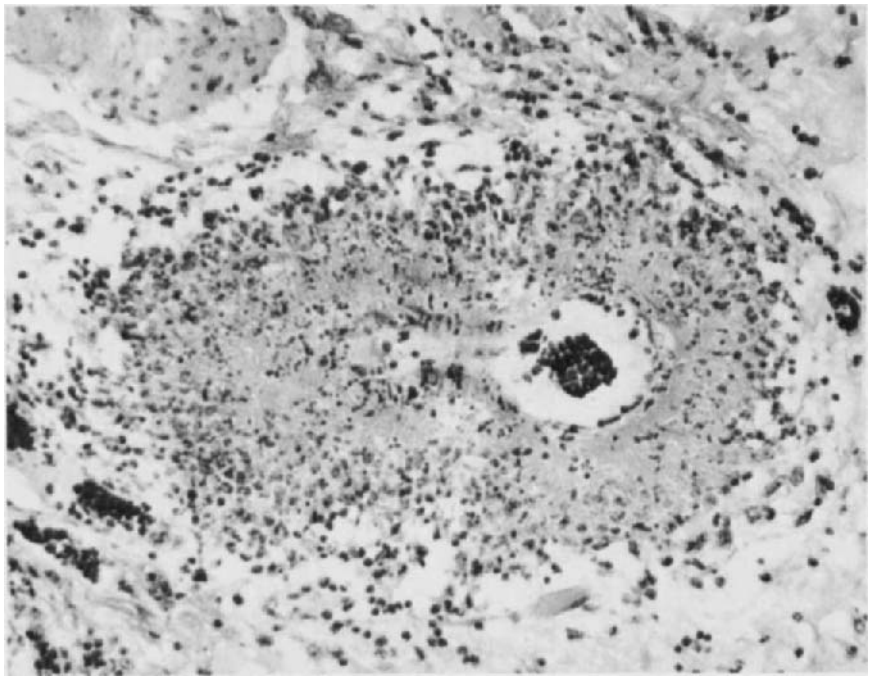


FIG. 9. The acute form of Aleutian disease (AD) arteritis is shown in a small artery in the bladder of an Aleutian mink infected with ADV 83 days previously. There is an accumulation of polymorphonuclear leukocytes and fibrinoid necrosis of the vessel wall. Hematoxylin and eosin; ×200.

cular arteries, especially those of the heart, brain, and kidney, is present in 10–20% of mink with AD. Only mink with the most severe AD have arteritis. About 10% of the lesions seen are acute (Fig. 9), with polymorphonuclear leukocytes, fibrinoid necrosis, and loss of the internal elastic membrane. The more usual lesions are subacute, with marked proliferation of endothelial cells and a dense perivascular collection of lymphocytes and plasma cells as shown in Figs. 10 and 11. The lesions may eventually heal and have areas of fibrosis, as shown in Fig. 12 (Henson *et al.*, 1966; Porter *et al.*, 1973). Immunofluorescence studies demonstrated extracellular deposits of Ig and C3, and after acid elution, viral antigen in areas of fibrinoid necrosis and between proliferating endothelial cells (Porter *et al.*, 1973). An example of the deposition of mink C3 in a vessel wall is shown in Fig. 13. The distribution and morphology of the lesions resemble those of serum sickness (Kniker and Cochrane, 1968).

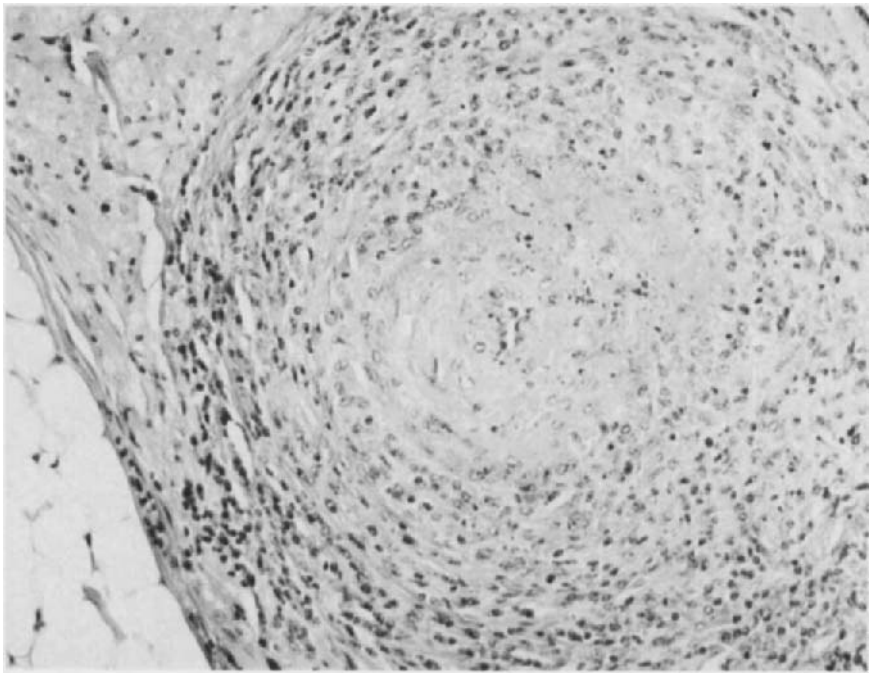


FIG. 10. A small coronary artery with the subacute form of Aleutian disease (AD) arteritis is shown from an Aleutian mink infected 83 days previously with ADV. The lumen is nearly obliterated by proliferation of endothelial cells, and the vessel is surrounded by a chronic inflammatory infiltrate of lymphocytes and plasma cells. Hematoxylin and eosin; ×200.

C. MANIPULATION OF LESION PRODUCTION

Continuous therapy of ADV-infected Aleutian mink with cyclophosphamide completely prevented the development of lesions of AD (Cheema *et al.*, 1972). The dosage of drug used partially suppressed the humoral immune response to bovine serum albumin, a relatively poor antigen in mink. Sufficient virus titrations were done to indicate that the blood ADV titers were not decreased as compared with untreated mink, but an increase in viral titers was not ruled out. Cessation of cyclophosphamide resulted in the development of lesions. When mink infected transplacentally with ADV are studied at 83 days of age and compared with mink infected as adolescents, there is a marked reduction in immunoglobulin and ADV antibody levels, a reduction of glomerulonephritis, an absence of arteritis, and eosinophils accumulate in the tissues (Porter *et al.*, 1977b). These animals

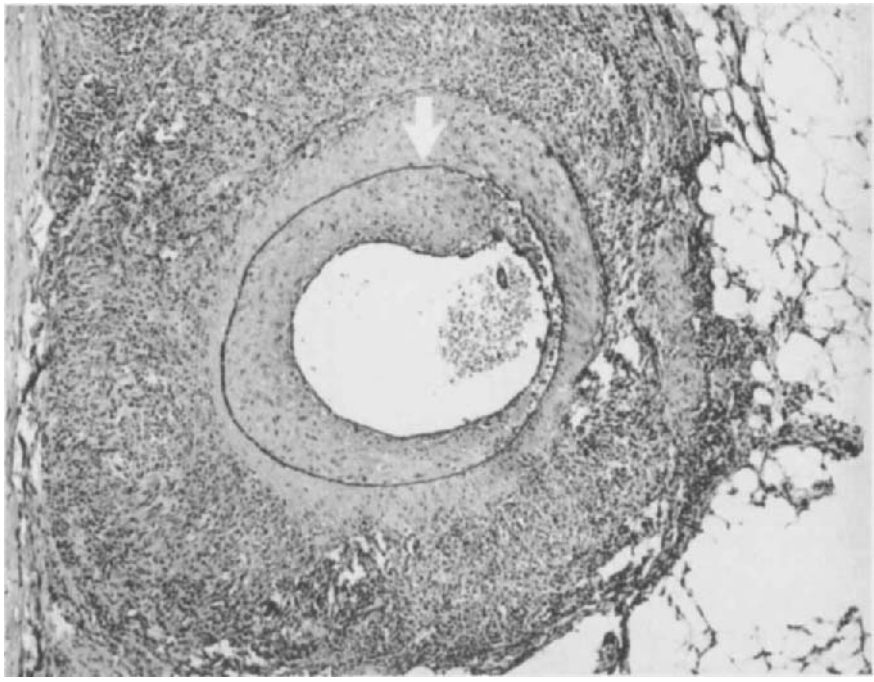


FIG. 11. The main left coronary artery of the same mink shown in Fig. 10 has severe subacute arteritis. The arrow indicates the internal elastic membrane of the artery, and the proliferation of endothelial cells is evident. Elastic tissue stain; ×80.

also have significantly higher ADV titers in their spleens and about 100-fold more infected cells than do mink infected as adolescents. Limited information suggests that by 6 months of age the transplacentally infected mink develop the full spectrum of AD lesions.

Mink immunized with a formalin-inactivated crude tissue ADV vaccine and then challenged with live ADV develop a markedly accelerated AD. A number of immunized non-Aleutian mink died or were moribund by 39 days after receiving live ADV, but unmanipulated mink of this genotype rarely died earlier than 5 months after infection (Porter *et al.*, 1972). This effect has not been seen in experiments with inactivated vaccine prepared from ADV grown in cell culture (unpublished). If mink are given this virus and 10 days later receive 1 gm of mink IgG containing ADV antibody, at the time of peak viral proliferation one sees foci of acute necrosis followed by chronic inflammatory cell infiltrates in the liver (Porter *et al.*, 1972).

Studies of the manipulation of the lesions of AD provide further evi-

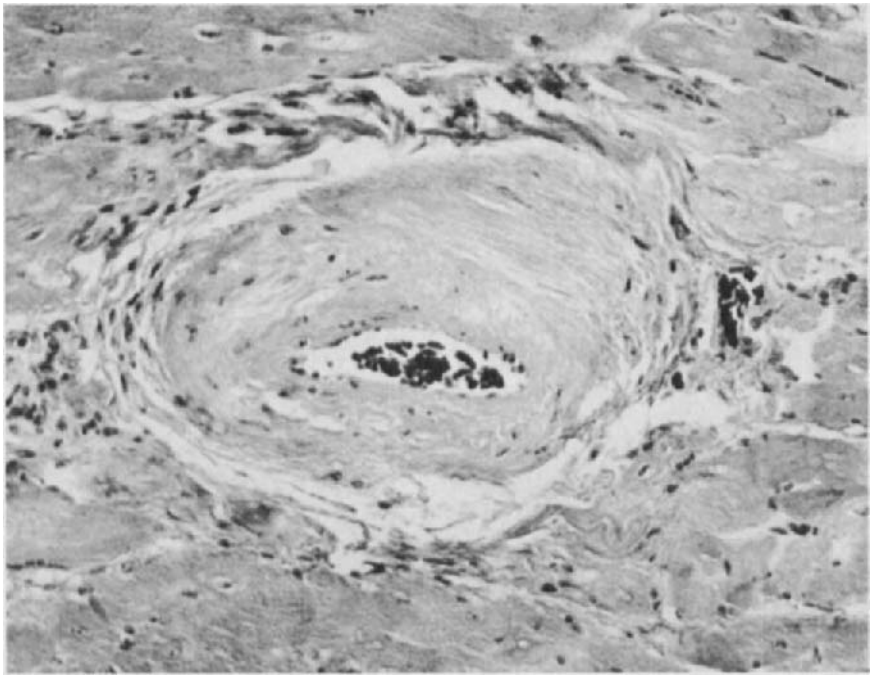


FIG. 12. A small coronary artery from the mink with Aleutian disease for 7 years is shown. An area of arteritis has healed with dense fibrosis. Hematoxylin and eosin; ×250.

dence that the lesions are wholly caused by the host immune response to the virus and ensuing immune complex formation. There is also a strong suggestion that the immune response to the virus decreases viral titers despite failure to demonstrate neutralizing antibody to ADV in mink.

D. OTHER LESIONS

McKay *et al.* (1967) described cyclic variations in the levels of clotting factors V and VIII and decreased fibrinogen levels and platelet counts in mink with AD. Unfortunately, no studies have been done to determine the magnitude of consumption of these clotting components, but it seems likely that intravascular coagulation may contribute to the lesions of AD. A marked proliferation of small bile ducts in the liver of mink with AD, occasionally with cyst formation, has been described, as shown in Fig. 14 (Leader *et al.*, 1963; Drommer and Trautwein, 1975). Viral antigens have not been seen in biliary epithe-

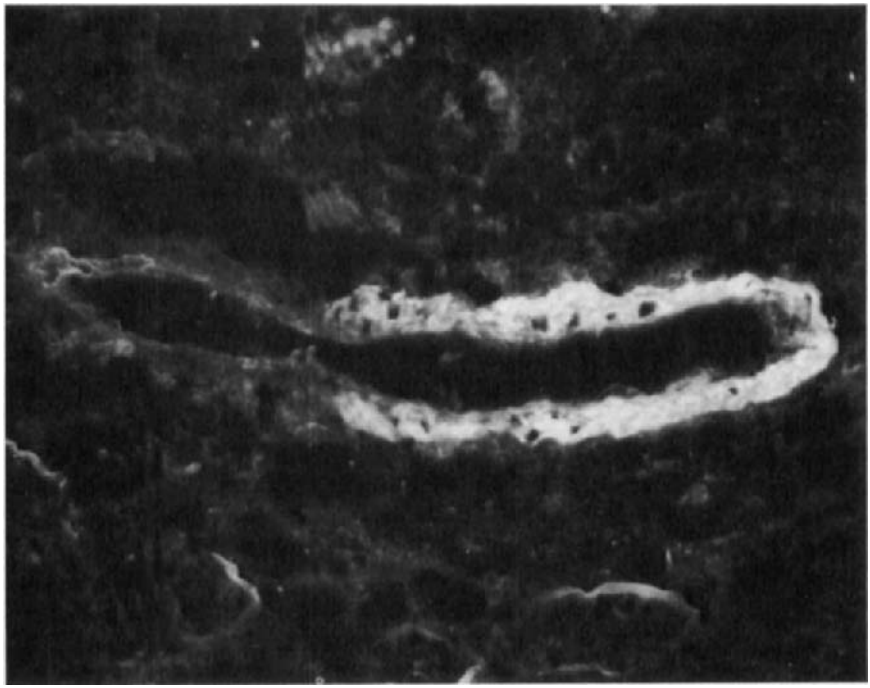


FIG. 13. An artery with subacute arteritis from the same mink as shown in Figs. 10 and 11 is shown stained for the presence of mink C3. Complement is deposited extracellularly beneath the endothelium along two-thirds of the circumference of the vessel. IgG and, after acid elution, Aleutian disease virus (ADV) antigen were present in a similar location in adjacent sections. Fluorescence micrograph; ×350.

lium. The nature of this lesion is not known, but immune complexes might be involved, since the lesion is less frequent in transplacentally infected mink (Porter *et al.*, 1977b). Cryoproteins have been noted in AD, but could not be correlated with specific tissue lesions (Porter and Larsen, 1964).

VI. Disease Control

Among the mink ranches observed, 1% to 75% of the mink were infected with AD (Porter and Larsen, 1964). Since AD is a major disease problem, attempts are being made to identify and pelt infected mink. The technically simple iodine agglutination test (Mallén *et al.*, 1950), which measures increased γ-globulin, was widely used, and a program of prophylactic sacrifice decreased the incidence of AD (Gorham

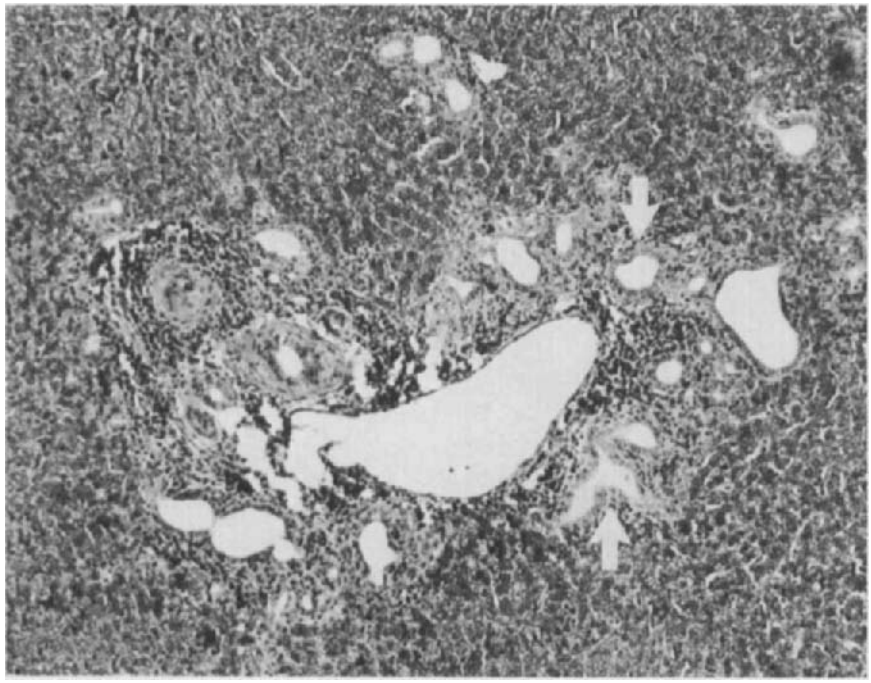


FIG. 14. The portal triad in the liver of an Aleutian mink infected for 83 days is shown. Proliferation of small bile ducts (arrows) is evident. Hematoxylin and eosin; ×90.

et al., 1976). However, the test identifies only 16–65% of mink that have ADV antibody (Greenfield *et al.*, 1973; Ingram and Cho, 1974; Gierloff and Thordal-Christensen, 1975) and thus is ineffective in eradicating AD. However, use of the specific counterelectrophoresis ADV antibody test and pelting of all animals that have the antibody has resulted in elimination of AD from at least seven commercial ranches (Cho and Greenfield, 1978; M. Hansen, personal communication). This method of disease control is both expensive and cumbersome, and further means of AD control should be sought.

VII. Discussion

The disease produced in mink by ADV is reasonably well understood. No disease seems to be caused by virus replication itself, but a large amount of viral antigen is produced. This induces a maximal immune response to viral antigen, a marked expansion of antibody-producing cells, and formation of circulating immune complexes of vari-

ous sizes. The complexes deposit in glomeruli and arteries and cause severe lesions similar to those produced by many other types of immune complexes (for a review see Cochrane and Koffler, 1973). The severity of AD is determined by the viral strain, the host genotype, and the age of the mink at the time of infection. No quantitative data have been obtained to define the proportion of the increased Ig that represents viral-specific antibody; this is the major remaining question about AD pathogenesis. Further studies on the extent to which each Ig class is involved in lesions, the nature of the liver lesion, and the extent of intravascular coagulation could yield interesting results.

Aleutian disease virus appears to be a parvovirus that does not require a helper virus for replication. The temperature sensitivity of *in vitro* viral replication is a particularly interesting property. This important pathogen should be extensively characterized. If adequate amounts of the individual ADV antigens can be prepared, it should be possible to fully define the specificity of the mink ADV antibody. It may be possible to learn why mink antibody does not neutralize ADV.

Several other persistent viral infections, such as hepatitis B (reviewed by Robinson, 1978), lymphocytic choriomeningitis (reviewed by Cole and Nathanson, 1974), equine infectious anemia (reviewed by Crawford *et al.*, 1978), and lactic dehydrogenase virus infection of mice (reviewed by Rowson and Mahy, 1975), have similarities in pathogenesis to AD, although none of the viruses themselves are similar. The reason why any of these viruses, including ADV, cause a persistent infection is unknown. However, there are several attractive possibilities for mechanisms that might result in ADV persistence. The temperature-sensitive nature of ADV replication could be linked to its ability to persist *in vivo*, since *in vitro* persistence of viruses has often been associated with the acquisition of a temperature-sensitive lesion in the viral genome (Preble and Youngner, 1975). ADV produces defective particles *in vivo*, and analogy to other viruses suggests that this could be a cause of persistence (Holland *et al.*, 1978). The formation of ADV–antibody complexes might lead to persistence by encouraging phagocytosis by macrophages in which the virus can replicate (Porter *et al.*, 1969). It is also possible that simple failure of the mink antibody to neutralize ADV leads to persistence. Although there is no present suggestion as to which, if any, of these mechanisms leads to persistence, it may be possible to devise experiments that can answer this central question concerning AD.

ACKNOWLEDGMENT

The authors thank Dr. Frank J. Dixon for encouraging and criticizing their efforts to understand Aleutian disease for the past 18 years.

REFERENCES

An, S. H., and Ingram, D. G. (1977). *Am. J. Vet. Res.* **38,** 1619.
An, S. H., and Ingram, D. G. (1978). *Am. J. Vet. Res.* **39,** 309.
An, S. H., DePauli, F. J., Wright, P., and Ingram, D. G. (1978). *Res. Vet. Sci.* **24,** 200.
Barnett, E. V., Williams, Jr., R. C., Kenyon, A. J., and Henson, J. E. (1969). *Immunology* **16,** 241.
Bloom, M. E. (1976). *Infect. Immun.* **13,** 281.
Bloom, M. E., Race, R. E., Hadlow, W. J., and Chesebro, B. (1975). *J. Immunol.* **115,** 1034.
Bowness, E. R. (1968). *Can. Vet. J.* **9,** 103.
Burger, D., Sriranganathan, N., and Gorham, J. R. (1978). *Abstr. Annu. Meet. Am. Soc. Microbiol.* p. 65.
Cheema, A., Henson, J. B., and Gorham, J. R. (1972). *Am. J. Pathol.* **66,** 543.
Chesebro, B., Bloom, M., Hadlow, W., and Race, R. (1975). *Nature (London)* **254,** 456.
Cho, H. J. (1977). *Can. J. Comp. Med.* **41,** 215.
Cho, H. J., and Greenfield, J. (1978). *J. Clin. Microbiol.* **7,** 18.
Cho, H. J., and Ingram, D. G. (1972). *J. Immunol.* **108,** 555.
Cho, H. J., and Ingram, D. G. (1973a). *Nature (London), New Biol.* **243,** 174.
Cho, H. J., and Ingram, D. G. (1973b). *Can. J. Comp. Med.* **37,** 217.
Cho, H. J., and Ingram, D. G. (1973c). *Infect. Immun.* **8,** 264.
Cochrane, C. G., and Koffler, D. (1973). *Adv. Immunol.* **16,** 185.
Coe, J. E. (1972). *Immunochemistry* **9,** 147.
Coe, J. E., and Hadlow, W. J. (1972). *J. Immunol.* **108,** 530.
Coe, J. E., and Race, R. E. (1978). *Proc. Soc. Exp. Biol. Med.* **157,** 289.
Cole, G. A., and Nathanson, N. (1974). *Prog. Med. Virol.* **18,** 94.
Crawford, T. B., McGuire,T. C., Porter, D. D., and Cho, H. J. (1977). *J. Immunol.* **118,** 1249.
Crawford, T. B., Cheevers, W. P., Klevjer-Anderson, P., and McGuire, T. C. (1978). *In* "Persistent Viruses" (J. G. Stevens, G. J. Todaro, and C. F. Fox, eds.), Vol. 11, ICN-UCLA Symposia on Molecular and Cellular Biology, p. 727. Academic Press, New York.
Davis, W. C., Spicer, S. S., Greene, W. B., and Padgett, G. A. (1971). *Lab. Invest.* **24,** 303.
Drommer, W. and Trautwein, G. (1975). *Vet. Pathol.* **12,** 77.
Eklund, C. M., Hadlow, W. J., Kennedy, R. C., Boyle, C. C., and Jackson, T. A. (1968). *J. Infect. Dis.* **118,** 510.
Ewer, R. F.(1973). "The Carnivores." Cornell Univ. Press, Ithaca, New York.
Gerell, R. (1970). *Oikos* **21,** 160.
Gierloff, B., and Thordal-Christensen, A. (1975). *Dansk Vet. Tidsskr.* **58,** 589.
Gorham, J. R., Leader, R. W., and Henson, J. B. (1964). *J. Infect. Dis.* **114,** 341.
Gorham, J. R., Leader, R. W., Padgett, G. A., Burger, D., and Henson, J. B. (1965). *In* "Slow, Latent, and Temperate Virus Infections" (D. C. Gajdusek, C. J. Gibbs, and M. Alpers, eds.), p. 279. NINDB Monograph No. 2, U. S. Government Printing Office, Washington, D.C.
Gorham, J. R., Henson, J. B., Crawford, T. B., and Padgett, G. A. (1976). *In* "Slow Virus Diseases of Animals and Man" (R. H. Kimberlin, ed.), p. 135. North-Holland Publ., Amsterdam.
Greenfield, J., Walton, R., and MacDonald, K. R. (1973). *Res. Vet.Sci.* **15,** 381.
Hahn, E. C., Ramos, L., and Kenyon, A. J. (1977). *Infect. Immun.* **15,** 204.
Hansson, A. (1947). *Acta Zool.* **28,** 1.
Hartsough, G. R., and Gorham, J. R. (1956). *Natl. Fur News* **28,** 10.

Helmboldt, C. F., and Jungherr, E. L. (1958). *Am. J. Vet. Res.* **19,** 212.
Henson, J. B., Gorham, J. R., Leader, R. W., and Wagner, B. M. (1962). *J. Exp. Med.* **116,** 357.
Henson, J. B., Leader, R. W., Gorham, J. R., and Padgett, G. A. (1966). *Pathol. Vet.* **3,** 289.
Henson, J. B., Gorham, J. R., and Tanaka, Y. (1967). *Lab. Invest.* **17,** 123.
Henson, J. B., Gorham, J. R., Tanaka, Y., and Padgett, G. A. (1968). *Lab. Invest.* **19,** 153.
Henson, J. B., Gorham, J. R., Padgett, G. A., and Davis, W. C., (1969). *Arch. Pathol.* **87,** 21.
Holland, J. J., Semler, B. L., Jones, C., Perrault, J., Reid, L., and Roux, L. (1978). *In* "Persistent Viruses" (J. G. Stevens, G. J. Todaro, and C. F. Fox, eds.), Vol. 11, ICN-UCLA Symposia on Molecular and Cellular Biology, p. 57. Academic Press, New York.
Hood, L., Gray, W. R., Sanders, B. G., and Dreyer, W. J. (1967). *Cold Spring Harbor Symp. Quant. Biol.* **32,** 133.
Ingram, D. G., and Cho, H. J. (1974). *J. Rheumatol.* **1,** 74.
Johansson, I., and Venge, O. (1951). *Acta Zool.* **32,** 255.
Johnson, M. I., Henson, J. B., and Gorham, J. R. (1975). *Am. J. Pathol.* **81,** 321.
Karstad, L., and Pridham, T. J. (1962). *Can. J. Comp. Med.* **26,** 97.
Kenyon, A. J. (1966). *Am. J. Vet. Res.* **27,** 1780.
Kenyon, A. J., Helmboldt, C. F., and Nielsen, S. W. (1963). *Am. J. Vet. Res.* **24,** 1066.
Kenyon, A. J., Magnano, T., Helmboldt, C. F., and Buko, L. (1966). *J. Am. Vet. Med. Assoc.* **149,** 920.
Kenyon, A. J., Howard, E., and Buko, L. (1967). *Am. J. Vet. Res.* **28,** 1167.
Kenyon, A. J., Kenyon, B. J., and Hahn, E. C. (1978). *Am. J. Vet. Res.* **39,** 1011.
Kindig, D., Spargo, B., and Kirsten, W. (1967). *Lab. Invest.* **16,** 436.
Kniker, W. T., and Cochrane, C. G. (1968). *J. Exp. Med.* **127,** 119.
Larsen, A. E., (1969). Ph.D. Dissertation, University of Utah, Salt Lake City.
Larsen, A. E., and Porter, D. D. (1975). *Infect. Immun.* **11,** 92.
Larsen, A. E., Porter, D. D., and Porter, H. G. (1971). *Proc. Soc. Exp. Biol. Med.* **136,** 430.
Leader, R. W., Wagner, B. M., Henson, J. B., and Gorham, J. R. (1963). *Am. J. Pathol.* **43,**33.
Ledoux, R. G., and Kenyon, A. J. (1975). *Comp. Biochem. Physiol.* **51A,** 213.
Lodmell, D. L., Hadlow, W. J., Munoz, J. J., and Whitford, H. W. (1970). *J. Immunol.* **104,** 878.
Lodmell, D. L., Bergman, R. K., Hadlow, W. J., and Munoz, J. J. (1971). *Infect. Immun.* **3,** 221.
Lodmell, D. L., Bergman, R. K., and Hadlow, W. J. (1973). *Infect. Immun.* **8,** 769.
McGuire, T. C., Crawford, T. B., Henson, J. B., and Gorham, J. R. (1971). *J. Immunol.* **107,** 1481.
McGuire, T. C., Perryman, L. E., and Gorham, J. R. (1979). *Vet. Microbiol.* **4,** 17.
McKay, D. G., Phillips, L. L., Kaplan, H., and Henson, J. B. (1967). *Am. J. Pathol.* **50,** 899.
Mallén, M. S., Ugalde, E. L., Balcazar, M. R., Bolívar, J. I., and Meyran, S. (1950). *Am. J. Clin. Pathol.* 20, 39.
Munoz, J., Lodmell, D. L., Race, R. E., Jackson, T. A., and Hadlow, W. J. (1974). *Z. Immunitaetsforsch.* **147,** 53.
Obel, A. -L. (1959). *Am. J. Vet. Res.* **20,** 384.
Ohshima, K., Shen, D. T., Henson, J. B., and Gorham, J. R. (1978). *Am. J. Vet. Res.* **39,** 653.

Padgett, G. A., Gorham, J. R., and Henson, J. B. (1967). *J. Infect. Dis.* **117,** 35.
Padgett, G. A., Reiquam, C. W., Henson, J. B., and Gorham, J. R. (1968). *J. Pathol. Bacteriol.* **95,** 509.
Pan, I. C.,Tsai, K. S., and Karstad, L. (1970). *J. Pathol.* **101,** 119.
Perryman, L. E., Banks, K. L., and McGuire, T. C. (1975). *J. Immunol.* **115,** 22.
Perryman. L. E., McGuire, T. C., and Banks, K. L. (1976). *J. Immunol.* **117,** 1981.
Porter, D. D. (1965). *Proc. Soc. Exp. Biol. Med.* **119,** 131.
Porter, D. D., and Cho, H. J. (1980). *In* "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), Vol. 17, Plenum, New York. In press.
Porter, D. D., and Dixon, F. J. (1966). *Am. J. Vet. Res.* **27,** 335.
Porter, D. D., and Larsen, A. E. (1964). *Am. J. Vet. Res.* **25,** 1226.
Porter, D. D., and Larsen, A. E. (1967). *Proc. Soc. Exp. Biol. Med.* **126,** 680.
Porter, D. D., and Larsen, A. E. (1968). *Perspect. Virol.* **6,** 173.
Porter, D. D., Dixon, F. J., and Larsen, A. E. (1965a). *J. Exp. Med.* **121,** 889.
Porter, D. D., Dixon, F. J., and Larsen, A. E. (1965b). *Blood* **25,** 736.
Porter, D. D., Larsen, A. E., and Porter, H. G. (1969). *J. Exp. Med.* **130,** 575.
Porter, D. D., Larsen, A. E., and Porter, H. G. (1972). *J. Immunol.* **109,** 1.
Porter, D. D., Larsen, A. E., and Porter, H. G. (1973). *Am. J. Pathol.* **71,** 331.
Porter, D. D., Larsen, A. E., Cox, N. A., Porter, H. G., and Suffin, S. C. (1977a). *Intervirology* **8,** 129.
Porter, D. D., Larsen, A. E., and Porter, H. G. (1977b). *J. Immunol.* **119,** 872.
Porter, D. D., Porter, H. G., and Larsen, A. E. (1977c). *Fed. Proc.* **36,** 1268. Abstract.
Portis, J. L., and Coe, J. E. (1979). *Am. J. Pathol.* **96,** 227.
Preble, O. T., and Youngner, J. S. (1975). *J. Infect. Dis.* **131,** 467.
Robinson, W. S. (1978). *In* "Persistent Viruses" (J. G. Stevens, G. J. Todaro, and C. F. Fox, eds.), Vol. 11, ICN-UCLA Symposia on Molecular and Cellular Biology, p. 485. Academic Press, New York.
Rowson, K. E. K., and Mahy, B. W. J. (1975). "Lactic Dehydrogenase Virus," Virology Monographs, Vol. 13 (S. Gard and C. Hallauer, eds.). Springer-Verlag, Berlin and New York.
Saison, R., Karstad, L., and Pridham, T. J. (1966). *Can. J. Comp. Med.* **30,** 151.
Sarich, V. M. (1969). *Syst. Zool.* **18,** 286.
Seal, U. S., Phillips, N. I., and Erickson, A. W. (1970). *Comp. Biochem. Physiol.* **32,** 33.
Shackelford, R. M. (1950). "Genetics of the Ranch Mink." Pilsbury Press, New York.
Shackelford, R. M. (1952). *Am. Natur.* **86,** 311.
Shahrabadi, M. S., and Cho, H. J. (1977). *Can. J. Comp. Med.* **41,** 435.
Shahrabadi, M. S., Cho, H. J., and Marusyk, R. G. (1977). *J. Virol.* **23,** 353.
Shen, D. T., Gorham, J. R., Harwood, R. F., and Padgett, G. A. (1973). *Arch. Gesamte Virusforsh.* **40,** 375.
Siegl, G. (1976). "The Parvoviruses," Virology Monographs, Vol. 15 (S. Gard and C. Hallauer, eds.), Springer-Verlag, Berlin and New York.
Suffin, S. C., Prince, G. A., Muck, K. B., and Porter, D. D. (1979). *J. Immunol.* **123,**6.
Tabel, H., and Ingram, D. G. (1970). *Can. J. Comp. Med.* **34,** 329.
Tabel, H., Ingram, D. G., and Fletch, S. M. (1970). *Can. J. Comp. Med.* **34,** 320.
Trautwein, G. W. (1970). *Zentralbl. Veterinaermed. Reihe B* **16,** 262.
Trautwein, G. W., and Helmboldt, C. F. (1962). *Am. J. Vet. Res.* **23,** 1280.
Trautwein, G. W., Schneider, P., and Ernst, E. (1974). *Zentralbl. Veterinaermed. Reihe B* **21,** 467.

ADVANCES IN IMMUNOLOGY, VOL. 29

Age Influence on the Immune System

TAKASHI MAKINODAN AND MARGUERITE M. B. KAY

Geriatric Research, Education and Clinical Center (GRECC), Veterans Administration Wadsworth Medical Center, and the Department of Medicine, University of California at Los Angeles, Los Angeles, California

I. Introduction 287
II. Age-Related Changes in Immune Function 288
A. Morphology 288
B. Cell-Mediated Immunity 289
C. Humoral Immunity 291
III. Mechanisms of the Age-Related Alterations in Normal Immune Functions 295
A. Cellular Milieu 295
B. Cellular Changes 296
C. Thymus 308
IV. Prevention and Restoration 312
A. Tissue Removal 312
B. Dietary Manipulation 314
C. Genetic Manipulation 315
D. Cell Grafting 316
E. Chemical Therapy 317
V. Concluding Remarks 321
References 322

I. Introduction

Aging is characterized by a decline in the ability of individuals to adapt to environmental stress. This is exemplified physiologically by an inability to maintain homeostasis. Consequently, elderly individuals are confronted with biomedically enforced curtailment of normal activities. The magnitude of this problem is reflected in the rising cost of nursing care and chronic medical care, which has been minimally estimated to be about 60 billion dollars in 1976 (Butler, 1978). This problem, which was not as apparent less than two decades ago, is becoming more critical. A U. S. Bureau of the Census publication (1976) shows that the fastest growing sector of the American populaton has been and will continue to be the group of people aged 65 years and older, at least until the year 2020. Thus, while there were only 4 million people in the over 65-year-old group in our country in 1900, there are 24 million today. This group is expected to increase in size, reaching a population of 32 million in 2000 and 45 million by 2020. Comparable socioeconomic problems are being faced by most, if not all,

ISBN 0-12-022429-1

countries throughout the world. Clearly, methods must be found that can delay the onset or lessen the severity of the disorders associated with aging, and thereby extend the *productive* life-span. This challenge, as well as the fact that aging is one of the principal unsolved problems in cellular and molecular biology, are attracting many researchers into the discipline of gerontology.

The immune system is one of the most attractive systems for studies on aging for the following reasons:

1. Our understanding of its differentiation and developmental processes at the cellular, molecular, and genetic levels is more comprehensive than perhaps that of any other physiological system.
2. Age-related homeostatic perturbations permit dissection of certain features of the immune functions, analogous to the developmental phase of life.
3. Immune functions can be approached mechanistically and are amenable to restorative manipulations.
4. Alteration in age-related immune functions may contribute to our understanding of the pathogenesis of those diseases that show peak incidence late in life—including neoplasia, infectious diseases, and autoimmune–immune complex diseases.

II. Age-Related Changes in Immune Functions

A. Morphology

The first hint that normal immune cell functions may decline with age came from the findings of classical morphologists. They showed that the thymic lymphatic mass decreases with age, primarily as a result of atrophy of the cortex. The onset of this decrease coincided with the attainment of sexual maturity, and was found in both laboratory animals and humans (Boyd, 1932; Andrew, 1952; Santisteban, 1960). Subsequently, atrophy of the epithelial cells (Hirokawa, 1977) and decreased levels of thymus hormone(s) (Bach *et al.*, 1975) have been observed. Histologically, the cortex of an involuted thymus is sparsely populated with lymphocytes, which are replaced by numerous macrophages filled with lipoid granules (Hirokawa, 1977). In addition, infiltration of plasma cells and mast cells can be observed in the medulla as well as the cortex. Although the size of lymph nodes and spleen remains about the same after adulthood in individuals without lymphatic neoplasia (Andrew, 1952), the cellular composition of these tissues shifts so that there are diminished numbers of germinal centers and increased numbers of plasma cells and macrophages, as well as

increased amounts of connective tissue (Chino *et al.*, 1971; Peter, 1973; Good and Yunis, 1974).

B. Cell-Mediated Immunity

Cell-mediated immunity has been analyzed *in vivo* and *in vitro*. Overall, these studies show that various T cell-dependent functions decline with age.

1. In Vivo

In general, T cell-dependent cell-mediated functions decline with age. However, there are conflicting reports in the literature concerning certain functions in aging humans and animals (Giannini and Sloan, 1957; Krohn, 1962; Baer and Bowser, 1963; Gross, 1965; Stjernsward, 1966; Teller *et al.*, 1964; Novick *et al.*, 1972; Stutman *et al.*, 1972; Menon *et al.*, 1974; Nielson, 1974; Pazmino and Yuhas, 1973; Stutman, 1974; Goodman and Makinodan, 1975; Grossman *et al.*, 1975; Walters and Claman, 1975; Gardner and Remington, 1977; Girard *et al.*, 1977; Perkins and Cacheiro, 1977). For example, some investigators report a decrease in delayed skin hypersensitivity to common test antigens to which individuals have been previously sensitized (such as purified protein derivative of tuberculosis, streptokinase–streptodornase, *Candida, Trichophyton*). Others report no decrease except in those elderly persons with acute illness. It could be argued that any decrease in delayed hypersensitivity seen in the elderly reflects aging of the skin rather than the immune system. However, results of tumor cell rejection tests in aging mice were comparable, and since tumor cells were injected intraperitoneally in these tests, aging of the skin was not the limiting factor (Goodman and Makinodan, 1975). It is more likely that the conflicting results could be attributed to (*a*) utilization of only one skin test antigen to assess T cell function; (*b*) selection of skin test antigens (e.g., since only a fraction of the United States population has been immunized to tuberculosis, a negative result cannot be interpreted as reflective of defective immunological memory); and (*c*) selection of the population samples (i.e., some studies used hospitalized patients and medical clinic populations). Based on these considerations, it is recommended that a battery of common test antigens be utilized for the assessment of secondary delayed skin hypersensitivity reactions.

In contrast to its controversial role in secondary delayed skin reactions, T cell function in primary delayed skin reactions in response to antigens to which the individuals have *not* been sensitized previously, such as dinitrochlorobenzene, declines with age (Baer and Bowser, 1963; Gross, 1965; Grossman *et al.*, 1975).

Studies performed with mice show that various T cell-dependent functions decline with age (Krohn, 1962; Baer and Bowser, 1963; Gross, 1965; Stjernsward, 1966; Stutman *et al.*, 1968; Teague *et al.*, 1970; Pazmino and Yuhas, 1973; Menon *et al.*, 1974; Nielson, 1974; Stutman, 1974; Goodman and Makinodan, 1975; Gardner and Remington, 1977; Girard *et al.*, 1977; Perkins and Cacheiro, 1977). Resistance to challenge with syngeneic and allogeneic tumor cells *in vivo* decreases dramatically with age (Stjernsward, 1966; Perkins and Cacheiro, 1977). In these studies, the decline in response to phytohemagglutinin (PHA) measured *in vitro* approximated the decline in graft-versus-host (GVH) and tumor cell challenge measured *in vivo* (Perkins and Cacheiro, 1977).

Results showing no decline with age have also been observed in experimental animals. The conflict appears to be due in part to the inappropriate choice of the age of young and old animals. Thus, the few investigators who reported either no change or an increase with age in *in vivo* cell-mediated immune responses used long-lived mice [mean life-span (MLS), 24–30 months] and only two age groups were employed: mice 2 or 3 months old and 14–20 months old as reference young and old mice, respectively. With regard to the choice of mice 2 or 3 months old, they may not have yet attained their peak activity; e.g., in our laboratory we have found that the anti-sheep red blood cell (RBC) response of long-lived mice (MLS, 30 months) peaks at 6 months of age (Makinodan and Peterson, 1962) and their response to PHA at 8 months (Hori *et al.*, 1973). This would mean that comparisons of old mice were being made with immunologically immature mice, not with mature mice as had been anticipated. With regard to the choice of mice 14–20 months old (MLS, 24–30 months), they cannot be considered old for long-lived mice, but rather middle-aged, for this age range is equivalent to 41–58 human years at most, i.e., (14/24) × 70 years to (20/24) × 70 years. It should also be noted that the MLS of even an inbred strain of mice can vary among laboratories; e.g., C57B1/6J mice with a MLS varying from 97 to 120 weeks (Storer, 1966; Walford, 1976). Thus, a 90-week-old C57B1/6J could be considered "old" in one laboratory and "middle-aged" in another. In view of these considerations, information on MLS and age of peak response should be included in age-related studies and thereby eliminate many of the apparently contradictory findings.

2. *In Vitro*

The findings from experiments performed *in vitro* show that the proliferative capacity of T cells of humans and rodents, in response to

PHA, concanavalin A (Con A), and allogeneic target cells, declines with age (Pisciotta *et al.*, 1967; Waldorf *et al.*, 1968; Adler *et al.*, 1971; Heine, 1971; Hallgren *et al.*, 1973; Hori *et al.*, 1973; Konen *et al.*, 1973; Roberts-Thomson *et al.*, 1974; Weksler and Hutteroth, 1974; Fernandez *et al.*, 1976; Bach, 1977; Inkeles *et al.*, 1977a; Kay, 1978a; Becker *et al.*, 1979). Some investigators have not observed a decrease in PHA or Con A response in humans with age (Kay, 1979b; Portaro *et al.*, 1978; Weiner *et al.*, 1978). There has been one report that the response to PHA in mice shows only a minimal decline with age (Shigemoto *et al.*, 1975). However, in this report, "middle-aged" rather than old C57B1/6J mice were used; i.e., mice 15–20 months old were used when the MLS of the mice in that laboratory was 24 months.

There are also conflicting reports in the literature on the effect of age on the mixed lymphocyte culture (MLC) reaction. Some investigators reported a marked decrease with age (Adler *et al.*, 1971; Hori *et al.*, 1973). Others reported that cells from old mice are at least as efficient as cells from young mice, as both responding and stimulating cells in the MLC reaction (Walters and Claman, 1975). However, the same cells showed a decreased GVH index (Walters and Claman, 1975). Some of these discrepancies may have arisen through the use of mitomycin-treated cells as the stimulating cells in the MLC reaction, for the possibility exists that mitomycin may have leaked from the stimulating cells, and responding cells from old animals may be more susceptible to this drug than are cells from young animals. It would seem, therefore, that the use of either X-rayed or hybrid cells from donors of one age group as the stimulating cells would be preferred to assess the MLC index when studying the effects of age on cell-mediated immunity.

C. Humoral Immunity

Normal B cell immune functions reflective of humoral immunity have been analyzed in terms of circulating levels of immunoglobulins (Ig), isoantibody, and natural heteroantibody, in terms of mitogen and antigen-induced immune response and in terms of avidity of antibodies.

The total number of B cells either does not change or increases with age in mice, depending on the strain and tissue (Adler *et al.*, 1971; Stutman, 1972; Mathies *et al.*, 1973; Makinodan and Adler, 1975; Callard *et al.*, 1977; Haaijman and Hijmans, 1978; Kay *et al.*, 1979a), and does not change appreciably with age in humans (Weksler and Hutteroth, 1974; Becker *et al.*, 1979). However, changes can occur among organs within individuals. Thus, Haaijiman and Hijmans (1978) re-

ported that the total number of B cells in the spleen and lymph nodes of long-lived CBA mice decreases significantly with age, but that the decrease is compensated by a proportional increase in the bone marrow. The number of B cells in spleen and lymph nodes of other strains and hybrids of mice tends to increase (Kay, 1979a). In humans, studies, which have been limited primarily to circulating B cells, indicate that the number of B cells remains relatively constant (Diaz-Jouanen *et al.*, 1974). Unfortunately, we do not know as yet whether the number of circulating B cells corresponds to the number in the spleen, lymph nodes, and bone marrow, the last of these appearing to be the major source of B cells of aging individuals (Turesson, 1976).

Subpopulations of B cells may fluctuate, as reflected in the levels of individual serum immunoglobulin classes. Thus, in humans, the level of serum IgA and IgG tends to increase with age, whereas that of serum IgM tends to remain constant or to decrease slightly (Haferkamp *et al.*, 1966; Lyngbye and Kroll, 1971; Radl *et al.*, 1975; Buckley *et al.*, 1974).

Studies on age-related change in secretory Ig, which have been sparse despite the obvious role of this Ig in respiratory diseases, suggest that the nasal wash of IgA concentration decreases with age in individuals both with and without respiratory infections (Alford, 1968). It is clear that more studies are needed in this important epidemiological area of aging.

An increasing frequency of homogeneous immunoglobulins in the sera of aging mice and humans without B cell malignancy has been repeatedly observed (Hallen, 1966; Zawadski and Edwards, 1972; Waldenstrom, 1973; Radl and Hollander, 1974; Radl *et al.*, 1975). This condition is most often designated as benign monoclonal gammopathy or idiopathic paraproteinemia (IP). In humans, there is a clear age-related increase from 0% in the third decade up to 19% in the tenth decade of life (Axelsson *et al.*, 1966; Englisova *et al.*, 1975; Radl *et al.*, 1975), and in mice from 0% (less than 3 months) to 60% (greater than 24 months) (Radl *et al.*, 1979b). The majority of the Ig belong to the IgG class, those of the IgM and IgA classes being less frequent. The etiology, mechanisms, and significance of this particular form of immunoglobulin production have not yet been resolved. However, Radl *et al.* (1979a) showed that spleen and bone marrow cells can be successively passaged serially for 3–4 times in either irradiated or unirradiated syngeneic young mice. This would indicate that IP is caused by impairment of intrinsic cellular factors, not to factors extrinsic to the immune system. Radl *et al.* (1979a) then demonstrated that the frequency of IP can be increased by neonatal and adult thymectomy

in both IP-susceptible C57B1 and IP-resistant CBA mice, suggesting that the impairment of intrinsic cellular factors may be reflective of alteration in the T cells.

Circulating levels of isoantibody and natural heteroantibody, which have been assessed systematically in the human, have been shown to decline with age, starting shortly after the thymus begins to involute (Friedberger *et al.*, 1929; Thomsen and Kettel, 1929; Paul and Bennell, 1932; Furuhata and Eguchi, 1955; Rowley *et al.*, 1968; Somers and Kuhns, 1972) and after the level of serum thymic hormone begins to decline (Bach *et al.*, 1973).

In contrast to the observed decline with age in iso- and heteroantibody levels, autoantibodies increase in frequency with age (Rowley *et al.*, 1968; Roberts-Thomson *et al.*, 1974; Diaz-Jouanen *et al.*, 1975). Autoantibodies to thyroglobulin, nuclear proteins and DNA, and globulin (rheumatoid factor) have been detected in aging humans (Rowley *et al.*, 1968; Roberts-Thomson *et al.*, 1974; Diaz-Jouanen *et al.*, 1975; Shu *et al.*, 1975). This increase in facultatively "pathologic" autoantibodies is not associated with an increase in the number of individuals with autoimmune disease (Kay and Makinodan, 1978). The effect of age on "physiologic" autoantibodies, such as those involved in the removal of senescent and damaged cells, has not yet been investigated (Kay, 1975a, 1977, 1978d; Kay and Makinodan, 1978).

Earlier studies on B cell mitogenic response to bacterial lipopolysaccharide (LPS) have revealed minimal decrease with age in mice (Makinodan and Adler, 1975). However, a more comprehensive study involving eight strains and hybrids subsequently revealed that the LPS response of lymph node and spleen cells can decrease, remain the same, or even increase with age, depending on the strain or hybrid tested (Kay, 1978a; Kay *et al.*, 1979a). An age-related change in response to B cell mitogens in humans has not been observed.

Aging rodents, which have been used primarily for the study of antibody responses, show that primary, but not necessarily secondary, antibody response decreases with age (Makinodan and Peterson, 1962; Goullet and Kaufman, 1965; Stjernsward, 1966; Metcalf *et al.*, 1966; Wigzell and Stjernsward, 1966; Morton and Seigel, 1969; Makinodan *et al.*, 1971b; Finger and Emmerling, 1973; Kishimoto *et al.*, 1976; Segre and Segre, 1976a,b; Callard and Basten, 1978). The apparent discrepancy in the secondary antibody response studies appears to be due not only to strain differences, but also to differences in the age, the dose of antigen, the interval between primary and secondary antigenic stimulation, and other variables. In any event, the onset of decline in antibody response can occur as early as when the

thymus begins to involute. This would suggest that with many types of primary antibody responses, aging may be affecting the T cells that regulate antibody response, but not necessarily the antigen-specific B cells. This suspicion has been verified partially by the subsequent demonstration that the antibody response of B cells to complex antigens generally requires the help of T cells (e.g., Claman and Chaperon, 1969); and that while the antibody response to the T-independent antigens type III pneumococcal polysaccharide, and LPS, does not decline appreciably with age in the relatively long-lived BALB/c and C3H mice (Smith, 1976), it does so in the relatively short-lived SJL/J mice. These latter mice, however, are predisposed to Hodgkin-like reticulum cell neoplasia and so they may not serve as good animal models for immunodeficiency as it occurs in long-lived mice and humans.

There have been exceptions to the "rule"; i.e., reports showing no decline in primary antibody response, especially against bacterial and viral vaccines (Sabin, 1947; Davenport *et al.*, 1953; Fulk *et al.*, 1970; Solomonova and Vizeb, 1973; Kishimoto *et al.*, 1976). Two explanations can be offered: (*a*) the individuals have been previously exposed to the antigen and therefore are, in fact, mounting a secondary response; and (*b*) the reaction is to antigens that do not require the participation of T cells; i.e., they are T-independent antibody responses.

Another exception is the observation by Naor *et al.*, (1976), who found that the T cell-independent humoral immune response can even increase with age in long-lived mice, as judged by their response to hapten-conjugated mouse red blood cells. This would suggest that, as mice age, they may be losing their capacity to suppress effectively the antihapten and antiself antigen responses. If so, the often observed age-related increase in the frequency of individuals with autoantibodies may be mediated in part to loss in the capacity to suppress effectively self antigens modified by naturally occurring agents, including viruses, fungi, and bacteria. Support of this notion comes from Meredith *et al.* (1979), who showed that *Escherichia coli* LPS, a polyclonal B cell activator, can stimulate more mouse red blood cell autoantibody secreting B cells from old than from young mice.

A significant decrease in the avidity of antibodies, as measured by the plaque inhibition assay, has been detected in aging mice (Goidl *et al.*, 1976; Kishimoto *et al.*, 1976; Naor *et al.*, 1976; Doria *et al.*, 1978; Fujiwara and Kishimoto, 1979) but not universally (Zharhary *et al.*, 1977). The discrepancy cannot be ascribed to differences in strains,

for the conflicting results among laboratories were observed even within an inbred strain. This would suggest that differences in the animal facility and the relative age of the animals could be the major contributory factors for the discrepancy.

III. Mechanisms of the Age-Related Alterations in Normal Immune Functions

The decline in immune capacity of aging mice could result from changes in the immune cells, changes in their milieu, or both. To differentiate between the influence of cells from that of their milieu, the cell transfer method was employed. In this assay, immunocompetent cells from young and old mice undergo anti-sheep red blood cell responses in immunologically inert old and young syngeneic recipients, respectively (Albright and Makinodan, 1966, 1976; Price and Makinodan, 1972a,b; Goodman and Makinodan, 1975). The results showed that although both types of change affect the immune response, much of the normal age-related decline can be attributed to changes in the immune cells (Price and Makinodan, 1972a,b).

A. Cellular Milieu

Systemic, noncellular factors were shown to influence the immune response (Price and Makinodan, 1972a,b). Spleen cells from young mice were cultured with the test antigen either in the young (or old) recipient's spleen by the cell transfer method, or in the recipient's peritoneal cavity by the cell-impermeable diffusion chamber method (Goodman *et al.*, 1972). A twofold difference in response was observed between young and old recipients at both sites, indicating that the factor(s) is systemic. The fact that the effect was observed in cells grown in cell-impermeable diffusion chambers further indicates that a noncellular factor is involved. A comparable twofold difference was observed also when bone marrow stem cells were assessed in the spleens of young and old syngeneic recipients (Chen, 1971), indicating that the systemic, noncellular factor(s) influences both lympho- and hematopoietic processes.

The factor(s) could be a deleterious substance of molecular or viral nature, or it could be an essential substance that is deficient in old mice. Factors of both types probably change with age, and, further, several factors of each type may exist. Unfortunately, this area of research has not progressed as rapidly as anticipated, because a simple, sensitive *in vitro* assay to analyze mouse sera has not yet been perfected. Thus, for example, it is unclear why normal adult mouse

serum, but not fetal bovine serum, is toxic for mouse immunocompetent cells grown *in vitro*.

B. Cellular Changes

It is well established that the rate and magnitude of immune responses are subject to a variety of cell regulatory controls. Therefore, precaution should be taken to identify the limiting cell type(s) in age-related altered immune responses. This can be done by assessing the cell type in question in the presence of optimal numbers of the other cell types, derived from reference young syngeneic donors that constitute the functional unit. Without such a precaution, it would be difficult to determine the extent to which the observed age changes are due to the effector cells and the extent to which they are due to the regulatory cells. Accordingly, the activity of reference immune cells from adult individual mice was assessed in the presence of immune cells from individual old mice (Makinodan *et al.*, 1976). A response of young–old cell mixtures that was less than the sum of the responses given by pure young cells and pure old cells would indicate that the decreased response of old individuals was due to an increase in suppressor cells. If the response of the mixture was comparable, it would indicate that the decreased response of old individuals was caused by a decrease in their functional efficiency. If the response of the mixture was higher, it would indicate that the decreased response of old individuals was caused by a selective loss of one type of immune cell that exists in excess in young individuals. The results (Fig. 1) showed that, indeed, all three types of responses can occur. About 60% of responses of the individual old mice with reduced activity appeared to have been caused by an increase in the number or activity of suppressor cells, about 10% by a decrease in the functional efficiency of effector cells or their progenitors, and about 30% by an absolute or relative loss of one or more cell types. This supports the contention that although there may be only one underlying mechanism responsible for the loss of immunologic vigor with age, it would be expressed differently by aging individuals, and this would contribute to the increased variability in immunologic performance with age. Furthermore, these results discourage the pooling of tissues from individual old mice, a practice routinely carried out by many for convenience—otherwise, biased sampling data will be generated.

These findings demonstrate the complexity of approaching the age effect of the immune system at the level of tissues. Studies of the four major immune cell types have proceeded further, as indicated subsequently.

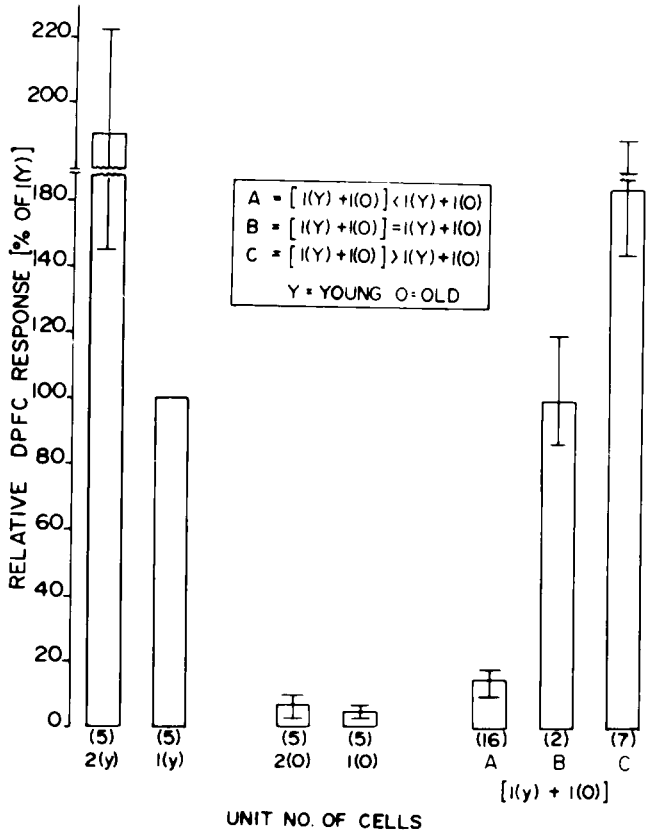


FIG. 1. Types of direct plaque-forming cell (DPFC) response by mixtures of young (Y) and old (O) spleen cells. A: Reduced response suggests that an increase in suppressor activity is responsible for reduced DPFC activity by old (O) spleen cells. B: Additive response suggests alteration(s) is responsible for reduced DPFC activity by old spleen cells. C: Elevated response suggests selective loss (number or function) of one or more of component cells is responsible for reduced DPFC activity by old spleen cells. 1(Y): 5×10^6 young spleen cells; 2(Y): 10×10^6 young spleen cells. Vertical brackets signify 1 SEM. Numbers in parentheses signify sample size. For comparative purposes, the response of 1(Y) was arbitrarily set at 100%. (From Makinodan *et al.*, 1976.)

1. Stem Cells

Stable, intrinsic alterations in the pluripotent stem cells will affect the lymphocyte population and, ultimately, its functions. Thus, there has been a continuing search for alteration in the stem cells. The number of stem cells with the ability to colonize in the spleen, commonly referred to as spleen colony-forming units (CFU-S) per unit bone marrow cells, decreases with age in some, but not all, strains of mice (Coggle and Proukakis, 1970; Chen, 1971; Davis *et al.*, 1971; Silini and Andreozzi, 1974; Kay, 1978a; Kay *et al.*, 1979a). Because the number of nucleated cells in the bone marrow tends to increase with age (Coggle and Proukakis, 1970; Chen, 1971; Silini and Andreozzi, 1974; Kay *et al.*, 1979a), the total number of CFU-S in the bone marrow does not change significantly in those strains showing a decrease in CFU-S concentration.

The transplantation potential of adult stem cells is less than that of young cells (Ogden and Micklem, 1976). This was demonstrated by serial transplantation studies in which stem cells from young and adult donor mice, each with distinct chromosome markers that allowed them to be differentiated from each other and from recipient cells, were passaged *in vivo*. Cells from adult donors exhibited impaired regenerative capacity several passages earlier than did young cells.

Unlike stem cells passaged *in vivo*, whose self-replicating ability can be exhausted (Cudkowicz *et al.*, 1964; Siminovitch *et al.*, 1964; Lajtha and Schofield, 1971), stem cells can self-replicate *in situ* throughout the natural life-span of an individual (Harrison, 1975). However, both the ability of stem cells to expand clonally and their rate of division decreases with age (Micklem *et al.*, 1973; Albright and Makinodan, 1976; Kay *et al.*, 1979a), as does their ability to repair X-ray induced damage (Chen, 1971), their ability to home into the thymus (Tyan, 1977), and their rate of B cell formation (Farrar *et al.*, 1974; Kishimoto *et al.*, 1976). In contrast to studies in which the self-replicating and differentiation activities of aging stem cells were assessed kinetically, studies in which the function of syngeneically transplanted old stem cells was assessed 3–12 months after transplanation indicate that old stem cells can reconstitute *unstressed* recipient mice to the same levels as those derived from young mice (Harrison *et al.*, 1977, 1978). However, these results should be taken with some degree of caution because they also showed that the variability of responses among individuals receiving young or old stem cells was severalfold greater than that *between* the two groups. Moreover, the bone

marrow of old mice is known to contain significantly greater numbers of B cells than the bone marrow of young mice (Farrar *et al.*, 1974; Haaijman and Hijmans, 1978), which may have contributed to the similarity of the response of the transplanted cells. In any event, the results of this study together with those of the kinetic studies indicate that the generation time of old stem cells and/or the differentiation time of their progenies are increased.

The observed kinetic alterations could be due in part to changes in the stem cells caused by the cellular milieu, for they are responsive to differentiation-homeostatic factors. Thus, for example, when attempts were made to reverse the age-related kinetic properties of old stem cells by enabling them to self-replicate in syngeneic young recipients for an extended period, they still behaved as old stem cells kinetically (Albright and Makinodan, 1976). However, when young stem cells were allowed to self-replicate in syngeneic old recipients for 1 month, they began to behave as old stem cells kinetically. These results suggest that once stem cells have undergone subtle, intrinsic kinetic alteration in an aging mouse, it cannot be reversed by tranplanting the cells in a young syngeneic recipient. Another observation reflective of an alteration intrinsic to the stem cells is that of Chen (1971), who found that old stem cells are inferior to young stem cells in their ability to repair X-ray induced DNA damage, even when they were allowed to grow in young recipients.

The nature of the cellular milieu is not known. However, lymphokines from a subpopulation of T cells may be involved in a positive manner. Evidence for this is derived from studies showing that hematopoiesis in heavily irradiated recipients reconstituted with parental stem cells is augmented by injection of T cells (Goodman and Shinpock, 1968; Pritchard *et al.*, 1975); and that an anti-theta sensitive regulatory cell, which is present in normal adult bone marrow, spleen, and thymus, is required for the promotion of differentiation of hematopoietic stem cells into erythrocytes (Wiktor-Jedrzejczak *et al.*, 1977). Thus, alterations in certain T cell subpopulations with age might explain some of the extrinsic changes that adversely influence stem cell kinetics.

As to the issue of whether age-related kinetic alterations of stem cells are likely to affect the immune capacity of old individuals, we believe that they are crucial to the old individual in stress. For example, immunologically immature young mice ($\leq$ 2 months of age) and immunologically inadequate old mice develop autoantibodies to erythrocytes following viral infection. However, the young mice with autoantibodies do not become anemic, whereas the old mice do (Kay,

1977, 1978b, 1979a; Kay *et al.*, 1979b). Thus, when stressed, as with an infection, old individuals may not be able to maintain homeostasis by increasing cellular production to compensate for increased cellular destruction. The kinetic limitations on stem cell reserve may also account for the clinical observation that elderly individuals with sepsis frequently do not have an elevated white cell count, although they may have a shift to less mature leukocytes in their peripheral blood smears. The significance of young stem cells was also demonstrated in an immunorestorative study (Hirokawa *et al.*, 1976), which demonstrated that the grafting into old mice of newborn thymus is effective in elevating their immunologic responsiveness for an extended period of time, *provided* young stem cells are present.

Finally, it should be noted that most stem cells are in a resting, noncyclic state at any given time (Epifanova and Terskikh, 1969; Lajtha and Schofield, 1971), which would mean that a significant number may never be called to self-replicate or differentiate until late in the life-span of the idividual. Hence, they would be as vulnerable to genetic damage as the oogonial cells, the neurons and the cardiac muscle cells. Some of these noncycling stem cells may lack the ability to repair genetic injuries, affecting their proliferative and differentiative performances. Such stem cells, in contrast to the cycling stem cells, are more likely to generate defective clones of effector immune cells. In view of these considerations, future studies on stem cell aging should differentiate those that are cycling from those in an extended noncycling state.

2. *Macrophages*

Many of the earlier studies on the mechanism of loss in immunologic vigor with age were focused on macrophages. Because macrophages confront antigens before the T and B cells, any defect in them could decrease immune functions without appreciable changes in the antigen-specific T and B cells. These studies showed that neither the number of macrophages in the peritoneum (Shelton *et al.*, 1970) nor their handling of antigens during both the induction of immune responses and phagocytosis is adversely affected by age (Perkins, 1971; Heidrick and Makinodan, 1973; Callard, 1978). For example, the phagocytic activity *in vitro* of peritoneal macrophages of old mice was equal to or better than that of young mice (Perkins, 1971); and the activity of lysosomal enzymes in splenic and peritoneal macrophages increased rather than decreased with age (Heidrick, 1972; Platt and Pauli, 1972). The latter property, especially, could be reflective of qualitative alterations in certain types of macrophages. The ability of

antigen-laden peritoneal macrophages of old mice to initiate primary and secondary antibody responses *in vitro* was comparable to that of young mice (Perkins, 1971). The capacity of splenic macrophages and other adherent cells to cooperate with T and B cells in the initiation of antibody response *in vitro* was unaffected by age (Heidrick and Makinodan, 1973). The ability of macrophages to support and regulate lymphocyte responses to mitogens and antigens was also unchanged with age (Callard, 1978). This would suggest that the preferential immunorestorative effect of 2-mercaptoethanol on old cells over that of young cells (Makinodan, 1979) may not be reflective of age-related alteration in the macrophages as suspected earlier.

When the antigen-processing ability of macrophages was indirectly assessed by injecting young and old mice with varying doses of sheep red blood cells, it was found that the slope of the regression line of the antigen dose-antibody response curve was lower, and the minimum dose of antigen needed to generate a maximum response was significantly higher, for old mice (Price and Makinodan, 1972a). Such results could be explained by assuming that the antigen-processing macrophages prevented antigen-sensitive T and B cells from responding maximally to limiting doses of the antigen (Lloyd and Triger, 1975). It is possible that an age-associated increase in phagocytic efficiency (Heidrick, 1972) contributed to the decrease in antigen-processing efficiency. The reduced antigen-processing activity is reflected by the failure of antigens to localize in the follicles of lymphoid tissues of antigen-stimulated old mice (Metcalf *et al.*, 1966; Legge and Austin, 1968). One clinical implication of these results is that the poor immune surveillance noted against low doses of certain syngeneic tumor cells (Penn and Starzl, 1972) could be due in part to the failure of T and B cells in an aging individual to confront neoplastic cells during their clonal emergence, because of the inability of macrophages to initially "process" the tumor antigens. It also could explain why the resistance to allogeneic tumor cell challenge can decline with age by more than 100-fold in mice manifesting only a 4-fold decline in T cell-mediated cytolytic activity against the same tumor cells (Goodman and Makinodan, 1975).

Because splenic dendritic cells can now be sorted and grown *in vitro* (Steinman *et al.*, 1979), it may be prudent to reinvestigate the role of old accessory cells in the regulation of immune responses by assessing these dendritic cells, rather than peritoneal phagocytic cells, as commonly done in the past. The reasons are 2-fold: (*a*) the dendritic cells, which are adherent but not phagocytic, may be more involved in the regulation of immune response *in situ* than phagocytic

cells; and (*b*) the decrease with age in follicular localization of antigens (Metcalf *et al.*, 1966; Legge and Austin, 1968) is reflective of diminished antigen trapping by the dendritic cells.

3. *B Cells*

As discussed earlier, the number of B cells in humans and mice does not change appreciably with age (Stutman, 1972; Weksler and Hutteroth, 1974; Callard *et al.*, 1977; Haaijman and Hijmans, 1978; Becker *et al.*, 1979). If anything, the number in the spleen and lymph nodes tends to increase slightly (Kay, 1978a; Kay *et al.*, 1979a). Although the total number of B cells remains relatively stable, the size of certain subpopulations of B cells appears to change with age. Support for this view comes from the observation that the number of B cells responsive to certain T-cell-independent antigens decreases slightly with age in a long-lived hybrid mouse (Price and Makinodan, 1972a); as well as from human cross-sectional and longitudinal studies in humans showing that serum IgG and IgA and benign monoclonal gammopathies tend to increase with age (Haferkamp *et al.*, 1966; Hallen, 1966; Kalff, 1970; Lyngbye and Kroll, 1971; Radl *et al.*, 1975; Waldenstrom, 1973; Buckley *et al.*, 1974; Radl and Hollander, 1974). An increase in IgG_1 and IgG_3 subclasses is responsible for the elevated IgG level (Kalff, 1970). An increase in IgG_1 and IgG_{2b} subclasses has been observed in a long-lived strain of mice (Haaijman *et al.*, 1977). Further, the number of colony-forming B cells in the peripheral blood of humans decreases with age (Kay, 1979b).

The responsiveness of B cells to stimulation with certain T cell-dependent antigens decreases strikingly with age (Makinodan and Peterson, 1962; Makinodan *et al.*, 1971a). The responses of young and of old mice were systematically evaluated by limiting dilution and dose-response methods (Price and Makinodan, 1972a,b). These studies revealed that the decline is caused by (*a*) a decrease with age in the number of antigen-sensitive immunocompetent precursor units (IPU), which are made up of two or more cell types in various ratios (e.g., T_1-M_1-B_1, T_2-M_1-B_1, etc.) (Groves *et al.*, 1970); and (*b*) a decrease in the average number of antibody-forming functional cells generated by each IPU, or the immunologic burst size (IBS). We do not know the cause(s) for the reduction with age in both the relative number of IPU and the IBS. It could be due to an increase in the number of regulatory cells which can inhibit the precursor cells making up the IPU from interacting with each other, as well as inhibiting the proliferation of B cells. This idea is supported by results demonstrating that the number of Ig-bearing B cells remains constant with age (e.g., Adler *et al.*,

1971; Mathies *et al.*, 1973; Stutman, 1972; Diaz-Jouanen *et al.*, 1974; Callard *et al.*, 1977) and that the proliferative capacity of mitogen-sensitive B cells also remains unaltered with age (Makinodan and Adler, 1975). It could also result from an alteration in the ability of certain B cells to interact with other cells making up the IPU and in their ability to respond to homeostatic factors during differentiation.

Qualitative changes in the B cells also appear to be occurring with age. For example, the ability of old B cells to respond to T cell-dependent antigens, even in the presence of young T cells, is impaired (Friedman and Globerson, 1976; Callard and Basten, 1978). The alteration may be at the membrane surface level. Thus, in their studies on Fc receptor-mediated immunoregulation, Scribner *et al.*, (1978) have found that the decline in B cell regulation with age is in part intrinsic to the receptor-mediated signaling mechanism. More recently, Woda and Feldman (1979) demonstrated that the rate of capping and shedding of cross-linked surface immunoglobulins by B cells was slower in old rats than in young rats, and this age-related kinetic alteration was associated with a decrease in the density of surface immunoglobulins. Decrease in the density of surface immunoglobulins may be characteristic of aging B cells, because Tada has also noted such a pattern of change with age in circulating B cells of humans (Tada *et al.*, 1978b). Interestingly, Biro and Beregi (1979) noted that B cells of old, but not of young, rats possess altered mitochondria; i.e., mitochondria that are often swollen, containing myelin-like fibers, with reduced numbers of cristae.

4. T Cells

As with B cells, all three possible changes that could cause decline with age in T cell functions have been detected: loss in number, shift in subpopulations, and qualitative change.

Decrease in the number of T cells has been detected in certain, but not all, strains and hybrids of aging mice examined; and, furthermore, the pattern and magnitude of change in the number of T cells are dependent upon the tissue and organ: i.e., whether it is in the spleen, lymph nodes, bone marrow, or thymus (Stutman, 1974; Kay, 1978a; Kay *et al.*, 1979a,b). Thus, Kay *et al.* (1979a), in their comprehensive analysis of eight different inbred strains and hybrid strains of aging mice, found that overall the number of T cells did not change with age, except for those in the thymus, which decreased.

In humans, the number of circulating T cells has been reported either to decrease progressively after adulthood (Carosella *et al.*, 1974; Foad *et al.*, 1974; Smith *et al.*, 1974) or remain the same (Weksler and

Hutteroth, 1974; Inkeles *et al.*, 1977b; Kay, 1979b). However, the absolute number of colony-forming, circulating T cells in humans decreases with age (55–82 years) to a level that is about 15% of that of young adults (21–35 years) (Table I) (Kay, 1979b).

The evidence for qualitative changes with age is increasing. At the membrane level, it would be anticipated that surface receptors would be scrutinized because cell-to-cell interactions in immune responses are mediated through surface receptors. Two observations support the view that surface receptors change with age—one indicating loss, and the other an emergence of new receptors. Thus, Brennan and Jaroslow (1975) noted that the surface density of theta receptors on T cells de-

TABLE I
EFFECT OF AGE ON HUMAN CLONABLE PERIPHERAL BLOOD T CELLS[a,b]

Age (years)	Number of colonies[c]	Age (years)	Number of colonies
I. Macrocolonies (256–2144 cells per colony)			
21	120 ± 18	55	64 ± 2
24	120 ± 2	56	6 ± 2
24	112 ± 4	58	19 ± 5
30	118 ± 2	59	28 ± 4
35	140 ± 13	59	30 ± 2
		65	3 ± 1
		68	0
		70	13 ± 11
		82	10 ± 2
II. Microcolonies (32–256 cells per colony)			
21	260 ± 3	55	586 ± 5
24	119 ± 16	56	142 ± 90
24	76 ± 8	58	199 ± 99
30	426 ± 6	59	25 ± 0
35	496 ± 28	59	44 ± 8
		65	20 ± 6
		68	21 ± 3
		70	289 ± 69
		82	179 ± 9

[a] From Kay (1979b).

[b] T cell clones were assessed kinetically on days 3, 6, 9, and 12 after initiation of culture (5×10^5 cell per culture). The peak response for each individual is presented.

[c] Data are presented as the mean number of colonies per 10^6 cells plated from 3–5 cultures per individual ± one standard error of the mean.

creased with age, as judged by immunofluorescence, and, further, that the theta receptors, visualized as a continuous ring in young cells, were patchy to faintly visible incomplete rings in old T cells. The possibility that such changes may be reflective of extrinsic rather than intrinsic changes comes from the observation of Rivnay *et al.*, (1979). They found that there can be a 20% increase with age in the microviscosity in the lymphocyte plasma membrane, which is associated with an increase with age in the serum cholesterol: phospholipid ratio, and that such changes as can be induced can decrease the responsiveness of T cells to Con A stimulation (Rivnay *et al.*, 1978).

Evidence for the emergence of new receptors comes from two observations. Gozes *et al.*, (1978) showed that young mice can undergo syngeneic GVH response by injecting into them syngeneic cells from old donors. Callard *et al.*, (1979) then showed that young mice can also undergo syngeneic mixed lymphocyte reaction and synthesize cytotoxic antibodies by injecting into them syngeneic cells from old donors. These latter observations have obvious implications for certain diseases of aging, particularly neoplasia and autoimmune–immune complex diseases, for it has been shown that continuous GVH-like reactions can increase the incidence of lymphoid tumor formation and autoimmunity (Gleichmann *et al.*, 1976).

Intracellular changes have also been detected. At the cytoplasmic level, both morphologic and functional changes have been observed. Thus, Beregi (1979) observed electron microscopically swollen mitochondria containing myelin-like structure with reduced numbers of cristae in sheep RBC-rosetting T cells of old, but not of young, humans. Functionally, there seems to be an imbalance in the level of cyclic adenosine 3′,5′-monophosphate and cyclic guanosine 3′,5′-monophosphate in resting and T cell-specific mitogen-stimulated cells in older mice (Heidrick, 1973; Tam and Walford, 1978). Since the adenyl and guanyl cyclases are activated by modulation of surface receptors (Earp *et al.*, 1977), these observations provide further evidence of membrane-cytoplasmic abnormalities in old T cells.

At the nuclear level is the demonstration that the frequency of PHA-responsive hypodiploid T cells increases with age, the loss of X and Y chromosomes being most prevalent (Jacobs and Court Brown, 1961; Hamerton *et al.*, 1965; Neurath *et al.*, 1970; Mellow *et al.*, 1974; Awa, 1975; Jarvik *et al.*, 1976). Another is the observation that the old PHA-stimulated T cells are not as efficient as young PHA-stimulated T cells in their capacity to bind actinomycin (Preumont *et al.*, 1978). This suggests that chromatin structures of PHA-responsive T cells are undergoing with age. Old PHA-stimulated T cells also show a decrease in

their ability to incorporate acetate (Oh and Conard, 1972), indicating that their histone metabolism may be undergoing a change with age that can affect their nuclear activities. Finally, at the nuclear-cytoplasmic level, Kay (1979b) observed that *clonable* circulating T cells exhibited a delay in initiating division and decreased proliferation capacity. Similarly, Tice *et al.* (1979) found that a mean cell cycle duration of circulating T cells of old humans is longer than that of young adults and that this is due to an extension of the G_1 phase of the cell cycle. Extension of the cell cycle duration due to an increase in the G_1 phase has also been detected in other turning-over cells, including the gut epithelium (Lesher *et al.*, 1961; Thrasher, 1971) and fibroblasts aged *in vitro* (Macieiro-Coelho *et al.*, 1966) and *in situ* (Schneider and Mitsui, 1976). The exact cause for the diminished proliferative capacity of T cells and other turning-over cells remains unsolved. However, with old T cells, it has been possible to enhance their proliferative capacity by mixing T cell-specific mitogens with 2-mercaptoethanol; i.e., the response to the mixture of stimulants is greater than that of the sum of the responses to the individual stimulants (Makinodan, 1979). This would indicate that the age-related qualitative changes responsible for the decline in the proliferative capacity of T cells with age can be reversed.

Evidence for the shift in subpopulations of T cells with age include the following findings.

1. The proportion of short-to-long lived theta-bearing T cells in the blood, lymph nodes, and spleen decreases with age in C3H mice (Olsson and Claesson, 1973). Since the total number of T cells does not change appreciably with age in this strain of mice (Kay *et al.*, 1979a) this suggests that the number of long-lived T cells is increasing at the expense of short-lived T cells.

2. The decrease in mitogenic response of mouse T cells with age does not appear to be caused by the decrease in the number of T cells with theta antigenic receptors, T cells with mitogen receptors, the number of mitogen receptors per T cell, or the mitogen receptor binding affinities (Hung *et al.*, 1975a,b; Callard and Basten, 1978), nor to an altered cell cycle time (Merhav and Gershon, 1977). These observations suggest that the number of T cells that are less responsive to T cell-specific mitogen is increasing with age at the expense of those which are more responsive.

3. In a long-lived hybrid strain of mice, the decline with age in the rate of mitogenic response to PHA is faster than that to Con A (Meredith *et al.*, 1975). This would suggest that the proportion of PHA-re-

sponsive T cells with surface receptors $Ly1^+2^+$, Ia^-, and Qal^+ is decreasing faster with age than the Con A-responsive T cells with surface receptors $Ly1^+2^-$, Ia^+, $Qa1^{+/-}$ (Hirst *et al.*, 1975; Neiderhuber *et al.*, 1976; Stanton *et al.*, 1978).

4. Density distribution analysis shows that the frequency of less dense cells increases at the expense of the more dense cells (Makinodan and Adler, 1975). This density shift within the lymphocyte population can also can be seen in young mice shortly after they are immunized with foreign red cells or allogeneic lymphocytes and in tumor-bearing mice. In these latter cases, however, the spleen cell number increases, whereas in unimmunized old mice it does not. This suggests that there is a relative increase with age in immature T cells at the expense of mature T cells.

5. Suppressor T cells have been seen either to decrease or increase with age. The evidence that suppressor T cell activity declines with age was derived from studies of short-lived NZB and related mice (Barthold *et al.*, 1974; Gerber *et al.*, 1974). A C-type virus infection, however, plays a major role in the pathogenesis of the disease of NZB. The causes and mechanisms of both the decline in normal immune functions and the increase in immunodeficient diseases in these animal models may be quite different from those occurring in long-lived mice. The reason for this reservation is that the relative number of supressor T cells increases with age in long-lived mice (Halsall *et al.*, 1973; Stutman, 1974; Goidl *et al.*, 1976; Makinodan, *et al.*, 1976; Segre and Segre, 1976a,b; Krogsrud and Perkins, 1977). Hence, we may be observing phenotypic caricatures of old-age immune deficiency in short-lived mice that are analogous to the phenotypic features of accelerated aging seen in progeric humans. Thus, although a decrease of suppressor T cell activity with age could account for the emergence of autoantibodies in the older short-lived mice, it may not account for the emergence of autoantibodies in the older long-lived mice.

In humans, T cell suppressor function is less well defined. As with the mouse, both a decrease and in increase in the number or activity of suppressor cells have been detected. The decrease has been detected in terms of an age-related decrease with age in Con A-stimulated suppressor activity (Hallgren and Yunis, 1977) and the increase in terms of both Con A-stimulated suppressor activity and the number of T cells with Fc receptors for IgG (T_{gamma}) (Antel *et al.*, 1978; Kishimoto *et al.*, 1978; Gupta and Good, 1979).

6. T helper function also declines with age. This has been demonstrated in intact animals as well as in assays performed both *in vivo* and *in vitro* (Price and Makinodan, 1972a,b; Segre and Segre, 1976a;

Krogsrud and Perkins, 1977; Callard and Basten, 1978); for example, the decrease with age in the carrier primed T cell populations, which can collaborate with reference hapten (2,4,-dinitrophenol)-primed young B cells, is dramatic (Callard and Basten, 1978). The possibility exists that this may not be reflective of a defect in a helper T cell subpopulation, because recent findings indicate that the T cell help required for T cell-dependent humoral response is mediated by a complex interaction of helper, suppressor, and amplifier subsets (Eardley *et al.*, 1978; Tada *et al.*, 1978a).

5. *Other Cells*

This category of cells comprises those that are not T cells, B cells, macrophages, or stem cells. Such cells with regulatory functions appear to increase in number and/or activity with age (Roder *et al.*, 1975; Michalski *et al.*, 1979). It is very likely that these cells are at some stage of maturation destined to become either T cell, B cell, or macrophage.

C. Thymus

Since the involution of the thymus, which occurs at about the time of sexual maturity in humans and mice (Boyd, 1932; Andrew, 1952; Santisteban, 1960) (Fig. 2), precedes the age-related decline in T cell-dependent immune responses, a "cause and effect" relationship has been suspected; i.e., thymic involution is responsible for the decline. This suspicion is supported by the following observations.

1. Long-lived mice attain peak thymus weight later in life and retain a greater thymus-to-body weight ratio later in life than do short-lived mice (Yunis *et al.*, 1973), and these changes precede the change in serum levels of thymic hormones (e.g., Bach *et al.*, 1975).
2. Thymic involution is associated with loss of cortical lymphocytes (Boyd, 1932; Andrew, 1952; Santisteban, 1960) and atrophic changes in the epithelial tissue (Hirokawa, 1977), that synthesizes thymic hormones (Hoshino, 1963; Clark, 1966; Dardenne *et al.*, 1974; Goldstein *et al.*, 1974).
3. Thymic terminal deoxynucleotidyl transferase, which has been postulated to be the intrinsic somatic mutagen for immunologic diversification (Baltimore, 1974), decreases in activity starting at adulthood in long-lived mice (Pazmino and Ihle, 1976).
4. Kinetic assessment of T cells in young adult, T cell-deprived (adult thymectomized, X-irradiated, and bone marrow reconstituted) recipients of thymic lobes from syngeneic donor mice 1 day to 33

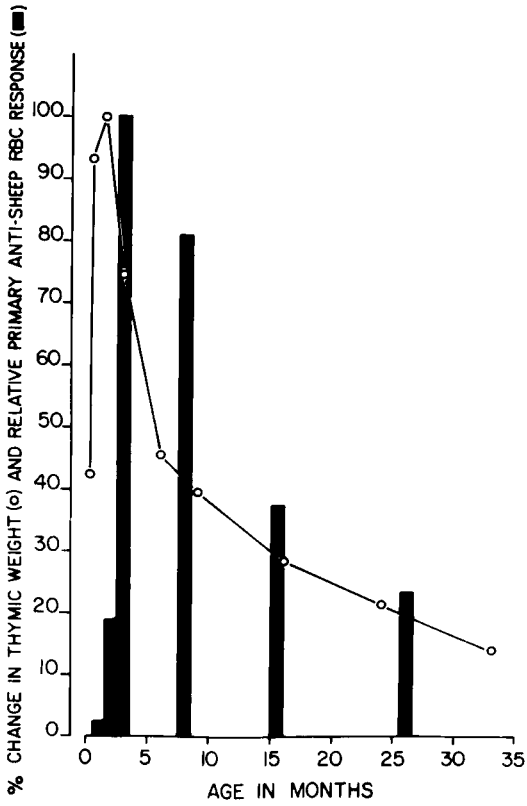


FIG. 2. The relationship between age-related changes in the thymus weight and the T cell-dependent anti-sheep red blood cell response of long-lived $BC3F_1$ mice. (From Makinodan, 1979.)

months old revealed that the ability of the thymus graft to influence the maturation of precursor T cells into functional T cells decreases with increasing age, starting as early as 1 month of age (Hirokawa and Makinodan, 1975; Hirokawa and Sado, 1978). Subsequent studies utilizing chromosome markers in parabiotic mice indicate that the movement of precursor T cells into the thymus ceases at about this time (Kay, 1978c). The cause for this age-dependent cell traffic phenomenon is not known, but it would appear, by process of elimination, that an extra thymic regulatory mechanism is operating, since these precursor T cells have the capacity to migrate into the thymus, and even a grafted involuting thymus is capable of accepting precursor T cells, under extraordinary conditions, as suggested by the thymus graft experiments of Hirokawa and Makinodan (1975).

These results indicate that shortly after 10–12 weeks of age, immature T cells in the thymus, and perhaps peripherally also, are the sole source of generators of functional T cells. This means that they must possess the capacity to self-replicate for a reasonable number of times, for otherwise a shift in subpopulations of T cells would ensue with advancing age.

5. Adult thymectomy of autoimmune-susceptible and autoimmune-resistant mice accelerates the decline in T cell-dependent humoral and cell-mediated immune responses (Metcalf, 1965; Miller, 1965; Taylor, 1965; Pachciarz and Teague, 1976), increases autoimmunity (Teague *et al.*, 1970; Yunis *et al.*, 1972; Carnaud *et al.*, 1979), and decreases longevity (Jeejeebhoy, 1971).

6. Dietary restriction which extends life expectancy in mice, slows the rate of immunologic maturation and the subsequent decline in immunologic vigor (Jose and Good, 1973; Walford *et al.*, 1974; Bell and Hazell, 1975; Gerbase-DeLima *et al.*, 1975a), and these functional alterations are associated with the stunting of thymus growth—specifically of the cortical tissue (Weindruch *et al.*, 1979; Weindruch and Suffin, in press).

7. Graft of thymus from young mice into syngeneic old mice can offer partial immunologic restoration (Yunis *et al.*, 1971; Hirokawa *et al.*, 1976; Fujiwara and Kishimoto, 1979), as does *in vivo* treatment of old mice and humans with thymic hormones (Weksler *et al.*, 1978).

8. The life-span of T cell-deficient hypopituitary dwarf mice can be extended from 4 to 12 months by injections of lymph node cells, but not of thymocytes or bone marrow cells (Fabris *et al.*, 1972; Piantanelli and Fabris, 1977). Comparable life prolongation was demonstrated by injection of growth factors and thyroxine in untreated, but not in thymectomized, dwarf mice. This suggests that (a)the immune system is closely linked, through the thymus, with the endocrine system for the sustenance of life.

In view of these observations, the thymus has been implicated as the aging "clock" of T cells. Because of the ability of T cells to regulate hematopoiesis, as mentioned earlier, some of the observed age-related alterations in stem cells and B cells could be due to changes in the thymus. Alternatively, changes in thymic activity could change the cellular milieu, since the thymus can modulate hormone levels (Fabris *et al.*, 1972; Besedovsky and Sorkin, 1975; Fabris, 1977; Piantanelli and Fabris, 1977). However, the mechanisms responsible for the changes in T cell function have not yet been elucidated, and therefore we do not know whether the changes are extrinsic or intrinsic to the thymus. The most likely extrinsic cause is a regulatory

breakdown in the hypothalamus–pituitary neuroendocrine axis in relation to the thymus. The most likely intrinsic cause is alteration of the thymic epithelial tissue, although alteration of the lymphoid component of the thymus cannot be excluded.

Support of the former possibility comes from various observations demonstrating that stress and other psychobiological factors can affect the immune response as measured by skin graft rejection and by primary and secondary antibody responses (Stein *et al.*, 1976). Moreover, lesions in the anterior basal hypothalamus, but not in the median or posterior hypothalamus, were found to depress delayed type hypersensitivity and reduce the severity of anaphylactic reactions (for review, see Stein *et al.*, 1976). Further, growth hormone and insulin have been shown to act preferentially on T cell-dependent immune functions, while thyroxine and sex hormones act on both T and B cell responses (for review, see Fabris, 1977; Piantanelli and Fabris, 1977). Finally, a single injection of anti-hypophysis serum into mice 25–35 days of age results in the development of a wasting disease and thymus atrophy in some, but not all, injected mice (Pierpaoli and Sorkin, 1967). The wasting disease observed in mice treated with antihypophysis serum resembles that seen after neonatal thymectomy (Pierpaoli and Sorkin, 1967). However, hormonal replacement therapy was not provided in these chemically hypophysectomized mice, and therefore thymic atrophy could have occurred secondary to the inability to maintain homeostasis as a result of inadequate thyroxine, corticosterone, growth hormone, etc. Interestingly, degranulation of acidophilic cells in the anterior lobe of the pituitary has been observed in neonatally thymectomized mice (Pierpaoli and Sorkin, 1967). Thus, it would appear that the thymus can modulate hormone levels (Besedovsky and Sorkin, 1975). The finding that grafting of neonatal thymus into old recipients corrects their abnormal serum levels of triidothyronine and insulin further supports this view (Piantanelli *et al.*, 1978).

Three possible mechanisms can be proposed to account for aging of the thymus, which can be triggered by intrinsic or extrinsic factors. One is clonal exhaustion (Hayflick, 1965); i.e., thymus cells have a genetically programmed clock mechanism to self-destruct and die after undergoing a fixed number of divisions. The mechanism would be similar to that of the Hayflick phenomenon, which is seen in fibroblasts *in vitro* (Hayflick, 1965). It would require that the thymus count the number of migratory thymocytes leaving it or the number of divisions thymocytes undergo, or both. Another possible mechanism is an alteration of thymus cell DNA, either randomly or through viral infec-

tion (Proffitt *et al.*, 1973). Various stable alterations of DNA can occur, including cross-linking and strand breaks (for review, see for example, Price and Makinodan, 1973). The third possible mechanism is a stable molecular alteration at the non-DNA level through subtle error-accumulating mechanisms (Strehler *et al.*, 1971).

IV. Prevention and Restoration

Efforts have been devoted to developing methods for delaying the onset of, lowering the rate of, or restoring the declining immune functions of aging animals for the following reasons.

1. An effective method with any of these capabilities can be used to resolve the issue of whether the loss of immunologic vigor with age predisposes an individual to diseases or whether age-associated diseases are responsible for immunologic aging.
2. An effective method may enable us to determine the pathogenesis of certain age-associated diseases.
3. An effective method can be used as a probe to study cellular and molecular mechanisms of immunologic aging.
4. An effective method could be used in geriatric preventive medicine.

To date, various methods have been attempted, of which five appear promising and will be discussed here. The findings, although preliminary, are encouraging, for they indicate that the immune functions of aging rodents are manipulable.

A. Tissue Removal

According to Denckla (1974), hypothyroidism can occur in aging individuals with normal levels of thyroid hormones because the pituitary begins to synthesize a substance(s) that blocks thyroid hormone receptors on target cells, including those of the immune system, which can account, in part, for the decline in immunologic vigor with age. Based on this theory, hypophysectomy plus maintenance hormone therapy should improve the immunologic status of old rats and have no effect on young rats. Accordingly, Bilder and Denckla (1977) and Scott *et al.* (1979) performed such a study and revealed that hypophysectomy did indeed have a pronounced immunorestorative effect on old, but not young, rats. Continued studies in this area should further broaden our understanding of the neuroendocrine–immune axis, of which we know very little. Of course, progress will hinge upon the success of purifying and identifying the pituitary "aging" substance.

Other tissue and organ removal has been attempted, but unfortunately no immunologic studies were performed, because the purpose was to control diseases that can disrupt normal immune functions and therefore shorten the life-span. This approach was first employed in aging most successfully by Furth over 30 years ago (Furth, 1946). Using a short-lived (MLS, 38 weeks), thymoma-susceptible AKR strain of mice, he thymectomized the mice at young adulthood after the thymus had completed its developmental lymphopoietic functions, but before the tumor became detectable histopathologically. As a consequence, the incidence of leukemia decreased drastically, and the mice lived significantly longer.

A similar approach was attempted by Albright *et al.*, (1969) with a long-lived (C57B1 × C3H)F_1 hybrid (MLS, 130 weeks). Based on preliminary studies that indicated that at old age many of these hybrid mice were dying with reticulum cell sarcoma originating in the spleen at about 100 weeks of age, an observation that has since been confirmed and extended (Chino *et al.*, 1971), these mice were either splenectomized or sham-splenectomized at 97 weeks of age. The results revealed that their *remaining* survival time after treatment at 97 weeks of age nearly doubled; i.e., the mean survival times (95% confidence limits) of splenectomized, sham-splenectomized, and untreated mice were 153 (144–159), 114 (111–129), and 129 (125–138) weeks, respectively. These results supported the view that the spleen of these mice at this age contained deleterious factor(s) that could contribute in part to their death. If this were so, then transfer of spleen cells from old mice into younger mice should shorten their life expectancy. The survival time of middle-aged (78 weeks) mice was therefore assessed after first exposing them to 400 R total body X-irradiation and then transferring spleen cells from donors 100 weeks, 76 weeks, and 12 weeks old. The recipients were exposed to a low-dose radiation to enable the donor cells to "take" and propagate efficiently. The results of this second experiment revealed that recipients receiving spleen cells from 100-week-old mice had a reduced survival time, as anticipated; i.e., the mean survival time was 129 (123–136) weeks, 113 (111–115) weeks, and 95 (95–99) weeks for the recipients receiving spleen cells from donors 12-, 76-, and 100-weeks old, respectively. The results of these two experiments, together with those demonstrating the increase in autoantibodies with age in these hybrid mice (Peterson and Makinodan, 1972), strongly suggest that removal of the spleen of these mice at this age eliminated the source of reticulum cell sarcoma and perhaps also of self-destructive autoimmune cells, thus enabling the mice to have a longer life.

The results of these latter experiments indicate that this method can be used to resolve the extent to which diseases emerging late in life compromise normal immune functions.

B. Dietary Manipulation

About 45 years ago, McCay *et al.* (1935) discovered that the life-span of rats can be extended significantly by restricting their caloric intake during growth. Later, more exhaustive studies were carried out demonstrating that early caloric restriction decelerates the aging rate, as judged by (*a*) delayed onsets of expected disease and biochemical change of old age (Tannenbaum, 1942; Berg and Simms, 1960; Ross, 1969; Ross and Bras, 1971), (*b*) slowed acceleration of mortality rate (Sacher, 1977), and (*c*) extension of maximal survivorship (Berg and Simms, 1961).

On the assumption that the life-prolonging effect of caloric restriction may be operating by retardation of immunologic aging, Walford and his co-workers (Walford *et al.*, 1974) then showed that the immune system of long-lived mice, subjected to the life-extending, calorically restricted but nutritionally supplemented diet, matured more slowly and began to age later in life and perhaps less rapidly. In their studies with short-lived, autoimmune-susceptible NZB mice, Fernandes *et al.* (1972, 1973) showed that a diet high in fat and relatively low in protein, which favors reproduction in experimental rodents, significantly decreased cell-mediated immunity, enhanced autoimmune manifestations, and shortened the life expectancy. In contrast, a diet low in fat and relatively high in protein, which is less favorable for reproduction, decreased autoimmune manifestations and prolonged the life expectancy of these mice. They then demonstrated that the life-span of these short-lived autoimmune-susceptible NZB mice could be dramatically extended by restricting their caloric intake (Fernandes *et al.*, 1973). The life-span of another relatively short-lived strain (DBA/2) was also extended by restricting their protein intake (Fernandes *et al.*, 1976). Other studies confirm the demonstration that the immune system of immature mice is highly susceptible to imposed nutritional stress, as reflected in the delay of maturation (Jose and Good, 1973; Bell and Hazell, 1975; Gerbase-DeLima *et al.*, 1975a; Weindruch *et al.*, 1979). Adult mice are also susceptible to nutritional stress, but, in contrast to immature mice, the nutritionally imposed reduced immune responsivity was shown to be associated with increases in supressor cell activity (Haffer *et al.*, in press). Three generalizations can be made from these studies.

1. The aging rate of mice with normal life spans can be retarded through appropriate caloric restriction manipulation by preserving their immunologic and related functional vigor, which, in turn, minimizes autoimmune diseases.
2. The life-span of short-lived mice can be extended significantly by delaying the expression of life-shortening disease, perhaps viral in origin, by appropriate dietary manipulation.
3. The fact that the life-prolonging effect of an appropriate dietary regimen has been demonstrable most dramatically during growth would strongly suggest that it could be mediated through the developing T cell arm of the immune system and the neuroendocrine system.

C. Genetic Manipulation

Genetic manipulation has been performed in long-lived and short-lived strains of mice in an attempt to control immunologic abnormalities associated with aging. Studies with long-lived mice stem from the hypothesis that (*a*) aging is influenced genetically by only a limited number of genes, which are regulatory rather than structural in type (Cutler, 1975); (*b*) the immune system plays a major role in aging (Walford, 1974); and (*c*) the major histocompatibility complex (MHC) system represents such a "super-regulatory gene complex system" of the immune system (Benacerraf, 1975), e.g., the *H-2* region of chromosome 17 in the mouse and the HLA region of chromosome 6 in the human, for it is known to have considerable influence on immune responsiveness and upon susceptibility to disease. Accordingly, strains of mice congenic for *H-2* region were assessed for their age-related immune functions, age-specific diseases and life-spans (Smith and Walford, 1978). The results revealed that variation in these parameters between congenic mice within an inbred strain was as great as that observed between different inbred strains of mice. If the MHC system in the mouse did not exert a significant influence upon aging and age-related immunologically associated abnormalities and life-span (i.e., decline in normal immune functions and increase in the number of immunologically related age-specific diseases), one would have expected a greater uniformity in these indices between congenic sets of mice within inbred strains than between inbred strains. Further investigations by Meredith and Walford (1977) with congenic mice revealed that the longest-lived strains tend to have the highest age-related functional preservation of mitogenic responsivity, especially of T cells. It follows, therefore, that the MHC system does play a major role in age-related immunologic abnormalities.

Studies with short-lived mice were centered on susceptibility to autoimmune-immune complex manifestations expressed early in life (Talal and Steinberg, 1974; Fernandes *et al.*, 1977). These revealed that susceptibility to autoimmune-immune complex diseases is not inherited from either inbred parents as a transmissible agent and suggest that more than one gene is involved. An example is reflected in the life-span of different inbred strains of mice and their hybrids; e.g., the MLS of NZB, NZW, CBA, (NZB × NZW)F_1 and (NZB × CBA)F_1 mice were 12, 20, 28, 9, and 27 months, respectively (Fernandes *et al.*, 1977).

In humans, numerous studies have demonstrated association between specific HLA antigens and a variety of disease states, many of which appear late in life, including T cell-deficiency-related autoimmune disorders (Dausset and Hors, 1975; van Rood *et al.*, 1975; Svejgaard *et al.*, 1975). More recently, Greenberg (1979) noted that both young and old females expressing B8 have lower response to T cell mitogens than do males of comparable age and females that are non-B8. How B8 influences T cell reactivity in females is not clear. However, it is interesting that there is a marked age-associated decrease in the A1-B8 haplotype frequency in females, but not in males, suggesting that the A1-B8 haplotype may predispose females to T cell deficiency in number and/or function. Either situation could account for the higher incidence of autoimmune disorders in females. Finally, Greenberg noted that A1-B8 haplotype appears to have survival advantage in males, but not females.

Obviously genetic manipulation will continue to serve as a powerful experimental tool to sort out the relative role genetic and environmental factors play on aging, from which practical methods could arise.

D. Cell Grafting

Cells and fragments of the thymus, spleen, lymph nodes, and bone marrow have been grafted individually or in combinations into genetically compatible old recipients with varying success in terms of immunologic restoration and extension of life expectancy.

The most impressive life-prolonging results were reported by Fabris *et al.* (1972) in growth-hormone deficient, short-lived hypopituitary dwarf mice. As discussed earlier, they demonstrated that the life-span of these mice could be extended three- to four-fold by injecting large doses of lymph node cells. A comparable life-prolonging effect was obtained by injecting growth hormone and thyroxine into dwarf mice with intact thymus, but not into dwarf mice whose thy-

muses had been removed beforehand. These results indicate that a two-way homeostatic relationship exists between the endocrine and immune system, the pituitary being capable of "turning on" the immune system through the thymus, and the T cells of "turning on" the endocrine system through the pituitary.

Cell grafting of autoimmune-susceptible, short-lived mice has had less spectacular results. Thus, although injection of young spleen or thymus cells or grafting of thymus lobes into old mice did delay the appearance of certain types of autoantibodies (Teague and Friou, 1969), it had a minimal life-prolonging effect (Kysela and Steinberg, 1973; Yunis and Greenberg, 1974); furthermore, age-associated pathological changes were unaltered (Yunis *et al.*, 1971).

Earlier studies involving the grafting of young thymus or bone marrow cells into old nonautoimmune-susceptible, long-lived mice were also unencouraging, as it did not extend their MLS appreciably (Albright and Makinodan, 1966; Metcalf *et al.*, 1966). Subsequent studies revealed that the loss of immunologic vigor with age is due in part to changes in the T cell population (Makinodan and Adler, 1975), in part to the reduced rate at which stem cells can expand clonally and generate progeny cells (Albright and Makinodan, 1976), and in part to the inability of involuted thymus to transform precursor cells into T cells efficiently (Hirokawa and Makinodan, 1975). Therefore, when this information became available, both young bone marrow stem cells and newborn thymic tissue were grafted into long-lived old mice (Hirokawa *et al.*, 1976). This treatment restored their immune functions to levels approaching those of adult mice, and the restorative effect was observed for at least 6 months after grafting in mice with a MLS of 27 months (an equivalent of 0.22 of a MLS, or about 15 human years), suggesting that the combined marrow-thymus grafting could have a long-lasting effect. Studies by Perkins *et al.* (1972) on susceptibility to infection have also generated encouraging data, showing that old mice can be made to resist lethal doses of virulent *Salmonella typhimurium* by pretreating them with spleen cells from young mice immunized beforehand with a vaccine.

Current studies in this area should resolve what effect, if any, grafting will have on the frequency and severity of diseases of the aged and whether cell grafting can alter the mean life expectancy of short-lived and long-lived mice.

E. Chemical Therapy

Only a few chemical agents have been shown to possess immunorestorative activity. These include double-stranded polynucleotide,

thymic hormones, certain free-radical inhibitors (antioxidants), levamisole, and mercaptoethanol.

Braun *et al.* (1970) were the first to demonstrate that double-stranded polynucleotides (e.g., polyadenlic–polyuridylic acid complexes) can restore the T cell-dependent antibody response of middle-aged mice to that of young adult mice. Han and Johnson (1976) not only confirmed this observation, but proceeded to demonstrate that the supernatant of cultures of thymocytes treated with double-stranded polynucleotides is equally effective as an immunorestorative agent. This would suggest that the double-stranded polynucleotide restores immunologic vigor of aging mice by acting on T cells. Further studies are required to determine the mechanism of its action on T and other immune cells, disease pattern and life-span. An insight into the possible mechanism of action at the membrane level comes from the observation of Schmidt and Douglas (1976), who found that double- and triple-stranded, but not single-stranded, polynucleotides increase the IgG binding activity of human monocytes *in vitro*. This would indicate that multistranded polynucleotides stimulate either by unmasking or promoting more synthesis of surface IgG binding receptors.

It would seem obvious that thymic hormones would be used to prevent immunologic aging or restore its effects, since the loss of immunologic vigor has been clearly shown to be associated with the failure of thymus to continue vigorously synthesizing T cell maturation hormone after sexual maturity (Bach *et al.*, 1973; Hirokawa and Makinodan, 1975). Surprisingly, however, there has been no systematic study on their effectiveness in preventing immunologic aging, and studies on their use as immunorestorative agents have been meager. Nevertheless, the results have been encouraging. Thus, Friedman *et al.* (1974) found that thymus humoral factor (THF), prepared by N. Trainin, can enhance the T cell dependent GVH activity *in vitro* of spleen cells of old, but not of young, mice. This would indicate that THF is not acting as a nonspecific adjuvant agent; otherwise, THF would also have enhanced the GVH activity of young spleen cells. Less encouraging are the preliminary findings of Bach (1977) using T cell-dependent lymphocyte-mediated cytotoxicity (LMC) as the assay. She found that circulating thymic factor (TF), prepared by J.-F. Bach, is effective *in vivo* in preventing the accelerated decline in LMC activity of adult thymectomized mice, but ineffective in normal young and middle-aged (75–86 weeks old) mice. It would appear that TF may be promoting the emergence of suppressor cells in these normal mice. Whether or not it would be effective in old mice, as demonstrated by Friedman *et al.* (1974), remains to be resolved.

Using another preparation of thymic hormones, thymopoietin, prepared by G. Goldstein, Weksler *et al.* (1978) were able to restore partially the antibody-forming capacity of old spleen cells by exposing them to it. In contrast, Martinez *et al.* (1978) failed to demonstrate the effectiveness of thymopoietin, ubiquitin and synthetic serum thymic factor, prepared by G. Goldstein, as judged by T cell mitogenic response and resistance to tumor cells. The test mice were thymectomized at neonatal age or at 1 month of age and then subjected to treatment with one of the hormones. No attempt was made to assess the effectiveness of a mixture of these hormones, which could have been effective, because it would appear that several different types of thymic differentiation hormones are necessary for the generation of mature functional T cells (Goldstein *et al.*, 1979).

Goldstein *et al.* (1979), using still another type of thymic hormone, thymosin, prepared by A. Goldstein, have found that repeated injection of the hormone can alleviate many of the symptoms in mice and humans manifesting immunodeficiency diseases. That repeated injection of thymosin may also have immunorestorative effect on elderly humans manifesting reduced T cell-dependent immune functions comes from a recent preliminary report showing that the number of T cells of old individuals can be increased by exposing their white blood cells to thymosin *in vitro* (Rovensky *et al.*, 1977). However, before assessing the effect of thymosin on elderly individuals, it would seem prudent to carry out animal studies assessing the effects of repeated injection into aging short-lived and long-lived mice on their normal immune functions, disease patterns, and life-spans.

The use of free-radical inhibitors stems from the hypothesis that aging is caused by somatic mutation (Strehler, 1977), and consequently high levels of free radicals should enhance aging and low levels delay aging (Harman, 1969). Harman *et al.* (1977) reasoned that free-radical inhibitors should enhance immune functions of aging individuals, since the immune system plays a major role in aging (Walford, 1969), and they tested this notion by incorporating these agents into the diet of aging mice. Their preliminary results indicate that vitamin E and other free-radical inhibitors can enhance the antibody response of adult and middle-aged (88 weeks) mice. Studies are being extended to assess the effectiveness of these agents on immune functions, disease pattern, and life-span of older mice. In a related study, Bliznakov (1978) found that coenzyme Q_{10}, a nonspecific stimulant of mitochondrial electron transport process of respiration, can enhance the level of antibody response of old mice to a level approaching that of adult mice. It is of interest that Biro and Beregi (1979) noted that old, but not young, T and B cells possess mitochondria with atrophic

manifestations. It would be of interest to resolve to what extent coenzyme Q_{10} can reverse the aging morphologic characteristics of mitochondria. These studies will require further investigations to identify the target cell(s) and the mechanism of action of these agents.

Various sulfhydryl compounds have been employed in enhancing various nonimmunologic and immunologic cellular activities (Fanger *et al.*, 1970; Lands *et al.*, 1971; Chen and Hirsch, 1972; Heber-Katz and Click, 1972; Broome and Jeng, 1973; Braun *et al.*, 1974; Johnson *et al.*, 1974). One of these that shows some promise is levamisole, an antihelminthic drug, which has been used successfully to restore the declining immune functions of aging mice (Renoux and Renoux, 1972; Morimoto *et al.*, 1979). However, its effectiveness is demonstrable only when administered *in vivo* over an extended period, but not *in vitro*. This would suggest that levamisole itself may not be exerting its enhancing effect directly on the immune cells, but rather through either another cell type or a metabolite.

Perhaps the most commonly used sulfhydryl compound for research by immunologists is 2-mercaptoethanol (2-ME). Studies on its immunorestorative actions on aging mice show that it enhances several of the immune responses of old mice preferentially over those of young mice (Halsall and Perkins, 1974; Makinodan *et al.*, 1975; Makinodan and Albright, 1979a,b). For example, in terms of its magnitude of enhancement of the antibody-forming capacity of an optimum number of spleen cells *in vitro*, the effect of 2-ME on old spleen cells was an order of magnitude greater than that on young spleen cells (500% vs 30% enhancement) (Makinodan and Albright, 1979a).

In terms of its ability to transform antigen-stimulated, non-antibody-responding cultures with limiting numbers of spleen cells into antibody-responding cultures, the effect of 2-ME on old spleen cells was 6.5 times greater than that on young spleen cells. That 2-ME is also an effective immunorestorative agent in *intact* old mice was demonstrated by restoring the T cell-dependent antibody responding capacity of long-lived old mice to that of young mice by giving 3 weekly injections of 4 μg of 2-ME (Makinodan and Albright, 1979b). These results would suggest that 2-ME and related chemicals may have practical applications.

The mode of action of 2-ME is not known. This is not surprising in view of the multitude of possible biochemical effects that sulfhydryl compounds can have on cell structure and functions, ranging from SH/SS exchange reactions at the membrane level, to the antioxidant and metal chelating effects (Chen and Hirsch, 1972; Broome and Jeng, 1973; Braun *et al.*, 1974). Moreover, various types of immunologic processes have been shown to benefit from the presence of 2-ME, in-

cluding antibody response (Chen and Hirsch, 1972), mitogenic response of T and B cells to plant lectins (Fanger *et al.*, 1970; Broome and Jeng, 1973; Goodman and Weigel, 1977), B colony formation (Metcalf, 1976), mixed lymphocyte reaction (Bevan *et al.*, 1974; Braun *et al.*, 1974), and cytolytic killer T cell formation (Engers *et al.*, 1975; Igarashi *et al.*, 1976). All three major cell types have been implicated as the target of 2-ME (Heber-Katz and Click, 1972; Broome and Jeng, 1973; Goodman and Weigel, 1977; Opitz *et al.*, 1977), as well as serum factor(s) in the tissue culture medium (Goodman and Weigel, 1977; Opitz *et al.*, 1977).

Finally, it should be emphasized that in previous immunoenhancing studies of 2-ME, the source of immune cells has been limited to young adult donors, whose magnitude of response in the presence of 2-ME does not increase by more than 100%. Logistically, this could make it difficult to resolve the molecular mechanism of the enhancing action of 2-ME. The use of immune cells of old donors is therefore of obvious advantage, for 2-ME, at a concentration as low as 4 μg/ml, can enhance the primary antibody response of old spleen cells by a much as 1100% (Makinodan and Albright, 1979a). For these reasons, 2-ME would appear to be a very promising molecular probe for study of the nature and mechanism of age-associated decline in normal immunologic activities.

Of the five model approaches, genetic manipulation and chemical therapy appear to be the most promising in serving as probes to understanding the biochemical nature and mechanism(s) of the decline. The former approach should enable one to determine which gene or genes are primarily responsible for the decline in normal immune functions with age and the diseases associated with it. The latter approach should enable one to determine the cell type or types most severely affected functionally and the nature of the changes associated with it at the subcellular level.

In terms of the practical application, dietary manipulation appears to be the most promising in controlling immunologic aging and the diseases associated with it. Chemical therapy could also serve as an effective preventive method. Perhaps the most effective practical approach in controlling immunologic aging may be a combination of dietary manipulation, chemical therapy, and cell grafting.

V. Concluding Remarks

An attempt has been made to summarize our present knowledge on the influence of age on immune functions and to show potential for future progress in the areas of mechanisms responsible for the age-re-

lated decline and diseases associated with declining immune function.

Normal immune functions can begin to decline as early as when an individual reaches sexual maturity. The decline is due to changes in the immune cells and their milieu. Cell loss, shift in the proportion of subpopulations, and qualitative cellular changes, the three possible types of changes that can cause the decline, have all been detected. The most visible cellular target of aging appears to be the T cells, changes in their regulatory subpopulations being most prominent. Since the changes are closely linked to the involution and atrophy of the thymus, an understanding of its changes could be the key to understanding immunosenescence.

The past phenomenologic studies are being replaced by mechanistic studies focused on the processes responsible for the disruption of cell-to-cell and intracellular communications essential for normal antigen-driven differentiation processes. These studies, in turn, are expanding into the more complex homeostatic network systems involving the immune-neuroendocrine axis. To complement these mechanistic studies, studies on prevention and restoration of the aging immune system are also evolving, for an effective manipulative method will not only contribute to our understanding of the field, but also open new avenues in the pathogenesis of aging.

As our knowledge of the aging immune system increases through further studies, it is anticipated that we may be able to develop methods for predicting, delaying, and minimizing debilitative processes associated with immunologic aging.

ACKNOWLEDGMENTS

This is publication No. 034 from GRECC, Wadsworth Medical Center. Work was supported by VA Research Service, NIH Grant HL 22671, and DOE Contract EY-76-S-03-0034.

REFERENCES

Adler, W., Takiguchi, T., and Smith, R. T. (1971). *J. Immunol.* **107,** 1357.
Albright, J. W., and Makinodan, T. (1976). *J. Exp. Med.* **144,** 1204.
Albright, J. F., and Makinodan, T. (1966). *J. Cell. Physiol.* **67,** Suppl. 3, 185.
Albright, J. F., Makinodan, T., and Deitchman, J. W. (1969). *Exp. Gerontol.* **4,** 267.
Alford, R. H. (1968). *J. Immunol.* **101,** 984.
Andrew, W. (1952). "Cellular Changes With Age." Thomas, Springfield, Illinois.
Antel, J. P., Weinrich, M., and Arnason, B. G. W. (1978). *Clin. Immunol. Immunopathol.* **9,** 134.
Awa, A. A. (1975). *J. Radiat. Res.* **16,** Suppl., 122.
Axelsson, U., Bachman, R., and Hallen, J. (1966). *Acta Med. Scand.* **179,** 235.

Bach, J.-F., Dardenne, M. and Salomon, J. C. (1973). *Clin. Exp. Immunol.* **14,** 247.
Bach, J.-F., Dardenne, M., Pleau, J. M., and Bach, M.-A. (1975). *Ann. N. Y. Acad. Sci.* **249,** 186.
Bach, M.-A. (1977). *J. Immunol.* **119,** 641.
Baer, H., and Bowser, R. T. (1963). *Science* **140,** 1211.
Baltimore, D. (1974). *Nature (London)* **248,** 409.
Barthold, D. R., Kysela, S., and Steinberg, A. D. (1974). *J. Immunol.* **112,** 9.
Becker, M. J., Farkas, R., Scneider, M., Drucker, I, and Klajman, A. (1979). *Clin. Immunol. Immunopathol.* **14,** 204.
Bell, R. G., and Hazell, L. A. (1975). *J. Exp. Med.* **141,** 127.
Benacerraf, B. (ed.) (1975). "Immunogenetics and Immunodeficiency." Univ. Park Press, Baltimore, Maryland.
Beregi, E. (1979). *Proc.* 6th *Eur. Symp. Basic Res. Gerontol., 6th,* Munich, 1979.
Berg, B. N., and Simms, H. S. (1960). *J. Nutr.* **71,** 255.
Berg, B. N., and Simms, H. S. (1961). *J. Nutr.* **74,** 23.
Besedovsky, H., and Sorkin, E. (1975). *Proc. Soc. Exp. Biol. Med.* **150,** 466.
Bevan, M. J., Epstein, R., and Cohn, M. (1974). *J. Exp. Med.* **139,** 1025.
Bilder, G. E., and Denckla, W. D. (1977). *Mech. Ageing Dev.* **6,** 153.
Biro, J., and Beregi, E. (1979). *Acta Gerontol.* **9,** 235.
Bliznakov, E. G. (1978). *Mech. Ageing Dev.* **7,** 189.
Boyd, E. (1932). *Am. J. Dis. Child.* **43,** 1162.
Braun, W., Yajima, Y., and Ishizuka, M. (1970). *J. Reticuloendothel. Soc.* **7,** 418.
Braun, W., Lichtenstein, W. M., and Parker, C. (eds.) (1974). "Cyclic AMP, Cell Growth and the Immune Response." Springer-Verlag, Berlin and New York.
Brennan, P. C., and Jaroslow, B. N. (1975). *Cell. Immunol.* **15,** 51.
Broome, J. D., and Jeng, M. W. (1973). *J. Exp. Med.* **138,** 574.
Buckley, C. G., Buckley, E. G., Dorsey, F. C. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 2036.
Butler, R. N. (1978). "Thoughts on Geriatric Medicine," NIA Science Writer Seminar Series. DHEW Publication No. (NIH) 78-1406. US Dept. Health, Education and Welfare, USPHS, NIH.
Callard, R. E. (1978). *J. Immunol.* **8,** 697.
Callard, R. E., and Basten, A. (1978). *Eur. J. Immunol.* **8,** 552.
Callard, R. E., Basten, A., and Walters, L. K. (1977). *Cell. Immunol.* **31,**26.
Callard, R. E., Basten, A., and Blanden, R. V. (1979). *Nature (London)* **281,** 218.
Carnaud, C., Charreire, J., and Bach, J.-F. (1977). *Cell Immunol.* **28,** 274.
Carosella, E. D., Mochanko, K., and Braun, M. (1974). *Cell. Immunol.* **12,** 323.
Chen, C., and Hirsch, J. G. (1972). *J. Exp. Med.* **136,** 604.
Chen, M. G. (1971). *J. Cell. Physiol.* **78,** 225.
Chino, F., Makinodan, T., Lever, W. E., and Peterson, W. J. (1971). *J. Gerontol.* **26,** 497.
Claman, H. N., and Chaperon, E. A. (1969). *Transplant. Rev.* **1,** 92.
Clark, S. L., Jr. (1966). *In* "Thymus: Experimental and Clinical Studies" (G. E. W. Wolstenholm and R. Porter, eds.), p. 3. Ciba Foundation Symposium, Churchill, London.
Coggle, J. E., and Proukakis, C. (1970). *Gerontolgia* **16,** 25.
Cudkowicz, G., Upton, A. C., Shearer, G. M., and Hughes, W. L. (1964). *Nature (London)* **201,** 165.
Cutler, R. G. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 4664.
Dardenne, M., Papiernik, M., Bach, J.-F., and Stutman, O. (1974). *Immunology* **27,** 299.
Dausset, J., and Hors, J. (1975). *Transplant. Rev.* **22,** 15.

Davenport, F. M., Hennessy, A. V., and Francis, T., Jr. (1953). *J. Exp. Med.* **98,** 641.
Davis, B. D., Dulbecco, R., Eisen, H. N., Ginsberg, H. S., and Wood, W. B., Jr. (1967). *In* "Microbiology," p. 1312. Harper (Hoeber), New York.
Davis, M. L., Upton, A. C., and Satterfield, L. C. (1971). *Proc. Soc. Exp. Biol. Med.* **137,** 1452.
Denckla, W. D. (1974). *J. Clin. Invest.* **53,** 572.
Diaz-Jouanen, E., Willians, R. C., Jr., and Strickland, R. G. (1975). *Lancet* **1,** 688.
Doria, G., D'Agostaro, G., and Poretti, A. (1978). *Immunology* **35,** 601.
Eardley, D. D., Hugenberger, J., McVay-Doudreau, L., Shen, F.-W., Gershon, R. K., and Cantor, H. (1978). *J. Exp. Med.* **147,** 1106.
Earp, H. S., Utsinger, P. D., Yount, W. J., Logue, M., and Steiner, A. L. (1977). *J. Exp. Med* **145,** 1087.
Engers, H. D., MacDonald, H. R., Cerottini, J. C., and Brunner, K. T. (1975). *Eur. J. Immunol.* **5,** 223.
Englisova, M., Englis, M., Kyral, V., Morell, A., and Hijmans, W. (1975). *Clin. Exp. Immunol.* **14,** 251.
Epifanova, O. I., and Terskikh, V. V. (1969). *Cell Tissue Kinet.* **2,** 75.
Fabris, N. (1977). *In* "Immunology and Aging" (T. Makinodan and E. Yunis, eds.), p. 73. Plenum, New York.
Fabris, N., Pierpaoli, W., and Sorkin, E. (1972). *Nature (London)* **240,** 557.
Fanger, M. W., Hart, D. A., Wells, J. V., and Nisonoff, A. (1970). *J. Immunol.* **105,** 1043.
Farrar, J. J., Loughman, B. E., and Nordin, A. A. (1974). *J. Immunol.* **112,** 1244.
Fernandes, G., Yunis, E. J., Smith, J., and Good, R. A. (1972). *Proc. Soc. Exp. Biol. Med.* **139,** 1189.
Fernandes, G., Yunis, E. J., Jose, D. G., and Good, R. A. (1973). *Int. Arch. Allergy Appl. Immunol.* **44,** 770.
Fernandes, G., Yunis, E. J., and Good, R. A. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 1279.
Fernandes G., Good, R. A., and Yunis, E. J. (1977). *In* "Immunology and Aging" (T. Makinodan and E. Yunis, eds.), p. 111. Plenum, New York.
Fernandez, L. A., MacSween, J. M., and Langley, G. R. (1976). *Immunology* **31,** 583.
Finger, H., and Emmerling, P. (1973). *Gerontologia* **19,** 22.
Foad, B., Adams, Y. Yamaguchi, and Litwin, A. (1974). *Clin. Exp. Immunol.* **17,** 657.
Friedberger, E., Bock, G., and Furstenheim, A. (1929). *Z. Immunitaetsforsch.*, 64, 249.
Friedman, D., and Globerson, A. (1976). *Isr. J. Med. Sci.* **12,** 1957.
Friedman, D. Keiser, V., and Globerson, A. (1974). *Nature (London)* **251,** 545.
Fujiwara, M., and Kishimoto, S. (1979). *J. Immunol.* **123,** 263.
Fulk, R. V., Fedson, D. S., Huber, M. A., Fitzpatrick, J. R., and Kasel, J. A. (1970). *J. Immunol.* **104,** 8.
Furth, J. (1946). *J. Gerontol.* **1,** 46.
Furuhata, T., and Eguchi, M. (1955). *Proc. Jpn. Acad.* **31,** 555.
Gardner, I. D., and Remington, J. S. (1977). *Infect. Immun.* **16,** 593.
Gerbase-DeLima, M., Liu, R. K., Cheney, K. E., Mickey, R., and Walford, R. L. (1975a). *Gerontologia* **21,** 184.
Gerbase-Delima, M., Meredith, P., and Walford, R. (1975b). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34,** 159.
Gerber, N. L. Hardin, J. A., Chused, T. M., and Steinberg, A. D. (1974). *J. Immunol.* **113,** 1618.
Giannini, D., and Sloan, R. S. (1957). *Lancet* **1,** 525.
Girard, J. P., Paychere, M., Cuervas, M., and Fernandes, B. (1977). *Clin. Exp. Immunol.* **27,** 85.

Gleichmann, E., Gleichmann, H., and Wilke, W. (1976). *Transplant. Rev.* **31,** 156.
Goidl, E. A., Innes, J. B., and Weksler, M. E. (1976). *J. Exp. Med.* **144,** 1037.
Goldstein, A. L., Hooler, J. A., Schulof, R. S., Cohen, G. H., Thurman, G. B., McDaniel, M. C., Whire, A., and Dardenne, M. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 2053.
Goldstein, A. L., Thurman, G. B., Low, T. L., Trivers, G. E., and Rossio, J. L. (1979). *In* "Physiology and Cell Biology of Aging" (A. Cherkin, C. E. Finch, N. Kharasch, T. Makinodan, F. L. Scott, and B. L. Strehler, eds.), p. 51. Raven, New York.
Good, R. A., and Yunis, E. J. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 2040.
Goodman, J. W., and Shinpock, S. G. (1968). *Proc. Soc. Exp. Biol. Med.* **129,** 417.
Goodman, M. G., and Weigel, W. O. (1977). *J. Exp. Med.* **145,** 473.
Goodman, S. A., and Makinodan, T. (1975) *Clin. Exp. Immunol.* **19,** 533.
Goodman, S. A., Chen, M. G., and Makinodan, T. (1972). *J. Immunol.* **108,** 1387.
Goullet, P., and Kaufman, H. (1965). *Experientia* **21,** 46.
Gozes, Y, Umiel, T., Meshover, A., and Trainin, N. (1978). *J. Immunol.* **121,** 2199.
Greenberg, L. J. (1979). *In* "Aging and Immunity" (S. K. Singhal, N. R. Sinclair, and C. R. Stiller, eds.), p. 43. Elsevier/North-Holland, Amsterdam.
Gross, L. (1965). *Cancer* **18,** 201.
Grossman, J., Baum, J., Fusner, J., and Condemi, J. (1975). *J. Allergy Clin. Immunol.* **55,** 268.
Groves, D. L. Lever, W. E., and Makinodan, T. (1970). *J. Immunol.* **104,** 148.
Gupta, S., and Good, R. A. (1979). *J. Immunol.* **122,** 1214.
Haaijman, J. J., and Hijmans, W. (1978). *Mech. Ageing Dev.* **3,** 375.
Haaijman, J., Berg, P., and Brinkhof, J. (1977). *Immunology* **32,** 923.
Haferkamp, O., Schlettwein-Gsell, D., Schwick, H. G., and Storiko, K. (1966). *Gerontologia* **12,** 30.
Haffer, K., Freeman, M. J., and Watson, R. R. (1979). *Mech. Ageing Dev.* **11,** 279.
Hallen, J. (1966). *Acta Med. Scand. Suppl.* **462,** 1.
Hallgren, H. M., and Yunis, E. J. (1977). *J. Immunol.* **118,** 2004.
Hallgren, H. M., Buckley, E. C., III, Gilbertsen, V. A., and Yunis, E. J. (1973). *J. Immunol.* **111,** 1101.
Halsall, M. H., Heidrick, M. L., Deitchman, J. W., and Makinodan, T. (1973). *Gerontologist* **3** (No. 3, Part II), 46.
Halsall, M. K., and Perkins, E. H. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 736.
Hamerton, J. L., Taylor, A. I., Angell, R., and McGuire, V. M. (1965). *Nature (London)* **206,** 1232.
Han, I. H., and Johnson, A. G. (1976). *J. Immunol.* **117,** 423.
Harman, D. (1969). *J. Am. Geriat. Soc.* **17,** 721.
Harman, D., Heidrick, M. L., and Eddy, D. E. (1977). *J. Am. Geriat. Soc.* **25,** 400.
Harrison, D. E. (1975). *J. Gerontol.* **30,** 279.
Harrison, D. E., Astle, C. M., and Doubleday, J. W. (1977). *J. Immunol.* **18,** 1223.
Harrison, D. E., Astle, C. M., and Delaittre, J. A. (1978). *J. Exp. Med.* **147,** 1526.
Hayflick, L. (1965). *Exp. Cell Res.* **37,** 614.
Heber-Katz, E., and Click, R. E. (1972). *Cell. Immunol.* **5,** 410.
Heidrick, M. L. (1972). *Gerontologist* **12,** 28.
Heidrick, M. L. (1973). *J. Cell Biol.* **57,** 139a.
Heidrick, M. L., and Makinodan, T. (1973). *J. Immunol.* **111,** 1502.
Heine, K. M. (1971). *Folia Haematol. (Leipzig)* **96,** 29.
Hirokawa, K. (1977). *In* "Immunology and Aging" (T. Makinodan and E. Yunis, eds.), p. 51. Plenum, New York.
Hirokawa, K., and Makinodan, T. (1975). *J. Immunol.* **114,** 1659.

Hirokawa, K., and Sado, T. (1978). *Mech. Ageing Dev.* **7,** 89.
Hirokawa, K., Albright, J. W., and Makinodan, T. (1976). *Clin. Immunol. Immunopathol.* **5,** 371.
Hirst, J. A., Beverley, P. C., Kisielow, P., Hoffman, M. K., and Oettgen, H. F. (1975). *J. Immunol.* **115,** 1555.
Hori, Y., Perkins, E. H., and Halsall, M. K. (1973). *Proc. Soc. Exp. Biol. Med.* **144,** 48.
Hoshino, T. (1963). *Z. Zellforsch. Mikrosk. Anat.* **59,** 513.
Hung, C.-Y., Perkins, E. H., and Yang, W.-K. (1975a). *Mech. Ageing Dev.* **4,** 29.
Hung, C.-Y., Perkins, E. H., and Yang, W.-K. (1975b). *Mech. Ageing Dev.* **4,** 103.
Igarashi, T., Okada, M., Kishimoto, T., and Yamamura, Y. (1976). *J. Immunol.* **118,** 1976.
Inkeles, B., Innes, J. B., Kuntz, M. M., Kadish, A. S., and Weksler, M. E. (1977a). *J. Exp. Med.* **145,** 1158.
Inkeles, B., Judith, B., Kuntz, M. M., Dadish, A. S., and Weksler, M. E. (1977b). *J. Exp. Med.* **145,** 1176.
Jacobs, P. A., Court Brown, W. M., and Doll, R. (1961). *Nature (London)* **191,** 1178.
Jarvik, L. F., Yen, F.-S., Fu, T.-K., and Matsuyama, S. S. (1976). *Hum. Genet.* **33,** 17.
Jeejeebhoy, M. F. (1971). *Transplantation* **12,** 525.
Johnson, N. Jessup, R., and Ramwell, P. W. (1974). *Prostaglandins* **5,** 125.
Jose, D. G., and Good, R. A. (1973). *Cancer Res.* **33,** 807.
Kalff, M. W. (1970). *Clin. Chim. Acta* **28,** 277.
Kay, M. M. B. (1975a). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 3521.
Kay, M. M. B. (1975b). *Nature (London)* **254,** 424.
Kay, M. M. B. (1976). *J. Am. Soc. Geriat.* **24,** 253.
Kay, M. M. B. (1977). *In* "Basic and Clinical Immunology" (H. Fudenberg, J. Caldwell, D. Stites, and J. Wells, eds.), p. 267. Lange Med. Publ., Los Altos, California.
Kay, M. M. B. (1978a). *In* "Genetic Effects on Aging" (D. Bergsma and D. Harrison, eds.), p. 213. Alan R. Liss, Inc., New York.
Kay, M. M. B. (1978b). *Proc. Soc. Expt. Biol. Med.* **158,** 326.
Kay, M. M. B. (1978c). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **37,** 1241.
Kay, M. M. B. (1978d). *J. Supramol. Struct.* **9,** 555.
Kay, M. M. B. (1979a). *Clin. Immunol. Immunopathol.* **12,** 301.
Kay, M. M. B. (1979b). *In* "Recent Advances in Gerontology" (H. Orimo, K. Shimada, M. Iriki, and D. Maeda, eds.), p. 442. Excerpta Medica, Amsterdam.
Kay, M. M. B., and Makinodan, T. (1978). *In* "Clinical Immunochemistry. Chemical and Cellular Bases and Applications in Disease" (S. Natelson, A. J. Pesce, and A. A. Dietz, eds.), p. 192. American Association for Clinical Chemistry, Washington, D. C.
Kay, M. M. B., Denton, T., Union, N., Mendoza, J., Diven, J., and Lajiness, M. (1979a). *Mech. Ageing Dev.* **11,** 295.
Kay, M. M. B., Mendoza, J., Hausman, S., and Dorsey, B. (1979b). *Mech. Ageing Dev.* **11,** 347.
Kishimoto, S., Takahama, T., and Mizumachi, H. (1976). *J. Immunol.* **116,** 294.
Kishimoto, S., Tomino, S., Inomata, K., Kotegawa, S., Saito, T., Kuroki, M., Mitsuya, H., and Hisamitsu, S. (1978). *J. Immunol* **121,** 1773.
Konen, T. G., Smith, G. S., and Walford, R. L. (1973). *J. Immunol.* **110,** 1216.
Krogsrud, R. L., and Perkins, E. H. (1977). *J. Immunol.* **118,** 1607.
Krohn, P. L. (1962). *Proc. R. Soc. London Ser. B* **157,** 128.
Kysela, S., and Steinberg, A. D. (1973). *Clin. Immunol. Immunopathol.* **2,** 133.
Lajtha, L.J., and Schofield, R. (1971). *Adv. Gerontol. Res.* **3,** 131.
Lands, W., Lee, R., and Smith, W. (1971). *Ann N. Y. Acad. Sci.* **180,** 107.
Legge, J. S., and Austin, C. M. (1968). *Aust. J. Exp. Biol. Med. Sci.* **446,** 361.

Lesher, S., Fry, R. J. M., and Kohn, H. S. (1961). *Gerontologia* **3,** 1976.
Lloyd, R. S., and Triger, D. R. (1975). *Immunology* **29,** 253.
Lyngbye, J., and Kroll, J. (1971). *Clin. Chem.* **17,** 495.
McCay, C. M., Crowell, M. F., and Maynard, L. A. (1935). *J. Nutr.* **10,** 63.
Macieiro-Coelho, A., Ponten, J., and Phillipson, L. (1966). *Exp. Cell Res.* **42,** 673.
Makinodan, T. (1979). *Mech. Ageing Dev.* **9,** 7.
Makinodan, T., and Adler, W. (1975). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34,** 153.
Makinodan, T., and Albright, J. W. (1979a). *Mech. Ageing Dev.* **10,** 325.
Makinodan, T., and Albright, J. W. (1979b). *Mech. Ageing Dev.* **11,** 1.
Makinodan, T., and Peterson, W. J. (1962). *Proc. Natl. Acad. Sci. U.S.A.* **48,** 234.
Makinodan, T., Chino, F., Lever, W. E., and Brewen, B. S. (1971a). *J. Gerontol.* **26,** 508.
Makinodan, T., Chino, F, Lever, W. E., and Brewen, B. S. (1971b). *J. Gerontol.* **26,** 515.
Makinodan, T., Deitchman, J. W., Stoltzner, G. H., Kay, M. M., and Hirokawa, K. (1975). *Proc. Int. Congr. Gerontol. 10th,* Vol. 2, p. 23.
Makinodan, T., Albright, J. W., Good, P. I., Peter, C. P., and Heidrick, M. L. (1976). *Immunology* **31,** 903.
Mandel, T. (1960). *Z. Zellforsch. Mikrosk. Anat.* **106,** 498.
Martinez, D., Field, A. K., Schwam, H., Tytell, A. A., and Hilleman, M. R. (1978). *Proc. Soc. Exp. Biol. Med.* **159,** 195.
Mathies, M., Lipps, L., Smith, G. S., and Walford, R. L. (1973). *J. Gerontol.* **28,** 425.
Mello, R. S., Kwan, D., and Norman, A. (1974). *Radiat. Res.* **60,** 482.
Menon, M., Jaroslow, B. N., and Koesterer, R. (1974). *J. Gerontol.* **29,** 499.
Meredith, P. J., and Walford, R. L. (1977). *Immunogenetics* **5,** 109.
Meredith, P. Tittor, W., Gerbase-DeLima, M., and Walford, R. L. (1975). *Cell Immunol.* **18,** 324.
Meredith, P. J., Kristie, J. A., and Walford, R. L. (1979). *J. Immunol.* **123,** 87.
Merhav, S., and Gershon, H. (1977). *Cell. Immunol.* **34,** 354.
Metcalf, D. (1965). *Nature (London)* **208,** 1336.
Metcalf, D. (1976). *J. Immunol.* **116,** 635.
Metcalf, D., Moulds, R., and Pike, B. (1966). *Clin. Exp. Immunol.* **2,** 109.
Michalski, J. P., McCombs, C. C., and Talal, N. (1979). *Eur. J. Immunol.* **9,** 440.
Micklem, H. S., Ogden, D. A., and Payne, A. C. (1973). *Haemopoietic Stem Cells, Ciba Found. Symp.* 13, p. 285. Excerpta Medica, Amsterdam.
Miller, J. F. A. P. (1965). *Nature (London)* **208,** 1337.
Morimoto, C., Abe, T., and Homma, M. (1979). *Clin. Immunol. Immunopathol.* **12,** 316.
Morton, J. I., and Seigel, B. V. (1969). *Immunology* **16,** 481.
Naor, D., Bonavida, B., and Walford, R. L. (1976). *J. Immunol.* **117,** 2204.
Neiderhuber, J. E., Frelinger, J. A., Dine, M. S., Schoffner, P., Dugan, E., and Shreffler, D. C. (1976). *J. Exp. Med.* **143,** 372.
Neurath, P., DeRemer, K., Bell, B., Jarvik, L., and Kato, T. (1970). *Nature (London)* **225,** 281.
Nielson, H. E. (1974). *J. Immunol.* **112,** 1194.
Novick, A., Novick, I., and Potoker, S. (1972). *J. Am. Geriat. Soc.* **20,** 455.
Ogden, D. A., and Micklem, H. S. (1976). *Transplantation* **12,** 287.
Oh, Y. H., and Conard, R. A. (1972). *Life Sci.* **11,** 677.
Olsson, L., and Claesson, M. H. (1973). *Nature (London)* **244,** 50.
Opitz, H. G., Opitz, U., Lemke, H., Flad, H. D., Hewlett, G., and Schlumberger, H. D. (1977). *J. Immunol.* **119,** 2089.
Pachciarz, J. A., and Teague, P. O. (1976). *J. Immunol.* **116,** 982.
Paul, J. R., and Bennell, W. W. (1932). *Am. J. Med. Sci.* **183,** 90.
Pazmino, N. H., and Ihle, J. N. (1976). *J. Immunol.* **117,** 620.

Pazmino, N. H., and Yuhas, J. M. (1973). *Cancer Res.* **33,** 2668.
Penn, I., and Starzl, T. E. (1972). *Transplantation* **14,** 407.
Perkins, E. H. (1971). *J. Reticuloendothel. Soc.* **9,** 642.
Perkins, E. H., and Cacheiro, L. H. (1977). *Mech. Ageing Devel.* **6,**15.
Perkins, E. H., Makinodan, T., and Seibert, C. (1972). *Infect. Immunol.* **6,** 518.
Peter, C. P. (1973). *J. Gerontol.* **28,** 265.
Peterson, W. J., and Makinodan, T. (1972). *Clin. Exp. Immunol.* **12,** 273.
Piantanelli, L., and Fabris, N. (1977). *In* "Genetic Effects on Aging" (D. Harrison, ed.), p. 315. Sinauer Assoc., Sunderland, Massachusetts.
Piantanelli, L., Basso, A., Muzzioli, M., and Fabris, N. (1978). *Mech. Ageing Dev.* **7,** 171.
Pierpaoli, W., and Sorkin, E. (1967). *Nature (London)* **215,** 834.
Pisciotta, A. V., Westring, D. W., Deprey, C., and Walsh. B. (1967). *Nature (London)* **215,** 193.
Platt, D., and Pauli, H. (1972). *Acta Gerontol.* **2,** 415.
Portaro, J. K., Glick, G. I., and Zighelboim, J. (1978). *Clin. Immunol. Immunopathol.* **11,** 339.
Preumont, A. M., Van Gansen, P., and Brachet, J. (1978). *Mech. Ageing Dev.* **7,** 25.
Price, G. B., and Makinodan, T. (1972a). *J. Immunol.* **108,** 403.
Price, G. B., and Makinodan, T. (1972b). *J. Immunol.* **108,** 413.
Price, G. B., and Makinodan, T. (1973). *Gerontologia* **19,** 58.
Pritchard, L. L., Shinpock, S. G. and Goodman, J. W. (1975). *Exp. Hematol.* **3,** 94.
Proffitt, M. R., Hirsch, M. S., and Black, P. H. (1973). *Science* **182,** 821.
Radl, J. (1979). *Clin. Immunol. Immunopathol.* **14,** 251.
Radl, J., and Hollander, C. F. (1974). *J. Immunol.* **112,** 2271.
Radl, J., Sepers, J. M., Skvaril, F., Morell, A., and Hijmans, W. (1975). *Clin. Exp. Immunol.* **22,** 84.
Radl, J., De Glopper, E., Schuit, H. R. E., and Zurcher, C. (1979a). *J. Immunol.* **122,** 609.
Radl, J., Hollander, C. F., Van den Berg, P., and De Glopper, E. (1979b). *Clin. Exp. Immunol.* **33,** 395.
Renoux, G., and Renoux, M. (1972). *C. R. Acad. Sci. Ser. D* **274,** 3034.
Rivnay, B., Globerson, A., and Shinitzky, M. (1978). *Eur. J. Immunol.* **8,** 185.
Rivnay, B., Globerson, A., and Shinitzky, M. (1979). *Mech Aging Dev.* **10,** 71.
Roberts-Thomson, I., Whittingham, S., Youngchaiyud, U., and Mackay, I. R., (1974). *Lancet* **2,** 368.
Roder, J. C., Bell, D. A., and Singhal, S. K. (1975). *J. Immunol.* **115,** 466.
Ross, M. H. (1969). *J. Nutr.* **97,** Suppl 1, part 2, 565.
Ross, M. H., and Bras, G. (1971). *J. Natl. Cancer Inst.* **47,** 1095.
Rovensky, J., Goldstein, A. L., Holt, P. J. T., Pwkarek, J., and Mistina, T. (1977). *Cas. Lek. Cesk.* **116,** 1063.
Rowley, M., Buchanan, H., and Mackay, I. R., (1968). *Lancet* **2,** 24.
Sabin, A. B. (1947). *Proc. Soc. Exp. Biol. Med.* **65,** 127.
Sacher, G. (1977). *In* "Handbook of the Biology of Aging" (C. E. Finch and L. Hayflick, eds.), p. 582. Van Nostrand-Reinhold, Princeton, New Jersey.
Santisteban, G. A. (1960). *Anat. Res.* **136,** 117.
Schmidt, M. E, and Douglas, S. D. (1976). *Proc. Soc. Exp. Biol. Med.* **151,** 376.
Schneider, E. L., and Mitsui, Y. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 3584.
Scott, M., Bolla, R., and Denckla, W. D. (1979). *Mech. Ageing Dev.* **11,** 127.
Scribner, D. J., Weiner, H. L., and Moorhead, J. W. (1978). *J. Immunol.* **121,** 377.
Segre, D., and Segre, M. (1976a). *J. Immunol.* **116,** 731.
Segre, D., and Segre, M. (1976b). *J. Immunol.* **116,** 735.
Shelton, E., Davis, S., and Hemmer, R. (1970). *Science* **168,** 1232.

Shigemoto, S., Kishimoto, S., and Yamamura, Y. (1975). *J. Immunol. 115,* 307.
Shu, S., Nisengard, R. J., Hale, W. L., and Beutner, E. H. (1975). *J. Lab. Clin. Med.* **86,** 259.
Silini, G., and Andreozzi, U. (1974). *Exp. Gerontol.* **9,** 99.
Siminovitch, L., Till, J. E., and McCulloch, E. A. (1964). *J. Cell. Physiol.* **64,** 23.
Smith, A. M. (1976). *J. Immunol.* **116,** 469.
Smith, G. S., and Walford, R. L. (1978). *In* "Genetic Effects on Aging" (D. Bergsma and D. E. Harrison, eds.), p. 281. Sinauer Assoc., Sunderland, Massachusetts.
Smith, M. A., Evans, J., and Steel, C. M. (1974). *Lancet* **2,** 922.
Solomonova, K., and Vizeb, S. (1973). Z. *Immunitaetsforsch.* 146, 81.
Somers, H., and Kuhns, W. J. (1972). *Proc. Soc. Exp. Biol. Med.* **141,** 1104.
Stanton, T. H., Calkins, C. E., Jandinski, J., Schendel, D. J., Stein, M., Schiavi, R. C., and Camerino, M. (1976). *Science* **191,** 435.
Stanton, T. H., Calkins, C. E., Jandinski, J., Schendel, D. J., Stutman, O., Cantor, H., and Boyse, E. A. (1978). *J. Exp. Med.* **148,** 963.
Stein, M., Schiavi, R. C., and Camerino, M. (1976). *Science* **191,** 435.
Steinman, R. M., Kaplan, G., Witmer, M. D., and Cohn, Z. A. (1979). *J. Exp. Med.* **149,** 1.
Stjernsward, J. (1966). *J. Natl. Cancer Inst.* **37,** 505.
Storer, J. B. (1966). *J. Gerontol.* **21,** 404.
Strehler, B. L. (1977). "Time, Cells and Aging," 2nd ed. Academic Press, New York.
Strehler, B. L., Hirsch, G., Gusseck, D., Johnson, R., and Bick, M. (1971). *J. Theor. Biol.* **33,** 429.
Stutman, O. (1972). *J. Immunol.* **109,** 602.
Stutman, O. (1974). *Fed Proc., Fed. Am. Soc. Exp. Biol.* **33,** 2028.
Stutman, O. (1975). *J. Immunol.* **114,** 1213.
Stutman, O., Yunis, E. J., and Good, R. A. (1968). *Proc. Soc. Exp. Biol. Med.* **127,** 1204.
Stutman, O., Yunis, E. J., and Good, R. A. (1972). *J. Exp. Med.* **135,** 339.
Svejgaard, A., Plaz, P., Ryder, L. P., Neilson, L. S., and Thomsen, M. (1975). *Transplant. Rev.* **22,** 3.
Tada, T., Takemori, T., Okumura, K., Nonaka, M., and Tokuhisa, T. (1978a). *J. Exp. Med.* **147,** 446.
Tada, T., Okamura, K., Ohmori, K., Kawata, M., Goto, M. (1979). *In* "Recent Advances in Gerontology" (H. Orimo, K. Shimada, M. Iriki, and D. Maeda, eds.), p. 442. Excerpta Medica, Amsterdam.
Talal, N., and Steinberg, A. D. (1974). *Curr. Top. Microbiol. Immunol.* **64,** 79.
Tam, C. F., and Walford, R. L. (1978). *Mech. Ageing Dev.* **7,** 309.
Tannenbaum, A. (1942). *Cancer Res.* **2,** 460.
Taylor, R. B. (1965). *Nature (London)* **208,** 1334.
Teague, P. O., and Friou, G. J. (1969). *Immunology* **17,** 665.
Teague, P. O., Yunis, E. J., Rodey, G., Fish, A. J., Stutman, O., and Good, R. A. (1970). *Lab. Invest.* **22,** 121.
Teller, M. N., Stohr, G., Curlett, W., Kubisek, M. L., and Curtis, D. (1964). *J. Natl. Cancer Inst.* **33,** 649.
Thomsen, O., and Kettel, K. (1929) Z. *Immunitaetsforsch.* **63,** 67.
Thrasher, J. D. (1971). *Exp. Gerontol.* **6,** 19.
Tice, R. R., Schneider, E. L., Kram, D., and Thorne, P. (1979). *J. Exp. Med.* **149,** 1029.
Turesson, I. (1976). *Acta Med. Scand.* **199,** 293.
Tyan, M. L. (1977). *J. Immunol.* **11,** 846.
U.S. Bureau of the Census. (1976). Series P-23, No. 59, USGPO, Washington, D. C., May, 1976.
van Rood, J. J., van Hooff, J. P., and Keuning, J. J. (1975). *Transplant. Rev.* **22,** 75.

Waldenstrom, J. G. (1973). *In* "Multiple Myeloma and Related Disorders" (H. A. Azar and M. Potter, eds.), p. 247. Harper New York.
Waldorf, D. S., Willikens, R. F., and Decker, J. L. (1968). *J. Am. Med. Assoc.* **203,** 111.
Walford, R. L. (1969). "The Immunologic Theory of Aging." Munksgaard, Copenhagen.
Walford, R. L. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Boil.* **33,** 2020.
Walford, R. L. (1976). *J. Immunol.* 117, 352.
Walford, R. L., Liu, R. K., Gerbase-DeLima, M., Mathies, M., and Smith, G. S. (1974). *Mech. Ageing Dev.* **2,** 447.
Walters, C. S., and Claman, H. N. (1975). *J. Immunol.* **115,** 1438.
Weindruch, R. H., Kristie, J. A., Cheney, K. E., and Walford, R. L. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 2007.
Weindruch, R. H., and Suffin, S. C. (1980). *J. Gerontol.* (in press).
Weiner, H. L., Scribner, D. J., Schocket, A. L., and Moorhead, J. W. (1978). *Clin. Immunol. Immunopathol.* **9,** 356.
Weksler, M. E., and Hutteroth, T. H. (1974). *J. Clin. Invest.* **53,** 99.
Weksler, M. E., Innes, J. B., and Goldstein, G. (1978). *J. Exp. Med* **148,** 996.
Wigzell, H., and Stjernsward, J. (1966). *J. Natl. Cancer Inst.* 37, 513.
Wiktor-Jedrzejczak, W., Sharkie, S., Ahmed, A., and Sell, K. W. (1977). *Science* **197,** 313.
Woda, B. A., and Feldman, J. D. (1979). *J. Exp. Med.* **149,** 416.
Yunis, E. J., and Greenberg, L. J. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 2017.
Yunis, E. J., Fernandes, G., and Stutman, O. (1971). *Am. J. Clin. Pathol.* **56,** 280.
Yunis, E. J., Fernandes, G., Teague, P. O., Stutman, O., and Good, R. A. (1972). *In* "Tolerance, Autoimmunity, and Aging" (M. Siegel and R. A. Good, eds), p. 62. Thomas, Springfield, Illinois.
Yunis, E. J., Fernandes, G., Smith, J., Stutman, O., and Good, R. A. (1973). *In* "Microenvironmental Aspects of Immunity" (B. D. Jankovic and K. Isakovic, eds.), p. 301. Plenum, New York.
Zawadski, Z. A., and Edwards, G. A. (1972). *Prog. Clin. Immunol.* **1,** 105.
Zharhary, D., Segev, Y., and Gershon, H. (1977). *Mech. Ageing Dev.* **6,** 385.

Subject Index

A

Aleutian disease virus
  disease control, 281–282
  host immune response to infection
    hypergamma globulinemia, 267–270
    immune complexes, 272
    specific responses, 270–272
    unrelated responses, 272–273
  infection and host range, 266–267
  pathogenesis of lesions
    general considerations and genetic effects, 273–275
    glomerulonephritis and arteritis, 275–278
    manipulation of lesion production, 278–280
    other lesions, 280–281
  properties, 266
  in vitro growth, 265–266
  in vivo growth, 263–265
Antibody
  antigenic modulation of virus-infected cells and, 221–224
  binding to infected cells
    effect on synthesis and expression of viral proteins, 224–226
  cell-mediated cytotoxicity of virus-infected cells and, 245–249
  to lymphokines, 109–110
    characterization, 110–112
    preparation of, 110
  lysis of virus-infected cells and complement-mediated, 235–237
    heterologous complement and, 230–231
  redistribution of viral antigens and, 215–220
  role in killing of virus-infected cells *in vivo*, 253–254
Antibody response
  to viral infection, 213–214
  lymphokines and, 85–91
Arteritis, Aleutian disease and, 275–278

B

Bactericidal activity, complement and, 32–34
B cell(s)
  adaptive differentiation and concept of interacting partner cell sets, 184–192
  monokines and
    activation, 119–120
    differentiation, 118–119
Bone marrow chimeras
  mechanisms of adaptive differentiation in, 154–160
  recent experiments with, 145–147

C

Cell(s), lysis, complement and, 35–37
Cell interaction
  basis for genetic restrictions on, 142–144
  concept of environmental restraint
    antiself receptor responses and, 181–184
    extrathymic environment influence on helper T lympocytes, 167–177
    teaching F_1T cells to help nonresponder B cells, 178–181
  immune response, 138–139
    genes and, 139–140
    major histocompatibility complex genes and, 140–142
Chemotaxis, lymphokines and, 73–76
Colony stimulation
  lymphokines and, 98–99
  monokines and, 121
Complement
  alternative pathway
    cytolytic, 30–32
    lysis of virus-infected cells and, 237–241
    molecular events, 27–29

C3b-dependent feedback and amplification, 15–17
generation of bactericidal activity and bacteriolysis, 32–34
inactivation of viruses and, 37–38
host-parasite relationship and, 38–40
initiation of alternative pathway
concentration dependence, 21–22
discrimination between activators and nonactivators by bound C3b, 24–27
initial enzyme, 19–20
restriction of control by activators, 22–24
stable five-protein system, 20–21
lysis of nucleated cells and, 35–37
metastable binding site, 17–19
nephritic factor: autoantibody to C3 convertase, 42–44
proteins, 2–4
BlH, 13
C3, 4–9
C3b inactivator, 13–14
factor B: C3 proactivator, 9–12
factor D: C3 proactivator convertase, 12–13
monokines and, 123–124
properdin and, 14–15
reaction products influencing cellular functions, 40–42
regulation of, 17
Cytotoxic activity, lymphokines and, 76–82
Cytotoxicity, antibody-dependent cell-mediated, of virus-infected cells, 245–249

D

Differentiation
adaptive
concept of, 144–145
explanation of statements 1–7, 149–151
extrathymic vs. intrathymic influences on, 160–167
mechanism in bone marrow chimeras, 154–160
mechanism in normal circumstances, 151–154
nature and function of *Ir* genes, 196–201
recognition mechanisms in immune system, 192–196
theoretical considerations, 147–149

F

Fc receptor, herpes simplex virus-induced, interaction with IgG, 226–227

G

Genes
immune response and, 139–140
major histocompatibility complex, cell-cell interaction and, 140–142
Genetic effects, of Aleutian disease, 273–275
Glomerulonephritis, Aleutian disease and, 275–278

H

Herpes simplex virus, Fc receptor, interaction of IgG with, 226–227
Hypergammaglobulinemia, Aleutian disease and, 267–270

I

Immune complexes, Aleutian disease and, 272
Immune function
age-related changes in
cell-mediated, 289–291
humoral, 291–295
morphology, 288–289
mechanisms of age-related alterations
cellular changes, 296–308
cellular milieu, 295–296
thymus, 308–312
prevention and restoration of age-related changes
cell grafting, 316–317
chemical therapy, 317–321
dietary manipulation, 314–315

genetic manipulation, 315–316
tissue removal, 312–314
Immune responses
to Aleutian disease virus infection
specific, 270–272
unrelated, 272–273
cell-cell interactions and, 138–139
genes and, 139–140
genes, nature and function of, 196–201
Immune system, recognition mechanisms in, 192–196
Immunobiology, of mink, 262–263
Immunoglobulin, binding, lymphokines and, 95–96
Immunoglobulin G, interaction with herpes simplex virus-induced Fc receptor, 226–227
Interferon, lymphokines and, 91–94

L

Lymphocytes, activation, monokines and, 115–118
Lymphokines
antibodies to, 109–110
characterization, 110–112
preparation of, 110
chemotactic factors, 73–76
colony-stimulating activity, 98–99
cytotoxic and cytostatic factors, 76–82
immunoglobulin binding factor, 95–96
interferon, 91–94
lymphocyte factors regulating antibody responses, 85–91
lymphocyte mitogenic factor, 82–85
macrophage activating factor, 70–73
measurement, clinical relevance of, 114–115
migration inhibitory factor, 58–70
osteoclast activating factor, 99–101
regulation and pharmacologic modulation
of action, 107–109
of production, 103–107
synthesis, cell types involved, 101–103
tissue factor: procoagulent activity, 96–98
in vivo significance, 112–114

M

Macrophages, activation, lymphokines and, 70–73
Major histocompatibility complex genes, cell-cell interactions and, 140–142
Migration, inhibition, lymphokines and, 58–70
Mink, biology and immunobiology of, 262–263
Mitogenic factor, lymphokines and, 82–85
Monocytes, pyrogen, monokines and, 120–121
Monokines
B cell activating factor, 119–120
B cell differentiating factor, 118–119
biologic significance of, 124–125
colony-stimulating activity, 121
complement proteins, 123–124
lymphocyte activating factor, 115–118
monocyte pyrogen, 120–121
mononuclear cell factor, 121–122
neutral proteinases, 122–123
thymic differentiation factor, 121
Mononuclear cells, monokines and, 121–122

N

Nephritic factor, complement and, 42–44

O

Osteoclasts, activation, lymphokines and, 99–101

P

Procoagulant activity, lymphokines and, 96–98
Properdin, alternative complement pathway and, 14–15
Proteinases, neutral, monokines and, 122–123
Pyrogen, monocyte, monokines and, 120–121

T

Thymus
  age-related alterations in, 308–312
  differentiation, monokines and, 121

V

Viral antigen
  antibody-induced redistribution on surface, 215–220
  expression on surface of infected cells, 211–213
  role of modulation *in vivo*, 252–253
Viral infection, antibody response to, 213–214
Viral proteins, synthesis and expression, antibody binding to infected cells and, 224–226
Viruses, inactivation, complement and, 37–38
Virus-infected cells
  antibody-induced antigenic modulation of, 221–224
  cytotoxicity, antibody-dependent cell-mediated, 245–249
  lysis
    analysis of requirement for alternative complement pathway, 237–241
    analysis of requirement for antibody in complement-mediated lysis, 235–237
    antibody and heterologous complement in, 230–231
    aspects of target cell structure and, 241–242
    complement and, 229–230
    human serum and, 232–235
  role of antibody-dependent killing *in vivo*, 253–254

CONTENTS OF PREVIOUS VOLUMES

Volume 1

Transplantation Immunity and Tolerance
M. HAŠEK, A. LENGEROVA, AND T. HRABA

Immunological Tolerance of Nonliving Antigens
RICHARD T. SMITH

Functions of the Complement System
ABRAHAM G. OSLER

In Vitro Studies of the Antibody Response
ABRAM B. STAVITSKY

Duration of Immunity in Virus Diseases
J. H. HALE

Fate and Biological Action of Antigen-Antibody Complexes
WILLIAM O. WEIGLE

Delayed Hypersensitivity to Simple Protein Antigens
P. G. H. GELL AND B. BENACERRAF

The Antigenic Structure of Tumors
P. A. GORER

AUTHOR INDEX–SUBJECT INDEX

Volume 2

Immunologic Specificity and Molecular Structure
FRED KARUSH

Heterogeneity of γ-Globulins
JOHN L. FAHEY

The Immunological Significance of the Thymus
J. F. A. P. MILLER, A. H. E. MARSHALL, AND R. G. WHITE

Cellular Genetics of Immune Responses
G. J. V. NOSSAL

Antibody Production by Transferred Cells
CHARLES G. COCHRANE AND FRANK J. DIXON

Phagocytosis
DERRICK ROWLEY

Antigen-Antibody Reactions in Helminth Infections
E. J. L. SOULSBY

Embryological Development of Antigens
REED A. FLICKINGER

AUTHOR INDEX–SUBJECT INDEX

Volume 3

In Vitro Studies of the Mechanism of Anaphylaxis
K. FRANK AUSTEN AND JOHN H. HUMPHREY

The Role of Humoral Antibody in the Homograft Reaction
CHANDLER A. STETSON

Immune Adherence
D. S. NELSON

Reaginic Antibodies
D. R. STANWORTH

Nature of Retained Antigen and its Role in Immune Mechanisms
DAN H. CAMPBELL AND JUSTINE S. GARVEY

Blood Groups in Animals Other Than Man
W. H. STONE AND M. R. IRWIN

Heterophile Antigens and Their Significance in the Host-Parasite Relationship
C. R. JENKIN

AUTHOR INDEX–SUBJECT INDEX

Volume 4

Ontogeny and Phylogeny of Adaptive Immunity
ROBERT A. GOOD AND BEN W. PAPERMASTER

Cellular Reactions in Infection
EMANUEL SUTER AND HANSRUEDY RAMSEIER

Ultrastructure of Immunologic Processes
JOSEPH D. FELDMAN

Cell Wall Antigens of Gram-Positive Bacteria
MACLYN MCCARTY AND STEPHEN I. MORSE

Structure and Biological Activity of Immunoglobulins
SYDNEY COHEN AND RODNEY R. PORTER

Autoantibodies and Disease
H. G. KUNKEL AND E. M. TAN

Effect of Bacteria and Bacterial Products on Antibody Response
J. MUNOZ

AUTHOR INDEX–SUBJECT INDEX

Volume 5

Natural Antibodies and the Immune Response
STEPHEN V. BOYDEN

Immunological Studies with Synthetic Polypeptides
MICHAEL SELA

Experimental Allergic Encephalomyelitis and Autoimmune Disease
PHILIP Y. PATERSON

The Immunology of Insulin
C. G. POPE

Tissue-Specific Antigens
D. C. DUMONDE

AUTHOR INDEX–SUBJECT INDEX

Volume 6

Experimental Glomerulonephritis: Immunological Events and Pathogenetic Mechanisms
EMIL R. UNANUE AND FRANK J. DIXON

Chemical Suppression of Adaptive Immunity
ANN E. GABRIELSON AND ROBERT A. GOOD

Nucleic Acids as Antigens
OTTO J. PLESCIA AND WERNER BRAUN

In Vitro Studies of Immunological Responses of Lymphoid Cells
RICHARD W. DUTTON

Developmental Aspects of Immunity
JAROSLAV STERZL AND ARTHUR M. SILVERSTEIN

Anti-antibodies
PHILIP G. H. GELL AND ANDREW S. KELUS

Conglutinin and Immunoconglutinins
P. J. LACHMANN

AUTHOR INDEX–SUBJECT INDEX

Volume 7

Structure and Biological Properties of Immunoglobulins
SYDNEY COHEN AND CESAR MILSTEIN

Genetics of Immunoglobulins in the Mouse
MICHAEL POTTER AND ROSE LIEBERMAN

Mimetic Relationships between Group A Streptococci and Mammalian Tissues
JOHN B. ZABRISKIE

Lymphocytes and Transplantation Immunity
DARCY B. WILSON AND R. E. BILLINGHAM

Human Tissue Transplantation
JOHN P. MERRILL

AUTHOR INDEX–SUBJECT INDEX

Volume 8

Chemistry and Reaction Mechanisms of Complement
HANS J. MÜLLER-EBERHARD

Regulatory Effect of Antibody on the Immune Response
JONATHAN W. UHR AND
GÖRAN MÖLLER

The Mechanism of Immunological Paralysis
D. W. DRESSER AND
N. A. MITCHISON

In Vitro Studies of Human Reaginic Allergy
ABRAHAM G. OSLER,
LAWRENCE M. LICHTENSTEIN,
AND DAVID A. LEVY

AUTHOR INDEX–SUBJECT INDEX

Volume 9

Secretory Immunoglobulins
THOMAS B. TOMASI, JR., AND
JOHN BIENENSTOCK

Immunologic Tissue Injury Mediated by Neutrophilic Leukocytes
CHARLES G. COCHRANE

The Structure and Function of Monocytes and Macrophages
ZANVIL A. COHN

The Immunology and Pathology of NZB Mice
J. B. HOWIE AND B. J. HELYER

AUTHOR INDEX–SUBJECT INDEX

Volume 10

Cell Selection by Antigen in the Immune Response
GREGORY W. SISKIND AND
BARUJ BENACERRAF

Phylogeny of Immunoglobulins
HOWARD M. GREY

Slow Reacting Substance of Anaphylaxis
ROBERT P. ORANGE AND
K. FRANK AUSTEN

Some Relationships among Hemostasis, Fibrinolytic Phenomena, Immunity, and the Inflammatory Response
OSCAR D. RATNOFF

Antigens of Virus-Induced Tumors
KARL HABEL

Genetic and Antigenetic Aspects of Human Histocompatibility Systems
D. BERNARD AMOS

AUTHOR INDEX–SUBJECT INDEX

Volume 11

Electron Microscopy of the Immunoglobulins
N. MICHAEL GREEN

Genetic Control of Specific Immune Responses
HUGH O. MCDEVITT AND
BARUJ BENACERRAF

The Lesions in Cell Membranes Caused by Complement
JOHN H. HUMPHREY AND
ROBERT R. DOURMASHKIN

Cytotoxic Effects of Lymphoid Cells *In Vitro*
PETER PERLMANN AND
GÖRAN HOLM

Transfer Factor
H. S. LAWRENCE

Immunological Aspects of Malaria Infection
IVOR N. BROWN

AUTHOR INDEX–SUBJECT INDEX

Volume 12

The Search for Antibodies with Molecular Uniformity
RICHARD M. KRAUSE

Structure and Function of γM Macroglobulins
HENRY METZGER

Transplantation Antigens
R. A. REISFELT AND B. D. KAHAN

The Role of Bone Marrow in the Immune Response
NABIH I. ABDOU AND MAXWELL RICHTER

Cell Interaction in Antibody Synthesis
D. W. TALMAGE, J. RADOVICH, AND H. HEMMINGSEN

The Role of Lysosomes in Immune Responses
GERALD WEISSMANN AND PETER DUKOR

Molecular Size and Conformation of Immunoglobulins
KEITH J. DORRINGTON AND CHARLES TANFORD

AUTHOR INDEX–SUBJECT INDEX

Volume 13

Structure and Function of Human Immunoglobulin E
HANS BENNICH AND S. GUNNAR O. JOHANSSON

Individual Antigenic Specificity of Immunoglobulins
JOHN E. HOPPER AND ALFRED NISONOFF

In Vitro Approaches to the Mechanism of Cell-Mediated Immune Reactions
BARRY R. BLOOM

Immunological Phenomena in Leprosy and Related Diseases
J. L. TURK AND A. D. M. BRYCESON

Nature and Classification of Immediate-Type Allergic Reactions
ELMER L. BECKER

AUTHOR INDEX–SUBJECT INDEX

Volume 14

Immunobiology of Mammalian Reproduction
ALAN E. BEER AND R. E. BILLINGHAM

Thyroid Antigens and Autoimmunity
SIDNEY SHULMAN

Immunological Aspects of Burkitt's Lymphoma
GEORGE KLEIN

Genetic Aspects of the Complement System
CHESTER A. ALPER AND FRED S. ROSEN

The Immune System: A Model for Differentiation in Higher Organisms
L. HOOD AND J. PRAHL

AUTHOR INDEX–SUBJECT INDEX

Volume 15

The Regulatory Influence of Activated T Cells on B Cell Responses to Antigen
DAVID H. KATZ AND BARUJ BENACERRAF

The Regulatory Role of Macrophages in Antigenic Stimulation
E. R. UNANUE

Immunological Enhancement: A Study of Blocking Antibodies
JOSEPH D. FELDMAN

Genetics and Immunology of Sex-Linked Antigens
DAVID L. GASSER AND WILLYS K. SILVERS

Current Concepts of Amyloid
EDWARD C. FRANKLIN AND DOROTHEA ZUCKER-FRANKLIN

AUTHOR INDEX–SUBJECT INDEX

Volume 16

Human Immunoglobulins: Classes, Subclasses, Genetic Variants, and Idiotypes
J. B. NATVIG AND H. G. KUNKEL

Immunological Unresponsiveness
WILLIAM O. WEIGLE

Participation of Lymphocytes in Viral Infections
E. FREDERICK WHEELOCK AND STEPHEN T. TOY

Immune Complex Diseases in Experimental Animals and Man
C. G. COCHRANE AND D. KOFFLER

The Immunopathology of Joint Inflammation in Rheumatoid Arthritis
NATHAN J. ZVAIFLER

AUTHOR INDEX–SUBJECT INDEX

Volume 17

Antilymphocyte Serum
EUGENE M. LANCE, P. B. MEDAWAR, AND ROBERT N. TAUB

In Vitro Studies of Immunologically Induced Secretion of Mediators from Cells and Related Phenomena
ELMER L. BECKER AND PETER M. HENSON

Antibody Response to Viral Antigens
KEITH M. COWAN

Antibodies to Small Molecules: Biological and Clinical Applications
VINCENT P. BUTLER, JR., AND SAM M. BEISER

AUTHOR INDEX–SUBJECT INDEX

Volume 18

Genetic Determinants of Immunological Responsiveness
DAVID L. GASSER AND WILLYS K. SILVERS

Cell-Mediated Cytotoxicity, Allograft Rejection, and Tumor Immunity
JEAN-CHARLES CEROTTINI AND K. THEODORE BRUNNER

Antigenic Competition: A Review of Nonspecific Antigen-Induced Suppression
HUGH F. PROSS AND DAVID EIDINGER

Effect of Antigen Binding on the Properties of Antibody
HENRY METZGER

Lymphocyte-Mediated Cytotoxicity and Blocking Serum Activity to Tumor Antigens
KARL ERIK HELLSTRÖM AND INGEGERD HELLSTRÖM

AUTHOR INDEX–SUBJECT INDEX

Volume 19

Molecular Biology of Cellular Membranes with Applications to Immunology
S. J. SINGER

Membrane Immunoglobulins and Antigen Receptors on B and T Lymphocytes
NOEL L. WARNER

Receptors for Immune Complexes on Lymphocytes
VICTOR NUSSENZWEIG

Biological Activities of Immunoglobulins of Different Classes and Subclasses
HANS L. SPIEGELBERG

SUBJECT INDEX

Volume 20

Hypervariable Regions, Idiotypy, and Antibody-Combining Site
J. DONALD CAPRA AND J. MICHAEL KEHOE

Structure and Function of the J Chain
MARIAN ELLIOTT KOSHLAND

Amino Acid Substitution and the Antigenicity of Globular Proteins
MORRIS REICHLIN

The *H-2* Major Histocompatibility Complex and the *I* Immune Response Region: Genetic Variation, Function, and Organization
DONALD C. SHREFFLER AND CHELLA S. DAVID

Delayed Hypersensitivity in the Mouse
ALFRED J. CROWLE

SUBJECT INDEX

Volume 21

X-Ray Diffraction Studies of Immunoglobulins
ROBERTO J. POLJAK

Rabbit Immunoglobulin Allotypes: Structure, Immunology, and Genetics
THOMAS J. KINDT

Cyclical Production of Antibody as a Regulatory Mechanism in the Immune Response
WILLIAM O. WIEGLE

Thymus-Independent B-Cell Induction and Paralysis
ANTONIO COUTINHO AND GÖRAN MÖLLER

SUBJECT INDEX

Volume 22

The Role of Antibodies in the Rejection and Enhancement of Organ Allografts
CHARLES B. CARPENTER, ANTHONY J. F. D'APICE, AND ABUL K. ABBAS

Biosynthesis of Complement
HARVEY R. COLTEN

Graft-versus-Host Reactions: A Review
STEPHEN C. GREBE AND J. WAYNE STREILEIN

Cellular Aspects of Immunoglobulin A
MICHAEL E. LAMM

Secretory Anti-Influenza Immunity
YA. S. SHVARTSMAN AND M. P. ZYKOV

SUBJECT INDEX

Volume 23

Cellular Events in the IgE Antibody Response
KIMISHIGE ISHIZAKA

Chemical and Biological Properties of Some Atopic Allergens
T. P. KING

Human Mixed-Lymphocyte Culture Reaction: Genetics, Specificity, and Biological Implications
BO DUPONT, JOHN A. HANSEN, AND EDMOND J. YUNIS

Immunochemical Properties of Glycolipids and Phospholipids
DONALD M. MARCUS AND GERALD A. SCHWARTING

SUBJECT INDEX

Volume 24

The Alternative Pathway of Complement Activation
O. GÖTZE AND H. J. MÜLLER-EBERHARD

Membrane and Cytoplasmic Changes in B Lymphocytes Induced by Ligand-Surface Immunoglobulin Interaction
GEORGE F. SCHREINER AND EMIL R. UNANUE

Lymphocyte Receptors for Immunoglobulin
HOWARD B. DICKLER

Ionizing Radiation and the Immune Response
ROBERT E. ANDERSON AND NOEL L. WARNER

SUBJECT INDEX

Volume 25

Immunologically Privileged Sites
CLYDE F. BAKER AND
R. E. BILLINGHAM

Major Histocompatibility Complex Restricted Cell-Mediated Immunity
GENE M. SHEARER AND
ANNE-MARIE SCHMITT-VERHULST

Current Status of Rat Immunogenetics
DAVID L. GASSER

Antigen-Binding Myeloma Proteins of Mice
MICHAEL POTTER

Human Lymphocyte Subpopulations
L. CHESS AND S. F. SCHLOSSMAN

SUBJECT INDEX

Volume 26

Anaphylatoxins: C3a and C5a
TONY E. HUGLI AND HANS
J. MÜLLER-EBERHARD

H-2 Mutations: Their Genetics and Effect on Immune Functions
JAN KLEIN

The Protein Products of the Murine 17th Chromosome: Genetics and Structure
ELLEN S. VITETTA AND
J. DONALD CAPRA

Expression and Function of Idiotypes on Lymphocytes
K. EICHMANN

The B-Cell Clonotype Repertoire
NOLAN H. SIGAL AND
NORMAN R. KLINMAN

SUBJECT INDEX

Volume 27

Autoimmune Response to Acetylcholine Receptors in Myasthenia Gravis and Its Animal Model
JON LINDSTROM

MHC-Restricted Cytotoxic T Cells: Studies on the Biological Role of Polymorphic Major Transplantation Antigens Determining T-Cell Restriction-Specificity, Function, and Responsiveness
ROLF M. ZINKERNAGEL AND
PETER C. DOHERTY

Murine Lymphocyte Surface Antigens
IAN F. C. MCKENZIE AND
TERRY POTTER

The Regulatory and Effector Roles of Eosinophils
PETER F. WELLER AND
EDWARD J. GOETZL

SUBJECT INDEX

Volume 28

The Role of Antigen-Specific T Cell Factors in the Immune Response
TOMIO TADA AND KO OKUMURA

The Biology and Detection of Immune Complexes
ARGYRIOS N. THEOFILOPOULOS AND
FRANK J. DIXON

The Human Ia System
R. J. WINCHESTER AND
H. G. KUNKEL

Bacterial Endotoxins and Host Immune Responses
DAVID C. MORRISON AND
JOHN L. RYAN

Responses to Infection with Metazoan and Protozoan Parasites in Mice
GRAHAM F. MITCHELL

SUBJECT INDEX

This Page Intentionally Left Blank