1977

Studies on the Complement Receptor of a Human Lymphoblastoid Cell Line

Gary John Hoffman

6

STUDIES ON THE COMPLEMENT RECEPTOR OF A HUMAN LYMPHOBLASTOID CELL LINE

A thesis submitted to the Faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Gary J. Hoffman, B.Med.Sc. M.A. B.M. B.Ch.

6 April 1977

The Rockefeller University

New York

TABLE OF CONTENTS

	Page
SUMMARY	vi
ACKNOWLEDGEMENTS	ix
CHAPTER I. INTRODUCTION	1
A. THE THIRD COMPONENT OF COMPLEMENT	1
B. RECEPTORS FOR COMPLEMENT	9
1. Historical Introduction	9
2. The C3 Receptors of Mouse and Rat Lymphocytes	13
3. The C3 Receptors of Human Lymphocytes	17
4. Human Lymphoid Lines	20
5. Human Lymphoid Line Raji	21
6, Human Lymphoid Line 8866	22
7. The Function of the Complement Receptor on Lymphocytes	23
8. The Definition of a Receptor	25
C. HUMAN LYMPHOID CELL LINES	27
1. Historical Survey	27
2. Features of Human Lymphoid Cell Lines	28
a. Source of the cell lines	28
b. Correlates with the ability to establish cell lines	29
c. General features of lymphoblastoid cell lines	30
d. Surface markers and secretory products of the lines derived from human peripheral blood	32
e. The homogeneity of lines	33
f. The stability of markers.	34
g. Products of lymphoid cell lines.	35
h. The karyotype of lymphoid cell lines	35
CHAPTER II. MATERIALS AND METHODS	37
A. CELLS: CULTURE AND CLONING	37
B. ROSETTING	45
C. IODINATION	45
D. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS	46
E. ELECTRON MICROSCOPY AND AUTORADIOGRAPHY	47
F. KARYOTYPING	47
G. SOLUTIONS AND REAGENTS	50

CHAPTER III. 8866 CELLS AND THE COMPLEMENT RECEPTOR 52

A. INTRODUCTION 52

1. General 52
2. 8866 and Complement Receptors 56

B. CLONING 61

1. Introduction 61
2. Semi-solid Substrate Cloning 63

a. Methodology 63
b. Results 63

C. PICKED SINGLE CELL CLONING 68

D. ON THE IDENTIFICATION OF CLONES 90

E. TRANSFER OF RECEPTORS FROM POSITIVE TO NEGATIVE CELLS 105

1. Mixing Experiments 105
2. Addition of Cell Growth Medium to Rosetting Mixtures 108

F. THE PHENOTYPIC INSTABILITY OF COMPLEMENT RECEPTOR EXPRESSION 109

CHAPTER IV. PROPERTIES OF THE LIGAND-RECEPTOR INTERACTION 117

A. INTRODUCTION 117

B. RESULTS 117

1. Ligand Specificity of the Receptor 117
2. Temperature Dependence of Rosetting 126
3. The Divalent Cation Independence of Rosetting 126
4. The Effect of Metabolic Inhibitors on Rosetting 126
5. The Effects of Colchicine and Cytochalasin B on Rosetting 133
6. The Effect of Immobilization of the Lymphoid Cell Membrane on Rosetting 136
7. The Effects of Reagents Reacting with Sulfhydryl Groups on Rosetting 136
8. The Nature of the Receptor 141

CHAPTER V. LACTOPEROXIDASE IODINATION OF 8866 CELLS 148

A. INTRODUCTION 148

B. METHODOLOGY 149

1. Iodination Protocol 149
2. Validation of the Localization of the Radio-iodination to the Cell Plasma Membrane 156

C. COMPARISON OF THE IODINATION PATTERNS OF RECEPTOR POSITIVE AND NEGATIVE CELLS 183

D. THE EFFECTS OF PROTEASES ON IODINATED MEMBRANE PROTEINS OF 8866 CELLS 189

E. THE TURNOVER OF IODINATED PROTEINS ON THE SURFACE OF 8866 CELLS 192

CHAPTER VI. THE SEARCH FOR THE C3 RECEPTOR 210

A. INTRODUCTION 210

B. METHODOLOGY 212

1. Glutaraldehyde-fixed Sheep Erythrocytes 212
2. Formaldehyde-fixed Sheep Erythrocytes 228
3. Zymosan Particles 228
4. Specifically Cross-linked Beads 237
5. Selection for Ligand-bound Molecules, Then Iodination 238

CHAPTER VII. DISCUSSION 241

A. 8866 AS A MODEL B-LYMPHOCYTE 241

B. VALIDITY OF THE CLONING METHOD 245

C. THE ANALYSIS OF THE COMPLEMENT RECEPTOR PHENOTYPE BY CLONING 247

D. COMPARISON OF THE COMPLEMENT RECEPTORS OF PERIPHERAL BLOOD LYMPHOCYTES AND 8866 CELLS 250

E. THE USE OF SURFACE IODINATION OF CELLS IN THE SEARCH FOR SURFACE RECEPTORS 252

1. Methods 252
2. Results 255

BIBLIOGRAPHY 266

SUMMARY

A human lymphoblastoid cell line, 8866, was used to investigate a surface marker, the C3d receptor. A method for cloning these cells at a relatively high efficiency was developed and C3d receptor bearing (CR+) and receptor free (CR-) subclones isolated. Receptor bearing cells were identified by their capacity to bind to complement coated sheep erythrocytes. Of the many clones isolated, only a few contained uniformly receptor positive or negative cells. Most expressed an intermediate character, not only in the percentage of CR+ cells, but also in the degree of resettability of the individual CR+ cells. Clones initially wholly CR+ remained CR+ both on prolonged culture and on recloning. CR-populatons always developed some CR+ cells with continued culture. Upon recloning, CR- clones gave rise to largely CR- clones, but also to CR+ clones, and to clones of intermediate character. Recloning of a CR+ clone derived from a CR- clone yielded mainly intermediate clones, but also CR- clones: the CR+ trait was unstable in such derivatives.

Having thus demonstrated the phenotypic stability of some clones, and the instability of others, attempts were made to induce or repress the expression of the receptor. A variety of compounds (BrdU, DMSO, 2-deoxy-glucose and sodium butyrate) had no effect. Neither did mixing CR+ and CR- cells, nor growing CR- cells in medium conditioned by CR+ cells. There was an apparent increase in the number of CR- clones produced by the picked single cell cloning method (in which cells grow for some time at suboptimal cell densities), and CR+ cells always appeared in cultures of cells kept at optimal density in the logarithmic phase of growth. These were the only conditions found to modify the expression of the receptor.

Since 1) the growth rates of CR+ and CR- cell types were similar, and 2) continued culture of clones containing mixtures of the two cell types

never became wholly CR+ or CR-, and 3) continued cultures varied the degree of receptor expression rapidly and significantly, it is considered unlikely that the conversion of CR- to a partially CR+ population could be explained simply by the emergence of a CR+ variant with a growth advantage. The ability of clonally derived cells to alter their degree of receptor expression indicates that the CR- cells are not mutants deficient in the structural gene necessary for the production of the receptor, nor are they bearers of a mutant structural gene which produces a receptor molecule with altered activity. Examination of karyotypes of the different clones has shown near diploid and near tetraploid clones of both CR+ and CR-types. Limited examination of individual karyotypes has not revealed any distinctive chromosomal abnormalities.

The nature of the receptor with respect to complement components was investigated. Using purified complement components, and specific antisera, it has been established that 8866 cells bear a C3d receptor exclusively. A number of physico-chemical studies were carried out to define the ligand-receptor interaction. It was concluded that the receptor may be regarded as representative of that present on the plasma membrane of normal peripheral blood lymphocytes.

The receptor is presumed to be an externally disposed protein, since its activity is destroyed by proteases, and its regeneration is inhibited by cycloheximide at concentrations which inhibit protein synthesis.

In an effort to identify the C3d receptor molecule(s), the lactoperoxidase-mediated iodination procedure of Hubbard and Cohn (1975a) was slightly modified for 8866 cells, and the major iodinatable surface proteins of these cells defined. Three major species, with molecular weights of approximately 11,000, 34,000, and 50,000 daltons were found, and eight other species were reproducibly present. No significant differences were found

between the iodine labelling patterns of membrane proteins of CR+ and CR- cell populations by this technique. Further studies with selective protease treatment, and affinity binding techniques were also unavailing in detecting the C3d receptor molecule(s). The proteolysis of iodinated cells did reveal a uniquely protease sensitive moiety of 35,000 daltons, which was cleaved by trypsin, chymotrypsin, papain and pronase, leading to the appearance of a new cell-associated moiety with a molecular weight of 22,000 daltons.

These studies have established the clonal character of the C3d receptor, and provided homogeneous populations of cells bearing a receptor like that on peripheral blood lymphocytes. In addition, an unstable system has been defined. This may prove useful in the investigation of the regulation of the expression of the C3d receptor.

ACKNOWLEDGEMENTS

These studies were performed in the laboratory of Dr. Samuel C. Silverstein. It is a pleasure to acknowledge the contributions of his breadth of interests and understanding, and his constant enthusiasm for ideas.

The Laboratory of Cellular Physiology and Immunology of Drs. James G. Hirsch and Zanvil A. Cohn provided a constant source of assistance, ideas and the comradeship to make carrying out studies not only possible, but also enjoyable. In addition to the heads of the laboratory, I must especially acknowledge the contributions of Drs. Paul Edelson, Celso Bianco and Ann Hubbard.

One of the special privileges of working at the Rockefeller University was the pleasure of learning from people in many different disciplines. I would particularly wish to thank Drs. T.P. King, Elaine Diacumakos, M.A. Schreiber, N-H Chua, and Dr. Purnell Choppin and the Virology laboratory.

Others outside the University were also generous in their advice and assistance. In addition to acknowledgements within the text, I would like to note my gratitude to some of these people here, viz. Dr. George Miller, of Yale University; Drs. Gordon Ross and Gretchen Darlington, of Cornell-New York Hospital; Drs. Dev and Tantravahi of Dr. O.J. Miller's laboratory at Columbia University; and Dr. Matthew Scharff of Albert Einstein College of Medicine.

Finally, I would like to extend my thanks to Miss Judy Adams, Mrs. Marion Johansen and Mrs. Grace Silvestri, members of the "Fourth Floor Family", for many kindnesses above and beyond the call of duty.

CHAPTER I

INTRODUCTION

The purpose of the work described in this thesis was to investigate the surface receptors of the plasma membrane of a lymphoblastoid cell line, 8866. The receptor chosen for characterization was that for C3d, a cleaved fragment of the third component of complement. It was found that erythrocytes coated with C3d bound to a variable proportion of the lymphoblastoid cells, and that this variability was independent of the cell density in culture and of the stage of the cells in the cell cycle. To further analyse this heterogeneity of C3d receptor activity, line 8866 was cloned, and the clones were characterized with respect to their growth properties, their membrane proteins, and chromosomal number and morphology, in addition to their ability to bind C3d -coated erythrocytes.

To place this work in perspective, it is necessary first to review the present knowledge of the third component of complement, receptors for this ligand on lymphocytes and lymphoid cell lines, and the properties of human lymphoid cell lines.

A. THE THIRD COMPONENT OF COMPLEMENT

The third component of complement is the most abundant of the complement species, being present in human serum at a concentration of approximately 1.2 mg/ml. It was first isolated by Müller-Eberhard and Nilsson in 1960, but the principal features of the molecule have only been elucidated in the last five years. The complete amino acid sequence of C3 has

not been determined. Consequently, there are still uncertainties regarding the order of the fragments of the molecule following limited proteolytic digestion, and the location of certain biological activities within the intact molecule. Human C3 has a molecular weight of approximately 195,000 daltons, and is made up of two subunits held together by disulfide bonds (Nilsson and Mapes, 1973). The larger subunit, alpha, has a molecular weight of 120,000 daltons, while the smaller, beta, has been assigned 75,000 daltons (Bokisch et al., 1975).

Activation of several of the components of the complement cascade involves a proteolytic cleavage of inactive precursors to smaller, active molecules. Several cleavages of the third component (C3) have been described, and account for most of the information we have concerning the structure of this molecule. The complex formed by the first, fourth and second components exhibits an enzymatic activity known as C3 convertase (Muller-Eberhard et al., 1967) which cleaves intact C3 into two fragments, C3a and C3b (See Fig. 1). This activity is mimicked by trypsin, yielding very similar products (Bokisch et al., 1969). The C3a fragment has a molecular weight of approximately 9,000 daltons and is responsible for the anaphylotoxic properties of the cleaved C3 (Silva et al., 1967). Hugli (1975) showed that the cleavage occurs uniquely between the 77th and 78th residues of the alpha chain. He demonstrated that C3a has a C-terminal arginine, whereas the C terminal residues of theintact C3 are not detectable by conventional means. Since the cleavage leads to shortening of the alpha, and not the beta chain, and both C3a and intact C3 have an N-terminal serine, the evidence indicates that C3a represents the N-terminus of the

alpha chain of the C3 molecule. The biological activity of the C3a is dependent on the presence of a high degree of secondary structure (alpha-helical), the maintenance of intrachain disulfide bonds, and the C-terminal arginine residue (Hugli et al., 1975a,b).

Excision of the C3a fragment produces a labile binding site on the rest of the C3 molecule. This binding site remains active for only a brief period (milliseconds), during which time it may bind strongly, through an unknown linkage, to a wide variety of cell surfaces, and to immunoglobulins. The labile binding site must be in that portion of the C3 molecule which becomes the C3d fragment, since, following subsequent cleavage to C3c and C3d, the C3d portion remains bound to the site of initial attachment, while the C3c fragment is released into solution. The mechanism of decay of the labile binding activity is not understood.

After loss of the short-lived, non-specific binding activity, the C3b fragment, either in solution, or bound to a surface, exhibits two stable sites capable of mediating binding to receptors present on a restricted range of cell types. One is the C3b, or Type I, receptor site, and the other the C3d receptor, or Type II site, which may be only partially expressed.

Additionally, the C3b molecule has two other activities. When complexed with C142 (the C3 convertase activity), a C5 convertase activity is generated. This cleaves C5 to C5b, which, in a complex with C6, binds to C3b (Hammer et al., 1976). In the alternative pathway, C3b interacts with Factors B and $\overline{D}$ to form a C3 convertase activity.

The C3b molecule is subject to cleavage by a heat-stable serum protease known as C3b inactivator (Tamura and Nelson, 1967) or conglutinogen activat-

ing factor (Lachmann and Müller-Eberhard, 1968). Gitlin and colleagues (1975) have presented evidence for rapid cleavage of C3b by this enzyme, yielding a product, $C3b^i$, which is extremely sensitive to further proteolytic cleavage, thereby becoming no longer functional in the alternative pathway. $C3b^i$ is made up of an alpha chain of 85,000 daltons, and a beta-chain of 70,000 daltons, thus appearing to result from the cleavage of the alpha chain alone. In the analytical system used by Gitlin and colleagues, the nature of the cleaved fragment could not be determined. Whereas the first cleavage took place in three minutes, the second cleavage occurred over a three hour period. The final product of this reaction was a disulfide-linked two-chain molecule resembling C3c. On disulfide reduction, this molecule yielded polypeptides of 70,000 daltons on SDS polyacrylamide gel electrophoresis. As in the analysis of $C3b^i$, however, the gel system used was such that small fragments would be run off the gel, and not detected. The same group (Stossel and others, 1975) has presented some evidence that $C3b^i$ may be the opsonically active portion of C3b.

Bokisch and colleagues (1975) have shown that C3b inactivator cleaves C3b into C3c, of 140,000 daltons, and C3d, of 25,000 daltons. Like Gitlin and co-workers, they found the C3c susceptible to subsequent cleavages, and showed the sensitive sites were in the alpha chain, with products of 25,000 and 20,000 daltons being produced in a ratio of two to one. The beta chain appeared to be unaffected. In this work there is no evidence of a single 15,000 dalton fragment to account for the Gitlin results, nor is there any

evidence of a species at the molecular weight of $C3b^i$, although no particular attempts were made to use very short times of incubation to look for an early conversion product. However, Bokisch notes a discrepancy of approximately 16,000 daltons between the sum of the fragments accounted for, and the initial molecular weight (C3a 9,000 + C3c 140,000 + C3d 25,000 = 174,000 vs 190,000): the discrepancy could be real, but also could be an artefact of straining the method of measuring molecular weights beyond its limits.

Reasoning from these data, Bokisch and colleagues (1975) have constructed a model of the C3 molecule. Unfortunately, insufficient data are available to allow the various cleavage products of the alpha chain to be arranged in order or to correlate the biological activities of the cleaved molecule with specific subfragments.

Like C3b, C3d has two distinct binding sites. One is the result of the initial activationof C3, and is manifest by the continued adherence of the C3d fragment to the site where the larger C3b molecule was initially bound. The second is retained after the cleavage of C3b, and is the ability to interact with specific receptors on a restricted range of cells. This ability to bind is long-lived, and will be discussed subsequently.

Figure I-1 is presented as a summary diagram of the major features of the C3 molecule and its cleaved products.

Thus the C3 molecule has a number of activities which remain to be accounted for in terms of specific amino acid sequences on the molecule. These include the labile binding site of newly cleaved C3b, the stable site of C3b which binds to receptors, the stable site of C3d which binds to another class of receptors, and the site or sites which enable C3b to

complex with C142 to form a C5 convertase, or with Factors B and $\overline{D}$ to form a C3 convertase. From the discussion above it is evident that the sites capable of binding to receptors are on different parts of the C3 molecule: it is not clear whether the enzymatic activities are located in one or two sites.

To complete a discussion of the C3 molecule, two points should be made. Antigenically similar molecules with different electrophoretic mobilities have been identified as polymorphic forms of C3. These have been used in population studies (see review: Seth and Seth, 1976). Secondly, there is now evidence of a linkage between levels of C3 in serum and the major histocompatibility region of the murine genome (Ferreira and Nussenzweig, 1975). This is the third complement component to be mapped to the major histocompatibility region: Fu et al. (1974) had described an association between human C2 deficiency and an HL-A haplotype, while Meo et al. (1975) reported the identity of Ss protein and C4 in mouse, thus placing the genes which encode, or regulate, C4 expression in the middle of the H2 complex.

It is relevant to compare C3 with another complement component, C4, with which it shares many interesting characteristics. The fourth component of complement is found in the serum at approximately 0.4 mg/ml. It is a protein with a molecular weight of 200,000 daltons. While C3 has two disulfide-linked chains, C4 has three, designated alpha, beta and gamma (Schreiber and Müller-Eberhard, 1974). Like C3, the intact C4 molecule is cleaved by proteolysis to an active component, 4b, which has a labile site capable of binding to cell membranes or to immunoglobulins. The activated

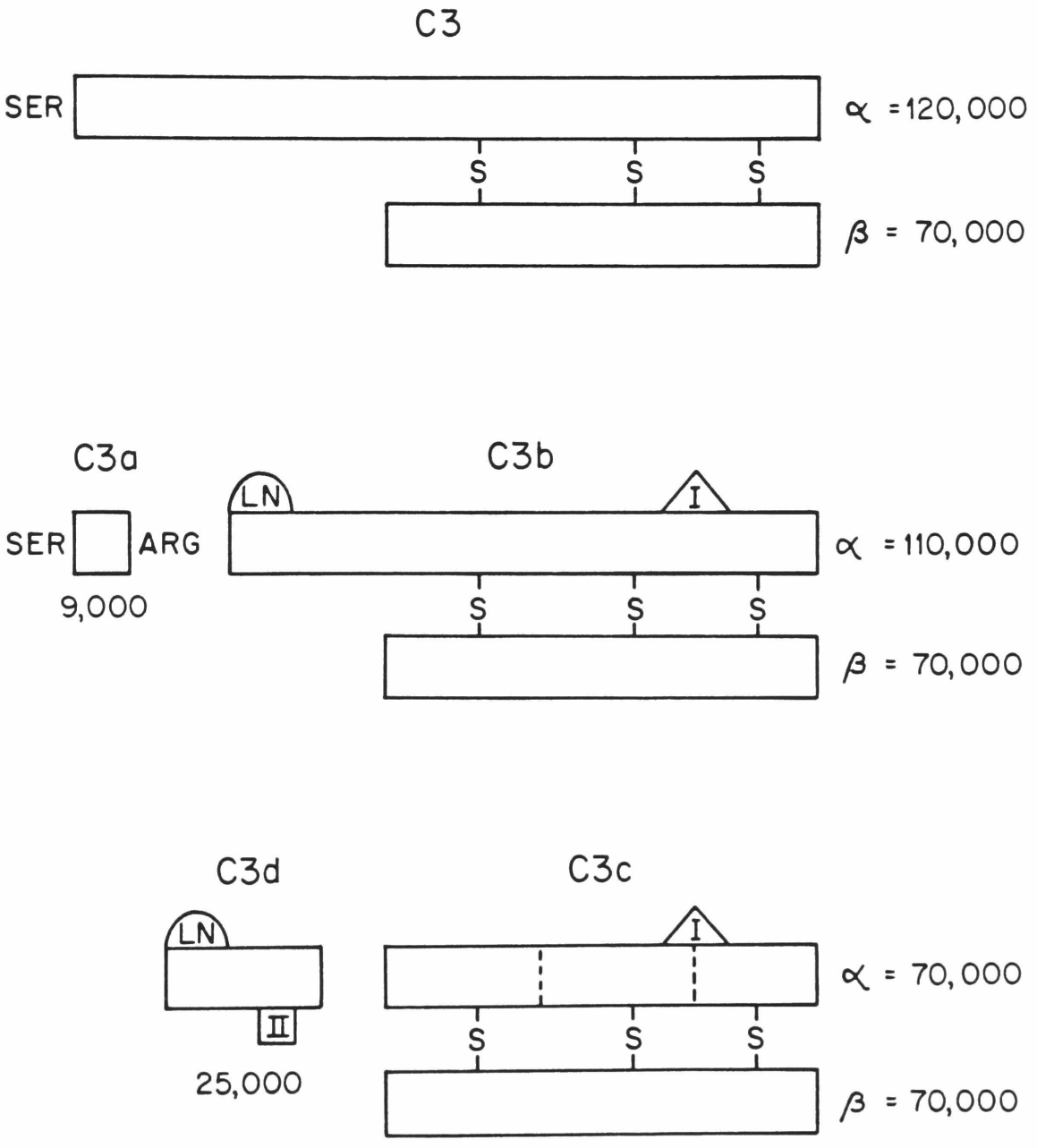
LEGEND: FIGURE I-1. A SCHEMATIC DIAGRAM OF C3 AND ITS CLEAVAGE PRODUCTS.

The intact C3 molecule (shown at the top of the figure) is formed of an alpha and a beta chain linked by three disulfide bonds. The exact location of these bonds is not known, though all are known to be present in the C3c fragment.

The C3b molecule is shown in the middle of the figure. The symbol "LN" represents the "Labile Non-Specific" site revealed by C3 convertase or trypsin. The Roman numeral "I" indicates the site which binds to the C3b receptor. The Roman numeral "II" indicates the site which binds to the C3d receptor.

On the C3a, the N-terminal serine and C-terminal arginine are indicated.

The two molecules shown at the bottom of the figure illustrate the fate of C3b after the action of C3b inactivator. The C3d fragment still bears the "LN" site, and the type II site. The C3c fragment carries the C3b receptor-binding site, as evidenced by its ability to block C3b-mediated rosetting. Upon reduction, the C3c runs as four bands on a dissociating SDS polyacrylamide gel.


C3
SER
α =120,000
S
S
S
β = 70,000
C3a
C3b
LN
I
SER
ARG
α =110,000
9,000
S
S
S
β = 70,000
C3d
C3c
LN
I
α = 70,000
II
S
S
S
25,000
β = 70,000

bound C4b has a short-lived enzymatic activity, partially stabilized by the binding of C2, and is rendered inactive by an inactivator indistinguishable from the C3 inactivator (Cooper, 1975). The transient activity, like that of C3b, is the ability to cleave the next component in the complement sequence. In addition to these structural similarities, it has been observed that C4b confers on particles to which it is bound the ability to bind to receptors for C3b (Cooper, 1969; Ross and Polley, 1975). This property will be considered at more length later.

For the purposes of this thesis the essential point is that, in the course of cleavage of the C3 molecule, two types of sites are revealed. One is a transient, non-specific site capable of binding firmly to a target particle such as a sheep erythrocyte, and unavailable for further interactions. The second type is stable, and capable of binding to surface receptors on specific cell types.

B. RECEPTORS FOR COMPLEMENT

1. Historical Introduction.

Two independent lines of research eventually converged with the clarification of the nature of complement receptors. The first began with Laveran and Mesnil (1901) and culminated in the work of Rieckenberg (1917) (see review by Nelson, 1963). These workers noted that serum induced adherence of a variety of microorganisms and protozoa to platelets and leukocytes. The second line began with Wright and Douglas (1903). They reported that fresh serum, as opposed to heat-inactivated serum, opsonized microorganisms for ingestion by leukocytes. In both cases, further work

was hindered by failure to differentiate the effects due to heat-labile components and those due to heat stable components of immune sera; that is, they failed to distinguish the effects of complement and antibody. It was the work of R. A. Nelson and his group (1953) which led to the first clear delineation of the complement-component dependent interaction between micro-organisms and human erythrocytes (Immune Adherence). However, after 10 years work in the area, D. S. Nelson, in his comprehensive state-of-the-art review (1963), was only able to catalogue a variety of phenomenological observations, many poorly controlled, with some simple physiochemical observations, and no definition of the complement components involved. Further progress of the field had to await the characterization of the complement components, and of the immunoglobulins. With these tools, it was possible to distinguish two phenomena. The first was the adherence of IgG-coated particles to nucleated cells mediated by the Fc portion of IgG. This was initially definitively described on murine macrophages by Berken and Benacerraf (1966), on murine polymorphonuclear leukocytes and macrophages (Lay and Nussenzweig, 1968), on murine lymphocytes (Uhr and Phillips, 1966) and on human monocytes (Huber and others, 1968). Basten and colleagues (1972a,b) are responsible for the definitive description of the Fc receptor of human lymphocytes. The remainder of this discussion will be concerned with the second phenomenon, namely the complement mediated process.

The role of C3 in attachment of sensitized target cells, and their phagocytosis, was first presented by three groups of workers in 1968 (Gigli and Nelson; Huber et al.; Lay and Nussenzweig). Gigli and Nelson used a system of guinea pig complement components and guinea pig leukocytes and

measured phagocytosis of sensitized sheep red cells. By this assay it was clearly shown that C3 was the determinant responsible for the enhancement of phagocytosis, but neither the nature of sensitizing antibody used, nor its quantitative relation to phagocytosis, were defined. Gigli and Nelson also demonstrated that, for guinea pig polymorphs, the C3 which mediated phagocytosis had to be in the form mediating Immune Adherence, since after exposure of sensitized particles to C3 inactivators in serum, the red cells were no longer able to bind to human red cells nor to be phagocytosed. Transposing this result to the findings with human complement, C3b and not C3d was the effective species.

Huber and colleagues studied human monocytes in monolayer cultures. They demonstrated that IgG sensitized sheep erythrocytes were opsonized, while IgM sensitized sheep erythrocytes were not opsonized until C3 was added by sequentially building a complex of purified human complement components consisting of C1423. The paper established two other important features of the interaction. Firstly, the binding site on the monocytes for C3 was trypsin sensitive, whereas the IgG site was not. Secondly, only 18% of monocytes phagocytosed red cells coated with IgM and complement, while 92% of monocytes ingested IgG coated red cells. Thirdly, the enhancing effect of C3 on phagocytosis occurred with many less C3 molecules per red cell when IgG was the sensitizing antibody than when IgM was used.

The most exhaustive study, and not the last chronologically, was that of Lay and Nussenzweig. These workers used murine cells, and assayed for attachment (rosetting) of sensitized sheep erythrocytes. They demonstrated the presence of receptors for complement sensitized target cells on mouse

peritoneal macrophages, polymorphonuclear leukocytes, and some lymph node lymphocytes, but not on thymocytes. They showed that sensitization occurred with C5 deficient serum, and hence the factor responsible must be earlier in the complement pathway. They also described the trypsin-sensitivity of the complement receptor. In addition, the binding to polymorphonuclear cells and macrophages, but not to lymphocytes, was magnesium ion dependent. A number of possible inhibitors of rosetting were tested, and heparin, N-acetylglucosamine, N-acetylgalactosamine, sodium azide, sodium merthiolate, actinomycin D, and puromycin were without effect.

The next historically significant step was the demonstration by Bianco and colleagues (1970,1971) in Nussenzweig's laboratory that the presence of complement receptors on murine lymphocytes was a feature of B lymphocytes, and that the ligand was C3. Receptor-bearing cells were found among both long and short lived lymphocytes. Within B cell types, it was found that receptor-bearing cells also bore surface immunoglobulin, and were not active secretors of immunoglobulin in the Jerne plaque assay.

The presence of the complement receptor on human lymphocytes was reported by Michlmayer and Huber (1970), but their finding that most chronic lymphocytic leukemic cells were receptor negative has not been supported by subsequent workers (Pincus, Bianco and Nussenzweig, 1972; Shevach et al., 1972; Ross and colleagues, 1973a). The present consensus is that the majority of chronic lymphocytic leukemias arise from B cell precursors.

Subsequent to these workers, the picture has become more complicated by the observations of Bokisch and Sobel (1974) and Ross and Polley (1975) that C4b will bind to some cells, and that some cells bind C3b while others

bind C3d (Okada and Nishioka, 1973; Eden et al., 1973; Ross et al., 1973b). The specific findings in relation to human lymphoid cells will be considered in detail in the next section. To complete the historical survey, it should be added that a receptor for C3a on rat mast cells has been described (ter Laan et al., 1974), and Theofilopoulos and colleagues (1974) have documented a receptor for intact C3 on a human lymphoblastoid cell line. Ross and Polley (1975) showed that the C3c fragment would inhibit rosetting mediated by C3b receptors, thus showing that the C3b receptor-binding site was intact on the cleaved C3c molecule, and capable of binding to the C3b receptor.

2. The C3 Receptors of Mouse and Rat Lymphocytes.

Following the initial characterization by Lay and Nussenzweig (1968), Bianco and colleagues (1970) established that B lymphocytes are responsible for binding the complement-sensitized erythrocytes. They showed that:

i. There were no rosettable cells in the thymus.

ii. Most of the cells bearing membrane immunoglobulin were also complement-receptor bearing.

iii. Depleting spleen cells of complement-receptor bearing cells did not deplete plaque-forming cells.

iv. Receptor bearing cells had a lower density than most non-receptor bearing lymphocytes.

v. The cells tended to bind to nylon wool columns.

In 1971, Bianco and Nussenzweig showed that anti-theta serum did not kill complement receptor bearing cells. When this evidence is added together, it seems conclusive that complement receptor bearing cells are a class of B cells. Arnaiz-Villena and Hay (1975) supplemented this evidence by showing that C3 receptor bearing cells with membrane immunoglobulin bore predominantly IgM, but also included IgG1, IgG2a, IgG2b, and IgA bearing cells.

The relationship of the C3 receptor to some other surface markers has been investigated. Bianco and Nussenzweig (1971) found that antibody directed against membrane immunoglobulin did not inhibit rosetting. Studies by Dickler and Sachs (1974) showed that anti-H2 antisera had no effect on rosetting, whereas Schlesinger and Chaout (1975) did find anti-H2 had an inhibitory effect. With human lymphocytes, Arnaiz-Villena and Festenstein (1976) found an inhibitory effect with a type-specific anti-HLA antiserum. However, Ferreira and colleagues (1976) have tested other sera with the same putative specificity and found no effect on rosetting. When to this evidence is added the finding of Parish and Hayward (1974a) that anti-major histocompatibility locus antiserum of the rat had no inhibitory effect on the binding of complement-coated red cells to rat lymphocytes, there seems to be little doubt that the membrane determinants of the major histocompatibility loci and the C3 receptor reside on different molecules.

Gelfand and colleagues (1974a,b) and Ferreira and Nussenzweig (1976) have studied the appearance of C3 receptor bearing lymphocytes in developing mice. The former group found that surface immunoglobulin appeared before the C3 receptor, and that, following sub-lethal irradiation,

ontogeny was recapitulated during recovery, with the membrane immunoglobulin bearing cells appearing in the spleens of mice before the C3 receptor bearing lymphocytes. Gelfand and colleagues also found that the rate of appearance, but not the final percentage of C3 receptor bearing cells in murine spleens was determined by two genetic loci, one of which mapped to the region of the major histocompatibility locus. Ferreira and Nussenzweig studied the same mouse strains, but made the crucial additional measurement of total spleen cell numbers, thus enabling them to convert percentages to total numbers of lymphocytes per spleen having a particular character. From such measurements they concluded it was the non-C3-receptor bearing cells which were varying from strain to strain, while the number of C3 receptor bearing cells in each strain at similar ages were remarkably similar. Taken together these two sets of data seem to indicate a genetic control of the type of lymphocytes present in the murine spleen, although the details of mechanism remain totally obscure.

Hämmerling and colleagues (1976) also studied ontogeny, but by means of in vitro rather than in vivo techniques. They used mouse bone marrow and spleen cells stimulated with bacterial lipopolysaccharide and examined the effects of specific depletion of cell populations by complement-mediated lysis using specific antisera directed against surface immunoglobulin (Ig), and an antigen of the immune response gene region (Ia). They used failure to alter the conversion of cells from prothymocytes ($Thy.1^-$) to thymocytes ($Thy.1^+$) as a control. They were able to demonstrate a sequence of four stages of differentiation: $Ig^-Ia^-CR^-$ --- $Ig^+Ia^-CR^-$ -- $Ig^+Ia^+CR^+$ (where CR stands for Complement Rosettability). Additionally, they advert to the

fact that they have preliminary evidence of a later step in which a plasma cell antigen appears, and the membrane immunoglobulin disappears. This sequence would account for the presence of Ig^+CR^- cells in the circulation, and the majority of CR^+ lymphocytes being Ig^+. The similarity of the model to spontaneous ontogeny is also persuasive evidence of its validity.

Most studies of complement receptors on rodent lymphocytes have utilized the red cell rosetting assay first described by Lay and Nussenzweig (1968). These investigators showed that binding of IgM and complement coated sheep red blood cells by mouse lymphocytes is temperature dependent. No rosettes were formed at 4°C, but that, once formed, the rosettes were stable in the cold. Similar findings regarding the temperature dependence of rosetting were reported by Parish and Hayward (1974a) using complement coated sheep erythrocytes and rat lymphocytes.

There are published reports of murine cells and sheep red cell-IgM-complement complexes rosetting in the cold, but there is generally insufficient evidence that the whole procedure was carried out at a constantly controlled temperature to be able to assess the significance of these reports, especially since it is clear that even warming to room temperature allows significant rosetting.

Lay and Nussenzweig (1968) showed that lymphocyte binding of complement

coated sheep red cells was unaffected by the presence of sodium azide, Actinomycin D, or puromycin in the incubation mixture, but was abolished by pretreatment of the lymphocytes with 0.1% trypsin for 15 minutes at 37°C. One surprising finding reported by Lay and Nussenzweig was that mouse lymphocytes treated with 0.1% sodium merthiolate, a concentration of drug sufficient to render virtually all these cells trypan blue permeable, were still able to bind target cells.

Gutierrez and coworkers (1976) showed that cytochalasin B, vinblastine, and colchicine did not inhibit the binding of complement coated red cells to human peripheral blood lymphocytes.

3. The C3 Receptors of Human Lymphocytes.

Normal human lymphocytes have been studied from two sources. Most of the material has been obtained by Ficoll-Hypaque gradient purification of peripheral blood, while some studies have been done on tonsillar cells. Ross et al. (1973a) showed the overlap between the populations of peripheral blood lymphocytes bearing membrane immunoglobulin and those showing complement rosettability, with the percentage of Ig^+ cells being greater than the CR^+. They also found a minor proportion of CR^+Ig^- cells. There was no overlap with those cells which were positive for the T cell test for human lymphocytes, namely the sheep cell rosette.

Studies have also been done on human leukemic cells. As already mentioned, the initial report of Michlmayr and Huber (1970) that chronic lymphocytic leukemic cells were predominantly complement receptor negative has not been borne out by many subsequent studies. Thus, Wilson and Nossal (1971), Grey and others (1971), Pincus and colleagues (1972), Shevach and

coworkers (1972) and Ross and others (1973a) have found that the leukemic lymphocytes in most patients with chronic lymphatic leukemia are complement receptor positive, although Shevach and colleagues (1972) found that acute lymphatic leukemic cells, and acute myelocytic cells were receptor negative. In the cases of chronic lymphatic leukemia there was no correlation between the presence of surface immunoglobulin and the presence of a complement receptor. Ross and others (1973a) also noted that the percentage of cells rosetting varied with the nature of the complement source used to sensitize the target cells. This phenomenon was further investigated by Dierich et al. (1974b), and shown to be valid, although the basis of inter-species differences has not been elucidated.

The synthesis of the above evidence on human peripheral blood lymphocytes is that the complement receptor is a marker for B lymphocytes, as was shown in the mouse system by Bianco et al. (1970), and that thence it is argued that most human chronic lymphatic leukemias are disorders of B cells.

In 1970, Bianco, Patrick and Nussenzweig demonstrated that C3 was the complement component responsible for binding to human peripheral blood lymphocytes. In 1973, Eden and colleagues, also using peripheral blood lymphocytes, and Ross and colleagues (1973a), using tonsillar lymphocytes, produced evidence that there were receptors for both C3b and C3d on the same cell. There is also evidence of the binding of C4 to the same receptor as C3b (Bokisch and Sobel, 1974; Ross and Polley, 1975) on peripheral blood lymphocytes and tonsillar cells respectively. Ross and others (1973b) extended the definition of the receptors by raising antibodies against cells

bearing C3d, C3b + C3d,and C3b (Immune Adherence positive erythrocytes) receptors. They were then able to combine the results obtained by varying the ligand on the target cell, using the selectively adsorbed antisera, and using soluble forms of cleaved C3 as inhibitors of rosetting. These led to the clear definition of the two receptors: one for C3b, also called CR I with a similar specificity and antigenicity as the Immune Adherence receptor of red cells, and with ability to bind cleaved C4 as well as C3b. The other had an ability to bind C3b and C3d, but was antigenically distinct from the C3b receptor, and was selectively blocked by C3d: this has been termed CR II. A discrepant note was added by the work of Simonian and others (1976) who found that peripheral blood lymphocytes and tonsillar cells bound fluid phase C3: in most cases intact C3 has been found to be a poor inhibitor of C3 mediated rosetting, and hence the demonstration of some binding by a fluorescent labelling technique may not be significant.

Having defined the two types of receptors, Ross and colleagues (1973b) were able to sort out some of the inconsistent findings on leukemic cells. They found 15/18 patients with chronic lymphatic leukemia had C3d receptors, and low percentages of C3b receptors. The array of leukemic patients' cells also enabled them (1973a) to show the independent expression of membrane immunoglobulin and complement receptors. Rothman and colleagues (1975) screened patients' erythrocytes for immune adherence. They found one case in which the red cells were receptor negative, but the patient's lymphocytes bore complement receptors, showing they were not controlled by the same mechanism.

The nature of the receptor-ligand interaction has not been extensively

studied with normal human cells. A temperature dependence of rosetting was demonstrated by Ross and others (1973a). Dierich and colleagues (1974a), using labelled C3 as a probe, reported decreased C3 receptor activity of lymphocytes treated with the sulfhydryl reagents, dithiothreitol, 2-mercaptoethanol, 2-aminoethylisothiouronium bromide hydrobromide, reduced glutathione, and L-cysteine (in order of decreasing effectiveness). On the other hand, they reported that iodo-acetamide did not affect C3 receptor activity. They concluded, therefore, that disulfide bridges were important for the activity of the C3b complement receptor.

4. Human Lymphoid Cell Lines.

Much of the work already described has been reproduced on lymphoid (lymphoblastoid) cell lines. The first studies were done by Shevach and colleagues (1972) at NIH, but the most extensive studies have been done by the group at the Scripps Clinic on the Raji line, and these will be given separate consideration, as will the limited existent data on the line to be studied, 8866.

Working with an array of cell lines from their own laboratory, Ross and colleagues (1973b) confirmed the finding of membrane immunoglobulin on some, but not all of their lines. All the lines were T-cell (i.e. unsensitized sheep erythrocyte) rosette negative, and hence the receptor seemed to indicate the B cell origin of most cell lines. However, the MOLT-4 line, developed by Minowada, Ohnuma and Moore (1972), and thought to be a T-cell line by virtue of its rosetting with sheep, pig, goat and horse cells, its absence of Epstein-Barr virus, and its absence of surface immunoglobulin, has been demonstrated to show complement mediated rosetting to the extent of

10-45% of cells, depending on the target cells (Huber and Wigzell, 1975; Theofilopoulos and Perrin, 1976). The first paper describing this anomalous behaviour (West and Herberman, 1974) also described the fact that the percentage of rosetting varied with the line of the same name carried by different workers, and within the line over time.

The lines have been shown to bear receptors for C3b + C3d, or for C3d alone (Ross et al., 1973b). Ross and Polley (1975) also showed that, as on normal cells, the C3b receptor of the lines bound E.IgM.$C14^b$.

Shevach and colleagues (1972) found no correlation between expression of complement receptor activity and the HL-A antigenic type, the production of immunoglobulin or its type, nor the expression of the Epstein-Barr virus antigens. Dierich and others (1974b), using other lines, found no correlation between complement receptor and beta-2-microglobulin expression: they also confirmed the lack of correlation with HL-A type. However, using human-mouse somatic cell hybrids, Curry and colleagues (1976) were able to show a linkage between expression of HLA-A and HLA-B and the complement receptor, suggesting that they are encoded on the same chromosome.

With the exception of Raji, the nature of the ligand bound by the receptors, and the critical conditions of the cell to allow rosetting, have been little studied. As will be shown, Raji represents a special case, and may not be representative of other lymphoid lines.

5. Human Lymphoid Line Raji.

Raji was derived from a patient with Burkitt's lymphoma (Pulvertaft,

1965). It is negative for sheep cell rosetting, and uniformly positive for complement receptors in some hands. Theofilopoulos and colleagues (1974) have shown that Raji binds intact C3, C3b, and E.IgM.C1423b. It was estimated that 4×10^5 molecules of C3 were bound per Raji cell. Soluble C3 and C3b not only inhibit binding, but are said to dissociate formed rosettes. These phenomena are not produced by the products of aged serum, viz. C3c and C3d. The same group showed that, after C3b had been bound to the lymphoid cells, the addition of fresh serum led to lysis. Similarly, the addition of fresh serum and cobra venom factor led to lysis of Raji cells, but not 8866 or Daudi cells (neither of which lines bore complement receptors). Thus, Raji is a peculiar line in that it binds intact C3, and bound C3b is capable of taking part in further activation of the complement system. Budzko and others (1976) made similar observations on Raji, and found chicken complement lysed Raji cells. Raji has also been shown to bind C4 (Bokisch and Sobel, 1974) and properdin (Theofilopoulos and Perrin, 1976).

Three other properties of the ligand-receptor interaction have been investigated. Theofilopoulos et al (1974) reported that the binding of C3-coated red cells was temperature dependent. The sensitivity to trypsin (sensitive) and to neuraminidase (insensitive; if anything, rosetting said to be slightly enhanced) was tested by Dierich and others (1974).

6. Human Lymphoid Line 8866.

The data on the line 8866 and complement receptors consists of a set of disconnected observations, by and large recording only the percentage

of cells rosetting in a particular assay. Thus Shevach et al. (1972) found that the line did not rosette with E.IgM. but that 80-90% were E.IgM.C-rosette positive. The line carried by the Scripps group, with the same designation, was reported as showing 5% of cells binding C3, 5% binding C3b, and 5% rosetting with E.IgM.C1423 (Theofilopoulos et al., 1974a). Dierich and colleagues (1974), from the same institution, found that E.IgM.C prepared with mouse, rabbit and human complement, all rosetted with less than 10% of the cells of the line. Theofilopoulos and others, in their 1976 publication, report that 8866 cells do not bind murine C3d. The same authors found 2% binding of E.IgM.C1-3 Mouse in 1974 (Theofilopoulos et al., 1974b). In the same paper it was reported that 8866 bound rabbit but not human C3 (83% of cells bound rabbit C3 compared with less than 5% rosetting with human or rabbit C3b, or binding human C3 or C3b).

7. The Function of the Complement Receptor on Lymphocytes.

The presence of receptors for cleaved products of C3 has been shown on erythrocytes, platelets, macrophages, polymorphonuclear leukocytes, and lymphocytes, as well as in the renal glomerular tuft (probably on the surface of endothelial cells) (Gelfand et al., 1975,1976; Matre and Tönder, 1976). A major area of investigation has been the role of the complement receptor on phagocytic cells. Ehlenberger and others (1976) have shown clearly the role of complement bound to complement receptors in enhancing phagocytosis of IgG-sensitized erythrocytes by monocytes and neutrophils, while complement by itself could only mediate attachment. Bianco and

colleagues (1975), investigating peritoneal macrophages obtained from mice, were able to show that E.IgM.C were bound to normal resident peritoneal macrophages, but were phagocytosed by activated macrophages. Thus there is clear evidence for a role for complement in the function of macrophages.

In the case of lymphocytes, however, the picture is confused by a series of poorly controlled experiments. Often, whole cell populations are taken, and care is not taken to distinguish macrophages from lymphocytes. In addition, since C3b and C3d receptors are known, it is now necessary to test for each type separately. It is clear that functions attributed to binding C3b-sensitized immune complexes may be transient because of the cleavage of the C3b by C3b inactivators in serum, for example (Brown et al., 1970; Tedesco et al., 1972; see review Nussenzweig, 1974). Possible roles for the complement receptor of lymphocytes include:

i. Localization of antigen (Dukor, Bianco and Nussenzweig, 1971; Papamichail and others, 1975).

ii. A second "trigger" necessary for the activation of B cells by thymus-independent antigens (Dukor and Hartmann, 1973; Hartmann, 1975). This work was done using antigens now known to be B cell mitogens, and the mitogenic response was independent of the ability of the antigen to activate the alternative complement pathway. Indeed, the mitogens can exert their effects in the absence of activatable complement (see review of evidence by Nussenzweig, 1974). Finally,

Alper and colleagues (1972) have described a patient with genetic absence of C3 but normal immunoglobulin levels in serum, and a normal immune reaction. Thus C3 cannot be an absolute requirement for the interaction of antigen and lymphocyte in the initiation of the immune response.

iii. Perlmann and others (1975) have presented some evidence for a role for complement receptor bearing lymphocytes in antibody-mediated cytolysis.

At present, however, there is no simple biological test analogous to those used in hormone studies for the interaction of cleaved complement and a lymphocyte, or lymphoblastoid cell, which would be a useful laboratory tool, nor is there clear evidence of the role of the receptor in vivo, obviously of more general interest.

8. The Definition of a Receptor.

The above discussion of the function of the complement receptor leads naturally into an effort to define the receptor. Consider a definition for a receptor proposed by pharmacologists (Hollenberg and Cuatrecasas, 1975):

i. The binding must show absolute structural and steric specificity pari passu with the known biological activity of the parent ligand, its structural analogues and its antagonists.

ii. The binding should demonstrate saturation within a concentration range that can be meaningfully related to that of

agonists which elicit the known biological response in intact biological systems.

iii. The binding interaction should reflect high affinity, in harmony with the sensitivity of the tissue to the physiologically active concentration range of the ligand.

iv. The presence of this binding should be restricted to the tissues (or species) known to be physiologically sensitive to the agonist.

v. The binding should in most if not all cases be reversible, in accord with the rapidity of several of the known biological responses upon removal of the agonist from the medium in the given system.

Hollenberg and Cuatrecasas summarize these criteria in a warning that care must be exercised in relating binding of a ligand as measured _in vitro_ with biological properties _in vivo_ or in an intact system. As was indicated at the end of the previous section, since there is no known physiological response to the interaction of C3b or C3d with lymphocytes, the criteria developed for the study of hormones are of little use at this stage of the art.

In this discussion, therefore, the definition of receptor will be an extremely limited one. It will be a species, present on the surface of cells, capable of interacting with a stable form of cleaved complement. This cleaved component will not interact non-specifically with all cell types. The control is a clone of cells lacking the receptor. The physicochemical measurements

made on the interaction are an attempt to further define the nature of the receptor so that comparisons can be made with other systems.

C. HUMAN LYMPHOID CELL LINES

1. Historical Survey.

The first successful long-term culture of human hematopoietic cells was achieved by Osgood and Brooke in 1955. The cells which persisted had a large cell blastic form, were lacking in distinctive granules, and were not adherent to the substratum. They may well have been lymphoblasts rather than myeloblasts, as the authors thought. In 1964 Pulvertaft, and Epstein and Barr, simultaneously reported the isolation of growing cultures from solid lymphoid tumors of African children, Burkitt's lymphoma, and these and similar lines have been much used as lymphoid cell models. In that same year, Iwakata and Grace, from the Roswell Park Memorial Institute, reported the establishment of the first of a long series of lines derived from the peripheral blood cells of donors with a variety of diseases. The first report concerned myeloid leukemic patients, and the authors believed, on morphologic grounds, that the resultant lines were myeloblasts. However, the description given of the electron microscopy and the growth characteristics is compatible with that of all subsequently derived lymphoblastoid lines. In addition, these authors demonstrated the presence of virus-like particles in the supernatant fluid of the cultures. Since then a number of lines have been found to produce infectious particles of the Herpes virus type indistinguishable from the virus of Burkitt's lymphoma, the Epstein-Barr Virus.

Following the success of Iwakata and Grace, many laboratories were able to establish similar lines, and there are now many hundreds of cell lines available. Three groups with large collections of lines have reviewed their experience (the Roswell Park group, Moore, 1972; the M.D. Anderson group, Wang and colleagues, 1975; and the Uppsala group, Nilsson and Ponten, 1975). In the following account, many of their findings will be presented in summary form for clarity. Many of the omitted citations will be found in the above summaries.

2. Features of Human Lymphoid Cell Lines.

a. Source of the cell lines.

The lines termed lymphoid or lymphoblastoid have been derived from normal donors, leukemics, patients with a variety of acute infectious diseases, patients with a variety of metabolic disorders and patients with a variety of malignancies. Excepting the lines derived from patients with specific metabolic diseases, the lines are similar in terms of culture conditions required, lag phase before establishment of lines, the morphology of the cells, and a battery of markers to be described. There is no evidence that leukemic blood gives rise to lines characteristic of the leukemic clones, but rather that a sub-population of normal cells gives rise to a typical lymphoid cell line. In hypogammaglobulinemias and paraproteinemias, the lines derived were generally not representative of the underlying disease: in particular, hypogammaglobulinemic patients gave rise to immunoglobulin secreting cell lines (Stites et al., 1971).

b. Correlates with the ability to establish cell lines.

In all reports of the establishment of human lymphoid lines it has been noted that there is a long and variable lag time between placing the cells into culture, and the appearance of lines. Initially, most of the cells die. If regular feeding is continued, and the cells are not disturbed, eventually clumping of the residual cells,and some acidification of the medium herald the beginning of a line. Moore (1972) found that the lag period varied from less than twenty days to more than one hundred and ten days, with the majority of lines being established between 30 and 90 days.

The nature of the donor of the cells was clearly pertinent. In some instances, repeated attempts to establish lines from healthy donors were unsuccessful. However, peripheral blood from the same individuals during acute febrile episodes gave rise to lines. Cells taken from the umbilical cord blood of the new born never gave rise to lines until cultures of these cells were infected with Epstein-Barr virus, derived from either the supernatant fluid of Epstein-Barr virus producer lines, or from the frozen and thawed pellets of cells from established lymphoid lines (Chang and Blankenship, 1973). Additional evidence for a role for the Epstein-Barr virus is the finding that lines were more readily established from cell donors who had antibody against the virus. Also, throat washings from patients with infectious mononucleosis, a known source of Epstein-Barr virus, enhanced the establishment of lines (Chang et al., 1971; see Chang et al., 1973, for other references).

Although Epstein-Barr virus is the only agent that has been found

associated with, and found to facilitate the establishment of, human lymphoid lines, it is possible that other viral agents can also perform this function. As noted above, lymphocytes from donors with unexplained febrile illnesses, presumably due to infections with viruses other than Epstein-Barr, have enhanced capacity to form cell lines. It is possible that these other viruses are responsible for initiating lymphoid cell lines directly, or indirectly through their capacity to stimulate lymphocytes to enter the cell cycle. This latter possibility is especially relevant in view of the reports of Wheelock and Edelman (1969) and Nowakowski and others (1973) that mitogen and antigen stimulated lymphocytes become markedly more permissive for viral replication.

c. General features of lymphoblastoid cell lines.

There are several features which differentiate lymphoid lines from most established cell lines. The most striking is the apparent immortality of lymphoid lines. They have been carried in continuous culture for many years without showing the aging phenomenon (Hayflick, 1965) seen in epithelial cultures.

Within lines, and even within clones of a single line, there is often marked anatomical polymorphism. The most extreme example is the line characterized by Drewinko and Trujillo (1972), in which some cells are firmly adherent to the substratum, while others, more characteristic of lymphoid lines, float in clumps in the culture medium. In most cultures, the cells form aggregates of various sizes, and remain unattached to the substratum, although, under various culture conditions, mild adhesion to

the plastic or glass may be noted. Within the lines, and within clones, some variety in cell size is seen, and the cells, while generally globular, are usually irregular in shape.

Lymphoblastoid cell lines present a monotonous appearance in the electron microscope. The cytoplasmic organelles are scanty, with a few mitochondria, little endoplasmic reticulum, few polyribosomes, a few fat droplets, and an occasional debris-filled lysosome. In lines derived from Burkitt's lymphoma, fat droplets may be a major feature. The plasma membranes of cells within clumps often approximate one another, but intercellular junctions are not seen. The nuclei of the cells are large, irregular, with clumped chromatin around the nuclear margin and within the nucleoplasm. Nucleoli are usually multiple and prominent.

Microcinematography of lymphoid cell lines has revealed active motility. This is borne out by observations of lymphoid cells on feeder layers of epithelial cells. The lymphoid cells are seen to become firmly attached to the feeder layers, and to move under the epithelial cells, separating them from the substrate. When so attached, the cells are difficult to detach: this is in marked contrast to the generally gentle mechanical force needed to disrupt cell clumps in culture.

Kammermeyer and colleagues (1968) showed that some cells of their lymphoid lines phagocytosed heat-killed staphylococci and polystyrene beads. Moore and Minowada (1973) anecdotally report having seen phagocytosis of dead cells by lymphoid lines.

A general feature of human lymphoid cell lines is their low cloning efficiency in soft agar. Thus, while Scharff's group (Coffino and others,

1972) has reported cloning efficiencies of over 90% for murine plasmacytoid lines, human lines usually clone with an efficiency less than 30%, and often under 10%. This is all the more dramatic in view of the much poorer viability of murine lymphoid cells in culture as compared with human peripheral blood lymphoid cells.

d. Surface markers and secretory products of the lines derived from human peripheral blood.

The nature of the cells forming the human lymphoblastoid cell lines has been defined by a series of markers. The conclusion is that the lines are lymphoid, with the vast majority being derived from B-cell precursors, and a few being of T-cell origin (Pattengale et al., 1974; Kaplan et al., 1974). The markers that identify these cells as of B-cell origin include a variety of secreted and surface immunoglobulins, a receptor for cleaved C3, a human B-cell antigen (serologically defined), and the absence of rosetting with sheep or horse erythrocytes (see Royston et al., 1974 for references). In contrast, the T-cell lines form mechanically fragile rosettes with sheep or horse erythrocytes, are positive for a serologically defined T-cell antigen, and are generally negative for the B-cell specific markers. An exception is that MOLT-4, a T-cell line of Minowada, Ohnuma and Moore (1972), has been reported to have a percentage of cells bearing a complement receptor (West and Heberman, 1974).

The B-cell lymphoid lines, unlike other human cell lines, have repeatedly been demonstrated to contain the genome of the Epstein-Barr virus, as detected by RNA-DNA hybridization, and to express a variety of virally determined antigens. The earliest expressed, and most commonly

found, is the nuclear antigen (EBNA) of Reedman and Klein (1973). It has also been found that lymphoid cell lines, like B-cells in human peripheral blood are able to be infected with EBV (Chang et al., 1976, for early references; Robinson and others, 1977). The resulting super-infection leads to expression of a different set of viral products than produced by the endogenous virus. There is now evidence from studies of peripheral blood lymphocytes and lymphoid cell lines that the relevant character for this infectibility is a surface property, the complement receptor (or a very closely related molecule), acting as the receptor for the virus (Jondal and others, 1976; Robinson et al., 1977). Jondal et al (1976) showed a close correlation between C3d receptor expression and the capacity of cells to be super-inected with EBV.

e. The homogeneity of lines.

It has been noted that both T- and B-cell lines can be derived from the peripheral blood of a single donor (one case: see Royston et al., 1974), but in most cases multiple cultures from the same donor give rise to multiple B-cell lines (Moore, 1972). In view of the wide range of cell numbers used by different authors in the initial inocula, it is highly likely that many of the lines originated from more than one cells, and are therefore heterogeneous in some respect. Drewinko and Trujillo (1972) studied the variable morphology of their line, and found it to be a stably transmitted trait rather than due to a mixed culture. Lerner, McConahey, and Dixon (1971) and Bloom et al. (1971) have used repeated cloning to examine the variability of immunoglobulin secretion. The latter group used a line expressing more than one class of immunoglobulin, and found that this

too was a heritable character, and not due to heterogeneity of the stock culture. Lines have not yet been examined for idiotype markers on their secreted immunoglobulins.

f. The stability of markers.

It has been shown that the HLA-A phenotype of the donor cells is maintained by cultured cells established as lines (Rogentine and Gerber, 1969: see Reisfeld and others, 1975, for review). Pious and colleagues (1973, 1976) have studied HLA-A and beta-2-microglobulin expression on cells in culture exposed to the selective pressure of specific anti-sera, and have been able to detect variants in which, in the case of HLA-A, a single serotype was lost, while in the case of beta-2-microglobulin, the whole molecule was lost. The HLA-A variants thus far isolated have proved stable on continued culture, while some of the beta-2-microglobulin variants were unstable, and reverted after exposure to dimethylsulfoxide. Strominger and colleagues have utilized the stability of HLA phenotypes in culture, and their resemblance to the molecule in the normal membrane, in a series of studies, culminating in a partial sequence of a portion of the molecule released into the supernatant by treatment with papain (Terhorst et al., 1976).

The use of isozymes is another tool for the detection of heterogeneity, but little use has been made of it. Conover and colleagues (1970) showed persistence of the donor phenotypes of phosphoglucomutase isoenzymes in twenty-eight lines. Povey and colleagues (1973) presented an extensive survey of isoenzymes in lines, and cloned them in the search for variants. There were very few spontaneous variants, but by use of ultraviolet

irradiation and mutagens variants were obtained. In addition, it should be noted that enzyme defects in the donors have been shown to be expressed in lymphoid lines, and use of this has been made to obtain suitable amounts of tissue to investigate such defects; the usual source of analytical material, skin fibroblasts, only live a few generations in culture, in contrast to the longevity of lymphoid lines. Examples of this are the isolation of lines with the characteristics of Chediak-Higashi syndrome by Blume and others (1969) and of Lesch-Nyhan syndrome by Choi and Bloom (1970b).

g. Products of lymphoid cell lines.

A variety of products have been found in the supernatant fluid of cultures of lymphoid cell lines. Glade and Chessin (1968) found serological evidence of the secretion of the third component of complement. Mundy and colleagues (1974) described a bone-resorbing activity. A variety of lymphokines have been described, and interferon has also been found (see reviews by Moore, 1972; Wang et al., 1975). In addition, lymphoid lines can be cytotoxic for a variety of cell types (see Wang et al., 1975). Reisfeld and colleagues (1975) have used the supernatants of lymphoid cultures as a source of HLA-A antigens.

h. The karyotype of lymphoid cell lines.

The karyotypic features of lymphoblastoid cell lines were a subject of considerable controversy in the late 1960's, when a marker chromosome in the C group was discovered in Burkitt's lymphoma cell lines (Kohn et al., 1967,1968; Macek et al., 1971). However, in 1970 two groups concluded it was not a specific marker (Huang and others; Whang-Peng and others), and

this conclusion seems to have stood the test of time. Many lines have been found to be diploid or pseudodiploid, and some aneuploid (also Steel, 1971; Povey and others, 1973). The latter authors studied cloning, karyotype and isozymes on a large number of cell lines. They correlated ease of cloning with aneuploidy, thus explaining the difficulty in cloning newly established diploid lines. They also found stability of most of their markers in the face of aneuploidy, with only a few spontaneous mutants occurring. No correlation between individual chromosomes and isozyme changes was seen. Venuat and Rosenfeld (1974) studied 13 cell lines over 2 years. Five lines were derived from normal donors and eight from leukemic patients. The authors report that the normal lines have remained euploid over this period, whereas the leukemic lines showed clonal evolution, with certain aneuploid cells bearing marker chromosomes showing a selective growth advantage. They believe this chromosomal instability is a feature of leukemic as opposed to normal cell lines. However, lines can be derived from patients with myeloid or lymphoid leukemias, and may not show the markers of the circulating leukemic cells.

Despite the dramatic progress in techniques for banding of cell chromosomes, there are technical difficulties with the use of these techniques on human lymphoblastoid cell lines, and these have prevented adequate cataloging of the many lines available. In many cases, even the chromosome numbers are not available, and studies of changes with time in culture have not been reported.

CHAPTER II

MATERIALS AND METHODS

A. CELLS: CULTURE AND CLONING

The 8866 cell line and derivative clones were grown in stationary cultures in plastic flasks (area 75 cm^2), containing approximately 50 ml of medium, and kept horizontal in an incubator with an atmosphere of 5% carbon dioxide in air saturated with water vapor. The cell concentration was maintained between 0.3 and 1.5 x 10^6/ml, and generally between 0.5 and 1.0 x 10^6/ml, by daily pouring out part of the mixture of cells and medium, and replacement with fresh medium. Cell counts were performed by mixing the flask well, pouring out an aliquot, and mixing 0.5 ml of cells with 0.5 ml of phosphate buffered saline containing 0.02% Trypan Blue. This mixture was counted in an eosinophil counting chamber, thus avoiding the correction for the dilution, and permitting more cells to be seen per chamber. The use of Trypan Blue allowed simultaneous assessment of viability and cell counts. By these means, cells were kept in the logarithmic phase of growth, and the condition of the cells constantly monitored microscopically for contamination and general appearance.

When aliquots of cells were to be frozen for storage, cells in the logarithmic phase of growth were pelleted at 250 xg for 5 minutes, and resuspended at between 1 x 10^7 and 5 x 10^7 cells/ml in a mixture of 10% glycerol, 10% fetal bovine serum and 80% medium. One milliliter aliquots were placed in 1.2 ml capacity freezing ampoules ("Cryules", Wheaton), sealed in by heat, and frozen at approximately 1°C/minute in a

biological freezer (BF-5 Linde) in the neck of a liquid nitrogen tank. The ampoules were then stored in liquid nitrogen. To thaw aliquots, the method described by the National Tissue Culture Collection was followed; viz. the ampoules were placed into a beaker of water at 37°C, and gently agitated until thawed. The outside of the ampoule was then sterilized with 70% alcohol, and the contents removed into a syringe containing 9 ml of medium. The mixture of cells and fresh medium was used to flush the ampoule several times, and the contents then placed into either a small flask ($25cm^2$, Falcon or Corning) or a large flask stored in the vertical position in the incubator. When the cell numbers or viability were low, recovery was enhanced by resuspending the cells in 20% fetal calf serum, pre-gassing the flask with 10% carbon dioxide in air, and sealing the cap, before storing the cells in the incubator.

The agarose cloning method was carried out following the mehtods learned in the laboratory of Dr. M.D. Scharff:

Method 1. Thirty ml of tissue culture grade sterile distilled water was added to 0.22 g of agarose (Seakem) in a 100 ml capped bottle, and the mixture was autoclaved. After autoclaving, and brief cooling in air, the still-liquid mixture was stored in a 45°C water bath. A final mixture was then made by adding to the 100 ml bottle, in order, 30 ml of twice-normal strength Dulbecco's medium at 37°C, 30 ml of fetal bovine serum at 37°C, and 10 ml of cells diluted to 5×10^3/ml in Dulbecco's medium containing 10% fetal bovine serum. The mixing was done out of the 45°C water bath. After pipetting to disperse the cells, 2 ml aliquots were dispensed into 35 mm petri dishes, placed on a bench at room temperature for approximately

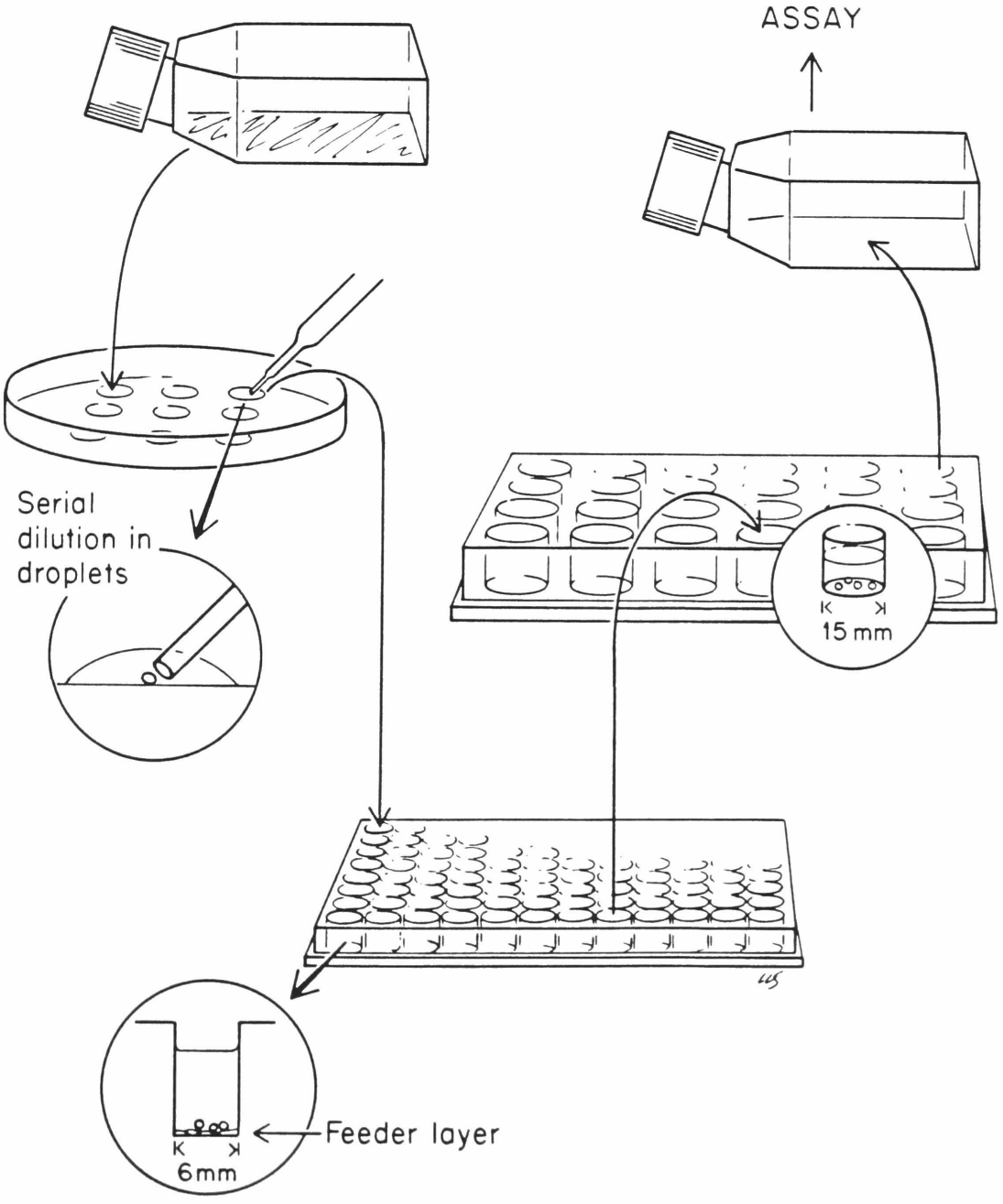
30 minutes to gel, and then stored undisturbed in the incubator at 37°C in an atmosphere of 5% carbon dioxide in air saturated with water vapor. After the appearance of colonies (approximately 1 week), some colonies were picked with a Pasteur pipette whose tip had been bent, and drawn out to less than 1mm external diameter. Under the control of an inverted microscope at approximately 40 times magnification, a colony was aspirated into a pipette already containing a drop of medium. The colony was then discharged into a 25 cm^2 flask containing approximately 2 ml of Dulbecco's medium supplemented with 20% fetal bovine serum, and stood on end in the incubator. The developing culture was fed by aspiration of about one half of the medium, and replacement with fresh medium twice weekly. Care was taken not to agitate the cell clusters until regular daily feeding was made necessary by the obvious acidification of the medium. The volume of the culture was then sequentially doubled, and the flask laid on its side. When 10 ml was reached, the colony was deemed established. It was transferred to a 75 cm^2 flask, and fed by addition of medium until 50 ml was reached. At this point, aliquots were taken for freezing if the clone had proved valuable. Otherwise, after testing, the 10 ml flask samples were frozen.

Method 2. Rat embryo fibroblasts from the third passage were removed from flasks with trypsin/EDTA, suspended in medium containing serum and plated out in petri dishes at near confluence. After 24 hours in culture, the feeder layer was overlaid with 0.4% agarose in medium

containing a final concentration of 20% fetal bovine serum. 10 ml were overlaid in 100 mm dishes, and 2 ml on 35 mm dishes. After this firmer layer had gelled, the same mixture as had previously been used without a feeder layer (i.e. 0.22% agarose and 30% fetal bovine serum) containing 10^3 cells/ml was dispensed onto it. 5 ml were added per 100 mm dish and 1 ml per 35 mm dish. The softer agarose was allowed to gel, and the plates were then stored in the incubator. After the appearance of colonies, the selection procedure was modified by the use of an initial culture in 16 mm diameter wells in Linbro dishes (containing approximately 1 ml of medium) before transfer to the smaller sized flask. This method proved easier for rapid feeding and examination of the developing colonies.

In the single cell picking method, features of several previous methods will be recognized. Rat embryo fibroblast cells were plated, and allowed to grow to near confluence in either Terasaki microwell dishes, or 6 mm diameter wells of Linbro dishes (containing approximately 0.3 ml of medium). The rest of the method is depicted in Figure II-1. Drops of a mixture of 70% Dulbecco's medium, 20% fetal bovine serum and 10% NCTC 109 medium (Microbiological Associates) were arranged in a 100 mm petri dish. Cells in the logarithmic phase of growth, and with high viability were dispersed with a pasteur pipette to give a single cell suspension. A drop of cells was serially diluted from droplet to droplet until examination by means of an inverted microscope showed that single cells occurred in each high-powered (100X) field. At this point, a pasteur pipette whose tip had been drawn out to a fine point (internal diameter in the range of 2-4 times the diameter of a single cell, and therefore about 50 micrometers),

Picked single cell cloning method



controlled by mouth via a plugged rubber tubing system, and pre-filled with some medium, was used to pick up one cell. The pipette was then taken to a droplet containing no cells, the single cell discharged, picked up again, and then transferred to a well containing the rat embryo fibroblast feeder layer and the same enriched medium just described. If there were any doubt about the number of cells taken up, as sometimes occurred in the presence of plastic particles or bubbles, or occasional doublets of cells, the pasteur pipette was discarded. The cells in the small wells were fed twice weekly by removal of approximately half the medium by aspiration via a steel needle flamed between wells to prevent contamination. Fresh medium was added through a fine needle from a syringe. When using the Terasaki dishes it proved critical to place pieces of filter paper soaked in sterile distilled water containing 0.2% sodium azide around the circumference of the dish, and to maintain the strips saturated throughout cloning. When this was not done, the presence of a saturated atmosphere in the incubator proved insufficient to prevent significant loss of fluid from the droplets in the wells, which contain approximately 10 microliters, and cell death of both lymphoid cells and feeder layers occurred.

When the lymphoid cells were seen to cover at least half of the bottom of the well, and acidification of the medium occurred daily, the clone was picked with a drawn-out pasteur pipette, and transferred to the next sized well. The well of origin was washed out with medium through the same pipette in order to maximize recovery, and then, as a safety measure, medium was replaced into the original well: usually sufficient cells remained to give rise to another culture. It should be noted that the lymphoid cells

were often very adherent to the feeder layer, and that in the transfers, fibroblasts were seen to be carried over for at least two passages, but disappeared with the transfer of the lymphoid cells to fresh vessels by pouring off the non-adherent cells.

In addition to Dulbecco's medium, the 8866 cells have been found to grow successfully in Minimal Essential Medium, the Alpha modification of Minimal Essential Medium, and RPMI 1640 (all supplied by Gibco).

B. ROSETTING

The reagents for rosetting were prepared as described by Bianco (1976) with the exception that, for some stages, Hanks' Balanced Salt Solution was substituted for the veronal buffered saline. The products appeared to be identical. The usual source of serum was mice of strain NCS, although AKR were sometimes used.

The rosetting procedure is illustrated in Figure II-2. Incubation was carried out in either V-bottomed wells or plastic V-bottomed capped capsules (Beem).

The alternative particles were zymosan (Nutritional Biochemicals or Sigma), latex particles (polystyrene, Dow Chemicals), Sepharose (Pharmacia), and P-2 beads (polyacrylamide beads, Biogel).

C. IODINATION

The iodination of the cells is described in the text, and is essentially that of Hubbard and Cohn (1975a), with Hanks' Balanced Salt Solution

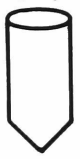
Rosetting method

Lymphoid cells
at ~ 1×10^6/ml
in Dulbecco 10% FCS

100 µl

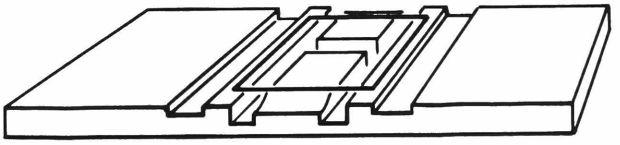
50 µl

1% EAC
in Dulbecco's MEM



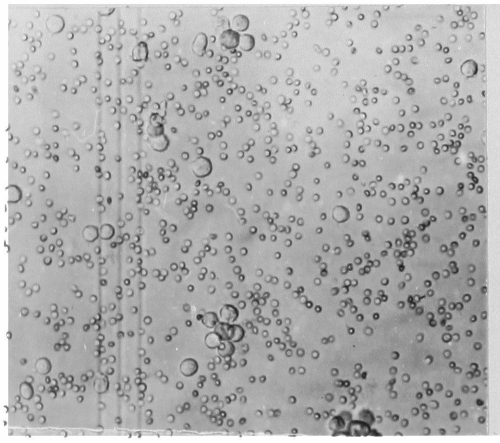
1hr, 37°C

Pipette
Add trypan blue

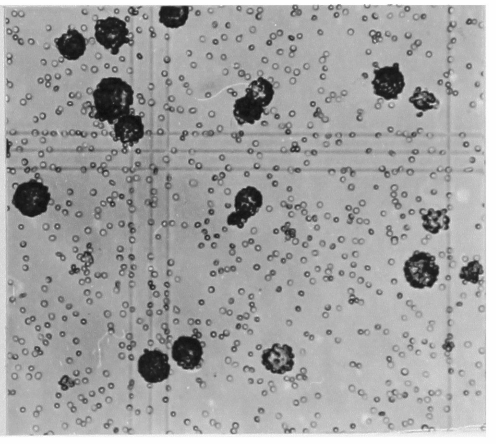


Haemocytometer

Score 200 live lymphocytes



Negative clone



Positive clone

substituting for the phosphate buffered saline which proved less satisfactory in maintaining the viability of lymphoid cells. The other changes are noted in the text (Chapter V).

The amount of iodine incorporated was also determined by the method of Hubbard (Thesis, 1973). 10% TCA was made 50 mM with potassium iodide and kept chilled. Aliquots of 1 ml were used to precipitate up to 100 micrograms of protein. When samples contained less than approximately 20 micrograms of protein, 50 micrograms of carrier fetal bovine serum were added to give a satisfactory precipitate. The sample was stored in an ice bucket for 1 hour, and the total counts then measured in a Packard 8220 gamma counter, with an efficiency for ^{125}I of approximately 60%. The sample was then dispensed onto a premoistened Millipore filter of 0.45 micrometers or a Whatman GF/C glass fiber filter paper, and washed three times with approximately 5 ml washes of 10% TCA/50 mM KI. The filter was aspirated to near dryness, then folded, and placed into a capped 5 ml plastic tube for counting of the TCA-precipitable counts.

The Chloramine-T iodination of proteins was carried out by the slight modification of the method of Greenwood and Hunter (1962) developed by Dr. Celso Bianco. In an ice bath 50 microliters of 0.8M Tris-HCl, pH 7.2; protein at approximately 1 mg/ml, 10 microliters; Sodium ^{125}iodide (carrier free, 20 mCi/ml), 5 microliters; and 50 microliters of Chloramine-T at 500 micrograms/ml in distilled water were added. The reaction was carried out for 10 minutes on ice, and then stopped by the addition of 50 microliters of sodium metabisulfite also at 500 micrograms/ml. The solution was then stored at -20°C until analyzed on SDS-polyacrylamide gels.

The solid-phase method of iodination was carried out using lactoperoxidase linked to Sepharose by the cyanogen bromide, N-hydroxysuccinimide method by Dr. S. C. Silverstein. In a solution of phosphate buffered saline sufficient to give a final volume of 0.2 ml, 5-10 microliters of soluble protein, 10 microliters of a slurry of lactoperoxidase-coupled beads, 10 microliters of 0.01M KI as carrier, 2 microliters each of carrier-free Na ^{125}I (in 0.1N NaOH) and 0.1N HCl were added and mixed. Two 5 microliter aliquots of hydrogen peroxide diluted in phosphate buffered saline to a final concentration of 44 micromolar were added 1 minute apart, and the mixture left 5 minutes. The reaction was stopped with 10 microliters of 0.6M sodium azide, and aliquots stored frozen at -20°C until analyzed on gels. For the labeling of the LPO and G.O., this was the protocol. For the labeled fetal bovine serum, the reaction was similar in proportion, but scaled up, and the resulting product extensively dialyzed against phosphate buffered saline containing 0.02% sodium azide until the material was approximately 100% acid precipitable.

D. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Samples were prepared for electrophoresis, and run in SDS-polyacrylamide gradient slab gels with discontinuous buffer systems of Laemmli (1970). The usual slab was approximately 15 cm x 15 cm x 1.5 mm. The stacking gel was generally 2.5%, and the gradient from 5 to 15%, although other gradients were tried, as described in the legends. All samples were boiled in SDS for 2 minutes in the presence of 10% 2-mercaptoethanol. Treated similarly, and run as standards, were beta-galactosidase (Sigma), human

transferrin (Nutritional), bovine serum albumin (Pentex), ovalbumin (Nutritional), and ribonuclease (Sigma). The typical gel was run at 10 mA constant current for 9.5 to 10 hours.

The gel was fixed in 7% acetic acid, 30% methanol for one hour, then stained in the same mixture containing 0.2% Coomassie Blue (Bio-Rad). After gentle rocking in the stain for 1-2 hours, the gel was destained by frequent changes of the fixation solution. The gel was dried onto filter paper by vacuum aspiration, mounted on Bristol board, and exposed to X-ray film (Cronex 2, Dupont) in an x-ray folder between sheets of mild steel and compressed by screw-adjusted boards in order to maintain close, constant proximity of gel and film. The film was developed in a clinical automatic developer.

E. ELECTRON MICROSCOPY AND AUTORADIOGRAPHY

Cells for fixation were washed in Hanks' Buffered Salt Solution, and then fixed either in the cold or at room temperature with 2.5% glutaraldehyde for 5 minutes. During the fixation stage, the cells were kept as a single cell suspension by pipetting and vortexing. After chilling, the cells were pelleted, and resuspended in a mixture at ice temperature of 2 volumes of osmium tetroxide (1% in 0.1M cacodylate buffer, pH 7.2) and 1 volume of glutaraldehyde (2.5% in the same buffer) for a total of 15 minutes, with one change of fixative. The cells were then post-fixed in the osmium tetroxide alone for 15 minutes. After two washes in 0.15M NaCl, and one wash in uranyl acetate (0.25% in 0.1M sodium acetate, pH 6.3), the sample was stained in the uranyl acetate for 30 minutes. Two further

washes in 0.15M NaCl were followed by sequential dehydration through 70%, 95% and 100% alcohol to propylene oxide. On some occasions, following the saline washes after the uranyl acetate staining, the cells were embedded as a pellet in melted agar, partially dehydrated in 70% alcohol, and then diced into fragments approximately 0.5 mm in their largest dimension, and then carried through the rest of the dehydration protocol. The propylene oxide was changed three times, then replaced by a mixture of one volume of propylene oxide and one volume of the epon mixture. This was incubated at 60°C for 30 minutes, then the samples were embedded in complete epon mixture, and cured at 60°C for three days. The epon mixture was according to the batch protocol described on the reagents purchased from Ernest Fullam. (This method is basically that of Hirsch and Fedorko, 1967).

For the light autoradiograms, thick sections (green, $\sim$ 0.4 μm) were cut, dried onto glass slides, and covered with a film of Ilford L4 emulsion by the dipping method of Caro and van Tubergen (1962). Exposures were for up to two weeks in the cold. Development was with D-19 (Kodak), and after fixation, the preparation was stained with 0.2% Azure A in 1% sodium borate.

For the electron microscopic autoradiography, thin sections (gold $\sim$ 100 nm) were cut on a Porter-Blum ultramicrotome, picked up with carbon-coated, formvar covered copper grids, and stuck to glass slides with transparent tape. The loop method of Caro and van Tunbergen (1962) was then used to apply a uniform film of Ilford L-4 emulsion. The slides were stored dessicated and in the cold. At periods up to one month slides were taken and developed. Development was with Microdol-X (Kodak). The

gelatin emulsion was removed by flotation of the grid on a drop of 0.1N NaOH for 20 minutes.

For all electron microscopic preparations, staining was with uranyl acetate and lead citrate by the method of Venable and Coggeshall (1965). The specimens were examined in a Siemens Model 1 microscope at 50 KV at various magnifications.

F. KARYOTYPING

8866 cells were prepared for karyotyping by maintenance in logarithmic phase. Colchicine was added to a final concentration of 0.5 µg per ml, and 15 ml cultures continued for four hours. At longer periods, the chromosomes became very condensed. During colchicine treatment, cultures were very sensitive to agitation, and placing even near-full tubes in a horizontal position on a wheel rotating slowly about a horizontal axis led to almost total loss of mitotic cells. After incubation, the cells were washed with Hanks' Balanced Salt Solution, then swelled in 10 ml of 0.075M KCl for 5 minutes at room temperature. Aliquots were checked by phase microscopy to find the time of maximum swelling of the cytoplasm, but preservation of the nuclei. This varied from clone to clone. The cells were then pelleted, resuspended in 1 ml 0.075M KCl, and drops to a total of 1 ml of a freshly prepared mixture of chilled Carnoy's fixative (3 volumes absolute alcohol, 1 volume glacial acetic acid) was added dropwise while gently vortexing the tube. After 10 minutes at room temperature, the cells were pelleted, and resuspended in approximately 4 ml of undiluted fixative. Following further fixation for 10 minutes, the cells were pelleted, and

1 ml of fixative added. The cell concentration was then adjusted empirically to give an optimal spread when a drop of cells in fixative was dropped approximately 1 meter onto a well-washed, chilled wet glass microscope slide. After drying, the smears were stained with dilute Giemsa's stain (one drop per 1 ml of distilled water buffered with a few drops of 0.1M sodium phosphate buffer, pH 7.2). The dried preparations were covered with Permount and a coverslip for permanent storage. Photography was carried out at 40X, 63X and 100X magnifications, using a green filter and Kodak Film 5060 on a Zeiss III Photomicroscope.

G. SOLUTIONS AND REAGENTS

Phosphate buffered saline is the solution of Dulbecco, containing NaCl 8.0g/l, KCl 0.2g/l, Na_2HPO_4 1.14g/l, KH_2PO_4 0.2g/l $MgCl_2$.$6H_2O$ 0.1g/l and $CaCl_2$ 0.1g/l at pH 7.2.

The Sodium RSB referred to is 0.01M NaCl, 0.01M Tris-HCl at pH 7.5.

All reagents for acrylamide gel preparation were from Bio-Rad, except the SDS which was from Pierce, and the 2-mercaptoethanol from Sigma.

The immunoglobulin fraction of rabbit serum was prepared by dialysing serum against 0.02M potassium phosphate at pH 7.2 (containing 0.02% sodium azide), and running it on a column of DEAE-cellulose (DE-52, Whatman) equilibrated with the same solution as the dialysis fluid. The immunoglobulin is that fraction of the protein which is not retarded by the column.

The Brunette and Till (1971) procedure required the preparation of a mixture of various solutions. The viscosity of the polyethylene glycol made it difficult to measure out accurately. The mixture's ingredients

were therefore calculated, so that the solids could be weighed out, and dissolved subsequently. The mixture is made of 40g of Dextran 500 (Pharmacia), Polyethylene Glycol 6000 30.9g, 331.1g distilled water, 333 ml of 0.22M sodium phosphate, pH 6.5. If the zinc chloride protocol was being used 80 ml of 0.01M $ZnCl_2$ were added. Otherwise, this volume was made up by distilled water. Sodium azide (0.14g), not in the original protocol, was added as a preservative. The mixture was dissolved by vigorous stirring in the cold, and then allowed to stand in a large separatory funnel for about 48 hours. At this point, phase separation was complete, and the upper and lower phases were collected. Precipitates at the interface and the bottom of the funnel were discarded.

The protocol for the formaldehyde fixation of sheep erythrocytes was learned from Dr. M.E. Fedorko. In the usual buffered formalin, the red cells lyse rapidly. A solution consisting of $NaH_2PO_4.H_2O$ 8.0g; Na_2HPO_4 13.0g; formaldehyde (37% solution) 200 ml; and distilled water 1800 ml was adjusted to pH 6.8 - 7.0 with 14 ml of 1N NaOH. Erythrocytes were washed twice in phosphate buffered saline, then gently swirled into suspension in a small amount of residual buffer. A large excess of fixative was then slowly added by gently pouring down the side of the container. The mixture was allowed to stand in the cold room for at least twenty-four hours undisturbed. Thereafter, part of the fixative was decanted and replaced with fresh fixative, but the cells were left in the fixative for many weeks. Aliquots were taken at intervals, and washed. The fixed cells were stable to washing after the initial twenty-four hours, but some lysis was noted if fixed cells were left in a buffer alone for long periods.

CHAPTER III

8866 CELLS AND THE COMPLEMENT RECEPTOR

A. INTRODUCTION

1. General.

The cell line used in this study was initiated at Roswell Park Memorial Institute, and Dr. Jun Minowada of that institute provided the following information about its origin. The donor of the cells was Virginia Burroughs, 50 year old white female (Roswell Park Memorial Institute No. 71070). Acute myeloid leukemia was diagnosed on 5/4/66. She died on 7/20/67 after suffering from bilateral bronchopneumonia and septic shock. On 5/17/66 Dr. George E. Moore set up white blood cell cultures. At that time her hematological data were: SBC count of 12,050, with a differential of 70% blast cells, 9% neutrophils, 18% lymphocytes, and 3% mono-myelocytic cells. The line was established after 80 days. Immunoglobulin synthesis was demonstrated. The patient was blood group type A+. No HL-A or karyotyping data were available from the early isolate.

Additional information concerning the line is available from several sources. Dr. Peter Wernet, formerly of the Rockefeller University, tissue typed the line, and reported (personal communication) it to be HL-A 2,3,7, and W5, although Dierich et al. (1974) made the assignment of HL-A 2,3,7, and 12. Lerner, McConahey and Dixon (1971) and Theofilopoulos and others (1974) reported surface immunoglobulin on this line, although subsequently, using other methodology, Lerner's laboratory (personal communication) has

not found a species compatible with membrane immunoglobulin, and Dr. Robert Winchester of the Rockefeller University, using Fab fragments directed against human immunoglobulins, found no membrane fluorescence on any of the stock culture of 8866 used in this work. Dr. Donald A. Pious, of the University of Washington, Seattle, found the line to be pseudo-diploid, with an extra chromosome in group C and one missing in group G, using only staining, but no banding techniques, in 1973 (personal communication).

Since the line 8866 is now widely dispersed, and has been continuously cultivated in several laboratories over a period of approximately ten years, the question of the definition of the line arises. This point will be considered further in the Discussion, but for the purpose of this work 8866 cells are those cells obtained directly from Drs. Peter Wernet and Siamon Gordon of the Rockefeller University. They had come from Dr. J. Fahey via Dr. Richard Lerner.

In our laboratory the cells have been carried virtually continuously in culture since October 1972. Over that period the following characteristics have been observed:

a. They will grow in RPMI 1640, Dulbecco's Modified Eagle's Medium or the alpha modification of MEM when supplemented with 10% heat inactivated fetal calf serum. In RPMI 1640, acidification takes place at about 24 hours, and viability begins to fall more rapidly than in the other media. There was some batch-to-batch variation in growth rate even within one medium.

b. Growth was distinctly density dependent. Diluting the cells

to less than 2 x 10^5/ml led to a marked slowing of doubling, and serial dilutions into wells containing 1 ml of medium showed that the minimal number of cells required was between 100 and 1,000 for reliable recovery.

c. Growth was affected by pH, being retarded by alkalinity greater than pH 7.6 more than by pH values of approximately 6.8. For sensitive cultures just recovered from freezing, optimal growth was obtained by adding 20% fetal calf serum to the medium, and gassing it with 10% carbon dioxide, both of which led to some acidification.

d. At optimal growth conditions the cells grew as clumps not adherent to the substratum. The size of the clumps varied with medium and serum batches. Failure to form clumps was an early sign of poor growth conditions.

e. When placed into either plastic or glass vessels in serum-free conditions, the cells tended to adhere to the walls. They did not grow as monolayers, and most adherent cells appeared to die within 24 hours.

f. When examined by phase microscopy, the cells showed many large surface protrusions, with some of the protrusions being almost as large as the cell body. As the cells became chilled the protrusions were retracted, and their final form tended to be globular. Electron microscopic examination also revealed surface projections. The cytoplasm contained a few mitochondria, scanty endoplasmic reticulum, and some free ribosomes. When fixation was performed at room temperature, the microtubules were preserved, and were seen radiating out from the centrosphere. The nuclei were large, with irregular borders, chromatin was coarsely clumped, and several

prominent nucleoli per cell were seen. Occasional large inclusions were seen. In some cells phagocytic vacuoles containing debris were a prominent feature.

In the medium of the cells grown to 1-2 x 10^6/ml, large vermiform sacs of cytoplasm were usually found. If the medium became more acidic than usual, these became more numerous. They presumably represent sloughing of the cytoplasmic protrusions.

g. On several occasions phagocytosis of target cells was observed by different populations of cells. Fresh erythrocytes were poorly phagocytosed. Aged erythrocytes coated with IgM and complement were phagocytosed best. Complement coated zymosan particles were also phagocytosed. The earliest time at which phagocytosis could be observed was 4 hours but was best seen after an overnight incubation at 37°C. As will be described, clones which were all complement receptor bearing were derived, and several were examined to see if any showed a high percentage of phagocytic cells; none was found. The discovery of such a line would have permitted the kind of membrane isolation using ingested latex particles so well exploited by Korn's group (Ulsamer et al., 1971) and by Hubbard and Cohn, 1975b.

h. The doubling time of the stock culture in November 1972 was 0.81 days, i.e. 19.4 hours. In general, the cells were divided 1:2 daily, and required only minor adjustments to keep them within the optimal growth range of 0.5-1 x 10^6/ml.

i. In some electron micrographs, contamination with mycoplasma was detected. Contaminated cultures were treated with Kånamycin and Lincomycin, and follow-up studies showed barely detectable levels of contamination. No regular

program of screening for this agent was followed. However, in spite of contamination, cell growth appeared unimpaired, and the property measured, namely rosetting with E.IgM.C did not appear different in contaminated and cleaned cultures.

2. 8866 and Complement Receptors.

In 1974, Theofilopoulos, Bokisch and Dixon reported that the lymphoblastoid cell line, 8866, bound both intact C3 and its cleavage product, C3b. They also showed that these cells rosetted with sheep red cells coated with antibody and complement. However, only 5% of the cells in their cultures manifested these properties. Theofilopoulos and Perrin (1976) confirmed the finding with C3, but demonstrated a marked difference between E.IgM.$C3b_{rabbit}$, which bound to 3% of cells, and E.IgM.$C3d_{mouse}$, which did not rosette at all. Properdin and Factor B of the alternative pathway of complement fixation were found not to bind. It was the finding that only a proportion of the cells in a lymphoid cell line rosetted that led to the work to follow.

The first experiments in this laboratory confirmed that not all cells of the stock culture rosetted when tested with sheep red cells sensitized with IgM anti-SRBC and then exposed to mouse serum as a source of complement. Erythrocytes alone, IgG sensitized SRBC, IgM sensitized SRBC, and IgM sensitized SRBC exposed to mouse serum inactivated by heating at 56°C for 30 minutes did not form any rosettes, and have failed to do so over a two year period.

Adding a vast excess of E.IgM.C relative to the number of lymphocytes did not cause all lymphoid cells to rosette, nor did incubating the cells overnight. In addition to the heterogeneity of not all cells binding target cells, there was a heterogeneity in the number of target cells which were bound, with some lymphoid cells resembling raspberries, being covered with erythrocytes, while others bound only one or two, and this heterogeneity also was not altered by increasing the E.IgM.C to lymphocyte ratio once a plateau value had been reached. It should be added that, even from the earliest observations, cells which would admit trypan blue would not rosette, and this has been confirmed under a wide range of conditions which kill cells, and cause permeability to trypan blue.

It was decided to investigate the nature of the heterogeneity of rosetting further, with the intent of producing pure populations of cells which were positive and negative with respect to the character of rosettability. Two likely explanations were considered. The first was that the receptor was only expressed for a limited portion of the cell cycle and the other was a heterogeneity of cells in the stock culture. The former was not considered highly likely, since there was such a marked difference between the percentage rosetting we observed (approximately 50%) and that observed by the La Jolla group and reported above. As Littlefield (1974) considered the methods of synchronizing lymphoid cells unreliable, an alternative approach was taken. Cells were set up in culture in logarithmic phase, and then allowed to grow without further feeding until they had passed through optimal density to a state where cell death was occurring, and, presumably, arrest of at least many of the cells in G1 phase had

occurred. Experiments comparing cultures in senescence with logarithmic phase cultures were done on several occasions, and no significant difference between them found. The most exhaustive experimental data are presented in Table III-1. Here, the stock culture, 8866, does show a fall in the percentage of rosetting cells. The value is still high, however, for expression in a limited and restricted part of the cell cycle; additionally, it has been found that, in other circumstances of progressive cell death, such as standing cells in cold balanced salt solutions, the ability to rosette was depressed more than was viability assessed by trypan blue exclusion. In the other columns of the table are presented data from some clones whose derivation from the stock culture will be described in the following pages. They show no significant effect due to passage through the cell cycle, unless there is a restriction in less than approximately 30 minutes, since the shortest time in which 100% rosetting was achieved with the 100% positive rosetting clone was 30 minutes. In addition to the lack of major effect shown by this technique, the results obtained by using an alternative theory, namely clones of different potential making up a mixed stock culture, seem to rule out a significant role for cell cycle dependent variation.

LEGEND: TABLE III-1. THE EFFECT OF CELL DENSITY-DEPENDENT ARREST OF THE CELL CYCLE ON ROSETTING.

Three cultures of cells were kept in suspension culture without division or feeding for the period of the experiment. Each day 1 ml aliquots were taken for cell counting and viability measurements, and for rosetting. These data were obtained using the stock culture, and two clones, derived from the stock culture, and whose nature and origin will be described in the section to follow, but which had the property of being approximately 100% rosette positive and negative, respectively.

TABLE III-1.

THE EFFECT OF CELL DENSITY-DEPENDENT ARREST OF CELL CYCLE ON ROSETTING

DAY	8866			Clone 34			Clone 23cl.		
	Cell Count[a]	Viability(%)	Live Cells Rosetting (%)	Cell Count	Viability(%)	Live Cells Rosetting (%)	Cell Count	Viability(%)	Live Cells Rosetting (%)
1	< 1	> 90	44	<1	> 90	93	<1	> 90	0
3	1.55	> 90	47.5	0.90	> 90	97.5	1.36	> 90	0
4	2.23	∿ 90	46.7	1.06	∿ 90	99	2.62	∿ 90	0
5	2.21	82	45.5	1.41	∿ 90	98.5	2.59		0
6	2.02	65	38.5	1.49	79	100	2.57	69	0
7	1.90	63	28.5	1.38	65	96.5	2.62	62	0

a = Cell count x 10^{-6}/ml.

B. CLONING

1. Introduction

The experiments just described made it unlikely that cell cycle controlled the variable expression of the complement receptor in a cell population. Two other possible explanations were entertained:

a. The cell population was a mixture of cells.

b. The cell population was phenotypically unstable.

The cell mixture could have arisen by contamination of one cell line with another line, by mutation of a cell line, or, since the line was derived from a large number of peripheral blood lymphocytes, the line could have been heterogeneous ab initio.

To approach these two possibilities, it was necessary to establish a reproducible method of cloning with high efficiency.

The simplest method of cloning, namely, serial dilution to limiting cell numbers, was not possible with this type of cell. Preliminary experiments showed that, when the cell count reached approximately 10^3/ml, in a medium otherwise satisfactory for growth, no proliferation was seen, and the cells died rapidly.

After this trial, a number of reported methods were tried without success. These included the single cell in an agarose drop method of Patuleia and Friend (1967), the microwell system of Choi and Bloom (1970a), the drop under silicone oil method of Paul (1959), the methyl-cellulose system used by Dr. B. Clarkson's laboratory (Dr. T. Kitahara, personal communication: the method was first described by Stoker et al., 1968) and the overlay on agarose method of Kuroki (1975). Various combinations of sera and media were tried to no avail. The methods which proved successful on several occasions will be the only ones described. They fall into two categories:

i. Dilute suspensions in semi-solid agar

ii. Picking of single cells.

Since the former method depends on the statistical probability of a clone arising from a single cell, and is dependent on a number of uncontrollable factors which make it possible that more than one cell could form the origin of a nest of cells picked as a clone, the second method provides valuable alternative support for the former, which, when successful, can provide larger numbers of clones with considerably less labor.

2. Semi-solid Substrate Cloning.

a. Methodology.

An extensive series of experiments were carried out over many months, and during this time, although many variables were tested in a systematic fashion, a totally reproducible system was not established. Initially, success was obtained without a feeder layer, but subsequently growth only occurred in the presence of second to fourth passages of primary rat fibroblasts provided and pre-tested by Dr. M. D. Scharff, from a stock purchased from Microbiological Associates. Unsuccessful feeder layers included L cells, the mouse fibroblast line; WI-38, the human embryo fibroblast line; E3, a minimum deviation rat hepatoma line; 1706, a rat embryo fibroblast line, 3T6, a mouse fibroblast line; and lethally X-irradiated (800 rads) 8866 cells. The results to be presented come from three series. The first two were without feeder layer, while the third was with the rat fibroblasts, as described in Methods.

b. Results.

The assessment of cloning efficiency with this method was difficult, if not impossible. Many colonies begin to grow, but abort at an early stage, yet remain visible to the naked eye, and could be counted as positive. In other cases, contiguous colonies coalesce, and would lead to an artificially low estimate: serial dilutions to the point where this

was statistically improbable gave rise to no colonies (less than 5 per 5,000 cells plated). Allowing for these problems, the cloning efficiency was less than 10% and usually less than 5%.

Colonies could be seen as early as one week, but some took up to three weeks to develop. By this time, some of the larger colonies were beginning to merge with neighboring colonies, and there was also evidence of involution of others. As a compromise, most colonies were picked at between 10 and 14 days after plating, although some were picked earlier and some later in order to be fully representative of the stock population. The subsequent testing of colonies picked at various times showed no clear differences of growth rate once established in liquid culture, nor any difference with regard to expression of the surface receptor.

The gross appearance of the colonies exhibited one of three forms: dense balls, loose straggling clusters, and sheets. Many of the sheet forms are the result of growth at the interface between the firmer under layer, and the soft over layer of the agarose. Colonies were selected to be representative of the different morphologies, excluding only those colonies appearing to be made up largely of dead cells. The subsequent growing up of colonies is described in Methods. Some colonies were tested directly from wells, while others were grown in flasks before testing. No correlation was found between colonial morphology and growth efficiency, growth rate, or the presence of the marker receptor.

Cells were screened for receptor activity by incubating 100µl aliquots of cells at 0.4-1.5 x 10^6/ml with 50µl of 0.5% suspensions of target cells

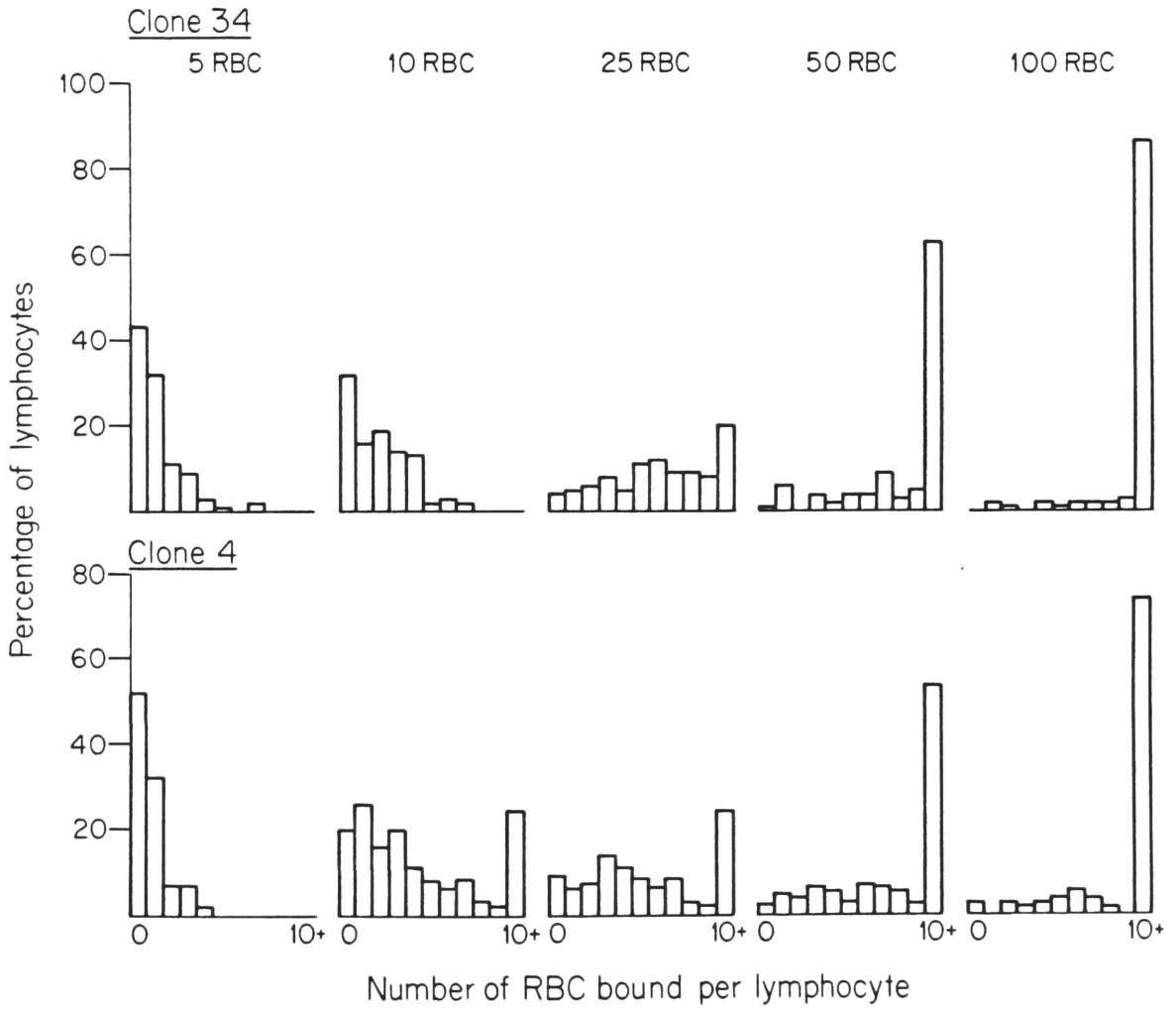
for one hour, then adding trypan blue, and scoring the percentage of live cells binding four or more target cells. Initially, the optimal number of target cells to add was not known, and also, in view of the large number of clones to be scored, it was not possible to do cell counts on all samples. The rule adopted was that there had to be a large excess of unbound target cells seen; otherwise, more target cells were added, and the samples retested. When the first clones showing the desired characteristics were isolated, a true dose/response study of target cells to lymphoid cells forming rosettes could be performed. Two clones, numbered 34 and 4 showed 100% rosette formation with excess target (E.IgM.C) cells. They were tested with increasing ratios of target cells to lymphoid cells, and incubated one hour. The results are shown in Figure III-1. For either clone at less than 100 E.IgM.C/lymphocyte, the system was not saturated. In subsequent work, E.IgM.C were added at 1% concentration so that there were 200 or more E.IgM.C/lymphoid cells. Additionally, Figure III-1 shows that there was a homogeneity of the response to the varying ratio of lymphocytes. With this kind of technique it is not possible to determine the number of binding sites per lymphoid cell.

The results of the three separate cloning experiments - in December 1974, April 1975 and February 1976 - are pooled, since the individual results resemble one another closely, and are shown in Figure III-2. The result is a distribution of clones with varying percentages of rosette-positive cells as defined by the arbitrary definition of four or more E.IgM.C bound per lymphocyte. Not shown in the figure is that, within the clones, as with the parent culture, the intensity of rosetting of individual

LEGEND: FIGURE III-1. DEPENDENCE OF ROSETTING ON TARGET CELL TO LYMPHOCYTE RATIO.

Cells of clones 34 and 4, both 100% positive rosetting with E.IgM.C, were grown in stationary suspension cultures. They were harvested in the logarithmic phase of growth, pelleted, and re-suspended in fresh Dulbecco's medium with 10% fetal calf serum to a concentration of 1×10^6/ml. E.IgM.C were suspended in medium at a concentration of 10^8 cells/ml. They were then serially diluted in serum-free Dulbecco's medium to give the requisite cell count. 100µl of each were then mixed and incubated 90 minutes at 37°C in a 5% carbon dioxide atmosphere. The cells were then chilled, and, just before counting in a hemocytometer, mixed with approximately 10µl of 0.4% trypan blue, and gently resuspended to disperse clumps. Approximately one hundred lymphoid cells were assessed for the enumeration of red cells bound at each cell ratio.

Dependence of rosetting on target cell to lymphocyte ratio



cells within a clone varied widely. Although most clones varied in both percentage and intensity of rosetting, a limited number of clones were obtained which were either uniformly intensely positive, or completely negative. Repeated testing of the clones proved the results to be reproducible for each clone tested within the limits of the method.

C. PICKED SINGLE CELL CLONING METHODS.

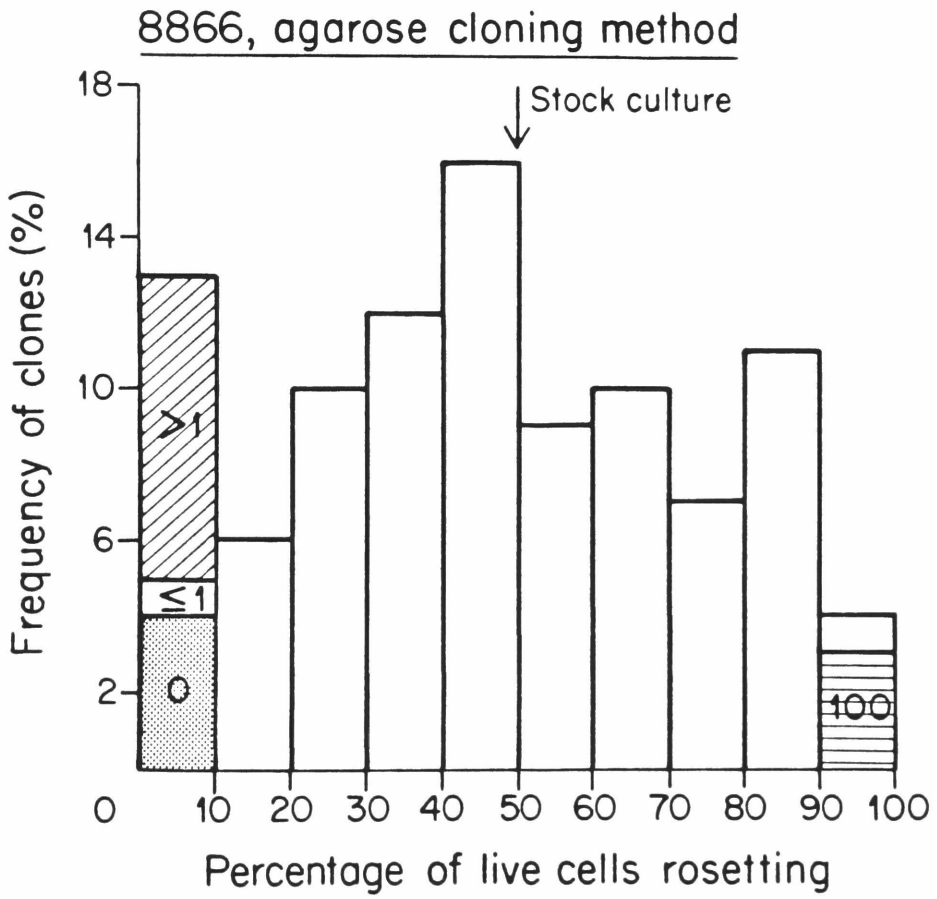
As already mentioned, simple serial dilution of cells into wells of various sizes showed that growth media capable of supporting regular doubling of cells in under 24 hours, and maintaining greater than 95% viability could not support cells at densities less than 1,000 cells/ml. Pretreating of the dishes with medium and serum or even serum alone did not "condition" the wells, and attempts to use medium "conditioned" by growing 8866 cells in it also were of no avail.

The picking of single cells in a finely drawn pasteur pipette was learned from Dr. Elaine Diacumakos, and proved reliable and relatively convenient. The method which enabled such cells to survive was acquired from an adaptation of the agarose method used by Dr. M. D. Scharff. In his latest agarose method, the medium is supplemented with 10% Medium NCTC 109, poured over a feeder layer of rat embryo fibroblasts. The fetal calf serum used was from a selected lot. Trials revealed that whether freshly plated, in growth phase or at confluence, the feed layer was equally effective in supporting clone growth. However, when added after the plating out of the single cell, no clones grew up.

LEGEND: FIGURE III-2. CLONES OF THE LYMPHOBLASTOID CELL LINE 8866 DERIVED BY THE SEMI-SOLID AGAROSE METHOD.

The histograms represent the pooled results of 98 clones picked from three independent clonings: on 25.xii.74, 6.iv.75 and 15.ii.76. One volume of cells at a concentration of 50,000 cells/ml, and extensively dispersed until careful microscopic screening revealed dispersal into single cells, was mixed with 9 volumes of a mixture of agarose and medium to give a final concentration of 0.22% agarose, 10% fetal bovine serum, all in Dulbecco's medium at the usual concentration. Two millililiters of this mixture were dispensed into 35 mm petri dishes. The dishes were kept at room temperature for approximately 30 minutes to allow the agarose to set, and then placed in an incubator at 37°C in an atmosphere of 5% carbon dioxide in air saturated with water vapor. The incubator was opened only once or twice daily during the development of clones. Colonies were picked, grown and tested as described in the text.

The bars indicate the frequency of clones having a given percentage of rosetting cells. In the range of less than 10% positivity, the data are subdivided to show, at the bottom of the bar, those completely negative clones; in the mid-portion, those clones with some positive cells, but less than 1% positivity; and the upper portion with those between 1% and 10%. Similarly, in the 90-100% region, the bar is divided to illustrate, in the lower portion, those completely positive clones, while the upper portion is for clones greater than 90% but less than 100%.


8866, agarose cloning method
Stock culture
Frequency of clones (%)
18
14
10
6
2
>1
≤1
0
100
0
10
20
30
40
50
60
70
80
90
100
Percentage of live cells rosetting

When a feeder layer was present, it was not possible to detect the presence of a single lymphoid cell, and, in the case of the sloping-walled Terasaki dishes, it was not always possible to detect a cell even when no feeder layer was present. To ensure that the method used was truly dispensing a single cell, an experiment was performed in which 6 mm diameter flat-bottomed wells were used without a feeder layer, and single cells picked and dispensed as described in Methods. The wells contained only a droplet of medium. After allowing a few minutes for settling, the droplets were examined at 100X magnification by means of an inverted microscope. Of 48 wells, three could not be scored because of the presence of air bubbles in the droplet. Of the remaining 45, 43 contained one cell, and in two no cell was seen. It was felt that this constituted a critical test of the method, giving no wells with more than one cell. In assessing cloning efficiency, it seemed the method might lead to a slight under-estimate.

With the single cell cloning method, the efficiency of each stage can be quantitated with reasonable accuracy, allowing for the fact that in any particular experiment the presence of an initial single cell cannot be determined directly. In Table III-2 some representative data on the efficiency of growth from single cells is presented. (It may be noted here that, after this series, the use of line 1706 was discontinued because of the morphological "transformation" to a non-adherent, no longer contact-inhibited form). As can be seen, there was a range of from 27% to 75%. This represents the efficiency of the first stage. No data are presented

LEGEND: TABLE III-2 CLONING EFFICIENCIES.

The table lists the macroscopically evident colonies visible up to four weeks after the seeding of a single cell into wells of two types of dishes: the 10 microliter capacity wells of the Terasaki dish (Falcon plates, 3034), or the microtiter II dish (Linbro, flat bottom, 6mm, 48 wells/dish). Most of the colonies were, in fact, picked at about 2 weeks, but observation was continued further, with bi-weekly aspiration of half the medium, and replacement with fresh.

1706 is the designation of an established line of rat embryo fibroblasts. REF refers to rat embryo fibroblasts in their second to fourth passages.

TABLE III-2. CLONING EFFICIENCIES

DATE OF ORIGIN	WELLS	FEEDER LAYER	CELL TYPE SEEDED	CLONING EFFICIENCY	
March 76	Terasaki	1706	8866	47/120	39%
			34	16/30	53%
			4	23/60	38%
			23	21/60	35%
			36	16/60	27%
March 76	6mm	REF	8866	55/96	57%
			34	20/47	42%
			23	20/48	42%
April 76	6mm	1706	23c1	30/96	31%
			23c2	16/24	67%
			36c1	10/24	42%
	6mm	REF	23c1	20/48	42%
			23c2	36/48	75%
			36c2	19/48	40%
			8866	34/48	71%

of the stage of transfer from small well to larger well, but this involved few losses. The stage of transfer from 16 mm wells to bulk culture is illustrated in Table III-3. Here the highly efficient recovery of clones is clearly demonstrated.

From some of the trial experiments the following features were found to be critical. Cells were fed regularly, at least once weekly, even in the early stages. Feeding was carried out by replacement of approximately half of the medium with fresh medium. The timing of transfer from one stage to another was determined by the regular acidification of the medium in 24 hours, and the presence of sufficient cells to cover at least half of the bottom of the well. Using this protocol, in one re-cloning of Clone 4b1, 11/12 seeded single cells were grown to bulk culture.

The data presented establish that mixed populations, positive clones and negative clones can all be recloned with an efficiency high enough to make it likely that the progeny are representative of the parent cultures. Additionally, the high yield of subclones means that the method is not restrictive in terms of the work required to yield sufficient clones.

The distribution of C3 receptor expression by the clones derived by the single cell picking method is illustrated in Figure III-3. Although there is a difference in the percentages of the cells within the various 10% blocks as compared with the agarose cloning data, there is the same finding that even single cells give rise to positive, mixed and negative clones.

In order to study this distribution further, several clones were recloned and some of the data are presented in Table III-4, and Figure III-6.

LEGEND: TABLE III-3. EFFICIENCY OF PASSAGE FROM 16mm WELLS TO BULK CULTURE.

The table records the efficiency of recovery of cells cloned in 16mm wells, with partial change of medium biweekly, or more frequently if marked acidification took place, until 50% of the surface area of the bottom of the well, or more, was covered by clumps of cells. At this point, an additional 1 ml was added to the well, and transferred to a 25 ml capacity tissue culture flask. The well was rinsed once with approximately 2 ml of medium to recover more cells, and these were added to the flask. One ml of medium was added to the well, and usually a few remaining cells grew up: this constituted a safety measure in case the flask was lost by way of contamination.

In those cases marked with asterisks, colonial growth was observed, but, on further culture, proved to be shed cells of the feeder layer. This fact was identified by the observations that these colonies became adherent when plated out, and did not show the characteristic free-floating clumps in the larger vessels.

TABLE III-3. EFFICIENCY OF PASSAGE FROM 16mm WELLS TO BULK CULTURE

DATE	CLONE	EFFICIENCY OF RECOVERY
5.iv.76	4b1	12/12
	4b2	12/12
	34c1	9/10
	34c2	9/9
	23c1	24/24
	23c2	10/17*
19.iv.76	23c2	36/36
	36c2	14/19**

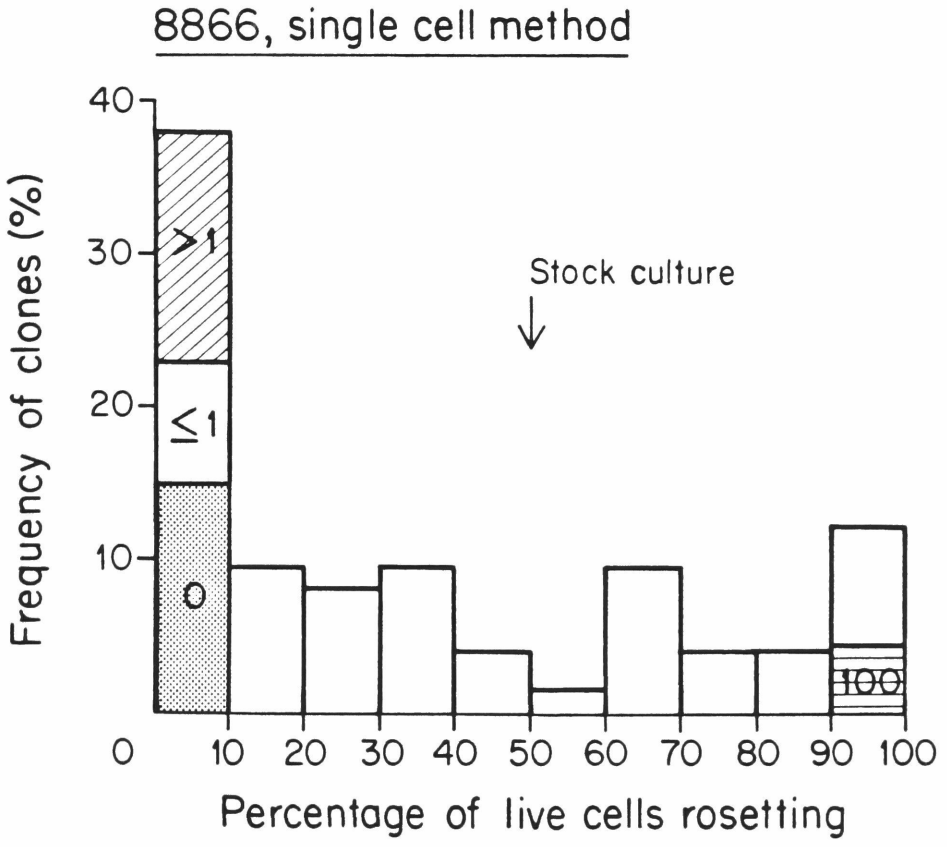
* = Of the 17 wells, 7 proved to be 1706 feeder layer growth only.

** = Of the 19 wells, 2 showed no growth, and 3 were not picked.

LEGEND: FIGURE III-3. CLONING OF 8866 BY THE PICKED SINGLE CELL METHOD.

The histograms depict the frequency of rosetting cells in clones derived from single cells picked and grown on two different feeder layers, rat embryo fibroblasts, and a rat embryo fibroblast line, 1706, in February and March of 1976. A total of 74 clones were grown up by the methods described in the text. The first testing for complement receptor activity is the one used in these data. All lines thought to be either 100% positive or negative were tested at least once more. The arrow indicates the percentage rosetting of the stock culture during the period of cloning.

As in the previous histogram of this type, the group of clones with less than 10% showing rosetting activity are subdivided into the totally negative, those falling between zero and 1%, and those which are totally composed of rosetting cells are differentiated from those with a rosetting percentage between 90% and 100%.


8866, single cell method
Frequency of clones (%)
40
30
20
10
0
>1
≤1
0
Stock culture
100
0 10 20 30 40 50 60 70 80 90 100
Percentage of live cells rosetting

The positive clones, initially picked from agarose, and re-cloned twice by the single cell method, have always given rise to positive progeny only. This is in keeping with the fact that samples of the clones, kept in almost continuous culture over nearly two years, have remained entirely positive; the apparent small changes to less than 100% appear to be the result of the reagents used in testing the rosetting rather than the cells as, when retested, it has always been found that 100% of live cells will rosette.

The negative clones have proved otherwise. Whereas early testing of progeny always gives a predominance of negative clones, some positive cells are seen at first testing, and if clones are kept in culture, all clones thus far tested have given rise to some positive cells within a period of approximately one month. Usually, the interval is less. In Figure III-4, the results of repeatedly taking different ampoules of totally negative cells out of frozen storage and growing them up can be seen. In all cases, positive cells appear. This is seen as the emergence of a population of cells which rosette weakly or with intermediate target cell numbers: it is rare to see very heavily rosetted cells. The conversion occurs at a time when the cells are doubling regularly, and of good viability: there is no evidence that the negative cells are selectively dying.

In addition to the appearance of positive cells in negative clones, there is the striking phenomenon of instability of phenotypic expression shown clearly in Figure III-4. Testing of the cells with two sets of different reagents on one day gave comparable results, and retesting at close intervals within experiments rule out the possibility of target cell reagent

LEGEND: TABLE III-4. RECLONING OF CELLS: PICKED SINGLE CELL TECHNIQUE.

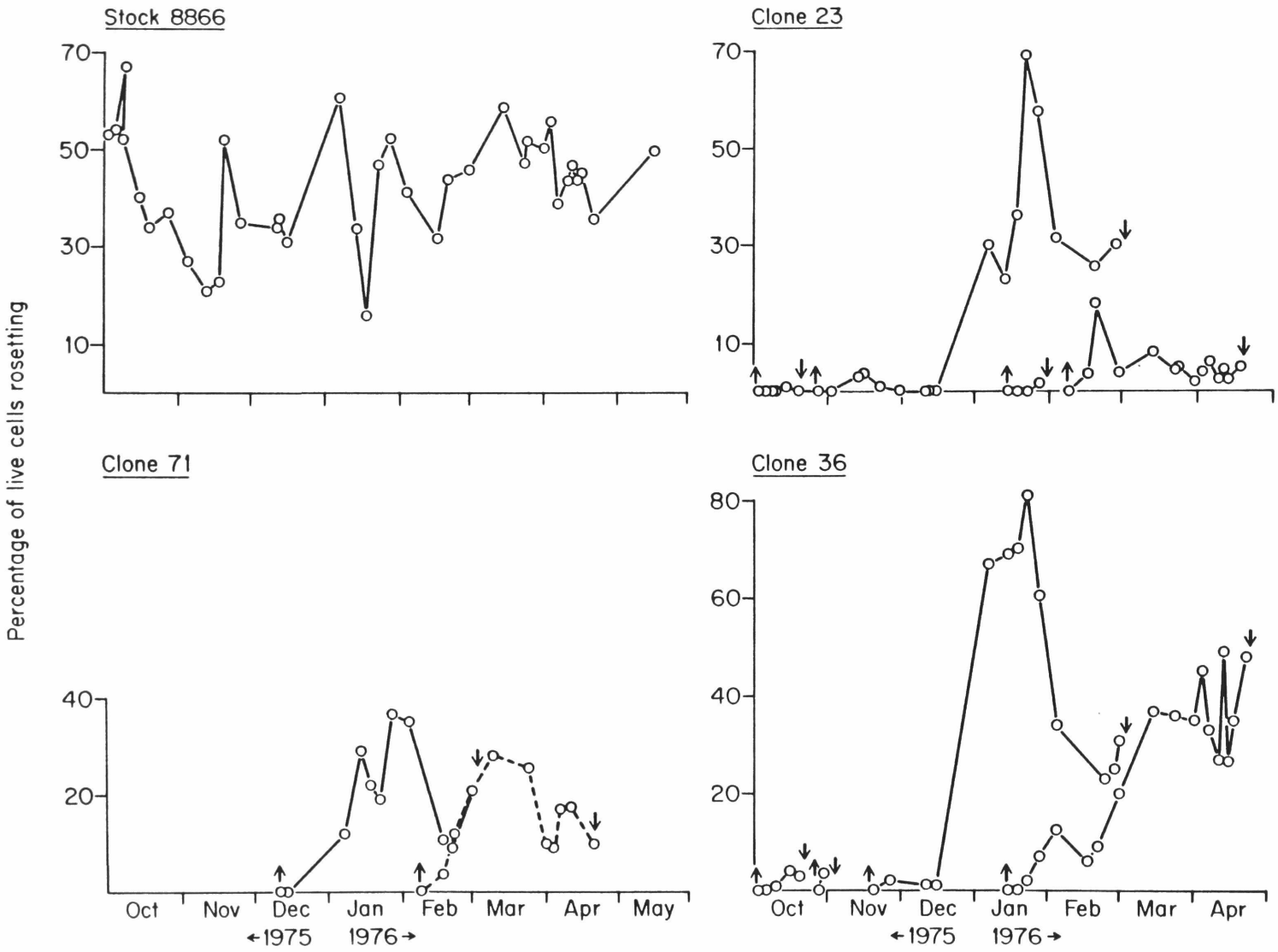
The table shows the results of the recloning of four clones originally derived by the agarose method. Those which gave absolutely clear cut results are presented in this table numerically. Those with some scatter are presented in the figures designated.

TABLE III-4. RECLONING OF CELLS (PICKED SINGLE CELL TECHNIQUE)

	PARENT CLONES		PROGENY CLONES	
	DESIGNATION	E.IgM.C ROSETTING	NUMBERS	E.IgM.C ROSETTING
SECOND CLONING	34	100%	13/13	100%
	4	100%	12/12	100%
	23	∿ 5%	SEE FIGURE III-5	
	36	∿ 35%		
THIRD CLONING	34c1	100%	9/9	100%
	34c2	100%	9/9	100%
	4b1	100%	8/8	100%
	4b2	100%	12/12	100%
	23c1	0%	10/10	0%
	23c2	0%	35/36	0%
			1/36	4.5%
	36c2	∿ 0%	13/17	0%
			4/17	< 1%
	36c4	∿ 0%	8/8	< 1%
	36c1	∿ 90%	SEE FIGURE III-6	

LEGEND: FIGURE III-4. SERIAL TESTING OF ROSETTING ON CONTINUOUS CULTURES: THE PHENOMENON OF PHENOTYPIC INSTABILITY.

During the months of October, 1975 to April, 1976, the four lines depicted, plus the two positive clones 34 and 4 were grown under similar conditions with daily feeding except when cell numbers indicated no need. Clones 34 and 4 remained at between 95 and 100% C3 receptor positivity on all testings during this period. The arrows pointing upward indicate the initiation of new cultures from frozen stocks. The arrows pointing downward indicate the termination of the culture. Between December 20th and mid-January there was a period of inexplicable slow cell growth, after which normal doubling time was recovered. At all other times, the cells required daily feeding, and showed better than 90% viability by the trypan blue method.


Stock 8866
Clone 23
Clone 71
Clone 36
Percentage of live cells rosetting
70
50
30
10
40
20
80
60
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
←1975
1976→

artefact. In addition, and not shown on the figure, two positive clones were always tested at each measurement and were always greater than 95% positive: such a result was a standard test for all E.IgM.C used in this thesis.

The results of the cloning and recloning studies may be summarized as follows:

i. Mixed populations yield a variety of clones - some mixed, some totally negative and some totally positive for the character of E.IgM.C binding. Like the starting population, the clones show variation not only in the percentage of cells which bind E.IgM.C, but also in the numbers of E.IgM.C bound per lymphocyte.

ii. 100% positive clones not only remain stable on prolonged cultivation, but on recloning also only give rise to 100% positive progeny. Some variation in the numbers of E.IgM.C bound by the cells of different positive clones has been observed. Thus clones 34 and 4 can bind many greater than 10 E.IgM.C, whereas clones d17 and d30, smaller cells, bind less than 10 even under supersaturating conditions of E.IgM.C/lymphoid cell. The reduction in E.IgM.C bound appears greater than the change in cell size. That is, clones with all cells binding E.IgM.C, but only to an intermediate degree, can be isolated. Therefore, in mixed populations of cells an intermediate degree of rosetting may represent a stable surface property or indicate a cell faining receptors.

iii. When initially negative lines have been cultured, and some complement rosetting cells appear, recloning gives rise to mixed, positive and negative clones, as can be seen in Figure 000-5. Clone 23 contained only 5% rosette-positive cells at recloning, and gave rise to predominantly negative clones, with some mixed. By contrast, Clone 36 contained

approximately 35% of rosetting cells. This gave rise to negative clones, but also to mixed clones, and to some 100% positive clones. One of these strongly rosetting clones, 36cl, which subsequently was about 95% positive, was selected for recloning. The results of this recloning are seen illustrated in Figure III-6. In spite of the starting population being 95% rosette-positive, the majority of the progeny were negative, with no 100% positive clones being found; the presence of the large number of mixed clones is also shown. In this study of a virtually entirely positive population, the statistically likely outcome has not been found, and phenotypic change from a positive to a negative character has been demonstrated. When the results of recloning Clone 36cl are added to the comparison of cloning in agarose or by the single cell method, it appears possible that the method of cloning may be a factor in the change. Note that no similar effect was observed with populations of 100% positive cells.

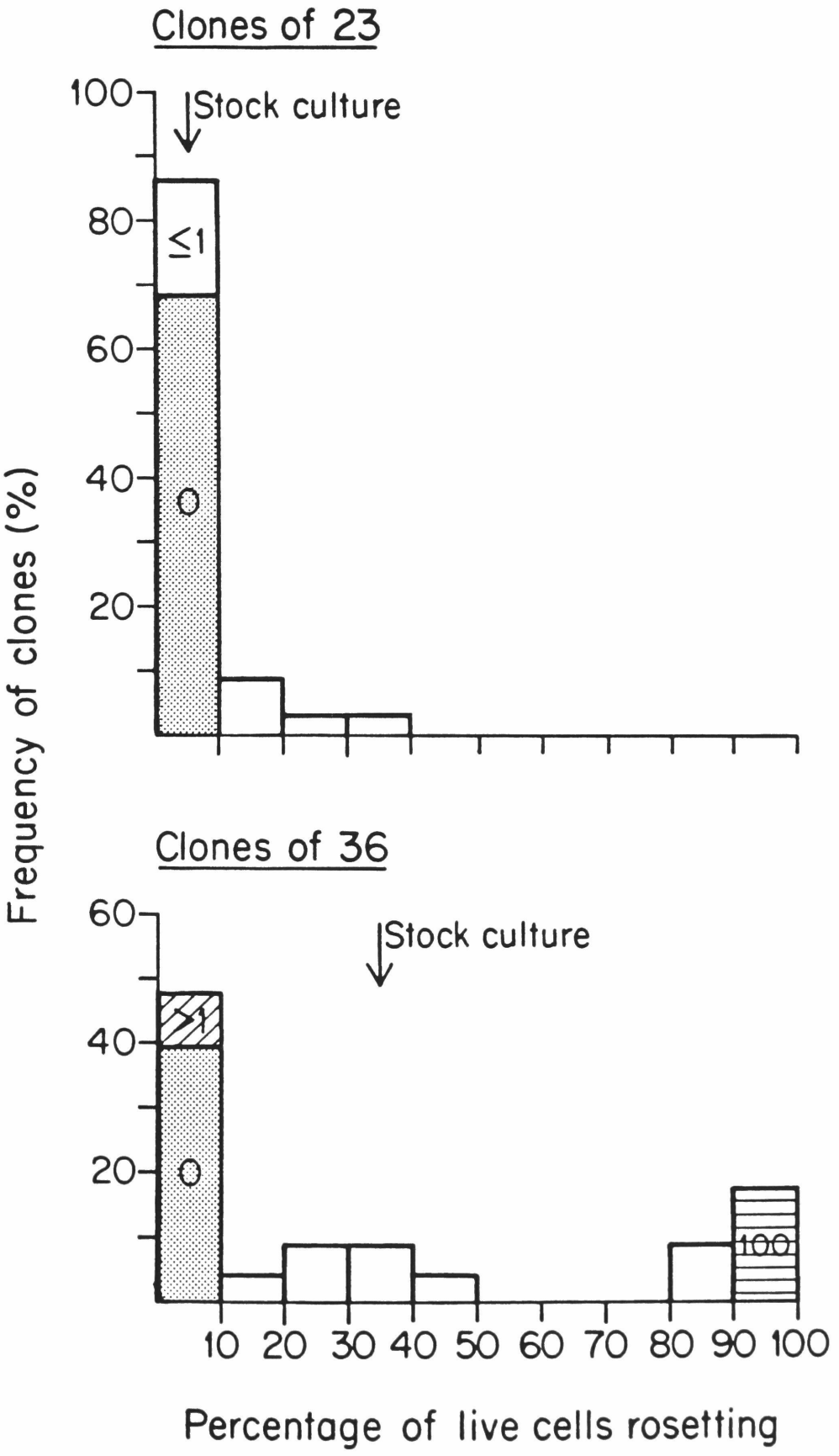
iv. In one case a phenotypic change from 100% positive to negative cells been observed. This is the case of Clone 36cl, which was initially picked as a negative clone, 36, and then as a 100% positive subclone. It was unequivocally thus phenotypically unstable in both directions.

To complete the description of these clones, some morphological observations should be added. The stock culture 8866 is made up of cells of very different diameters. During cloning, lines have been derived of large and small cells, and positivity and negativity found in both size classes, showing that size was not correlated with the presence of the complement receptor.

LEGEND: FIGURE III-5. RECLONING OF INITIALLY NEGATIVE CLONES 23 AND 36.

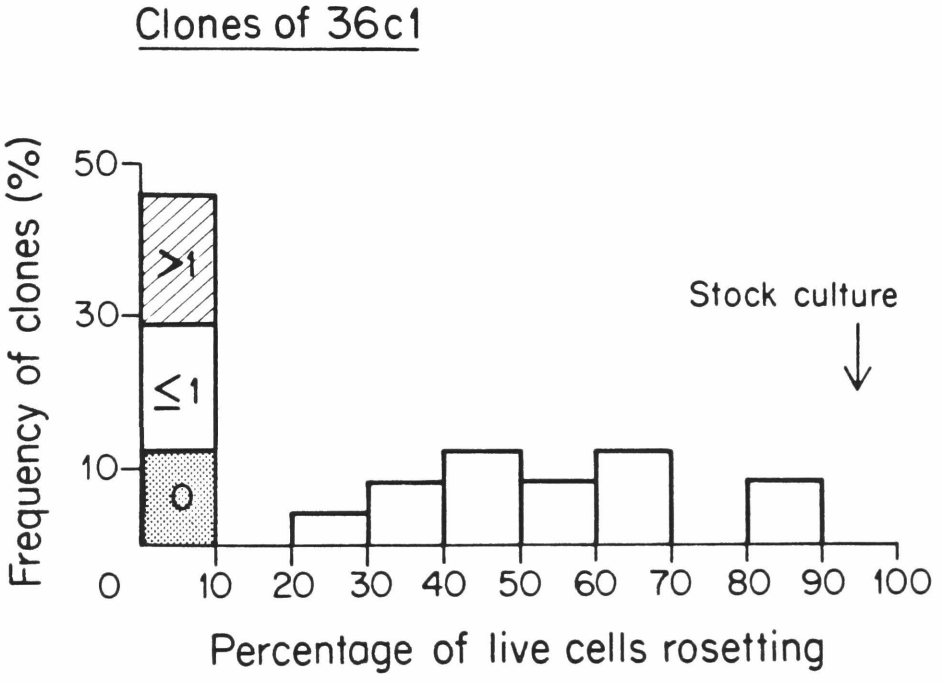
The two histograms depict the distribution of clones derived from initially 100% negative clones which had drifted since their selection in December 1974. The degree of drifting is indicated by the arrow and the designation Stock Culture. In the case of clone 23, the results of 2 clonings are pooled, and a total of 35 clones shown. For clone 36, also two cloning series were used, and 23 clones picked.

The blocks of clones less than 10% and greater than 90% are subdivided as in other similar histograms.


Clones of 23
100
Stock culture
80
≤1
60
40
0
20
Clones of 36
60
Stock culture
40
>1
20
0
100
10 20 30 40 50 60 70 80 90 100
Frequency of clones (%)
Percentage of live cells rosetting

LEGEND: FIGURE III-6. RECLONING OF CLONE 36cl.

The histogram depicts the distribution of 24 clones picked from the recloning of clone 36cl, which was 90-95% rosette positive, as indicated by the arrow and the designation, Stock Culture. The subdivision of the block of the histogram below 10% is as in all similar histograms.


Clones of 36c1
Frequency of clones (%)
50
30
10
0
>1
≤1
0
Stock culture
0
10
20
30
40
50
60
70
80
90
100
Percentage of live cells rosetting

In addition, in mixed clones, and in mixing experiments, both positive and negative cells could be found within a single cell cluster, demonstrating that the presence of the receptor was not a determinant for cell aggregation.

In Figure III-7 the interrelationships just considered are depicted graphically in qualitative terms. The variability of the results obtained with the negative and mixed clones precludes any exact quantitation of rates of interconversion from the data available, but a rough assessment is given by the thickness of the arrows.

D. ON THE IDENTIFICATION OF CLONES

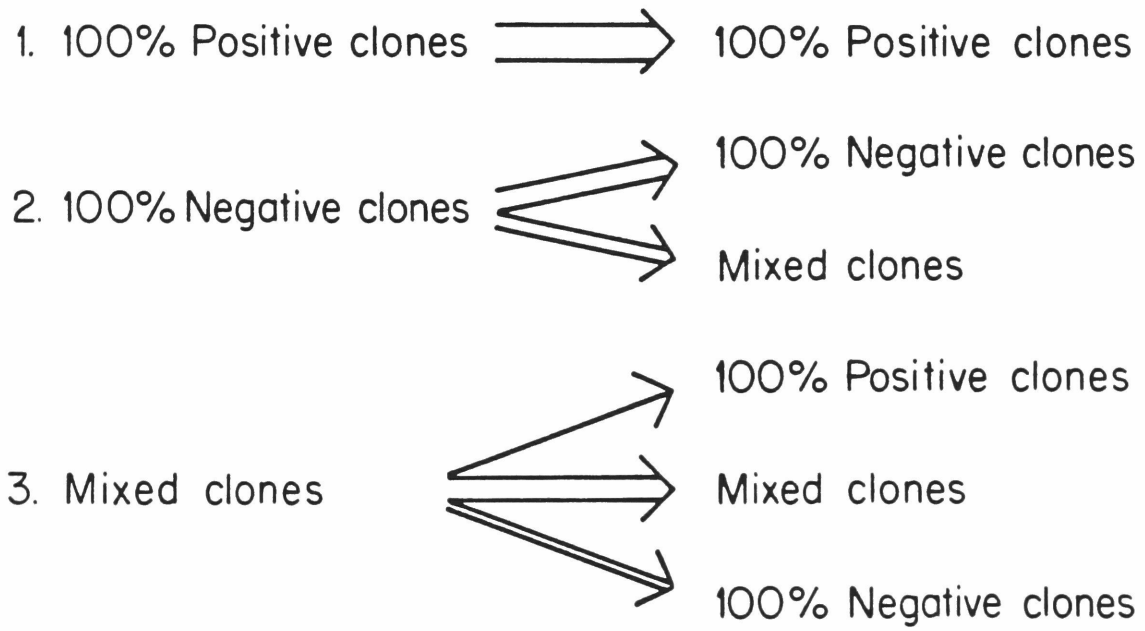
It seems necessary to provide some evidence that the clones derived from the stock culture are, in fact, derived from the same individual as the bulk of the cells in the stock. As mentioned in the Introduction, there is ample evidence for multicellular origin of lines, so that strict criteria suitable for the definition of bacterial mutants are inappropriate until some initial cloning has been done. Recent evidence on the widespread contamination of mammalian cell lines by HeLa cells has delineated the problem clearly. With lymphoblastoid cells, there is the additional difficulty that the cells have been maintained in continuous culture for many years in laboratories which carry a variety of morphologically similar lymphoblastoid cells. Moreover, the tools for distinguishing between cells of very similar origin, morphology, and properties are limited.

As an initial step, and based on the evidence of Pious et al. (1973)

LEGEND: FIGURE III-7. THE RESULTS OF CLONING STUDIES.

A schematic, semi-quantitative depiction of the results obtained from the serial reclonings of 8866 cells. The thickness of an arrow indicates the likelihood of a change occurring.

Behavior of the phenotypic character C3d-rosettability during cloning of 8866 cells



that the HL-A antigens are relatively stable markers, a set of the initial agarose clones and the stock culture were HL-A typed by Dr. T. Fuller of the Massachusetts General Hospital. The data from the cytotoxicity tests are presented in Tables III-5A and B. All cells were compatible with the types 2,3 and 7 previously assigned, although the fourth designation is still in doubt, but Dr. M. Fotino, of the N.Y. Blood Center, believes it to be 40.

A second way of assessing the homogeneity of the clones was to analyze the chromosomes. In spite of extensive attempts using several different techniques, and even using the special facilities of two laboratories specializing in karyotyping, no successful banding system was found for 8866 cells. As a final resort, the simplest method was used, namely Giemsa staining of the chromosomes, and then simply counting them. The results are depicted in Figure III-8. The stock culture has a clear modal number of 46 chromosomes, but with much greater scatter than is found in primary isolates from peripheral blood. In particular, the number of spreads showing 4n (i.e. 92) chromosomes should be noted. In Parts B and C of the figure, the chromosome numbers of 100% positive and 100% negative clones are shown, and reveal that near diploidy and near tetraploidy are found in both classes. Therefore, ploidy alone cannot account for the presence or absence of the complement receptor. It may be noted that the two totally stable positive clones, 34 and 4, and the strikingly unstable negative clone, 36, are all near tetraploid, so that stability is not simply correlated with chromosome number either. The conclusion is clear that the chromosome counts support the claim that the cell strains are true clones.

LEGEND: TABLES III-5 A and B. HISTOCOMPATIBILITY TESTING OF 8866 AND CLONES.

The tables show the results of using two trays of test sera by the Amos, two-stage, cytotoxicity assay. The scoring system is modified from the NIH in that, instead of using the number 5 to indicate inadequate cell numbers for scoring, a confusing convention suggesting maximal cell death, the asterisk has been substituted. Otherwise, the convention is the same, namely:

0 = 0-15% kill

1 = 15-30% kill

2 = 30-60% kill

3 = 60-80% kill

4 = > 80% kill

The fresh rabbit serum used as a complement source showed some anti-34 effect in the orange plate negative control.

Virtually all of the assessments were subjective rather than actual cell counts.

TABLE III-5A.

SERUM ABBR.	HL-A SPEC.	RESULTS 8866 34 4 23 36
ORANGE PLATE:		
POS. CON.	---	2 4 2 3
NEG. CON.	---	2 1
Burt/6	1	1
Glo 2/5	1	2 1
Land	1	1 3 2 1 2
MSN	1+9?	2
NS	1+11	2 3 1 2 4
BC 1/10	3	3 1 4
Gall	3	4 4 2 3 3
Tuc/2	3	4 4 4 2 4
394	3	4 3 4 2 4
BT-11	11	3 3 1 1
Davies	11	4 2
JPO/12	11	3 2
R. Ross	11	3 1
RSS	11	2 2 1 1
Con	2	3 4 2 2
Cron	2	2 4 2 3
Hu-1	2	1 3 1 1
Pin-08	2	3 4 3 2
PB 482	2	1
Mang	2	2 3
Roz	2	1 2 2
Seavy	2	3 1 2 1
WM	2+28+	4 4 3 2 2
Ayotte	28+5	3
HAR	28	2 2
V. Allen	28	2 2 1 2
Vereen	28,W10	1
Cardin	28,10	1 1 2
Card I	9+12	4 3 2 1 4
Dmi	9+5 ?	2 1
Grub	9	3
Jones	9	3 3 1
Higg	24	3 1
Bim	24	1 1
Eva/6	10	3 2 1
Fe 5/8	10	3 4 2 1
MS	10	2 1
Todd	10	1 1 1
9225 3/2	25	2 1
Mailleux	25	2 1
13504.3	26	1 1
1142.4	26	3
Phe	10+32	2
CC23.2	30+31+32	3 3 2 2
Quin	31+32	2 1
Thomp.	31+32	2 1 1 1 1
24089.2/2	30+31	3
Karschbaum	29,30,31	2 4 1 2
Brown	29,32	3 2
Schmidt	29	2 1
Hib 2/8	29	2 2
Hib 1	29	1
Rc Pool6/8	29	1 2 1
ABR	32	1 1
998	30?	2 2
CC40	32,25	1 1
135727	32,25,4	3 *
D66	5	2 1 *
Fe 28/8	5	2
Fmc	5	2
Tha	32	2 2 2
Rm 4/2	5,28,10?	4 4 2 4
131-4	5+HR	3 1
SA8-69	5+5+18	2 1 2
Bon/4	5,5,18	2 1
Ham	5+5	1 1
Ho-2	5 +10 ?	1 2 2 1
Mcm	5+5+18	2 1 2
Da-1	18	3 2 2 1 3
Sts	29	1 2 2

TABLE III-5B.

SERUM ABBR.	HL-A SPEC.	RESULTS 8866	34	4	23	36
YELLOW PLATE:						
NEG. CON.	---					1
Martin	18+14	*	1			1
Martin ABS	18+5^{W}		3		2	1
As-71	7		3	4	2	3
Cut II	7+22	4	3	3	1	3
MGH#4	7		3	2		1
76	7	2	1	2	1	
378	7	1	2	3		1
768	7+22		3	2	2	1
Ceol	22+17	3	2		1	2
Loe	22+W10					1
Lul	7+22		2			
Mmm	22		1			
710	22		1	1		
Knowles	22	2	3	4	3	2
Bell/4	27	*		1		
BT-27	27	1	1	2	1	
Suth	27+17		2	3	2	
Chiara	8		2	3	1	1
CW-2	8	2	2	3	2	1
Hunt	8		2	1		3
CUR	8	*	1			
GBA2/6	14+27		3	2	2	
Fe 16/3	14+18		1			2
BT-14	14		2			2
Strangewick	14					
Fe 7/4	12					
JDP/1.5	12	2			1	
Johnson	12				1	
Rm 1/4	12				1	3
SLA/2	12	1	2			3
Esk	13		2			
Gesvain	13		3	1		1
HK	13		2			1
MAD	13		2	2		

SERUM ABBR.	HL-A SPEC.	RESULTS 8866	34	4	23	36
Neu	13		1			1
BT-13	13		1			1
Elliot	W10		2			
McK	W10	2	2			1
Cole	W10+7^{S}	3	2	1	1	
Gr-2	W10	4	2	2	3	
1033	W15		2			
Tuit	W15		1			1
640.3	W15/W6		2			
1543.5	W15	2	2		2	
BT-W15	W15	3	3	1	2	
Hib 2/2	W16		3	2		
RC 3/3	W16	*	1	2	1	1
19643.5	W16		1			
Ba 4/74	W16.5	1	4	2		
AWA	W17,AWA	*		1		
Eames	W17		1			2
Fry	17+10		3			
Lou	17		1			2
RT	17		2	2		
3346.04	17		1			
765	17+		4	2	3	3
Rintelman	17		2	1		
JCA/2	A078?21?		3		1	
Ket-2	21		3	1		
F.Cannady	21		1			
Agst	W4		2	1		3
23944.2	W4		2	1		3
20971.4	W4		*			1
Heek	W4		3		1	2
To 28/01	W4		3			
West	W6	4	4	2	4	2
196633	W6	4	3	3	4	3

A start has been made on further analysis of the qualitative variation of chromosomes, even without banding. In Figure III-9 representative spreads of different clones are presented. At this level of resolution, no gross abnormalities of the chromosomes have been detected. In Figures III-10 and III-11 two formal karyotypes (prepared by Mr. Edwin Sanchez in the laboratory of Dr. Gretchen Darlington) are presented. They were selected preparations with 46 chromosomes. It was found that they could be easily typed by the normal criteria, with one exception which merits further attention. In the B group of both clones there is one member of a pair larger than normal. It is possible this is a useful marker chromosome for 8866 cells in general, but this requires further study, and some form of banding will be necessary to assign the nature of the abnormality.

In the light of these findings of chromosome numbers in reclonings, some samples of the original agarose selected clones were examined. Examination of limited numbers of spreads reveals that the numbers for the agarose clones were the same as their derivative subclones, namely, Clones 34 and 4 were made up of 4n spreads, whereas Clone 23 was largely 2n. This supports the clonal nature of the agarose selected colonies.

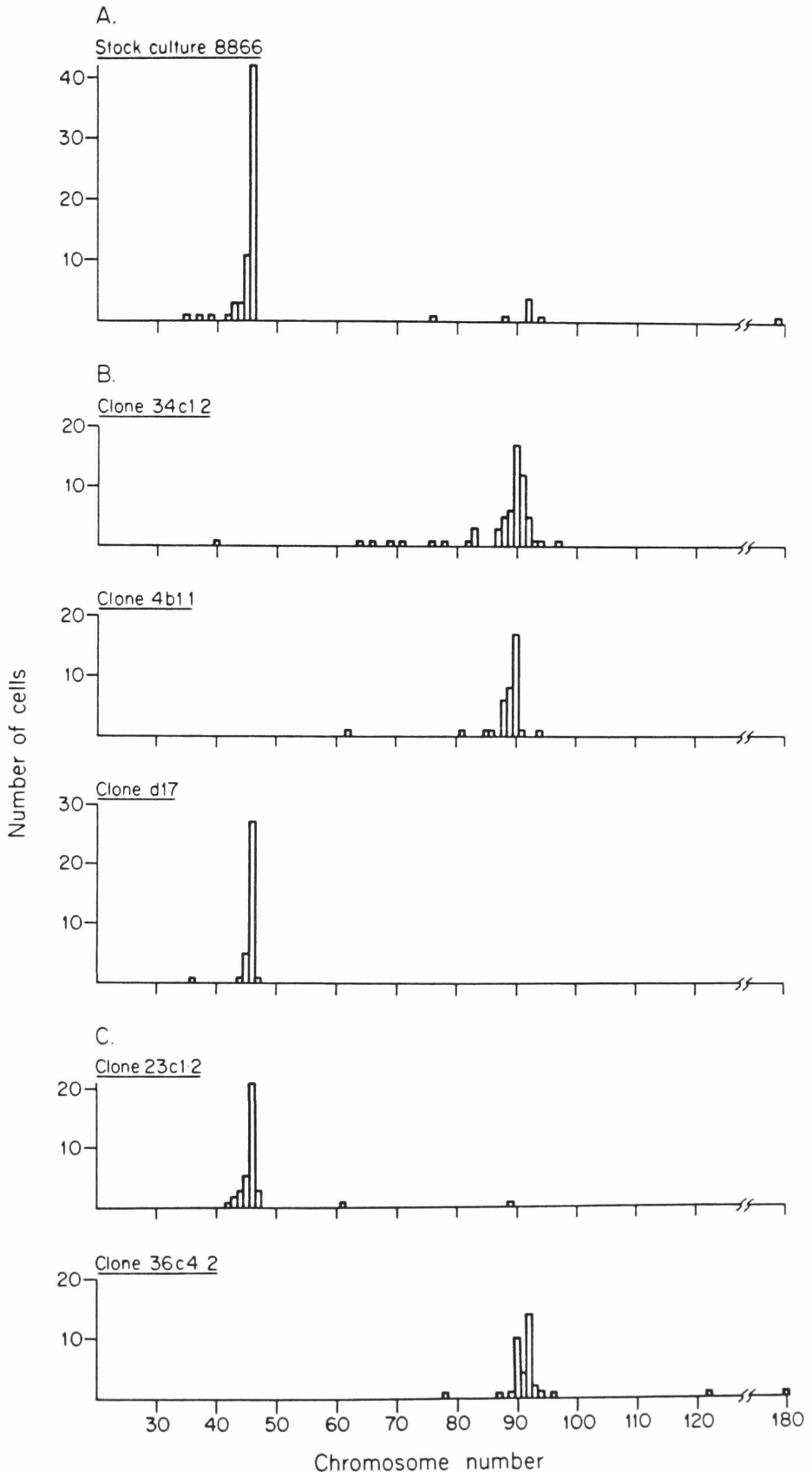
A third method of assessing clonal nature was to examine for expression of the character of surface immunoglobulin. This was performed by Dr. Robert Winchester using his Fab anti-immunoglobulin reagents. No significant staining was seen in the stock culture, nor on any of the original four clones - 34,4,23 and 36. This test was therefore non-contributory.

LEGEND: FIGURE III-8. DISTRIBUTION OF CHROMOSOMAL NUMBERS IN 8866 AND CLONES.

The histograms show the distribution of chromosomes as assessed after colchicine mitotic arrest, hypotonic swelling, acetic acid/ethanol (1:3 v/v) fixation and Giemsa staining. The spread cells were photographed, and counts of chromosomes made from the photographs.

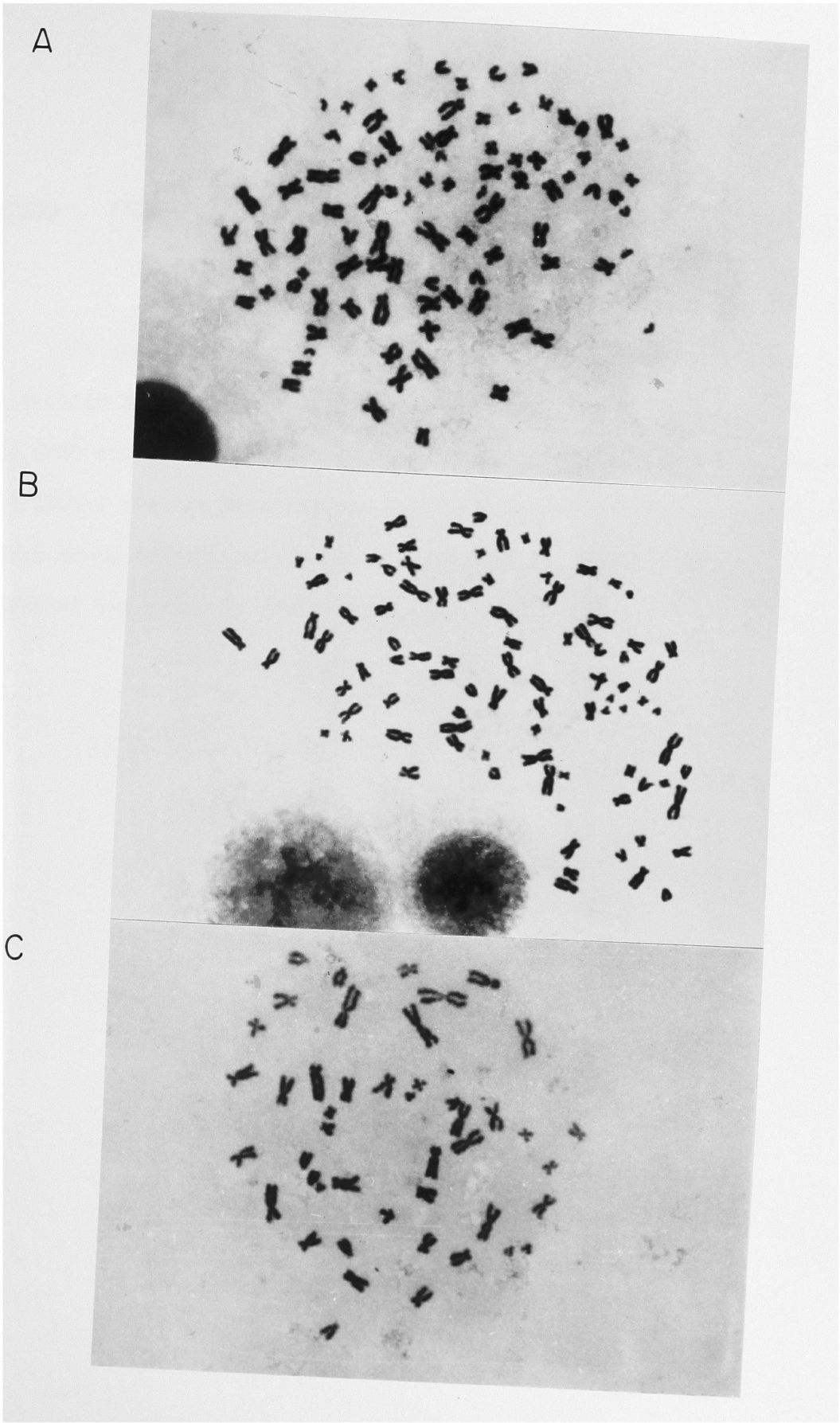
The total numbers of spreads counted were:

8866	71
34c1.2	62
4b1.1	37
d17	36
23c1.2	38
36c4.2	38


A.
Stock culture 8866
40
30
20
10
B.
Clone 34c1 2
20
10
Clone 4b1.1
20
10
Clone d17
30
20
10
C.
Clone 23c1 2
20
10
Clone 36c4 2
20
10
Number of cells
30
40
50
60
70
80
90
100
110
120
180
Chromosome number

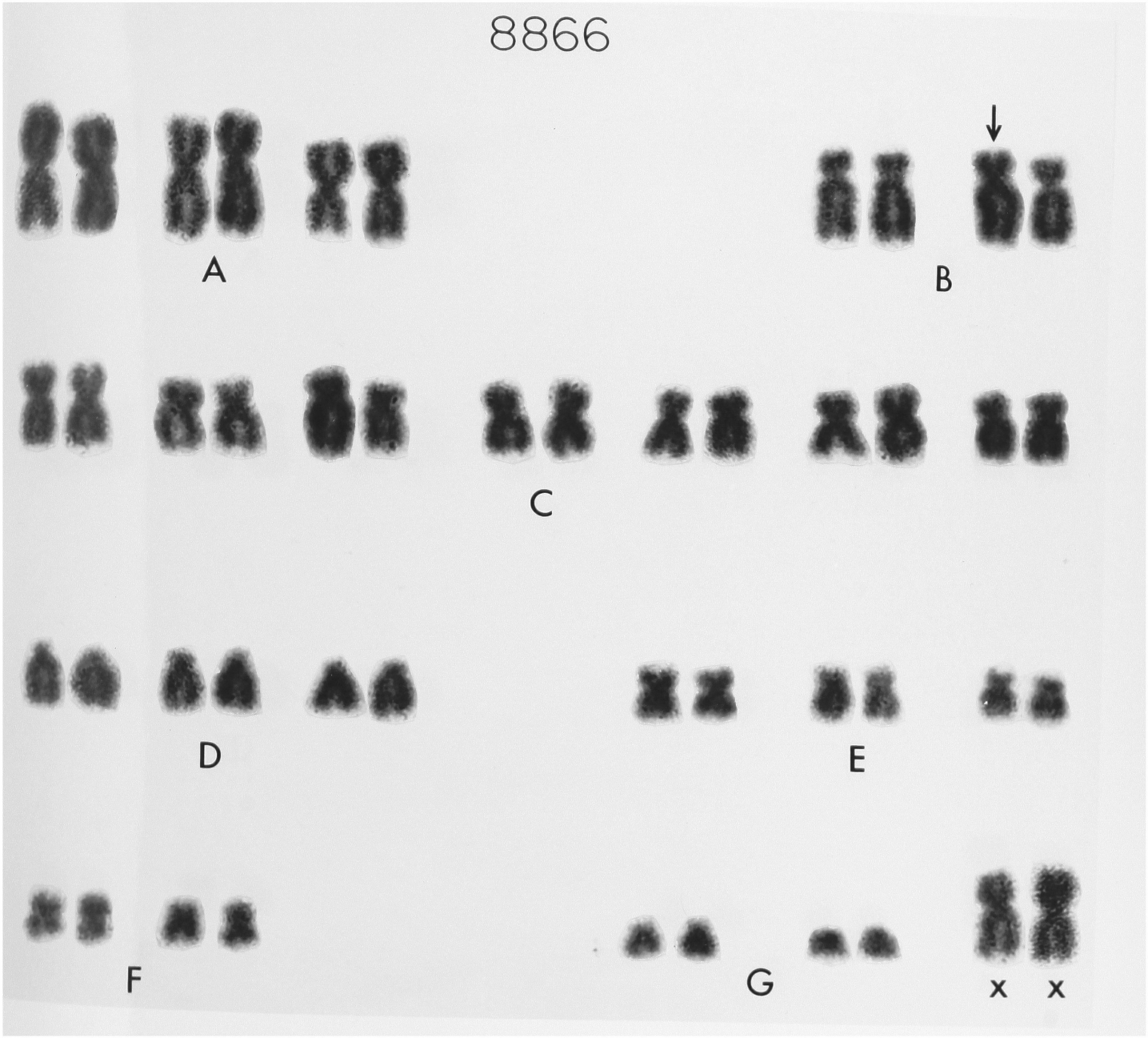
LEGEND: FIGURE III-9. REPRESENTATIVE CHROMOSOME SPREADS.

In order to illustrate the absence of gross abnormalities in the chromosomes, three spreads from different clones are presented. In A, the 92 chromosomes of clone 4b1.1, in B the 92 chromosomes of 36c4.2, and in C the 46 chromosomes of 23c1.2 are presented. Magnification has been varied in order to present prints of uniform size, rather than maintaining uniform size of chromosomes.

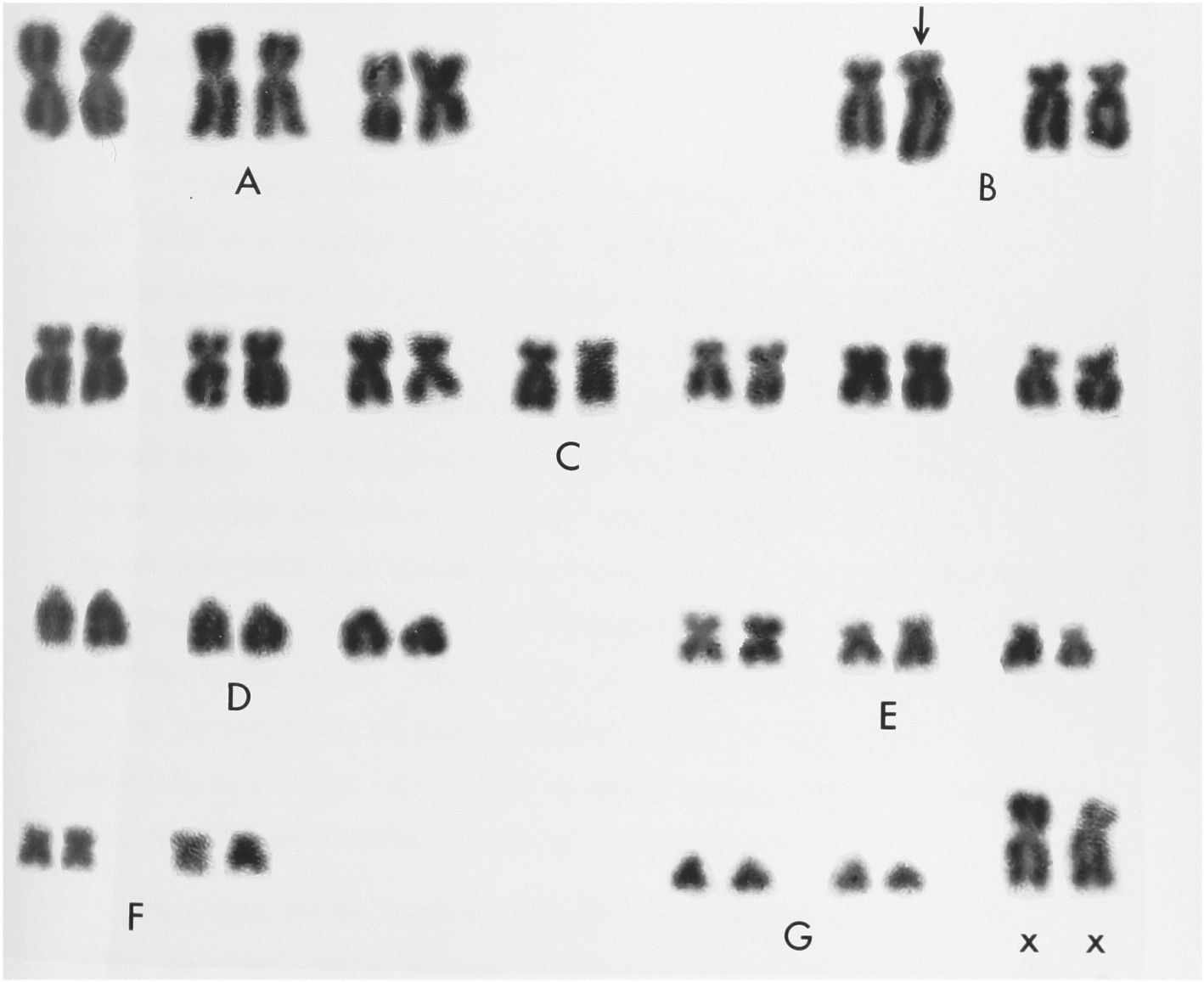

A
B
C

LEGEND: FIGURES III-10 and III-11. FORMAL KARYOTYPES OF TWO CELL PREPARATIONS.

Well spread cells, with no overlapping of chromosomes, and no distortion of the arms, were chosen for formal karyotyping. Both the 8866 stock culture cell and the clone d17 cell had 46 chromosomes. The Giemsa stained preparations are laid out in the standard form. In the B group of both karyotypes one chromosome larger than the other three of the group is indicated by an arrow.


8866
A
B
C
D
E
F
G
X X

d 17



E. Transfer of Receptors from Positive to Negative Cells.

It was considered possible that there might be transfer of receptor from positive to negative cells by either direct cell-to-cell contact, or via membrane fragments, or soluble material in the medium. These possibilities were tested by two kinds of experiments.

1. Mixing Experiments.

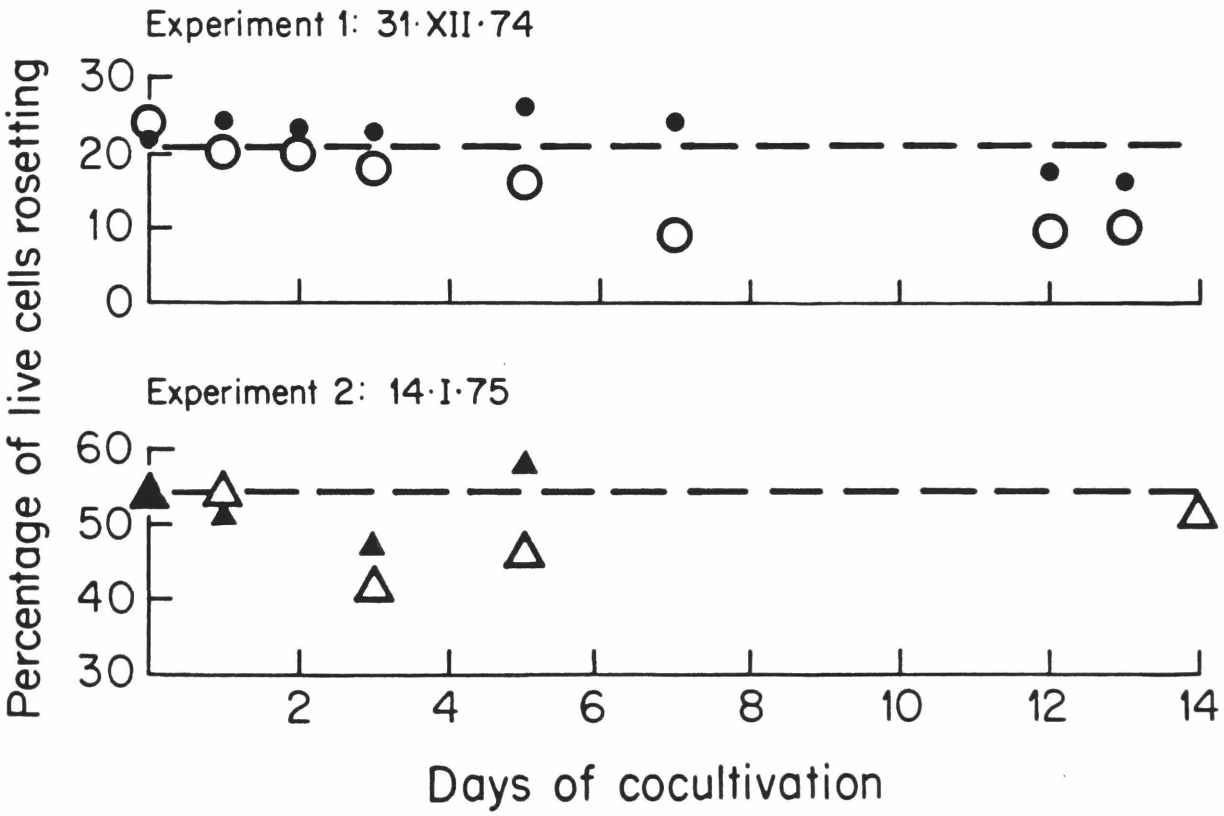
In these experiments, complement-receptor positive and negative cell lines were mixed to give a predicted percentage of positive cells, and the cells were then grown as a mixture, and tested at intervals. A relatively low percentage of positive cells was chosen in the first experiment in order to facilitate detection of small increases in phenotypically altered cells. In the second experiment, to increase the possibility of change, a higher percentage of positive cells was added. The results of the two experiments are presented as Figure III-12. The points represent the changes in cultures of entirely independent mixtures of clones, as explained in the legend. Over 14 days in culture there is no significant rise in the percentage of positive cells; indeed, in Experiment 1 there appears to be a slight fall. Based on these results, there is no evidence positive cells can transfer positivity to negative cells.

The slight shifts in these short-term experiments, as well as the larger phenotypic shifts observed in the mixed populations might be due to differential growth rates of positive and negative cells. To test this, the following experiment was done. Cells of different clones in the logarithmic phase of growth were adjusted to the same cell concentration.

LEGEND: FIGURE III-12. MIXING OF POSITIVE AND NEGATIVE CLONES.

The figure depicts two independent experiments each with independent cell mixtures. In Experiment 1, cell mixtures were made to give 21% positive cells. The open circles represent a mixture of clones 34 and 23, while the closed circles represent clones 4 and 36. In Experiment 2, the open triangles represent a mixture of clones 34 and 36 calculated to give 52% rosetting, while the open triangles represent clones 4 and 23 mixed to give a calculated 49% rosette-positive cells. The closed triangle culture was discontinued after day 5.

Mixing of positive and negative clones



By daily removal of medium and cells, and replacement with fresh medium, the cell concentration was brought back to the starting concentration. From the cell counts, growth curves for positive and negative clones were constructed. The rate of increase in cell number proved to be linear over 5 days. The mixed culture had a doubling time of 0.7 days. The positive clones 34 cl.2 and 4bl.1 had doubling times of 1.0 and 0.9 days, while the negative lines 23cl.2 and 36c4.2 doubled in 0.8 and 1.0 days respectively. Within the error of the method, no differences could be detected.

2. Addition of Cell Growth Medium to Rosetting Mixtures.

Two complete experiments of this type were done. Cells were allowed to grow to confluence, and then low speed centrifugation used to pellet out whole cells. E.IgM.C sufficient to rosette 100 microliters of lymphoid cells of a positive clone (i.e. 10^5 lymphoid cells) were mixed with supernatant from 15 mls of cells at approximately 10^6 lymphoid cells per ml, and incubated for 30 minutes at 37°C. The target cells were then pelleted and added to complement-receptor bearing lymphocytes. Rosetting proceeded normally, whether the medium tested was from positive or negative clones. Similarly, rosetting was performed on positive cells in their growth medium, or in fresh medium, and no difference noted.

On several occasions cells were grown in medium made up of 50% conditioned medium taken from logarithmically growing cultures, and 50% fresh

medium. The conditioned medium was usually from positive clones. No effect on the rosettability of the cells grown was noted.

These experiments show that the presence of either type of cell, or the supernatant medium from either type of cell, is not able to alter the rosettability of positive or negative cells.

F. The Phenotypic Instability of Complement Receptor Expression.

Having observed the phenomenon of phenotypic instability occurring spontaneously in culture, the question arose as to whether some compounds might be found which would modulate the expression of the C3 receptor on the cultured cells. The series of compounds tested were chosen because of their effects on tissue culture model systems which might be analogous with the one under study.

Dimethylsulfoxide is an inducer of hemoglobin synthesis in the Friend erythroleukemia system (Friend et al., 1971) and of beta-2-microglobulin production in some suppressed variants of human lymphoid lines (Pious, 1976). Sodium butyrate has been found to affect growth rate, spreading, enzyme content and morphological differentiation of a variety of tissue culture systems (see review of Prasad and Sinha, 1976). Bromodeoxyuridine has been shown to activate the synthesis of antigens of the Epstein-Barr Virus in lymphoblastoid cells (Hampar et al., 1972; Gerber, 1972), and to have effects on the tumorigenicity of the melanoma cell line system of Silagi (Christman et al., 1975). Prednisolone has been used by

Lotem and Sachs (1974,1976) to induce differentiation in a mouse myeloid leukemia model. A similar hormone, hydrocortisone, has been found to affect the expression of the Epstein-Barr Virus, and interferon production by human lymphoid cell lines (Joncas and others, 1973).

With this background, all of these compounds were tested in the dose range and the time scale found effective in other systems.

The results of the experiments are depicted in Tables III-6, III-7, and III-8. As can be seen, no compound was effective in altering the expression of the receptor except insofar as they affected cell viability. The protocols of the individual experiments are explained in the legends.

Not depicted in tables are the results using 2-deoxyglucose. Two experiments were done with this agent. In the first the 2-deoxyglucose was made up to 10^{-3}M in Dulbecco's medium containing 2.5 x 10^{-2}M glucose, and clone 34 (receptor positive) cells grown in this medium for seven days. No alteration in receptor expression was found. In the second experiment, the alpha modification of Modified Eagle's Medium was used. This has a glucose level of 5.5 x 10^{-3}M. Various doses of 2-deoxyglucose up to 5 x 10^{-2}M were used. After three days in culture, growth of the clone 34 cells had ceased, and viability was compromised, but the remaining cells rosetted normally. Thus, no specific effect was noted.

LEGEND: TABLE III-6. DIMETHYLSULFOXIDE AS AN INDUCING OR REPRESSING AGENT.

Tabulation of serial testing of cultures either with treatment with 5% DMSO in medium at room temperature for 30 minutes, or controls kept in medium alone for 30 minutes at room temperature. Growth was observed in both cultures, and viabilities remained comparable.

TABLE III- 6. DMSO AS AN INDUCING OR REPRESSING AGENT

CELL LINE	DMSO 5%	% ROSETTED CELLS DAY 1	DAY 3	DAY 5	DAY 7	DAY 9
8866	+	29	44	42.5	44	45.5
	—		33	62.5	43	39.5
34	+	87	93	97.5	95.5	91
	—		93	100	98.5	94.5
23	+	3	3	2.5	4.5	2.5
	—		2	10	3	4.5
23c1	+	0	0	0	0	0.5
	—		0	0	0	0
36	+	24	27	46	27	35
	—		22.5	46	37	37.5
36c2	+	0	0	0	0	0
	—		0	0	0	0

LEGEND: TABLE III-7 SODIUM BUTYRATE AND DIMETHYLSULFOXIDE AS INDUCING OR REPRESSING AGENTS.

The data depict the effect on rosetting of culture of two clones of 8866 cells in the continuous presence of the pharmacological agent.

TABLE III-7. BUTYRATE AND DMSO AS INDUCING OR REPRESSING AGENTS

CELL LINE	DRUG		% LIVE CELLS ROSETTED				
	Butyrate	DMSO	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
4b1.1	Nil		100	97	∿ 100	∿ 100	∿ 100
	$5x10^{-4}M$		100	99	∿ 100	∿ 100	∿ 100
	$2x10^{-3}M$		100	98	∿ 100	∿ 100	∿ 100
	$5x10^{-3}M$		100	100	∿ 100 *	∿ 100 *	+ *
		3%	100	100	∿ 100 *	∿ 100 *	+ *
23c1.2	Nil		0	0	0	1%	0
	$5x10^{-4}M$		0	0	0	0	0
	$2x10^{-3}M$		0	0	0	0	0 *
	$5x10^{-3}M$		0	0	0 *	0 *	- *
		3%	0	0	0 *	0 *	- *

+ = The few viable cells seen rosetted.

* = These samples contained few viable cells.

LEGEND: TABLE III-8. PREDNISOLONE AND BROMODEOXYURIDINE AS INDUCING OR REPRESSING AGENTS.

The results show the serial testing for rosetting of cell grown in the continuous presence of the agents at the concentrations shown, and fed daily when cell growth and acidification of the medium required it. In the higher levels of prednisolone, very little growth occurred, and dead cells increased as the experiment continued.

TABLE III- 8. PREDNISOLONE AND BrdU AS INDUCING OR REPRESSING AGENTS

CELL LINE	DRUG: Prednisolone	DRUG: Bromodeoxyuridine	% LIVE CELLS ROSETTED: DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
34c1.2	NIL		∿ 100	∿ 100	100	100	100
	0.1µg/ml	-	∿ 100	100	100	100	100
	1 µg/ml	-	∿ 100	100	100 *	100 *	100 *
	10 µg/ml	-	∿ 100	100	100 *	100 *	100 *
		0.1µg/ml	∿ 100	100	100	100	100
		1 µg/ml	∿ 100	100	100	100	100
		10 µg/ml	∿ 100	100	100	100	100
23c1.2	NIL		1	2	1	2	2
	0.1µg/ml		0	0.5	1	0	1
	1 µg/ml		1	0	0.5	0.5 *	1.5 *
	10µg/ml		0.5	1.5	0.5	1 *	0 *
		0.1µg/ml	0.5	2.5	4.5	2	4
		1 µg/ml	0	1	1	2.5	2
		10µg/ml	0	2	2	1.5	3

* Decreased viability noted.

CHAPTER IV

PROPERTIES OF THE LIGAND-RECEPTOR INTERACTION

A. Introduction

In some model systems, as in pure, isolated chemical systems, direct measurements can be made of the rate constants of binding and dissociation, and of the number of sites involved in the interactions. In the present system, this is not the case and the only assay used in this study is one involving the interaction between a large particle bearing multiple ligands (C3b and C3d) with a living cell bearing multiple receptors.

Most of the experiments to be presented were performed on cloned cells. In many cases, similar work has been done on the mixed (parent) population, and qualitatively similar results obtained. Thus it appears that the cloned cell lines are representative of the stock culture.

B. Results

1. Ligand Specificity of the Receptor.

In the absence of a series of purified components on which binding can be measured, the ligand responsible for binding has been narrowed down by a series of experiments using the classical techniques of complement studies.

Most of the work was done with particles coated with whole mouse serum as a source of complement, and consistent findings were as follows:

a. E, E.IgG, E.IgM, and zymosan particles showed no binding at all to any 8866 cells.

b. Serum pretreated at 56°C for 30 minutes does not confer binding capacity when subsequently incubated at 37°C with E.IgG or E.IgM, but fresh unheated mouse serum does. Aged serum stored more than two months at -70°C also showed a diminution if not abolition of the ability to confer binding capacity. Thus, there is a heat labile component, which is also unstable on storage. This is consistent with a complement component, and unlike any antibody.

c. When AKR mouse serum, known to be deficient in C5, and thus defective in activating the late components (C6-9), is used to prepare E.IgM.C, rosetting proceeds normally. Thus C5 and later components do not act as ligands in the system.

d. Zymosan activates complement largely if not wholly by the alternative pathway. Incubation of zymosan particles in fresh, but not heat inactivated serum, produces particles capable of binding to C3 receptor bearing cells. Prior adsorption of human serum in the cold with zymosan particles did not remove the ability of the serum to sensitize zymosan when subsequently incubated at 37°C. Thus, the component of relevance is involved in both the alternative and the classical pathway. It therefore cannot be C1,2 or 4. Taken with the AKR serum result, only C3 is left as the responsible component.

e. To further define the system, experiments were done with purified human complement components. The results of two sets of experiments are shown in Tables IV-1 and IV-2. E.IgM.C14 were prepared with

LEGEND: TABLE IV-1. COMPARISON OF E.IgM.C14$_{Hu}$ AND E.IgM.C1423$_{Mo}$ AS TARGET CELLS.

The E.IgM.C14$_{Hu}$ were made with purified components of human complement by Dr. Gordon Ross, and tested for immune adherence by him, by the methods described in Ross and Polley (1975). The E.IgM.C1423$_{Mo}$ were made with NCS mouse serum as described in Methods. Target cells were mixed at a ratio of 200:1 with lymphoid cells, and incubated at 37°C for one hour. Results are given by the criteria described in Methods. All the test cells except 8866, the stock culture, were cloned derivatives made up of cells 100% of which rosette with the standard erythrocytes sensitized with rabbit IgM and mouse serum.

TABLE IV-1.

COMPARISON OF E.IgM.C14$_{Hu}$ AND E.IgM.C1423$_{Mo}$ AS TARGET CELLS

	TARGET CELLS					
	E.IgM.C14$_{Hu}$					E.IgM.C1423$_{Mo}$
μg of C4/10^9 E.IgM.C1	3.4	48	96	192	384	
E.IgM.C14 bound/100 RBC$_{Hu}$ (i.e. Immune Adherence)	0	36	115	95	119	
TEST CELLS:						
8866	0*	0	1	0	0	65
34c1.2	0	0	4.5	10	5	96
4b1.1	0	0	0	0	2.5	95
d17	0	0	1.5	2	2.5	91
d30	0	0	12.5	7	13.5	93

* = Percentage of live cells rosetting.

LEGEND: TABLE IV-2. COMPARISON OF HUMAN C3b AND C3d AS LIGANDS.

The target cells were made from purified human components by Dr. Celso Bianco according to the methods he described (1976), and the immune adherence assay was also performed by him, and scored semi-quantitatively. The rosetting was performed by the usual methods.

TABLE IV-2.

COMPARISON OF HUMAN C3b and C3d AS LIGANDS

BINDING CELL	TARGET CELLS			
	E.IgM	E.IgM.C14	E.IgM.C1423b$_{Hu}$	E.IgM.C1423d$_{Hu}$
HUMAN RBC	0	+	+++	0
34c1.2	0*	0	78	90
4b1.1	0	0	12.5	90.5

* = Percentage of live cells rosetting.

increasing amounts of C4, and assessed for Immune Adherence on human erythrocytes, by Dr. Gordon Ross. A clear increase in binding with increasing C4 is seen. The E.IgM.C14 were then tested in our laboratory on the parent cell line and various clones of 8866. The rosetting results are seen in the second section of the table and compared with results of rosetting with E.IgM.C made with fresh whole mouse serum. With E.IgM.C14, the maximum percentage of cells rosetting was 3-5%. Although some binding was seen with E.IgM.C14 and met the definition of a rosette, namely four or more erythrocytes per lymphocyte, in all cases the rosettes were weak, never being more than 10 erythrocytes/lymphocyte. In contrast, the mouse serum E.IgM.C rosettes on the positive clones gave intense rosettes, and it was not possible to score the cell ratio. Thus C4 plays little role in the binding of serum-sensitized particles.

C2 was not specifically tested in these experiments, but indirect evidence suggests it plays no role in rosetting. Firstly, it is not activated in the alternative pathway, and therefore would not lead to rosetting of zymosan particles. Secondly, E.IgM.C remain stable as rosetting target cells for at least one week. This is incompatible with the known instability of C2.

As described earlier, not only has the intact C3 molecule been found to bind to some lymphocytes, but some lymphocytes show specificity for the cleaved fragments, C3b or C3d. Therefore, in order to test for the specificity of the third component of complement, the various forms must be

prepared and tested. In Table IV-1 a set of reagents prepared by Dr. Celso Bianco from purified components was tested on two positive clones. The E.IgM and E.IgM.C14 (with small amounts of C4) showed no binding, confirming the previous result. Both the C3b and C3d forms of C3 resulted in binding, although the C3b was strikingly less efficient on Clone 4b1.1 than was the C3d form, while the two reagents were comparable when tested on Clone 34c1.2. It is concluded that the major ligand is the C3d bound to sensitized erythrocytes.

f. To confirm this result, blocking studies were performed. These were done with whole mouse serum as a source of C3, and using cobra venom factor to generate the cleavage products. Heat inactivated and fresh serum are both known to contain high levels of uncleaved C3. In the course of heat inactivation the C3 proactivator, the substrate for the cobra venom factor, is destroyed, and thus heat inactivated serum treated with cobra venom factor should also contain C3 in the uncleaved state. Only fresh serum after cobra venom factor treatment had any inhibiting effect on rosetting. When blocking was carried out with the highest concentration used, approximately 1:3 dilution of serum, the rosetting was reduced from 90% to 49% of lymphocytes; additionally there was a marked reduction in the number of target cells bound per lymphoid cell. The effect was detected out to the third 1:2 dilution. This experiment showed that the blocking agent was generated by the cleavage of C3 by cobra venom factor, and was stable in solution after the action of the venom, since addition of EDTA prevented the

production of fresh cleavage products during the blocking test. It is therefore compatible with C3d.

In another experiment, heat inactivated serum was added to a final concentration of 40%, and no inhibition of rosetting was seen.

g. The data thus far presented are in accord with a C3d receptor as defined by Ross and colleagues (1973b). As further confirmation, antisera raised in primates against the two receptor types were kindly provided by Dr. Gordon Ross. They were tested on Clone 34, a positive clone. An antiserum raised against human erythrocytes, and hence the C3b (immune adherence) receptor, had no blocking effect. An anti-human spleen serum with anti-C3d receptor activity, showed distinct inhibition to a titer between 1:20 and 1:40. Repeated intravenous injection of cells from rosette-positive clones into rabbits in this laboratory led to the production of one serum with pronounced blocking activity at the same titer as the Ross antiserum: this activity could not be adsorbed by human erythrocytes, used as a source of C3b receptors. Pre-immunization serum and sera from other rabbits similarly immunized and showing cytotoxic activity, did not show blocking activity. Thus, control serum and serum with anti-human cell activity did not contain non-specific blocking activity. The purified IgG of cytotoxic non-blocking and blocking were tested: only the IgG of blocking serum inhibited rosetting.

h. In the course of these studies, particles were made coated with mouse, guinea pig and human complement. All showed strong binding.

In summary, the receptor under investigation has specificity for the

cleavage product of the third component of complement, C3d.

2. Temperature Dependence of Rosetting.

Rosetting is sensitive to the temperature at which the reaction is performed. Lymphoid cells and the E.IgM.C were brought to the temperature of reaction, then mixed, and allowed to rosette. In repeated experiments the only difference from the results shown in Figure IV-I has been that the degree of rosetting at 0°C is usually less than 1%. To ensure the effect was on the reaction, and not to some independent effect on the lymphocyte or the E.IgM.C, the experiment shown in Table IV-4 was performed. Lymphocytes and E.IgM.C were pre-treated at different temperatures, and then mixed at either 37°C or 0°C for rosetting after being equilibrated at that temperature. Except for the fact that 56°C pretreatment lysed the E.IgM.C and killed the lymphocytes, it is seen that it is the temperature at the time of rosetting, and not pretreatment, which determines the degree of rosetting.

3. The Divalent Cation Independence of Rosetting.

The requirement for a divalent cation for rosetting was tested for and the results shown in Table IV-4. Using the same solutions as Nussenzweig (Lay and Nussenzweig, 1968), it was found that these cells show no requirement for either calcium or magnesium, and rosette equally well in buffer and buffer containing the chelator, EDTA.

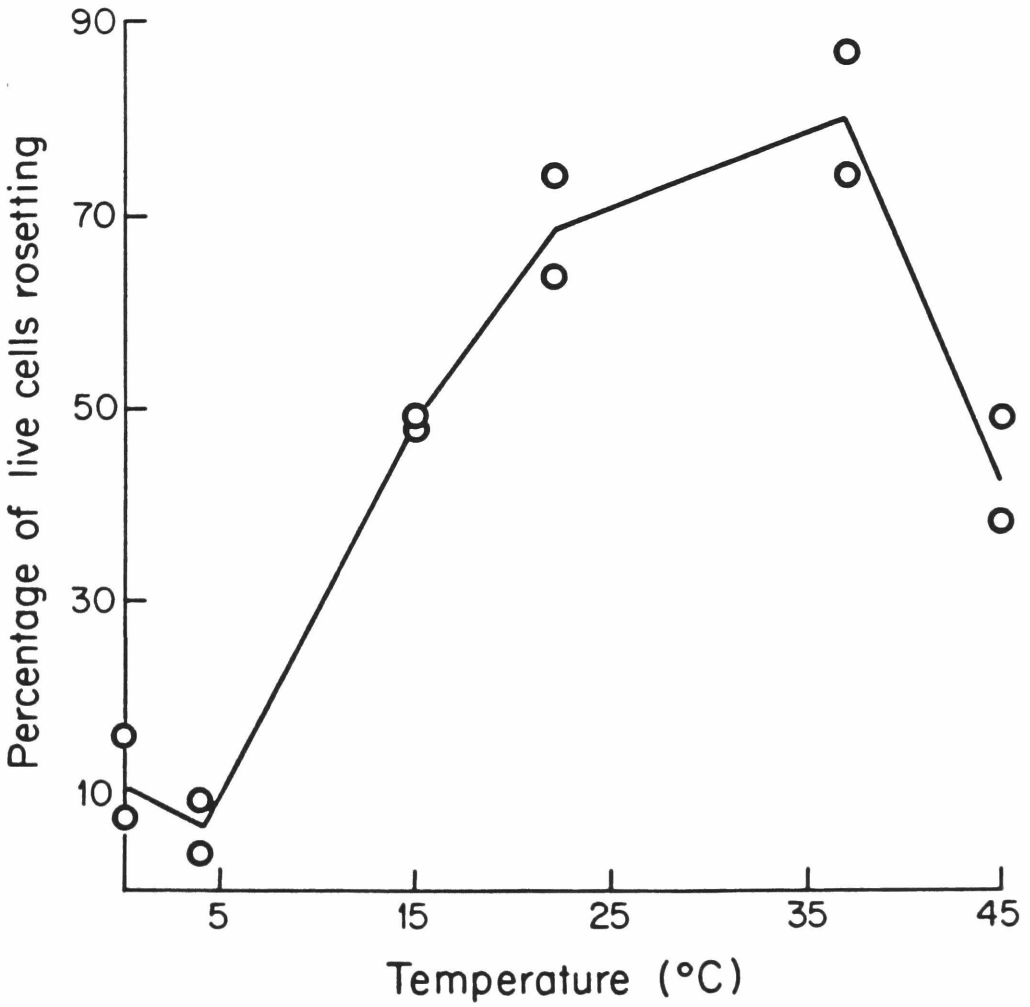
4. The Effect of Metabolic Inhibitors on Rosetting.

The mechanism of rosetting involves the interaction of ligands

LEGEND: FIGURE IV-1. THE TEMPERATURE DEPENDENCE OF ROSETTING.

E.IgM.C and Clone 34 cells were pre-equilibrated at the various temperatures, then mixed, and incubated at the same temperature, for 60 minutes. The tubes were then plunged into ice-water mixture, and scored for viability and rosetting.

Temperature dependence of rosetting



LEGEND: TABLE IV-3. TEMPERATURE EFFECTS ON CLONE 34, E.IgM.C AND ROSETTING.

The experiment was performed by pre-treating Clone 34 cells in Dulbecco's medium with 10% FCS, or E.IgM.C in Dulbecco's medium, at various temperatures for 1 hour, then rosetting at the indicated temperature after 15 minutes to bring the cells to either 0°C or 37°C. The rosetting time was 60 minutes.

TABLE IV-3.

TEMPERATURE EFFECTS ON CLONE 34, E.IgM.C, AND ROSETTING

Pretreatment of Lymphoid Cells	Pretreatment of E.IgM.C	Lymphoid Cell Viability (%)	% Live Cells Rosetting (37°C)	% Rosettes (0°C)
0°		75	100	
24° (room temp.)		74	100	
37°	0°	71	100	
45°		69	100	
56°		0	0	
	0°		100	
	37°		100	
37°	45°		100	
	56°		**	
	0°			1.5 #
	37°			3 #
0°	45°			2 #
	56°			**

* = All lymphoid cells dead. No rosetting seen.

** = All E.IgM.C lysed - ghosts seen under phase microscopy. No rosetting by ghosts was detected.

\# = These values were rechecked after 4 hours further rosetting. No change found.

LEGEND: TABLE IV-4. THE DIVALENT CATION INDEPENDENCE OF ROSETTING.

The experiment depicted follows that of Lay and Nussenzweig (1968). Cells were suspended in 0.12M NaCl, 0.005M KCl, and 0.025M Tris at pH 7.4. Calcium was added to 1mM, magnesium to 1mM and EDTA to 5mM where indicated. Rosetting was carried out for 45 minutes at 37°C. Viability fell to approximately 85% in the absence of any protein in the solution, but rosetting of viable cells was strong, as well as of 100% cells in those cases where rosetting is recorded as positive.

TABLE IV-4.

THE DIVALENT CATION INDEPENDENCE OF ROSETTING

LYMPHOID CELLS	TARGET CELLS	PERCENTAGE LIVE CELLS ROSETTED			
		BUFFER	BUFFER + Ca^{++}	BUFFER + Mg^{++}	BUFFER + EDTA
	E	0	0	0	0
Clone 34	E.IgM	0	0	0	0
	E.IgM.C	100	100	100	100
	E	0	0	0	0
Clone 4	E.IgM	0	0	0	0
	E.IgM.C	100	100	100	100

on one cell with receptors on another. Since it seems likely that more than one interaction must occur for stable binding, and since the reaction is inhibited in the cold, it seemed it might be comparable with the capping reaction (Taylor et al., 1971). It was therefore decided to examine the energy requirement of the lymphoid cells during rosetting; since zymosan particles, the carbohydrate ghosts of yeast cells, can rosette, there was clearly no requirement for active processes by the target cell. The experiments involved the pre-incubation of lymphoid cells in a metabolic inhibitor for 30 minutes, then incubation for an additional hour in the presence of inhibitor and E.IgM.C. The results of a typical experiment are shown on Table IV-5. It can be seen that most of the cells remained viable through the procedure, and that most of the live cells rosetted. Virtually all dead cells showed no bound E.IgM.C. Thus, 2-deoxyglucose, sodium cyanide, sodium fluoride, and a combination of the latter two inhibitors had no effect on rosetting. In another experiment sodium azide was tested at doses from 10^{-6} to 10^{-2}M, and a slight depression of rosetting was noted only at the highest concentration.

5. The Effects of Colchicine and Cytochalasin B on Rosetting.

Since there was no evidence of energy dependence, it was of interest to see if agents which affect the motility of cells would have any effect on the rosetting phenomenon. Therefore, cells were pre-incubated for one hour in colchicine or cytochalasin B at doses ranging from 10^{-3} to 10^{-9}M, with a control of DMSO used at the same dilution as the highest dose necessary to dissolve the cytochalasin B. The cells were then mixed with pelleted

LEGEND: TABLE IV-5. THE EFFECT OF METABOLIC INHIBITORS ON ROSETTING.

Clone 34 cells were used. They were pelleted and resuspended in Dulbecco's medium with 10% FCS plus inhibitor at the indicated concentration, so that the cells were at a concentration of 1×10^6/ml. The mixture was incubated at 37°C for 30 minutes, then E.IgM.C were added and the incubation continued a further hour in the presence of the inhibitor.

TABLE IV-5.

THE EFFECT OF METABOLIC INHIBITORS ON ROSETTING

INHIBITORS	CONCENTRATION	CELL VIABILITY (%)	% LIVE CELLS ROSETTING
None	0	86*	91*
		87	95
2-Deoxyglucose	5×10^{-2}M	82	95
		85	91
Sodium Cyanide	10^{-2}M	75	89
		88	91
Sodium Cyanide	10^{-2}M		
+		77	81
Sodium Fluoride	10^{-2}M	81	90

* = Determinations on independent replicates. The viability corresponds with the rosetting value on the same horizontal line.

E.IgM.C and incubated a further hour, still in the presence of the drug. No inhibition of rosetting was observed at any drug dose.

6. The Effect of Immobilization of the Lymphoid Cell Membrane On Rosetting

To investigate the possibility that mobility of some of the surface molecules might be necessary for the formation of rosettes, two kinds of experiments were done. The first kind consisted of various fixation regimens. Many variations of time, temperature, and fixative concentration were tried, but in all cases it was found that inhibition of rosetting was in proportion to cell killing as assessed by Trypan Blue permeability. The results of a typical experiment are presented in Table IV-6, with the protocol presented in the legend.

The second kind of experiment was to make cell spreads on glass microscope slides, mimicking the conditions used for frozen sections. Air drying alone, or air-drying followed by acetone or alcohol fixation, or smears directly fixed in acetone or alcohol were tried. No protocol gave rosettable preparations.

Thus, it has not proved possible to immobilize the cell membrane and retain the ability to rosette.

7. The Effect of Reagents Reacing with Sulfhydryl Groups on Rosetting

Dierich and colleagues (1974) suggested a specific role for compounds reacting with sulfhydryl groups in inhibiting the C3 receptor. The effects of dithiothreitol, N-ethyl maleimide, iodoacetamide, and a combination of the

LEGEND: TABLE IV-6. THE EFFECT OF FIXATION OF LYMPHOID CELLS ON ROSETTING.

Cells of Clone 34 were washed three times in Hanks' Balanced Salt Solution, then fixed in formaldehyde at the concentrations shown made by diluting a stock solution of 10% in 0.1M Cacodylate buffer, pH 7.4, in the same buffer. The cells were in fixative at ice-water mixture temperature for 15 minutes, with mixing each 5 minutes, then washed in Hanks' Buffered Salt Solution, treated with 0.1M Glycine/0.05M Tris- HCl pH 7.4 for 15 minutes at 0°C, then washed twice with Hanks' Buffered Salt Solution twice before testing for rosetting.

TABLE IV-6.

THE EFFECT OF FIXATION OF LYMPHOID CELLS ON ROSETTING

FIXATIVE	VIABILITY (%)	ROSETTED LIVE CELLS (%)	ROSETTED DEAD CELLS (%)
Formaldehyde 1%	0	0	0
Formaldehyde 0.5%	22	85	7
Formaldehyde 0.25%	45	89	12
Formaldehyde 0.1%	31	97	10
Formaldehyde 0.1% / Glutaraldehyde 0.1%	55	81	12

LEGEND: TABLE IV-7. THE EFFECT OF SOME REAGENTS REACTING WITH SULFHYDRYL GROUPS ON ROSETTING.

Cells of Clone 34 were made to 2 x 10^6/ml in Hanks' Balanced Salt Solution after two washes to remove protein. The inhibitors at twice the indicated strength in HBSS were then mixed with an equal volume of cells, and incubated at 37°C for 15 minutes. The samples were placed in an ice-water bath, and in the relevant sample iodoacetamide sufficient to alkylate the DTT was added, and incubated in the ice temperature for 15 minutes. The mixtures were diluted with Dulbecco's medium, the cells pelleted, washed once with Dulbecco's medium, then resuspended in medium and mixed with E.IgM.C. The rosetting was carried out for 1 hour at 37°C, after which viability and rosettability were assessed simultaneously.

TABLE IV-7.

THE EFFECT OF SOME REAGENTS REACTING WITH SULFHYDRYL GROUPS ON ROSETTING

REAGENT	CONCENTRATION (mM)	VIABILITY (%)	ROSETTES AS % OF LIVE CELLS
Control		90	82
		89	82
DTT	100	67	80
	50	73	70
	10	81	81
	1	85	72
NEM	100	0	0
	50	2	0
	10	1	0
	1	0	0
IAA	100	11	0
	50	10	0
	10	53	20
	1	88	67
DTT then IAA 0°C	10	76	64
	1	89	91
Incubate then IAA 0°C	200	15	7
	100	45	10
	20	90	62
	2	91	70

reducing action of dithiothreitol followed by alkylation with iodoacetamide were therefore examined for their effects on 8866 cells. The results are shown in Table IV-7. DTT had no significant effect. N-ethylmaleimide was toxic even at 1mM. Iodacetamide appeared to have no effect separable from its toxicity, and the DTT followed by iodacetamide was similarly non-selective. From these findings it seems likely that the reported capacity of sulfhydryl reagents to inhibit rosetting merely reflects their ability to kill cells, since measurement of rosetting without simultaneously measuring Trypan Blue exclusion would lead to an erroneous impression of blocking.

8. The Nature of the Receptor.

Previous workers had demonstrated that the receptor for complement was susceptible to the action of trypsin. The action of a variety of proteases was therefore tried with the possibility of finding a selective protease which might act on other surface proteins but leave the complement receptor intact. The results of testing trypsin, chymotrypsin, papain and pronase are shown in Figure IV-2. At a range of doses, it can be seen that the receptor is inactivated by all of these enzymes. The specificity of the trypsin has been shown by the use of TPCK-trypsin, and showing inhibition by the presence of Soybean trypsin inhibitor at stoichiometric concentration. The papain was inhibited by alkylation, or failure to activate with a sulfhydryl reagent.

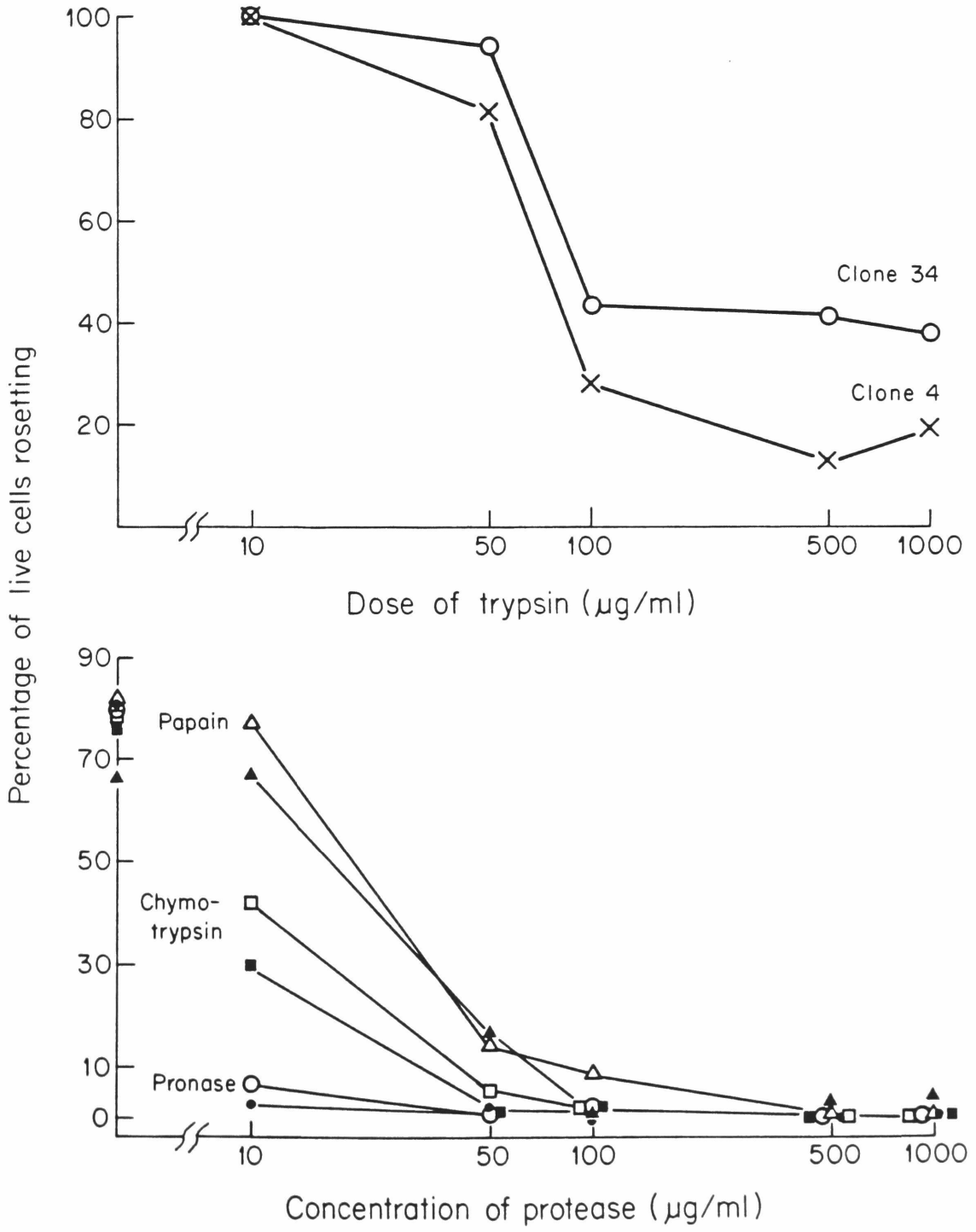
To determine that the receptor activity was dependent on some intrinsic protein of the cell, and not merely on adsorbed material, experiments were

LEGEND: FIGURE IV-2. THE EFFECT OF PROTEASES ON ROSETTING OF POSITIVE CLONES OF 8866.

Cells of Clones 34 and 4 were washed twice in Hanks' Balanced Salt Solution and then incubated in the same solution with the cells at a concentration of 10^6/ml, and trypsin at the concentration indicated for 15 minutes at 37°C. Further proteolysis was stopped by adding 3 volumes of cold Dulbecco's medium containing 20% fetal calf serum. The cells were pelleted, resuspended in Dulbecco's medium containing 5% fetal calf serum, and incubated at 37°C for one hour with samples of E, E.IgM, or E.IgM.C. The E and E.IgM showed no rosetting with any of the cells. Viabilities remained greater than 90%. All studies were carried out in siliconized glass tubes.

In the second experiment, the cells were at a concentration of 0.5×10^6 per milliliter in Hanks' Balanced Salt Solution. Otherwise, the protocol was exactly as in the trypsin experiment. Viabilities and recoveries remained high in all tubes. For each pair of symbols representing a protease, the open symbol represents Clone 34 cells, while the closed symbol represents Clone 4 cells.

The effect of proteases on rosetting of positive clones



done on the ability of the cells to recover rosetting following protease treatment. One experiment is depicted in Figure IV-3. Cells of Clone 4b1.1 (100% receptor positive) were treated with trypsin until the percentage of cells rosetting was 20% of control. Cells were then washed, resuspended in medium containing 10% fetal calf serum, and incubated at 37°C. It was found that 50% recovery (i.e. to 60% rosetting cells) took 7-8 hours. In other experiments in which trypsinized cells were cultivated, a similar time-course of recovery of receptor activity was found.

In a preliminary experiment it was found that 10 μg/ml of cycloheximide inhibited the uptake of radiolabelled leucine by greater than 90% after 1 hour at 37°C. Therefore, control

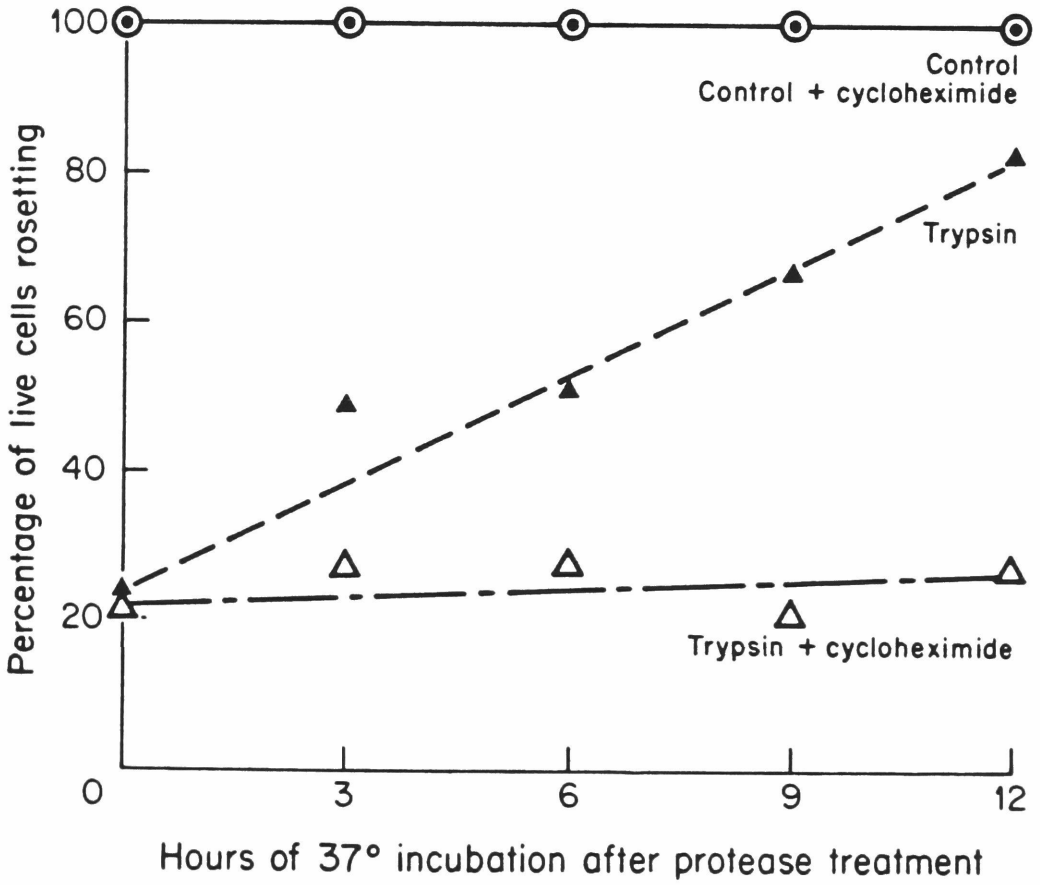
and trypsinized cells were cultured in the presence of this dose of the protein-synthesis inhibitor. The results are shown by the open symbols on Figure IV-3. There is total inhibition of recovery of the receptor over 12 hours, and no effect on the rosetting of cells not previously treated with protease.

These experiments showed that recovery of receptor activity was dependent on ongoing protein synthesis. It was still possible that the receptor activity was dependent on the binding of a protein contained in fetal bovine serum to a protein of the cell surface. To rule this out, an experiment was performed in which the cells of Clone 4b1.1 were treated with trypsin at 1 mg/ml for 15 minutes at 37°C, reducing the rosetting from 100% to 3%. The protease action was stopped by chilling and dilution in Dulbecco's medium containing 10% lactalbumin hydrolysate and 1% bovine serum albumin. The cells were washed, then resuspended in the same medium at 37°C and cultured. After 12 hours, the percentage of live cells rosetting was 47%.

LEGEND: FIGURE IV-3. THE EFFECT OF CULTIVATION OF ROSETTE-POSITIVE CELLS AFTER PROTEASE TREATMENT.

Clone 4b1.1 cells in logarithmic phase of growth were washed twice with HBSS, and divided into equal aliquots, calculated to contain 2×10^6 cells/ml. To one was added an equal volume of HBSS +0.5% FCS. To the other was added an equal volume of HBSS + 1 mg/ml trypsin (Millipored through 0.45 µl filter). Both mixtures were incubated 20 minutes at 37°C. The reaction was stopped by adding 1/4 volume of fetal calf serum, and spinning out the cells, which were then resuspended in Dulbecco's medium containing 10% fetal calf serum. These samples were divided again into two, pelleted, and one of the replicates resuspended in medium/FCS, while the other was resuspended in medium/FCS plus 10 µg/ml of cycloheximide. The mixtures were then dispensed as 1 ml aliquots into tubes, and placed in the water at the bottom of a 37°C, 5% carbon dioxide in air, incubator. Samples were taken for cell counts, viabilities, and rosetting at the times indicated. Viabilities and recoveries remained high in all samples, although from three hours onwards the cycloheximide cultures began to contain Trypan Blue-excluding granular refractile cells which did not rosette. The viabilities were all greater than 90%.

The effect of cultivation of clone 4b1·1 cells after protease treatment



When correction was made for the cell count and the lowered viability found after both trypsin treatment and culture in this less than optimal medium, it was found that the number of rosetting cells had increased ten-fold, showing that there was true regeneration of receptors, and that the effect was therefore totally serum independent.

Before leaving the discussion of proteases and the surface of 8866 cells, it should be noted that experiments were performed in which cells were examined before and after protease treatment for their ability to rosette with E.IgG; that is to say, the cells were examined for Fc receptor activity. None was found.

CHAPTER V

LACTOPEROXIDASE IODINATION OF 8866 CELLS

A. INTRODUCTION

Having isolated a series of clones with a distinctive membrane property, namely the presence or absence of a receptor for C3d, the next step was an attempt to use these clones to identify the molecular species responsible for the receptor activity. By definition, part or all of the receptor molecule(s) must be exposed on the external surface of receptor bearing cells. The capacity of proteases to destroy the C3 receptor activity, and the inhibitory effect of cycloheximide upon its regeneration following proteolysis suggest that the receptor is protein in nature. The finding that receptor negative clones spontaneously regain receptor activity suggested that these negative clones retained the genetic information encoding the C3d receptor. It seemed likely that these negative clones did not contain mutant structural genes, encoding non-functional receptor molecules, but rather, failed to express the receptor genes. For these reasons efforts were directed toward identifying one or more of the protein species present in clones bearing C3d receptors but absent from clones lacking these receptors.

A simple calculation indicates the difficulties of trying to identify these molecules in whole cell homogenates, or in isolated plasma membranes. 10^9 cells (one liter of cells at 10^6/ml) contains 100 mg of protein. Assuming that 1% of the total cell protein is membrane and that the receptor constitutes 1% of the membrane protein, then, at 100% efficiency of recovery, 100 mg of material would yield 10 μg of receptor. Labeling these molecules internally with radioactive amino acids seemed unlikely to enhance the ability to differentiate them from other cellular proteins.

However, methods which selectively label externally disposed membrane proteins seemed ideally suited for this purpose. The lactoperoxidase mediated radio-iodination method of Hubbard and Cohn (1972,1975a) provided a highly sensitive, gentle procedure which has been demonstrated to have this desired specificity. The labeled species can then be separated from one another by SDS-polyacrylamide gel electrophoresis. The basic method of Hubbard and Cohn required only minor modification for the study of the membranes of 8866 cell clones.

B. METHODOLOGY

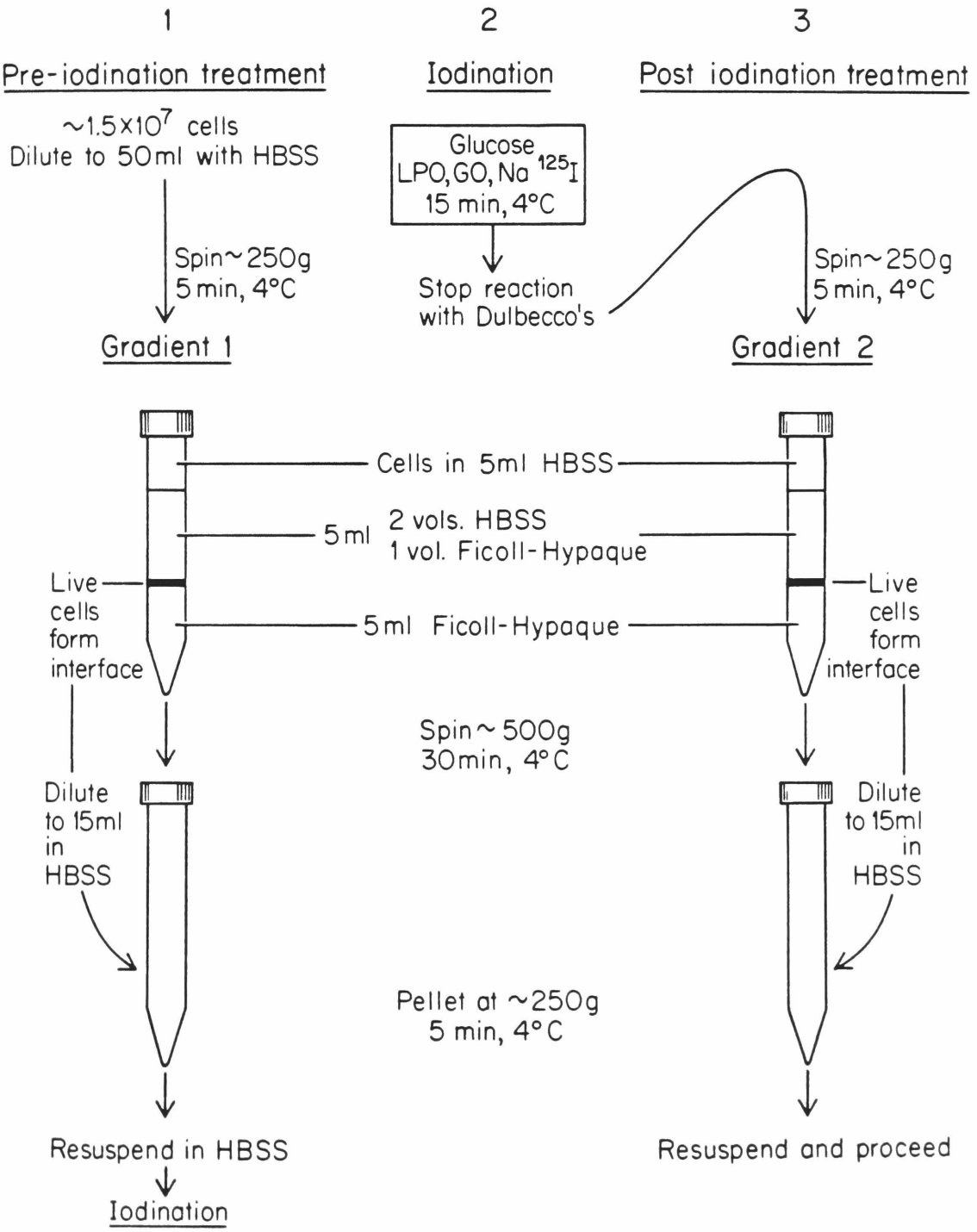
1. Iodination protocol.

A small percentage of non-viable cells are contained in each culture of 8866 cells. Examination of autoradiograms of epon embedded thick sections of 8866 cells labeled by the $Na^{125}I$/glucose oxidase/lactoperoxidase method of Hubbard and Cohn showed that a small percentage (approximately 5%) of the cells were very heavily labeled (10-100 fold as many grains) compared with the rest of the population. When examined as thin-section autoradiograms in the electron microscope, it was found that these cells showed diffuse grains throughout the cytoplasm and nucleus, and comprised a population of disrupted, non-viable cells. Therefore, the following method (illustrated in Figure V-1) was devised to remove dead cells both before and after radio-iodination in order to minimize contamination with labeled intracellular proteins. The method also replaces the multiple washings required by the Hubbard and Cohn procedure to remove loosely bound serum proteins, and proved to give higher post-washing viabilities than

LEGEND: FIGURE V-1 THE IODINATION OF 8866 LYMPHOBLASTOID CELLS

The Figure depicts a typical experimental protocol. The 1.5×10^7 cells were found to yield approximately 10^7 cells for the iodination step. From gradient 1, only the interface cells are transferred to the second tube, where the cells and the Ficoll-Hypaque carried over are diluted with HBSS: the same procedure is followed in the exactly parallel second, post-iodination gradient. The order of the reagents in the box for iodination is not the order of addition. As indicated in the text, the order was cells or diluent where applicable, glucose, lactoperoxidase, sodium 125-iodide, and then glucose oxidase, all being at the temperature of melting ice. The reaction is stopped by the addition of Dulbecco's medium.

Iodination of 8866 lymphoblastoid cells



repeated pelletings.

8866 cells in Dulbecco's medium containing 10% fetal calf serum were pelleted by centrifugation at 250g for 10 minutes at 4°C. The cells were resuspended in Hanks' Balanced Salt Solution (HBSS) at a concentration of approximately 5 x 10^6 cells/ml, and 5 mls of this suspension was layered over a step gradient containing an upper layer of 5 mls of 2 volumes of HBSS and 1 volume of a Ficoll-Hypaque mixture, and a lower layer of 5 ml of the same Ficoll-Hypaque mixture (density 1.077 g/cc). The Ficoll-Hypaque mixture is the one described by Böyum (1968). The gradients were centrifuged at 500g for 30 minutes at 4°C. The live cells band at the interface between the two Ficoll-Hypaque layers, while the dead cells pellet to the bottom of the tube. To recover the live cells, the overlying fluid was removed to just short of the interface, and the cells then aspirated into a pasteur pipette, resuspended in HBSS, pelleted by centrifugation at 250g for 10 minutes at 4°C, and then resuspended in HBSS at 2 x 10^7 cells/ml. The yield and viability of cells treated in this manner is shown in Table V-1A.

Following the washing step, the cell suspension was brought to a final concentration of 10^7/ml by the addition of glucose to a final concentration of 45mM, 10 mU of lactoperoxidase, 0.1 to 1mCi of carrier-free sodium ^{125}iodide, and 10 mU of glucose oxidase in that order. Sufficient HBSS was added to the cells before the addition of the glucose so that the final volume was one milliliter. In experiments with more cells, all constituents of the mixture were increased proportionately. The reaction was carried out in a horizontal tube rotating slowly about a horizontal axis

LEGEND: TABLE V-1. FICOLL-HYPAQUE GRADIENTS: REMOVAL OF DEAD CELLS

The two parts of the Table show experiments in which the Ficoll-Hypaque step gradient depicted in Figure V-1 was used to separate the cells of populations either from a growing culture, or after iodination.

In Part A, cells were treated as shown in an iodination except that the iodination step itself was omitted; instead, the cells were kept in buffer in the cold for the same length of time as it would take to add the constituents of the iodination mixture, and carry out the procedure. Both viabilities and cell recoveries are shown.

In Part B, the viabilities from a series of experimental iodinations are presented as representative of the method in routine use over the time of the studies presented in this thesis.

TABLE V-1.

FICOLL-HYPAQUE GRADIENTS: REMOVAL OF DEAD CELLS

PART A.

	Total Cells ($x10^{-6}$/ml)	Viable Cells ($x10^{-6}$/ml)	Viability (%)
Initial Aliquot	32.7	30.3	94
Post First Gradient	21.8	21.2	97
After 30' in HBSS at 4°C (Mock iodination)	20.2	19.0	94
Post Second Gradient	17.1	16.9	99

PART B.

	Percentage Viable Cells			
	Expt 1 24.v.75	Expt 2 17.vi.75	Expt 3 30.vi.75	Expt 4 28.iv.75
Initial cell population	91.4	84.4	93.8	95.5
After gradient purification, iodination, and the second gradient purification.	96	94	97.3	97.6

for 15 minutes at 4°C, and was terminated by the addition of 9 mls of cold Dulbecco's medium. This medium contains many low molecular weight iodinatable competing substrates (including tyrosine, histidine and phenol red), reducing agents (e.g. cystine), and also dilutes the reactants. Following iodination, the cells were pelleted, resuspended in HBSS, and submitted to the same Ficoll-Hypaque gradient protocol as before. The cells at the interface were collected, washed, and studied further. The amount of ^{125}I incorporated into macromolecular products was assayed as described in Methods. The incorporation into acid-precipitable counts varied between 1 and 12% of the label added, and it was found that increasing the amount of radio-iodine increased both the percentage of label incorporated, and the absolute amount of radio-iodine incorporated. Omission of lactoperoxidase from the reaction mixture reduced the amount of label incorporated to less than 1% of the amount found in the presence of the enzyme.

The effect of the entire iodination procedure on cell viability is demonstrated by the results presented in Figure V-1B.

To confirm that the serum proteins were not carried through the washing procedures and gradient purification steps, a sample of fetal bovine serum was iodinated as described in Methods, by the solid phase enzymatic method (David, 1972; David and Reisfeld, 1974), and 25 microliters of serum (containing approximately 10^6 cpm) were added to 4 ml of cells in medium containing 10% fetal bovine serum. This mixture was incubated for 15 minutes at 37°C, layered over a Ficoll-Hypaque step gradient as previously described, and centrifuged at 500g for 30 minutes at 4°C. The cells at the interface were washed

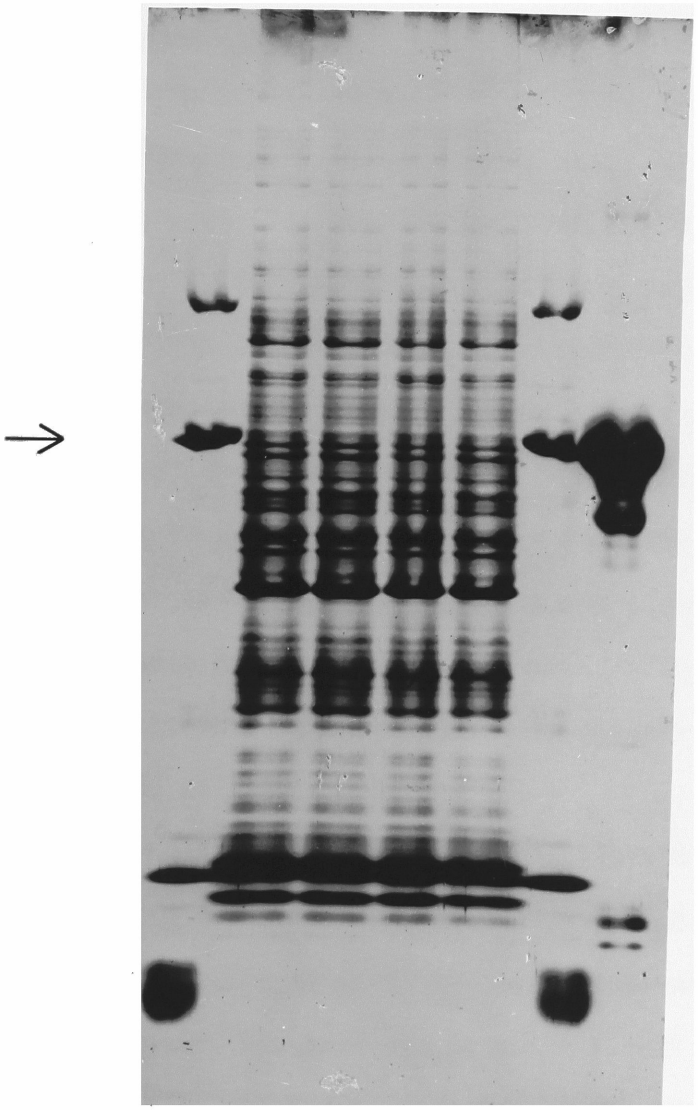
twice by centrifugation in HBSS, and assayed for cell-associated ^{125}I. The cells contained no significant radio-label (15 cpm above a background of 100 cpm). Virtually all of the added radiolabeled protein was recovered in the supernatant of the step gradient. Assuming a specific activity of the fetal bovine serum of 4 x 10^4 cpm/mg protein, at most 0.4 micrograms of serum protein (or approximately 1 part in 10^5 of the initial load) could have been carried along with the cells. Although 0.4 micrograms of a heterogeneous group of serum proteins was unlikely to influence the pattern of cellular proteins analyzed on polyacrylamide gels, the presence of 0.4 micrograms of one protein species might be of consequence. To rule out this possibility samples of 8866 cells were taken at several steps in the washing procedure, and the cells and associated proteins assessed by SDS-polyacrylamide electrophoresis. As shown in Figure V-2, no change in the gel pattern is evident with serial gradient centrifugations, and no major cell-associated protein co-migrates with serum albumin, the major protein species of fetal bovine serum. Thus it seems unlikely that weakly adsorbed serum proteins constitute a significant proportion of the cell surface components being iodinated.

2. Validation of the localization of the radio-iodination to the cell plasma membrane.

In these studies the chemical nature of the products of iodination were not examined: the evidence of others that, under mild conditions, the product is largely iodo-tyrosine was accepted as being true also in this case. However, it was of critical importance to establish the topographical localization of the iodinated species to the cell plasma membrane

LEGEND: FIGURE V-2. FICOLL-HYPAQUE GRADIENTS: REMOVAL OF SERUM PROTEINS

This Figure depicts the efficiency of the Ficoll-Hypaque step gradient regimen illustrated in Figure V-1 in removing the serum proteins from cultured cells. Duplicate cultures of 1.5×10^7 cells were treated as shown, except that, in lieu of iodination, the cells were kept in HBSS on ice for 30 minutes. After the first gradient and subsequent pelleting, aliquots were taken for gels. After the 30 minutes on ice, the second gradient and washing, a second aliquot was taken. Duplicate aliquots of 100 μg of protein were then loaded into slots of a 5-15% slab gel with a 2.5% stacking gel. Marker proteins were run either side of the samples: these were β-galactosidase (134,000), bovine serum albumin (68,000), ribonuclease (13,400), and insulin. In the rightmost column is 1 μl of fetal calf serum, showing the predominant species in BSA. The arrow to the left indicates the position of BSA.



There are two basic methods in use for establishing such localization. The first is electronmicroscopic autoradiography. The second is cell fractionation.

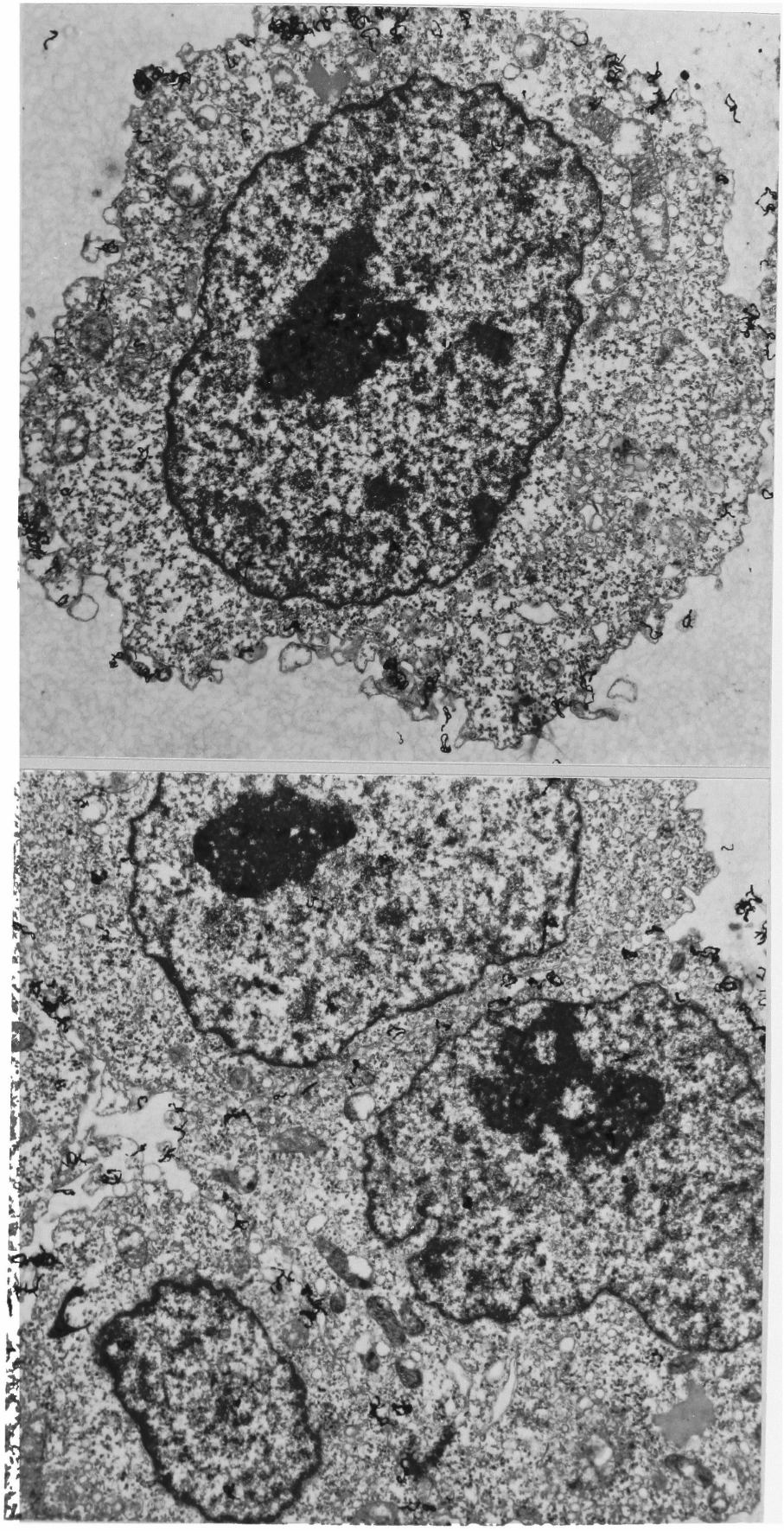
a. Autoradiographic evidence.

Cells iodinated by glucose oxidase/lactoperoxidase after simple washings in Hanks' Balanced Salt Solution were fixed in the cold as in Methods, pelleted in molten agar, gelled, embedded in Epon, and cut for thin and thick section autoradiography by the techniques described. Examples of the typical appearance of live cells are shown in Figure V-3. The autoradiograms show a clear concentration of grains over the periphery of the cells, and also the difficulty sometimes encountered in deciding whether overlapping tortuous grain tracks are due to one or more radioactive disintegration event. Another source of difficulty is the close apposition of cell membranes, so that a grain could be associated with one of two cells. In general, efforts were made to quantitate only clearly separate grains over clearly separated, well-preserved cells.

The criterion used to score whether or not a grain is associated with the plasma membrane must take into account the size of the track of the grain at the magnification at which the photograph was taken. As an adaptation of the Salpeter, Bachmann and Salpeter (1969) technique, a plastic sheet with circles of different diameters was used. For each grain, that circle which just enclosed the grain was found. For any grain to be scored as membrane associated, a circle of the same diameter as that enclosing the grain, placed so as to touch the circle around the grain, had to touch or include the plasma membrane. Using this criterion, the

LEGEND: FIGURE V-3. ELECTRON MICROSCOPIC RADIO-AUTOGRAPHIC LOCALIZATION OF IODINATED SPECIES.

The Figure is made up of two representative pictures of the silver grains indicating the localization of iodinated species in cells iodinated, diluted with medium, washed in HBSS, and then processed as described in Methods. The pictures were chosen to depict one isolated cell and one cluster of apposed cells. In both cases the marginal distribution of grains is evident, as is the tortuosity of the grains. With longer exposures it became impossible to separate individual grains with certainty. Hence this type of density was used in determining grain counts.



data of Table V-2 were obtained. These show approximately 80% of grains were clearly in the vicinity of cell plasma membrane. The remainder of the grains were randomly scattered over cytoplasm and nucleus. No correction was for the background density, which was generally negligible.

b. Cell fractionation evidence. To evaluate the cell fractionation and other biochemical evidence on the localization of the labeled species, it is necessary to present the pattern of labeled species seen on autoradiograms of polyacrylamide gradient slab gels of SDS-solubilized whole cells, the basic technique used in these studies. This is a versatile method allowing the identification of protein species between 1,000,000 daltons and approximately 1,000 daltons, and when not deliberately overrun, also showing label in the fast moving species which include small peptides and phospholipids. Since the whole cell was applied to the gel, it should be the most inclusive way of assessing the iodinated species, excluding only those molecules higher than 10^6 daltons in mass, or aggregates not solubilized by boiling in SDS and 2-mercaptoethanol.

In Figure V-4 is depicted the autoradiogram of a single slab gel with independent iodinations of different cell clones in each of the six slots. It is representative of other gels, and includes all the major bands seen in gels in the two years of running such preparations. The reproducible bands are identified by letters A to K. As shown on this gel, the bands of one slot line up identically with those in neighbouring slots, and slight differences in mobility can therefore be regarded as significant.

LEGEND: TABLE V-2. MEMBRANE-ASSOCIATED GRAINS ON ELECTRON MICROSCOPIC RADIO-AUTOGRAPHS.

The Table includes data from 15 different prints of different cells at varying magnifications. The definition of membrane-associated was as described in the text. Only cells which contained a large cross-section of nucleus were included, so as to exclude bias against cytoplasmic and nuclear iodination.

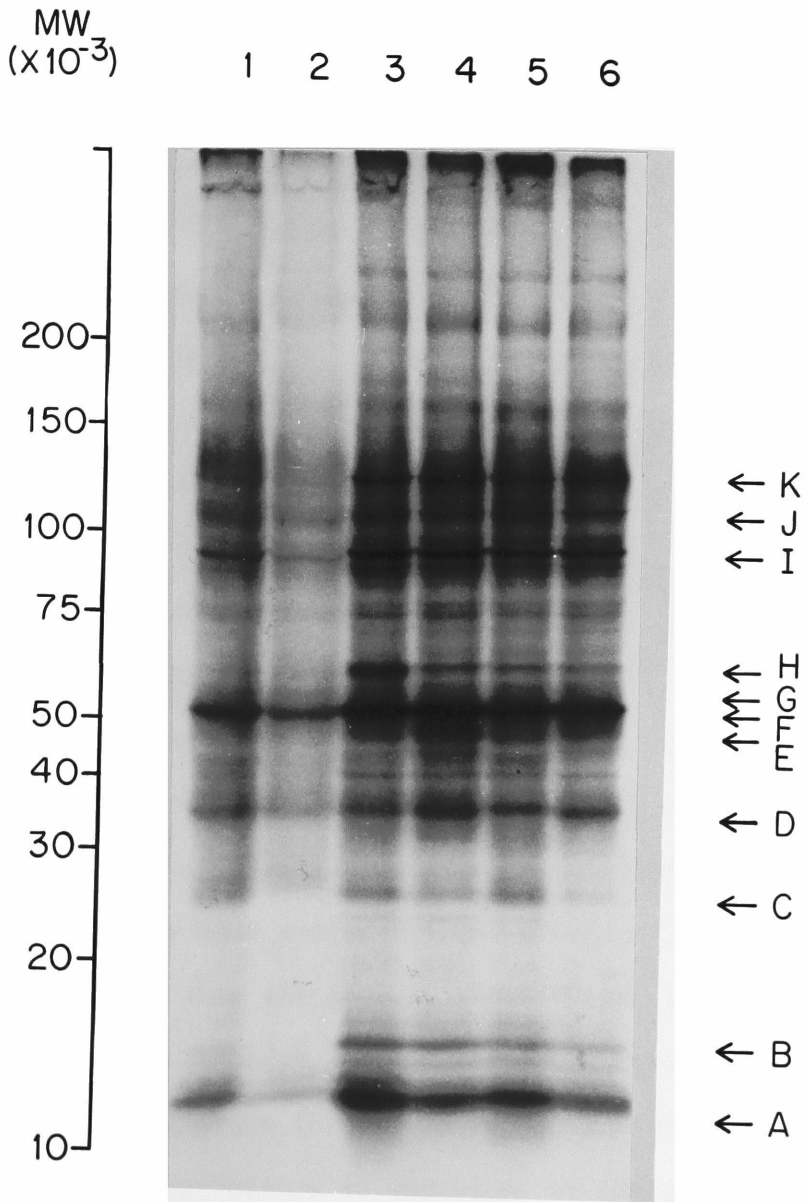
TABLE V-2.

MEMBRANE-ASSOCIATED GRAINS ON E.M. AUTORADIOGRAMS

Plate No.	Membrane Associated	Non-Membrane Associated	Total	%
1	64	14	78	82
2	45	11	56	80.3
3	53	13	66	80.3
4	49	9	58	84.5
5	52	11	63	82.5
6	49	16	65	75.4
7	69	14	83	83.1
8	62	9	71	87.3
9	64	22	86	74.4
10	45	19	64	70.3
11	41	4	45	91.1
12	75	18	93	80.6
13	67	17	84	79.8
14	23	7	30	90
15	49	9	58	84.4
	807	193	1000	80.7

LEGEND: FIGURE V-4. THE REPRODUCIBLE IODINATION PATTERN OF 8866 CELLS

The Figure illustrates the patterns of six different cell cultures iodinated by the method described, then reduced and denatured for application to gels. Those bands which recur on a large number of autoradiograms are identified by letters on the right hand side. The bands above K are variable in position, and not well resolved by this 5-15% gradient slab gel technique. The numbers designate the cell cultures: 1 is Clone 34; 2 is Clone 4; 3 is Clone 23 containing 57% rosette-positive cells; 4 is Clone 23 containing 1% rosette-positive cells; 5 is Clone 36 containing 60% rosette positive cells; 6 is Clone 36 containing 7% rosette-positive cells.


MW
(X10-3)
1
2
3
4
5
6
200
150
100
75
50
40
30
20
10
K
J
I
H
G
F
E
D
C
B
A

The species of molecular weights above 100,000 proved to be generally weak in intensity and variable in migration. Often the bands were too weak to be detected. Additionally, they fall outside the range for which useful molecular weight markers exist. For these reasons they are not treated any further. Below the major band at approximately 11,000 (labeled A), there was sometimes an extended blurred zone of low intensity. (See Figure V-5). From staining patterns, this probably represented phospholipids, but was a very minor component, and not further analysed. The other feature not shown on this gel pattern, but seen whenever the cell viability was lower than usual during iodination, was a series of bands between the 10,000 and 20,000 molecular weight markers. These corresponded to the Coomassie Blue stained bands of isolated nuclei, as seen in Figure V-6, and were taken to be labeled nuclear proteins. There are weak bands of this type on Figure V-5. When these bands were prominent, the preparation was regarded as unsatisfactory.

The gel of Figure V-4 shows the constancy of slots within one preparation. Table V-3 shows that the band pattern within a series of gels was very constant, whereas there were differences in the estimates of molecular weights. Undoubtedly this represents the limits of resolution of molecular weight by such gels: they are most accurate in the lower molecular weight zone, and less so in the high zones. It should be noted that usually the regression lines were drawn through four points, from 134,000 to 13,400. This might not show variations in the slope of the gel gradient, and certainly cannot be accurately extrapolated beyond those limits. In the case of the 7.5-15% gradient gels made according to the Neville protocol

LEGEND: TABLE V-3. MOLECULAR WEIGHT ESTIMATES OF IODINATED SPECIES ON SDS-POLYACRYLAMIDE GEL GRADIENT ELECTROPHORESIS.

On the Table are molecular weight estimates obtained by calculator-generated least-squares linear regression lines using protein markers run in parallel slots on each gel. The usual markers were: beta-galactosidase (134,000), transferrin (90,000) ovalbumin (43,000) and ribonuclease (13,400). Measurements were made to the nearest 0.5mm with a ruler. The measurements of the fronts of the bands were used in the calculations. The gels used in the construction of the Table are representative of gels run over the course of a one year period, and are of independent iodinations and gels.

TABLE V-3.

MOLECULAR WEIGHT ESTIMATES OF IODINATED SPECIES ON SDS-POLYACRYLAMIDE GEL GRADIENT ELECTROPHORESIS

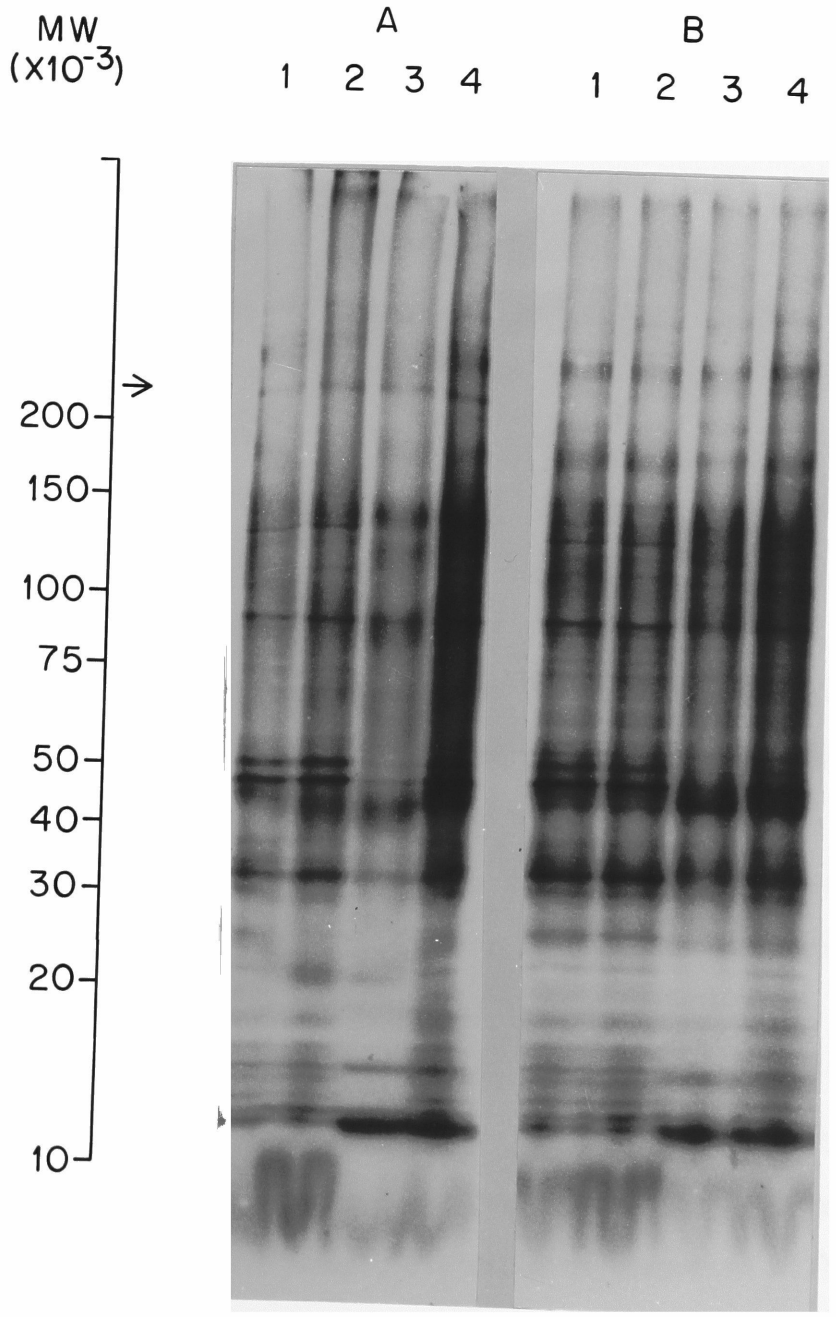
BAND	AVER-AGE	NUMBER OF GEL								
		1	2		3	4	5	6	7	8	9
			+ clone	- clone							
K_{b*}	122	16**	123	123	129	118	123	122	122	124	124
J_s	108			105	111	103	109	102	107	107	108
I_s	92				92	89	94		92	92	91
H_s	57		55					55	61	59	
G_b	52	48	52						56 55.5	54	
F_s	50	46	46	46	46	53	53	48	51	51	50
E_b	45		42	42	42	48	49	53	47	46	
D_s	34	34	32	32	33	34	35	34	34	35	36
C_b	25	25	24	24	27.5 25.7 24	24	25	24	27	25	26
B_b	14.5			14		14.5	14.9		14.1	(15 14	
A_s	11	11.2	11.4	11.4	11.8	9.8	9.7	11.2	11.1	11.5	11.4

* = The subscript refers to the type of band; "b" refers to blurred edges, "s" designates sharp.

** = The number is the apparent molecular weight in thousands of daltons. At the higher levels of the gels, the estimates are to the nearest thousand; in the lower range, where distances can be measured with more certainty, estimates are to hundreds.

LEGEND: FIGURE V-5. COMPARISON OF REDUCED AND UNREDUCED IODINATED CELL PREPARATIONS.

The Figure is a comparison of the autoradiograms of radio-iodinated clones of 8866 cells run on a 5-15% gradient slab gel. In Part A the samples were boiled in SDS. In Part B the cells were boiled in SDS and 2-mercaptoethanol before loading onto the gel. The numbers refer to four different cell clones iodinated in parallel: namely clone 34, 4, 23, and 36. The arrow at the left indicates a high molecular weight species evident in the unreduced samples, but not seen in the reduced. No new species could be discerned in the reduced sample.


MW
(X10-3)
A
B
1 2 3 4
1 2 3 4
200
150
100
75
50
40
30
20
10

(and presented later), there was a clear non-linearity above 100,000 and below 20,000 (data not shown). In spite of these limitations, assignments can be made on the basis of molecular weights except in the E to H region, i.e. 40,000 to 60,000. This is a series of bands which are separated by their order, and whether they are broad, blurred bands or narrow, sharply defined bands. In some cases there appears to be splitting into more than one species, as is seen in three cases on the Table. Attempts to resolve these by a series of constant percentage gels were not successful: the bands became blurrier, and merged.

From the gels tabulated and others, the approximate molecular weight assignments are A = 11,000; B = 14,500; C = 25,000; D = 34,000; E,F = 45-50,000; G = 52,000; H = 54,000, I = 92,000; J = 108,000 and K = 122,000. However, in the light of the variability and the fact that these assignments have not been confirmed by either a range of different gel systems, or alternative methods, they will be referred to provisionally by the alphabetical designations, awaiting confirmation by the other methods (see Discussion).

In Figure V-5 a gel autoradiogram is shown to illustrate the effect of reduction of disulfide bonds on the iodination pattern. Only a species at greater than 200,000 Daltons is seen to be affected: the products of reduction are too weak to be detected. The major bands described in the previous figure remain unchanged. Because of the superior solubilization achieved with 2-mercaptoethanol, all patterns presented will be of reduced preparations.

With a knowledge of the pattern of iodination, it is now possible to consider whether the pattern is of the plasma membrane. The small number of major bands is promising. The number of minor bands is dependent on

the specific activity of the preparation and hence the intensity of the bands. Two kinds of evidence will be considered bearing on the origin of the iodinated bands. The first is the possibility of extrinsic protein contamination. The second is labeling of intracellular species. These will be taken in order.

The likeliest candidates for extrinsic molecules to be labeled are the most abundant species in the medium in which the cells were grown, namely bovine serum albumin, and the two proteins present during the iodination reaction, glucose oxidase and lactoperoxidase. A comparison of labeled cells and these species is made in Figure V-6. Two kinds of evidence are contained within the figure. Firstly, the iodination pattern appears identical whether glucose oxidase was present to generate the peroxide, or whether peroxide was added exogenously. Secondly, glucose oxidase and lactoperoxidase are seen to run quite differently from any of the major named bands. The lactoperoxidase band, at greater than 95,000 daltons, is differentiated from Band I on this method of separation. For comparison, the labeling pattern of bovine serum is included. It was concluded that BSA, LPO and GO did not contribute to the apparent labeling pattern. What could not be excluded, since there are so many species in the labeled FCS, is that there is a minor component of serum which has special affinity for the membrane, and is labeled.

The second comparison is between labeled whole cell preparations, and various fractions. Many futile attempts were made to develop pure plasma membrane preparations using the methods of Warren, Glick, and Nass (1966). In particular, the methods which resulted in large membrane pieces

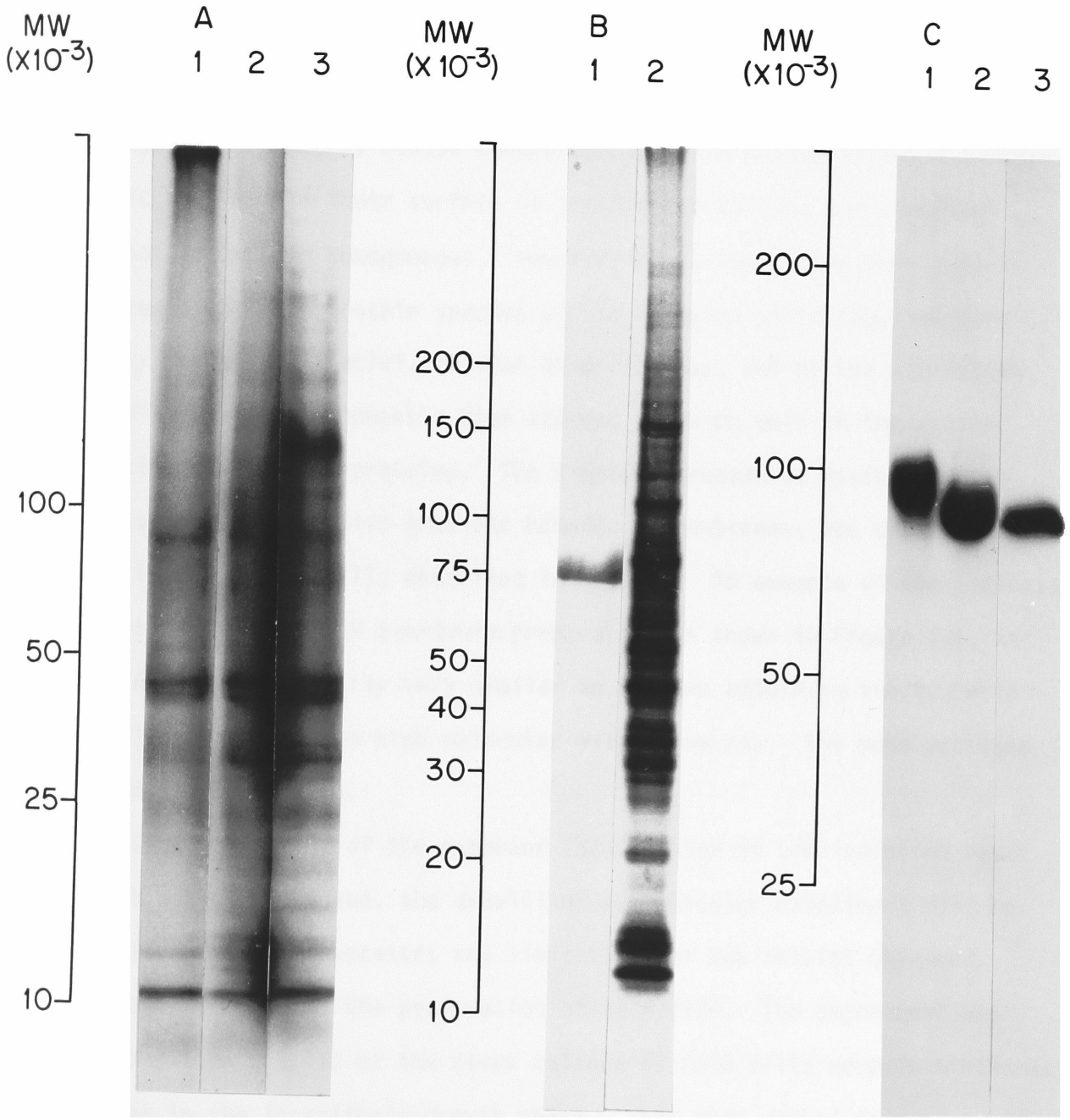
LEGEND: FIGURE V-6. COMPARISON OF IODINATED SPECIES: CELLULAR AND SOLUBLE PROTEINS.

This Figure is a composite to compare the gel patterns of various iodinated preparations. All are autoradiograms of 5-15% gradient slab gels, with 2.5% stacking gels.

Part A shows three strips from a single gel. The aliquots represent 1. the pattern of whole cells labeled by the glucose oxidase/lactoperoxidase technique; 2. an aliquot of cells labeled on the same day in the same experiment, but, instead of glucose oxidase, two aliquots of hydrogen peroxide added at 5 minute intervals to give a final concentration of 44 μM; 3. an aliquot of membranes prepared by the Brunette and Till procedure from cells iodinated by the lactoperoxidase/glucose oxidase method.

Part B shows the pattern of 1. Glucose oxidase and 2. Fetal bovine serum iodinated using lactoperoxidase immobilized on Sepharose beads, and added hydrogen peroxide.

Part C shows 1. Lactoperoxidase, 2. Glucose oxidase, and 3. Bovine serum albumin labeled by the Chloramine T method.


MW
(X10-3)
A
1
2
3
100
50
25
10
MW
(X 10-3)
B
1
2
200
150
100
75
50
40
30
20
10
MW
(X10-3)
C
1
2
3
200
100
50
25

were tried. These used either zinc chloride or fluorescein mercuric acetate as 'hardening' agents, and after gentle douncing, yielded almost intact plasma membranes reminiscent of the ghosts of erythrocytes, with a hole at the site of the extrusion of the nucleus. Screening by phase and electron microscopy always showed considerable contamination with cytoplasmic tags on the inner surface of these preparations, and thus they were not considered homogeneous. However, the preparations were clearly depleted of soluble protein species by the repeated pelleting, and were grossly depleted of nuclei, as seen by microscopy, and by the diminution of the intensity of Coomassie Blue stained bands on gels in the region of nuclear-associated proteins. The simplest procedure, giving similar results to more extensive gradient banding of membranes, was that of Brunette and Till (1971), described in Methods. An example of the iodinated species present in such a membrane preparation is shown in Figure V-6, as track A-3. It is clearly very similar to the two adjoining tracks, with some slight loss of the high molecular weight species - the most variable portion of the pattern.

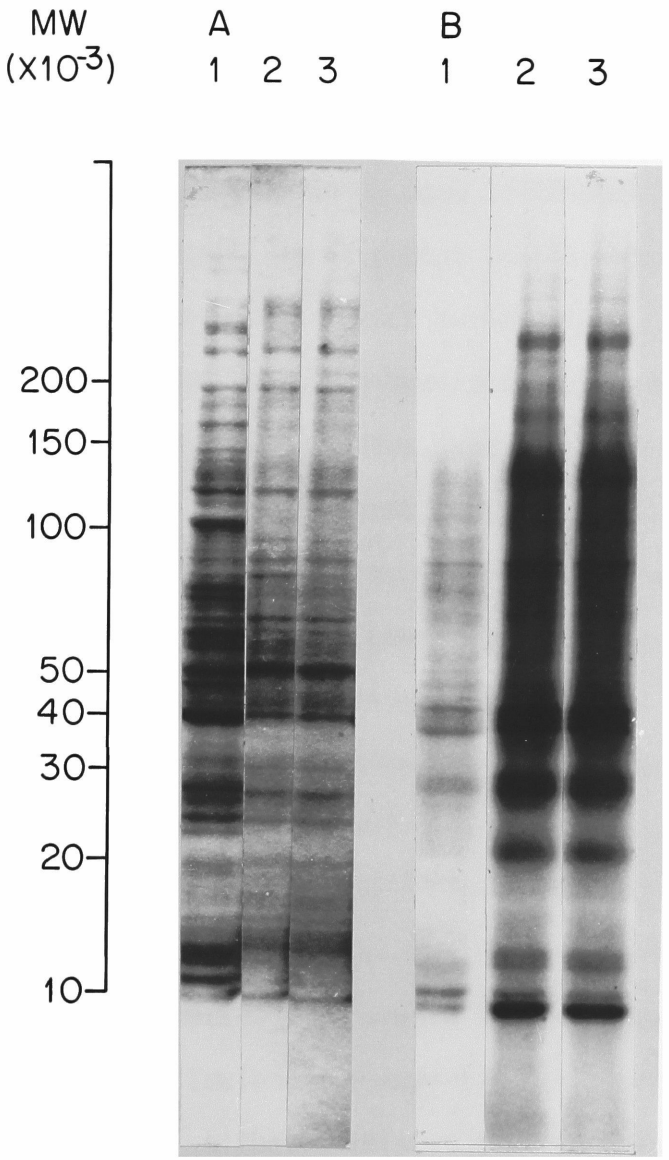
Since the proof of the membrane localization of the iodinated bands is such a critical issue, the details of a particular experiment will be given to detail the successes and limitations of the results obtained. The example chosen is the preparation of 11.v.1974. The experiment was done as follows: Cells of the stock culture of 8866 cells were in stationary cultures in the logarithmic growth phase. They were washed four times in HBSS, and iodinated at 5 x 10^7 cells/ml with 2 mCi of $Na^{125}I$, 20 mU of LPO and 20 mU of GO in a 2 ml reaction mixture. A control mixture, lacking

LPO, incorporated 0.02% of the label into TCA-insoluble material, while the test mixture gave 1.66% incorporation. After washing in HBSS containing 0.1 mM KI, the cells were swollen in 0.077M NaCl, and treated with fluorescein mercuric acetate according to the Warren, Glick and Nass (1966) procedure. The exceptionally slow douncing yielded large ghosts typical of a successful experiment. Also typical of these preparations was the granular material seen under phase microscopy on the cytoplasmic aspect of the membranes. The ghosts were pelleted with the nuclei, and then banded in the two-phase system of Brunette and Till. The interface was rebanded in a fresh two-phase system. The second interface was then washed in 0.02M Tris-HCl, pH 7.4, and divided into replicates. One was washed in the same Tris buffer containing 0.02% sodium azide. The other was washed in 0.5M KCl, with the intention of removing extrinsic, loosely attached species from both faces of the membrane. The pellets were then resuspended and assayed for protein, TCA-precipitable counts, and then aliquots stored frozen at -20°C for gels. There proved to be little difference in the specific activity of the two preparations: 5,970 cpm/μg of protein for the low salt washed material versus 6,456 cpm/μg for the 0.5M KCl washed membranes. A sample of the whole cell homogenate had a specific activity of 1,263 cpm/μg, giving an enrichment factor of approximately 4.8.

The gel patterns of the two membrane preparations are compared with whole cell homogenate in Figure V-7. In Part A the Coomassie Blue stained tracks show the simpler pattern of the membrane preparations, especially in the 10,000-15,000 Dalton region. In the second section, the limitation of protein loading prevented the whole cell preparation being of equal

LEGEND: FIGURE V-7. COMPARISON OF WHOLE CELL AND ISOLATED MEMBRANE COOMASSIE BLUE STAINED GEL AND AUTORADIOGRAPHY.

This Figure shows a comparison of a whole cell sample and a membrane isolates after iodination run as samples on a 5-15% gradient slab gel, with a 2.5% stacking gel. In Part A, column 1 represents whole cells, column 2 represents membranes toughened with fluorescein mercuric acetate then isolated by the Brunette and Till procedure, and washed with 0.02% NaN_3, while column 3 shows the same membrane isolate washed with 0.5M KCl. In Part B, the same three samples are represented in the same order, but this time the picture is of the autoradiogram.

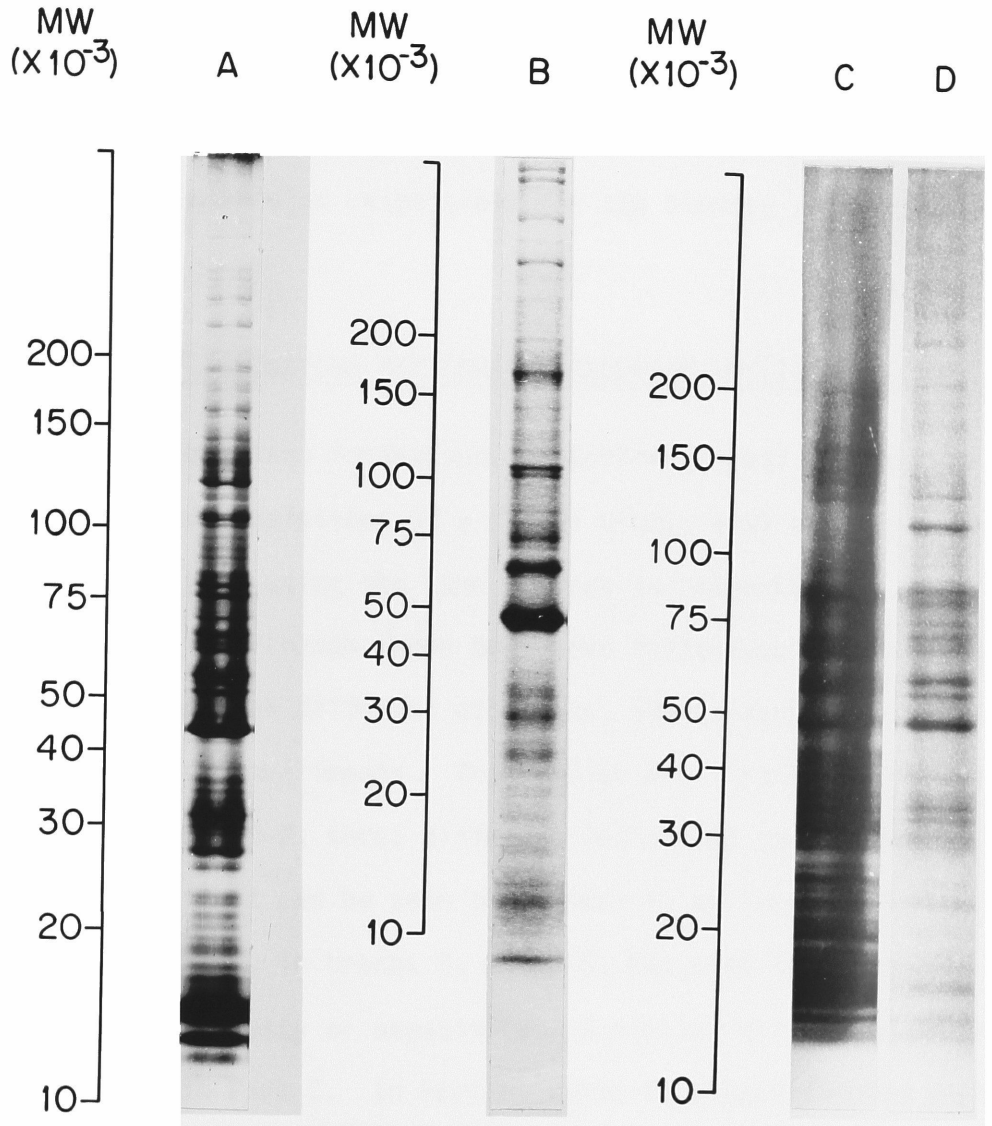

MW
(X10-3)
A
1 2 3
B
1 2 3
200
150
100
50
40
30
20
10

intensity on the radio-autogram. However, the similarity of the major bands is clear, and no major band is present in whole cells and absent on the membrane isolates. Put the other way, it appears that the iodinated species seen in whole cell preparations are also contained in partially purified plasma membrane preparations.

An alternative analysis, to be used in Chapter VI, was done by solubilizing iodinated cells in detergent in the presence of divalent cations. This procedure leaves intact nuclei which can be pelleted out with low speed centrifugation. Two non-ionic detergents, NP-40 and Emulphogene, were used in such studies, and gave identical results. An illustrative example is the experiment of 11.iv.1975. Iodinated cells were solubilized in 1% Emulphogene, the nuclei pelleted and washed, and the supernatant spun at 100,000g for 1 hour. In Figure V-8 is presented a comparison of the Coomassie Blue stained gel patterns of whole cells, a Brunette and Till membrane preparation, the Emulphogene pelletable material, and the soluble supernatant from Emulphogene. The protein loads are comparable (100-150 μg) but not identical. The simplicity of the Emulphogene pattern approaches that of the membranes. The pelletable material is more complex, and shows the marked increase in lower molecular weight species. An autoradiogram of the Emulphogene supernate is presented in the next section in Figure VI-3. Although this is the remaining supernatant after a selective procedure, it is identical with whole supernate. The band pattern shows the major named bands seen in whole cell preparations, and in apparently identical ratios. This does not establish the membrane origin, but does show that the iodinated species are fully represented in the

LEGEND: FIGURE V-8. A COMPARISON OF THE COOMASSIE BLUE-STAINED PATTERNS OF VARIOUS CELLULAR PREPARATION.

All preparations were approximately 100 μg of protein run on 5-15% gradient slab gels after denaturation in SDS and reduction with 2-mercaptoethanol. A: Whole cells. B: Plasma membranes prepared by the Brunette and Till procedure. C: The pellet obtained from Emulphogene 1% solubilized whole cells by centrifugation for 1 hour at 100,000g. D: The 1% Emulphogene supernatant after centrifugation.


MW
(X10-3)
A
MW
(X10-3)
B
MW
(X10-3)
C
D
200
150
100
75
50
40
30
20
10
200
150
100
75
50
40
30
20
10
200
150
100
75
50
40
30
20
10

detergent solubulized cytoplasmic extract, and that significant amounts of them remain in solution after centrifugation at 100,000g for 1 hour.

This concluded the evidence on the localization and nature of the iodinated species. It was now pertinent to ask if iodination and resolution on SDS-polyacrylamide gel gradient electrophoresis could identify the molecule or molecules responsible for the binding of C3d.

C. COMPARISON OF IODINATION PATTERNS OF RECEPTOR POSITIVE AND NEGATIVE CELLS

Several comparison iodinations of different cell clones, and phenotypically altered populations of a single clone, have been done over the course of a year using the same iodination reagents.

Although there have been differences between positive and negative clones on different occasions, the differences have not been consistent between experiments. The results are illustrated in three figures. In Figure V-9, three different iodination comparisons are presented. In Part A it can be seen that there is an increase in Band B, at about 15,000 Daltons, in tracks 3, 4 and 5, but that this does not correlate with receptor positivity or negativity. A similar difference is present in Part B, but not in Part C. In Part B, there is a band present in tracks 1 and 2 at just greater than 50,000 Daltons. This band is much weaker in tracks 3 and 4. In Part C is depicted a set of iodinations which appear virtually identical. However, when brief exposure autoradiograms were analyzed on a Joyce-Loebl microdensitometer, there was evidence of an altered ratio of the 48,000 and the 50,000 Dalton peaks between the positive and the negative clones. This was also visible to the naked eye. With

LEGEND: FIGURE V-9. IODINATION PATTERNS OF COMPLEMENT RECEPTOR POSITIVE AND NEGATIVE CLONES.

All are autoradiograms of iodinated cells denatured in SDS and 2-mercaptoethanol, run on 5-15% gradient gels with 2.5% stacking gels.

A: Preparation of 28.i.76

1 = Cl 34;2 = Cl 4;3 = Cl 23 with 57% cells rosette positive

4 = Cl 23 with 1.5% cells rosette positive, 5 = Cl 36 with 60% cells rosette positive,

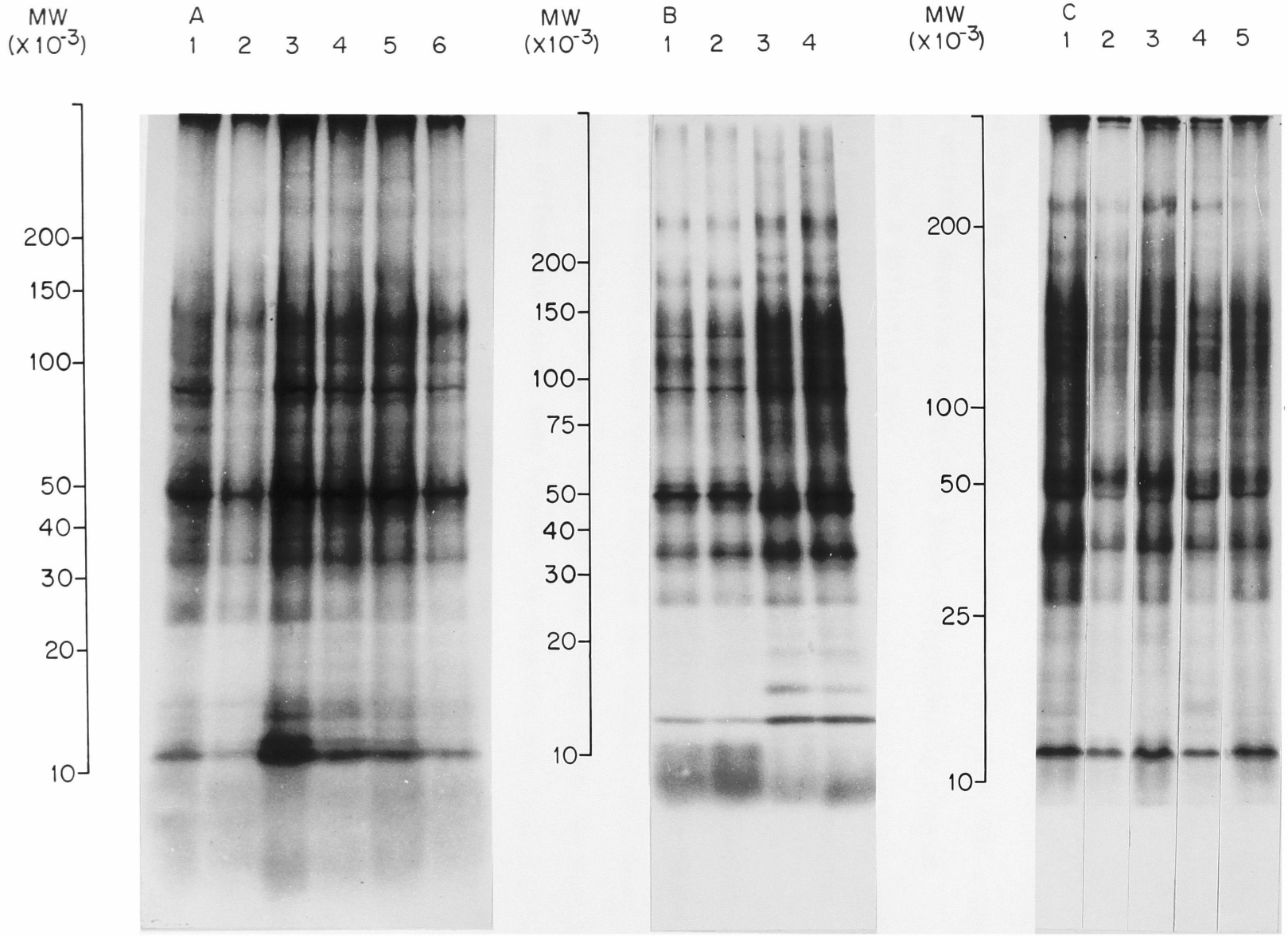
6 = Cl 36 with 7% cells rosette positive.

B: Preparation of 16.iv.75

1 = Cl 34; 2 = Cl 4; 3 = Cl 23 (<1% rosette positive); 4 = Cl 36 (approx. 2% rosette positive).

C: Preparation of 14.ii.75

1 = uncloned 8866 cells; 2 = Cl 34; 3 = Cl 4; 4 = Cl 23; 5 = Cl 36.


MW (X10-3)
A
1 2 3 4 5 6
200
150
100
50
40
30
20
10
MW (X10-3)
B
1 2 3 4
200
150
100
75
50
40
30
20
10
MW (X10-3)
C
1 2 3 4 5
200
100
50
25
10

increasing exposure of the x-ray film, this difference was lost. Using the densitometer to scan autoradiograms of both low and high intensity of other preparations revealed no such difference.

Since the positive and negative clones were picked from an uncloned population, it was always possible that there were more differences between the clones than the one character being assessed. It was felt that a comparison of entirely negative clones with their phenotypically altered progeny would be a definitive test. It seems unlikely that multiple variations would arise independently at the rapid rate of change found for the complement receptor.

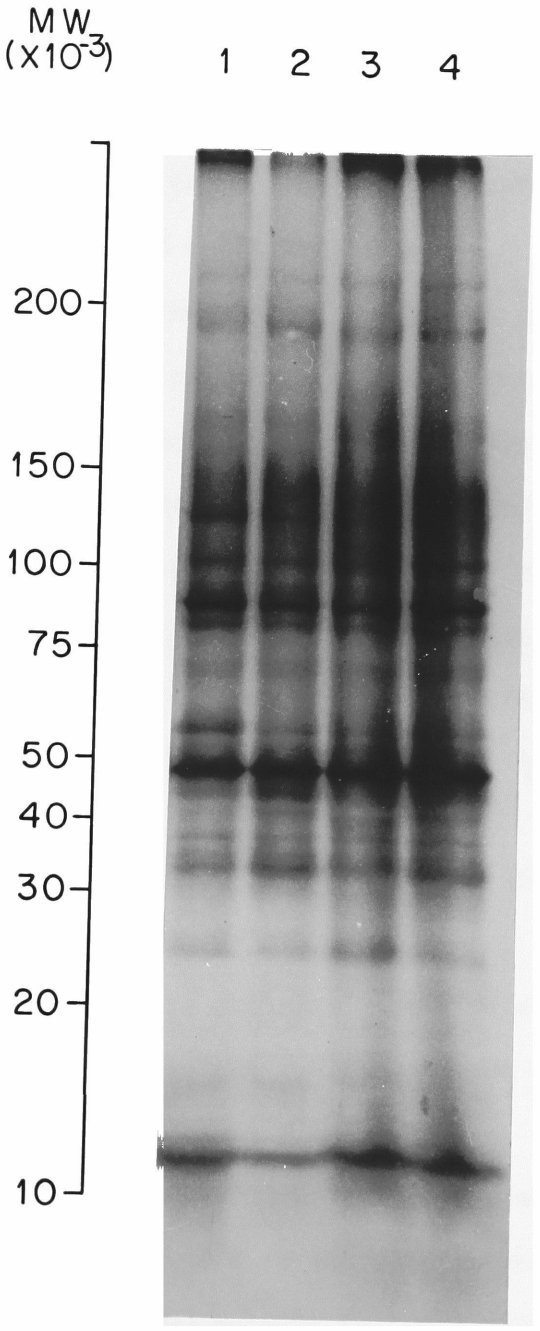
The results of comparative iodinations are shown in Figures V-4 and V-10. In both of these, cells recently thawed out, and therefore predominantly negative are compared with very markedly positive populations grown from the same frozen stock, but kept in culture. No differences can be seen between the negative and part-positive cultures. Microdensitometry was performed on the autoradiograms, but still no differences were detected.

The conclusion from these studies is that the technique used was incapable of distinguishing receptor positive and negative cells directly.

Two additional types of studies were performed using surface iodination. The first concerned the relative susceptibility of iodinated proteins to proteases. The second was concerned with the biological life-span of the labeled species. These results will be considered here before continuing the direct line of argument, namely the attempt to identify the complement receptor.

LEGEND: FIGURE V-10. COMPARISON OF NEGATIVE CLONES WITH POSITIVE PROGENY OF THOSE CLONES.

Autoradiogram of a 5-15% slab gradient gel with 2.5% stacking gel. At the time of iodination, track 1 = Cl 23 with 36% rosette positive cells; 2 = Cl 23 with no positive cells; 3 = Cl 36 with 70% positive cells; 4 = Cl 36 with approximately no positive cells.


MW
(X10-3)
1
2
3
4
200
150
100
75
50
40
30
20
10

D. THE EFFECTS OF PROTEASES ON IODINATED MEMBRANE PROTEINS OF 8866 CELLS

From the evidence presented in Chapter IV it was known that several proteases were able to destroy the complement receptor in a dose-dependent fashion without killing the cells. Using this information, the effects of these proteases on iodinated cells were examined. Cells were iodinated, treated with protease, and then washed. Aliquots taken for rosetting and for analysis on polyacrylamide gels. Although no protease had been found which, at an appropriate concentration, did not destroy complement receptor activity, nevertheless, it was hoped that by employing low doses of proteases, the complexity of the radio-iodination pattern of 8866 cell membrane proteins could be reduced sufficiently to reveal differences between receptor positive and negative clones. Figure V-11 shows three series of positive and negative clones treated with varying amounts of trypsin. The main findings illustrated in this figure are that:

i. The control material kept on ice for 15 minutes resembles other iodination series.

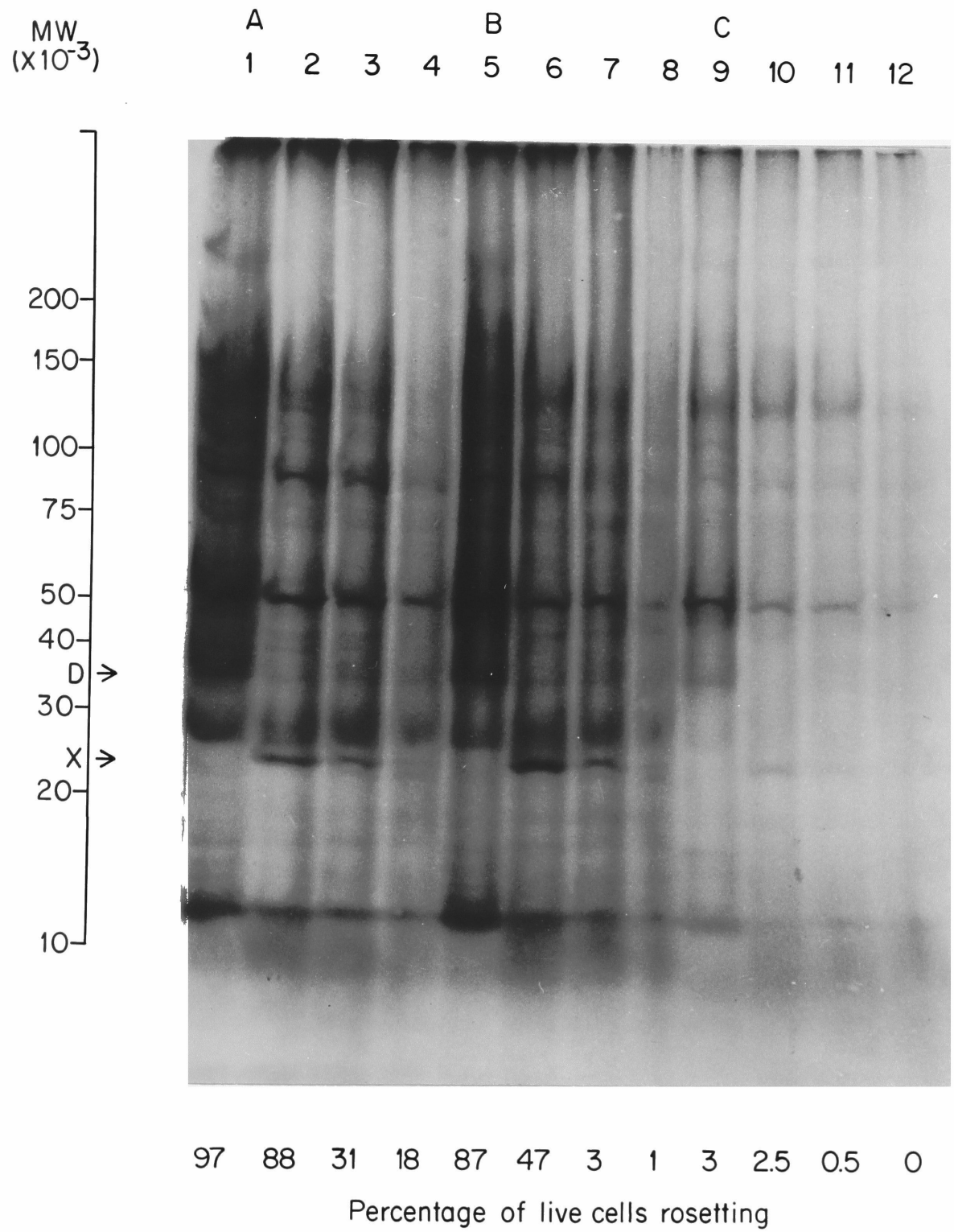
ii. With the action of protease, there is a generalized loss of bands in the high molecular weight range, and some simplification of the pattern.

iii. Band D, at about 35,000 Daltons, is progressively lost.

iv. A new band, labeled X in the figure, and with a molecular weight of approximately 22,000 appears concomitantly with the loss of Band D in the low doses of trypsin, but is diminished at the highest doses of trypsin. At the highest dose, the band appears split into two, with

LEGEND: FIGURE V-11. THE EFFECT OF TRYPSIN ON IODINATED SPECIES OF 8866 CELLS.

Autoradiogram of 5-15% gradient slab gel, with 2.5% stacking gel. A, B, and C refer to clones 34, 4, and 36. 1, 2, 3, and 4 refer to cells at 10^6/ml exposed to 0, 10, 100, and 1000 μg/ml of trypsin for 15 minutes at 37° C. Reactions were stopped with 1 ml of fetal bovine serum, diluted with Dulbecco's medium, pelleted and washed. Aliquots were tested for rosetting, and the remainder of the sample was boiled in SDS and 2-mercaptoethanol and layered onto gel slots.


MW
(X10-3)
A
B
C
1 2 3 4 5 6 7 8 9 10 11 12
200
150
100
75
50
40
D
30
X
20
10
97 88 31 18 87 47 3 1 3 2.5 0.5 0
Percentage of live cells rosetting

the second band being approximately 1,000 Daltons smaller than X.

v. The loss of Band D, and the appearance of Band X, occur in both positive and negative cells.

vi. A major effect is seen on Band D with virtually no change in the percentage of cells rosetting EAC.

The findings of this study led to the testing of other proteases. The material was run on a Neville (1971) gel, and is shown in Figure V-12. Here only one cell type, Clone 4b1.1 was used, but four proteases were tested: trypsin, chymotrypsin, papain and pronase. The resultant autoradiogram confirms the simplification of the pattern, the loss of high molecular weight species, the loss of band D, and the appearance of Band X. However, the different resolution of the gel system, plus the presence of more bands in the low molecular weight region, especially near the critical Band D, do not show the clear correlation between the loss of Band D and the appearance of Band X seen in figure V-11. As in that figure, however, the other major bands are preserved in approximately the same amounts and ratios. Thus, it appears that there is one major iodinated species on the membrane of 8866 cells which is particularly sensitive to proteases, and that, with four different proteases it is cleaved to yield a membrane-associated species of similar size. With more extensive proteolysis, the new species is further cleaved. The cleavage of Band D is not associated with the loss of receptor activity.

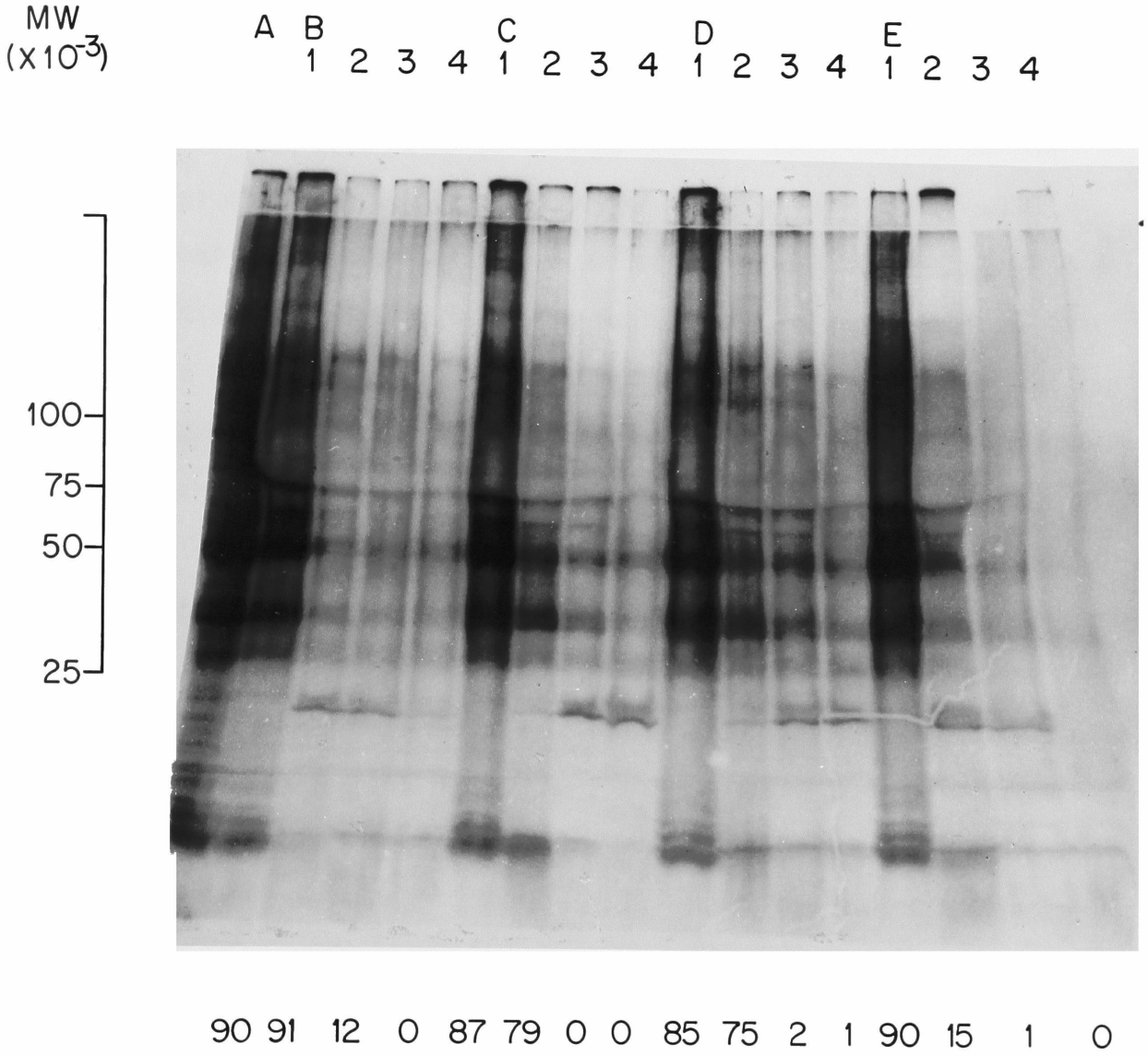
E. THE TURNOVER OF IODINATED PROTEINS ON THE SURFACE OF 8866 CELLS

The purpose of this study was to examine the rate of turnover of the iodinated protein species on the lymphoblastoid cell lines, and to see if

LEGEND: FIGURE V-12. THE EFFECTS OF PROTEOLYSIS ON THE IODINATION PATTERNS OF 8866 CELLS.

An autoradiogram of a 7.5-15% gradient slab gel, with a 6% stacking gel, made after the method of Neville (1971). The samples are iodinated whole cells. Sample A is a control of Clone 4b1.1 cells after iodination and gradient purifications described earlier. B,C,D, and E refer to treatment with proteases trypsin, papain, chymotrypsin and pronase, respectively, for 15 minutes at 37°C. The numbers 1,2,3 and 4 refer to the doses of 0, 10, 100 and 1000 μg/ml of protease, all for 10^6 cells/ml. The reactions were stopped with Soybean trypsin inhibitor in excess added to the trypsin, iodoacetamide added to the papain, and fetal calf serum added to all samples. After dilution and pelleting, aliquots were taken for rosetting, and the remainder of the cells boiled in SDS and 2-mercaptoethanol, and applied to the gel. The numbers at the bottom of each slot refer to the percentage of the cells applied to that slot which were rosettable.

Note: There is an artefact due to tearing of the gel at ∿ 20,000 Daltons in panels D and E.


MW
(X10-3)
A B C D E
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
100
75
50
25
90 91 12 0 87 79 0 0 85 75 2 1 90 15 1 0

there were differences in the rates between species.

It was particularly important to establish if there were any very rapidly turning-over species which might be lost from the surface in experiments to be done next, in which iodinated cells would be rosetted for up to two hours at 37°C in serum containing medium. Such rapidly turning-over species might not be identified in an affinity-selection process.

Several trial experiments were unsuccessful because the cells did not go into active growth phase following iodination. It had been found that even simple but multiple washing in cold buffer resulted in an initial lag period before normal doubling times were achieved, but in the type of study described above, it is clearly necessary that the cells be growing normally and maintaining high viability. In the trial experiments either growth or viability vitiated the findings. One successful experiment was performed, and this will be presented.

Cells of Clones 34c1.2 and 4b1.1 were iodinated by the usual protocol, with 400 μCi of sodium 125-iodide for 2×10^7 cells in a 2 ml reaction mixture. A parallel control iodination without LPO was carried out. The control incorporation into TCA-precipitable counts was 0.014%, while the corresponding values for clones 34c1.2 and 4b1.1 were 2.37% and 3.14%: i.e. the ratio of specific to non-specific iodination in the two clones was 169 and 224 respectively. After the gradient and wash step post-iodination, the cells were suspended in Dulbecco's medium plus 10% fetal calf serum at 37°C at a concentration of 0.5×10^6/ml, and 1 ml aliquots dispensed into glass, capped tubes, and incubated in a humidified 5% carbon dioxide incubator, except for a control aliquot which was immediately put onto ice. For each clone duplicate

aliquots were analyzed for each time point. In an initial analysis, the data was treated as four independent series, but it was found that the duplicates were highly reproducible: data from each iodination series and the pooled series for each clone will be presented.

At each time point, the 1 ml aliquots were analyzed as follows:

a. 100µl for cell count and viability.

b. 100µl added directly to 1 ml of cold 10% TCA/50mM KI for total TCA-precipitable counts.

c. 100µl was placed into Hanks'Balanced Salt Solution/1% fetal bovine serum. This material was washed onto a GF/C filter and kept moist until the addition of ice cold 10% TCA/50mM KI. It was therefore a measure of the TCA-precipitable material in the form of cells or cell-derived fragments large enough to be retained by the filter.

d. The cells in the remaining 700µl were pelleted at 1200 rpm for 5 minutes at 4°C, and a 100µl aliquot of the supernatant added to 1 ml of 10% TCA/50mM KI. From this the TCA-precipitable but non-cell associated counts could be obtained.

e. The cell pellet was resuspended in 5 ml of Hanks' Balanced Salt Solution, repelleted at 1200 rpm for 5 minutes at 4°C, the supernatant was removed and the cell pellet was then frozen at -20°C until prepared for SDS-polyacrylamide gel electrophoresis.

These samples should thus allow assessment of the retention of total TCA-precipitable count, cell associated TCA-precipitable counts, and non-cell associated TCA-precipitable counts simultaneously, allowing accurate "book-keeping" on the fate of incorporated iodide, while the gel should allow a

a qualitative assessment and semi-quantitative assessment of the distribution and relative amounts of the iodinated species.

The key interest clearly lies with the cell associated TCA-precipitable counts, and these results on two of the four series are presented in Figure V-13: the results of the other two series are virtually identical. The open symbols show the raw data plotted on a semi-logarithmic scale. The line drawn through them is a calculator-generated least squares linear regression line on the points from 4 to 48 hours. The value of this line at time points less than four hours

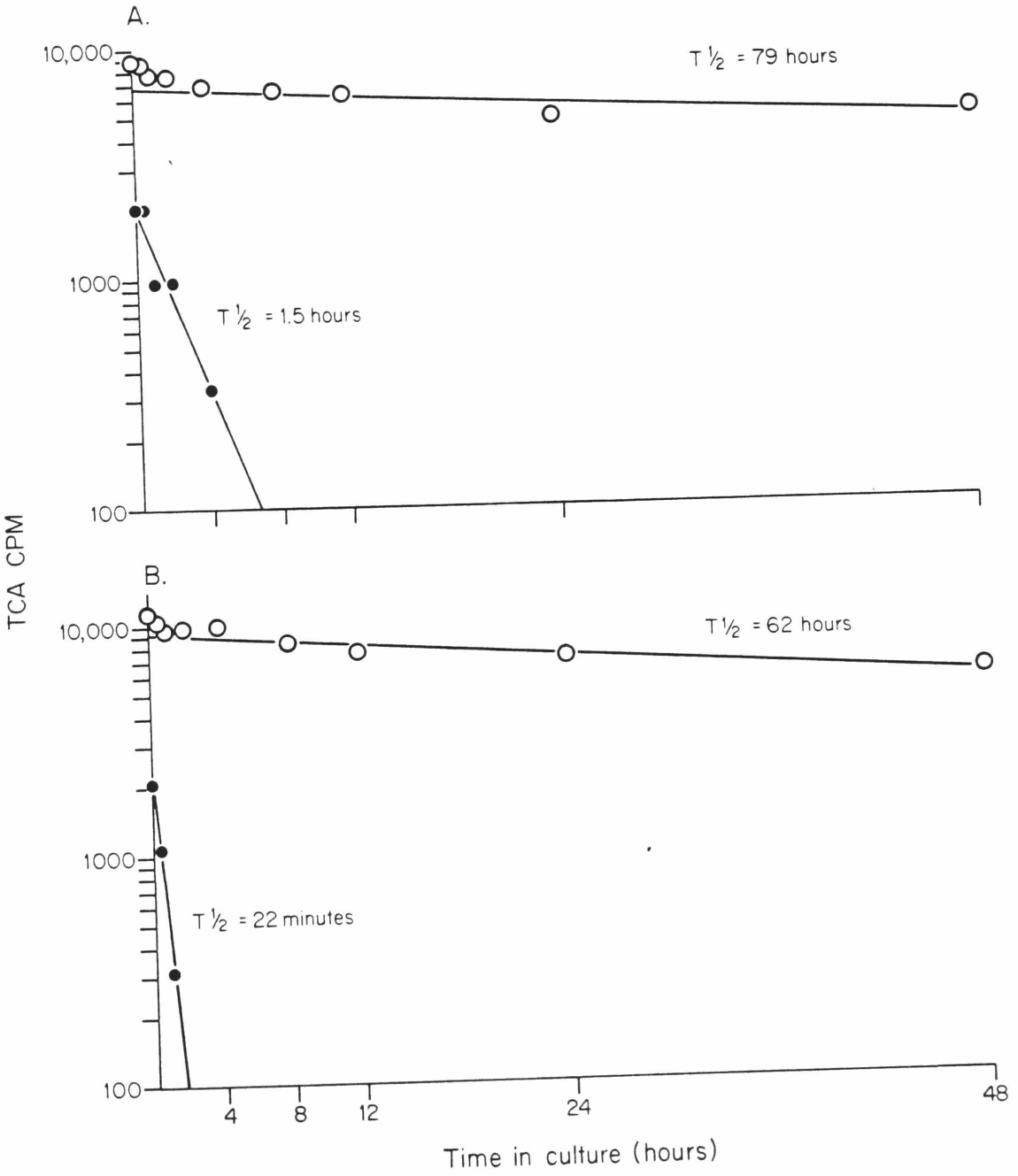
was subtracted from the early time point measured values, and the result plotted in solid symbols in the lower part of each graph. Linear regression lines were then calculated for these points only as long as the points showed a clear downward trend. Thus, after the two hour time point in Part B, there was a flattening out of the subtraction derived curve, and therefore the subsequent points were omitted: this biased the result in favor of the shortest half life, and the maximal estimate, for the short-lived component. In view of the paucity of measurements, it was felt that this limited two-component analysis was an adequate mathematical description of the data. The conclusions are clear. The majority of the iodinated species have a long half life, while a small proportion of the material has an extremely short half life. In Table V-4, the same plots are represented numerically, along with the other two independent series, plus the results obtained by pooling the data from 4 to 48 hours, and generating another linear regression line. This table, by comparing the $t_{1/2}$ for 0-48 hours and 4-48 hours, gives convincing evidence for

LEGEND: FIGURE V-13. TURNOVER OF IODINATED SPECIES ON THE MEMBRANES OF 8866 CELLS.

The plots are of those components of 100 µl/aliquots which were retained by GF/C filters when washed with HBSS and then 10% TCA/ 50mM KI, and counted in a gamma counter. The curves are linear regression lines derived by calculator by the least-squares method for the 4-48 hour time points, and then a second least-squares line drawn through those points at less than four hours which appeared to show a consistent decline when the 4-48 hour extrapolated value was subtracted from the measured value. The short half-life is therefore the maximum value to be obtained, rather than necessarily the most probable.

A = values for one set of clone 34 cells.

B = values for one set of Clone 4 cells.


A.
10,000
1000
100
T ½ = 79 hours
T ½ = 1.5 hours
B.
10,000
1000
100
T ½ = 62 hours
T ½ = 22 minutes
TCA CPM
4
8
12
24
48
Time in culture (hours)

LEGEND: TABLE V-4. ANALYSIS OF IODINATED SPECIES OF 8866 CELLS AFTER SUBSEQUENT CULTURE.

The Table demonstrates the half-lives of filter-retained, TCA-precipitable counts from 100 µl samples of cell cultures. The half-lives were calculated by the least-squares method of linear regressions using a calculator. The results were obtained from duplicate cultures derived from a single iodination of each of Clones 34 and 4 carried in parallel. The upper panel includes the data from one of the replicates at each time point arbitrarily assigned the designation a or b. In the lower panel, the replicates were all used to calculate the regression line.

TABLE V-4.

ANALYSIS OF IODINATED SPECIES OF 8866 CELLS AFTER SUBSEQUENT CULTURE

CELLS	$t_{1/2}$		
	0 - 48 Hours	4 - 48 Hours	0 - 4 Hours
34 a	54 hours	79 hours	1.5 hours (23.5%)*
34 b	56 hours	68 hours	1.2 hours (12.6%)
4 a	52 hours	62 hours	0.4 hours (19.6%)
4 b	45 hours	57 hours	0.45 hours (26.2%)
Pooled Data			
34		73 hours	
4		59 hours	

* = The percentage of total counts which represents the maximum estimate of the fast component in a two component analysis.

a short-lived component, and shows the estimate of that component to be between 13 and 26 percent of the incorporated cell-associated counts. It also confirms the long survival of approximately 80% of the label.

To examine the origin and fate of the short-lived component, the data of Figure V-14 are presented. In this the filter-retained TCA-precipitable material, the TCA-precipitable material in the low-speed centrifugation supernatant, and the sum of the two are shown. It can be seen that the initial rapid fall in the cell-associated counts was exactly correlated with the appearance of labelled macromolecules in the supernatant. Thereafter, there is apparently concomitant loss of TCA-precipitable counts from both compartments at the slow rate which characterizes the slow component for the 4 to 48 hour period of study.

The amount of the fast component in the medium is also seen to be very closely correlated with the loss from the cells, and is initially TCA-precipitable. Thus the fast component represents the transfer of macromolecules bearing the iodide label from the cells to the medium but the slow component includes a loss of TCA-precipitable counts from both compartments.

To evaluate the possibility that the initial loss of label from the surface of the cells and the subsequent slow rate of turnover of the cell surface components might reflect the death of a subset of cells, cell growth

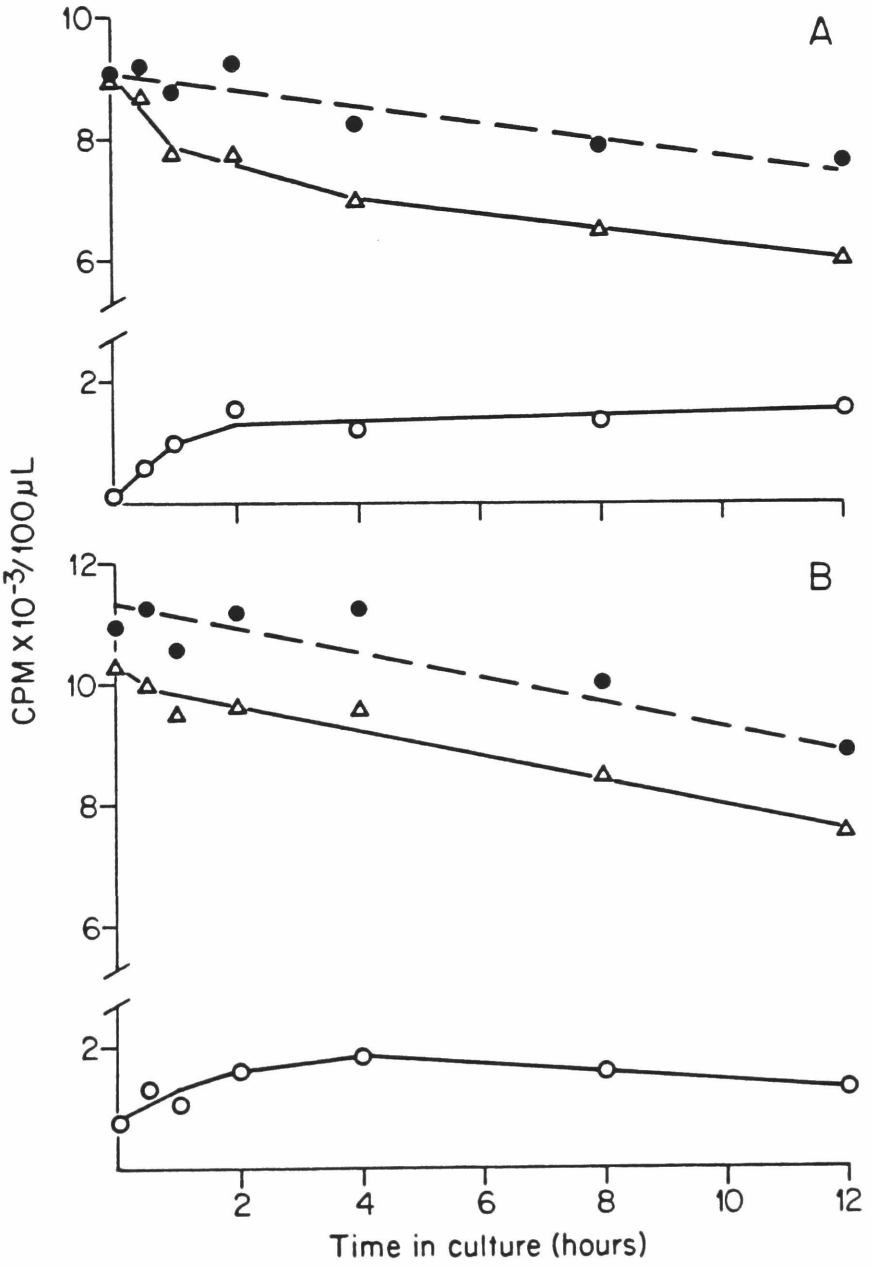
LEGEND: FIGURE V-14. ANALYSIS OF THE NATURE OF THE RAPIDLY TURNING-OVER IODINATED SPECIES OF 8866 CELLS.

The plots represent the filter-retained TCA-precipitable iodinated material, the low-speed supernatant, and the sum of the two for A: clone 34 and B: clone 4 cells.

Δ = counts retained by a GF/C filter washed with HBSS and then with TCA. It therefore represents large particulate, mainly cell-associated iodinated species.

o = TCA-precipitable counts in the supernatant after the cells were spun down at approximately 250 g x 5 minutes.

● = The sum of the two above components, as assessed for a 100 μl aliquot.


A
B
10
8
6
2
12
10
8
6
2
CPM X 10⁻³/100 µL
2
4
6
8
10
12
Time in culture (hours)

and viability were carefully monitored from the time of iodination onward. As shown in Table V-5, cell viability remained high throughout the 48 hours of the experiment. There was no significant change in cell viability in the first 4 hours, indicating that cell death could not account for the initial loss of iodinated macromolecules from the cells. Unfortunately, little cell growth occurred during the following 44 hours in this experiment. Similar failure to grow occurred in several additional experiments done in an effort to obtain better growth after iodination and processing (results not shown). Moreover, there was a small but significant decrease in cell viability during this time period. Thus it cannot be determined whether the slow decrease in cell-associated iodinated proteins reflects processes regulated by active cellular metabolism, the death of a small number of cells, or some combination of the two.

To determine whether there are differences in the rates of turnover of different iodinated species, cells harvested at the different time points after iodination were analyzed by autoradiography of SDS-polyacrylamide slab gels (Figure V-15). As is evident, all major iodinated species turn over at similar rates. The labeled proteins released into the growth medium could not be analyzed because of the vast excess of serum proteins present.

In summary, these findings show that there is no rapidly turning-over subset of surface proteins.

LEGEND: TABLE V-5. THE TURNOVER OF IODINATED SPECIES: CELL COUNTS AND VIABILITIES.

At each time point 100 microliters of cell suspension were taken with a disposable micropipette, mixed with an equal volume of 0.1% Trypan blue diluted in Hanks' Balanced Salt Solution, and pipetted up and down to disperse clumps. Aliquots were then counted in eosinophil counting chambers, and both live and dead cells scored. The four squares on the diagonal across the chamber were counted, and, if there were discrepancies greater than 10% between corners, a second chamber was counted. The total number of cells counted was always greater than 200.

TABLE V-5.

THE TURNOVER OF IODINATED SPECIES: CELL COUNTS AND VIABILITIES

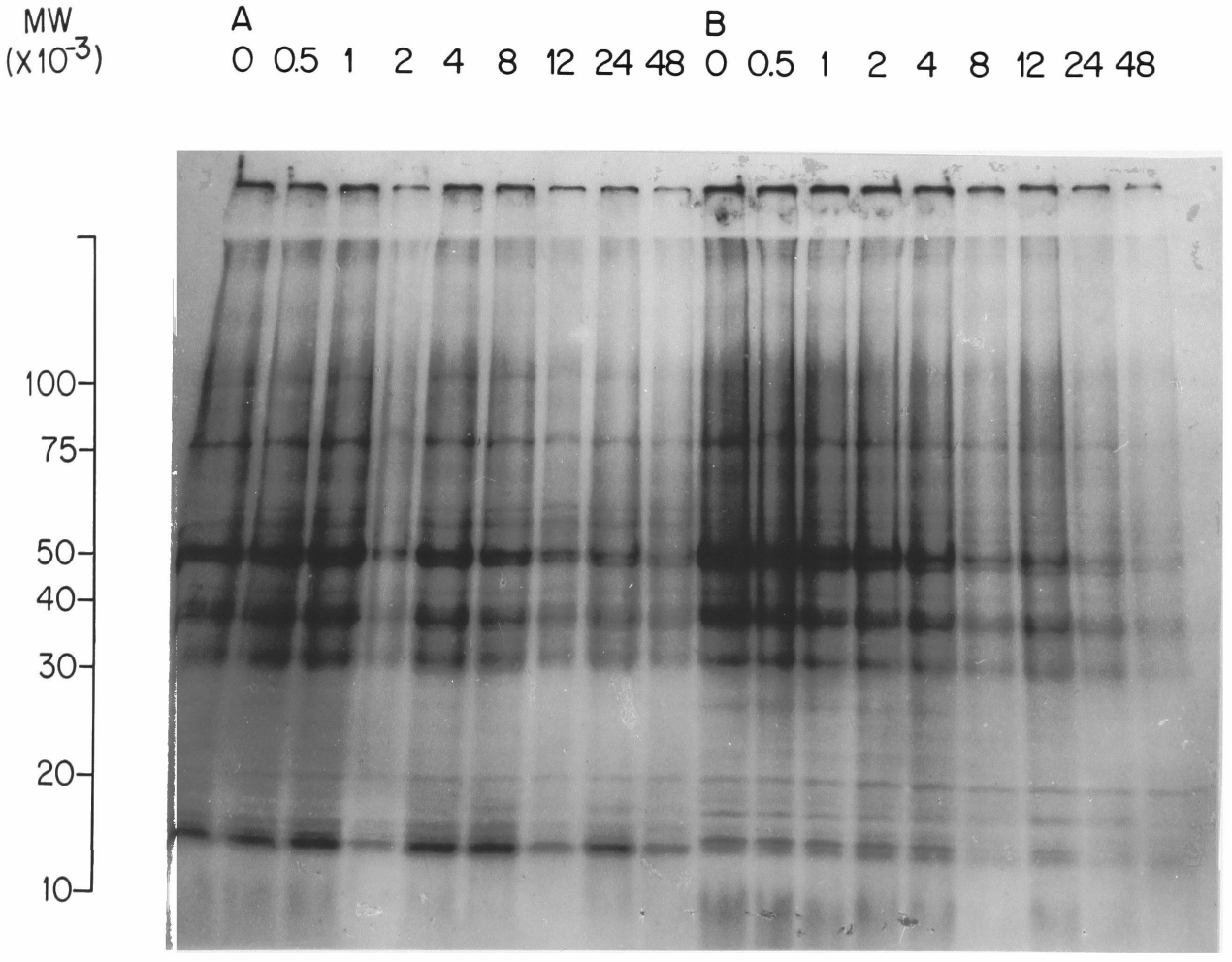
	TIME IN CULTURE (IN HOURS)								
	0	0.5	1	2	4	8	12	24	48
Clone 34 a Count*	0.59	0.78	0.72	0.80	0.63	0.65	0.54	0.51	0.77
Viability**	86.6	93.6	91.0	87.8	90.6	85.5	74.5	82.0	80.0
Clone 34 b Count	0.69	0.69	0.71	0.76	0.58	0.60	0.60	0.89	0.57
Viability	92.0	94.5	87.9	88.5	88.4	83.2	84.3	82.3	61.1
Clone 4 a Count	0.74	0.72	0.98	0.89	0.85	0.58	0.83	0.92	1.30
Viability	94.6	94.1	94.4	90.2	91.4	89.3	86.1	73.4	84.6
Clone 4 b Count	0.85	1.08	0.96	0.86	0.86	0.79	0.79	1.04	1.07
Viability	95.3	96.8	94.5	87.6	94.5	89.0	90.2	86.4	84.2

* = Total cell count in millions of cells per milliliter.

** = Percentage of trypan blue negative cells out of the total cell population.

LEGEND: FIGURE V-15. ANALYSIS OF IODINATED SPECIES OF 8866 CELLS AFTER CULTURING.

This Figure is the autoradiogram of a 7.5-15% gradient slab gel (after the method of Neville (1971)),with a 6% stacking gel. At each time point, after 3 x 100µl samples had been taken for analysis, the remainder of the 1 ml culture was washed with HBSS,stored frozen, and then boiled in SDS and 2-mercaptoethanol. In Part A are the pooled samples of Clone 34 duplicates. In Part B are pooled aliquots of Clone 4 duplicates. The numbers over the slots refer to the number of hours in culture after being placed into Dulbecco's medium with 10% FCS at 37°C.


MW
(X10-3)
A
0 0.5 1 2 4 8 12 24 48
B
0 0.5 1 2 4 8 12 24 48
100
75
50
40
30
20
10

CHAPTER VI

THE SEARCH FOR THE C3 RECEPTOR

A. INTRODUCTION

Having failed to identify the receptor directly by using the combination of the lactoperoxidase-mediated iodination and the positive and negative clones, an alternative strategy using the affinity of the receptor for its legend. These experiments were predicated on three major assumptions which could not be independently tested before beginning the study:

1. The receptor can be labeled with ^{125}I by the glucose oxidase/ lactoperoxidase method.
2. The affinity of the receptor for the ligand is sufficient to resist the effects of solubilization in non-ionic detergents.
3. The off-rate of the interaction between ligand and receptor is slow enough to allow isolation of the complex.

In order to be able to use an affinity technique, there are three basic requirements. The first is to have a biologically active ligand firmly bonded to a particle. In these studies, the ligand was derived by activating complement components in whole serum, rather than by converting isolated C3 to an active cleaved product, and then bonding it to a particle. Biological activity was assessed by examining the ability of the C3 coated particles to rosette. In general, antibody was reacted with aldehyde fixed erythrocytes, and then mixed with a complement source. As will be demonstrated, the complement bound to the red cells under these conditions was resistant to elution with non-ionic detergents.

The second requirement is to have an inert particle which does not bind cell proteins unless the particle is coated with the specific ligand. It is preferable that the particle does not have interstices of such size as to trap proteins, as is the case with some of the Sephadex and Sepharose beads. Additionally, the particle should be small enough that many particles can bind to the surface of a lymphocyte, thereby engaging significant numbers of receptors.

Thirdly, one needs to have a means of dissociating the membrane to yield either single proteins or limited protein complexes without disrupting the ligand-receptor bond. The methods used in these studies were non-ionic detergents known not to disrupt antibody-antigen complexes in salt solutions of approximately physiological ionic strength and pH. In some cases sonication was used, although the likelihood of this technique alone breaking membranes down to single protein species is remote. However, it was hoped to produce small enough pieces of membrane that enrichment of a particular protein species might be detectable.

Most of the experiments were done by a modification of a system that had hitherto proved effective. That is, sheep red cells were used as the target particle, but in these experiments the cells were first fixed, and then coated with antibody and complement. Two different approaches were then tried. 1. The target cells were mixed with lymphocytes, rosetted, and then disruption attempted, 2. The lymphocytes were lysed, the supernate cleared of particulate material by high speed centrifugation, and then this supernatant was mixed with target cells.

In the presentation to follow it will be shown that no success was achieved in identifying a probable C3d receptor. Therefore, rather than

review many negative experiments, an account of the methods and results will be given in summary form, with some illustrative examples presented in detail.

B. METHODOLOGY

1. Glutaraldehyde-fixed Sheep Erythrocytes.

These experiments were initiated with a batch of erythrocytes fixed by Dr. Celso Bianco, and stored for many months. When tested as washed test particles, they showed occasional adherent erythrocytes bound to lymphocytes, but rarely more than one or two, and only to a small percentage of lymphocytes. Pilot studies were carried out on this batch, and showed they could be sensitized with IgM directed against sheep erythrocytes, and that they would subsequently fix complement. Once complement was fixed, the particles rosetted well, although not as strongly as fresh erythrocytes: this may be correlated with the fact that a lower dose of IgM agglutinated fixed as opposed to fresh erythrocytes, and hence a smaller amount of IgM was added per cell. With this pilot batch of cells it was also found that the fixed erythrocytes were resistant to boiling in 10% SDS, and hence could be applied to the surface of polyacrylamide gels without protein overload resulting from the large amount of hemoglobin added.

After most of this test batch had been used, many fixation protocols were tried to produce the same fixed erythrocyte, but a recurrent problem was non-specific adherence of the fixed cells to lymphocytes. In an attempt to overcome this, a variety of agents known to bind excess aldehyde groups were used without success. These included glycine, Tris-glycine buffer,

mixed amino acids in the form of culture medium, bovine serum albumin, and heat inactivated serum. No totally negative preparation was ever achieved. A typical result is shown in Table VI-1, where the results of Clone 34 show about one fourth of the percentage of lymphocytes binding non-sensitized as opposed to sensitized red cells, but binding a significantly smaller number of target cells.

Arising incidentally out of attempts to test E_{glu} preparations was an anomaly which remains unexplained. In two tests on one target cell preparation,different lymphoid cell clones were used, and discordant results were obtained. A summary of a more systematic survey of the anomaly is shown in Table VI-2. This extends Table VI-1, but also shows that receptor positive cells consistently bound more glutaraldehyde fixed red cells than did negative lines. Evidence that this correlation of rosette positivity and ability to bind glutaraldehyde fixed cells is significant is supported by the evidence in Part B of Table VI-2. Here there is a time lapse between measurements during which the negative lines experessed 20-30% positivity, and the binding of fixed red cells rose to the same extent. In Part A of the same table is shown the result of prior trypsinization of the red cells before fixation. It is known that trypsinization removes the surface species responsible for the T cell rosetting. However, in this case, no change in binding resulted from this treatment. In spite of this phenomenon, it was decided to use the glutaraldehyde fixed erythrocyte as a test particle with the particles without antibody or with antibody alone as controls for the E_{glu}IgM.C.

LEGEND: TABLE VI-1. ROSETTING WITH GLUTARALDEHYDE-FIXED ERYTHROCYTES.

The Table presents data of the rosetting of glutaraldehyde fixed erythrocytes compared with fresh erythrocytes. Testing was done by incubating lymphoid cells in Dulbecco's medium with a final concentration of 5% fetal calf serum at 37°C for approximately 1 hour with an erythrocyte to lymphoid cell ratio of approximately 200:1.

TABLE VI-1.

ROSETTING WITH GLUTARALDEHYDE-FIXED ERYTHROCYTES

LINES TESTED	% LIVE CELLS ROSETTING			
	E_{Glu}	$E_{Glu}IgM$	$E_{Glu}IgM.C$	E.IgM.C
Cl 34	20 (4-8)*	22 (4-8)	86 (> 8)	97 strong
Cl 23	3 (weak)	1 (weak)	5 (2 strong)	8

* = Numbers of erythrocytes bound per lymphocyte.

LEGEND: TABLE VI-2. EVIDENCE FOR DIFFERENTIAL BINDING OF GLUTARALDEHYDE-FIXED ERYTHROCYTES TO COMPLEMENT-RECEPTOR BEARING LYMPHOCYTES.

The Table presents the results of rosetting two positive clones, and two initially entirely negative clones, with a variety of target cells. Note that the cells were kept in continuous culture between the two testings, and that the percentage of receptor-positive cells in the initially negative clone has increased with time. The abbreviations for the target cells, which are all ovine, are:

E = native erythrocytes.

E.IgM.C = erythrocytes sensitized with rabbit IgM anti-sheep erythrocyte and mouse serum as a source of complement.

E^{Emul}_{Glu} = erythrocytes fixed in 2% glutaraldehyde in phosphate buffered saline for 15 minutes at 37°C, washed in buffered saline, in Dulbecco's medium, and in 2% bovine serum albumin, then stored in buffered saline containing 0.2% sodium azide for two weeks. At that time the cells were washed once in 1% Emulphogene in phosphate buffered saline, then washed twice in buffered saline.

E^{Emul}_{Glu}.IgM = same cells as above treated with a sub-agglutinating dose of rabbit IgM anti-sheep erythrocyte.

E^{Emul}_{Glu}.IgM.C = the cells above exposed to a 1:20 dilution of NCS mouse serum at 37°C for 30 minutes.

E^{Tryp}_{Glu} = erythrocytes at 5% concentration in Hanks' Balanced Salt Solution were treated with an equal volume of 0.2% Trypsin in the same solution for 60 minutes at 37°C. They were then washed, and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate at pH 7.2 overnight in the cold. They were subsequently washed multiply with HBSS, Dulbecco's medium, and stored in phosphate buffered saline containing 0.2% sodium azide.

TABLE VI-2.

EVIDENCE FOR DIFFERENTIAL BINDING OF GLUTARALDEHYDE-FIXED ERYTHROCYTES TO COMPLEMENT-RECEPTOR BEARING LYMPHOCYTES

TARGET CELLS	PERCENTAGE AND STRENGTH OF ROSETTING			
	Clone 34	Clone 4	Clone 23	Clone 36
A. 11.iv.1975				
E	0	0	0	0
E.IgM.C	100,++	100,++	< 1,+	∿ 2,+
E^{Emul}_{Glu}	96,++	96,++	45,+	34,+
E^{Emul}_{Glu}. IgM	85,++	95,++	21,+	28,+
E^{Emul}_{Glu}. IgM.C	89,++	90,++	6,+	10,+
E^{Tryp}_{Glu}	85,++	94,++	2	1
E_{Glu}	75,++		10,+	
B. 30.v.1975				
E	0	0	0	0
E.IgM.C	100,++	100,++	10,++	30,++
E_{Glu}	85,++	85,++	20,++	30,++

As indicated in the introduction, a variety of experiments was done. Test cells were tried after pre-washing in detergent. Cell lysates were used after high speed centrifugation to remove particulates. Two non-ionic detergents, NP-40 and Emulphogene at 0.5, 1 and 2% were used as solubilizing agents. If rosetting was performed first, and then detergent used to lyse the cells, it was found that the nuclei pelleted with the target cells, and grossly contaminated the pellets. It was therefore necessary to develop a means of separating the nuclei from the fixed red cells. This was done by labeling an aliquot of cells with ^{3}H-thymidine, lysing in detergent and then placing the lysate on a Hypaque gradient. The erythrocytes banded sharply in a 10-45% Hypaque gradient, and the nuclei pelleted to the bottom, as indicated by the radioactivity. If detergent were present in the gradient, a phenomenon found even without the detergent became more marked: the bare nuclei tended to be disrupted in the high salt solutions, and the chromatin strands resulting clumped the red cells together. Rather than use DNAse, which would have necessitated a 37°C incubation, sonication in the cold was used on some occasions to break up these strands. That this had some effect was shown by the marked decrease in the intensity of Coomassie Blue stained bands on gels in the 10-15,000 Dalton region. However, at no time was there any evidence of a significant increase in binding of ^{125}I-label to particles coated with complement over controls, and there was no evidence of a change of pattern in the autoradiogram of the E.IgM.C versus the E and E.IgM.

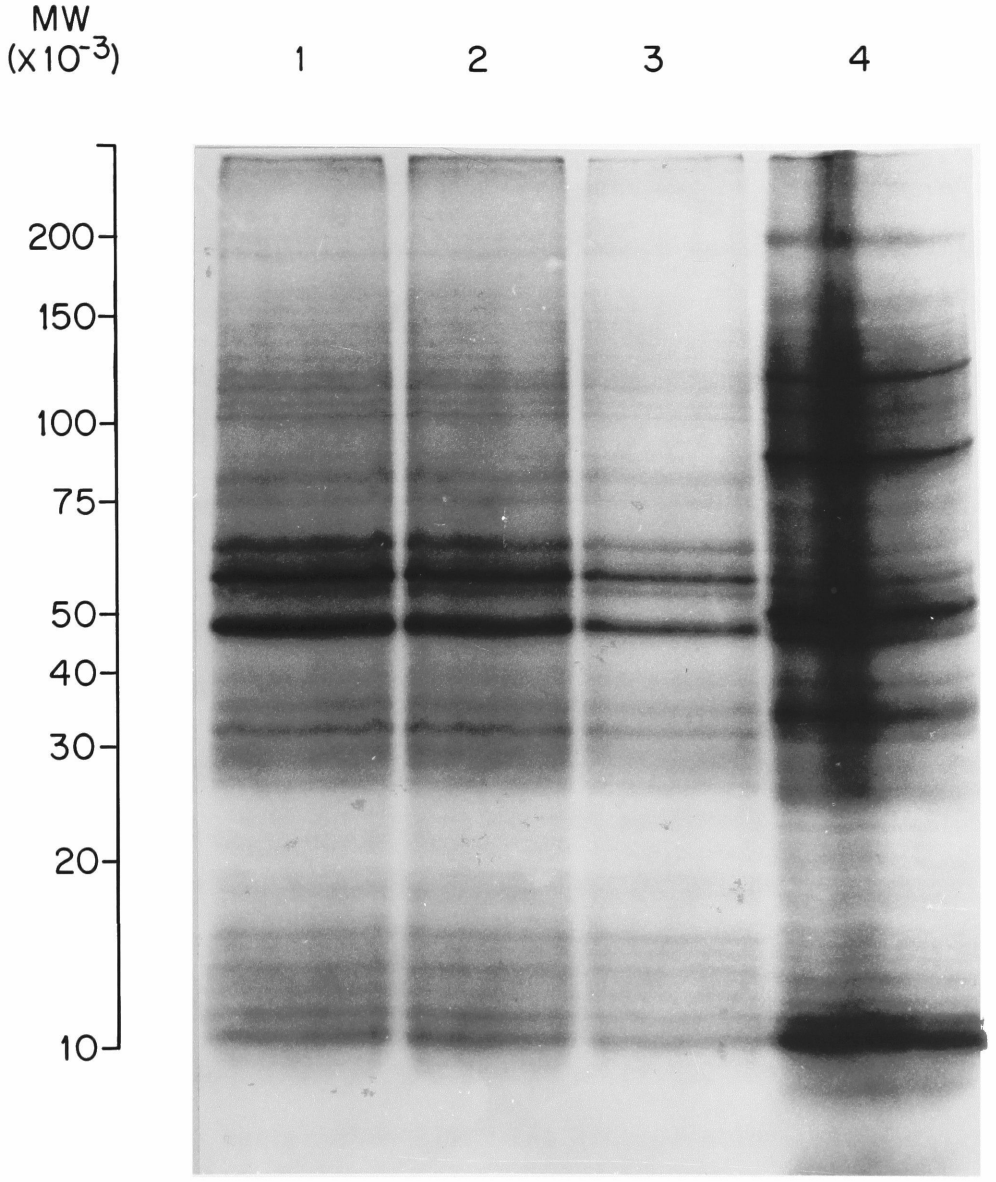
Two illustrative examples of experiments are presented in detail. The first shows the use of rosetting followed by lysis, while the second utilizes lysis followed by selection from the lysate.

a. Iodinate, Rosette, then Lyse Experiment.

On 19.v.1975 10^7 cells of Clone 34 were washed by the step-gradient protocol, and iodinated with 500 microcuries of sodium 125-iodide, 10mU LPO and 10 mU GO. A parallel procedure was done without LPO, as a control for iodination. The control incorporated 0.003% of label into TCA-precipitable counts, while the test mixture incorporated 8.17%. The cells were washed by the second step gradient, pelleted, and resuspended in 6 ml of Dulbecco's medium containing 1% FCS. The cells were 90% viable. Aliquots of 2 mls were added to tubes in which 1 ml of 1% solutions of E_{glu}, E_{glu}.IgM or E_{glu}.IgM.C had been pelleted. The two cell types were mixed by pipetting, and allowed to rosette at 37°C for 2 hours. At the end of that time the rosetting percentages were 86, 83 and 89 respectively, and of approximately equal strength (i.e. about the same numbers of red cells/lymphocyte). After pelleting, the rosettes were washed once with HBSS, and then suspended in 2 ml of 0.5% NP-40 in sodium RSB at room temperature. Under phase microscopy the nuclei were seen to be clean and intact, and the red cells well dispersed (i.e. not showing aggregation as if bound together by membrane fragments). After 5 minutes the cells were pelleted and resuspended in 2 ml of the same buffer-detergent, and then overlaid on gradients formed from 5 ml of 10% Hypaque and 5 ml of 45% Hypaque adjusted with sodium RSB concentrates to be equal to sodium RSB. The gradients were spun at 20,000 g_{max} for 30 minutes at 10°C. The erythrocyte bands, in which the cells tended to be clumped together by chromatin strands, were washed in

LEGEND: FIGURE VI-1. ANALYSIS OF MATERIAL OBTAINED BY ROSETTING AND THEN NP-40 LYSIS.

An autoradiogram of a 7.5-15% gradient slab gel with 2.5% stacking gel. The samples demonstrate the iodinated species precipitated with glutaraldehyde-treated erythrocytes in a band in 10-45% Hypaque gradients buffered with NaRSB, and containing 0.5% NP-40. Track 1 was obtained with E_{glu}.,Track 2 was obtained with E_{glu}.IgM, Track 3 with E_{glu}.IgM.C, and Track 4 is a sample of whole cells after iodination.


MW
(x10-3)
1
2
3
4
200
150
100
75
50
40
30
20
10

RSB-NP 40, aspirated, suspended in 20 microliters of 10% SDS, and stored frozen at -20°C for gels, as were control samples of cells from the iodination mixture.

The samples were applied to 7.5-15% gradient slab gels, and the 8 day autoradiogram is shown in Figure VI-1. This shows striking enhancement of some bands, and loss of others relative to the whole cell mixture, but no difference between the slots derived from the different target cells. The major band is not merely the major band of the whole cell pattern displaced by some effect of the target cells. This was demonstrated by mixing whole cells with fixed cells, and running them on a gel next to a control slot of iodinated cells alone. This is illustrated in Figure VI-4. No mobility change can be attributed to the presence of the fixed cells. The molecular weight estimates of the major bands are 11,000, 35,000, 40,000, 56,000 and 62,000, with many minor bands.

b. Iodinate. Lyse, and Selectively Precipitate Experiment.

The E_{glu} used in this experiment were made on 11.iv.1975, and their testing is shown in Table VI-2, Part B, as the Emulphogene washed fixed erythrocytes. On the same date four clones of 8866 were iodinated in a parallel set of mixtures containing 10^7 cells, 500 microcuries of sodium 125-iodide, 10 mU of LPO and 10 mU of GO. Post-iodination cell viabilities were better than 94%. The incorporations of label into TCA-precipitable counts were 13.19, 13.57, 28.37 and 28.89% respectively, unusually high values. Run at the same time as controls were samples of BSA with and without LPO. The control incorporation was 0.056%, while the test was 68.4%, which were values within the usual range. The cells were treated by the standard two Ficoll-Hypaque gradients. After the wash step,

the cell pellets were resuspended in 2 ml of 1% Emulphogene in PBS for 5 minutes, then spun at 35,000 rpm in a 40 rotor in a Beckman ultracentrifuge ($100,00g_{max}$) at 4°C for 1 hour. The supernate was stored frozen at -20°C. The pellets were resuspended in 2 ml of the same detergent-buffer mixture, and spun again, then stored frozen also for gels.

The following day the supernates were thawed, and 200 microliters added to the pellets of 100 microliters of 1% solutions of the three target cell types. The mixtures were suspended and incubated 15 minutes at room temperature with occasional shaking. They were then pelleted in a micro-centrifuge, and the supernates collected. The pellets were washed twice with cold 1% Emulphogene in PBS with 0.02% sodium azide, and then stored frozen for gels.

In Figure VI-2 is shown the autoradiogram of the 5-15% gradient slab gel of the pelletable material. The Figure shows four sets of triplicates of E, E.IgM, and E.IgM.C precipitated aliquots. No differences can be seen within the sets of three. The samples are arranged with two positive clones on the left and two negative clones on the right. The major finding is of the qualitative similarities between them, although there may be a difference in the region of 44,000 Daltons. However, this was not a consistent finding in other preparations. The molecular weights of the major species are 11,000, 24,000, 45,000, 57,000, 58,000 and 130,000. Note that there are species at 11,000, 45,000 and 56,000 in both Figures VI-1 and VI-3.

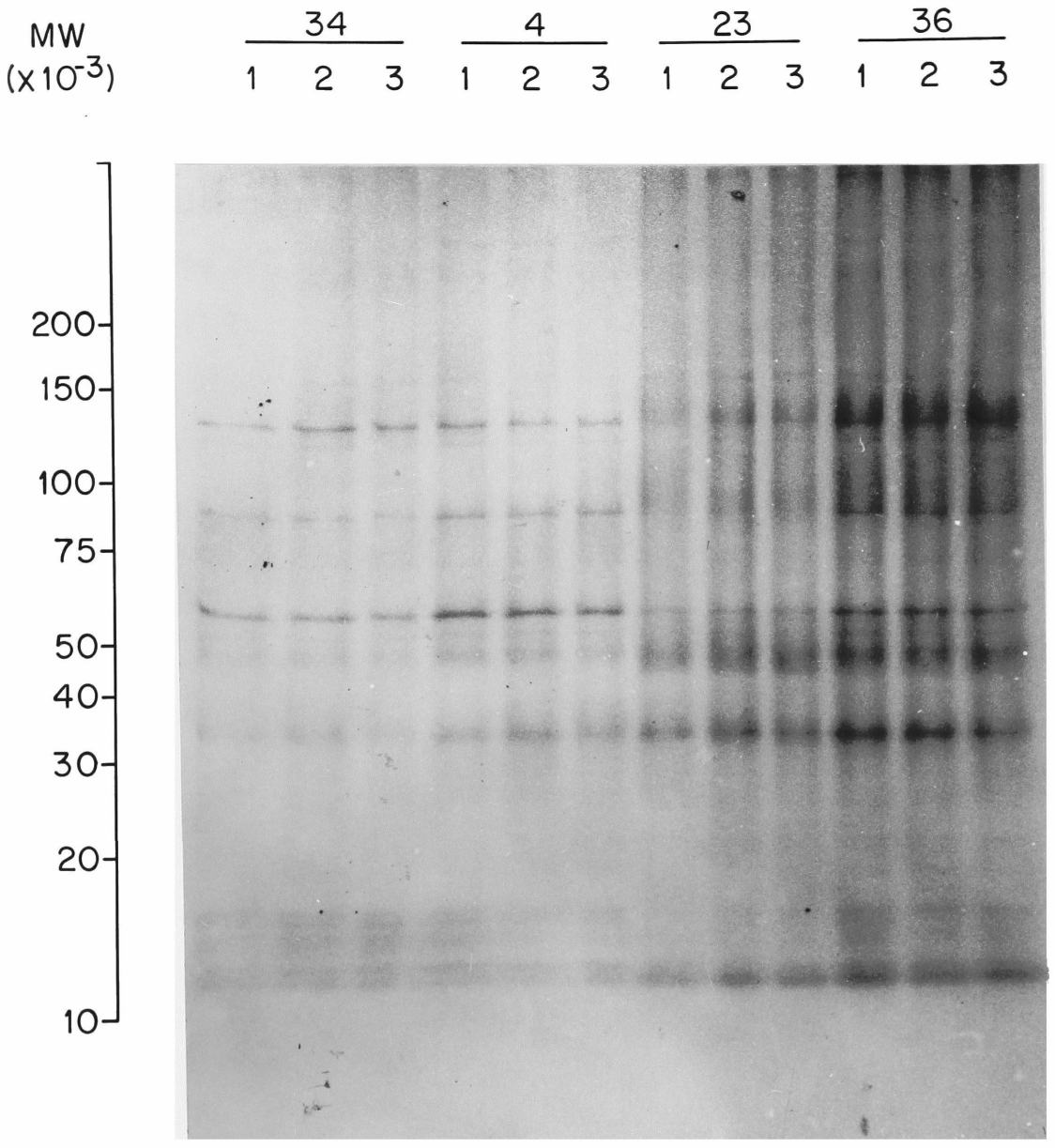
In Figure VI-3 is presented the autoradiogram of the gel of the material left after the selection process, which should reveal any selective depletion. Within the triplets of samples selected by E, EIgM and E.IgM.C there are no differences. The presence of a band in the 15,000 Dalton range

LEGEND: FIGURE VI-2. ANALYSIS OF MATERIAL OBTAINED BY SELECTIVE PRECIPITATION FROM AN EMULPHOGENE LYSATE OF IODINATED CELLS.

Cells of the clones listed at the top of the Figure (34, 4, 23 and 36) were iodinated, gradient washed, lysed in 1% Emulphogene in PBS, and clarified at 100,000g for 1 hour at 4°C. Equal aliquots of the supernatant were then mixed with glutaraldehyde-treated sheep erythrocytes (E_{glu}), E_{glu}IgM, or E_{glu}IgM.C for 15 minutes at room temperature. The erythrocytes were pelleted and washed in 1% Emulphogene/PBS x 2, then boiled in SDS and 2-mercaptoethanol and applied to gels.

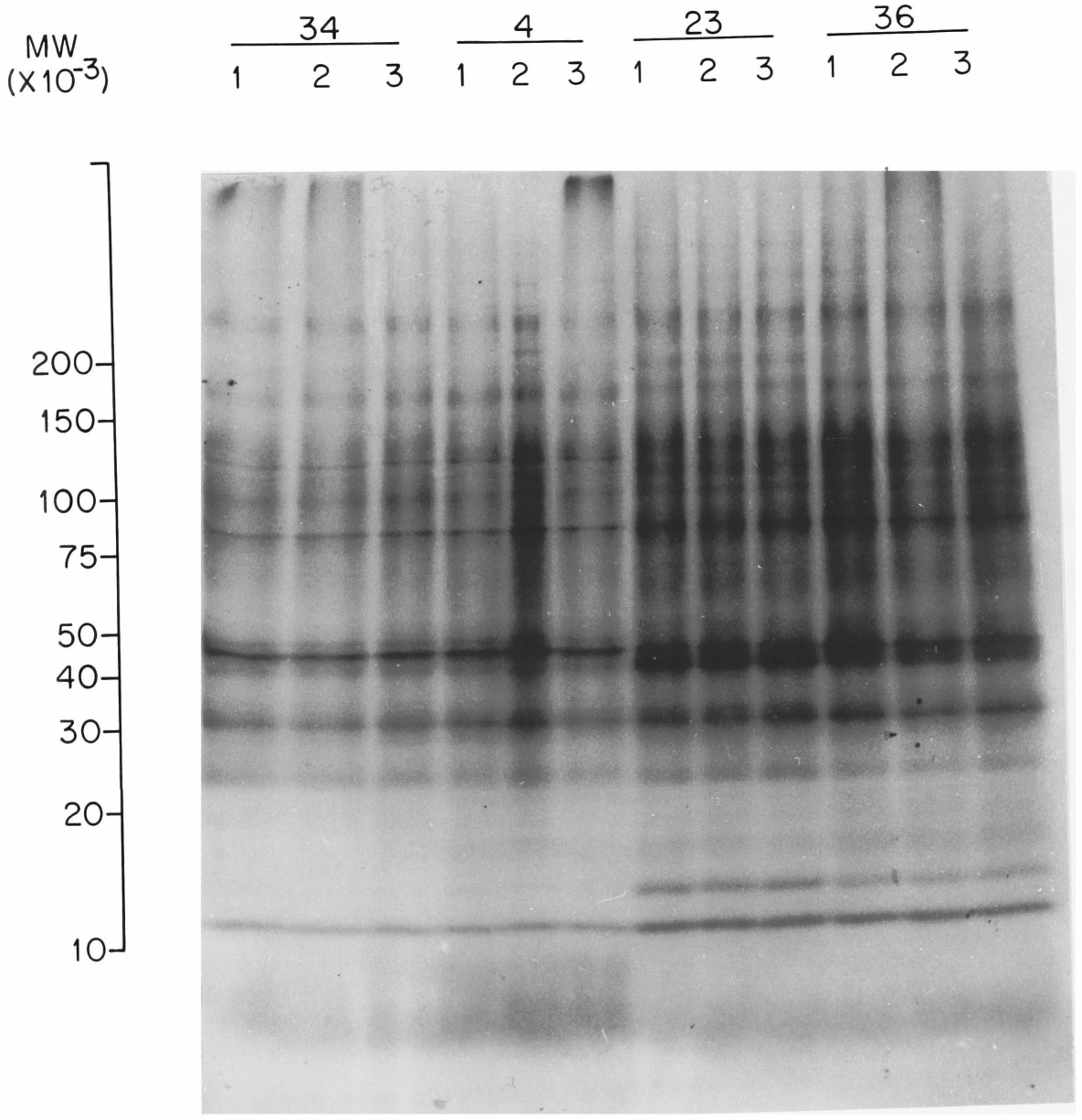
1 = E_{glu} 2 = E_{glu}IgM 3 = E_{glu}IgM.C

The Figure is an autoradiogram of a 5-15% gradient slab gel, 25% stacking gel.


MW
(x10-3)
34
4
23
36
1 2 3 1 2 3 1 2 3 1 2 3
200
150
100
75
50
40
30
20
10

LEGEND: FIGURE VI-3. ANALYSIS OF MATERIAL REMAINING AFTER SELECTION FROM EMULPHOGENE LYSATE OF IODINATED CELLS.

The Figure is an autoradiogram of aliquots of the supernatant fluid from the samples depicted in Figure VI-2 applied under the same conditions to a similar gel, i.e. 5-15% gradient slab gel with a 2.5% stacking gel. The designations at the top of the gel are the same as in Figure VI-2.


MW
(X10-3)
34
1 2 3
4
1 2 3
23
1 2 3
36
1 2 3
200
150
100
75
50
40
30
20
10

is seen in the negative clones, and is barely detectable in the positive clones. Otherwise the samples are indistinguishable. Thus, there is no evidence of specific depletion.

When experiments of this type were done, the total counts precipitated by the different types of particles were counted before the samples were boiled for gels. There was no consistent pattern suggesting an increase in labeled species binding to the complement coated particles.

2. Formaldehyde-fixed Sheep Erythrocytes.

Erythrocytes were fixed by the protocol in Methods, and left fixing for up to two months in the cold. After extensive washing, including Dulbecco's medium, 10% fetal calf serum, and many changes of HBSS, there was still significant binding to both positive and negative cell lines. In the most extensive study it was calculated that the non-specific binding was approximately 5% of the specific binding. This technique was not used further.

3. Zymosan Particles.

Zymosan, the cell wall ghosts of Brewer's Yeast cells after extensive treatment to remove all components except the carbohydrate polymer, was bought from suppliers who used the protocol of Pillemer and colleagues (1956) in its preparation. Following the work of Pillemer it has been established that the zymosan particles fix complement by the alternative pathway during which some complement components become adherent to the particles. Among these is cleaved C3. Zymosan was therefore assayed by two functional tests. The first was the reduction in hemolytic titer of

fresh human serum after incubation with zymosan, and the second was the conversion of the zymosan from particles with absolutely no affinity for 8866 cells into rosetting particles. A variation in batches of zymosan was noted, and affected both assays similarly. One satisfactory batch was obtained and used. It was found that, in the preparation of the particles, similar results were produced by particles suspended by autoclaving, boiling, or simple washing combined with mild sonication to disperse clumps.

No preparation of zymosan particles showed any binding to lymphoid cells, and the same was true for zymosan treated with heat-inactivated serum. In Table VI-3 is illustrated a test of zymosan treated with fresh serum in comparison with standard E.IgM.C. The percentages of cells showing rosetting are clearly similar, although, in all cases, the number of zymosan particles binding per lymphoid cell was never more than ten, and always less than the number of E.IgM.C. binding.

Two attempts were made to augment the rosettability of zymosan particles by utilizing the classical pathway of complement fixation in addition to the alternative pathway. An anti-mannan antibody was provided by courtesy of Dr. Peter Owen of Dr. M.J.R. Salton's laboratory at N.Y.U. School of Medicine. When this was mixed with the particles prior to addition of the complement source, no enhancement of rosetting in terms of zymosan particles/8866 cell was noted.

As an example of a zymosan-selective experiment, that performed on 30.vii.75, is presented. The target cells were made on 29.vii.75, using washed zymosan incubated with a 1:5 dilution of fresh or heat-inactivated

LEGEND: TABLE VI-3. THE BINDING OF ZYMOSAN-COMPLEMENT COMPLEXES TO LYMPHOID CELLS.

The Table shows the rosetting of zymosan particles exposed to whole fresh mouse serum at a 1:20 dilution in Hanks' Balanced Salt Solution for 60 minutes at 37°C as compared with zymosan alone, and with E.IgM.C made in the usual way with mouse serum. Rosetting was carried out for 1 hour at 37°C in Dulbecco's medium containing 5% heat-inactivated fetal bovine serum. Not shown in the Table is that, as in all other such experiments, the number of ZC binding per lymphoid cell is considerably less than the number of E.IgM.C bound to the same cell type. There was no evidence of any binding of untreated zymosan particles.

TABLE VI-3.

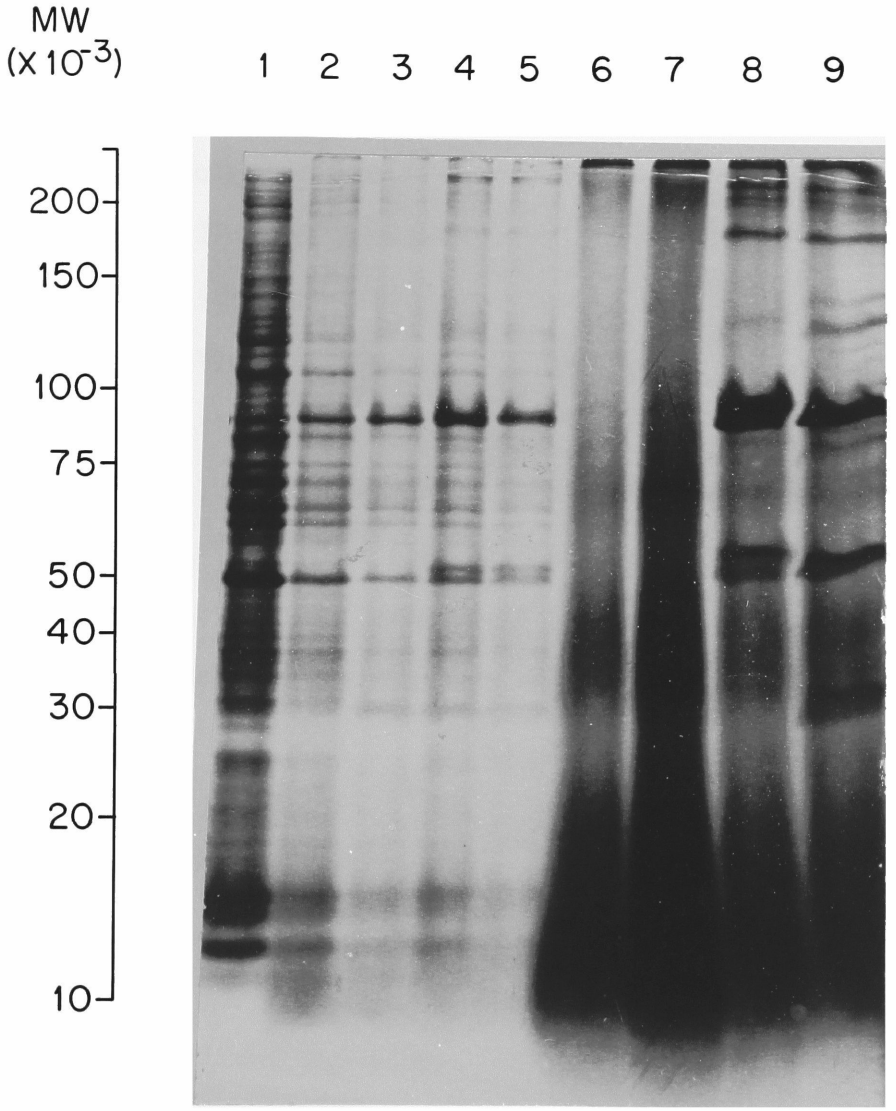
THE BINDING OF ZYMOSAN-COMPLEMENT COMPLEXES TO LYMPHOID CELLS

		PERCENTAGE OF LIVE CELLS ROSETTING		
TARGET CELLS:		Z	ZC	E.IgM.C
LYMPHOID CELLS:	Clone 34	0	100	100
	Clone 4	0	95	100
	Clone 23	0	10	5
	Clone 36	0	50	67

LEGEND: FIGURE VI-4. ANALYSIS OF SELECTION OF PROTEIN SPECIES OF 8866 CELLS USING ZYMOSAN PARTICLES.

The Figure is a Coomassie Blue stained 5-15% gradient slab gel, with a 2.5% stacking gel. Cells of Clone 34 were iodinated, gradient washed, and then aliquots taken for incubation with Zymosan, Zymosan exposed to serum heat inactivated at 56°C for 30 minutes, or Zymosan treated with fresh human serum, both for 1 hour at 37°C. After approximately 1 1/2 hours, aliquots were taken and frozen. The remainder of each was pelleted, and samples of Z, Z-HI, and Z-C were resuspended in PBS. The samples were sonicated until all cells and nuclei were broken. The Zymosan was then pelleted through a cushion of 45% Hypaque buffered with NaRSB and washed with PBS/0.1% NP-40 or PBS, according to the initial treatment. Washing was repeated four times to achieve constant counts. Some sticking together of particles, due to DNA, was observed even after sonication. The pelleted Zymosan was then boiled in SDS and 2-mercaptoethanol, as were the total samples.

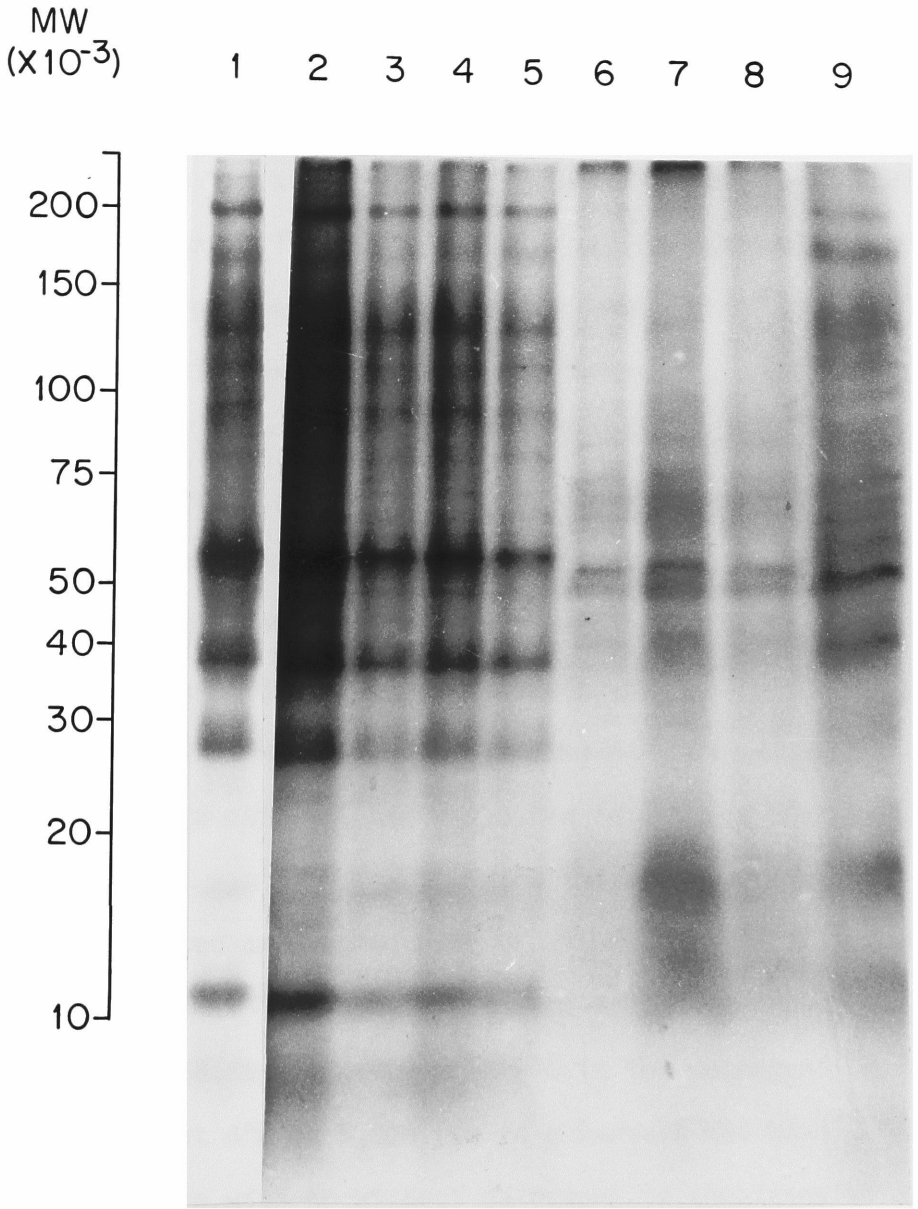
Track 1 = whole iodinated cells. 2,3,4 and 5 are aliquots of cells and Z, Z-HI, Z-C and Z-C respectively. Tracks 6,7,8 and 9 are the material pelleted after sonication with Z, Z-HI and Z-C treated with NP-40, and Z-C with PBS alone.


MW
(X10-3)
1 2 3 4 5 6 7 8 9
200
150
100
75
50
40
30
20
10

LEGEND: FIGURE VI-5. ANALYSIS OF SELECTION OF IODINATED SPECIES OF 8866 CELLS USING ZYMOSAN PARTICLES.

This Figure is an autoradiogram of the preceding Figure, VI-4. The slots are identical:

1. Whole cells
2. Cells plus Z
3. Cells plus Z-HI
4. Cells plus Z-C
5. Cells plus Z-C
6. Pelleted Z from NP-40 and sonication
7. Pelleted Z-HI from NP-40 and sonication
8. Pelleted Z-C from NP-40 and sonication
9. Pelleted Z-C from PBS and sonication


MW
(X10-3)
1
2
3
4
5
6
7
8
9
200
150
100
75
50
40
30
20
10

human serum prepared on the same day. When tested for rosetting, the zymosan (Z) and zymosan-heat inactivated serum (ZHI) showed no adherence, while the zymosan-fresh serum (ZC') adhered strongly to Clone 34 cells. On the 30.vii.75 three samples of approximately 10^7 Clone 34 cells were iodinated, one lot being without LPO. The amount of $Na^{125}I$ used was 1mCi per sample, with the usual 10 mU of each of the enzymes in the final 1ml incubation mixture. The incorporations were 0.054% for the control, and 5.53 and 7.34% for the two mixtures with LPO. After the gradient wash, the lymphoid cells were resuspended in Dulbecco's medium containing 1% FCS, and divided into four equal aliquots. These were added to pellets of Z, ZHI or two separate tubes of ZC'. The aliquots were gassed with 10% CO_2 and gently rocked in the warm room (37°C). After incubation, aliquots of each were pelleted and frozen for gel samples. The rest were pelleted, and three resuspended in 2 ml of PBS/2% NP-40, while the duplicate ZC' was merely suspended in HBSS. After standing at room temperature for five minutes, all were chilled, and sonicated to destroy the DNA clumping. Phase microscopy was used to determine the absence of nuclei. The samples were then pelleted and washed with or without detergent according to the earlier treatment. The suspensions were pelleted through 45% Hypaque buffered with RSB, and containing NP-40 in the detergent treated samples, for 10 minutes at 4°C at 5,000 rpm in a Sorvall HB4 head. The pellets were washed again with or without detergent to constancy of counts in the pellets (six times). Fifty microliters of 10% SDS were then added to the pellets, and they were frozen at -20°C until used on gels. In Figure VI-4 is shown the Coomassie Blue stained pattern of the 5-15% gradient slab

SDS-polyacrylamide gel which gave rise to the autoradiogram presented as Figure VI-5. The gel shows that the presence of the zymosan particles did not affect the electrophoretic mobility of the proteins of the cells.

The additional bands of Tracks 8 and 9 are the results of the fixation of complement-components to the particles.

In Figure VI-5 are shown the autoradiographic patterns of whole cells, cells plus target particles, and the material selected by particles. This demonstrates no changes in pattern resulting from the use of non-ionic detergent, no changes due to the different types of test particles, and confirms the fact that this type of affinity method has selected out, as its major products, iodinated species other than the major species of the whole cell preparation, but that the selection was not related to the presence or absence of the critical probe, C3d. The major proteins identified are 11,000, 21,000, 26,000, 45,000, 58,000, 67,000, 70,000 and a series of bands between 70,000 and 100,000 Daltons. Thus, comparing these results with the glutaraldehyde fixed cells, one finds that there are certain bands in common: at about 11,000, 25,000, 45,000 and 57,000 being the most marked. As with the main patterns of whole cells, the high molecular weight bands are generally weak, and are beyond the range of marker proteins, so that inter-gel comparison is difficult, but no major band in the greater than 100,000 Dalton class is regularly seen.

4. Specifically Cross-linked Beads.

Attempts were made to use solid-phase immunoabsorption by means of antibody covalently linked to Sepharose beads as used by Gottlieb et al.,

(1975). A model system was attempted, utilizing a rabbit serum with known activity against human beta-2-microglobulin, and the immunoglobulin fraction of a high titer sheep serum against rabbit immunoglobulin. No evidence of specific precipitation was obtained. Other solid-phase covalently linked systems also tested were polystyrene beads, and polyacrylamide beads (P2) aminoethylated by the method of Inman and Dintzis (1969) and then coupled to the IgG fraction of sheep anti-rabbit immunoglobulin by the method of Weston and Avrameas (1971). The latex beads were rendered active by incubation in cobra venom factor in serum free solution, and then addition of fresh serum as a complement source. This system rendered them specifically able to bind to complement receptor bearing cells, but only weakly. The ability of the polyacrylamide beads to bind rabbit immunoglobulin was also weak. Neither method was effective as a selective agent.

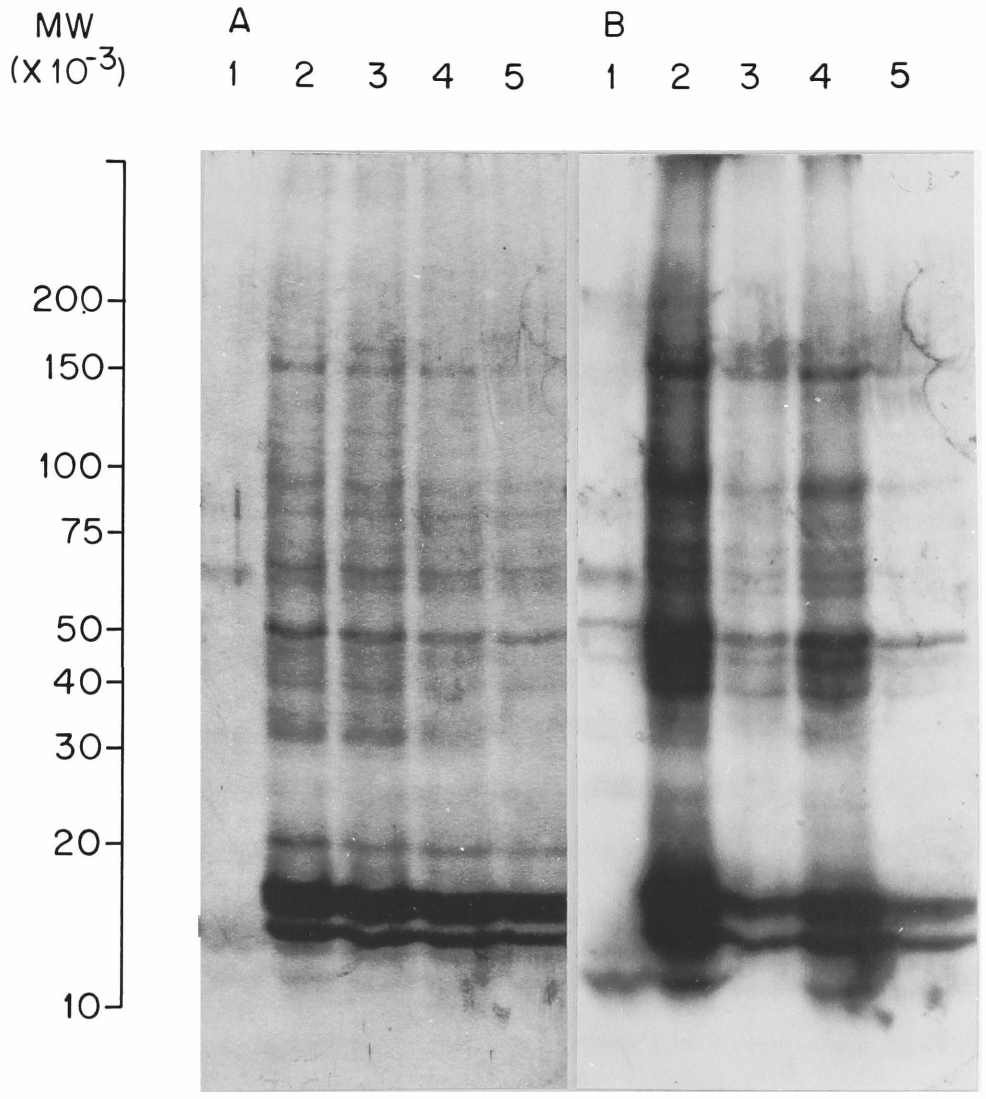
5. Selection for Ligand-bound Molecules, Then Iodination.

It was possible that the various selective methods being used were, in fact selecting the receptor, but, that, since only enzymatically iodinated molecules were being sought, the receptor was not being recognized. To exclude this, an experiment was done with E_{glu}.IgG.C rosetted at either 0°C (as a control) or 37°C. After rosetting, the cells were lysed in NP-40, the fixed red cells purified on a Hypaque gradient, washed, and then iodinated by the Chloramine T method modified from Hunter and Greenwood (1962) as described in Methods. The results are presented in Figure VI-6. The Coomassie Blue stained gel is too complex for analysis. The autoradiogram does not show any bands preferentially gained by rosetting at 37°C. Thus, if it were being bound, the receptor is either present in small amounts, or is being masked under a major, non-specific band.

LEGEND: FIGURE VI-6. ANALYSIS OF CHLORAMINE-T IODINATABLE SPECIES BOUND TO PREPARED GLUTARALDEHYDE-TREATED SHEEP ERYTHROCYTES.

The Figure shows a sample of a Coomassie Blue stained gel (A) and the corresponding autoradiogram (B) of a 5-15% gradient slab gel, with 2.5% stacking gel. The samples were obtained by preparing glutaraldehyde fixed sheep erythrocytes and sensitizing these with IgG and fresh mouse serum as a source of complement. Clone 34 and Clone 4 cells were mixed with target cells at 0°C (both samples pre-chilled in melting ice) or at 37°C. After 90 minutes, when the 37°C cells showed good rosetting, and the 0°C cells showed virtually none, the cells were pelleted and lysed in NaRSB/0.5% NP-40. The E_{glu} were spun onto a 10-45% Hypaque gradient, washed with NaRSB/NP-40, and then sonicated to break up remaining DNA strands. After washing in distilled water, the cells were iodinated by a modification of the method of Greenwood and Hunter (see Methods), washed, and prepared for gels. Control E.IgG.C never exposed to cells were also iodinated. The samples are labeled:

1. E.IgG.C alone
2. Cl 34, 0°C
3. Cl 34, 37°C
4. Cl 4, 0°C
5. Cl 4, 37°C


MW
(X 10-3)
A
1 2 3 4 5
B
1 2 3 4 5
200
150
100
75
50
40
30
20
10

CHAPTER VII

DISCUSSION

This work was directed towards the development of a model system for the investigation of the surfaces of lymphoid cells. The initial surface moiety chosen for study was a receptor for complement. It must therefore be shown that the cell line 8866 is a suitable model of a B lymphocyte, that an appropriate method of isolating representative clones has been developed, and that the receptor being investigated is representative of the receptors on circulating B lymphocytes. Finally, attempts to exploit these cloned populations to identify the complement receptor will be discussed.

A. 8866 AS A MODEL B-LYMPHOCYTE

The first question is to show that 8866 is a representative of lymphocytes of B cell type. It is recognized that B cells undergo maturation, and thus their surfaces may vary during their life cycle. A set of surface markers identified on lymphoid cells is listed in Table VII-1, and the markers of 8866 cells may be compared with it. Of course, the histocompatibility antigens are not restricted to B-lymphocytes, but have been found on all lines examined, and including 8866. Membrane immunoglobulin and secreted immunoglobulin were reported associated with 8866 previously (Lerner et al., 1971), but the same workers have not found surface immunoglobulin more recently (personal communication), and it was not demonstrable on the cells used in these studies. No Fc receptor was found, and the cells did not rosette with sheep erythrocytes (a T-cell

marker). The cell line has not been tested for a monkey erythrocyte receptor activity. A complement receptor is present, and consideration of it will constitute much of this Discussion. In unreported studies still in progress, 8866 cells have been shown to be superinfectible with Epstein-Barr Virus and hence it is deduced that the cells bear a receptor for the virus. (In cells not superinfected, the EBNA has been demonstrated, indicating the presence of the viral genome in the cells used in these studies. This was the only EBV-associated antigen demonstrated in 8866 cells.) Receptors for the hormones have not been sought. Of the enzymes, preliminary assays were done on 5'-nucleotidase, and some activity was detected.

In addition, the morphology, pattern of growth, and intolerance of low cell density indicate that 8866 is typical of B-lymphocyte-type lymphoblastoid cell lines (Nilsson and Pontén, 1975).

When comparing results obtained in different laboratories on the same cell line, one faces a problem of the definition of the cell line. Even obtaining cells from a common source has given rise to problems (see Moore and Woods, 1976; Ferrone and colleagues, 1976). The possibilities for confusion arise at many different levels:

a) Lymphoid cell lines are derived from large numbers of peripheral blood lymphocytes. There is a likelihood that lines are not monoclonal even from their inception. Royston and others (1974) have presented convincing evidence of heterogeneity in a newly established line.

b) Lines may become contaminated by other cell lines. Nelson-Rees and Flandermeyer (1976) have shown that many widely distributed tissue

TABLE VII-1.

SOME SURFACE MARKERS OF HUMAN LYMPHOCYTES

1. HISTOCOMPATIBILITY ANTIGENS:	HLA	Rogentine and Gerber (1969)
	HLB	Winchester et al. (1975)
2. MEMBRANE-ASSOCIATED IMMUNOGLOBULINS.		
3. RECEPTORS:		
	Fc	Berken and Benacerraf (1966)
	SRBC Sheep RBC	Coombs et al. (1970)
	MRBC Monkey RBC	Pellegrino et al. (1975)
	C3b, C3d	Ross et al. (1973b)
	EBV	Jondal and Klein (1973)
	Insulin	Gavin et al. (1972)
	Growth Hormone	Lesniak (1973)
	Calcitonin	Marx et al. (1974)
	Histamine	Plaut et al. (1975)
	LDL	Ho et al. (1976)
4. ENZYMES:		
	5'-nucleotidase	Crumpton and Snary (1974)
	Nucleotide pyrophosphatase, Alkaline phosphodiesterase	Abney et al. (1976)
5. IDENTIFIED BY MOLECULAR WEIGHT ONLY:		
	39,000; 34,000; 29,000 daltons	Humphreys et al., (1976)
	30,000 daltons	Fujita and others (1975)
6. LIGANDS BOUND TO ANY OF THE SPECIFIC RECEPTORS:		

culture lines are, in fact, HeLa cells. In the course of their prolonged culture, it is highly likely that many lymphoid lines have been cross-contaminated with other lines with similar morphology and growth characteristics. In order to define cell lines one requires markers. An ideal marker would be quantitatively and qualitatively stable in culture, show a range of types in a cell donor population, and be applicable to screening large numbers of samples. The most sensitive screen would probably also be assessed on a cell-by-cell basis. No such marker is presently in use.

Iso-enzymes have proved to be stable and applicable to many cell types. Conover and others (1970) showed that lymphoid cell lines persistently expressed the phosphoglucomutase type of the donor. Povey and colleagues (1973) examined lines for many isozymic variants, but found the phenotypes were stable in the absence of mutagens. Nelson-Rees and Flandermeyer (1976) screened many lines, and were able to catalog them according to their isozymes.

Histocompatibility antigens have also been used. Pious and others (1973) have shown HLA-A antigen types to be stably expressed on lines over long periods, and Strominger and his group (see Terhorst et al., 1976) have verified this stability by extracting chemically homogeneous HLA molecules from lymphoid cell lines.

Although many papers have suggested a stability of the types of heavy and light chains of the immunoglobulin molecules a lymphoid cell line may synthesize, Litwin and Hartman (1976) found marked variations in the amounts and chain types secreted by bulk cultures over the course of a few months.

Imamura and others (1970) found that growth rates, cloning efficiency, chromosome constitution and immunoglobulin production by clones were representative of a parent culture; that is, these characteristics remained stable over the period of observation.

Thus, there are no ideal markers available, and the critical characters of many lines have never been published, so that comparisons with a standard set by the lines' originators is usually not possible.

In the particular case of 8866, the cells of the stock culture have always been heterogeneous in size, and have been found to have considerable variation in chromosome number although with a clear dominant modal number. The HLA-A typing is consistent with published data, and compatible with a single donor, but does not rule out the possibility the culture was derived from more than one donor cell, not that there is a minor subpopulation with another histocompatability type. The behavior of two surface markers, namely, the surface immunoglobulin and the complement receptor, indicates these are not sufficiently stable to be reliable markers for the identification of cell lines. The suggestive evidence of a marker chromosome will have to be followed up. No isozymal studies on 8866 have been reported.

B. THE VALIDITY OF THE CLONING METHOD

A method has been established for the cloning of 8866 cells. This method gave a higher efficiency of cloning than any found in the literature, and was capable of reliably producing an efficiency such that progeny clones may reasonably be regarded as representative of the parental stock. The

method was critically dependent on the choice of feeder layer, on the batch of fetal bovine serum used, on regular feeding of cells, and on the gradual, controlled dilution of the cells as growth occurred. By the use of a feeder layer of primary heterologous cells, problems of contamination of the clones with feeder layer cells were avoided. In the unlikely event of transformation of a feeder layer cell, karyotyping or examination of surface antigens would have enabled contamination to be readily detected.

The proof that the progeny were true clones seems unequivocal in the case of the picked single cell method, both by the method used, and from the pilot study which showed no multiple cells seeded. This evidence is supported by the homogeneity of the chromosome numbers, especially since some of the clones have a modal number which occurs at low frequency in the stock culture. In addition, from mixed populations, clones which are made up of purely complement-receptor positive or negative cells were derived, and the characteristic was maintained through two subsequent clonings. It was also observed that the cloned cells were much more homogeneous in size than the parent culture, although cell cycle changes in size, and occasional polykaryocytes, were noted.

In addition to the evidence regarding the picked single cell clones, the agarose clones seem to be shown to be genuine clones also as judged by the criteria of modal chromosomal number and homogeneity of the complement receptor presence or absence in some clones.

C. THE ANALYSIS OF THE COMPLEMENT RECEPTOR PHENOTYPE BY CLONING

The initial intention of the study was to derive stable positive and negative clones for further study, but the nature of the results of the early studies, and the marked fluctuations of mixed cultures maintained in culture, demanded further exploration of the phenomena. The results can be recapitulated simply. Four 100% complement receptor positive clones were recloned, and also maintained in culture for some months. Recloning yielded only positive progeny, and maintained cultures remained totally positive. All receptor negative cultures eventually showed some degree of expression of complement receptor activity but the final percentage of positive cells, and the strength of rosetting varied. In no case did an initially totally negative population become completely positive, nor revert to total negativity. When originally negative clones, grown until they contained some rosette-positive cells, were recloned, most progeny clones were rosette negative, although a few positive clones were obtained. One such clone (36c1) was 100% positive when first tested, but became progressively less positive on continued culture, and, upon recloning, gave rise to mostly negative clones. In most cases, cloning from mixed cultures gave rise to a majority of clones containing cells of various percentages of rosettable cells, with different strengths of rosetting for those cells which were positive. This compares with the clear demonstration of an apparently unimodal distribution of rosettability of the two positive clones, 34 and 4 (Figure III-1).

From these results it was possible to conclude that the C3d receptor of 8866 cells is: 1) able to be expressed on all, some or none of the cells in a population; 2) able to be expressed to a different extent on different cells within a cloned population; 3) an unstable phenotypic marker on most clones, though not on the two clones which were consistently made up of receptor-expressing cells; 4) regulated in its expression by a heritable control. The last point deserves further consideration. The positive clones isolated were all stable, while all other clones were not. Since initially receptor-free clones were shown to fluctuate in their expression of the receptor, it seems clear that the cells do not lack the genomic information encoding the receptor. It is also unlikely that they are sometimes expressing a variant, functionally-defective molecule, and sometimes an active receptor, as might be the case if alleles on pairs of chromosomes were switching on and off. The most likely explanation is some form of regulatory control. However, no exogenous means of affecting this regulation was found.

Analogous systems were sought in the literature. Pious and colleagues (1976) have described a set of beta-2-microglobulin-deficient variants of 8866. They noted that, after a short time in culture, the variant cells began to express the microglobulin. Growth in the presence of anti-B-2-mircoglobulin selected against microglubulin expression, and re-expression, was induced by DMSO. No significant bidirectional changes in extent of expression of beta-2-microglobulin were described.

Dividson (1972) studied a melanoma cell line. Upon cloning both pigmented and unpigmented clones were found. When clones were recloned,

pigment-free clones gave rise to both pigmented and unpigmented clones, whereas unpigmented clones remained unpigmented. A striking finding was that the proportion of pigmented clones derived in successive clonings proved to be heritable, and varied from 20% to 80%. This may be interpreted as inheritance of an alteration in regulatory function of the genome rather than the structural components responsible for pigmentation.

Lotem and Sachs (1974) described a murine leukemia cell line which give rise to a variety of progeny, and in which the ability to be induced to differentiate proves to be a heritable characteristic.

Drewinko and Trujillo (1972) have studied a culture derived from a human lymph node. Both adherent and non-adherent cells were found, and many morphological varieties were seen. Upon cloning the derivative cultures were also found to be mixed populations. This variability has also been a common feature of the EBV induced marmoset lymphoid cell lines of Dr. George Miller (personal observations), but the variable ability to adhere to glass and plastic has not been explained.

Thus, the reversibility described in the 8866 cells does not seem to have an exact parallel, and, in the absence of an effective inducing or repressing agent, remains simply a phenomenological description. The only factors which seem to affect the system reproducibly is the continuous growth of negative cultures, leading to positivity, and the single cell cloning system, which results in more negative clones from mixed cultures than would be predicted by chance.

The mechanism of regulation of expression of the C3d receptor has not been explained. The present studies did not investigate critically the

question of how many molecules might be regulated simultaneously with the C3 receptor, though the radio-iodination studies did not show major changes in the cell surfaces, and the methods of analysing the chromosomal structure do not seem to offer a reasonable approach at this time. It has been found that the presence or absence of the complement receptor does not affect the expression (qualitatively) of the HLA antigens. However, using a human-murine hybrid system, in which human chromosomes are selectively deleted, Curry and colleagues (1976) have found evidence that the complement receptor is encoded on the same chromosome as the HLA antigen.

D. COMPARISON OF THE COMPLEMENT RECEPTORS OF PERIPHERAL BLOOD LYMPHOCYTES AND 8866 CELLS

Having derived homogeneous populations of cells bearing the complement receptor of 8866 cells, it was possible to characterize this receptor in order to be able to compare it with other receptors, and particularly with that of the peripheral blood lymphocyte, for which this system was to be a model. It has been clearly shown that the receptor is a C3d receptor by the reactions with purified human complement components, and by the blocking with the specific antisera of Dr. Gordon Ross. The overlapping binding of C3b with the C3d receptor is as described by Ross and colleagues, and has been shown to be due to partial expression of the CRII (C3d type) site on the C3b molecule, and not to partial expression of C3d on the target particles (Ross and Polley, 1975). In addition, the variability in the rosetting by C3d-receptor bearing lymphoid lines with target cells sensitized with purified human C3b has been confirmed by Ross (personal communication), and remains unexplained, as does the variation in affinity for the different receptors for complement fragments from different species.

In the case of 8866, the high affinity for the C3d receptor for cleaved mouse C3, largely in the form of C3b (since it binds to mouse macrophages which bear only a C3b receptor), parallels the behavior of human peripheral blood lymphocytes. The advantage of the model system 8866 is that only one type of receptor is present. This is in contrast to some peripheral blood lymphocytes, tonsillar lymphocytes, and the Raji cell used by Dierich, Bokisch, Perrin and colleagues.

The assay used throughout these studies was rosetting with sheep erythrocytes sensitized with purified IgM anti-sheep erythrocyte, thus avoiding any interaction of the antibody with an Fc receptor. The complement source was AKR or NCS mouse serum diluted 1:20 in Hanks' Balanced Salt Solution, so that the major product was C3b, as already indicated. This assay has proved reproducible and semi-quantitative. Zymosan particles proved similar in behavior, but fewer particles bound per lymphoid cell, and counting of the translucent particles was more difficult. They were therefore little used.

The alternative assays depend on the use of purified components, with identification of binding either by iodination of the C3, or by use of fluorescent-tagged anti-C3. Yount and colleagues (1976) have published the only study comparing methods. They did find some striking discrepancies between fluorescent labeling and rosetting, but cound not account for it. It also remains to be shown that the binding of cleaved C3 in solution has the same affinity as bound cleaved C3, although it is clear that the soluble form will compete with the bound form. The possibility is raised by the results with temperature dependence of rosetting, and the inability of cell smears of 8866 (or Trypan Blue permeable cells) to bind target cells, that rosetting requires some movement in the membrane of the receptors not required for the binding of solitary soluble molecules.

The other properties of ligand-receptor binding measured included confirmation of the cation independence, a finding of energy independence, a finding of independence of microtubules and microfilaments, and a discrepancy from a previous report of a specific sensitivity to sulfhydryl reagents. The latter seems to have been acounted for by the simultaneous counting of viability, and showing the loss of rosetting exactly paralleled the cell death. It did not prove possible to fix the lymphoid cell membrane, even by the shortest protocol, without abolishing rosetting. This result was reminiscent of the finding of Rutishauser and Sachs (1974,1975) that the lymphoid cell membrane required maintenance of its fluidity for lectin induced agglutination.

It was clearly established that the receptor for C3d is a protein. Not only was it sensitive to a variety of proteases, but its regeneration was blocked by a protein synthesis inhibitor. That it was not dependent on any serum component was demonstrated by regeneration in a serum-free medium.

E. THE USE OF SURFACE IODINATION OF CELLS IN THE SEARCH FOR SURFACE RECEPTORS

1. Methods

Having established a model cell type, with both receptor positive and negative cells available, and having shown the protein nature of the receptor, attention was turned to attempting to identify the molecule responsible for receptor activity. As indicated in Chapter V, the method which offered the most reasonable prospects was that of lactoperoxidase mediated iodination as described by Hubbard and Cohn (1972, 1975a). Alter-

native surface labeling procedures have been reviewed by Hubbard and Cohn (1976), but for lack of cell toxicity, and for specificity for cell surface proteins, the iodination protocol still seems the method of choice. The method required modification only in the pre- and post-iodination washing steps since 1) 8866 cells did not tolerate the multiple pelletings in phosphate buffered saline as well as did L-cells and 2) the presence of dead cells proved to be a source of significant contamination of the labeled whole cells which, in turn, was important since there was not a satisfactory method of isolating the cell plasma membrane of 8866 cells. The observation of labeling of dead cells has also been made by Juliano and Behar-Bannelier (1975). They overcame it by a cell fractionation protocol which removed the labeled intracellular proteins. In most other reports of cell iodinations, the whole cell labeling pattern is not shown, so that the problem of dead cell labeling is not only not addressed, but cannot be assessed by the reader. In most cases, selective immune precipitates are shown, and there is not sufficient evidence of the membrane localization of the iodide to interpret the highly selected data.

The state of the art methods for the assessment of membrane localization have been presented. It is felt that the autoradiographic evidence is clear-cut, and as convincing as could be expected. The cell fractionation does not show the degree of enrichment of membrane that other workers have achieved (see review by Crumpton and Snary, 1974), and it is not clear whether this is due to the choice of membrane marker, namely iodide, not being strictly confined to the membrane, or to the limitations of the membrane purification procedure itself. Not only did the scale of the isolation restrict the measurement of enzyme markers, but the presence of Zn^{++} or Hg^{++} blocked many activities. It is only since these studies were

completed that Crumpton's group have shown that 5'-nucleotidase,which is blocked by Zn^{++}, is a suitable membrane marker for lymphoid cells, and Abney and others (1976) have demonstrated the presence of nucleotide pyrophosphatase and alkaline phosphodiesterase limited to the plasma membrane. However, the results of the cell fractionation did show an approximately 5-fold enrichment of the iodide in the membrane fraction over the whole cell homogenate.

Canellakis and colleagues (Tsai et al., 1974) have proposed a third criterion for the restriction of label to the plasma membrane. They include erythrocytes in the reaction mixture with nucleated cells, then separate the two cell types after iodination, and show that iodination pattern of the erythrocytes is the same as that under optimal conditions for erythrocytes. However, this seems to be based on a spurious assumption, namely that the erythrocyte has a membrane either directly comparable or more delicate membrane than the nucleated cell with which it is being compared. Not only do they fail to show this for their particular case, but the available evidence will not support this as a generalization, and therefore this criterion was not used in these studies.

The labeling pattern of the cells was of some use in assessing the limitation of iodide to the plasma membrane. In cases where there was evidence of reduced viability, the number of labeled bands on the autoradiograms of slab gels was increased, and, in particular, there were more bands in the 14,000 to 20,000 Dalton region. This is the region enhanced in nuclear pellets, and correlates with the Coomassie Blue stained bands attributed to nucleoproteins. The supernatant fluid of iodinated cells lysed with non-ionic detergents, from which nuclei have been pelleted, show the same limited number of bands as found in the best whole cell prep-

arations, supporting the claim that major iodinated species are of membrane origin.

The evidence for the validity of the gradient washing technique as a means of removing serum proteins, and of enhancing cell viability has been presented. Attention may be drawn to the fact that the method is less effective at removing dying cells, having approximately the same density and diameter as live cells, as compared with the removal of shriveled, pyknotic cells.

2. Results

The method of analysis of the iodinated species was the gradient slab gel of SDS-polyacrylamide after the protocol of Laemmli (1970). This has the advantage of including all species from approximately 500,000 to approximately 2,000 daltons when used as a 5-15% gradient, and not over-run. Additional experiments were peformed with lower starting concentrations of the gradient gels to ensure that even larger species were not present in significant amounts. In several figures, the running front of the gel is presented to show that there are no significant amounts of label incorporated in very low molecular weight peptides or phospholipids. Although there are some deviations from the direct correlation of migration with molecular weights in this procedure (see review by Maddy, 1976), and even the type of SDS used may have some effect (Swaney and colleagues, 1974), the method is reproducible when the procedure is standardized, as was shown in Chapter V, although ultimate reliance may not be given to a particular molecular weight estimate. In particular, glycoproteins may behave anomalously (Rodbard and

Chrambach, 1971). Several attempts were made to run a Ferguson series according to the method of these authors, but were unsuccessful. In all cases the DNA present led to unacceptable smearing of the bands. It was therefore decided on these bases to use the lettering code of designating iodinated bands until confirmatory evidence is forthcoming. In support of this stance is the difficulty in assigning the bands in the 45,000-55,000 dalton region. The pattern was constant but there were overlapping changes in estimates of molecular weight. Since this region contains the larger subunit of the HLA molecule, it is vital to analyse it more carefully. It may be that this is a group of totally disparate protein species, or may be evidence of the heterogeneity of the four molecules which make up the HLA phenotype.

In comparing the lettered bands identified on the gels in these studies with known protein species, it is found that the catalogue of the latter is very limited. It is presented in Table VII-2. Thus, for the human lymphocytes, only two molecular weights can be assigned, and in one case, viz. the 44,000 dalton species, it is not clear which of the E,F,G complex is the HLA, since E, the nearest in molecular weight, is not usually the strongest band, and this might be expected for the group of major histocompatibility determining molecules.

When human lymphocytes are compared with a variety of other cell types, such as 3T3 mouse cells (Hogg, 1974), Sarcoma 180 (Shin and Carraway, 1973), or L cells (Hubbard and Cohn, 1975a) it is found that the patterns are totally different. When compared with two published patterns for whole human lymphocytes, in Table VII-3, similarities and differences can be seen. Kennel and Lerner (1973) used the hydrogen peroxide method instead of the glucose oxidase/glucose system, but carefully validated

their results as being membrane labeling. Unfortunately, however, they used the gel system of Weber and Osborne (1969), and this would not resolve some of the bands found in the Laemmli (1970) gel system. On the other hand, the Trowbridge data (Trowbridge and others, 1976) were run on the high resolution gel system, but, although the authors claim to be using the method of Hubbard and Cohn (1975a), examination of their methods reveals that they used the enzymes at much higher levels than recommended. Whereas Hubbard and Cohn used 3.6mU/ml of both glucose oxidase and lactoperoxidase, Trowbridge used 125mU/ml and 625mU/ml respectively. As was shown by Edelson and Cohn (1973) concentrations of both enzymes in excess of 30mU led to significant cytotoxicity as assayed by radiolabeled chromate release, a system they showed to be more sensitive than the Trypan Blue exclusion test. In no study of human lymphoid cells can the ultimate test of failure to inflict cell damage by iodination be tried. This was done by Hubbard and Cohn (1975a) when they measured plating efficiency after iodination, and found it to be 95% for L cells. Unfortunately, no plating assay of similar sensitivity is known for lymphoid cells. In these studies post-iodination dye exclusion tests always found >90% of cells viable, but attempts to grow the cells revealed that the population was not able to grow as if 100% of cells were indeed viable. A lag period was always seen.

The number of very similar species found by Trowbridge and colleagues, and this study support the idea of the similarity of the surfaces of B lymphocytes. It seems likely that Bands A,C,D,E, and I are common. The differences may well be due to differences in surface species between lines,

TABLE VII-2.

SOME IDENTIFIED LYMPHOCYTE SURFACE MOLECULES

SOURCE		M.W.(daltons)	AUTHORS
MURINE:			
	H2	47,000 11,500	Silver & Hood (1974)
	β_2-microglobulin-like	12,000	Vitetta, Uhr & Boyse (1975)
	TL	45,000	Vitetta, Uhr & Boyse (1975)
	Thy-1	25-30,000	Trowbridge et al.(1975b,c)
	T lymphocyte antigen	200,000	Trowbridge et al.(1975a)
HUMAN:			
	HL-A	44,000 12,000	Springer et al. (1974)
	β_2-microglobulin	12,600	Poulik and Motwani (1972)

TABLE VII-3.

COMPARISON OF IODINATED SPECIES IDENTIFIED ON HUMAN LYMPHOID CELLS

KENNEL & LERNER WIL-2 LINE	TROWBRIDGE CELL LINES	PRESENT STUDY 8866 LINE
	220,000	
		122,000 (K)
		108,000 (J)
	95,000	92,000 (I)
> 70,000*		
70,000	75,000	
60,000		
		55,000 (H)
		52,000 (G)
		50,000 (F)
40-50,000 (broad peak)	45,000	45,000 (E)
	35,000	34,000 (D)
	27,000	
		25,000 (C)
		14,000 (B)
11,000	11,000	11,000 (A)

* = Data insufficient for an accurate assessment of molecular weight.

and remain to be identified. The striking differences are in the absence of the FGH cluster and Bands J and K on the Trowbridge lines, and conversely, the absence of the 75,000 and 220,000 dalton bands on 8866. It should be noted that Kennel and Lerner found a broad peak in the 40-50,000 dalton region, supporting one side, and an approximately 70,000 dalton band, in agreement with the other, on their WIL-2 line.

When the data from iodination are compared with results obtained by other means, it is of note that Fujita and colleagues (1975), using a cell fractionation protocol, found a B-cell specific band at approximately 30,000 daltons. This may well be equivalent to the 34-35,000 band, in view of the different gel system they used. Humphreys and others (1976) also found a 34,000 dalton species as part of a human B lymphocyte surface antigen complex. However, neither the 39,000 nor the 29,000 dalton species forming the rest of the complex are seen as part of the iodination pattern.

When the effects of proteases on radio-iodinated cells were examined, it was noted that there was some simplification of the radioautographic pattern. This correlated with loss of dead cells from the pelleted cells used for application to the gels, and may well represent loss of contaminating cytoplasmic and nuclear labeling. The loss of high molecular weight species was also noted, but not explained. An intriguing finding was the cleavage of Band D by four different proteases, with the disappearance of the 35,000 dalton band, and the appearance of a new band (X) at approximately 22,000 daltons. Although the autoradiographic technique is only semi-quantitative, it is clear that the portion of the molecule which remains cell associated retains most of the radiolable present on the intact molecule.

This phenomenon might best be explained in one of two ways. Either most of the Band D protein is exposed in the hydrophilic, external aspect of the membrane but has a uniquely sensitive region, such as one not covered by carbohydrate, or alternatively, only about 13,000 daltons of the protein are outside the cell membrane, and these include the few iodinatable species situated very close to the membrane. In the latter case, when cleavage occurs, the tyrosines remain attached to the membrane, and the internal portions of the molecule are protected from the proteases. Even in this second case, it may well be that there is a specially sensitive portion of the molecule so that the striking similarity of molecular weights of the proteolysed products can be accounted for. Attempts at examining the cell membrane by iodination after proteolysis were not successful: the cells were sensitive to the gradient washes, and became permeable, leading to non-membrane labeling.

The turnover studies presented are limited by the poor cell growth after iodination. However, the clear positive findings are that there were no rapidly turning-over species identified, and that the major iodinated bands were degraded at the same rate (within the limits of the semi-quantitative nature of the autoradiograms). This rate was much slower than the expected synthetic rate, namely replacement of the cell membrane in the doubling time of the cells, 20 hours. The rapidly lost material was accounted for in the supernatant medium in the first few hours, and was thereafter degraded at a slow rate. The nature of this acid-precipitable, rapidly shed material has not been defined. It is quantitatively small enough to be accounted for by the breakdown of damaged cells but this is not proven.

The slow turnover rate can be compared with other data obtained by the same methodology. Hubbard and Cohn (1975b) showed that the L-cell membrane proteins were degraded in a biphasic pattern, with 5-20% of the initial counts having a half life of 0.75-3 hours, and 80-95% having a range of half life of 22-35 hours. They also found that the degradation of species was uniform except when the membrane was interiorized into phagolysosomes, in which case some selectivity was evident. They also found that the early component was delayed by storing cells in the cold, but that after the delay, the same proportion of material was lost at an even more rapid rate.

Tweto and Doyle (1976) studied a hepatoma tissue culture cell line by surface iodination and internal labeling. They showed that the membrane was degraded with a half life of about 100 hours, as compared with the half life of 50-60 hours for total cell protein in cells with a doubling time of about 30 hours. The results from intrinsic and external labeling were comparable. They did not examine early time points after iodination, and so would not detect a rapidly turning-over component. The iodide and leucine labels used gave no evidence of heterogeneity in turnover time for membrane proteins, although there was heterogeneity with a fucose label. A significant point raised by this paper is that contamination of membrane with intracellular protein, that is, contamination of a homogeneously turning-over group of proteins with a heterogeneously turning-over group, would give rise to an appearance of heterogeneity of turnover for membrane proteins.

The evidence of Hubbard and Cohn (1975b), Tweto and Doyle (1976) and the present studies therefore suggests a homogeneous rate of degradation of membrane proteins which can be iodinated. Since the membrane remains

intact in logarithmically growing cultures, it suggests further synchronous synthesis of these proteins. However, some protein species, such as membrane immunoglobulin, appear capable of being stripped off selectively and rapidly, and proteases are able to cause selective loss of surface species without loss of membrane integrity. This problem has been extensively reviewed (see Siekevitz, 1972; Fox, 1972), and is now being considered at the level of individual membrane protein constituents (Edelson and Cohn, 1976a,b; Doljanski and Kapeller, 1976; Raff, 1976), but the ability to regulate the protein constituents of the plasma membrane at approximately constant proportions in the face of perturbations remains an unexplained phenomenon.

Not previously discussed here are results relating to surface immunoglobulin of human lymphoid cells. Grundke-Iqbal and Uhr (1974) present data on the turnover of the iodinatable and immunoprecipitable immunoglobulin of the lymphoid line, Daudi. When their data are taken only relating to cells in the physiological state, and extrapolated beyond the short time course they measured, a turnover rate approximating that of this study is obtained. That is, in a human cell line, as opposed to murine cells, there is no evidence that membrane immunoglobulin is a rapidly turning-over species. Because of the shortness of the time course studied, this must remain a tentative conclusion.

The use of labeling of clones bearing the C3d receptor did not have the desired result of identifying the receptor molecule. In contrast, Trowbridge and Hyman (1975a,b) have presented good evidence for the identity of Thy-1 by labeling lines with and without this antigen: they did not have to resort to the use of clones of a single line. They were able to confirm

the identity of the Thy-1 by immune precipitation with an appropriate antiserum. As an alternative approach this study attempted to utilize a type of affinity chromatography, with modifications which seemed appropriate for the particular situation. As has been shown, in spite of these variations the receptor was not identified.

Considering some of the possible complications, care was taken to use salt concentrations and a physiological pH range which allowed ligand-receptor interaction. Non-ionic detergents known to permit antibody-antigen reactions, a group of reactions known to have a wide range of affinities, were used. Binding was attempted at temperatures at which rosetting occurred, and further processing carried out at low temperatures at which formed rosettes are known to be stable. The possibility that the ligand-receptor interaction is less stable when only one ligand binds to one receptor cannot be excluded. It is also possible that the receptor has been isolated but was masked by one of the bands which precipitated in the presence or absence of C3d. The Chloramine-T experiment seems to exclude the possibility that a major protein species was isolated, but unrecognized because it was not iodinated. The known sensitivity of Band D to low doses of proteases was felt to be a good control for the presence of proteolytic activity during the isolation procedure. Examination of the supernatants after selection does not show conversion of Band D to X, and therefore proteolysis is an unlikely cause for failure to isolate the receptor.

The use of zymosan as well as glutaraldehyde-fixed erythrocytes demonstrated that the problem of "non-specific" precipitation was not due solely to the peculiar properties of the latter particle. The similarity

of some of the species selected by both particles is intriguing but unexplained.

The propensity of glutaraldehyde fixed erythrocytes to rosette with complement receptor bearing cells more than with receptor-negative cells remains unexplained also. A variety of agents which should have prevented cross-linking by aldehyde groups failed to abolish the binding. The phenomenon is analogous to the findings of Rabinovitch, 1969; Rabinovitch and de Stefano, (1973) that aldehyde fixed erythrocytes were more readily attached to and ingested by murine macrophages and fibroblast lines. In their hands, the behavior was modified by pretreatment of the nucleated cell membrane with trypsin, a procedure also known to affect the complement receptor. However, this cannot be the full explanation, since the fibroblast cells do not bear a complement receptor, and a similar, although not selective, "non-specific" adherence has been observed for a variety of cells when exposed to latex particles. Direct comparison with the results of Rabinovitch is not possible, since they did not use short time exposures, and distinguish adherence from phagocytosis.

The ability to make a particle of the same size as an erythrocyte, and to covalently bind a variety of complement components to it still seems a desirable way to go about selecting the complement receptor. The problems of non-complement dependent precipitation may be solved by the method used by the Uhr group in their immune selection studies, namely a preliminary precipitation by beads or other particles to which some indifferent species is attached. This may reduce the background. An alternative approach is to attempt to use the antibody raised in rabbits against 8866 cells, and which blocks rosetting. Although of low titer, it may be possible to bind sufficient to identify the receptor.

REFERENCES

Abney, E.R., Evans, W.H., and Parkhouse, R.M.A. (1976). Biochem. J. 159:293-299.

Alper, C.A., Colten, H.R., Rosen, F.S., Rabson, A.S., Macnab, G.M., and Gear, J.S.S. (1972) Lancet ii:1179-1181.

Arnaiz-Villena, A., and Hay, F.C. (1975). Immunol. 28:719-729.

Arnaiz-Villena, A., and Festenstein, H. (1976) Nature (London) 258:732-734.

Basten, A., Miller, J.F.A.P., Sprent, J., and Pye, J. (1972a). J. Exp. Med. 135:610-626.

Basten, A., Warner, N.L., and Mandel, T. (1972b). J. Exp. Med. 135:627-642.

Berken, A., and Benacerraf, B. (1966). J. Exp. Med. 123:119-144.

Bianco, C., Patrick R., and Nussenzweig, V. (1970). J. Exp. Med. 132:702-720.

Bianco, C. and Nussenzweig, V. (1971). Science 173:154-156.

Bianco, C., Griffin, F.M. Jr., and Silverstein, S.C. (1975). J. Exp. Med. 141:1278-1290.

Bianco, C. (1976) in "In Vitro Methods in Cell Mediated and Tumor Immunity," (Eds.) B.R. Bloom and J.R. David, Academic Press, New York.

Bloom, A.D., Choi, K.W., and Lamb, B.J. (1971). Science 172:382-383.

Blume, R.S., Glade, P.R., Gralnick, H.R., Chessin, L.N., Haase, A.T., and Wolf, S.M. (1969). Blood 33:821-832.

Bokisch, V.A., and Sobel, A.T. (1974). J. Exp. Med. 140:1336-1347.

Bokisch, V.A., Müller-Eberhard, H.J., and Cochrane, C.G. (1969). J. Exp. Med. 129:1109-1130.

Bokisch, V.A., Dierich, M.P., and Müller-Eberhard, H.J. (1975). Proc. Nat. Acad. Sci. (USA) 72:1989-1993.

Böyum, A. (1968). Scand. J. Clin. & Lab. Invest. Suppl. 97:77-89.

Brown, D.L., Lachmann, P.J., and Dacie, J.V. (1970). Clin. Exp. Immunol. 7:401-421.

Brunette, D.M., and Till, J.E. (1971). J. Membrane Biol. 5:215-224.

Budzko, D.B., Lachmann, P.J., and McConnell, I. (1976). Cell Immunol. 22:98-109.

Caro, L.G., and van Tubergen, R.P. (1962). J. Cell Biol. 15:173-188.

Chang, R.S., and Golden, H.D. (1971). Nature (London) 234:359-360.

Chang, R.S., Lewis, J.P., and Abildgaard, C.F. (1973).
New Eng. J. Med. 289:1325-1329.

Chang, R.S., and Blankenship, W. (1973). Proc. Soc. Exp. Biol. Med. 144:337-339.

Chang, R.S., Fillingame, R.A., Paglieroni, T., and Glassy, F.J. (1976)
Proc. Soc. Exp. Biol. Med. 153:193-196.

Chen, K-Y., Tsai, C-M., and Canellakis, E.S. (1975).
Cancer Res. 35:2403-2412.

Choi, K.W., and Bloom, A.D. (1970a). Nature (London) 227:171-173.

Choi, K.W., and Bloom, A.D. (1970b). Science 70:89-90.

Coffino, P., Baumal, R., Laskov, R., and Scharff, M.D. (1972).
J. Cell Physiol. 79:429-440.

Cone, R.E., and Marchalonis, J.J. (1974). Biochem. J. 140:345-354.

Cone, R.E. (1976). J. Immunol. 116:847-853.

Conover, J.H., Hathaway, P., Glade, P.R., and Hirschhorn, K. (1970).
Proc. Soc. Exp. Biol. Med. 133:750-753.

Coombs, R.R.A., Gurner, B.W., Wilson, A.B., Holm, G., and Lindgren, B.
(1970). Arch. Allergy Appl. Immunol. 39:658-663.

Cooper, N.R. (1969). Science 165:396-398.

Cooper, N.R. (1975). J. Exp. Med. 141:890-903.

Crumpton, M.J., and Snary, D. (1974). Contemp. Topics Molec. Immunol. 3:27-56.

Curry, R.A., Dierich, M.P., Pellegrino, M.A., and Hoch, J.A. (1976).
Immunogenetics 3:465-471.

David, G.S. (1972). Biochem. Biophys. Res. Commun. 48:464-471.

David, G.S. and Reisfeld, R.A. (1974). Biochemistry 13:1014-1021.

Davidson, R.L. (1972). Proc. Nat. Acad. Sci. (USA) 69:951-955.

Dickler, H.B., and Sachs, D.H. (1974). J. Exp. Med. 140:779-796.

Dierich, M.P., Ferrone, S., Pellegrino, M.A., and Reisfeld, R.A. (1974a).
J. Immunol. 113:940-947.

Dierich, M.P., Pellegrino, M.A., Ferrone, S., and Reisfeld, R.A. (1974b).
J. Immunol. 112:1766-1773.

Doljanski, F., and Kapeller, M. (1976). J. Theor. Biol. 62:253-270.

Drewinko, B., and Trujillo, J.M. (1972). In Vitro 7:344-358.

Dukor, P., Bianco, C., and Nussenzweig, V. (1971). Eur. J. Immunol. 1:491-494.

Dukor, P., and Hartmann, U. (1973). Cell Immunol. 7:349-356.

Edelson, P.J., and Cohn, Z.A. (1973). J. Exp. Med. 138:318-323.

Edelson, P.J., and Cohn, Z.A. (1976a). J. Exp. Med. 144:1581-1595.

Edelson, P.J., and Cohn, Z.A. (1976b). J. Exp. Med. 144:1596-1608.

Eden, A., Miller, G.W., and Nussenzweig, V. (1973).
J. Clin. Invest. 52:3239-3242.

Ehlenberger, A.G., and Nussenzweig, V. (1977). J. Exp. Med. 145:
357-371.

Epstein, M.A., and Barr, Y.M. (1964). Lancet i:252-253.

Ferreira, A., and Nussenzweig, V. (1975). J. Exp. Med. 141:513-517.

Ferreira, A., and Nussenzweig, V. (1976). J. Immunol. 117:771-773.

Ferreira, A., Fotino, M., and Nussenzweig, V. (1976).
Eur. J. Immunol. 6:832-833.

Ferrone, S., Pellegrino, M.A., and Cooper, N.R. (1976). Science 194:480.

Fox, C.F. (1972) in "Membrane Molecular Biology" (Ed.) C.F. Fox and
A. Keith, Sinauer Assoc., Inc., Stamford, Conn., p. 345-385.

Friend, C., Scher, W., Holland, J.G., and Sato, T. (1971).
Proc. Nat. Acad. Sci.(USA) 68:378-382.

Fu, S.M., Kunkel, H.G., Brusman, H.P., Allen, A.F. Jr., and Fotino, M.
(1974). J. Exp. Med. 140:1108-1111.

Fujita, S., Litwin, S.D., and Hartman, N. (1975). J. Exp. Med. 142:1416-1424.

Gavin, J.R. III, Roth, J., Jen, P., and Freychet, P. (1972)
Proc. Nat. Acad. Sci. (USA) 69:745-751.

Gelfand, M.C., Elfenbein, G.J., Frank, M.M., and Paul, W.E. (1974a).
J. Exp. Med. 139:1125-1141.

Gelfand, M.C., Sachs, D.H., Lieberman, and Paul, W.E. (1974b)
J. Exp. Med. 139:1142-1153.

Gelfand, M.C., Frank, M.M., and Green, I. (1975). J. Exp. Med. 141:1221-1226.

Gelfand, M.C., Shin, M.L., Nagle, R.B., Green, I., and Frank, M.M. (1976). New Eng. J. Med. 295:10-14.

Gigli, I., and Nelson, R.A. Jr. (1968). J. Exp. Med. 141:1221-1226.

Gitlin, J.D., Rosen, F.S., and Lachman, P.J. (1975). J. Exp. Med. 141:1221-1226.

Glade, P.R., and Chessin, L.N. (1968). Int. Arch. Allergy 34:181-187.

Gormus, B.J., Crandall, R.B., and Shands, J.W. Jr. (1974). J. Immunol. 112:770-775.

Gormus, B.J., and Shands, J.W. Jr. (1975). J. Immunol. 114:1221-1225.

Gottlieb, A.B., Seide, R.K., and Kindt, T.J. (1975). J. Immunol. 114:51-54.

Grey, H.M., Rabellino, E.M., and Pirofsky, B. (1971). J. Clin. Invest. 50:2368-2375.

Grundke-Iqbal, I., and Uhr, J.W. (1974). Eur. J. Immunol. 4:159-163.

Gutierrez, C., Papamichail, M., and Faulk, W.P. (1976). Clin. Exp. Immunol. 23:258-263.

Hammer, C.H., Abramowitz, A.S., and Mayer, M.M. (1976). J. Immunol. 117:830-834.

Hämmerling, U., Chin, A.F., and Abbott, J. (1976). Proc. Nat. Acad. Sci. (USA) 73:2008-2012.

Hartmann, U. (1975). Transplant. Rev. 23:98-104.

Hayflick, L. (1965). Exp. Cell Res. 37:614-636.

Helenius, A., and Simons, K. (1975). Biochim. Biophys. Acta 415:29-79.

Hirsch, J.G., and Fedorko, M.E. (1967). J. Cell Biol. 38:615-627.

Ho, Y.K., Brown, M.S., Kayden, H.J., and Goldstein, J.L. (1976). J. Exp. Med. 144:444-445.

Hogg, N.M. (1974). Proc. Nat. Acad. Sci. (USA) 71:489-492.

Hollenberg, M.D., and Cuatrecasas, P. (1975) in "Handbook of Psychopharmacology", (Eds) L.L. Iversen, S.D. Iversen, S.H. Snyder, Plenum Press, New York, Vol. 2, p. 129-177.

Honig, R., Billing, R., Matsuyama, M., and Terasaki, P.I. (1976). J. Immunol. 117:1300-1302.

Huang, C.C., Minowada, J., Smith, R.T., and Osunkoya, B.O. (1970). J. Natl. Cancer Inst. 45:815-829.

Huang, C-C., Tsai, C-M., and Canellakis, E.S. (1974). Biochim. Biophys. Acta 332:59-68.

Hubbard, A.L., and Cohn, Z.A. (1972). J. Cell Biol. 55:390-405.

Hubbard, A.L., and Cohn, Z.A. (1975a). J. Cell Biol. 64:438-460.

Hubbard A.L., and Cohn, Z.A. (1975b). J. Cell Biol. 64:461-479.

Hubbard, A.L., and Cohn, Z.A. (1976) in "Biochemical Analysis of Membranes", (Ed.) A.H. Maddy, Chapman Hall, London, p. 427-501.

Huber, H., Polley, M.J., Linscott, W.D., Fudenberg, H.H., and Müller-Eberhard, H.J. (1968). Science 162:1281-1283.

Huber, C., and Wigzell, H. (1975). Eur. J. Immunol. 5:432-435.

Hugli, T.E. (1975). J. Biol. Chem. 250:8293-8301.

Hugli, T.E., Vallota, E.H., and Müller-Eberhard, H.J. (1975a). J. Biol. Chem. 250:1472-1478.

Hugli, T.E., Morgan, W.T., and Müller-Eberhard, H.J. (1975b). J. Biol. Chem. 250:1479-1483.

Humphreys, R.E., McCune, J.M., Chess, L., Herrman, H.C., Malenka, D.J., Mann, D.L., Parham, P., Schlossman, S.F., and Strominger, J.L. (1976). J. Exp. Med. 144:98-112.

Hunter, W.M., and Greenwood, F.C. (1962). Nature (London) 194:495-496.

Hynes, R.O. (1973). Proc. Nat. Acad. Sci. (USA) 70:3170-3174.

Hynes, R.O. (1976). Biochim. Biophys. Acta 458:73-107.

Imamura, T., Huang, C.C., Minowada, J., Takahashi, M., and Moore, G.E. (1970). J. Natl. Cancer Inst. 44:845-854.

Inman, J.K., and Dintzis, H.M. (1969). Biochemistry 8:4074-4082.

Iwakata, S., and Grace, J.T. Jr. (1964). N.Y. State J. Med. 64:2279-2282.

Joncas, J., Boucher, J., Boudreault, A., and Granger-Julien, M. (1973). Cancer Res. 33:2142-2148.

Jondal, M., and Klein, G. (1973). J. Exp. Med. 138:1365-1378.

Jondal, M., Klein, G., Oldstone, M.B.A., Bokisch, V.A., and Yefenof, E. (1976). Scand. J. Immunol. 5:401-410.

Juliano, R.L., and Behar-Bannelier, M. (1975).
Biochim. Biophys. Acta 375:249-267.

Juliano, R.L., and Stanley, P. (1975). Biochim. Biophys. Acta 389:401-406.

Kaplan, J., Shope, T.C., and Peterson, W.D. Jr. (1974).
J. Exp. Med. 139:1070-1076.

Kammermeyer, J.K., Root, R.K., Stites, D.P., Glade, P.R., and Chessin, L.N.
(1968). Proc. Soc. Exp. Biol. Med. 129:522-527.

Kennel, S.J., and Lerner, R.A. (1973). J. Mol. Biol. 76:485-502.

Kohn, G., Mellman, W.J., Moorhead, P.S., Loftus, J., and Henle, G. (1967).
J. Natl. Cancer Inst. 38:209-222.

Kohn, G., Diehl, V., Mellman, W.J., Henle, W., and Henle, G. (1968).
J. Natl. Cancer Inst. 41:795-804.

Kuroki, T. (1975) in "Methods in Cell Biology", (Ed.) D.M. Prescott,
Academic Press, New York, Vol. 9, pp. 157-178.

Lachman, P.J., and Müller-Eberhard, H.J. (1968). J. Immunol. 100:691-698.

Laemmli, U.K. (1970). Nature (London) 227:680-685.

Laveran, A., and Mesnil, F. (1901). Ann. Inst. Pasteur 15:673-714, cited
in Nelson, D.S. (1963).

Lay, W.H., and Nussenzweig, V. (1968). J. Exp. Med. 128:991-1009.

Lerner, R.A., McConahey, P.J., and Dixon, F.J. (1971). Science 173:60-62.

Lesniak, M.A., Roth, J., Gorden, P., and Gavin, J.R. III (1973).
Nature (New Biol.) 241:20-22.

Littlefield, J. (1974) in "Somatic Cell Hybridization", (Ed.) R.L. Davidson
and F. de la Cruz, Raven Press, New York, pp. 207-209.

Litwin, S.D., and Hartman, N. (1976). Scand. J. Immunol. 5:789-794.

Lotem, J., and Sachs, L. (1974). Proc. Nat. Acad. Sci. (USA) 71:3507-3511.

Lotem, J., and Sachs, L. (1976). J. Immunol. 116:580-586.

Macek, M., Seidel, E.H., Lewis, R.T., Brunschwig, J.P., Wimberly, I., and
Benyesh-Melnick, M. (1971). Cancer Res. 31:308-321.

Maddy, A.H. (1976. J. Theor. Biol. 62:315-326.

Marchalonis, J.J., and Cone, R.E. (1973). Transpl. Rev. 14:3-49.

Marx, S.J., Aurbach, G.D., Gavin, J.R. III, and Buell, D.W. (1974). J. Biol. Chem. 249:6812-6816.

Matre, R., and Tönder, O. (1976). Scand. J. Immunol. 5:437-441.

Mendes, N.F., Miki, S.S., and Peixinho, Z.F. (1974). J. Immunol. 113:531-536.

Meo, T., Krasteff, T., and Schreffler, D.C. (1975). Proc. Nat. Acad. Sci. (USA) 72:4536-4540.

Michlmayer, G., and Huber, H. (1970). J. Immunol. 105:670-676.

Minowada, J., Ohnuma, T., and Moore, G.E. (1972). J. Natl. Cancer Inst. 49:891-895.

Moore, G.E. (1972). J. Surg. Oncol. 4:320-353.

Moore, G.E., and Minowada, J. (1973). New Eng. J. Med. 288:106.

Moore, G.E., and Woods, L.K. (1976). Science 194:480.

Müller-Eberhard, H.J., and Nilsson, U. (1960). J. Exp. Med. 111:217-234.

Müller-Eberhard, H.J., Polley, M.J., and Calcott, M.A. (1967). J. Exp. Med. 125:359-380.

Mundy, G.R., Luben, R.A., Raisz, L.G., Oppenheim, J.J., and Buell, D.N. (1974). New Eng. J. Med. 290:867-871.

Nelson, D.S. (1963). Adv. Immunol. 3:131-180.

Nelson, R.A. (1953). Science 118:733-737.

Nelson-Rees, W.A., and Flandermeyer, R.R. (1976). Science 191:96-98.

Neville, D.M. Jr. (1971). J. Biol. Chem. 246:6328-6334.

Nilsson, U., and Mapes, J. (1973). J. Immunol. 111:293 (Abstract).

Nilsson, K., and Pontén, J. (1975). Int. J. Cancer 15:321-341.

Nowakowski, M., Feldman, J.D., Kano, S., and Bloom, B.R. (1973). J. Exp. Med. 137:1042-1059.

Nussenzweig, V. (1974). Adv. Immunol. 19:217-259.

Okada, H., and Nishioka, K. (1973). J. Immunol. 111:1444-1449.

Osgood, E.E., and Brooke, J.H. (1955). Blood 10:1010-1022.

Ostberg, L., Rask, L., Wigzell, H., and Peterson, P.A. (1975).
Nature (London) 253:735-737.

Papamichail, M., Gutierrez, C., Embling, P., Johnson, P., Holborow, E.J., and Pepys, M.B. (1975). Scand. J. Immunol. 4:343-347.

Papenhausen, P., Papageorgiou, P., and Hirschhorn, K. (1975).
J. Immunol. 114:519-521.

Parish, C.R., and Hayward, J.A. (1974). Proc. Roy. Soc. (London) B 187:47-63.

Pattengale, P.K., Smith, R.W., and Gerber, P. (1974).
J. Natl. Cancer Inst. 52:1081-1086.

Patuleia, M.C., and Friend, C. (1967). Cancer Res. 27: 726-730.

Paul, J. (1959) in "Cell and Tissue Culture," E. & S. Livingstone, Edinburgh, p. 195-197.

Pellegrino, M.A., Ferrone, S., and Theofilopoulos, A.N. (1975).
J. Immunol. 115:1065-1071.

Perlmann, P., Perlmann, H., and Müller-Eberhard, H.J. (1975).
J. Exp. Med. 141:287-296.

Pillemer, L., Blum, L., Lepow, I.H., Wurz, L., and Todd, E.W. (1956).
J. Exp. Med. 103:1-13.

Pincus, S., Bianco, C., and Nussenzweig, V. (1972). Blood 40:303-310.

Pious, D., Hawley, P., and Forrest, G. (1973).
Proc. Nat. Acad. Sci. (USA) 70:1397-1400.

Pious, D., Soderland, C., Weagant, S., and Poulik, D. (1976).
J. Immunol. 116:570-572.

Plaut, M., Lichtenstein, L.M., and Henney, C.S. (1975).
J. Clin. Invest. 55:856-874.

Poulik, M.D., and Motwani, N. (1972). Clin. Res. 20:795. (Abstract)

Povey, S., Gardiner, S.E., Watson, B., Mowbray, S., Harris, H., Arthur, E., Steel, C.M., Blenkinsop, C., and Evans, H.J. (1973).
Ann. Hum. Genet.(London) 36:247-266.

Prasad, K.N., and Sinha, P.K. (1976). In Vitro 12:125-132.

Pulvertaft, R.J.V. (1964). Lancet i:238-240.

Rabinovitch, M. (1969). Exp. Cell Res. 54:210-216.

Rabinovitch, M., and De Stefano, M.J. (1973). J. Immunol. 110:695-701.

Raff, M.C. (1976). Nature (London) 259:265-266.

Reedman, B.M., and Klein, G. (1973). Int. J. Cancer 11:499-520.

Reisfeld, R.A., Pellegrino, M.A., Dierich, M.P., and Ferrone, S. (1975). In Vitro 11:173-185.

Rieckenberg, H. (1917). A. Immunitätsforsch. 26:53-64, cited in Nelson, D.S. (1963).

Robinson, J.E., Andiman, W.A., Henderson, E., and Miller, G. (1977). Proc. Nat. Acad. Sci. (USA) 74:749-753.

Rodbard, D., and Chrambach, A. (1971). Anal. Biochem. 40:95-134.

Rogentine, G.N. Jr., and Gerber, P. (1969). Transplantation 8:28-37.

Ross, G.D., Rabellino, E.M., Polley, M.J., and Grey, H.M. (1973a). J. Clin. Invest. 52:377-385.

Ross, G.D., Polley, M.J., Rabellino, E.M., and Grey, H.M. (1973b). J. Exp. Med. 138:798-811.

Ross, G.D., and Polley, M.J. (1975). J. Exp. Med. 141:1163-1180.

Rothman, I.K., Gelfand, J.A., Fauci, A.S., and Frank, M.M. (1975). J. Immunol. 115:1312-1315.

Royston, I., Smith, R.W., Buell, D.N., Huang, E-S., and Pagano, J.S. (1974). Nature (London) 251:745-746.

Rutishauser, U., and Sachs, L. (1974). Proc. Nat. Acad. Sci.(USA)71:2456-2460.

Rutishauser, U., and Sachs, L. (1975). J. Cell Biol. 66:76-85.

Rogentine, G.N. Jr., and Gerber, P. (1969). Transplantation 8:28-37.

Salpeter, M.M., Bachmann, L., and Salpeter, E.E. (1969). J. Cell Biol. 41:1-20.

Salpeter, M.M., Fertuck, H.C., and Salpeter, E.E. (1977). J. Cell Biol. 72:161-173.

Schlesinger, M., and Chaouat, M. (1975). Eur. J. Immunol. 5:27-32.

Schreiber, R.D., and Müller-Eberhard, H.J. (1974). J. Exp. Med. 140:1324-1335.

Seth, P.K., and Seth, S. (1976). Hum. Genet. 33:201-211.

Shevach, E.M., Herberman, R., Frank, M.M., and Green, I. (1972). J. Clin. Invest. 51:1933-1938.

Shields, R. (1975). Nature (London) 256:257-258.

Shin, B.C., and Carraway, K.L. (1973). Biochim. Biophys. Acta 330:254-268.

Siekevitz, P. (1972). Ann. Rev. Biochem. 41: 117-140.

Silagi, S. (1971). In Vitro 1:105-114.

Silva, W.D., Eisele, J.W., and Lepow, I.H. (1967). J. Exp. Med. 126:1027-1048.

Silveira, N.P.A., Mendes, N.R., and Tolnai, M.E.A. (1972). J. Immunol. 108:1456-1460.

Silver, J., and Hood, L. (1974). Nature (London) 249:764-765.

Simonian, S., Molenaar, J.L., Zeijlemaker, W.P., Knape, J.T.A., Bakker, S. and Pondman, K.W. (1976). Eur. J. Immunol. 6:52-56.

Springer, T.A., Strominger, J.L., and Mann, D. (1974). Proc. Nat. Acad. Sci. (USA) 71:1539-1543.

Steel, C.M. (1971). Nature (London) 233:555-556.

Stites, D.P., Levin, A.S., Austin, K.E., and Fudenberg, H.H. (1971). J. Immunol. 107:1376-1381.

Stoker, M., O'Neill, C., Berryman, S., and Waxman, V. (1968). Int. J. Cancer 3:683-693.

Stossel, T.P., Field, R.J., Gitlin, J.D., Alper, C.A., and Rosen, F.S. (1975). J. Exp. Med. 141:1329-1347.

Swaney, J.B., Vande Woude, G.F., and Bachrach, H.L. (1974). Anal. Biochem. 58:337-346.

Tamura, N., and Nelson, R.A. Jr. (1967). J. Immunol. 99:582-589.

Taylor, R.B., Duffus, W.P.H., Raff, M.C., and De Petris, S. (1971). Nature (New Biol.) (London) 233:225-229.

Tedesco, F., Corrocher, R., and Brown, D.L. (1972). Clin. Exp. Immunol. 10:685-696.

Terhorst, C., Parham, P., Mann, D.L., and Strominger, J.L. (1976). Proc. Nat. Acad. Sci. (USA) 73:910-914.

Ter Laan, B., Molenaar, J.L., Feltkamp-Vroom, T.M., and Pondman, K.W. (1974). Eur. J. Immunol. 4:393-395.

Theofilopoulos, A.N., Bokisch, V.A., and Dixon, F.J. (1974a). J. Exp. Med. 139:696-711.

Theofilopoulos, A.N., Dixon, F.J., and Bokisch, V.A. (1974b). J. Exp. Med. 140:877-894.

Theofilopoulos, A.N., and Perrin, L.H. (1976). J. Exp. Med. 143:271-289.

Trowbridge, I.S., Ralph, P., and Bevan, M.J. (1975a). Proc. Nat. Acad. Sci. (USA) 72:157-161.

Trowbridge, I.S., Weissman, I.L., and Bevan, M.J. (1975b). Nature (London) 256:652-654.

Trowbridge, I.S., and Hyman, R. (1975c). Cell 6:279-287.

Trowbridge, I.S., Hyman, R., and Mazauskas, C. (1976). Eur. J. Immunol. 6:777-782.

Tsai, C-M, Huang, C-C., and Canellakis, E.S. (1974). Biochim. Biophys. Acta 332:47-58.

Tsai, C-M., Chen, K-Y., and Canellakis, E.S. (1975) Biochim. Biophys. Acta 401:196-212.

Tweto, J., and Doyle, D. (1976). J. Biol. Chem. 251:872-882.

Uhr, J.W., and Phillips, J.M. (1966). Ann. N.Y. Acad. Sci. 129:793-798.

Ulsamer, A.G., Wright, P.L., Wetzel, M.G., and Korn, E.D. (1971). J. Cell Biol. 51:193-215.

Venable, J.H., and Coggeshall, R. (1965). J. Cell Biol. 25:407-408.

Venuat, A.M., and Rosenfeld, C. (1974). In Vitro 9:378. (Abstract)

Vitetta, E.S., and Uhr, J.W. (1975a). J. Exp. Med. 139:1599-1620.

Vitetta, E.S., and Uhr, J.W. (1975b). Biochim. Biophys. Acta 415:253-271.

Vitetta, E.S., Uhr, J.W., and Boyse, E.A. (1975). J. Immunol. 114:252-254.

Wang, C-H., Sinkovics, J.G., Kay, H.D., Györkey, F., and Shullenberger, C.C. (1975). Texas Repts. on Biol. and Med. 33:213-250.

Warren, L., Glick, M.C., and Nass, M.K. (1966). J. Cell Physiol. 68:269-288.

Weber, K., and Osborn, M. (1969). J. Biol. Chem. 244:4406-4412.

West, W., and Herberman, R.B. (1974). Cell Immunol. 14:139-145.

Weston, P.D., and Avrameas, S. (1971). Biochem. Biophys. Res. Comm. 45:1574-1580.

Whang-Peng, J., Gerber, P., and Knutsen, T. (1970).
J. Natl. Cancer Inst. 45:831-839.

Wheelock, E.F., and Edelman, R. (1969). J. Immunol. 103:429-436.

Wilson, J.D., and Nossal, G.J.V. (1971). Lancet ii:788-791.

Winchester, R.J., Fu, S.M., Hoffman, T., and Kunkel, H.M. (1975).
J. Immunol. 114:1210-1212.

Wright, A.E., and Douglas, S.R. (1903). Proc. Roy. Soc. (London) 72:357-370.

Yount, W.J., Utsinger, P.S., Hutt, L.M., Buchanan, P.D., Korn, J.H.,
Fuller, C.R., Logue, M., and Pagano, J.S. (1976)
Scand. J. Immunol. 5:795-810.

Yu, A., and Cohen, E.P. (1974a). J. Immunol. 112:1285-1289.

Yu, A., and Cohen, E.P. (1974b). J. Immunol. 112:1296-1307.

End