GENE EXPRESSION AND DIFFERENTIATION IN ANTIBODY FORMING CELLS

A thesis presented for the
degree of
DOCTOR OF PHILOSOPHY

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by

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ABBREVIATIONS

Abbreviations used in this thesis are those recommended in the Biochemical Journal Instructions to Authors (revised 1978), with the following additions:

General

ABC	Antigen binding cell(s)
BL	Burkitt's Lymphoma
BSA	Bovine serum albumin
CHO	Carbohydrate, Carbohydrate Moiety
EC, IC	Extracellular, Intracellular
fl	Fluorescent
H-2	Murine Major Histocompatibility Complex
HLA	Human Major Histocompatibility Complex
LPS	Lipopolysaccharide (B-cell mitogen)
MLR	Mixed lymphocyte reaction
NP-40	Nonidet P-40 (nonionic detergent)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
poly $(A)^+$ RNA	RNA species containing poly(A) sequences at their 3' termini, functionally equivalent to mRNA in this study
RER, SER	Rough, Smooth endoplasmic reticulum
SDS	Sodium dodecyl sulphate
T,G,A-L	A synthetic polypeptide antigen
3D-TKM	3-Detergent, Tris-potassium-magnesium buffer

Chemicals

DATD	Diallyltartardiamide
DEAE	Diethylamino ethyl
DEP	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNP	Dinitrophenyl
DTT	D,L-Dithiothreitol
EACA	ε-Amino-n-caproic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IAA, IAM	Iodoacetic acid, Iodoacetamide
PCA	Pyrrolidone carboxylic acid
PMSF	Phenylmethylsulphonyl fluoride
PPO	Diphenyloxazole
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
TLCK	N-Tosyl-L-lysine chloromethyl ketone

Serological

Ig, cIg, sIg	Immunoglobulin, cytoplasmic Ig, cell surface Ig
IgX	Immunoglobulin of a nonspecified class
H, L-chain	Immunoglobulin Heavy, Light chains
V, C-region	Immunoglobulin Variable, Constant regions
C, N-terminal	Carboxy, Amino terminal ends of a polypeptide chain
F(ab), F(ab)', $F(ab)'_2$, Fc, Fd	Immunoglobulin H-chain or H+L-chain fragments produced by specific proteolytic cleavage
GARIgG, GARIg	Goat anti rabbit immunoglobulin G
NoRIgG	Normal rabbit immunoglobulin G
RAHIgA, RAHIgG, RAHIgM	Rabbit anti human IgA, IgG, IgM

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SUMMARY

Considerable interest is attached to the biochemical events which characterize B-lymphocyte gene expression and differentiation. The expression of Ig is central to these processes. Among the events which are important for an understanding of Ig expression are: 1) the mode of integration of H-chain V and C-region genes, 2) the expression of Ig H-chain genes during isotype diversification, 3) the mode of surface binding and signal transmission of the several receptor isotypes, 4) the pathways which regulate the expression of cell surface and secretory Ig, and 5) the mechanisms involved in amplification of Ig synthesis. Many of these processes can be studied at the level of the unstimulated B-lymphocyte. However, model systems of homogeneous B-lymphocyte cell lines have only recently been considered for detailed biochemical analyses.

The aim of this thesis work was first to characterize a series of human lymphoma and lymphoblastoid cell lines with respect to Ig expression. It was hoped that these cell lines would be characteristic of a series of early stages in B-cell differentiation. Secondly, using these cell lines as model systems, an investigation was undertaken to determine the cell surface binding and biosynthetic pathway of cell surface associated Ig.

Many of the lymphoma cell lines investigated were found to synthesize predominantly cell surface IgM (sIgM), with varying degrees of IgM secretion as well. One lymphoma cell line (U698M) coexpressed cell surface IgM and IgD. Another cell line (Raji) synthesized IgM, but secreted none, and deposited very little on the cell surface. The

lymphoblastoid cell lines synthesized and secreted IgM, with very little surface deposition. One lymphoblastoid cell line (Bec-11) secreted IgG and retained a significant quantity of IgG on the cell surface. It was concluded that these cell lines were analogous in their Ig expression to many of the cell types described in B-cell ontogeny and during mitogen stimulation.

SDS-PAGE analysis of cell surface and secretory IgM μ-chains revealed a reproducible relationship in the molecular sizes of the isolated chains. Cell surface μ-chains were found to be larger than either secretory or intracellular μ-chains from IgM secreting cells. Cell surface IgG from Bec-11 cells was also found to have a larger γ-chain than its secreted counterpart.

The basis for the μ-chain size differences was further investigated in light of previous reports of the same phenomenon, and of a current hypothesis that cell surface μ-chains may have a C-terminal extra hydrophobic sequence for integral membrane binding. Evidence was obtained which strongly supports this hypothesis. Thus, the higher molecular weight μ-chain (putative surface form) was shown by carboxypeptidase digestion to have a different C-terminal amino acid residue than secretory μ-chain. This was the first actual correlation of a C-terminal difference with the higher molecular weight form of μ-chain.

A working hypothesis, that retention of the putative N-terminal precursor sequence on surface μ-chains was involved in membrane binding, was also tested. This hypothesis was disproved on the basis of N-terminal microsequencing analysis (performed in collaboration with Dr. Donald Capra),

which indicated the presence of unblocked V_{HIII} N-terminal sequences on both Daudi and Raji cell surface μ-chains. However, evidence was obtained which suggested a less efficient processing of N-terminal precursors in nonsecretory cells.

Further analysis of the Ig H-chains (and L-chains) from nonsecretory and secretory cells was carried out by comparative SDS-PAGE of the mature cellular products, and their nonglycosylated and cell-free synthesised counterparts. This analysis revealed that the size difference which related to surface deposition or secretion was the property of the polypeptide chains, and not the carbohydrate moieties. However, evidence for variable glycosylation of the nonsecretory μ-chains was obtained. There was no reason to conclude that this variable glycosylation was involved in surface deposition or secretion of the IgM molecules.

SDS-PAGE analysis of nonglycosylated surface μ-chains from Daudi and Raji cells revealed both the surface and secretory forms to be present in a characteristic ratio for each cell line. In vitro synthesis of μ-chains from these cell lines also revealed the two forms in similar relative proportion. It was concluded that these μ-chain forms are encoded by separate messenger RNA species, differing from one another by the presence or absence of a sequence coding for the C-terminal extra peptide. An hypothesis is proposed which integrates this concept into the current understanding of the biosynthetic pathways leading to Ig secretion and surface deposition.

Routine radioiodination and immunoprecipitation of sIgM from Daudi cells

revealed the presence, by SDS-PAGE, of a polypeptide chain (p33) in an unusual covalent association with the sIgM. Analysis of the size, radioiodination characteristics, SDS-PAGE behaviour, cysteine content, and isoelectric focusing spectrum of this polypeptide was carried out. The results clarified earlier reports which considered this component to be an unusual L-chain. Instead, it has been proposed based on these findings that p33 may represent the Daudi I_a antigen heavy chain. Some preliminary serological evidence is cited in support of this proposition. The idea of the Daudi I_a antigen heavy chain being in covalent association with its sIgM is discussed in the light of the known aberrant expression of HLA molecules by this cell line.

INTRODUCTION

1. CELL SURFACE IMMUNOGLOBULIN AS THE B-LYMPHOCYTE ANTIGEN RECEPTOR

1.1. Clonal Selection and the Receptor Hypothesis

Ehrlich (1900) first proposed the idea that precursors of antibody secreting cells possessed a specific receptor for antigen. Ehrlich's model further proposed that this antigen receptor was a cell surface bound form of the antibody molecule itself. It was not until over fifty years later, however, that Ehrlich's original concepts were returned to. The selection theories (Jerne, 1955; Talmage, 1957) were the earliest attempts to restate and amplify Ehrlich's original hypothesis in the light of the existing experimental evidence. The Clonal Selection Theory of Acquired Immunity (Burnet, 1959) was the first comprehensive statement of these ideas. According to the theory, each precursor cell, or small lymphocyte, is precommitted to the synthesis of only one specific antibody. This precommittment is expressed by the lymphocyte in that it possesses surface receptors of only this one specific type, which are capable of binding to a complementary antigen. Upon interaction of these receptors with antigen, the precursor cell becomes stimulated to rapid clonal expansion and the progeny cells in the clone undergo differentiation to increased synthesis and secretion of the specific antibody molecule. Ehrlich's model indicating the Ig nature of the antigen-specific receptor was amplified into the Receptor Hypothesis (Mitchison, 1969).

Clonal Selection and the Receptor Hypothesis are now supported by ample evidence. They explain in a general sense the process by which B-lymphocytes, which are the subset of small lymphocytes responsible for the production of humoural antibody (Claman & Chaperon, 1969;

Miller & Mitchell, 1969), respond to foreign antigens. The evidence supporting clonal selection and the receptor hypothesis has developed along several lines. Thus, it has been shown that: 1) B-lymphocytes possess receptors for antigen, 2) B-lymphocytes have readily detectable sIg, and 3) the sIg and the antigen receptor are the same molecule. In addition, it has been shown that interactions which affect antigen receptors or sIg also affect immune system function in ways which would be predicted from the Clonal Selection Theory.

1.2. Detection of Antigen Binding Cells

To investigate the nature of lymphocyte-antigen interaction, early efforts were directed at the detection of antigen binding cells (ABC) and the elucidation of their functional roles in the immune system.

1.2.1. The Use of Particulate Antigens

The adherence of large particulate antigens to lymphoid cells was the earliest recognized method of directly visualising antigen binding. These studies used as antigens either bacteria (Hayes et al., 1951; Mäkelä & Nossal, 1961; Russel & Diener, 1970) or erythrocytes (Nota et al., 1964; Zaalberg, 1964); the latter technique, referred to as 'rosette formation', found by far the most general application. Thus, rosette forming cells (RFC) could be detected in the lymphoid tissues of both immunised and unimmunised animals, using either heterologous erythrocytes or homologous erythrocytes coated with various soluble antigens, including proteins, polysaccharides and haptens (reviewed by Bach, 1973).

1.2.2. The Use of Radiolabelled Antigens to Enumerate ABC

The use of radiolabelled soluble antigens to detect ABC has been extensively studied in several laboratories (Byrt & Ada, 1969; Sulitzeanu & Naor, 1969; Ada et al., 1970; Dwyer & Mackay, 1970; Humphrey & Keller, 1970). In the original studies of Naor & Sulitzeanu (1967), radiolabelled BSA and autoradiography were used to enumerate BSA-reactive cells in normal mouse splenocytes. It was found that 0.3/1000 cells bound the radiolabelled antigen. Other studies using similar techniques have established the proportion in the lymphoid tissues of ABC for a variety of antigens (reviewed by Warner, 1974). The low amounts of specifically antigen-reactive cells in the normal lymphoid tissues, i.e. 0.002 to 0.07% or an average of about 25,000/spleen (Warner, 1974), are consistent with the predictions of the Clonal Selection Theory.

1.2.3. Other Methods of ABC Detection

Several alternative methods of detecting ABC have been developed. Enzyme antigens, such as β-galactosidase, have been used and the ABC detected by the action of the enzyme on its substrate (Miller et al., 1971; Sercarz et al., 1971). Columns of antigen-coated beads have been used to selectively deplete ABC from lymphoid cell populations (Wigzell & Andersson, 1969; Wigzell, 1970).

1.2.4. Significance of ABC in the Immune Response

In addition to the detection and enumeration of ABC, studies using either the RFC technique or radiolabelled soluble antigens have demonstrated that these techniques are: 1) antigen specific, 2) that binding can be blocked by excess unlabelled antigen, 3) that one cell

binds only one antigen, and 4) that immune animals often show a pronounced increase in numbers of ABC (reviewed by Roelants, 1972). It has been further demonstrated that at least some ABC are precursors of antibody secreting cells. Thus, in the original experiments of Ada & Byrt (1969), it was shown that normal splenocytes which were previously subjected to highly radioactive (125-I) flagellin were unable to produce a specific anti-flagellin response when transferred to syngeneic lethally-irradiated mice. It was concluded that the antigen-reactive lymphocytes had been killed by radioactive damage, by virtue of their having bound the labelled antigen. Similar such 'antigen suicide' experiments were performed in other laboratories using, for example, fowl γ-globulin (Basten et al., 1971) or the synthetic antigen T,G,A-L (Humphrey & Keller, 1970; Humphrey et al., 1971). Unanue (1971) showed that antigen suicide could be induced with bone marrow cells as well, indicating the presence of some ABC in this less-differentiated lymphocyte population.

1.3. Detection of sIg on Lymphoid Cells

The readily detectable presence of sIg on antigen binding cells provided early circumstantial evidence for postulating the antigen receptor to be sIg. Various methods, both direct and indirect, have established the presence of sIg on lymphoid cells. At the core of these methods is the use of specific anti Ig reagents to bind to the sIg. This approach has required careful consideration of the following sources of erroneous results: 1) lack of antiserum specificity, 2) binding of aggregated Ig to cellular F_c receptors, and 3) the possibility that the sIg detected was cytophilic Ig and not native to the cells it was detected on. Many methods have been used to

detect sIg on lymphoid cells (Warner, 1974). In the following sections, only the use of fluorescent antibody will be reviewed, as this method has become widespread and has contributed greatly to the characterisation of sIg.

1.3.1. Direct Visualisation by Fl-anti Ig Binding

The use of fluorescent (fl) labelled antibody was first introduced by Möller (1961) in studies of the mouse H-2 surface antigens. Möller noticed the staining of some cells with fl-anti mouse Ig even in the absence of sandwiched anti H-2, and suggested that this was due to the presence of sIg on the mouse lymphocytes. Raff and coworkers (Raff et al., 1970) characterised the technique with respect to the quantitative and qualitative factors which influence fl-antibody binding. Subsequently, fl-antibody has been used in many studies to determine both the proportion of sIg-bearing cells and the class distribution of sIg in the lymphoid tissues. In addition, fl-antibody has been used to: 1) detect the presence of different classes of sIg and cytoplasmic Ig (Pernis et al., 1970) or sIg only (Pernis et al., 1971) on the same cell; 2) to demonstrate the membrane distribution and movement of sIg molecules (see below); 3) to show that sIg H and L-chains are associated (Takahashi et al., 1971) and 4) to show that sIg and antigen-specific receptors co-cap in the lymphocyte membrane (Raff et al., 1973).

1.3.2. Detection of Patch and Cap Formation with Fl-anti Ig

The reaction of fl-anti Ig on mouse lymphoid cells was observed to yield a characteristic 'patch' formation caused by apparent clumping of Ig determinants in the plasma membrane (Pernis et al., 1970).

This was originally thought to reflect the distribution of sIg in the membrane, however further investigations in which monovalent fl-F(ab) antibodies were used (Loor et al., 1972; Taylor et al., 1971; dePetris & Raff, 1973) showed only a diffuse pattern of labelling. These latter findings were interpreted to indicate that the distribution of sIg was in fact diffuse, and that patching was only induced by the crosslinking effect of divalent antibody. The patching of sIg determinants was shown, in metabolically active cells, to be followed by 'capping' of these determinants to one pole of the cell (Raff, 1970). Investigations from many laboratories (Taylor et al., 1971; Loor et al., 1972; dePetris & Raff, 1972; Unanue et al., 1973) have established that capping is linked to microfilament activity. The 'patching to capping' phenomenon is one of the most important observations to be considered in concepts of lymphoid cell membrane dynamics (Singer, 1974).

1.3.3. Lymphocyte Stimulation with anti Ig

In an important series of experiments, it was first demonstrated that blast transformation and stimulation of DNA synthesis occurred in lymphocytes treated in vitro with antiserum to Ig determinants. The original studies showed that either anti L-chain allotype (Sell & Gell, 1965) or anti class specific heteroantisera (Sell, 1967) could stimulate rabbit lymphocytes in culture. These and other similar studies not only indicated the presence of sIg on lymphocytes, but also demonstrated important aspects about the class representation on sIg and the requirements for lymphocyte stimulation. Thus, anti L-chain antisera was shown to stimulate human lymphocytes (Greaves et al., 1969) and to be generally more effective than class

specific anti H-chain antisera (Greaves, 1970). In both human and rabbit cultures, F(ab) or F(ab)' fragments of anti Ig antibody do not stimulate (Greaves et al., 1969; Fanger et al., 1970), whereas $F(ab)'_2$ fragments do stimulate (Fanger et al., 1970; Froland & Natvig, 1970), indicating a requirement for multipoint binding or crosslinking in the membrane.

1.3.4. Quantitation of sIg

Various methods of quantitating sIg on homogeneous populations of lymphoid cells have been developed. The technique of haemagglutination inhibition was first applied by Klein et al. (1970) to quantitate sIgM on human lymphoid cell lines. This technique was developed into a useful routine method for the qualitative and quantitative determination of sIg on lymphoblastoid cell lines (Steel et al., 1974). Radioimmunoassay techniques have also been used on human (Lerner et al., 1971) and mouse (Rabellino et al., 1971) lymphoid cells. Cell surface iodination by the lactoperoxidase technique (Marchalonis et al., 1971) has also been used to quantitate sIg, but has found much wider application in the biochemical analysis of isolated sIg (discussed below).

1.4. Correlation of sIg with the Antigen Specific Receptor

The ABC detecting techniques described above demonstrated the presence of antigen receptors on lymphoid cells, and the various techniques using anti Ig binding have demonstrated the presence of Ig on the surface of these cells. One major approach to determining whether the antigen receptor and sIg are the same molecule has been to attempt to interfere with antigen binding or B-lymphocyte function using anti Ig antisera.

1.4.1. Blocking of Antigen Binding with anti Ig

Pretreatment of ABC with anti Ig has generally shown that virtually all ABC are inhibited (Byrt & Ada, 1969; Warner et al., 1970; Dwyer & Mackay, 1970 and reviewed by Warner, 1974). In contrast, pretreatment with anti lymphocyte serum (Ada, 1970; Walters & Wigzell, 1970; Roelants et al., 1973) or anti H-2 serum (Hammerling & McDevitt, 1974) does not inhibit antigen binding. Studies of this kind also provided early evidence for the H-chain class of antigen receptors. Thus it was shown (Warner et al., 1970; Dwyer & Mackay, 1970) that only anti L-chain or anti μ gave significant blockage of antigen binding, indicating that the major class of cell surface antigen-binding Ig was IgM.

Anti Ig blocking of ABC was open to the criticism that it may be detecting mere steric hinderance of the antigen receptor, and not indicating that surface Ig and the receptor are the same molecule (Marchalonis, 1975). However, the demonstration that receptors and surface Ig co-capped in the lymphocyte membrane (Raff et al., 1973), whereas most other surface molecules move independently, provided convincing evidence against steric hinderance as an explanation of blocking.

1.4.2. Blocking of B-Lymphocyte Function with anti Ig

The binding of anti Ig to ABC has also been shown to interfere with the transfer of immunity into lethally irradiated syngeneic mice simultaneously injected with antigen (Mitchison, 1967; Warner et al., 1970; Takahashi et al., 1971). In addition, inhibition with anti Ig sera of primary in vitro responses to sheep erythrocytes has been reported by various groups (Fuji & Jerne, 1969; Greaves, 1970; Lesley & Dutton, 1970; Warner & Dwyer, 1971). In other investigations of

anti Ig blocking Pierce et al. (1972a, 1972b, 1973) showed that treatment of unprimed cells with anti μ suppressed responses of all Ig classes, whereas anti γ_1 and γ_2 suppressed only IgG_1 and IgG_2 responses, and anti α suppressed only IgA responses. In primed cells, anti μ suppressed only IgM and IgA responses and anti γ_1 and γ_2 suppressed only IgG_1 and IgG_2 responses. Experiments such as these have led to a clearer understanding of the sequential expression of sIg classes in lymphocyte differentiation. This subject is reviewed in greater detail below (section 5).

1.4.3. Direct Visualisation of sIg-Antigen Binding

In one investigation (Rolley & Marchalonis, 1972), direct visualisation of sIg-antigen binding was achieved in the following way. Spleen cells from unimmunised mice were radioiodinated by the lactoperoxidase method in order to label their sIg. These cells were then incubated with 125-I labelled DNP-haemoglobin. The authors then immunoprecipitated complexes of receptor plus sIg using either antibody to the antigen (DNP-haemoglobin) or antibody to the sIg. It had been previously shown (Lawrence et al., 1973) that a high percentage (0.1-1%) of unimmunised splenocytes bind this antigen, and so limitations normally imposed by the vanishingly small amounts of specific antigen-reactive cells available for analysis could be overcome in this system. It was shown that labelled sIg was present in the anti DNP-haemoglobin precipitate, and vice versa, indicating that sIg was directly interacting with the antigen on the lymphocyte cell surface.

2. PHYSICAL CHARACTERISTICS OF sIgM AND ITS MEMBRANE ASSOCIATION

2.1. Molecular Size of Cell Surface IgM

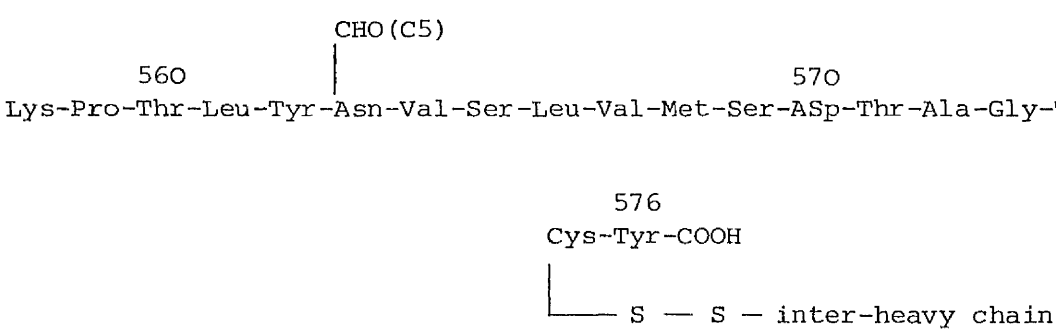
The earliest biochemical analysis of cell surface IgM (sIgM) consisted of G-200 chromatography and centrifugation studies of the sIgM from a human Burkitt's lymphoma (BL) cell line, Daudi (Eskeland & Klein, 1970). These studies showed the sIgM molecule to be an 8S structure. The observation that sIgM was an 8S monomeric molecule was confirmed on BL cells and extended to mouse splenocytes by lactoperoxidase iodination (Baur et al., 1971; Klein & Eskeland, 1971; Vitetta et al., 1971; Marchalonis et al., 1972; Sherr et al., 1972) or biosynthetic labelling (Melchers & Andersson, 1973) and SDS-PAGE analysis of unreduced sIgM isolated by immunoprecipitation. In a more systematic investigation, Kennel & Lerner (1973) showed that SDS-PAGE of iodinated sIgM from a human lymphoblastoid cell line (Wil_2-A3) gave an apparent molecular weight of 265,000 $\pm$ 15,000. This value was considerably higher than the expected value of 180 - 200,000 for monomeric IgM, a point which the authors considered, and which has not yet been adequately explained.

2.2. The Polypeptide Chain Structure of Monomeric IgM

As reviewed in the preceeding section, there is very strong evidence that cell surface IgM is essentially identical to the monomeric form of secretory IgM. Thus, to understand the biosynthesis and membrane presentation of sIgM, it is of value to review a few features of the structure of monomeric IgM.

The basic structure of the human IgMs polypeptide chains is reviewed by Putnam et al. (1973), who also presented the first complete sequence

of the μ-chain (from macroglobulinemia protein Ou). The subunit consists of two μ heavy chains and two light chains (κ or λ). One disulphide bond connects the C-terminal cys of each light chain to cys 140 of each μ-chain. Two disulphide bonds (cys 337 and cys 575) connect the μ-chains to one another. Another sulphydryl (cys 414), which interconnects the subunits into the 19S pentamer, is thought to be reversibly blocked in the monomeric sIgM molecule, based on observations of in vitro polymerisation reactions (Askonas & Parkhouse, 1971). In this context, Stott & Feinstein (1973) have reported that two μ-chain sulphydryls are free in the intracellular IgMs subunit and suggest that a rearrangement of free sulphydryls may occur upon polymerisation. In addition, the μ-chain has 10 cysteine residues which form 5 intrachain disulphide bridges — one in each of the 5 homology regions (V_H, $C_{\mu 1}$, $C_{\mu 2}$, $C_{\mu 3}$, $C_{\mu 4}$). The V_H region ends and the C_μ region begins at approximately position 125. The Fd' segment of Fabμ terminates at Lys-213 and the Fcμ begins at Gly-326. The region in between is degraded to small peptides by trypsin cleavage at 60°C, and includes most of $C_{\mu 2}$ and the area identified as the μ-chain 'hinge' region. The C-terminal end of the μ-chain has a nonadecapeptide 'tail' as shown below, for which there is no counterpart in γ- or ε-chains, but an identical counterpart in α-chains (Kehoe et al., 1973; Moore & Putnam, 1973).



As can be seen from the amino acid sequence, the tail portion is not particularly hydrophobic. The tail sequence (and the entire μ-chain sequence) terminates with Tyr-576.

2.3. Structure and Location of Carbohydrate Moieties

Human monomeric IgM has been shown to contain carbohydrate attached at 5 sites (C1 - C5) in the constant region of each μ-chain (reviewed by Metzger, 1970). The oligosaccharides are of two types: complex and simple. The complex oligosaccharides are present in glycopeptides C1, C2 and C3, and the simple oligosaccharides in glycopeptides C4 and C5 (Spragg & Clamp, 1969). Shimizu et al.(1971) and Putnam et al. (1973) have determined the oligosaccharide compositions and locations, respectively, on the sequenced μ-chain Ou. The glycopeptide C1 is in the Fd' fragment of the μ-chain at position 120. C2 was first reported near position 230 in the 'hinge' region (Shimizu et al., 1971) but was later localised to position 332 in the $C_{\mu 3}$ domain immediately carboxy-terminal to the beginning of the Fcμ (Putnam et al., 1973). This discrepancy was not explained in the latter paper which came from the same laboratory as the earlier report. C3 and C4 are also located within the $C_{\mu 3}$ domain, at positions 395 and 402 respectively, which makes this domain particularly rich in carbohydrate (i.e. two complex and one simple oligosaccharide in a space of 70 amino acid residues). Both the C3 (complex) and C4 (simple) oligosaccharides are within the intra-chain disulphide loop in $C_{\mu 3}$, and are close to the inter-subunit and one of the inter-heavy chain disulphide bridges. C5, a simple oligo-saccharide, is located in the C-terminal 'tail' region, at position 563.

The complex oligosaccharides (C1, C2 and C3) contain one residue of fucose, 2-3 of mannose, 2 of galactose, 3-5 of N-acetylglucosamine and 0-2 of sialic acid in a highly branched structure. The simple oligosaccharide C4 contains 5 residues of mannose and 2 of N-acetylglucosamine, whereas C5 contains considerably more mannose residues (8-11) and 2-3 residues of N-acetylglucosamine. Both of the simple oligosaccharides are linear (unbranched) structures.

2.4. Attachment of sIgM to the Lymphocyte Membrane

Hypotheses concerned with the mode of attachment of sIgM to the lymphocyte membrane must address the critical problem of how a basically hydrophilic molecule like monomeric IgM can be adapted to the hydrophobic membrane environment.

Early explanations of this interaction envisaged an unusual folding of the sIgM molecule, perhaps mediated by a specific pattern of carbohydrate attachment, in which hydrophobic regions of the polypeptide chains were exteriorized forming loops which could interact with the membrane bilayer (Vitetta & Uhr, 1975b). This model would predict a rather weak binding, and support for this was found in the apparent ease of 'shedding' of sIgM as opposed to cell surface H-2 (murine alloantigen) molecules (Vitetta & Uhr, 1973).

More recent hypotheses have considered sIgM as either an integral or peripheral membrane protein respectively, according to the definitions proposed by Singer & Nicolson (1972). Integral membrane proteins, for example glycophorin (Segrest *et al.*, 1972), cytochrome b_5 (Spatz & Strittmatter, 1971) and probably HLA (Springer & Strominger, 1976),

possess C-terminal hydrophobic sequences in their polypeptide chains which extend into, and often through, the membrane lipid bilayer. Secretory IgM μ chain contains no such hydrophobic peptide. However, to examine the possibility that surface μ-chains do have such an extra anchoring peptide, Melcher & Uhr (1973) compared the size of surface iodinated and secreted μ-chains by SDS-PAGE. Their results showed heterogeneous but lower molecular weights for surface μ-chains, which instead was interpreted as favouring the conformational change model; conformational change being induced by under-glycosylation of the surface μ-chain (Vitetta & Uhr, 1975b). Subsequent similar investigations by the same and other workers, however, arrived at the opposite conclusion. Thus, Lisowska-Bernstein & Vassalli (1975) and Melcher & Uhr (1976) both found murine splenic cell surface μ-chains to migrate more slowly than secreted μ-chains, with a molecular weight difference of 1700 reported by the latter group. This finding was consistent with an extra hydrophobic peptide on surface μ-chains capable of integral membrane binding.

Further investigations of cell surface IgM were also consistent with a partial hydrophobic nature of the molecule relative to secreted IgM. Melcher et al. (1976) showed that when nonionic detergent (NP-40) was removed from a cell lysate containing labelled murine sIgM, a proportion of the sIgM molecules became insoluble. In contrast, secreted IgM remained totally soluble in the absence of detergent. These results suggested that sIgM has hydrophobic portions which require detergent for solubility. Moreover, it was shown (Melcher & Uhr, 1977) that sIgM has a lighter buoyant density (by isopycnic centrifugation in caesium chloride gradients) in the presence of nonionic detergents than secreted IgM, consistent with the firm binding of detergent molecules to the sIgM.

Recent hypotheses which view sIgM as a peripheral membrane protein, point to the possible interaction of sIgM with an 'anchor' protein. No such anchor protein has been definitively identified, although a protein similar to the Fc-receptor (Ramasamy et al., 1974) has been implicated. New, if somewhat dubious, impetus for this hypothesis has come from the studies of McIlhinney et al. (1978), who failed to find a difference in the C-terminus of surface versus secreted IgM μ-chains by carboxypeptidase analysis. These studies are open to objection, however, principally due to the lack of convincing evidence that they were indeed analysing actual surface IgM. In contrast to the above findings, Williams & Grey (1978) have investigated by similar methods the well-established sIgM-producing human lymphoma cell Daudi, and reported a distinct C-terminus difference relative to secreted μ-chain.

2.5. Topography of Cell Surface IgM on the Membrane

IgM is a relatively soluble molecule with 5 carbohydrate moieties attached to each μ-chain (Metzger, 1970; Shimizu et al., 1971). Based on thermodynamic considerations (Singer & Nicolson, 1972), these carbohydrate moieties restrict the portions of the μ-chain that could be buried in the plasma membrane. In addition, good evidence that sIgM μ-chains have a C-terminal hydrophobic sequence for membrane binding (reviewed above) eliminates the need to postulate any close interaction between the major portion of sIgM and the plasma membrane.

Evidence indicating the orientation and exposure of sIgM on the lymphocyte membrane derives from experiments testing the susceptibility of sIgM to iodination, proteolysis, and to anti Ig reagents. Uhr & Vitetta (1973) reported that the ratio of μ to L-chain labelling of sIgM

was the same whether the cells were labelled by iodination in the membrane or in solution, implying nearly complete exposure of the sIgM on the cell membrane. However, it was pointed out by Marchalonis & Cone (1973), based on the findings of Bayse & Morrison (1971), that iodination by diffusable short-lived iodine free radicals or molecular iodine can occur in lactoperoxidase-catalysed reactions. This would therefore make relatively buried parts of the sIgM molecule accessible to iodination.

In contrast to the above discussion, a series of well-documented experiments has shown that fl-anti Fcμ antibodies do not stain sIgM-containing lymphocytes very effectively (Pernis et al., 1970; Froland & Natvig, 1972). Fu & Kunkel (1974) showed that the last 50 residues (C-terminal) of sIgM from human leukemia cells were blocked to fl-anti Fcμ antibody. These findings imply that the Fc portion of the sIgM μ-chains are not available for antibody binding.

Evidence examining the relative susceptibility of sIgM to proteolysis also argues in favour of considerable protection of the sIgM molecule. It has been shown that sIgM on intact cells is much more resistant than secretory IgM to degradation by papain (Vitetta & Uhr, 1976), trypsin, α-chymotrypsin and pronase (Hough et al., 1977), although one report from Ferrarini et al. (1976) claims that pronase treatment does result in the loss of cell surface IgM. Hough et al. (1977) found that the sIgD Fcδ fragment, which is normally stable to further degradation (Jefferis, 1975), was removed from intact cells by treatment with papain (in agreement with Vitetta & Uhr, 1976), α-chymotrypsin and pronase; whereas sIgM was left intact. This implies

that only sIgM, and not sIgD, may be 'protected' by the plasma membrane.

Taken as a whole, the present evidence would seem to indicate that while sIgM is probably not deeply buried in the plasma membrane, a large portion of the molecule is nonetheless protected from interaction with large macromolecules such as antibodies or enzymes. The possible modes of protection have been discussed previously (Vitetta & Uhr, 1976). Thus, protection could be rendered by conformational changes in the sIgM molecule to secretory IgM or by interaction of the sIgM with other membrane proteins.

3. BIOSYNTHESIS, ASSEMBLY AND TRANSPORT OF IMMUNOGLOBULIN

3.1. Synthesis on Membrane-Bound Polyribosomes

It is now widely accepted that the endoplasmic reticulum is the subcellular organelle responsible for the processing of secretory and plasma membrane associated proteins (reviewed by Palade, 1975; Shore & Tata, 1977). Accordingly, proteins of this type are considered to be synthesised primarily on polyribosomes bound to rough ER membranes (reviewed by Sabatini & Kreibich, 1976). The first demonstration of this was with the pancreas secreted protein, α-chymotrypsin (Siekevitz & Palade, 1960). Other examples of secretory proteins include rat serum albumin, thyroglobulin, major fractions of milk proteins from mammary glands, chick embryo collagen, egg-white proteins synthesised in the hen oviduct, and immunoglobulins synthesised in plasma cells (reviewed by Shore & Tata, 1977).

A variety of approaches have been used to demonstrate synthesis of

Ig H and L-chains on membrane-bound polyribosomes, including 1) autoradiography coupled with electron microscopy (Scharff & Laskov, 1970; Uhr, 1970) 2) analysis by immunoprecipitation of polypeptides isolated with free or membrane-bound polyribosome fractions (Sherr & Uhr, 1970; Cioli & Lennox, 1973) 3) analysis of in vitro synthesised products from fractionated polyribosomes (Lisowska-Bernstein et al., 1970) or from mRNA derived from fractionated polyribosomes (Blobel & Dobberstein, 1975a and 1975b). Several of these studies also demonstrated significant synthesis associated with free polyribosomes (Lisowska-Bernstein et al., 1970; Sherr & Uhr, 1970), which has been attributed to either artifactual disruption of polyribosomes or alternatively to an occurrence of physiological significance, having to do with the signal hypothesis of polyribosome-membrane attachment (see below).

3.2. Polyribosome-Membrane Interactions in Ig Synthesis

What is the mechanism(s) by which secretory and cell surface proteins become selected for synthesis on membrane-bound polyribosomes? Studies of Ig biosynthesis have complemented numerous similar investigations in other systems to provide a partial understanding of the mechanisms involved. Three types of probable binding interactions have been identified: 1) direct binding of the ribosomal 60S subunit to the rough ER membrane, mediated either by ribosomal proteins, ribosomal RNA, membrane receptor sites, or all of these, 2) binding of the nascent chains to membrane sites, and 3) binding of the mRNA (3'end) to membrane sites.

Polyribosomes can either be completely membrane bound (i.e. each ribosome on the messenger is membrane-bound) or 'dangling' (some but not all

of the ribosomes on the messenger are membrane-bound). This distinction was made by Rosbash & Penman (1971a, 1971b). Rat liver polyribosomes are an example of the completely bound type, since limited digestion (of only mRNA) with pancreatic ribonuclease removes only 10% of the ribosomes from liver rough microsomal fractions (Blobel & Potter, 1967). Adelman et al. (1973b) have further shown that puromycin treatment, which dissociates nascent chains from the ribosome, releases a negligible amount of 60S subunits. This approach shows that direct ribosomal binding is occurring in rat liver microsomes, but does not rule out the possibility that nascent chain - mediated binding is also occurring.

Investigations using myeloma cells have led to the conclusion that Ig synthesizing polysomes are of the 'dangling' type (Harrison et al., 1974a, 1974b), and have further shown that the tightly bound ribosomes are anchored by a nascent chain - membrane interaction (Harrison et al., 1974b). The distinction between this type of interaction and one in which only direct binding is involved is based on the development of a method for removing 60S subunits (Adelman et al., 1973a, 1973b; Harrison et al., 1974a, 1974b) using puromycin and high concentrations of KCl. Thus, ribosomes bound by nascent chains are removed with puromycin and KCl treatment, but not KCl alone. Harrison et al. (1974b) have also demonstrated that KCl/puromycin treatment removes almost all the poly(A)$^+$ Ig mRNA from the rough ER, which argues against direct binding of Ig mRNA to the membrane in myeloma cells. Based on their results with ribosome binding, Harrison et al. (1974b) have suggested that loosely bound ribosomes associated with Ig mRNA are precursors of the tightly bound fraction. In support of this idea, Mechler & Vassalli (1975) have shown that loosely bound myeloma cell

ribosomes (i.e. those released by limited ribonuclease digestion) contain nascent polypeptide chains which are on average 2-3 times shorter than chains associated with the tightly bound fraction.

3.3. The 'Signal Hypothesis' and Vectorial Release

The implication that nascent chain binding to rough ER membranes is a mechanism of polyribosome attachment was supported by the original observation of Milstein et al. (1972) that Ig L-chains synthesised in vitro had an hydrophobic extra peptide at the N-terminus. Schmeckpeper et al. (1975) showed that a product the same size as the L-chain precursor could be isolated from the intact myeloma cells, but only with very short labelling periods and in the presence of the protease inhibitor, TLCK. This indicated that the precursor was not an artefact of in vitro synthesis, and that it was probably removed during or immediately after translation. Similar hydrophobic precursors of Ig L-chains (Blobel & Dobberstein, 1975; Schechter & Burstein, 1976) and more recently H-chains of the mouse α (Jilka & Pestka, 1977) and γ_{2a} type (Singer & Williamson, in preparation) have been detected and sequenced. Examples of precursors detected on other secreted proteins include: insulin (Chan et al., 1976; Duguid et al., 1976), α-lactalbumin (Craig et al., 1976), melittin (Suchanek, 1975), parathyroid hormone (Kemper et al., 1976) and pancreatic secretory proteins (Devillers-Thiery et al., 1975).

The 'signal hypothesis' as originally proposed, (Blobel & Sabatini, 1971; Milstein et al., 1972) and in its more elaborated form (Blobel & Dobberstein, 1975a, 1975b), incorporates many of the above observations into an attractive model. According to the model, initiation

of translation occurs on messenger RNAs free in the cytoplasm. Binding of the messenger-ribosome complexes occurs as the growing N-terminus, with its hydrophobic segment, interacts with the membrane. Evidence that this interaction may be through special receptor proteins has been taken from investigations showing that mild treatment of rough microsomal membranes with trypsin either releases ribosomes or interferes with binding of ribosomes to previously stripped membranes (Shires et al., 1973; Bailley et al., 1974; Jothy et al., 1975). The model is also consistent with the evidence of Rosbash (1972) that newly-synthesised mRNA was arrested in small cytoplasmic polyribosomes upon treatment with the translation inhibitor, cycloheximide. Upon removal of the cycloheximide, the small polyribosomes could be 'chased' into a fraction of larger, membrane associated polyribosomes.

An important feature of the 'signal hypothesis' is that the hydrophobic signal sequence which initially binds the polyribosome to the rough ER membrane is also thought to lead the growing nascent chain through the membrane and into the microsomal compartment (cisternal space). Such a transfer across the microsomal membrane has been termed 'vectorial release', and is an essential element in the intracellular life-history of secretory or cell surface molecules such as Ig. It has been possible to test this hypothesis by the use of cell-free translation systems incorporating exogenously added polyribosomes and native or stripped microsomes. Thus, Blobel & Dobberstein (1975a, 1975b) have demonstrated that L-chain mRNA directs the synthesis of mature L-chains in a cell-free system containing microsomes. This L-chain could not be degraded by exogenous proteases, indicating that it had been segregated into the microsomal cisternae. In contrast,

globin mRNA translated in the same system was not resistant to proteolysis. The authors were able to conclude from these experiments that the information for microsomal binding and vectorial release of nascent polypeptides was encoded by the mRNA (i.e. the N-terminal hydrophobic sequence) and was not inherent in the protein synthetic machinery. Previous results with a similar in vitro microsomal system (Vassalli et al., 1971) also indicated that newly synthesised L-chain is segregated into the cisternal space of microsomes.

3.4. Ig Assembly and Early Events in the Microsomal Compartment

Williamson & Askonas (1967) first showed that Ig H and L-chains are synthesised on separate polyribosomes. As reviewed above, ample evidence established that these H and L-chain synthesising polyribosomes 1) become bound to the cytoplasmic side of the microsomal membrane; 2) that this binding is mediated by the hydrophobic N-terminal precursor segment on the nascent chains; 3) that the growing nascent chains are transferred vectorially through the membrane and into the microsomal lumen, perhaps through a 'pore' created by the prior passage of the precursor segment; and 4) that inside the lumen the polypeptide chains fold into their native soluble conformation and, once this occurs, they cannot be transferred back through the membrane.

Are the rates of synthesis of H and L-chains coordinated, or is one polypeptide chain type synthesised in excess? Studies from different laboratories and using different cell types (i.e. myeloma tumours or lymph node cells) give somewhat varying results. It is clear that small L-chain pools exist in both tumour (Askonas & Williamson, 1966) and lymph node cells (Askonas & Williamson, 1967a), and that the

L-chain pool is involved in the assembly of Ig in both cell types (Williamson & Askonas, 1968; Stott, 1972). Very little, if any, free H-chain has been detected in intracellular pools (Askonas & Williamson, 1966; Scharff et al., 1967; Schubert, 1968). However, these findings are not inconsistent with a balanced synthesis of H and L polypeptide chains (Askonas & Williamson, 1967a). To the contrary, the apparent steady-state nature of the L-chain pool provides evidence in favour of balanced synthesis. However, the studies of Baumal & Scharff (1973), using both lymph node cells from hyperimmunised mice and a panel of myeloma tumours, are in contrast to the above investigations. These authors found balanced synthesis of H and L-chains in only a small number of cases. In all cases where unbalanced synthesis occurred, L-chain was the one synthesised in excess.

Besides glycosylation (see below), two post-translational processing events, 1) removal of H and L-chain precursor segments and 2) cyclisation of N-terminal glutamine residues present in some V_H subgroups to PCA, are known to affect Ig polypeptide chains. It is generally assumed that the hydrophobic precursor segment is rapidly removed from the growing nascent chain once it enters the microsomal compartment, although this has been shown conclusively only for the L-chain (Milstein et al., 1972; Blobel & Dobberstein, 1975b). PCA formation in a mouse IgG_{2a} myeloma protein (from Adj PC5 plasmacytoma line) has been investigated by Stott (1972). Cyclisation to PCA, which affects only H-chains in this tumour line, was barely detectable on nascent polypeptides precipitable with anti Fc antibody; and even after 1 or 5 hours only 60-66% of the H-chains have N-terminal PCA. It was concluded from this investigation that cyclisation was, in fact, a post-trans-

lational processing event, but not necessarily an early one in the Ig biosynthetic pathway. This finding is compatible with either rapid or delayed removal of the H-chain hydrophobic precursor segment.

The next stage in the intracellular life history of Ig is the assembly of H and L-chains into the characteristic Ig H_2L_2 structure. It is evident that appreciable assembly of the chains does not occur until after they are released from the ribosomes. The early investigations of Williamson & Askonas (1967a) on Ig nascent chains in the mouse myeloma tumour line 5563 indicated that only insignificant amounts of L-chains could be detected by specific immunoprecipitation of H-chain nascent polypeptides. However, in subsequent investigations, these authors found that 131-I-labelled L-chains can associate *in vitro* with nascent H-chains (Askonas *et al.*, 1969). The L-chain binding was spread across a broad range of polyribosome sizes, but was equivalent to only one L-chain per polyribosome complex (14-18 ribosomal units). Pulse-chase labelling of myeloma tumour cells and normal lymph node cells (Shapiro *et al.*, 1966; Baumal *et al.*, 1971; Laskov *et al.*, 1971) indicated that nascent H and L-chains were fully released 2 minutes after a 1-3 minute pulse. It was shown that during this time very little noncovalent or covalent assembly occurred. Moreover, 7 minutes after the chase, only 37% of the newly synthesised chains had been assembled into H_2L_2 structures. Lymph node cells were found to assemble H and L-chains more rapidly than the myeloma cells, although assembly in both cell types was not totally complete, with free L-chains and various intermediate forms also being secreted. Thus, while association of H and L-chains prior to release from the ribosomes can, and possibly does, occur to a very limited degree, this association is apparently

not a very significant aspect of the whole assembly process.

The pathways of assembly of H and L-chains vary depending primarily on the structure (i.e. class) of the H-chain, and probably to a lesser extent on the species investigated. In Balb/c tumours, IgG_1, IgG_{2a}, and IgA assemble predominantly through H_2 and then H_2L intermediates, while IgG_{2b} and IgM assemble primarily through the HL half molecule. The intermediates formed in these pathways also reflect the order of noncovalent association of the polypeptide chains (Askonas et al., 1969; Baumal et al., 1971; and reviewed by Scharff, 1974). The assembly, and possibly the surface deposition, of IgM as half molecules in human cells is also suggested by the findings of Kennel & Lerner (1972). These authors detected IgM half molecules on the surface of cells of the lymphoblastoid line Wil_2-A3 using lactoperoxidase iodination and SDS-PAGE analysis.

Are the pathways of Ig assembly mediated by cellular processes, or simply by the physical properties of the H and L-chains involved? The experiments of Petersen & Dorrington (1974) and Percy et al. (1975) support the latter view. Thus, these authors found that the in vitro assembly kinetics and pattern of several mouse and human Igs is identical to the in vivo situation.

3.5. Glycosylation

Immnoglobulin molecules are glycoproteins. Carbohydrate is covalently attached to one or more specific sites in the constant region of each class of H-chain. Carbohydrate moieties are also found attached to H-chain and L-chain V-regions, although much less frequently. The composition,

method of assembly and subcellular sites of assembly of Ig carbohydrate follows the general pattern observed for other glycoproteins. These aspects are discussed below.

For both H and L-chains, the carbohydrate moiety consists of a 'core' region of linearly arranged N-acetylglucosamine and mannose subunits, attached to an asparagine residue via the N-acetylglucosamine. Recent evidence (Behrens et al., 1973; Hsu et al., 1974; Lennarz, 1975) has indicated that this 'core' region can be transferred in vitro, as a preassembled oligosaccharide, from a dolichol phosphate carrier. Eagon et al. (1975) have reported that such a transfer occurs in vitro to nonglycosylated MOPC 46 L-chain obtained by 2-deoxyglucose inhibition. The 'core' region linkage is specific for the tripeptide sequence asn-X-ser (or thr), and apparently also for certain undefined factors (i.e. protein conformation), since glycosylation does not occur at every asn-X-ser (or thr) tripeptide sequence (Shimizu et al., 1971).

Carbohydrate moieties on Ig molecules are of two general types: simple and complex. Simple carbohydrate moieties consist only of the $(glcNAc)_2$-$(mannose)_x$ 'core' region, which may be added as a preassembled oligosaccharide in a single step, as described above. Complex carbohydrate moieties consist also of the 'core' region, to which are attached other 'side chain' sugars such as additional glcNAc, galactose, and terminal fucose or sialic acid (reviewed by Jentoft et al., 1976 and Molnar, 1976). These additional sugars (and possibly some of the core sugars as well) are added by sequential transfer of individual sugars to the growing oligosaccharide side chains. These reactions are catalysed by a series of membrane-associated enzymes known collectively

as the multiglycosyl-transferase system (MGT) (Schachter et al., 1970). Each enyzme catalyses the transfer of a specific sugar from a sugar nucleotide to the oligosaccharide chain. Each enzyme requires the product of the preceeding enzyme as its substrate, and in turn generates the substrate for the next transferase. In addition to recognising the preceeding sugar as substrate, there is evidence that transferase enzymes (i.e. sialyltransferase) may also recognize the peptide sequence around the linkage amino acid, since the IgM μ-chain oligosaccharides C2 and C3 are apparently identical, except that C2 contains two terminal sialic acid residues, whereas the C3 contains only one (Baenziger & Kornfeld, 1974). The acceptor specificity exhibited by the glycosyl transferases in general appears quite stringent, and it is this stringency which is thought to determine the precise structure of a particular oligosaccharide (reviewed by Baenziger & Kornfeld, 1974).

A number of groups have used a variety of ultrastructural and biochemical methods to determine the subcellular biosynthetic sites and order of addition of carbohydrate residues to L-chains (Choi et al., 1971ab),to IgG γ-chains (Melchers & Knopf, 1967; Swenson & Kern, 1968; Melchers, 1970; Melchers, 1971a, 1971b), and to IgM μ-chains (Schenkein & Uhr, 1970a, 1970b; Uhr, 1970; Uhr & Schenkein, 1970; Zagury et al., 1970; Parkhouse & Melchers, 1971).

The results of these studies, which are virtually identical to those obtained in investigations of the biosynthesis of non-Ig glycoproteins, are briefly outlined below. The glcNAc and mannose core sugars are incorporated either prior to polypeptide chain completion or shortly after release of completed polypeptides into the cisternae of the rough

ER (Uhr, 1970). These sugars can also be incorporated at a slightly later stage in the sequential passage of the polypeptide chains through the various membrane compartments - i.e. at the level of the smooth ER or Golgi complex. Sugars which make up the side chains of complex oligosaccharides, such as galactose and/or additional glcNAc, are incorporated by membrane-bound transferases in the smooth ER and primarily in the Golgi complex. The terminal fucose and sialic acid residues appear to be added very close to the time of secretion (Parkhouse & Melchers, 1971; Melchers, 1972).

3.6. Secretion

The final stages of transport of Ig through the cisternal spaces of the endoplasmic reticulum (i.e. RER, SER, Golgi, and post-Golgi compartments) are poorly understood, as are the mechanisms which enable Ig, of all the intracisternal proteins, to be selectively secreted. An hypothesis has been proposed by Uhr (1970), which provides a basis for further understanding. According to the hypothesis, Ig molecules are transferred from the Golgi complex to the cell surface within vessicles formed from the endoplasmic reticulum. These vessicles are then thought to fuse with the plasma membrane, thereby releasing the Ig to the outside by reverse pinocytosis. For Ig to be selectively secreted according to the model, it would have to be free inside the vessicle, whereas non-secretory proteins would presumably be bound to the vessicular membrane. It has been proposed (Uhr & Vitetta, 1973) that Ig molecules are initially membrane-bound, and that at some point in their transport a 'signal' is read which results in their release into the vessicle. There is no direct evidence to support this hypothesis, although Choi et al. (1971a, 1971b) have observed that sonication of microsomal

fractions, which is thought to disrupt vessicles but leave the structure of the membrane basically intact, results in the selective release of Ig.

It has been argued that glycosylation of Ig is important for secretion, perhaps providing the 'signal' necessary for release of vesicular membrane-bound Ig (Uhr et al., 1974). However, nonglycosylated proteins such as L-chains or albumin can be secreted normally (Schachter, 1974). Melchers (1973) used 2-deoxyglucose to selectively inhibit glycosylation of Ig in several myeloma tumours. Cell fractionation studies revealed that 2-deoxyglucose inhibited Ig transport from the polysomes to the RER, and from the RER to the SER, but Ig in the SER was quantitatively secreted. Thus it was concluded that addition of the terminal sugars fucose and sialic acid, and perhaps also galactose, was not required for secretion. Investigations of a variant cell line which synthesises a small (42,000 daltons) γ heavy chain, half the molecules of which are nonglycosylated (Weitzman & Scharff, 1976), indicated that both the glycosylated and nonglycosylated γ-chains were assembled and secreted with similar kinetics.

IgM and IgA molecules are further polymerised before secretion. In the case of IgM, the polymerised product consists of five IgMs subunits arranged radially and disulphide bonded to one another. In both Ig types, a third polypeptide chain, J-chain, has been found attached to the penultimate cysteine residue of the μ-chain (Halpern & Koshland, 1970; Mestecky et al., 1971). J-chain has a molecular weight of approximately 15,000, and is present in a ratio of one chain per polymer molecule (Mestecky et al., 1972; Halpern & Koshland, 1973).

The function of J-chain is not clear, although it has been suggested that it may play a part in the assembly of the polymer (Della Corte & Parkhouse, 1973; Parkhouse & Della Corte, 1973; Chapuis & Koshland, 1974). However, Stott (1976) has shown that J-chain can be added to fully polymerised IgM in the myeloma tumour line Y5781, and is absent from intracellular IgMs in mitogen-stimulated spleen cells. These results therefore suggest that J-chain is not necessary for the polymerisation of IgM.

4. BIOSYNTHETIC PATHWAY OF CELL SURFACE IgM

4.1. Introductory Remarks

Experiments designed to investigate the synthesis and intracellular transport of cell surface IgM, as distinct from secretory IgM, have used either small murine splenic lymphocytes or human lymphoma cell lines as model systems. These experiments begin with the synthesis of Ig H and L-chains on membrane-bound polyribosomes, and follow the incorporation of ^{3}H-sugars and the intracellular compartmentalisation of the Ig to the point of surface deposition. It has emerged that many of the basic steps of this process are the same for surface IgM as for secretory IgM. However, many questions concerning the control of the two pathways still remain to be answered. The details are reviewed below.

4.2. Synthesis and Early Intracellular Transport

The human lymphoma cell line Daudi (Klein et al., 1968) has been used for many of these investigations. Daudi cells have been shown to synthesize (Sherr & Uhr, 1971; Sherr et al., 1971) but not secrete

(Klein et al., 1968; Nadkarni et al., 1969; Sherr & Uhr, 1971; Sherr et al., 1971; Grundke-Iqbal & Uhr, 1974) an IgMκ molecule. Instead, cell surface IgMκ is expressed, which is readily detectable by conventional techniques (Klein et al., 1967, 1968, 1970; Takahashi et al., 1969; Hammond, 1970). By electron microscopic examination (Hammond, 1970; Sherr & Uhr, 1971) Daudi cells have a paucity of membrane-bound polyribosomes and lack a well developed endoplasmic reticulum. Because of these characteristics Daudi cells have been considered a neoplastic counterpart and good experimental model for resting B-lymphocytes.

In the investigations of Sherr & Uhr (1971) and Sherr et al. (1971), Daudi IgM was detected by 3H-leucine incorporation and antibody precipitation from various subcellular fractions. Although there was little membrane-bound protein synthesis in these cells, IgM was preferentially synthesized on membrane-bound polyribosomes. The newly synthesized IgM rapidly entered the microsomal compartment where it accumulated linearly for at least 90 minutes. The labelled microsomal IgM could not be chased into the cell sap during the 90 minute incubation with unlabelled leucine, indicating that any presumed degradation of IgM either acts on a very large intracellular pool; or alternatively, that degradation is the final step in an ordered intracellular transport of newly synthesized IgM which takes more than 90 minutes to complete.

4.3. Glycosylation and Transport to the Golgi Complex

As part of the same experiments described above (Sherr & Uhr, 1971; Sherr et al., 1971), the incorporation of 3H-galactose was shown by autoradiography and electron microscopy to be localised in the Golgi

complex. Galactose and fucose were also incorporated into IgM H and (putative) L-chains, which were isolated by immunoprecipitation from the microsomal fraction of the cells. These results were consistent with the addition of these sugars to Ig chains in the smooth ER or Golgi complex, similar to the situation observed with glycosylation of secreted Ig.

The pattern of glycosylation of cell surface IgM in mouse splenocytes has been more controversial, possibly due to the heterogeneity of the splenic lymphocyte population. Melchers & Andersson (1973) could not label slowly turning over (i.e. cell surface) IgM from mouse splenocytes with 3H-galactose. Consistent with this, Melcher & Uhr (1973) found splenocyte surface IgM μ-chains to be of variable and lower molecular weight than secreted IgM μ-chains, from which they argued indirectly that glycosylation of sIgM may not be as complete as that of secreted IgM.

In contrast to their previous findings (Melcher & Uhr, 1973) and those of Melchers & Andersson (1973), Vitetta & Uhr (1974) reported that galactose and fucose were added to splenic cell surface IgM. These experiments involved the isolation of biosynthetically labelled (3H-amino acids and 3H-sugars) IgM from the surface of intact cells by immunoprecipitation. It was also shown by this method that labelled IgM appeared on the cell surface with a latent period of approximately 1-2 hours (Vitetta & Uhr, 1974; Uhr et al., 1974). Subsequent investigations on the size of splenic cell surface IgM (Melcher & Uhr, 1976) and Daudi lymphoma cell surface IgM (this thesis) provide additional indirect evidence that the glycosylation of sIgM is almost, if not

entirely, equivalent to that of secreted IgM.

4.4. Post-Golgi Transport and Surface Deposition

As is the case for secretory Ig, the mechanism of post-Golgi transport and surface deposition of sIgM is not well understood. A model that has been put forward (Uhr, 1970; Uhr & Vitetta, 1973) proposes that the mechanism thought to exist for secretory Ig — that is, transport within post-Golgi vessicles, fusion of these vessicles with the inside of the plasma membrane, and reverse pinocytosis to release Ig into the extracellular environment — is basically similar for cell surface IgM. The distinction is only that cell surface IgM remains attached to the membrane in the interior of the transport vessicle, rather than being free inside the vessicle. Thus, when reverse pinocytosis occurs, the sIgM molecules end up attached to the outer plasma membrane.

This model is consistent with the little available data, however it leaves many questions unanswered. The mode of attachment of sIgM to the microsomal membrane is not known, although ideas advanced to explain the mode of attachment of sIgM to the plasma membrane (section 2) presumably apply. A key feature of the Uhr (1970) model (also elaborated in Uhr & Vitetta, 1973; Vitetta & Uhr, 1975a) is that molecules destined both for surface deposition and secretion start off in the rough ER and progress to the Golgi complex bound, for a period during that migration, to intracellular membranes. This is presumed to be necessary for the addition of carbohydrate by membrane-bound glycosyl transferases.

During Golgi processing and post-Golgi transport, it is proposed that

a 'signal' is read which results in release into the Golgi vessicle of the IgM destined for secretion, with the IgM destined for surface deposition being retained in the membrane. The nature of this hypothesised signal for the release of bound Ig in the Golgi complex has not been resolved. (The original idea of the models' authors, that the addition of terminal carbohydrate residues such as fucose to the secretory IgM molecule signaled its release (Uhr et al., 1974), is inconsistent with their own earlier data from Daudi cells, and subsequent data from splenocytes indicating fucose addition to sIgM as well.)

Do these synthetic pathways occur in the same or different subcellular compartments? The answer to this question is of course important in assessing the Uhr model. The kinetics of appearance of pulse labelled cell surface IgM (Uhr et al., 1974; Vitetta & Uhr, 1974) and secretory Ig (Scharff et al., 1967; Melchers, 1970; Uhr, 1970; Parkhouse, 1971) are relevant here. Experiments from the above laboratories have indicated that secretory Ig appears with a latent period of 20-30 minutes; whereas the previously cited investigation of Daudi cells indicated a latent period of 1-2 hours for the appearance of sIgM. A cursory comparison of these results appears to argue for different compartmentalisation, and against the precise formulation of the Uhr model. However, the interpretation is complicated because the kinetics were measured in very different cell types (i.e. mouse myeloma cells and Daudi human lymphoma cells). Moreover, the greater sensitivity generally required to detect sIgM by biosynthetic labelling may have resulted in an over-estimate of its latent period.

5. Ig EXPRESSION DURING B-LYMPHOCYTE MATURATION

5.1. Introduction

The question of Ig expression during lymphocyte maturation has recently received considerable attention. This increased interest has derived principally from recent studies on the generation of Ig isotype diversity, both *in vivo* and in mitogen stimulated cultures (for recent reviews see Nossal *et al.*, 1977; Parkhouse & Cooper, 1977; Kearney & Abney, 1978). These studies have re-emphasised other maturation-associated phenomena of Ig expression as well, such as the change from sIg to secretory Ig and the amplification of Ig synthesis upon stimulation. The picture presented for Ig expression is one in which both pre-programmed control at the virgin B-lymphocyte level and antigen and/or accessory cell-dependent control appear to be operating. Forms of Ig expression which have been described, and which can occur in the progeny of a single precursor cell, include: 1) synthesis of cytoplasmic Ig (cIg) without surface deposition, 2) surface deposition without appreciable cIg or secretory Ig synthesis, 3) surface deposition *with* cIg and secretory Ig and 4) cIg synthesis and secretion without appreciable surface deposition.

5.2. Isotypes and the Evidence for Isotype Diversity

The evidence that Ig H-chains (and L-chains) are composed of two functionally separate regions, termed V-region and C-region, is well established. In the H-chain, the V_H region determines the antibody specificity (in combination with V_L), while the C_H region determines the Ig class (i.e. μ, γ, α, δ, and ε) and subclass (γ_1, γ_2, γ_3 etc.) of the molecule. Thus, it was first recognized from amino terminal

sequence analysis of Igs (Hood et al., 1966; Edelman & Gally, 1967; Pink et al., 1972) and from comparison of rabbit V-region (a) allotypes (Todd, 1963; Koshland, 1967; Todd & Inman, 1967) that Ig H-chains from most of the known classes share similar V-regions.

Further investigations revealed biclonal myeloma Igs which, although of different classes, share identical idiotypic determinants and apparently identical V-region sequences. Of such biclonal myelomas, those of the Ig classes IgM plus IgG_2 (Wang et al., 1969; Wang et al., 1970ab; Fudenberg et al., 1971), IgM plus IgG_3 (Penn et al., 1970), IgM plus IgA (Seon et al., 1973; Yagi & Pressman, 1973), and IgA plus IgG (Sledge et al., 1976) have been described. In these cases of rare myelomas, it was generally agreed that transformation had occurred in a lymphocyte clone which, although precommitted to the synthesis of only one V_H region, was in the process of changing C_H expression from one class to the other.

The predictions of clonal selection, together with the observation that normal immune responses 'mature' from IgM antibody to IgG (or IgA) antibody production, provided an early basis for the concept of H-chain constant region shifts within a clone of differentiating lymphocytes. The term 'isotypes' is used for the Ig products of such clones, denoting the fact that these Igs possess identical V_H regions associated with different C_H regions. (In contrast, both the V_L and C_L regions of the L-chain are known to remain fixed during clonal differentiation.)

It is now firmly established that isotypes are generated during the life cycle of B-lymphocytes. The existence of biclonal myeloma proteins

provided strong early support for this concept. In addition, various investigations have led to the conclusions that 1) the progeny of single antibody binding cells have identical idiotypes as the receptor Ig (Press & Klinman, 1973; Eichmann & Rajewsky, 1975; Gearhart et al., 1975; Trenkner & Riblet, 1975), 2) a small percentage of antibody forming cells secrete both IgM and IgG antibodies (Nossal et al., 1964; Ivanyi & Dresser, 1970; Nordin et al., 1970; Nossal et al., 1971), 3) cells double staining for different classes of cellular Ig and surface Ig by immunofluorescence can be detected (Pernis et al., 1971; Jones et al., 1973; Litwin & Cleve, 1973), and 4) sIgM and sIgD receptors (discussed below) on a single cell have the same idiotype (Salsano et al., 1974; Fu et al., 1975; Goding & Layton, 1976) and antigen specificity (Pernis et al., 1974).

5.3. Early Observations and Models of Ig Isotype Diversification

Many investigations of the suppression of Ig production (Kincade et al., 1970; Kincade et al., 1971; Herrod & Warner, 1972; Lawton et al., 1972; Kincade & Cooper, 1973; and reviewed by Lawton & Cooper, 1974) and the suppression of specific antibody production (Pierce et al., 1972a, 1972b, 1973) by heterologous anti Ig treatment of B-lymphocytes have established that Ig isotypes are sequentially expressed in vivo. Since IgM is the major detectable antigen receptor on B-lymphocytes, the concept emerged that IgG and IgA-producing cells arise in clones where the cells originally bore sIgM.

In a series of classical experiments, it was clearly demonstrated that anti μ treatment of lymphocytes in embryonic chickens resulted , later in life, in a profound depression of serum IgM, as well as IgG (Kincade

et al., 1970) and IgA (Kincade & Cooper, 1973) levels. Similar anti μ treatment at hatch, however, depressed only serum IgM levels, but not serum IgG levels (Kincade et al., 1971). These studies were extended to neonatal germ-free mice, where it was shown that anti μ treatment was only effective in eliminating IgG and IgA production if it was administered at a certain critical time during neonatal development (Lawton et al., 1972).

When heterologous anti Ig was used to suppress primary in vitro antibody responses, it was found that anti μ suppressed responses of all Ig classes, whereas anti γ_1, anti γ_2, and anti α suppressed only IgG_1, IgG_2, and IgA responses. In contrast, when primed cells were tested for a secondary in vitro response, it was found that anti μ treatment had progressively less effect with time after priming (Pierce et al., 1972a, 1972b). The authors concluded from these results that some sIgM-bearing cells are precommitted to differentiate into cells secreting one of the other Ig isotypes.

The studies of Pierce et al. (1972a, 1972b) provided evidence for antigen-driven isotype diversification, or the so-called isotype 'switch' model (Nossal et al., 1964, 1971 ; Wang et al., 1970a; Warner, 1972; Pierce et al., 1973; Vitetta & Uhr, 1975a). The alternative model of isotype diversification, the 'minimal receptor' model (Mäkelä, 1970; Mäkelä & Cross, 1970) postulates that separate IgM, IgG and IgA precursors exist. The results cited previously, from Kincade, Lawton, Cooper and coworkers, provided support for this latter model, and further suggested that the generation of separate precursors occurred by sequential activation of receptor isotypes (IgM → IgG → IgA) during

embryogenesis (Cooper et al., 1972a, 1972b). Both models therefore have in common the concept of isotype switch. They differ, however, in whether the switch is antigen driven (Model 1) or antigen-independent (Model 2).

Many of the observations of Ig isotype diversity appeared to be better explained by one or the other of the above models (Lawton & Cooper, 1974; Warner, 1974). Thus, the concept of sequential embryonic development of IgM → IgG → IgA-bearing cells, which then become the precursors of cells secreting the respective isotypes, was inconsistent or less consistent with the following observations, which have been reviewed above: 1) the presence of a small percentage of cells secreting both IgM and IgG antibodies; 2) the phenomenon of biclonal myeloma proteins; 3) the stable persistence of sIgM in cells staining by fluorescence also for cytoplasmic IgG; and 4) various observations which link IgA and IgM expression, rather than IgA and IgG expression (such as the IgA plus IgM biclonal myeloma proteins, the cytoplasmic IgA, sIgM containing Peyer's patch cells of the rabbit, and the effectiveness of anti μ and not anti γ in suppressing IgA responses (Pierce et al., 1972b)).

5.4. Ig Expression in Ontogeny and in Mitogen-Stimulated Cells: Current Concepts

Recent advances in the understanding of Ig isotype diversification have come mainly from immunofluorescence studies, which have identified Ig isotypes on lymphocytes both during ontogeny and in mitogen-stimulated cultures (Abney et al., 1975; Kearney & Lawton, 1975a, 1975b; Melchers et al., 1975a; Andersson et al., 1976; Kearney et al., 1976a,

1976b; Raff et al., 1976; Askonas & North, 1977; Kearney et al., 1977a, 1977b; Parkhouse & Cooper, 1977; Pernis et al., 1977; Kearney & Abney, 1978). It has become evident that sequential expression of receptor Ig isotypes is the rule for B-cell maturation, and that this receptor isotype diversification is independent of specific antigen or accessory cell influence. The ontogenetic and mitogen-stimulated expression of Ig isotype receptors has been incorporated, along with observations of the physical, functional and kinetic characteristics of subpopulations of antibody forming cells (Nossal et al., 1977), into an overall picture of B-lymphocyte differentiation. Such a picture includes other aspects of Ig expression aside from isotype diversification. The details are reviewed below.

5.4.1. Pre-B Cells: IgM-Synthesising, $sIgM^{-ve}$

Ontogenic studies in several laboratories have demonstrated the presence, in 10-12 day fetal liver or adult bone marrow, of cells which stain weakly for cytoplasmic IgM, but are negative for sIg (Melchers et al., 1975a; Osmond et al., 1976; Phillips & Melchers, 1976; Raff et al., 1976; Phillips et al., 1977; Rosenberg & Parish, 1977). These (pre-B) cells preceed by 4-5 days the appearance of the first detectable $sIgM^{+ve}$ B-cells (see below). Morphologically, the pre-B cells are large cells. They may, as suggested by Nossal et al. (1977), express very low amounts of rapidly turning over sIgM. In addition, the following observations are relevant to an understanding of the role of pre-B cells: 1) pre-B cells exhibit allelic exclusion (Lawton et al., 1977), 2) at the earliest detected appearance of a clonotype, it is represented by at least 10 individual cells (Metcalf et al., 1977), and 3) the bone marrow small lymphocytes show some cells which

are also sIg^{-ve}. Based on the above, Osmond & Nossal (1974 a, 1974b) have suggested that early (cycling) pre-B cells develop into more mature (non-cycling) cells by reduction division mitosis, accounting for observation (3). Cells at the pre-B stage are thought not to be generating clonotype diversity (based on the demonstration of allelic exclusion). Instead, the production of pre-B progeny cells by limited reduction-division would serve to amplify individual clones (Nossal et al., 1977), accounting for observation (2) above.

5.4.2. Early Primary B Cells: $sIgM^{+ve}$

Pre-B cells develop into primary B cells in the fetal liver or adult bone marrow through the acquisition of sIgM. That IgM is the first isotype expressed on developing B cells has been shown in several independent laboratories (Abney & Parkhouse, 1974; Melchers et al., 1975a; Vitetta et al., 1975; Vossen & Hijmans, 1975; Gathings et al., 1976; Gupta et al., 1976; Raff et al., 1976), and is considered to be well established. These $sIgM^{+ve}$ cells: 1) respond to LPS and 2) can easily be tolerised by antigen in vitro, and are thus considered likely candidates for in vivo tolerance induction (Nossal & Pike, 1975; Vitetta & Uhr, 1975a; Stocker & Nossal, 1976; Stocker, 1977). It has been further suggested that early primary B-cells (i.e. probably those positive for sIgM only) may, if they fail to acquire another receptor isotype, develop into non-switching clones, producing IgM antibody to relatively thymus independent antigens (Vitetta & Uhr, 1975a, 1977; Nossal et al., 1977).

5.4.3. Maturation of Primary B Cells: Generation of Multiple Receptor Isotypes

The first indication of the existence of multiple isotypes on the surface of B-lymphocytes was the identification of IgD as a major receptor on human (van Boxel et al., 1972; Knapp et al., 1973; Rowe et al., 1973; Kubo et al., 1974; Lobo et al., 1975) and on mouse B-cells (Abney & Parkhouse, 1974; Melcher et al., 1974).

Ontogenic studies using carefully purified fluorescent anti Ig antibodies have recently described the existence of murine B-lymphocytes also bearing IgG_1, IgG_2, IgG_3 and IgA isotypes (Parkhouse & Cooper, 1977; Kearney & Abney, 1978). These studies demonstrated that a small but significant percentage of cells in spleens from 3 day old mice arise bearing the IgG_1, IgG_2, IgG_3 or IgA isotypes. These isotypes arise only on those cells previously $sIgM^{+ve}$, thereby creating doubles (i.e. $sIgM^{+ve}$, $sIgX^{+ve}$). Likewise, sIgD also arises on $sIgM^{+ve}$ cells at this stage of development. This latter point was independently established by anti μ suppression, which was found to inhibit the development of $sIgD^{+ve}$ cells (Parkhouse & Cooper, 1977). Thus, it was concluded that the early primary B-cell ($sIgM^{+ve}$ only) is the pivotal cell for the development of isotype diversity. An important further conclusion from these studies was that only one of the additional isotypes (IgG_1, IgG_2, IgG_3. and IgA) could arise on a single $sIgM^{+ve}$ cell (i.e. no IgG_1^{+ve}, IgG_2^{+ve}-type cells were found). This was interpreted to indicate a precommittment to the synthesis of only one of these additional isotypes within a clone (Kearney & Abney, 1978). In contrast, the acquisition of sIgD was found to be an independent event, which occurs on a large percentage (20-30%) of $sIgM^{+ve}$, $sIgX^{+ve}$

doubles, thereby creating trebles, and also on a large percentage (approximately 50%) of $sIgM^{+ve}$-only cells, thereby creating $sIgM^{+ve}$, $sIgD^{+ve}$ doubles. That these events were antigen-independent was shown by Lawton & Cooper (1974) and Vitetta & Uhr (1975a), who demonstrated the identical sequence of isotype acquisition in germ-free mice.

5.4.4. LPS Stimulation to Ig Secreting Blast Cells

Recent improvements in LPS-stimulation techniques have enabled workers in several laboratories to obtain differentiation on IgG and IgA synthesising plasma cells (Kearney & Lawton, 1975a, 1975b; Melchers et al., 1975a; Askonas & North, 1977; Pernis et al., 1977). From these studies a general picture of antigen-driven isotype expression, based on the LPS model, has emerged. The major observations are reviewed below.

It was shown that as soon as $sIgM^{+ve}$ cells appear in the fetal liver (day 16-17) they could be stimulated by LPS (Kearney & Lawton, 1975b). When surface Ig isotypes were examined, it was found that LPS rapidly induced the expression of other isotypes on a small percentage of $sIgM^{+ve}$ cells, thereby creating doubles. This was completely analogous to the situation observed with ontogenetic expression. The expression of new receptor isotypes was independent of proliferation, but dependent on mRNA and protein synthesis, as determined from the use of the metabolic inhibitors cytosine arabinoside and hydroxyurea, actinomycin D, and cycloheximide respectively (Kearney & Abney, 1978).

When $sIgG_2^{+ve}$, $sIgM^{+ve}$ doubles were examined it was found that, coincident with the time of maximum cell proliferation (35 -96 hours), the sIgM

was rapidly lost from the cells, leaving a population of $sIgG_2^{+ve}$-only cells. Coincident with the transition of these cells to $sIgG_2^{+ve}$-only, the appearance of cytoplasmic (and presumably secretory) IgG_2 was detected (Kearney & Abney, 1978). It was also observed by several investigators (Askonas & North, 1977; Pernis et al., 1977; Kearney & Abney, 1978), that some of these cells could retain both cytoplasmic and sIgM during transition to IgG_2 expression, and thus be double isotype producers (and presumably secretors). In a series of parallel experiments, Kearney et al. (1977b) demonstrated that the persistence of cytoplasmic (and surface) IgM in cells differentiating to IgG_2 expression was enhanced by inhibition of cell proliferation. From these and similar studies (Askonas & North, 1977) it was concluded that cellular proliferation is required for the development of Ig class restriction in LPS responses, insofar as the loss of the primary receptor isotypes (sIgM and sIgD) and cytoplasmic IgM is concerned (Kearney & Abney, 1978). Cell proliferation also resulted in an increase in the absolute numbers of IgG-synthesising cells. Upon removal of $sIgG_2^{+ve}$ cells prior to culture with the fluorescence-activated cell sorter, Kearney & Abney (1978) reported a failure to deplete LPS-stimulated production of IgG_2. The authors suggested from this result that there is a recruitment of single $sIgM^{+ve}$ cells as precursors for IgG_2-synthesising cells.

The overall suggestion from the above results is that the apparent antigen-driven 'switch' phenomena, such as seen in LPS cultures and inferred from biclonal myelomas, can be explained fully in terms of the recruitment and stimulation of isotype-committed precursor cells. A key distinction here seems to be the concept that recruitment of $sIgM^{+ve}$

cells to the synthesis and secretion of other isotypes (limited to only one other isotype per clone) is different from antigen-driven switch in that the cells first pass through an intermediate stage of $sIgM^{+ve}$, $sIgX^{+ve}$. Since this intermediate stage has been shown to be an expression of the inherent differentiation capacity of B-lymphocytes, the actual isotype switch is reasoned not to be antigen-driven. It has been recognized, however, that B-lymphocyte differetiation is likely to be considerably more complicated than originally anticipated, and it would therefore be premature to attempt to fit all the observations into too rigid a conceptual framework (Nossal et al., 1977; Kearney & Abney, 1978).

MATERIALS

AND

METHODS

1. GENERAL MATERIALS

1.1. Cell Culture Materials

RPMI 1640 Medium powder	Gibco Bio-Cult Ltd. Paisley, Scotland.
Foetal Calf Serum	Flow Laboratories Ltd. Irvine, Scotland
Penicillin, Streptomycin	Glaxo Pharmaceuticals Ltd. London, England

In addition, tissue culture flasks, petri dishes, tubes, and pipettes were supplied by Falcon Plastics Inc., Oxnard, California or by Corning Glass Works, Corning, New York.

1.2. Radiochemicals

The following radiochemicals were supplied by the Radiochemical Centre, Amersham, England:-

Amino Acids	Specific Activity
L- {4,5-^{3}H} Leucine	55 - 60 Ci/mmole
L- {^{35}S} Methionine	500 - 1200 "
L- {4-^{3}H} Phenylalanine	12 "
L- {3,4(n)-^{3}H} Proline	40 "
L- {3-^{3}H} Serine	29 "
L- {3,5-^{3}H} Tyrosine	43 "
L- {3,4(n)-^{3}H} Valine	36 "

Nucleotides	Specific Activity
{5,6-^{3}H} Uridine	40 - 60 Ci/mmole

Other Radionuclides	Specific Activity
Iodine-125	Carrier-free
Iodine-131	"
Phosphorous-32	30 - 100 Ci/mmole

In addition, L- {^{35}S} Cystine (20-60 Ci/mmole) was supplied by New England Nuclear Inc., Boston, Massachusetts.

1.3. Photographic and Liquid Scintillation Spectrometry Materials

X-Omat-R Film	Kodak Canada Ltd., Toronto, Ontario
DX-80 Developer	Kodak UK Ltd., London, England
FX-40 X-Ray Liquid Fixer	"
Lightproof Vinyl Film Bags	H.A. West (X-ray) Ltd. Edinburgh, Scotland
2,5 Diphenyloxazole (PPO)	Koch-Light Laboratories Ltd. Colnbrook, England
Toluene, AR Grade	"
Protosol	New England Nuclear Inc. Boston, Massachusetts
Triton X-100	Rohm & Haas (UK) Ltd. Jarrow, England

1.4. Enzymes

Creatine Kinase	Boehringer Corporation (London) Ltd., Lewes, East Sussex
Lactoperoxidase	Sigma (UK) Ltd., London, England
Carboxypeptidase A	Worthington Biochemical Corporation, Freeport, New Jersey

1.5. Wheat Germ

Texan wheat germ, obtained from Mrs. H. Singer, was a gift from Dr. J. Tata, National Institute of Medical Research, London.

Israeli wheat germ, obtained from Mrs. H. Singer, was a product of the Bar-Rav Mill, Tel Aviv, Israel.

1.6 Fine Chemicals

The following fine chemicals were supplied by Sigma (UK) Ltd., London, England:-

Agarose

Creatine phosphate

Cycloheximide

Deoxycholic acid, Na salt

Diethyl pyrocarbonate (DEP)

Dimethyl sulphoxide (DMSO)

D,L - Dithiothreitol (DTT)

N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)

Iodoacetamide

Iodoacetic acid, Na salt

Phenylmethylsulphonyl fluoride (PMSF)

Spermidine, free base

N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)

Tris (hydroxymethyl) aminomethane (TRIZMA BASE)

The following fine chemicals were supplied as indicated:-

2-Mercaptoethanol	Koch-Light Laboratories Ltd., Colnbrook, England
3-methyl-1-butanol (isoamyl alcohol)	"
Triton X-100, purified	"
Adenosine 5'-triphosphate	P-L Biochemicals Inc. Milwaukee, Wisconsin
Guanosine 5'-triphosphate	"
N,N' Methylene bisacrylamide	Eastman Kodak Company Rochester, New York
Nonidet P-40	Shell Chemicals UK Ltd. London, England
Sucrose, ribonuclease free	Schwartz/Mann Orangeburg, New York

Formamide	Fluka AG Chemische Fabrik Buchs, Switzerland
N,N'-Diallyltartardiamide (DATD)	Serva Feinbiochemica Heidelberg, W. Germany
Absolute Alcohol, AR Grade	James Burrough Ltd., London England
Pepstatin-A	Protein Research Foundation Osaka, Japan
Heparin, mucous, freeze dried	Evans Medical Ltd., Liverpool,England
Ampholine, carrier ampholytes	LKB Produkter AB, Bromma, Sweden
L-Amino Acids, A Grade	Calbiochem Ltd., Bishops-Stortford, England

All other chemicals were, whenever possible, "AnalaR" grade reagents, supplied by BDH Chemicals Ltd., Poole, Dorset, England.

1.7. Chromatography and Other Materials

Oligo dT Cellulose	Collaborative Research Inc., Waltham, Massachusetts
Sepharose 4B	Pharmacia (Great Britain) Ltd. London, England
Sephadex G-25 coarse	"
Sephadex G-200 medium	"
DEAE Sephadex	"
QAE Sephadex	"
Nitrocellulose filters	Millipore Corporation Bedford, Massachusetts

In addition, materials for ultrafiltration were supplied by Amicon Corporation, Lexington, Massachusetts, or by Schleicher & Schuell GMBH, Dassel, W. Germany.

2. STANDARD SOLUTIONS

TABLE 1

COMPOSITION OF RPMI 1640 MEDIUM

Amino Acids	mg/litre
L-Arginine (free base)	200.0
L-Asparagine	65.0
L-Aspartic Acid	20.0
L-Cystine (2 HCl)	65.0
L-Glutamic Acid	20.0
Glycine	10.0
L-Histidine (free base)	15.0
L-Hydroxyproline	20.0
L-Isoleucine (Allo free)	50.0
L-Leucine (Methionine free)	50.0
L-Lysine HCl	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline (Hydroxy-L-Proline free)	20.0
L-Serine	30.0
L-Threonine (Allo free)	20.0
L-Tryptophane	5.0
L-Tyrosine	28.94
L-Valine	20.0

Inorganic Salts	mg/litre
$Ca(NO_3)_2.4H_2O$	100.0

Table 1 continued

Inorganic Salts	mg/litre
KCl	400.0
$MgSO_4$	48.84
NaCl	6000.0
Na_2HPO_4 (anhyd.)	800.0

Vitamins	mg/litre
Biotin	0.2
D-Ca pantothenate	0.25
Choline Cl	3.0
Folic Acid	1.0
i-Inositol	35.0
Nicotinamide	1.0
Para-aminobenzoic acid	1.0
Pyrodoxine HCl	1.0
Riboflavin	0.2
Thiamine HCl	1.0
Vitamin B_{12}	0.005

Other Components	mg/litre
Glucose	2000.0
Glutathione (reduced)	1.0
Phenol red	5.0
*$NaHCO_3$	2000.0

*added separately to dissolved medium

The pH was adjusted with concentrated HCl to 7.0. The media was sterilised by filtration and stored at $4^{o}C$.

2.1. Cell Culture Medium

The composition of RPMI 1640 powdered medium is shown in Table 1. The complete tissue culture medium was prepared immediately before use and consisted of:-

80% (by volume)	RPMI 1640 medium
20% "	Foetal calf serum
10^5 units/l	Penicillin
100 mg/l	Streptomycin
2.0 mM	Glutamine

The foetal calf serum was heat inactivated by incubation at 56°C for 1 hr and stored at -20°C.

The penicillin/streptomycin and glutamine were stored as separate 100x solutions at -20°C.

I am grateful to the tissue culture staff, and in particular to Mrs. Irene Gall and Mrs. Jacqui Grieves, for the routine preparation of the above cell culture medium and stock solutions.

2.2. Phosphate Buffered Saline

Phosphate buffered saline, solution A, consisted of:-

PBS-A

170 mM NaCl

3.4 mM KCl

10 mM Na_2HPO_4

1.8 mM KH_2PO_4 pH 7.2

2.3. Scintillation Spectrometry Solutions

Toluene-PPO

4 g/l PPO in toluene

Triton-Toluene-PPO

67% (by volume)	Toluene
33% "	Triton X-100
4 g/l	PPO

3. HAEMATOPOIETIC CELL LINES

The human lymphoma cell lines under investigation in this thesis, and some of their general characteristics are shown in Table 2.

The lymphoblastoid and lymphoma cell lines were obtained as indicated below:-

Bec-11, Bri-8, Mich RPMI-1788	A generous gift from Searle Research Laboratories, Ltd., High Wycombe, England
Tay-3	A generous gift from Dr. C.M. Steel, MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh, Scotland
U698M	A generous gift from Dr. C. Sundström, Karolinska Institute, Stockholm, Sweden
BJAB, Daudi, Namalwa, Raji-ATG	A generous gift from Dr. G. Clements, Department of Pathology, Western Infirmary, Glasgow, Scotland

4. SEROLOGICAL REAGENTS

4.1. Immunoglobulins

The immunoglobulin reagents used in this thesis, their designations, classes and methods of preparation are summarized in Table 3.

Immunoelectrophoretic analyses for purity and light chain class of the various reagents are shown in Fig. 1 and Fig. 2 respectively.

TABLE 2

CHARACTERISTICS OF LYMPHOMA CELL LINES

Designation	Origin	Surface Markers	References
BJAB	Burkitt's Lymphoma	sIgM, (F_c), (C3)	Klein et al., 1974 Menèzes et al., 1975
Daudi	"	sIgM, F_c, C3	Klein et al., 1968
Namalwa	"	sIgM, F_c, C3	Reedman & Klein, 1973
Raji-ATG	"	(sIgM), (F_c), C3	Pulvertaft, 1965 Epstein et al., 1966 Nyormoi et al., 1973
U698M	Lymphosarcoma (tonsil)	sIgM, (sIgD)	Nilsson & Sundström, 1974

Abbreviations: F_c, F_c receptors; C3, complement (C3) receptors

( ) = weakly positive

TABLE 3

SUMMARY OF IMMUNOGLOBULIN PREPARATIONS

Designation, Source	Class	Method of Preparation
Human Immunoglobulins		
GM, Wald. Macro.	IgM_K	BA, G-200
7A, myeloma serum	IgM_K	BA, G-200
3F, myeloma serum	IgMλ	BA, G-200
2L, myeloma serum	IgMλ	BA, G-200
Rh IgMs, rheumatoid sera	IgMκλ	BA, G-200
GA, myeloma serum	IgA_K	AS, DEAE
3F, myeloma serum	IgA_K	AS, DEAE
2K, myeloma serum	IgA_K	AS, DEAE
3H, myeloma serum	IgAλ	AS, DEAE
8L, myeloma serum	IgA_K	AS, DEAE, G-200
2P, myeloma serum	IgAλ	AS, DEAE, G-200
Ford, myeloma serum	IgGλ	AS, QAE
Budapest, myeloma serum	IgDλ	AS
AI, myeloma serum	IgDλ	AcA 34, DEAE*
Rabbit Immunoglobulins		
NoRIgG, normal rabbit serum	NA	AS, G-200
RbIgG, Nordic Immunological Laboratories Ltd., Maidenhead, Berks, England	NA	

*R. Jefferis, Journal Immunol. Methods 9, 231-234 (1976)

(BA) boric acid precipitation; (AS) ammonium sulphate precipitation; (G-200) G200 Sephadex Chromatography; (DEAE) DEAE Sephadex Chromatography; (QAE) QAE Sephadex Chromatography; NA, not applicable

Fig. 1

Immunoelectrophoretic Analysis for Purity of Immunoglobulin Reagents

Immunoelectrophoresis was carried out essentially as described by Scheidegger (1955). A solution of 50 mM Veronal buffer, pH 8.6, containing 1% w/v agarose was pipetted onto microscope slides at 60°C and allowed to cool. Wells and troughs were cut in the agarose gel using a cutting template. The wells were filled with the Ig sample to be tested (approximately 1 µl) and the gels electrophoresed at 6 V/cm for 1-2 hr. After electrophoresis the troughs were filled with antisera as indicated (50-100 µl) and the gels incubated in a humid chamber (overnight at 20°C) to develop the precipitin arcs. The gels were then washed at 4°C in three changes of PBS-A and finally stained in a solution of 0.1% w/v Coomassie Blue dye in methanol:water:acetic acid (45:45:10).

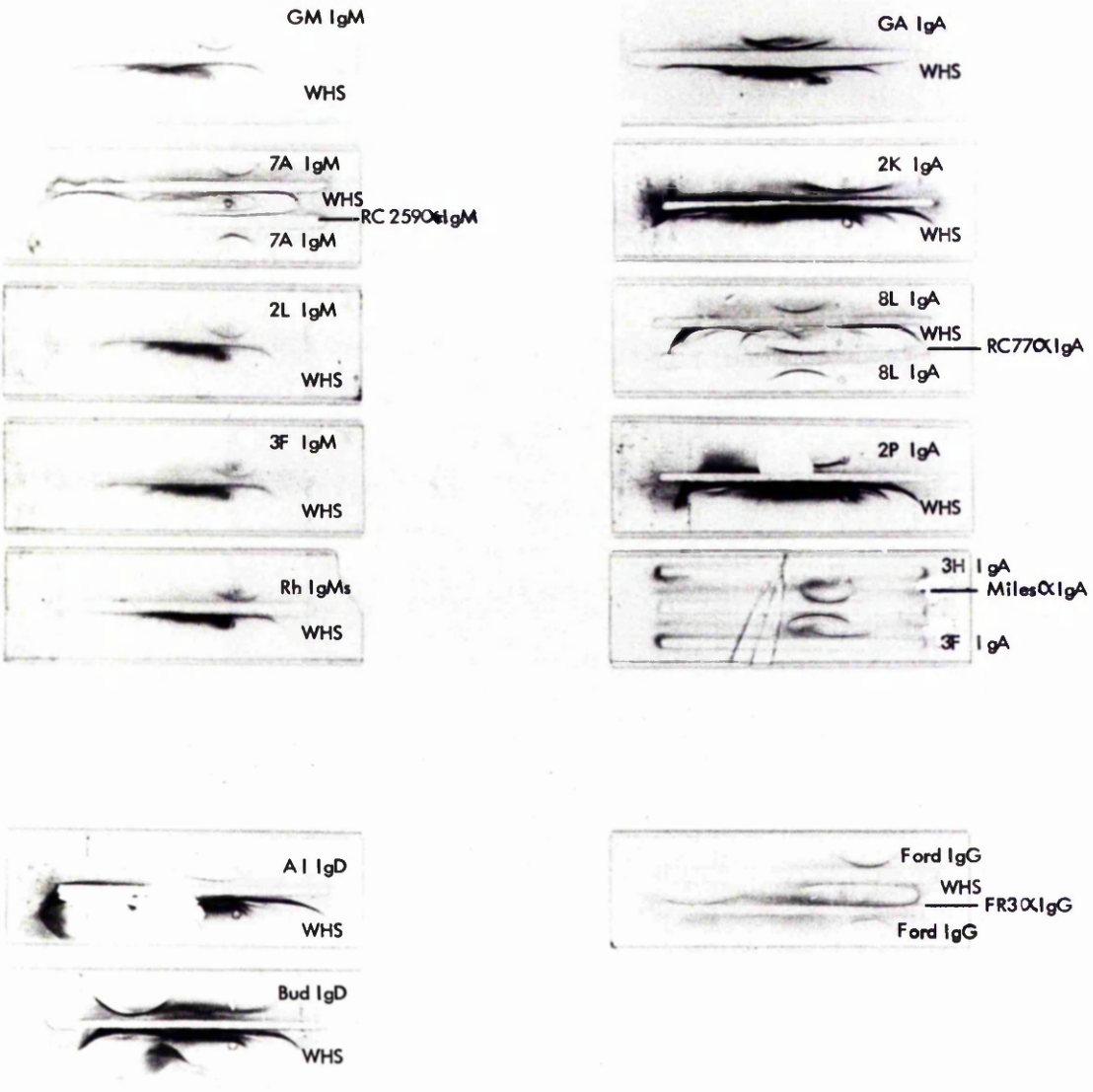
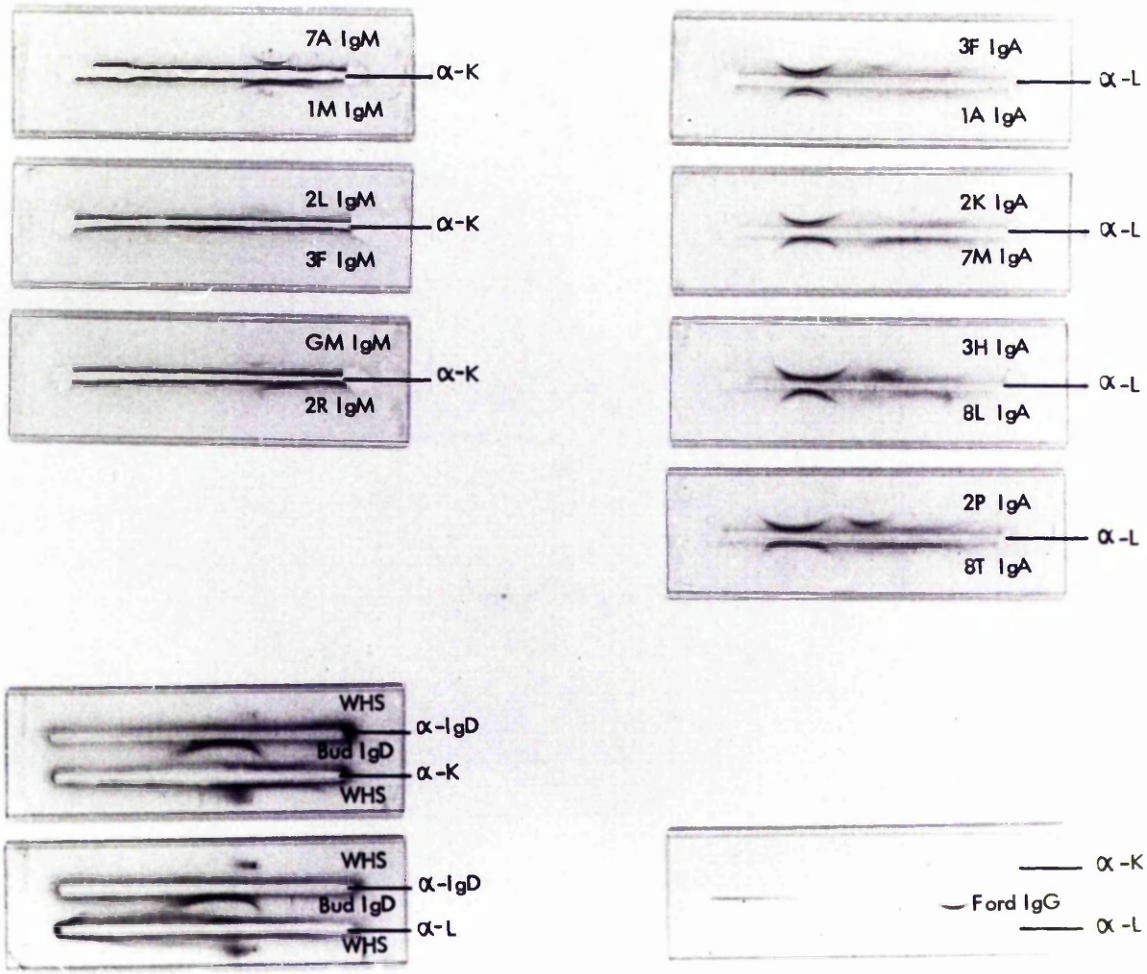

GM IgM
WHS
7A IgM
WHS
RC 2590αIgM
7A IgM
2L IgM
WHS
3F IgM
WHS
Rh IgMs
WHS
GA IgA
WHS
2K IgA
WHS
8L IgA
WHS
RC770αIgA
8L IgA
2P IgA
WHS
3H IgA
Miles α IgA
3F IgA
Al IgD
WHS
Bud IgD
WHS
Ford IgG
WHS
FR3αIgG
Ford IgG

Fig. 2

Immunoelectrophoretic Analysis to Determine the Light Chain Class of Various Immunoglobulin Reagents

Immunoelectrophoresis was carried out as described in the legend to Fig. 1. The results are summarised in Table 3.


7A IgM
α-K
1M IgM
2L IgM
α-K
3F IgM
GM IgM
α-K
2R IgM
3F IgA
α-L
1A IgA
2K IgA
α-L
7M IgA
3H IgA
α-L
8L IgA
2P IgA
α-L
8T IgA
WHS
α-IgD
Bud IgD
α-K
WHS
WHS
α-IgD
Bud IgD
α-L
WHS
α-K
Ford IgG
α-L

Ford myeloma serum was obtained from Dr. B. Thomas, NIMR, Mill Hill, London, England.

Budapest myeloma serum was a kind gift from Dr. J. Gergeley, Budapest, Hungary.

The AI IgD myeloma protein, in highly purified form, was a kind gift from Dr. R. Jefferis, University of Birmingham Medical School, Birmingham, England.

All other serum samples were obtained from Dr. Eva Kirkwood, Department of Immunology, Western Infirmary, Glasgow, Scotland.

4.2. Antisera and Antibody Preparations

The following antisera against human immunoglobulins were obtained as indicated:-

Rabbit anti-IgA (α -specific)	Miles Laboratories Research Products, Ltd., Stoke Poges, Slough, England
Rabbit anti-IgM (μ -specific)	"
Rabbit anti-IgD (δ -specific)	"
Rabbit anti-κ (Bence-Jones)	"
Rabbit anti-λ (Bence-Jones)	"
Rabbit anti-IgD (R.33, unabsorbed)	A kind gift from Dr. J. Gergeley Budapest, Hungary
Rabbit anti-IgD (R.164, δ-specific)	"
Rabbit anti-IgD (R.410, $F_c\delta$ - specific)	A kind gift from Dr. R. Jefferis, University of Birmingham Medical School, Birmingham, England

In addition, several antisera were raised to the immunoglobulin reagents described in the previous section (4.1), and from these

TABLE 4

DERIVATION AND PREPARATION OF PURIFIED ANTIBODIES

Antibody	From Antiserum	Against Ag	Selected on Immunoadsorbent
RAHIgM			
prep. 1	RC 79 (AS)	IgMκ (GM, 7A)	IgMλ (3F, 2L)
prep. 2	RC 259 (G-200)	IgMκ (7A)	IgMκλ (Rh)
prep. 3	RC 259 (W)	IgMκ (7A)	IgMκλ (Rh)
RAHIgG			
prep. 1	FR 1 (DEAE, G-200)	IgGλ (Ford)	IgGλ (Ford)
prep. 2	FR 1 (DEAE, G-200)	IgGλ (Ford)	IgGλ (Ford)
RAHIgA			
prep. 1	RC 77 (AS)	IgAκ (GA, 8L)	IgAλ (2P, 3H)
prep. 2	RC 479 (W)	IgAκ (3F, 8L)	
GARIgG			
prep. 1	G 320 (AS)	Rb IgG (Nordic)	NoRIgG (NRS)
prep. 2	G 315 (G-200)	Rb IgG (Nordic)	NoRIgG (NRS)
prep. 3	G 315 (AS)	Rb IgG (Nordic)	NoRIgG (NRS)

(AS) ammonium sulphate globulin fraction; (G-200), G-200 Sephadex IgG fraction; (DEAE) DEAE Sephadex IgG fraction; (W) whole antiserum.

antisera purified antibody preparations were made. The relevant information is summarized in Table 4.

Immunoelectrophoretic analyses of the specificity of each antisera or antibody preparation used are shown in Fig. 3.

5. CELL CULTURE METHODS

5.1. Maintenance Culture Method

Cell lines were grown in continuous suspension culture in complete RPMI 1640 medium (section 2.1) in 250 ml plastic flasks in a 5% CO_2-95% air atmosphere. Subculturing was done every third or fourth day by replacing 80-90% of the cell culture with fresh medium.

5.2. Bulk Culture Method

Cells were grown in continuous suspension culture in sealed 2.5 l glass burlers (400 ml/burler).

5.3. Cell Viability Determination

A 0.2% w/v solution of trypan blue was added to an equal volume of culture fluid, and cell numbers determined by counting in a Neubauer type haemocytometer.

Trypan Blue Dye Solution

4 parts	1% w/v NaCl
1 part	1% w/v Trypan blue dye

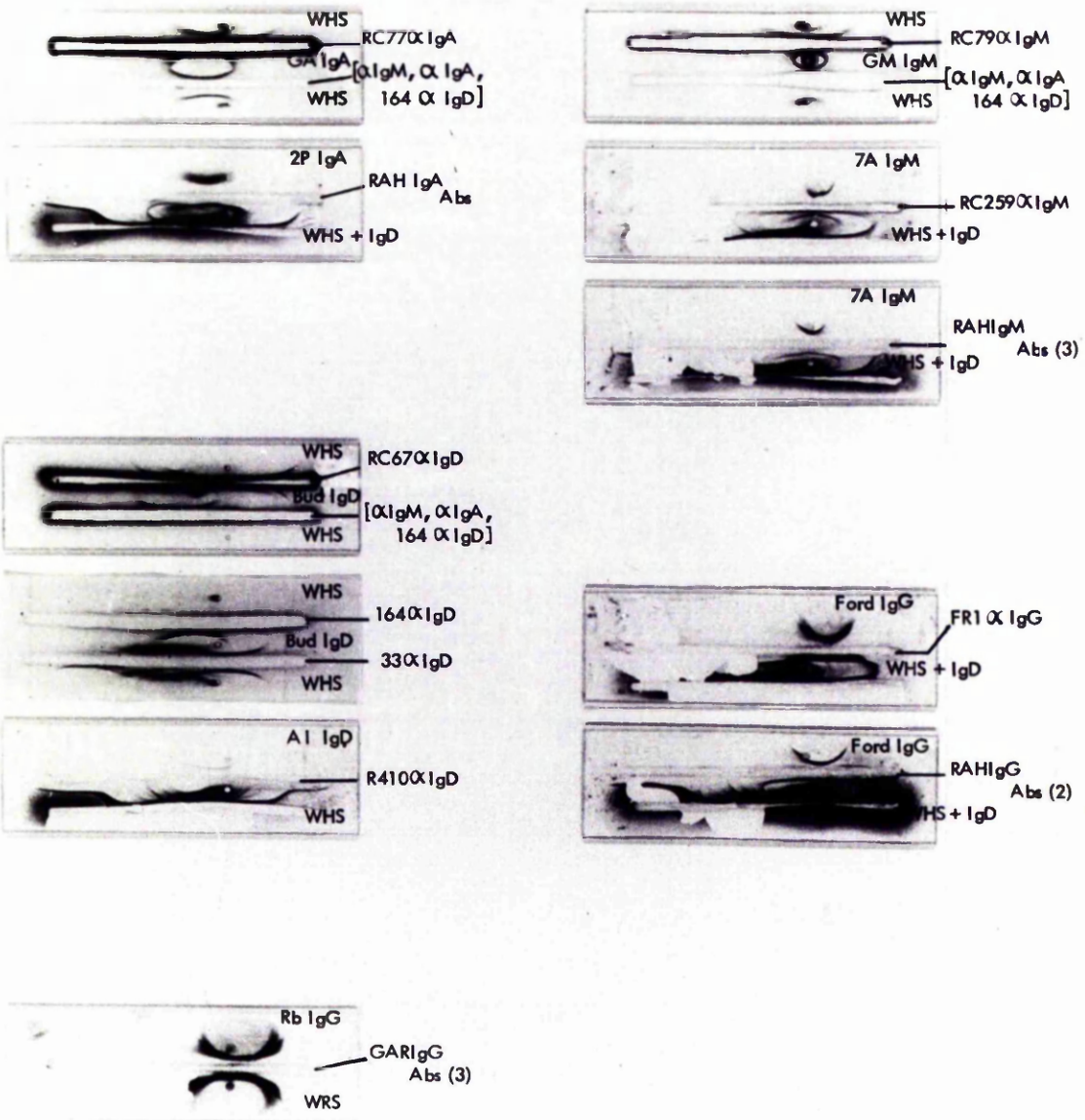
5.4. Contamination Checks

5.4.1. Bacterial

Fig. 3

Immunoelectrophoretic Analysis of Antiserum and Antibody Specificities

Immunoelectrophoretic analysis was carried out as described in the legend to Fig. 1. The sources of the various antisera analysed, and their designations are summarised in Table 4.


WHS
RC770αIgA
GA IgA
[αIgM, αIgA,
164 αIgD]
WHS
WHS
RC790αIgM
GM IgM
[αIgM, αIgA
164 αIgD]
WHS
2P IgA
RAH IgA
Abs
WHS + IgD
7A IgM
RC259αIgM
WHS + IgD
7A IgM
RAHIgM
Abs (3)
WHS + IgD
WHS
RC67αIgD
Bud IgD
[αIgM, αIgA,
164 αIgD]
WHS
WHS
164αIgD
Bud IgD
33αIgD
WHS
Ford IgG
FR1 α IgG
WHS + IgD
A1 IgD
R4100αIgD
WHS
Ford IgG
RAHIgG
Abs (2)
WHS + IgD
Rb IgG
GARIgG
Abs (3)
WRS

Blood-agar plates were prepared to the following formulation:-

For 10 Plates	
50 ml	4% w/v Blood-agar, "Difco Bacto"
5 ml	Horse blood

Concentrated cell suspensions were spread on blood-agar plates and incubated at 37°C in a humid chamber. If positive, colonies developed in 2-5 days.

5.4.2. PPLO

PPLO-agar plates were prepared to the following formulation:-

For 10 Plates	
25 ml	3.5% w/v PPLO-agar, "Difco Bacto"
7.5 ml	Horse serum
3.75 ml	0.5% w/v Yeast extract
3.75 ml	Tryptose phosphate broth, 29.5 g/l
0.4 ml	1.25% w/v Thallous acetate
0.15 ml	Penicillin, 2.5×10^5 units/ml

Agar plates were pierced several times with a pasteur pipette containing concentrated cell suspensions and the plates placed in a sealed jar in a 5% CO_2-95% N_2 atmosphere. If positive, colonies developed in 3-7 days.

5.5. Frozen Storage of Cell Lines

Cell freezing medium was prepared to the following formulation:-

Freezing Medium		
40%	(by volume)	2 x RPMI 1640 (minus $NaHCO_3$)
10%	"	DMSO
20%	"	Foetal calf serum (heat inactivated)
30%	"	Distilled water

Cells were centrifuged (500 x g for 5 min) and resuspended at approximately 5 x 10^6 cells/ml in cold Freezing Medium. The temperature of the culture was then reduced slowly (1°C per min), and the frozen cells stored in liquid nitrogen.

To thaw, the frozen cells were quickly brought to 37°C by immersion in a water bath and diluted into 10 volumes of complete RPMI 1640 medium. The cells were centrifuged (500 x 5 for 5 min) and resuspended in fresh medium for initiation of normal growth.

I am grateful to the tissue culture staff, and in particular to Mrs. Irene Gall for carrying out contamination checks and for the frozen storage and recovery of cell lines.

6. PREPARATION OF SEROLOGICAL REAGENTS

6.1. General Preparation Methods

6.1.1. Ammonium Sulphate Fractionation

All steps were done at room temperature. The serum sample was centrifuged (10,000 x g for 15 min) to remove insoluble material, and then diluted with an equal volume of PBS-A. Saturated ammonium sulphate (53.6% w/v at 20°C) was added dropwise, with mixing, until 40% saturation was obtained. After standing a minimum of 2 hr, the precipitate was collected by centrifugation, washed once with 40% saturated ammonium sulphate, dissolved in PBS-A and dialysed.

6.1.2. Boric Acid Precipitation of Euglobulins

All steps were done at room temperature. The serum sample was centrifuged to remove insoluble material, and then added dropwise

with mixing into a solution of 2% w/v boric acid (20:1 v/v). After standing for a minimum of 2 hr, the precipitate was collected by centrifugation, dissolved in PBS-A and dialysed.

6.1.3. DEAE Sephadex Chromatography

The sample was dialysed extensively against Column Buffer, centrifuged to remove insoluble material, and applied as a narrow band to the top of a column (1.25 cm i.d. x 25 cm,bed volume 40 ml) of DEAE Sephadex equilibrated in Column Buffer. A linear gradient of increasing buffer concentration (usually 0.01 M - 0.40 M potassium phosphate, pH 6.5) was applied to the column using an LKB Ultrograd gradient maker at the rate of 20 ml/hr for a total of 16 hr. Fractions were collected and the appropriate peak fractions pooled on the basis of absorbancy (A_{280}) measurements.

DEAE Column Buffer

0.01 M Potassium phosphate, pH 6.5 (Ref: Methods in Enzymology 1:143)

6.1.4. QAE Sephadex Chromatography

The sample was dialysed extensively against Column Buffer, centrifuged to remove insoluble material, and applied as a narrow band to the top of a column (1.25 cm i.d. x 25 cm, bed volume 40 ml) of QAE Sephadex equilibrated in Column Buffer. Buffer was applied to the top of the column, and fractions collected and monitored by their absorbency at 280 nm. IgG was the only significant protein eluted under these conditions.

QAE Column Buffer

0.1 M Tris-HCl, pH 6.5

I am grateful to Mrs. Jacki Bennett for the QAE Sephadex purification of Ford IgG.

6.1.5. Sephadex G-200 Chromatography

A column (2.5 cm i.d. x 100 cm,bed volume 500 ml) was packed with Sephadex G200 equilibrated in Column Buffer and de-gassed.

G200 Column Buffer

0.1 M Tris-HCl, pH 8.0

0.2 M NaCl

The sample was dialysed extensively against Column Buffer, centrifuged to remove insoluble material, and applied to the top of the gel bed under the buffer solution. The column was connected by an airtight system to a buffer reservoir, and a flow rate of 10-15 ml/hr achieved by adjustment of the hydrostatic pressure head. Fractions were collected and the appropriate peak fractions pooled on the basis of absorbance (A_{280}) measurements.

6.2. Raising of Antiserum

6.2.1. Primary Immunisations

Immunoglobulin preparations for injection (0.5-1 ml) were mixed with an equal volume of complete Freund's adjuvant and the mixture emulsified by sonication. Rabbits were given two primary injections intramuscularly at times separated by one week. From 0.2 to 1.0 mg of protein antigen was used per injection.

6.2.2. Booster Immunisation

Booster injections were given every four weeks, beginning four weeks after the last primary injection. Approximately 0.2 mg of antigen was used and the injections given at multiple sites subcutaneously.

6.2.3. Collection of Antiserum

Rabbits were bled from the ear vein, beginning six weeks after the last primary injection, and thereafter at 1-2 week intervals. Twenty to 50 ml of blood was taken at each bleeding, and allowed to clot at 37°C for 30 min and overnight at 4°C. The serum was sterilised by filtration and stored at -20°C.

I am grateful to Mrs. Liz Blakely for preparation of much of the antisera used in this thesis.

6.3. Sepharose Immunoadsorbents (Axen et al., 1967)

Sepharose 4B was washed thoroughly with distilled water and reacted with cyanogen bromide (100 mg CNBr/ml packed Sepharose) in aqueous solution of approximately 25% v/v Sepharose. The reaction was carried out inside a fume cupboard at room temperature with gentle mixing. The pH was monitored and adjusted continually to 11.0 - 11.5 by addition of 5 N NaOH and the reaction stopped when the pH stabilised. The activated Sepharose was washed with an ice cold solution of 0.1 M $NaHCO_3$ and immediately resuspended in approximately 5 volumes of the same buffer. The immunoglobulin sample to be coupled (1-10 mg Ig/ml activated Sepharose) was then added and the reaction allowed to proceed at 4°C with gentle mixing for 16 hr. The Sepharose was then washed with 0.1 M $NaHCO_3$, the absorbance (A_{280}) of the eluates measured and used to determine the total uncoupled Ig. The amount of coupled Ig was then calculated according to the following formula:-

$$\text{Coupled Ig} = (\text{Total Ig}) - (\text{Uncoupled Ig})$$

The Sepharose was then reacted at 4°C for 16 hr with a molar equivalent (relative to the CNBr) of glycine to 'quench' any remaining

active sites. The Sepharose was then washed with PBS-A, suspended in PBS-A containing 0.05% sodium azide as preservative and stored at 4°C.

6.4. Preparation of Purified Antibody

Antiserum was passed slowly through a small column of Sepharose immunoadsorbent at room temperature. After thorough washing of the column with PBS-A, the bound antibody was eluted with one of the following buffers:-

Elution Buffers

(i) 0.2 M Glycine-HCl, pH 2.5

(ii) 0.1 M Acetic acid

(iii) 1.0 M Propionic acid

The elution was monitored by absorbance measurements at 280 nm, and the fractions containing the antibody were pooled and immediately neutralised with Trizma Base. The antibody was then dialysed against PBS-A, concentrated and stored as aliquots at -20°C. Sepharose immunoadsorbents were regenerated by washing with PBS-A and stored at 4°C in PBS-A containing 0.05% sodium azide as preservative. They could be used repeatedly with only moderate progressive loss of absorption capacity.

6.5. Radioiodination of Immunoglobulins

Immunoglobulins were radioiodinated by the chloramine-T method of Hunter and Greenwood (1962). The reaction mixture was held on ice inside a fume cupboard. All reactants (except the radioiodine) were prepared in PBS-I.

PBS-I

50 mM	Sodium phosphate, pH 7.2 (Ref: Methods in Enzymology 1:143)
150 mM	NaCl

Generally, 500 µg of Ig (5 mg/ml) was placed in an Eppendorf polypropylene tube (1.6 ml capacity) to which the following was added, in order:-

1 mCi	Iodine-125, or Iodine-131 (5-10 µl)
20 µl	Chloramine-T, freshly prepared, 1 mg/ml

and, after 3 min of vigorous mixing:-

20 µl	Sodium metabisulphite, freshly prepared, 1 mg/ml
10 µl	100 mM KI

The iodinated Ig sample was then dialysed extensively against PBS-A and stored in aliquots at -20°C.

7. SEROLOGICAL PRECIPITATION METHODS

Several methods were employed to analyse the parameters of indirect immunoprecipitation, as they apply to the isolation of cellular immunoglobulin. In this section these methods are described and illustrated.

7.1. Equivalence Titrations Against GARIgG

Equivalence titrations against Goat anti-Rabbit IgG were done for each rabbit antiserum in order to establish the most efficient precipitation conditions for each system. GARIgG antibody (100 µg) was mixed with increasing amounts of a rabbit anti-human Ig antiserum in a constant volume (0.2-0.5 ml) of PBS-A. After incubation for 1 hr

at 37°C and overnight at 4°C, the precipitates were collected by centrifugation (8,000 x g for 2 min) and washed three times with ice cold PBS-A. The washed precipitates were dissolved in 0.2 N NaOH and the absorbance (A_{280}) determined. The amount of precipitate was plotted against the amount of rabbit antiserum reacted, yielding a titration curve similar to that shown in Figure 4A. The point of maximum precipitation is the equivalence point for the system. The equivalence points for many of the serological precipitation systems used in this these are shown in Table 5.

7.2. Indirect Immunoprecipitation Using GARIgG

7.2.1. Effect on Antigen Binding of Non-Equivalence Precipitations

Since it was not always practical to use exact equivalence of rabbit antiserum and GARIgG, the efficiency of primary antigen (human Ig) precipitation under non-equivalence conditions was investigated. A trace amount of I-125 labelled human IgM (0.2 µg; 33,000 cpm) was introduced into an equivalence titration with GARIgG antibody (100 µg) and rabbit anti-human IgM antiserum. Precipitation of the human IgM was monitored by gamma counting of the washed immuno-precipitates, and plotted on a normalised scale against the amount of rabbit antibody reacted. The results are illustrated in Fig. 4A, and show precipitation of primary antigen (human IgM) to be essentially complete in a broad region from antibody (GARIgG) excess to antigen (Rb anti-IgM) excess.

7.2.2. Binding Capacity of the System of Equivalence

Increasing amounts of human IgM were aliquoted into a series of small test tubes and a constant amount of I-125 labelled human IgM (0.2 µg; 33,000 cpm) added. Indirect immunoprecipitations were then carried out using anti-human IgM and GARIg antibody and the

Fig. 4

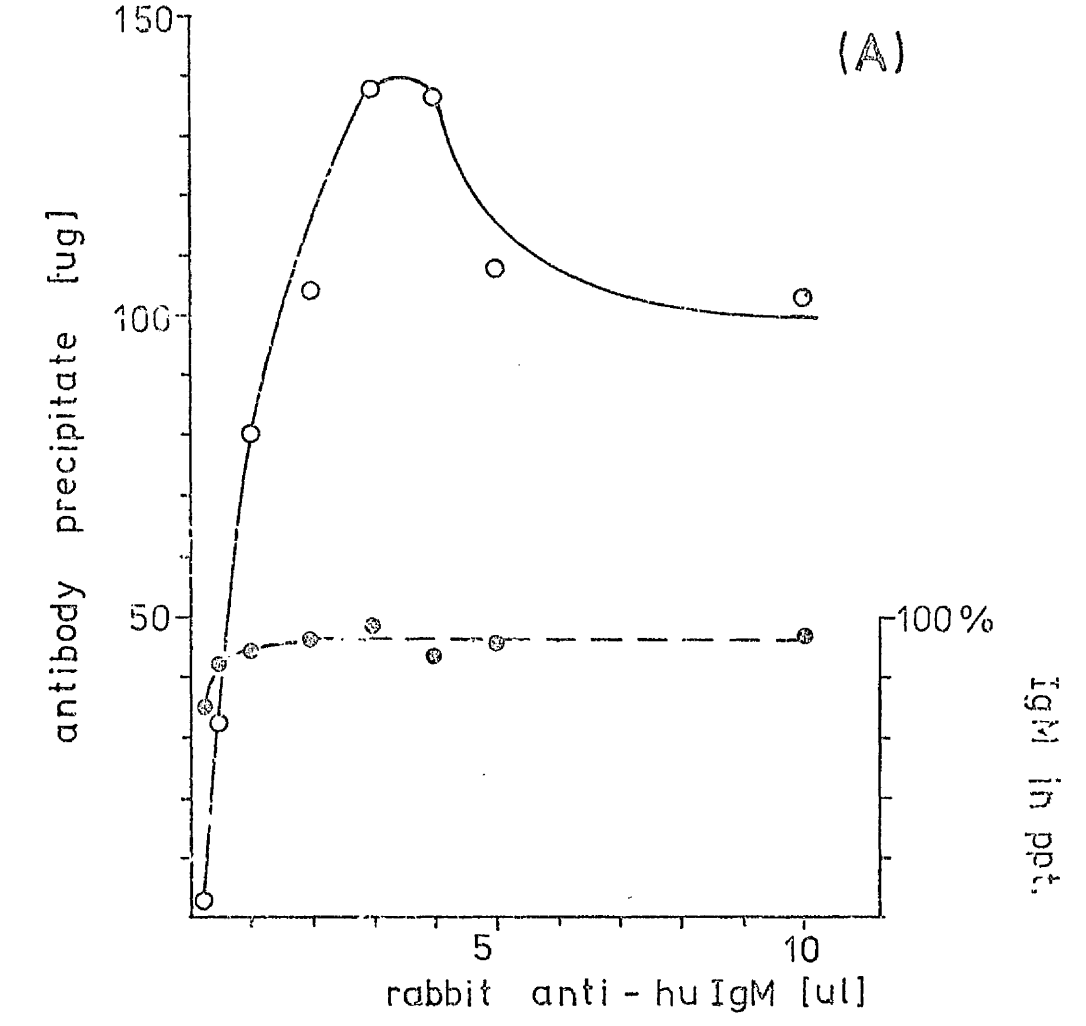
Titration Curves for Indirect Immunoprecipitation

(A) Precipitation of IgM as a Function of Anti-IgM: GARIgG Equivalence

A trace amount of I-125 labelled human IgM (0.2 µg; 33,000 cpm) was added to a series of tubes containing increasing amounts of Rb anti-human IgM antiserum (Miles Laboratories) in a constant volume of 0.2 ml PBS-A. Goat anti-Rb IgG (GARIgG) antibody (100 µg in 10 µl PBS-A) was then added and incubation for 1 hr at 37°C and overnight at 4°C allowed. The immunoprecipitates were collected by centrifugation (8000 x g for 2 min) and washed three times with ice cold PBS-A. The washed precipitates were dissolved in 1 ml of 0.2 N NaOH and the absorbency (A_{280}) determined. The radioactivity in the dissolved immunoprecipitates was then determined by gamma counting. The amount of immunoprecipitate (1.4 mg/ml Ig = 1 A_{280} unit) and the percentage of labelled IgM precipitated are plotted versus the amount of Rb antiserum used.

(B) Capacity of Anti-IgM and GARIgG at Equivalence to Precipitate IgM

Increasing amounts of IgM were aliquoted into two identical sets of tubes and label introduced by addition of a constant trace amount (0.2 µg; 33,000 cpm) of I-125 labelled IgM. Indirect immunoprecipitations were then carried out by addition of either 1 µl anti-IgM (1 hr at 37°C) followed by 40 µg GARIgG (overnight at 4°C) to one set of tubes, or 3 µl anti-IgM followed by 120 µg GARIgG to the other set of tubes. The immunoprecipitates were collected and washed as described above, and the radioactivity determined by gamma counting. The percentage of labelled IgM precipitated is plotted versus the amount of unlabelled IgM present in the reaction mixture.


(A)
150
100
50
antibody precipitate [ug]
100 %
IgM in ppt.
5
10
rabbit anti-hu IgM [ul]

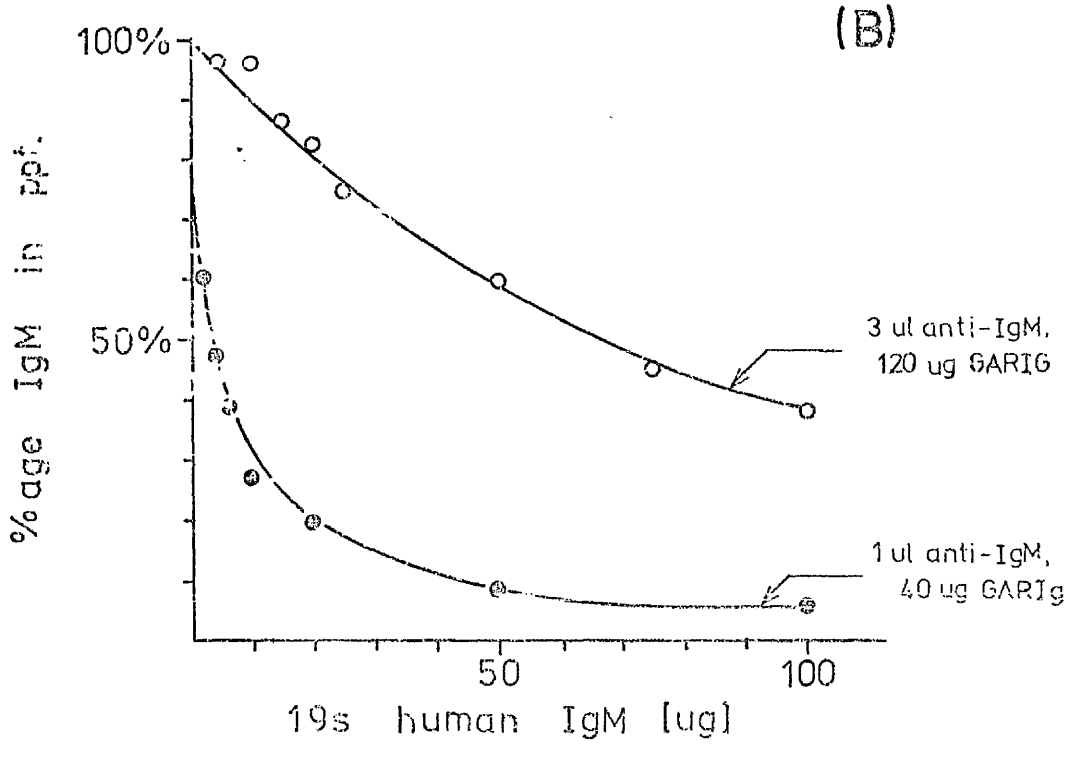

(B)
100%
50%
%age IgM in ppt.
3 ul anti-IgM,
120 ug GARIG
1 ul anti-IgM,
40 ug GARIg
50
100
19s human IgM [ug]

TABLE 5

EQUIVALENCE AMOUNTS OF VARIOUS ANTISERA AND ANTIBODY PREPARATIONS WITH GARIgG

Antisera, Antibody Designation	Equivalence for 100 µg GARIgG Antibody
RAHIgM Antibody	20-25 µg
RAHIgG Antibody	20-25 µg
RAHIgA Antibody	20-25 µg
Miles Anti-Human IgM	2.5 µl
Miles Anti-Human IgA	3.5 µl
Miles Anti-Human IgD	Not used
Miles Anti-Human Bence Jones κ	2.0 µl
Miles Anti-Human Bence Jones λ	2.0 µl
R. 33 Anti-Human IgD	Not used
R. 164 Anti-Human IgD	Not Used
R. 410 Anti-Human IgD (AI)	2.0 µl
Normal Rabbit IgG	20-25 µg

precipitation of IgM monitored by gamma counting of the washed immunoprecipitates. The percent of IgM precipitated was plotted against the amount of IgM present in the reaction mixture. The results are illustrated in Fig. 4B, and show greater than 75% binding of up to 30 µg of IgM (equivalent to 6 µg of IgG) using 3 µl of antiserum.

7.3. Indirect Immunoprecipitation Using S. Aureus Immunoadsorbent (Kessler, 1975)

A 10% v/v solution of formalin-fixed S. aureus (Cowan I strain) was added in place of GARIg antibody in immunoprecipitations using rabbit antiserum (or antibody) to human immunoglobulins in the first stage. Fifty microliters of S. aureus solution was used per 10 µg of rabbit antibody, or per 1 µl of antiserum.

8. LABELLING AND ANALYSIS OF CELLULAR PROTEINS

8.1. Biosynthetic Labelling

Aliquots (1-5 x 10^6 cells) of log phase cultures were centrifuged and the cells washed once with, and resuspended in, labelling medium containing the radioactive amino acid to be used (lyophilised).

Labelling Medium

RPMI-1640 medium, without serum and minus the labelling amino acid

8.1.1. Pulse Labelling

Incubation in the labelling medium was allowed to proceed at 37°C for 5-90 min.

8.1.2. Pulse-Chase Labelling

Following the incubation as above, complete RPMI-1640 medium (with serum) was added, and the incubation continued for a further 4-18 hrs, depending on the individual experimental protocol.

For details of labelling conditions for individual experiments, see figure legends.

8.2. Radioiodination of Cell Surface Proteins

The lactoperoxidase method of Marchalonis et al. (1971) was used, with minor modifications. An aliquot of healthy log phase cells (1×10^7 cells, greater than 95% viable) was taken. The cells were centrifuged (500 x g for 5 min), washed twice with ice cold serum-free medium, twice with ice cold PBS-I (section 6.5) and then resuspended in 50 µl of PBS-I in an Eppendorf polypropylene tube (1.6 ml capacity). The following reagents, all prepared in PBS-I (except the radioiodine), were added in order:-

50 µl	Lactoperoxidase, 1 mg/ml
1-2 mCi	Iodine-125 or Iodine-131 (5-10 µl)
10 µl	H_2O_2, freshly prepared as a 1:1000 dilution

The reaction mixture was incubated at 30°C for 3 min, and the reaction stopped by addition of 1 ml ice cold PBS-I containing 5 mM potassium iodide. The cells were centrifuged and washed twice with 1 ml aliquots of PBS-I, and once with 1 ml of TKM buffer.

TKM Buffer

100 mM	Tris-HCl, pH 8.2
100 mM	KCl
5 mM	$MgCl_2$

8.3. Cell Lysis

The labelled cells were centrifuged (500 x g for 5 min) and resuspended in 20 µl TKM buffer. A small volume (0.2-1 ml) of ice cold lysis buffer was then added and the mixture held on ice for 10 min. Lysis buffers were made to the following formulations:-

NP-40 Lysis Buffer

25 mM	Tris-HCl, pH 8.2
150 mM	NaCl
1% v/v	NP-40
(0.1% v/v)	PMSF, 1M in dimethyl formamide
(100 mM)	Iodoacetate,sodium salt.

The NP-40 lysis buffer was used in early experiments, either with or without the protease inhibitors PMSF and iodoacetate.

3-D TKM Lysis Buffer (Dr. T.Mosmann, personal communication)

1 part	5% w/v SDS
1 part	10% w/v Deoxycholate, sodium salt
1 part	10% v/v Triton X-100
1 part	10 X TKM buffer
6 parts	Distilled water
(0.1% v/v)	PMSF, 1M in dimethyl formamide
(0.1% v/v)	Pepstatin-A, 0.7 mg/ml in 50% ethanol

The 3-D TKM lysis buffer was used in later experiments, either with or without the protease inhibitors PMSF and pepstatin-A. When used, the PMSF and pepstatin-A were added to the lysis buffers immediately before use. The cell lysates were centrifuged twice - first to pellet the nuclei (2000 x g for 10 min) and then to pellet any cellular debris (30,000 x g for 1 hr). The final supernatants were used for all subsequent extractions of cellular proteins. Post-nuclear and/or final supernatants were sometimes stored at -70°C but were then given an additional high speed centrifugation (30,000 x g for 1 hr) upon thawing.

The lysis conditions used in some experiments differed from the standard method outlined above. For details of individual experiments, see figure legends.

8.4. Analysis of Cellular Proteins

8.4.1. Acetone Precipitation

Aliquots of cell lysate were precipitated by addition of acetone (5:1 v/v) at room temperature. The precipitate was collected by centrifugation (8,000 x g for 2 min) and air dried.

8.4.2. TCA Precipitation

An aliquot of cell lysate (10-50 µl) was mixed thoroughly with 50 µg of carrier protein (NoRIgG) and precipitated by the addition of 1 ml ice cold 10% w/v TCA. The precipitate was allowed to form for a minimum of 2 hr on ice and then collected by centrifugation (8,000 x g for 2 min).

8.4.3. Indirect Immunoprecipitation

Rabbit anti- human Ig antiserum (or purified antibody) was added to the cell lysate, and a 5-15 min incubation at room temperature allowed. An

equivalence of GARIgG antibody (see Table 5) or S.aureus immunoadsorbent (Section 7.4) was added, and the immunoprecipitate allowed to form for 30 min at 37°C and overnight at 4°C. The immunoprecipitate was collected by centrifugation (8,000 x g for 2 min) and washed three times with 1 ml aliquots of lysis buffer.

The details of immunoprecipitations are given for individual experiments in the figure legends.

9. LABELLING AND ANALYSIS OF CELLULAR RNA

9.1. 32P Labelling

Approximately 50 ml of a log phase culture was taken 16 hr before harvesting and the cells centrifuged (500 x g for 5 min). The cells were resuspended in an equal volume of Phosphate-Free Medium containing 5 mCi of 32P and incubated at 37°C until harvesting for polysome preparation.

Phosphate-Free Medium

80%	(by volume)	RPMI-1640 medium minus Na_2HPO_4
20%	"	Foetal calf serum (dialysed vs. normal saline) plus Penicillin/Streptomycin and Glutamine (section 2.1.)

9.2. 3H-Uridine Labelling

Approximately 16 hr before harvesting, 10 mCi of ^{3}H-Uridine was added to one burler (400 ml) of log phase cells. Incubation was continued until harvesting.

9.3. Preparation of Polysomes

Cells were harvested in mid log phase at a viability of greater than 95%. Just prior to harvesting, the cell culture was made to 0.1 mg/ml cycloheximide and swirled gently for 2 min at 37°C. All steps from this point were done at 4°C. The cells were centrifuged (500 x g for 10 min), the medium thoroughly removed, and the cells washed twice with 200 ml aliquots of ice cold PBS-A containing 0.1 mg/ml cycloheximide.

9.3.1. Method of Fitzmaurice, Bennett, and Williamson (in press)

Polysome extraction solutions were prepared immediately before harvesting of cells, to the following formulations:-

Lysis Buffer A

0.88 M	Sucrose
1 mg/ml	Heparin
0.1 mg/ml	Cycloheximide
0.08% v/v	Triton X-100

Made to volume with Solution C

Lysis Buffer B

0.88 M	Sucrose
1 mg/ml	Heparin
0.1 mg/ml	Cycloheximide
0.08% v/v	Triton X-100
0.2 M	KCl
0.06% w/v	Deoxycholate, sodium salt

Made to volume with Solution C

Solution C

50 mM	Tris-HCl, pH 7.4
25 mM	NaCl
5 mM	Magnesium acetate
7 mM	2-Mercaptoethanol

The washed cell pellet was homogenised in a Dounce homogeniser in

5 volumes of Lysis Buffer A, with two strokes of a loose-fitting pestle. The lysate was centrifuged (10,000 x g for 20 min) and the supernatant (Fraction I) removed and held on ice. The resulting nuclear pellet was homogenised as above in 5 volumes of Lysis Buffer B. The second lysate was centrifuged (50,000 x g for 20 min) and the supernatant (Fraction II) removed. Fractions I and II were combined, centrifuged (10,000 x g for 10 min) to remove debris and then layered onto 10 ml cushions of 1.5 M sucrose (prepared in Solution C, containing 0.1 mg/ml cycloheximide, 1 mg/ml heparin), in screw cap polycarbonate tubes to fit the Spinco 60 Ti rotor. The polysomes were pelleted through the sucrose cushion by centrifugation in a Beckman L5-65 Ultracentrifuge (45,000 rpm for 3 hr).

9.3.2. Modified Method (T. Mosmann, unpublished communication)

Polysomes were extracted using Lysis Buffer A', prepared to the following formulation:-

Lysis Buffer A'

0.88 M	Sucrose
0.1 mg/ml	Heparin
0.1 mg/ml	Cycloheximide
2% v/v	Triton X-100

Made to volume with Solution C

The washed cell pellet was homogenised in 5 volumes of Lysis Buffer A' by vigorous whirlimixing and the lysate centrifuged (10,000 x g for 3 min). The supernatant was removed, made to 1 mg/ml heparin, layered onto 10 ml sucrose cushions and centrifuged (45,000 rpm for 3 hr) to pellet the polysomes. For both methods, the final polysome pellet was

washed by gentle swirling in 0.25 M sucrose, and dissolved in a small volume (1-2 ml) of the same solution. The polysome concentration was determined by dissolving a 10 µl aliquot in 1 ml of 1% w/v SDS (1:100 dilution) and measuring the absorbancy at 260 nm and 280 nm. Polysomes were stored at -70°C.

9.3.3. Sucrose Density Gradient Centrifugation of Polysomes

Linear sucrose gradients of 0.5 to 1.25 M sucrose in Solution C were poured into 12 ml polyallomer tubes to fit a Spinco SW41 rotor. Gradients were overlayed with 5-10 A_{260} units of polysomes (50-100 µl), and centrifuged at 4°C in a Beckman L5-65 Ultracentrifuge (40,000 rpm for 90 min). A_{260} profiles were obtained by pumping the sucrose gradient through a spectrophotometer flow cell of 1 mm path length.

9.4. Phenol Extraction of Polysomal RNA

The method of Mendecki et al. (1972) was used. Polysomes were dissolved in phenol extraction buffer at a concentration of approximately 10 A_{260} units/ml, and shaken vigorously at room temperature with an equal volume of buffer-saturated phenol (redistilled):chloroform: isoamyl alcohol (50:50:1). The phases were separated by centrifugation (10,000 x g for 5 min), the aqueous phase removed, and the phenol phase again extracted with an equal volume of buffer. The two aqueous phases were combined and extracted with an equal volume of fresh phenol: chloroform:isoamyl alcohol. The final aqueous phase was removed carefully, 1/10th volume of 4 M NaCl added, and the RNA precipitated overnight at -20°C with 2 volumes of ethanol.

Phenol Extraction Buffer

0.1 M	Tris-HCl, pH 9.0
0.1 M	NaCl

1	mM	EDTA
2%	w/v	SDS

9.5. Oligo dT Cellulose Chromatography

The method of T. Hunt (unpublished communication) was used. Polysomal RNA which had been twice ethanol precipitated, was centrifuged (10,000 x g for 15 min) and the pellet dried under a stream of nitrogen. The polysomal RNA was dissolved in a small volume (1-5 ml) of Loading Buffer and applied to a column of oligo dT cellulose (bed volume 0.5-1 ml) which was equilibrated in the same buffer. The column was washed thoroughly, the absorbance (A_{260}) of the eluant monitored, and the washing stopped when the absorbance was less than 0.04. The column was next washed with Intermediate Buffer and the washing stopped when the absorbance (A_{260}) was essentially nil. The poly(A) RNA was then eluted in a small volume of Elution Buffer. One tenth volume of 4 M NaCl was added to the eluate, and the RNA precipitated overnight at -20°C with 2 volumes of ethanol. Ethanol precipitation was repeated and the poly(A) RNA was finally dissolved in distilled water at 100 µg/ml (A_{260}= 2.2) and stored at -70°C.

Loading Buffer

10	mM	Tris-HCl, pH 7.5
1	mM	EDTA
0.1%	w/v	SDS
0.5	M	LiCl

Intermediate Buffer

10	mM	Tris-HCl, pH 7.5
1	mM	EDTA
0.1%	w/v	SDS
0.1	M	LiCl

Elution Buffer

10	mM	Tris-HCl, pH 7.5
1	mM	EDTA

10. WHEAT GERM CELL-FREE TRANSLATION SYSTEM

10.1. Preparation of Wheat Germ Extract

The wheat germ extract was prepared according to the method of Marcu & Dudock (1974). All glassware was baked at 200°C to destroy ribonuclease, and all steps were done quickly at 4°C. Untoasted wheat germ (2 g) was ground in a mortar for 60 sec with an equal weight of powdered glass. Extraction Buffer (4 ml) was then added, to make a paste which was then removed and centrifuged (30,000 x g for 10 min). The supernatant (approximately 1 ml) was applied to a column (1.4 cm i.d. x 25 cm) of coarse Sephadex G25 (treated overnight in 0.1% v/v DEP to destroy ribonuclease) and washed thoroughly with Column Buffer.

Extraction Buffer		Column Buffer	
20 mM	HEPES buffer	20 mM	HEPES buffer
1 mM	Magnesium acetate	5 mM	Magnesium acetate
100 mM	KCl	120 mM	KCl
2 mM	$CaCl_2$	6 mM	2-Mercaptoethanol
6 mM	2-Mercaptoethanol	Adjusted to pH 7.6 with KOH, yielding final potassium concentration of 132 mM	
Adjusted to pH 7.6 with KOH			

The wheat germ supernatant was eluted with Column Buffer, and the turbid fractions collected, pooled and centrifuged (30,000 x g for 20 min). The supernatant was immediately frozen in small aliquots and stored in liquid nitrogen.

The following formulation and assay conditions were developed by Mrs. H. Singer (Singer and Williamson, in preparation). I am also grateful to Mrs. H. Singer for making available to me wheat germ extracts and supplementary solutions and for running many of my assays.

10.2. Supplementary Solutions

The following supplementary solutions were prepared and stored in small aliquots at -20°C (except the creatine kinase).

ATP Mixture

Sixty mg of ATP was dissolved in 2 ml distilled water and neutralised with KOH. To this was added 0.2 g of creatine phosphate and 1 mg GTP. The volume was adjusted to 5 ml.

DTT Mixture

A solution of 0.12 M DTT was prepared, and deaerated by bubbling N_2 through it for 30 min.

Creatine Kinase Solution

A solution of 0.5 mg/ml creatine kinase was prepared just prior to preparation of the assay mixture.

Spermidine Solution

A solution of 4 mM spermidine was prepared.

Salt Solution

0.7 M	HEPES buffer, pH 7.8 (adjusted with KOH)
1.6 M	KCl

10.3 Preparation of Assay Mixture

An Energy Mixture was prepared immediately before the assay, according to the following formulation:-

Energy Mixture

25 µl	ATP mixture
10 µl	DTT mixture
5 µl	Creatine kinase solution

10 µl	Salt solution
50 µl	Distilled water
100 µl	Total volume

The Assay Mixture was then prepared to the following formulations:-

Assay Mixture	
15 µl	Wheat germ extract
10 µl	Energy mixture
5 µl	Spermidine solution
5 µl	Poly(A) RNA, 100 µg/ml
-----	Radiolabelled amino acid(s), lyophilised
15 µl	Distilled water
50 µl	Total volume

The assays were incubated at 25°C for 90 min in sealed Eppendorf polypropylene tubes (1.6 ml capacity).

10.4. Analysis of Cell-Free Translation Products

After the incubation period, assays were diluted with 4 volumes of 3-D TKM Buffer (section 8.3), and debris pelleted by centrifugation (8,000 x g for 2 min). Aliquots were then removed for TCA, acetone, or serological precipitation.

10.4.1. Acetone Precipitation

Acetone precipitations were done as described in section 8.4.1.

10.4.2. TCA Precipitation

To determine the amount of incorporation of radiolabelled amino acid(s), a 25 µl aliquot (10%) of each diluted assay was removed, and an equal volume of 0.2 N NaOH added. Incubation was allowed to proceed for 15 min at 37°C to hydrolyse charged tRNA molecules. Twenty µg of carrier protein (NoRIgG) was added, and the proteins precipitated at 4°C by addition of 50 µl of 25% w/v TCA followed immediately by 1 ml of 10% w/v TCA. The precipitates were allowed to form on ice for 1 hr, collected by centrifugation (8,000 x g for 2 min), and washed three times with 10% w/v TCA.

10.4.3. Indirect Immunoprecipitation

Indirect immunoprecipitation was done as described in section 8.4.3.

11. DISCONTINUOUS SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Discontinuous SDS-PAGE using a Tris-glycine buffer system was done according to the method of Laemmli (1970). Slab gels (20 x 25 x 0.15 cm) were prepared from stock solutions as follows:-

11.1. Stock Solutions

Solution A

30%	w/v	Acrylamide
0.8%	w/v	N,N' Methylene bisacrylamide

Deionised, filtered and stored at 4°C

Solution B

1.5	M	Tris-HCl, pH 8.8
0.13%	v/v	TEMED

Filtered and stored at 4°C

Solution C

0.65 M Tris-HCl, pH 6.8

Filtered and stored at 4°C

11.2. Separating Gel Preparation

The following table was used to prepare 100 ml of separating gel solution of the required percentage acrylamide:-

	Percentage Acrylamide				
	5%	7.5%	10%	12.5%	15%
Solution A (ml)	16.7	25	33.3	41.7	50
Solution B (ml)	25	25	25	25	25
10% w/v SDS (ml)	1	1	1	1	1

Distilled water to 100 ml

The gel solution was degassed and ammonium persulphate added to 1 mg/ml. The gel was poured immediately and left to polymerise at room temperature for 1 hr.

11.3. Stacking Gel Preparation

Gel solution for 5% polyacrylamide stacking gel was prepared as follows:-

4 ml	Solution A
2.4 ml	Solution C
0.24 ml	10% w/v SDS
24 µl	TEMED
Distilled water to 24 ml	

The gel solution was degassed and ammonium persulphate added to 1 mg/ml. The gel was immediately poured on top of the separating gel and left to polymerise around a 26-place PTFE (teflon) 'well' template.

11.4 Sample Preparation and Electrophoresis

Protein samples to be electrophoresed were dissolved in 50 µl of Sample Buffer, and heated at 100°C for 2 min.

Sample Buffer

1 part	Solution C
1 part	Glycerol
2 parts	10% w/v SDS
(100 mM)	DTT
6 parts	Distilled water, containing 0.01 mg/ml bromophenol blue

The samples were loaded into the sample wells, under the Reservoir Buffer, and electrophoresed overnight at 4°C at 15-20 mA/gel constant current.

Reservoir Buffer, pH 8.8

0.025 M	Trizma base
0.192 M	Glycine
0.1% w/v	SDS

12. CONTINUOUS SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Continuous SDS-PAGE using a phosphate buffer system was done according to the method of Summers et al. (1965). Either slab gels (20 x 25 x 0.15 cm) or cylindrical gels (0.55 cm i.d. x 6 cm) were prepared from stock solutions as follows:-

12.1. Stock Solutions

Solution A, A'

30% w/v	Acrylamide
0.8% w/v(A)	N,N' Methylene bisacrylamide
1.2% w/v (A')	N,N' Diallyltartardiamide (DATD)
6 M	Urea

Deionised, filtered and stored at 4°C

Solution B

0.4 M	Sodium phosphate, pH 7.2
6 M	Urea, deionised

Filtered and stored at 4°C

12.2. Gel Preparation

The following table was used to prepare gel solutions of the required percentage acrylamide:-

	*3%	5%	6%	10%
Solution A or A' (ml)	10	16.7	20	33.3
Solution B (ml)	25	25	25	25
10% w/v SDS (ml)	1	1	1	1

6 M Urea, deionised, to 100 ml

*agarose to a final concentration of o.5 % w/v was incorporated into the gel solution by heating to 80°C in the 6 M Urea.

The gel solution was degassed and ammonium persulphate added to 1 mg/ml. Gels were poured immediately. Cylindrical gels were overlayed with water and left to polymerise at room temperature. Slab gels were polymerised around a 26-place PTFE (teflon) 'well' template.

12.3. Sample Preparation and Electrophoresis

Protein samples to be electrophoresed were first dissolved in a small volume (10- 100 µl) of Sample Buffer by heating at 100°C for 2 min.

Sample Buffer

0.25 part	Solution B
2 parts	10% w/v SDS
1 part	Glycerol
6.75 parts	9M Urea, deionised

Bromophenol blue to 0.01 mg/ml final concentration

Samples to be reduced and alkylated were treated by addition of 1/20th volume of 1M DTT (30 min at 37°C) followed by 1/10th volume 1.25M iodoacetamide (30 min at 20°C).

The samples were loaded on top of cylindrical gels, or into the sample wells of slab gels, under the Reservoir Buffer, and electrophoresed at 4°C at 10 mA/cylindrical gel, or 50 mA/slab gel.

Reservoir Buffer

0.1 M	Sodium phosphate, pH 7.2
0.1% w/v	SDS

13. POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA

Polyacrylamide gel electrophoresis of RNA in 99% formamide was done according to the method of Duesberg and Vogt (1973).

13.1. Gel Preparation

The gel solution was made as follows:-

10 mM	Na_2HPO_4
10 mM	NaH_2PO_4 (pH 7.0)

Made to volume with formamide, dissolved with stirring overnight at $4^{o}C$ with light excluded.

3% w/v	Acrylamide (recrystallised)
0.5% w/v	N,N' Methylene bisacrylamide (recrystallised)

The gel solution was degassed and ammonium persulphate added to 1 mg/ml. Cylindrical gels (0.6 cm i.d. x 8 cm) were poured immediately and left to polymerise at room temperature.

13.2. Sample Preparation and Electrophoresis

The RNA samples to be electrophoresed were dissolved in 20 µl buffered formamide containing 0.01 mg/ml bromophenol blue, and 5 µl glycerol added with vigorous mixing. The samples were applied to the tops of the gels, under a 2 cm layer of buffered formamide, and electrophoresed at 100 V constant voltage for 6 hr.

Reservoir Buffer

0.04 M	Sodium phosphate, pH 7.0

14. ISOELECTRIC FOCUSING (IEF)

14.1. Stock Solutions

Solution A

30% w/v	Acrylamide
0.8% w/v	N,N' Methylene bisacrylamide

10 M Urea, deionised, to volume, filtered and stored at 4°C.

Solution B

10 M Urea, deionised, filtered and stored at 4°C

14.2. Gel Preparation (Awdeh et al, 1968)

The following table was used to prepare gel solutions of the required percentage acrylamide:-

	5%	6%	7.5%	10%
Solution A (ml)	1	1.2	1.5	2
40% w/v Ampholine carrier ampholytes (ml)	0.3	0.3	0.3	0.3
Solution B	To 6 ml			

TEMED to 0.1% v/v and ammonium persulphate to 1 mg/ml were added and the gel solution introduced as a 1 mm thick layer between a siliconized glass plate and a chromic acid washed glass plate (7.5 cm^2) so as to adhere to the latter plate.

14.3. Sample Preparation and Focusing

Gels were placed face down onto carbon electrodes in a humidified chamber and prefocused for 1 hr at 50 V constant voltage. The electrodes were first coated lightly with the following solutions:-

Anode: 2% v/v Phosphoric acid

Cathode: 5% v/v TEMED

Samples were dissolved in 9 M urea (deionised) and applied to the pre-focused gel as small volumes (10-20 µl) soaked into 0.5 cm x 1 cm filter papers. The samples were focused at 50 V for 1 hr, and then

150 V overnight at room temperature.

15. RECOVERY OF PROTEINS FROM POLYACRYLAMIDE GELS

15.1. Localisation of the Protein Bands

Dried slab gels were overlayed with their respective autoradiographs, and the two carefully aligned using the fine edge markings of the gel which could be seen on the autoradiograph. The positions of the bands were marked on the gel by puncturing through with a drawing pin. The protein bands were then sliced out with a scalpel, and the paper backing scraped off.

15.2. Elution of Proteins

Proteins were eluted by overnight incubation of gel slices at 60°C in 0.2-1ml of Elution Buffer.

Elution Buffer

250 mM	Tris-HCl, pH 8.2
2% w/v	SDS
(20 mM)	DTT

The progress of the elution was monitored (with iodinated proteins) by gamma counting of both eluate and gel slice. When not directly measured, the elution efficiency was inferred from the above results. After sufficient protein was eluted (usually 50-80%), the gel slices were removed and carrier protein (50-250 µg/ml) added to the eluates. A further incubation at 37°C for 30 min was allowed and alkylation was then carried out as described in section 12.3. For charge-shift titration the specific conditions of alkylation are shown in the legend to Fig. 3.7.

15.3. Preparation of Proteins for Microsequencing and Carboxypeptidase Digestion

After alkylation, the eluted proteins were precipitated with acetone (5:1 v/v) at room temperature, and washed once with acetone:water (5:1).

The dried protein pellet was redissolved in 0.1 ml of 30% v/v glacial acetic acid and then diluted to 1.0 ml. Aliquots were removed for determination of radioactivity and the samples lyophilised.

15.4. Preparation of Proteins for IEF - Removal of SDS

After alkylation, the eluted proteins were precipitated with acetone (5:1 v/v) at room temperature. The precipitates were redissolved in 200 µl of 9 M urea containing 10 mM Tris-HCl (pH 8.2) and again precipitated with acetone. This cycle of dissolution and acetone precipitation was repeated twice more. The samples were then dissolved in 9 M urea for IEF.

16. CARBOXYPEPTIDASE DIGESTIONS (Winstead & Wold, 1964)

16.1. Sample Preparation

Lyophilised samples containing 0.5 mg BSA and the radiolabelled (3H-tyrosine) polypeptides to be analysed were dissolved in 0.9 ml Digestion Buffer by heating at 100°C for 2 min. Insoluble material was removed by centrifugation (8,000 x g for 2 min) and the pH adjusted to 8.0-8.2 with 0.5 N HCl (5-10 µl).

Digestion Buffer

0.05 M $NaHCO_3$

0.2 M LiCl

16.2. Enzyme Preparation

Crystals of carboxypeptidase A were washed with two successive 1 ml aliquots of distilled water and just before use dissolved in 10% w/v LiCl and the concentration adjusted to 0.2 mg/ml using its absorbency at 280 nm ($A_{280}/2.3$ = mg/ml). The enzyme solution was kept on ice until use.

16.3. Digestions

An aliquot of sample (75 µl) was taken for determination of total radioactivity, brought to 0.2 ml by addition of Digestion Buffer and counted in 20 ml of Triton-Toluene-PPO scintillation liquid. A second aliquot (150 µl) was taken for determination of TCA soluble radioactivity before addition of enzyme (Time 0), added to 50 µl of 40% w/v TCA and kept on ice.

Digestion at an enzyme-substrate ratio of 1:100 was initiated by addition of 10 µl enzyme solution (2 µg carboxypeptidase, mol.wt. 34,400) to remaining sample at room temperature. Aliquots (150 µl) were removed at specified times of incubation (0.2, 1, 2, 5 min), added to 50 µl of 40% TCA and kept on ice for a total of 30 min. The resulting TCA precipitates were centrifuged (8,000 x g for 2 min) and the supernatants (200 µl) carefully removed and counted in 20 ml of Triton-Toluene-PPO scintillation liquid.

17. DETECTION OF RADIOACTIVITY

17.1. Scintillation Counting of Aqueous Samples

Aqueous samples for liquid scintillation counting were from the following sources:-

(i) Acetone precipitated cellular or wheat germ proteins, dissolved in 2% w/v SDS, 9 M urea, or SDS-PAGE sample buffer.

(ii) TCA precipitated cellular or wheat germ proteins, dissolved in 0.2 N NaOH and neutralised with 1 M acetic acid.

(iii) Serological precipitates dissolved in 2% SDS, 9 M urea, or SDS-PAGE sample buffer.

Aqueous samples were counted in a Beckman LS 333 Scintillation Counter as either 1% or 10% v/v solutions in Triton-Toluene-PPO scintillation liquid. Comparisons were made only between samples counted in an identical manner.

17.2. Scintillation Counting of Polyacrylamide Gel Slices

Gel slices of polyacrylamide crosslinked with N,N' methylenebis-acrylamide were solubilised by incubation at 60°C for 2 hr with 0.5 ml Protosol in tightly capped scintillation vials. Toluene-PPO scintillation liquid (5 ml) was then added with vigorous mixing. Gel slices of polyacrylamide crosslinked with DATD were solubilised by room temperature incubation for 2 hr with 0.5 ml 2% periodic acid. Triton-Toluene-PPO scintillation liquid (5 ml) was then added. Samples were counted in a Beckman LS 333 Scintillation Counter. The counting efficiencies were: 1) ^{3}H, 35% 2) ^{35}S, 90%

17.3. Autoradiography of Slab Gels

Slab gels were washed with three changes (500 ml each) of 20% v/v ethanol and dried on to Whatman 3MM paper under vacuum. The dried gels were held in contact with Kodak X-Omat-R film between two glass plates, and stored for the required exposure time in light-proof

bags at room temperature.

17.4. Fluorography of Slab Gels (Bonner & Laskey, 1974)

Slab gels were washed in three successive 250 ml volumes of dimethyl sulphoxide (DMSO) for 30 min each and then impregnated with PPO by gentle agitation in a solution of 20% w/v PPO in DMSO (40 g PPO + 160 g DMSO) for 45 min. Excess PPO-DMSO solution was decanted off, and the gels flushed continuously with water for 1 hr. Gels were dried on to Whatman 3MM paper under vacuum and the dried gels held in contact with Kodak X-Omat-R film between two glass plates, and stored for the required exposure time in light-proof bags at -70°C.

RESULTS

AND

DISCUSSION

CHAPTER I

SYNTHESIS, SURFACE DEPOSITION, AND SECRETION OF IMMUNOGLOBULIN IN SELECTED HUMAN LYMPHOID CELL LINES

1.1 EARLY STUDIES OF LYMPHOMA CELL SURFACE IgM

1.1.1. Purpose of Investigations

The use of lactoperoxidase catalysed radioiodination as a specific means of labelling cell surface proteins for biochemical analysis has been described by Marchalonis et al. (1971). One of the early aims of this project was to assess the efficacy of this method, combined with SDS-PAGE analysis of specific immunoprecipitates, for the identification of surface Ig (sIg) on lymphoid cells.

1.1.2. Surface IgM Detection on Cell Lines Daudi and Raji

Two lymphoma-derived cell lines, Daudi and Raji, which were available to me in the beginning of my investigations were analysed for the presence of sIg. Previous workers had shown by immunofluorescence that Daudi cells expressed high levels, and Raji cells extremely low levels of sIgM (see Materials and Methods, Table 2). In addition, Sherr et al. (1972) and Kennel (1974) had identified Daudi sIgM by SDS-PAGE analysis.

In the present investigation, the cells were surface radioiodinated and the labelled sIgM analysed by immunoprecipitation and SDS-PAGE on phosphate-buffered 6% polyacrylamide tube gels. Figure 1.1 shows the profiles obtained from the reduced immunoprecipitates. The Daudi sIgM (Fig. 1.1 (A)) was readily identified by the presence of a large peak of radioactivity comigrating with the μ marker protein. In addition, a small light chain peak (κ type) and a large peak of slightly slower mobility were resolved. This latter peak is discussed in Chapter 3. The SDS-PAGE profile shown in Fig. 1.1 (A) was in

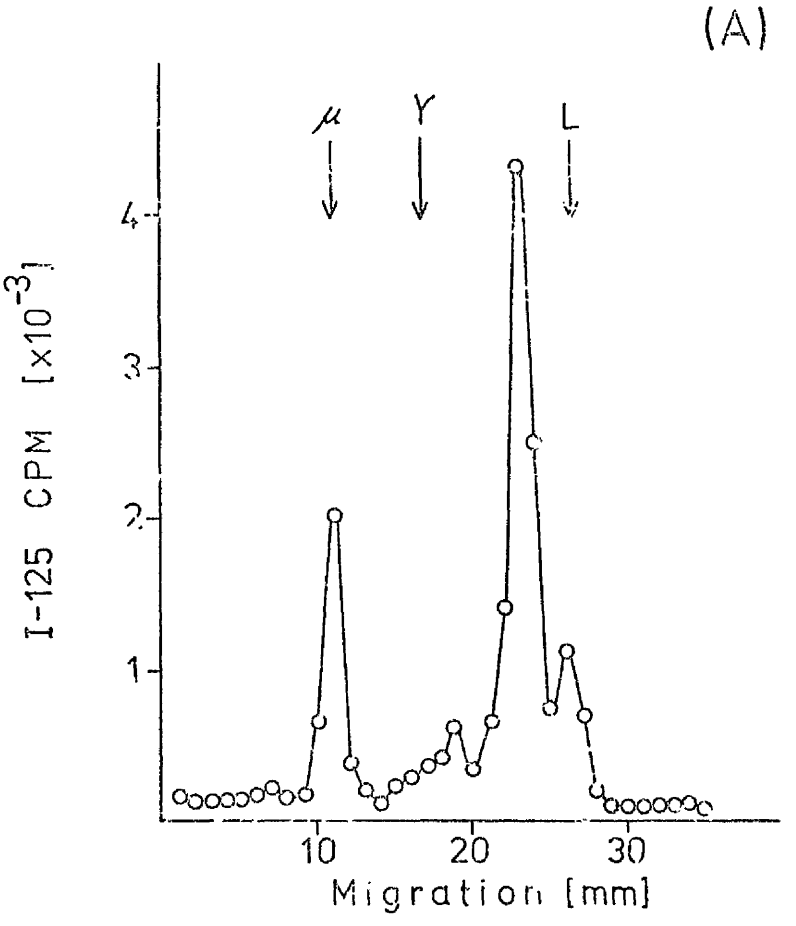
Fig. 1.1

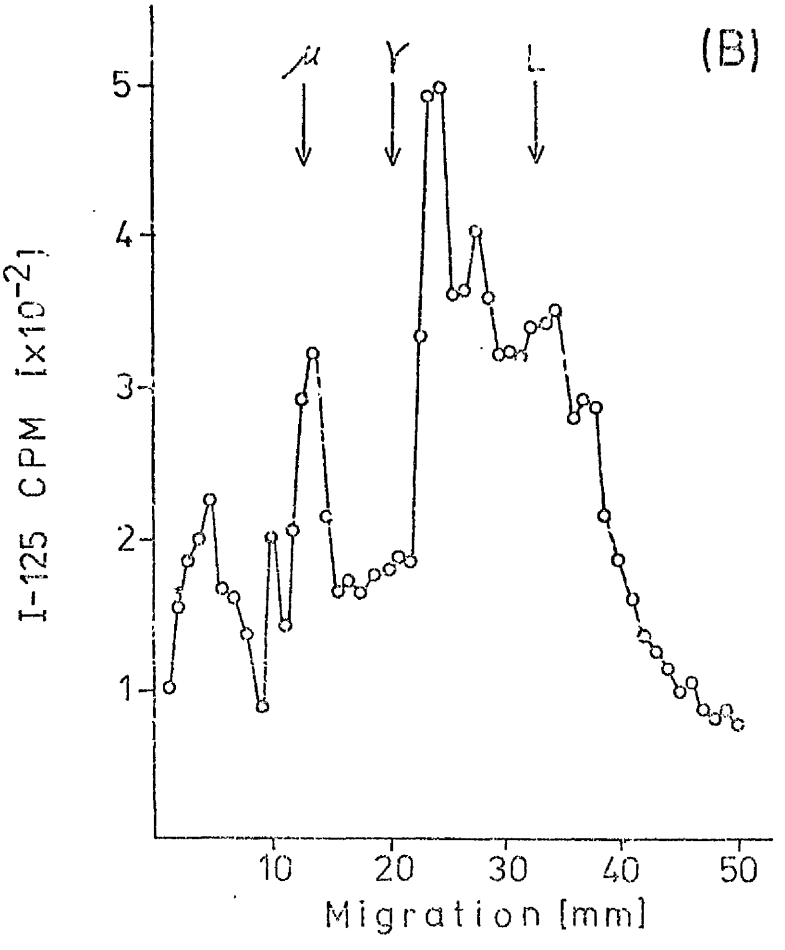
SDS-PAGE Profiles of Daudi and Raji Cell Surface IgM

Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-125, 0.25 mCi) as described in Methods, section 8.2, and the cells lysed in 1 ml NP-40 lysis buffer containing PMSF and iodoacetate (Methods, 8.3). Immunoprecipitations from the lysates were carried out by addition of 2 µl anti-IgM (15 min at 4°C) followed by 100 µg GARIgG (15 min at 4°C). Washed immunoprecipitates were dissolved in 75 µl SDS-PAGE sample buffer and half removed for reduction and alkylation (Methods 12.4). Electrophoresis was in phosphate-buffered tube gels of 6% polyacrylamide (Methods, 12). The radioactivity in individual gel slices was determined by gamma counting. Molecular weight marker µ, γ, and light chains were electrophoresed in a separate tube gel.

(A) Daudi sIgM (Reduced)

(B) Raji sIgM (Reduced)


(A)
μ
γ
L
I-125 CPM [x10⁻³]
1
2
3
4
10
20
30
Migration [mm]


(B)
μ
γ
L
I-125 CPM [x10⁻²]
1
2
3
4
5
10
20
30
40
50
Migration [mm]

good agreement with those obtained by Sherr et al. (1972) and Kennel (1974).

The Raji SDS-PAGE profile (Fig. 1.1 (B)) proved more difficult to interpret. While a peak of μ mobility was resolved, the radioactivity in this peak was only slightly above background levels. Moreover, the background radioactivity in the Raji profile itself resolved into non-specific peaks which contained in some cases more label than the putative μ peak.

1.1.3. Conclusions: Limitations of Methodology

These preliminary results indicated that sIgM could be readily detected by SDS-PAGE when it was expressed at high levels. However, the limitations of the methodology for the biochemical analysis of extremely low levels of sIgM (as with Raji cells) were also clearly illustrated. These limitations had also been reported by workers applying this methodology to similar problems such as the detection of putative T-lymphocyte sIg (Haustein et al., 1975). Modifications in the methodology which significantly improved the sensitivity of SDS-PAGE analysis of sIg are discussed below.

1.2. METHODOLOGICAL CONSIDERATIONS FOR THE SDS-PAGE ANALYSIS OF IMMUNOGLOBULIN EXPRESSION

1.2.1. Background Radioactivity in SDS-PAGE Profiles of Immunoprecipitates

The peaks of background radioactivity seen in the Raji SDS-PAGE profile (Fig. 1.1 (B)) are typical of results from many other laboratories performing similar analyses. Much of this background radioactivity

is generally accepted to be the result of non-specific binding of cellular proteins to immunoprecipitates (see Melchers & Andersson, 1973, for detailed analysis). However, some debate exists as to whether all of the peaks are truly non-specific. The SDS-PAGE profiles of the B-lymphocyte F_c receptor (Basten et al., 1972; Cline et al., 1972), for example, have striking features in common with the non-specific background profiles, notably the broad peaks of radioactivity migrating with proteins of approximately 40-50,000 molecular weight. It has also been suggested in work leading up to this thesis investigation (Premkumar et al., 1975a, 1975b) that certain of these peaks may represent proteins specifically associated with sIgM in the outer cell membrane, perhaps as sIgM anchor proteins.

1.2.2. Characterisation of Background Radioactivity by High Resolution SDS-PAGE

Using high resolution SDS-PAGE on slab gels (Laemmli, 1970), experiments were undertaken to better resolve and identify the background radioactivity in immunoprecipitates. In one such experiment, surface radioiodinated and biosynthetically labelled samples were prepared from several IgM-producing cell lines. Samples of labelled IgM were isolated by immunoprecipitation and electrophoresed adjacent to samples of total cell lysate from the corresponding cells in a 12.5% polyacrylamide slab gel (Fig. 1.2). After fluorographic exposure the tracks containing the immunoprecipitates (even numbered) clearly showed the expected background radioactivity, as well as the specifically precipitated μ and (in some cases) light chains.

The SDS-PAGE profiles shown in Fig. 1.2 revealed several features of the background radioactivity as well. Thus, the background profiles consisted of bands representing most of the labelled proteins in the

Fig. 1.2

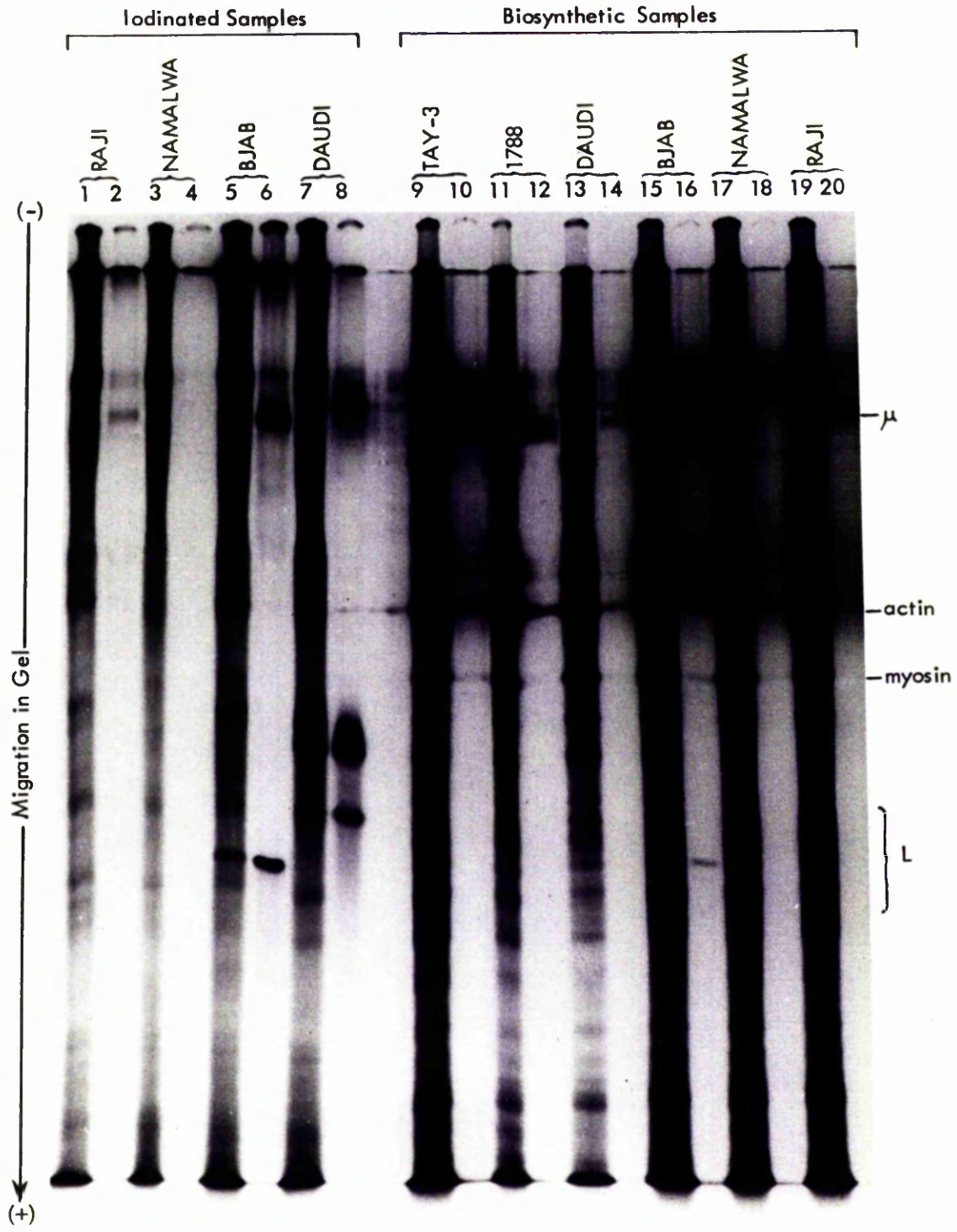
SDS-PAGE Analysis of Background Radioactivity in Immunoprecipitates

Cells surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-131, 2 mCi) as described in Methods, section 8.2, and the cells lysed in 0.5 ml NP-40 lysis buffer containing PMSF (Methods, 8.3). Aliquots of lysate were removed for acetone precipitation (Methods, 8.4.1). Immunoprecipitations were then carried out by addition of 25 µg RAHIgM plus 25 µg NoRIgG (15 min at 20°C) followed by 200 µg GARIgG (1 hr at 20°C, overnight at 4°C).

For biosynthetic labelling, 1 x 10^6 cells from log phase cultures were incubated for 1 hr in 0.2 ml labelling medium containing 100 µCi 35S-methionine. Complete medium (0.8 ml) was then added and a further 6 hr incubation allowed (Methods 8.1). The cells were separated from the culture medium and lysed in 0.25 ml NP-40 lysis buffer as described above. Aliquots of lysate were removed for acetone precipitation. Immunoprecipitations were carried out by addition of 5 µg RAHIgM plus 45 µg NoRIgG (15 min at 20°C) followed by 200 µg GARIgG (1 hr at 20°C, overnight at 4°C). Washed acetone and immune precipitates were dissolved in 100 µl 2% SDS. Five microlitre to 10 µl aliquots of immunoprecipitates (10% of sample) and acetone precipitates (2% of iodinated and 5% of biosynthetic samples) were added to 50 µl reducing sample buffer for discontinuous SDS-PAGE in a 12.5% polyacrylamide slab gel (Methods, 11). The gel was processed for fluorography (Methods, 17.4) and the fluorogram developed after an exposure of 24 hr. The µ and light chains from the cells investigated were used as mobility markers.

Odd Numbered Tracks: Acetone Precipitates (Total Cellular Proteins)

Even Numbered Tracks: Immunoprecipitates


Iodinated Samples
Biosynthetic Samples
RAJI
NAMALWA
BJAB
DAUDI
TAY-3
1788
DAUDI
BJAB
NAMALWA
RAJI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
(-)
(+)
Migration in Gel
μ
actin
myosin
L

cell lysates. Most bands were labelled in the background profiles approximately proportional to their labelling intensity in the cell lysate, as expected in the absence of any specific binding. Moreover, the prominent peaks of radioactivity with mobilities between those of γ and light chain (see Raji profile, Fig. 1.1 (B)) could be seen in Fig. 1.2 to correspond to the highly labelled cellular proteins actin and myosin (indicated by arrows).

1.2.3. Identification of Specific Immunoglobulin Bands Against a Constant Background

With the SDS-PAGE analysis shown in Fig. 1.2, it was possible to detect extremely low levels of IgM synthesis and surface deposition against a high background of radioactivity. Thus, the radioiodinated (track 2) and biosynthetically labelled (track 20) Raji μ bands could be readily distinguished. In contrast, a non-producer mutant of cell line Namalwa showed only a background profile in both the radioiodinated (track 4) and biosynthetically labelled (track 18) samples. Although in this experiment the background radioactivity was quite high, the principle of identifying specifically enriched immunoprecipitated bands was clearly illustrated. With immunoprecipitates having lower background radioactivity, this type of analysis gives a considerably greater sensitivity than previously possible.

1.2.4. Conclusions Concerning Non-Specific Binding to Immunoprecipitates

The above results were therefore inconsistent with there being any specific binding to immunoprecipitates, other than that of the antigenic μ and light chains to which the antiserum was directed. The single unique exception to this conclusion was found to be the p33

protein associated with Daudi sIgM (track 8). Further investigation of this phenomenon is discussed in detail in Chapter 3.

1.2.5. Methods for Improved Sensitivity and Specificity of Immunoprecipitation and SDS-PAGE: A Summary

In the previous section, results were cited which suggested the cause of background radioactivity to be non-specific binding to the precipitating immune complexes. Over the course of many experiments subsequent to this finding, steps were taken to reduce this unwanted background radioactivity. Following is a summary of the modifications in the methodology which were found to be useful.

1.2.5.1. Reduction in Amount of Immunoprecipitate

As background radioactivity was found to be approximately proportional to the amount of immunoprecipitate, steps were taken to minimise the amount of antiserum or antibody used. Complete immunoprecipitation of cellular and/or secreted Ig produced by 1×10^7 cells under normal conditions of labelling was found to require 10-50 µg of specific antibody. In order to deliver this quantity with the minimum of rabbit Ig, affinity purified antibody was used. Immunoprecipitates formed by the subsequent addition of GARIgG were as much as tenfold smaller (i.e. 40-200 µg) than if whole rabbit antiserum was used.

1.2.5.2. Reduction in Cell Lysate and Labelling Medium Volumes

This step was necessary for efficient immune complex formation with the lesser quantities of antibody employed. Accordingly, 1×10^7 cells (as for radioiodination) were generally lysed in 0.5 ml lysis buffer. For biosynthesis, 1×10^6 cells were labelled in 0.2 ml medium, and lysed in 0.25 ml lysis buffer. In the case of biosynthetically labelled cells, proportionately less antibody was required, and

therefore used.

1.2.5.3. Use of Three-Detergent Lysis Buffer

Immunoprecipitations from cell lysates and wheat germ extracts in 3D-TKM lysis buffer (Materials and Methods, section 8.3) were found generally to have somewhat less non-specific binding. It was further found that washing of immunoprecipitates with 3D-TKM buffer reduced background binding.

1.2.5.4. Use of Fixed S.Aureus Immunoadsorbent

A very useful reduction in amount of immunoprecipitate was achieved in later experiments using minimal amounts of purified antibody in a sandwich precipitation with fixed S.aureus immunoadsorbent(Kessler, 1975) instead of GARIgG. Because of the inherently low non-specific binding of the S.aureus immunoadsorbent, this method significantly reduced the background radioactivity. This approach also had other important advantages. First, because it does not rely on formation of precipitable immune complexes, the reaction is both fast and relatively independent of reactant (i.e. antigen, antibody) concentrations. Secondly, because of the reduced protein recovered from immunoprecipitates, larger proportions of each precipitate could be loaded onto polyacrylamide slab gels. This decreased the exposure time required to see specific bands, and therefore increased the sensitivity of the methodology.

1.2.5.5. Use of High Energy, High Specific Activity Isotopes

High specific activity biosynthetic labelling was achieved using less cells (1×10^6) than commonly reported in the literature, in a small volume (0.2 ml) of labelling medium. Relatively large amounts of isotope were added to the labelling medium by prior lyophilisation. The use of the high energy β-emitters 131-I and 35S-methionine gave

a considerable increase in sensitivity when combined with fluorographic development of slab gels.

1.2.5.6. Minimal Loading of Polyacrylamide Slab Gels

Slab gel wells with cross-sectional dimensions of 0.15 x 0.5 cm were loaded with a maximum of 10-15 µg of immunoprecipitate. Larger loadings were found to cause distortion of the bands and high background radioactivity at a mobility equivalent to that of the antibody γ-chains.

1.3. HIGH RESOLUTION SDS-PAGE OF LYMPHOMA CELL SURFACE IgM

1.3.1. Introductory Remarks

The identification of sIgM on lymphoid cells was greatly improved in later studies by adoption of the system of high resolution SDS-PAGE (Laemmli, 1970) on polyacrylamide slab gels. The lymphoma cell lines described below, which were initially screened for sIgM expression, represent an enlarged selection used for many of the subsequent investigations to be described in this thesis. The cell lines BJAB, Daudi, Namalwa and Raji were all derived from patients with Burkitt's lymphoma. The cell line U698M was derived from a patient with lymphosarcoma of the tonsils (see Materials and Methods Table 2 for details).

1.3.2. Detection of µ-Chain Bands

Figure 1.3 (A) shows the SDS-PAGE profiles obtained in a 12.5% polyacrylamide slab gel from the reduced anti-IgM immunoprecipitates of the above cell lines. These cell lines had all been previously reported to have surface immunoglobulin of the IgM class and the results

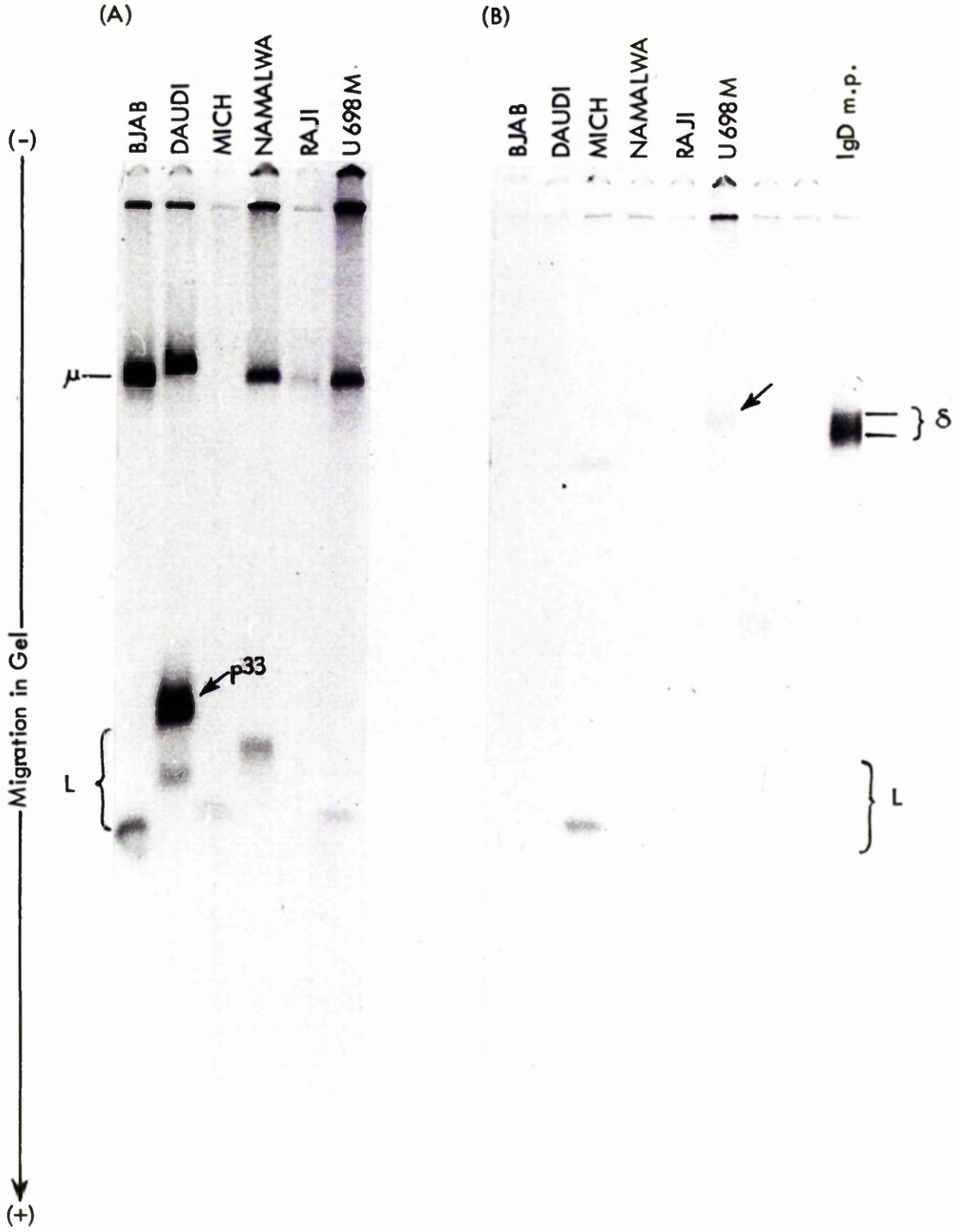
Fig. 1.3

SDS-PAGE Screening of Several Lymphoid Cell Lines for Cell Surface IgM and IgD Expression

Cell surface proteins from 2 x 10^7 cells were labelled by lactoperioxidase iodination (I-131, 1 mCi) as described in Methods, section 8.2, and the cells lysed in 0.5 ml NP-40 lysis buffer containing PMSF (Methods, 8.3). Immunoprecipitations were carried out by addition of 10 µg RAHIgM to 40% of each lysate and 0.5 µl R.410 anti-IgD to 20% of each lysate (15 min at 20°C) followed by either 50 µg or 25 µg respectively of GARIgG (1 hr at 20°C, overnight at 4°C). Washed immunoprecipitates were dissolved in 9M urea (RAHIgM precipitates) or 2% SDS (anti-IgD precipitates), and aliquots (25%) precipitated with acetone and redissolved in reducing sample buffer for discontinuous SDS-PAGE in a 12.5% polyacrylamide slab gel (Methods, 11). The gel was processed for fluorography (Methods 17.4) and the fluorogram developed after an exposure of 4 days. The µ and light chains from the cells investigated, and the δ-chains from radioiodinated AI IgD were used as mobility markers.

(A) Anti-IgM Precipitates

(B) Anti-IgD Precipitates


(A)
BJAB
DAUDI
MICH
NAMALWA
RAJI
U 698 M
(-)
Migration in Gel
(+)
μ
p33
L
(B)
BJAB
DAUDI
MICH
NAMALWA
RAJI
U 698 M
IgD m.p.
δ
L

obtained were consistent with those reports. Thus bands of radioactivity corresponding to the mobility of μ-chain were clearly resolved (indicated by arrow). As expected, all the μ-chain bands comigrated (except Daudi μ, discussed below).

1.3.3. Relative Labelling of μ-Chains by Surface Iodination

Because the conditions of labelling and analysis of the cell surface IgMs were identical, the observed intensities of the μ bands in Fig. 1.3 (A) accurately reflects the relative amounts and/or susceptibility to radioiodination of the sIgMs. Thus, the sIgM from BJAB, Daudi, Namalwa, and U698M was always found to be very highly labelled, whereas the Raji sIgM was only weakly labelled. These results were consistent with the observations of other workers, (see Materials and Methods, Table 2) using mainly immunofluorescence to visualise the sIgM of these lymphoma cells, and seems to indicate a similar susceptibility of the sIgM to labelling by both techniques.

1.3.4. Higher Apparent Molecular Weight of Daudi Surface μ-Chains

High resolution SDS-PAGE revealed that the μ-chain of Daudi sIgM migrated with a markedly higher molecular weight than other radioiodinated μ-chains (Fig. 1.3 (A)). This finding had been reported previously by Kennel (1974), although not as well documented with respect to a panel of other surface radioiodinated μ-chains. As shown in Fig. 1.3 (A) and confirmed in other studies, Daudi surface μ-chains were unique in their higher apparent molecular weight. An estimate of the molecular size of Daudi surface μ-chains could be made by extrapolation from the semi-log plot of molecular weight versus SDS-PAGE mobility shown in the inset to Fig. 3.2 (Chapter 3) and the data shown in Fig. 2.1 (Chapter 2). Thus, Daudi surface μ-chain was determined to have an apparent molecular wiehgt of 88-90,000. This

value is 3-5000 daltons larger than the other surface μ-chains (see Chapter 2).

In addition, the Daudi sIgM profile shows the presence of a novel polypeptide of approximately 33,000 molecular weight (see Chapter 3) and an unusually large κ-chain (discussed below).

1.3.5. Variable Mobilities of Light Chains on Discontinuous SDS-PAGE

As can be seen from Fig. 1.3. (A), individual light chains (indicated by brackets) showed a range of mobilities in this SDS-PAGE system. This phenomenon has been noticed by other workers (T. Mosmann, personal communication). In my investigations the mobility of each individual light chain was found to be characteristic and reproducible. The relative mobilities of the light chains investigated during the course of this thesis work are shown in Table 1.1. In general λ-chains were found to migrate more slowly than κ-chains (λ-chains, 5.4-5.9 cm; κ-chains, 5.9-6.2 cm). It seems likely that amino acid differences in the constant regions of the λ and κ-chains are responsible for the class specific mobility differences observed. Variations among individual light chains of the same class may arise from either amino acid or carbohydrate differences in the variable regions.

1.3.6. Higher Apparent Molecular Weight of Daudi κ-Chains

As shown in Table 1.1, Daudi κ-chains migrated significantly slower than the other κ-chains (5.7 cm as compared with 5.9 - 6.2 cm for the other κ-chains). It has been suggested by the work of Kennel (1974) that Daudi κ-chains contain carbohydrate, although these authors may instead have been detecting the carbohydrate associated with the Daudi p33 protein. However, further investigations of

TABLE 1.1 Relative Mobilities of Ig Light Chains by Discontinuous SDS-PAGE[1]

Cell Line	Light Chain Type	Relative Mobility (cm)
Namalwa	λ	5.4
Bec-11	λ	5.7
Daudi	κ	5.7 (5.8)[2]
1788	λ	5.8 (5.9)[3]
Raji	κ	5.9
Mich	κ	6.0
Tay-3	κ	6.0
U698M	κ	6.0
Bri-8	κ	6.1
BJAB	κ	6.2

1. Mobilities represent approximate relative values obtained by SDS-PAGE in a 12.5% polyacrylamide slab gel.

2. This faster mobility was often obtained with biosynthetically labelled Daudi κ.

3. This faster mobility was obtained with one preparation of 1788 λ (see Fig. 2.3).

Daudi κ-chain and its cell-free translated precursor (see Chapter 2, section 2.4.6) are indeed consistent with the presence of carbohydrate moieties on the cellular κ-chains which would confer on them a higher molecular weight. It has not been possible, however, to determine whether ordinary, or perhaps excessive levels of glycosylation are involved.

1.4. HIGH RESOLUTION SDS-PAGE OF LYMPHOMA CELL SURFACE IgD

1.4.1. Introductory Remarks

Much evidence has accumulated in recent years to firmly establish cell surface IgD as an important receptor on B-lymphocytes (for review see Immunological Reviews, 37, 1977). According to most schemes of B-cell differentiation, cell surface IgD first appears on early B-lymphocytes which are already expressing cell surface IgM. A large proportion of these B-cells then continue to express both sIgM and sIgD on their cell surfaces, as determined by immunofluorescence studies.

Morphologically as well as by the criteria of sIgM expression, the Burkitt's lymphoma-dervied cell lines are ideal human cells to investigate by SDS-PAGE for the presence of sIgD. In addition, the cell line U698M had been previously demonstrated by immunofluorescence to have sIgD (Sundström, 1977).

1.4.2. Detection of δ-Chain Band from U698M Cells

Several of the Burkitt's lymphoma-derived cell lines were surface radio-iodinated and the cell lysates immunoprecipitated with a rabbit anti-human IgD antiserum. Figure 1.3 (B) shows the SDS-PAGE profiles of the reduced immunoprecipitates. Of the cell lines investigated

(BJAB, Daudi, Namalwa, Raji, and U698M) the U698M cells alone showed a readily detectable band of radioactivity (indicated by arrow in Fig. 1.3 (B)) which approximately comigrated with the δ marker protein. This result agreed with the finding of sIgD on U698M cells by Sudström (1977). However, due to suspected cross-reactivity in the anti-IgD antiserum, and the ambiguity of δ-chain SDS-PAGE mobility (see section 1.4.4.2), the result shown in Fig. 1.3 (B) was not sufficient to prove the SDS-PAGE detection of sIgD δ-chain.

1.4.3. Confirmation of sIgD and sIgM on U698M Cells

To determine whether the band in the U698M profile (Fig. 1.3 (B)) was indeed δ-chain, another aliquot of U698M cells was labelled by lactoperoxidase iodination and the resulting cell lysate immunoprecipitated with either anti-IgM or anti-IgD in the presence or absence of competing unlabelled myeloma proteins. Figure 1.4 shows the SDS-PAGE profiles obtained from the reduced immunoprecipitates. The anti-IgM profile (track 1) shows the presence of the U698M sIgM μ and κ-chains, as expected. The anti-IgD profile with competing IgA, IgG and IgM but without competing IgD (track 2) was identical to the anti-IgD profile shown in Fig. 1.3 (B), except that the longer exposure in Fig. 1.4 also revealed an apparent degradation product of the δ-chain, as well as the U698M κ-chain. In contrast, the inclusion of IgD in the mixture of competing Ig eliminated all the radioactive bands from the profile (track 3).

The above result confirmed the finding by radioiodination and SDS-PAGE of both sIgD and sIgM on the U698M cell line. Spiegelberg (1977) has recently reported a similar finding on a human lymphoblastoid cell line (Wil-2WT), but has not presented any data.

Fig. 1.4

SDS-PAGE Analysis of U698M Cell Surface IgM and IgD

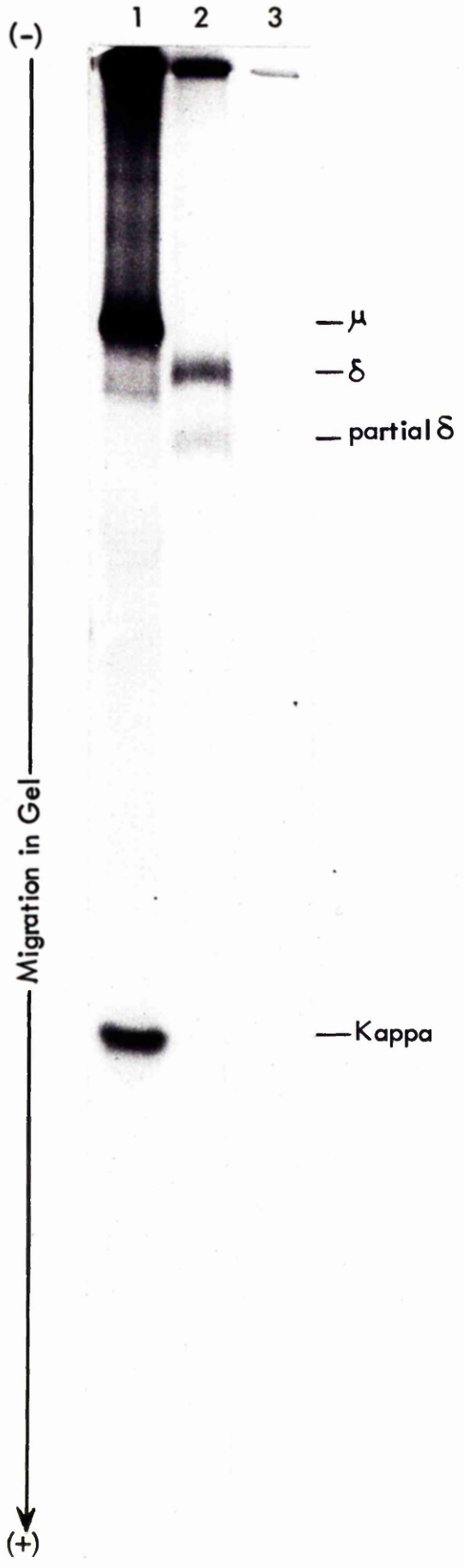
Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-131, 0.8 mCi) as described in Methods, section 8.2, and the cells lysed in 0.5 ml NP-40 lysis buffer containing PMSF (Methods, 8.3). The lysate was divided into three aliquots and immunoprecipitations carried out in the presence of competing unlabelled antigen by addition of either: (1) 10 µg each IgAk and IgGλ, followed by 4 µg RAHIgM; (2) 10 µg each IgAk, IgGλ, and IgMλ, followed by 0.4 µl R.410 anti-IgD; or (3) 10 µg each IgAk, IgGλ, IgMλ, and IgDλ, followed by 0.4 µl R.410 anti-IgD. Incubation for 15 min at 20°C was followed by addition of 20 µg GARIgG (overnight at 4°C). Washed immunoprecipitates were dissolved in reducing sample buffer for discontinuous SDS-PAGE in a 12.5% polyacrylamide slab gel (Methods, 11). The gel was processed for fluorography (Methods, 17.4) and the fluorogram developed after an exposure of 2 days. The U698M µ, δ, and κ-chains; as well as the putative cleavage product of U698M surface δ are indicated by the arrows.

Track

(1) Anti-IgM Precipitate

(2) Anti-IgD Precipitate

(3) IgD-Competed Anti-IgD Precipitate


1
2
3
(-)
μ
δ
partial δ
Migration in Gel
Kappa
(+)

1.4.4. SDS-PAGE Mobility of U698M Surface δ-Chains

1.4.4.1. Comparison to Surface μ-Chain

The SDS-PAGE profiles shown in Fig. 1.4 demonstrated U698M cell surface δ-chains to migrate markedly faster than the surface μ-chains. This finding is in agreement with those of Ferrarini et al. (1976) and Spiegelberg (1977) who also used surface radioiodination on chronic lymphatic leukemia (CLL) and tonsil cells, or on CLL and normal peripheral blood lymphocytes, respectively. In addition, Spiegelberg (1977) also reported the same finding upon investigation of a human lymphoblastoid cell line (Wil-2WT). Finkelman et al. (1976), however, have reported that surface μ and δ-chains of normal adult peripheral blood and cord blood lymphocytes migrate identically.

1.4.4.2. Comparison to Serum δ-Chains

Indirect evidence, derived from the fact that 90% of IgD myeloma proteins are λ (Jancelewicz et al., 1975) while the majority of surface IgD is κ (Rowe et al., 1973), has been interpreted to suggest that δ-chains from these sources may be of different subclasses. Possible support for this idea comes from the work reported by Spiegelberg (1977) where surface δ-chains were shown to be of higher apparent molecular weight than their serum counterparts by SDS-PAGE, although these workers also point out that this difference may instead be related to the attachment of surface IgD to the cell membrane.

From the results of the present investigation, it was possible to compare the SDS-PAGE mobilities of surface and serum δ-chains. Figure 1.3 (B) shows that the myeloma IgD δ-chain used as a molecular weight marker migrated as two bands (indicated by arrows). This was

in agreement with the observation of Goyert et al. (1977), who showed this to be the result of spontaneous degradation of the serum δ-chain at the carboxy-terminal end. According to the reported findings of Kennel et al. (see Spiegelberg (1977)), the U698M surface δ would be expected to migrate more slowly (by a reported 2500 dalton difference) than undegraded serum δ. Goyert et al. (1977) reported an apparent molecular weight difference of 3000 daltons for the two serum δ species, and so a similar mobility difference would be expected for serum versus surface δ. As can be seen in Fig. 1.3 (B), this difference in mobility was not observed.

One likely explanation for the above finding would be that the U698M surface δ-chains were partially degraded during their isolation. In this regard the previously mentioned authors used the protease inhibitors EACA, benzamidine, and PMSF during cell lysis and immunoprecipitation. In the present investigation, however, only PMSF was used. Moreover, direct evidence for some spontaneous degradation (in the form of a second, faster-migrating δ-band) can be seen in the profile shown in Fig. 1.4 (track 2).

1.5. BIOSYNTHESIS AND FATE OF IgM IN NONSECRETORY AND SECRETORY LYMPHOID CELLS

1.5.1. Introductory Remarks

Over the course of many experiments, the rate of synthesis and secretion of IgM in several lymphoid cell lines representing different stages of differentiation was investigated. Although synthesis and secretion naturally varied somewhat from experiment to experiment, it

was possible to distinguish patterns of IgM expression, based on (i) the percentage synthesized relative to total cellular proteins, (ii) the distribution (cellular or extracellular) after pulse-chase labelling, and (iii) the molecular form (8S or 19S) of the extracellular IgM. In addition, the pattern of IgM expression (secretion or surface deposition) was reflected in the molecular size of the biosynthetic μ-chains (discussed in detail in Chapter 2).

The results to be described are from a single biosynthetic labelling experiment, which illustrated well the general pattern of IgM synthesis and secretion observed from a composite of experimental results. Cells were allowed to incorporate 35S-methionine for 1 hr in methionine-free medium, and then for a further 4 hr in complete RPMI 1640 medium. After incubation, the cell lysates and culture media were analysed for labelled IgM by immunoprecipitation and SDS-PAGE of the reduced immunoprecipitates (Fig. 1.5).

1.5.2. Biosynthesis of IgM in the Nonsecretory Lymphoma Cell Lines Daudi and Raji

Daudi and Raji cells were consistently found to be nonsecretory, as illustrated by the complete lack of extracellular IgM in the culture medium Fig. 1.5,(tracks 6 and 10, respectively). The lack of secretion from Daudi cells was consistent with the findings of Sherr & Uhr (1971) and Grundke-Iqbal & Uhr (1974), who also used incorporation of labelled amino acids and immunoprecipitation to detect intracellular IgM.

The SDS-PAGE profiles of intracellular IgM from Daudi and Raji (tracks 5 and 9, respectively) show distinct bands of μ mobility,

Fig. 1.5

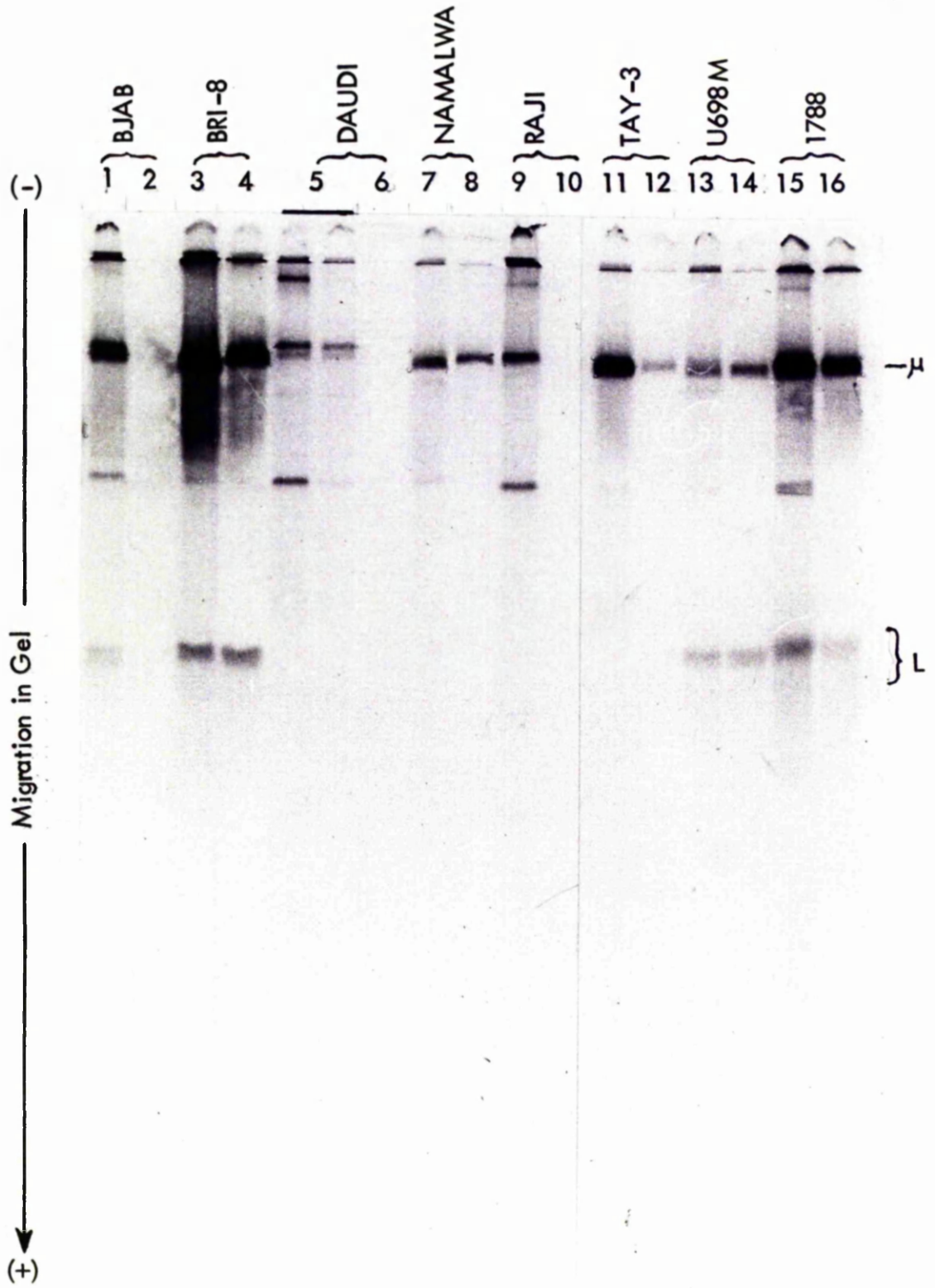
SDS-PAGE Analysis of Biosynthetically Labelled Intracellular and Secreted IgM from Several Lymphoid Cell Lines

For biosynthetic labelling, 1 x 10^6 cells from log phase cultures were incubated for 1 hr in 0.2 ml labelling medium containing 100 µCi 35S-methionine. Complete medium (0.8 ml) was then added and a further 4 hr incubation allowed (Methods, 8.1). The cells were separated from the culture medium and lysed in 0.25 ml NP-40 lysis buffer containing PMSF (Methods, 8.3). The medium was centrifuged to remove residual cells (500 x g for 10 min) and debris (30,000 x g for 30 min). Immunoprecipitations were carried out by addition of 4 µg RAHIgM to the lysates and 8 µg to the medium (15 min at 20°C), followed by either 20 µg or 40 µg respectively of GARIgG (6 hr at 4°C). In the case of U698M, 0.2 µl R.410 anti-IgD was also added to the lysate and 0.4 µl to the medium, followed by addition of extra GARIgG (10 µg and 20 µg extra respectively).

Washed immunoprecipitates were dissolved in sample buffer for discontinuous SDS-PAGE in a 12.5% polyacrylamide slab gel (Methods, 11). The gel was processed for autoradiography (Methods, 17.3) and the autoradiogram developed after an exposure of 8 hr. The mobilities of the µ and light chains from the various cell lines are indicated by the arrows and brackets.

Odd Numbered Tracks: Intracellular IgM

Even Numbered Tracks: Secreted IgM


BJAB
BRI-8
DAUDI
NAMALWA
RAJI
TAY-3
U698M
1788
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
(-)
(+)
Migration in Gel
μ
L

as expected. The labelled intracellular IgM in these cells was consistently 0.2 to 1% of the total labelled cellular proteins - consistently the lowest value found among the cell lines investigated (see Table 1.2).

1.5.3. Biosynthesis and Secretion of IgM in the Lymphoma Cell Lines BJAB, Namalwa, and U698M

The three lymphoma cell lines - BJAB, Namalwa, and U698M - which express high levels of surface IgM (and sIgD in the case of U698M), were also found to synthesize and secrete moderate amounts of IgM (see Table 1.2). Figure 1.5 shows the SDS-PAGE profiles of IgM immunoprecipitated from the culture medium of these cell lines. Of the three, BJAB (track 2) shows the lowest level of secretion (just visible in the figure). This observation is reflected upon quantitative analysis as well. Thus, in several experiments, after a 4-6 hr incubation in complete medium (following a 1 hr pulse labelling) only 10 to 25% of the labelled IgM from BJAB cells was secreted. In contrast, both Namalwa and U698M cells were generally found to secrete 25 to 50% of their labelled IgM under the same conditions.

The intracellular anti-IgM precipitates from these cell lines often revealed two bands of μ mobility on SDS-PAGE - a phenomenon apparently related to the large quantities of surface IgM expressed. Thus, track 1 clearly shows two bands of μ mobility in the BJAB cell lysate, the slower band migrating with the surface μ-chains of Daudi and Raji cells and the faster band migrating with the intracellular μ-chains from the lymphoblastoid cells, i.e. Bri-8 (see Chapter 2 for detailed discussion). An equivalent, weakly labelled μ band of slower mobility

TABLE 1.2 Summary of Secretion and Surface Deposition of IgM by Lymphoma and Lymphoblastoid Cells

Cell Line	IgM Synthesis (% of Total Protein)	Secretion*	Surface** Deposition
Lymphoma Cells			
Daudi	0.25 - 1%	-	+++
Raji	0.25 - 1%	-	+-
BJAB	0.5 - 2%	+	+++
Namalwa	2 - 3%	++	+
U698M	2 - 3%	++	+
Lymphoblastoid Cells			
Bri-8	5 - 10%	+++	+-
Tay-3	5 - 10%	+++	+-
1788	5 - 10%	+++	+-

**Surface Deposition

(+++) 3-5% of Total Surface Proteins

(+) 1% of Total Surface Proteins

(+-) 0.1-1% of Total Surface Proteins

*Secretion

(+) 19S IgM in medium, 10-25% secreted after 5 hr chase

(++) 19S IgM in medium, 25-50% secreted after 5 hr chase

(+++) 19S IgM in medium, 50-90% secreted after 5 hr chase

can also be seen, although barely, in the Namalwa (track 7) and U698M (track 13) profiles. In addition a weak band corresponding in mobility to δ-chain can just be detected in the profile from U698M cell lysate (track 13), which was also immunoprecipitated with anti-IgD.

1.5.4. Biosynthesis and Secretion of IgM in the Lymphoblastoid Cell Lines Bri-8, Tay-3, and 1788

The three lymphoblastoid cell lines - Bri-8, Tay-3, and 1788 - which were found to express extremely low levels of surface IgM, were generally the most active in the synthesis and secretion of IgM. This is illustrated by the relatively intense μ bands in the SDS-PAGE profiles (Bri-8, tracks 3 & 4; Tay-3, tracks 11 & 12; 1788, tracks 15 & 16) of immunoprecipitates from both cell lysates and culture media.

Quantitatively, 50 to 90% of the labelled IgM in these cells was generally secreted during 4-6 hr labelling periods (see Table 1.2). In some cases, however, (for example see tracks 11 & 12) the secretion was found to be anomalously low, which may reflect the growth phase or general health of the cell cultures at the time of labelling.

1.5.5. Conclusions

In general, the investigations of IgM biosynthesis and secretion indicated that the lymphoid cell lines studied could be divided with respect to IgM expression into either (i) nonsecretors or (ii), secretors. This distinction between nonsecretors and secretors was found to be strict, whereas the secretory cell lines varied continuously from low to high rate IgM synthesis and secretion. The lymphoma-derived cell lines, all of which express high levels of

surface IgM, except Raji (Fig. 1.3), were either nonsecretors or low rate secretors. The lymphoblastoid cell lines, all of which were found to have very low levels of surface IgM (data not shown), were only secretors (generally high rate). Moreover the overall level of IgM synthesis, expressed as a percentage of total labelled cellular proteins increased steadily with the progression from nonsecretors to high level secretors. The relevant information is summarized in Table 1.2.

1.5.6. Molecular Size of Secretory IgM Investigated

To establish whether the IgM isolated from the culture medium was of the normal 19S secretory form, SDS-PAGE analysis of the unreduced molecules was performed. Figure 1.6 shows the results from three of the lymphoblastoid cell lines (Bri-8; Tay-3; 1788) and one of the lymphoma-derived cell lines (BJAB). The major peak of radioactivity in each case migrated with the 19S IgM marker protein (indicated by arrow). In addition, small peaks of approximately 8S were resolved, consistent with the findings of Kennel & Lerner (1973), and probably indicative of either turnover of surface IgM (in the case of BJAB), or leakage of unpolymerised IgM from ruptured cells. This latter explanation would appear the most likely in the case of the lymphoblastoid cells, because of their negligible levels of sIgM. It was not possible, however, to distinguish between these possibilities on the basis of the available data.

1.6. SYNTHESIS, SURFACE DEPOSITION, AND SECRETION OF IgG BY BEC-11 LYMPHOBLASTOID CELLS

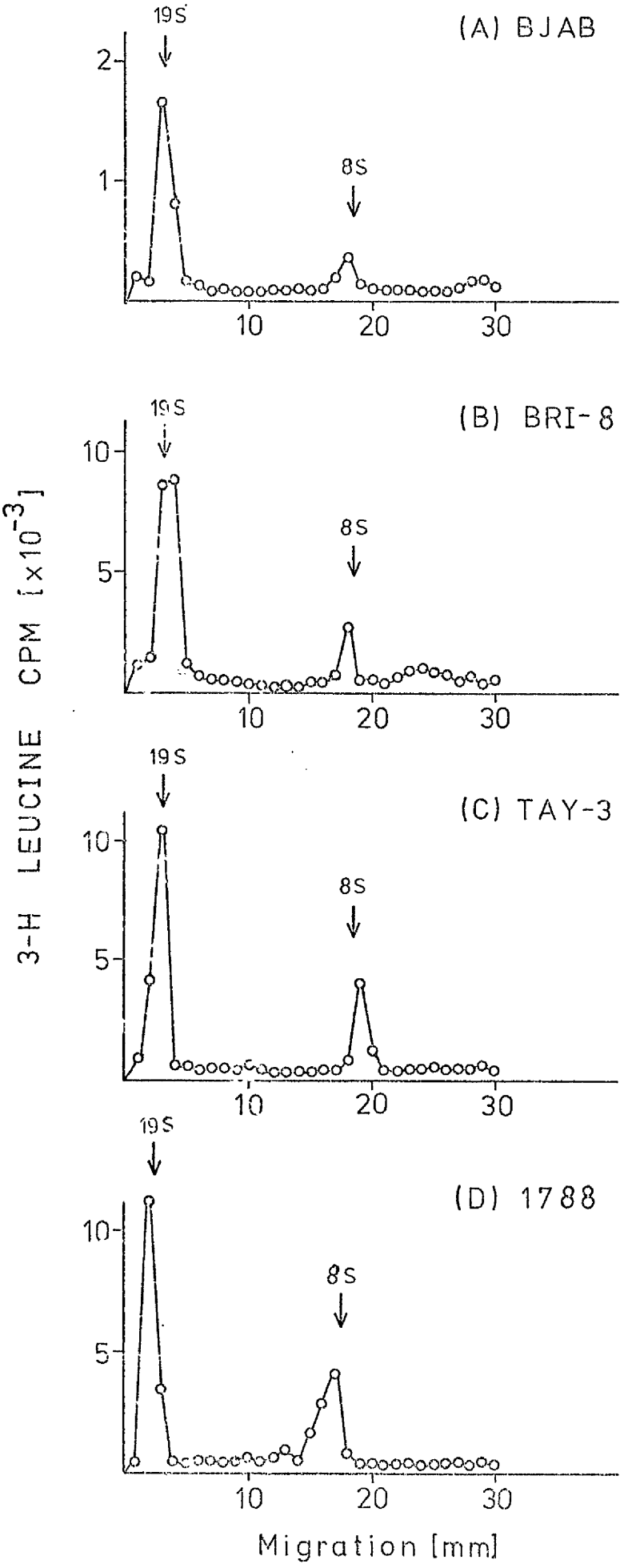
1.6.1. Early SDS-PAGE Analysis

In a screening experiment designed to determine the surface and

Fig. 1.6

SDS-PAGE Analysis Showing Secreted IgM to be 19S

The anti-IgM immunoprecipitates from the labelling media of several cell lines were obtained as described in the legend to Fig. 1.5. Aliquots (10%) of each immunoprecipitate were added to non-reducing SDS-PAGE sample buffer and electrophoresed in phosphate-buffered tube gels of 3% polyacrylamide-0.5% agarose. The radioactivity in individual gel slices was determined by scintillation counting of the Protosol-solubilised slices (Methods, 17.2). Marker 19S IgM and 7S IgG (radioiodinated as described in Methods, 6.5) were electrophoresed in a separate tube gel.


(A) BJAB
19S
8S
2
1
10
20
30
(B) BRI-8
19S
8S
10
5
10
20
30
(C) TAY-3
19S
8S
10
5
10
20
30
(D) 1788
19S
8S
10
5
10
20
30
3-H LEUCINE CPM [x10^{-3}]
Migration [mm]

secretory Ig expression of several cell lines, Bec-11 cells were either surface radioiodinated or biosynthetically labelled for 6 hrs with 3H-leucine. Labelled Ig was isolated from the cell lysate and culture medium (biosynthetic labelling) or from the cell lysate alone (surface radioiodination) by immunoprecipitation using a mixture of anti-IgG and anti-IgM antiserum. The SDS-PAGE profiles of the reduced immunoprecipitates are shown in Fig. 1.7.

Bec-11 cells had been previously characterised as IgG-secreting (F. Cramer, personal communication) and the biosynthesis results shown in Fig. 1.7(A) and (B) confirmed this. Thus, the SDS-PAGE profile of Bec-11 secreted Ig (Fig. 1.7(B)) shows a broad, symmetrical peak comigrating with the γ marker protein (indicated by arrow), as well as a light chain peak. Intracellularly (Fig. 1.7(A)) the γ and light chain peaks are also clearly seen, although the γ peak has a distinct shoulder, suggesting the presence of another peak of slower mobility.

The profile obtained from the Bec-11 surface radioiodinated Ig (Fig. 1.7(C)) had a predominant peak of radioactivity migrating approximately midway between the γ and μ markers. This SDS-PAGE mobility has been reported by several laboratories (Ferrarini et al., 1976; Spiegelberg, 1977) to be characteristic of δ-chain from human cell surface IgD. Thus, the detection of sIgD on Bec-11 cells by antiserum cross-reactivity was an attractive hypothesis to explain the foregoing result.

1.6.2. Identification of Surface IgG on Bec-11 Cells

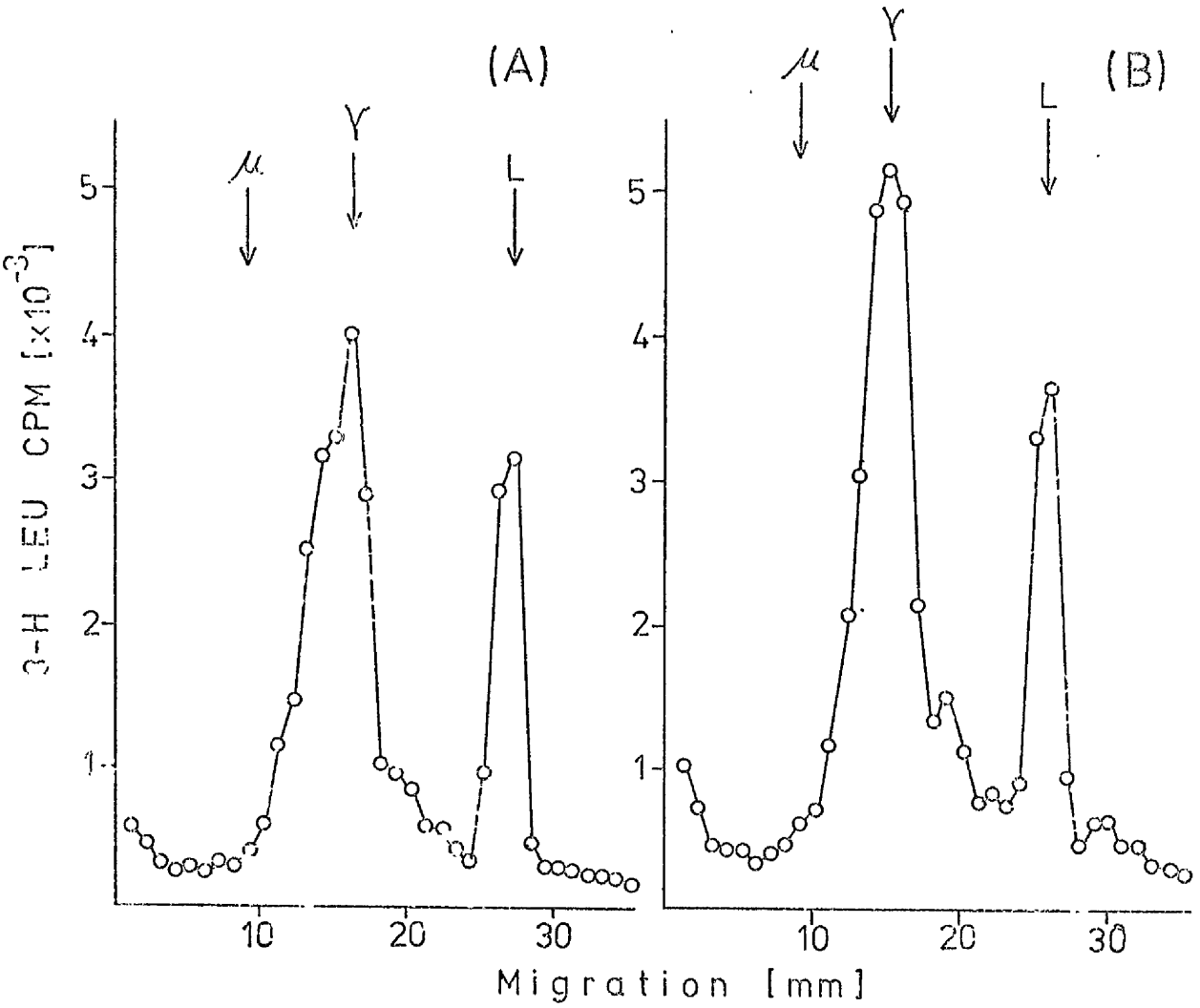
As shown in Fig. 1.7(C), cell surface Ig heavy and light chains were detected on Bec-11 cells by immunoprecipitation with anti-IgG plus anti-IgM antiserum. The major heavy chain species detected had a

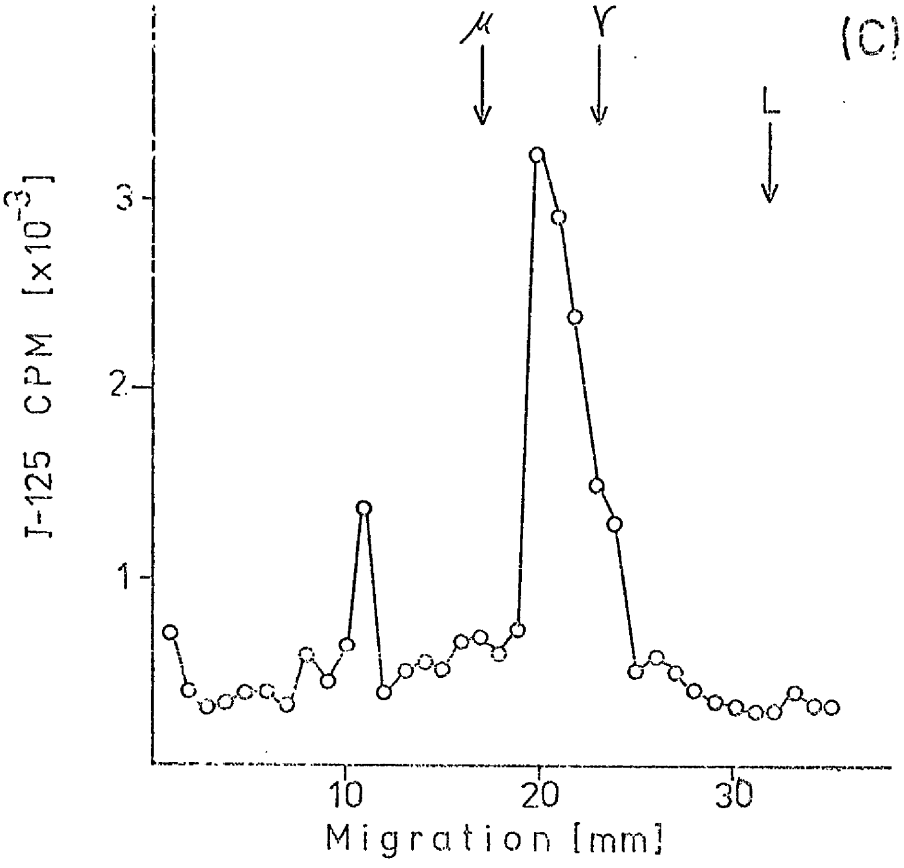
Fig. 1.7

SDS-PAGE Profiles of Intracellular, Secreted, and Cell Surface Ig of Bec-11 Cells

For biosynthetic labelling, 5 x 10^6 cells from log phase cultures were incubated for 6 hr in 1 ml labelling medium containing 62.5 µCi 3H-leucine (Methods 8.1). The cells were separated from the culture medium and lysed in 1 ml NP-40 lysis buffer containing PMSF and iodoacetate (Methods 8.3). Immunoprecipitations from both lysate and medium were carried out by addition of 2 µl each anti-IgG and anti-IgM (15 min at 4°C) followed by 200 µg GARIgG (15 min at 4°C). Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-125, 0.25 mCi). Cell lysis and immunoprecipitation were carried out as described above.
Washed immunoprecipitates were dissolved in 75 µl SDS-PAGE sample buffer and half removed for reduction and alkylation (Methods 12.4). Electrophoresis was in phosphate buffered tube gels of either 10% polyacrylamide (DATD crosslinked) for the biosynthetic samples, or 6% polyacrylamide for the radioiodinated sample (Methods 12). Radioactivity in individual gel slices was determined for biosynthetic samples by scintillation counting of periodic acid-solubilised slices (Methods 17.2), or for radioiodinated samples by gamma counting. Molecular weight marker µ, γ, and light chains were electrophoresed in separate 6% or 10% polyacrylamide tube gels.

(A) Intracellular Ig (biosynthetic)
(B) Secreted Ig (biosynthetic)
(C) Cell Surface Ig (radioiodinated)


(A)
(B)
μ
γ
L
3-H LEU CPM [x10⁻³]
1
2
3
4
5
10
20
30
Migration [mm]


(C)
μ
γ
L
I-125 CPM [x10⁻³]
1
2
3
10
20
30
Migration [mm]

mobility approximately equal to that reported for human δ-chains, and in further experiments was also shown to be precipitable by anti-IgD antiserum (data not shown).

To determine the heavy chain class of the Bec-11 sIg, the following experiment was performed. Bec-11 cells were surface radioiodinated and the NP-40 lysate divided into aliquots for immunoprecipitation with polyvalent anti-Ig (including anti-IgD). Before addition of antisera, however, separate aliquots were treated by addition of excess unlabelled IgA, IgM and IgD (1); IgD alone (2); IgA, IgM and IgG (3) or IgG alone (4). The SDS-PAGE profiles of the resulting reduced immunoprecipitates are shown in Fig. 1.8, tracks 1-4. Excess unlabelled IgD alone (track 2), or in combination with IgA and IgM, (track 1) did not compete successfully with the Bec-11 labelled sIg; whereas IgG alone (track 4) or in combination with IgA and IgM, (track 3) did compete successfully, and therefore it was concluded that the surface Ig detected on Bec-11 cells was IgG.

1.6.3. Comparison of SDS-PAGE Mobilities of Bec-11 Surface and Secretory IgG γ-chains

As indicated in Table 1.2, when Bec-11 cells were labelled biosynthetically generally 2-5% of the total labelled cellular proteins were IgG. Moreover, after 4-6 hr labelling periods, 50-90% of this IgG was generally found secreted into the culture medium. High resolution SDS-PAGE analysis of Bec-11 secretory IgG (Fig. 1.8, track 5) showed it to consist of two molecular forms of γ-chain, as well as λ-light chain. That both of the observed heavy chain bands were indeed γ-chain was confirmed by antigen competition experiments similar to those described in the previous section. Thus excess

Fig. 1.8

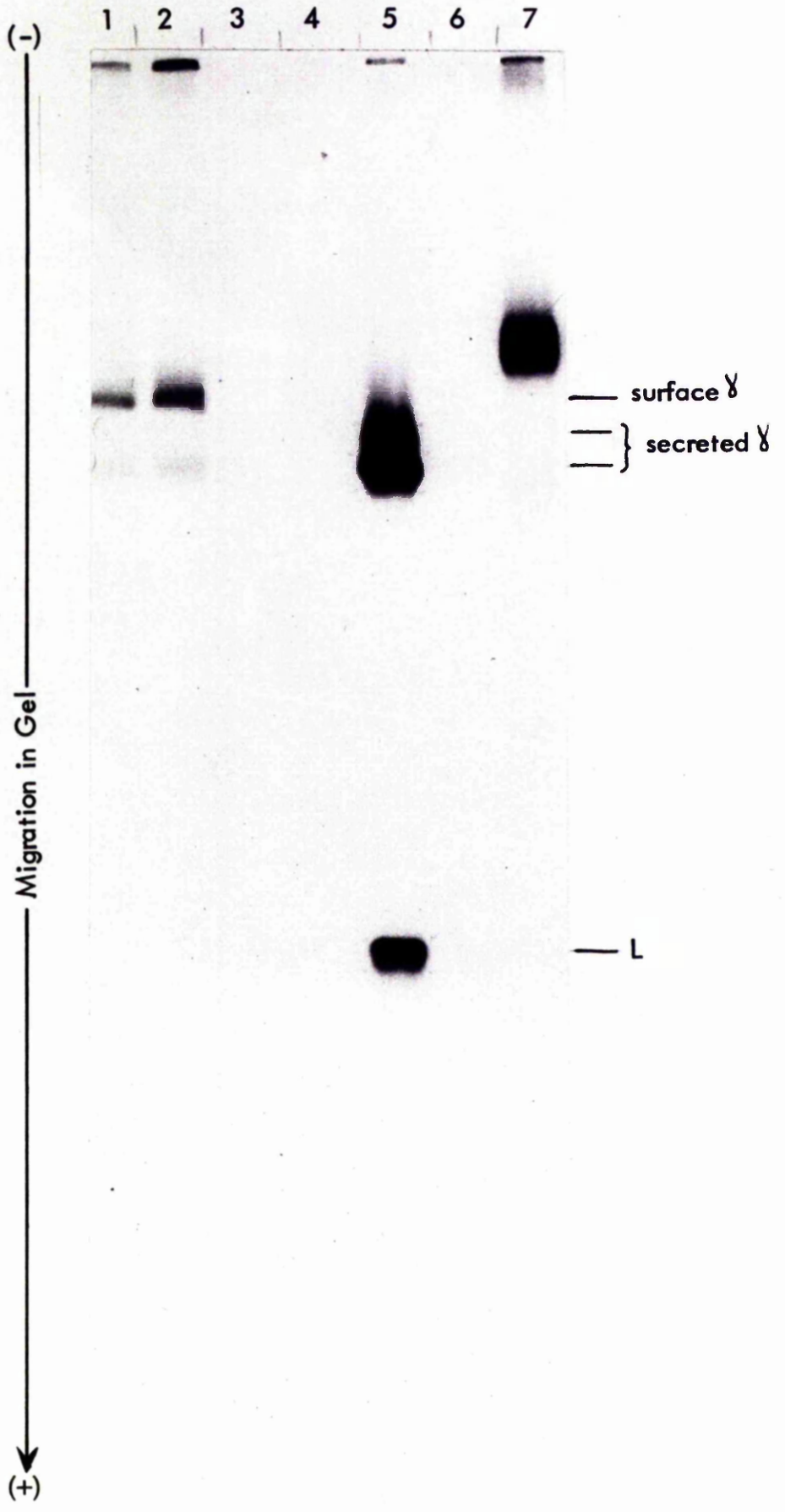
SDS-PAGE Analysis Identifying Bec-11 Cell Surface IgG, and Comparing its Mobility to that of Secreted IgG

The cell lysate obtained as described in the legend to Fig. 1.3 was used for the isolation of iodinated cell surface Ig. The remaining lysate (40%) was divided into four aliquots (50 µl each) and immunoprecipitation carried out in the presence of competing unlabelled antigen by addition of either (1) 10 µg each IgAκ, IgMλ and IgDλ ; (2) 10 µg IgDλ; (3) 10 µg each IgAκ, IgMλ and IgGλ ; or (4) 10 µg IgGλ - followed in each case by 1 µl of combined anti-IgM, IgG, IgA, IgD. Incubation for 15 min at 20°C was followed by addition of 50 µg GARIgG (overnight at 4°C).

For the isolation of biosynthetically labelled secreted Ig, 1 x 10^6 cells were incubated for 1.5 hr in 0.2 ml labelling medium containing 200 µCi 35S-methionine. Complete medium (1 ml) was then added and a further 18 hr incubation allowed (Methods, 8.1). The cells were separated from the culture medium, and the medium centrifuged to remove residual cells (500 x g for 10 min) and debris (30,000 x g for 30 min). Immunoprecipitation was carried out on aliquots (25%) of medium by addition of either (5) 5 µg RAHIgG or (6) 100 µg IgGλ followed by 5 µg RAHIgG. Incubation for 15 min at 20°C was followed by addition of 25 µg GARIgG (overnight at 4°C).

Washed immunoprecipitates were dissolved in sample buffer for discontinuous SDS-PAGE in a 12.5% polyacrylamide slab gel (Methods, 11). The gel was processed for fluorography (Methods, 17.4), and the fluorogram developed after an exposure of 24 hr. The mobilities of δ-chain from radioiodinated AI IgD, normal cell surface µ-chains (bands not shown), and Bec-11 light chain are indicated by the arrows.

Surface Iodinated Samples	Tracks
	(1) Anti-Ig(Polyvalent), Competed by IgA,IgM,IgD
	(2) " " " " IgD
	(3) " " " " IgA,IgM,IgG
	(4) " " " " IgG
Biosynthetic Samples	Tracks
	(5) RAHIgG Precipitate, Uncompeted
	(6) RAHIgG Precipitate, Competed by IgG
Markers	Track
	(7) AI serum IgD


1
2
3
4
5
6
7
(-)
Migration in Gel
(+)
surface
secreted
L

unlabelled IgG added to the cell lysate before immunoprecipitation with anti-IgG selectively eliminated both heavy chain bands and the λ band from the SDS-PAGE profile (Fig. 1.8, track 6). In this regard, neither IgD, IgM, or IgA myeloma proteins were able to compete with the Bec-11 secretory immunoglobulin in immunoprecipitations using anti-IgG (data not shown). The nature of the two forms of Bec-11 γ-chain is currently under further investigation.

Despite the unusual banding pattern of Bec-11 secretory IgG γ-chains, a comparison of the SDS-PAGE profiles in Fig. 1.8 clearly shows the surface γ-chain to be of higher apparent molecular weight than either of the secretory forms. Moreover, when biosynthetically labelled intracellular IgG from Bec-11 cells was analysed (data not shown), a γ band corresponding to the surface form, as well as those corresponding to the two secretory forms could be demonstrated.

1.7. CONCLUDING REMARKS

Considerable interest is attached to the biochemical events which underly B-cell maturation. Among these events are: 1) the mode of integration of H-chain V and C region genes, and their expression during isotype diversification; 2) the mode of surface binding and signal transmission of the several receptor isotypes; 3) the pathways which regulate the expression of cell surface bound and secreted Ig; and 4) the mechanisms involved in amplification of Ig synthesis. Many of these events take place at the level of the B-lymphocyte, and efforts are being made to investigate them in homogeneous B-lymphocyte model systems.

Spontaneously derived murine B-lymphomas (Shevach et al., 1972;

Ramasamy & Munro, 1974; Kim et al., 1978), or B-lymphomas induced by Abelson virus transformation (Premkumar et al., 1975b) have been identified and partially characterised. In contrast to the situation with fresh splenic or peripheral blood lymphocytes, these lymphoma cell lines are not readily stimulated to maturation by antigen or mitogen. This makes biochemical analysis of maturation-associated events difficult. Recently, two new approaches have been described: 1) the use of mitogen plus T-cells to induce in vitro maturation of human lymphoma and lymphoblastoid cell lines (Kishimoto et al., 1978), and 2) hybridisation of murine lymphoma and myeloma cells in order to stimulate the maturation of lymphoma cell-associated processes, such as IgM expression (Laskov et al., 1978).

A third approach is the use of differentiation-arrested lymphoma cell lines. Because of their inherently stable phenotypic expression, such continuous culture cell lines are ideally suited for biochemical investigations — particularly those concerned in the first instance with molecular structure. The work described in this section was undertaken to characterise several human lymphoma cell lines with respect to Ig synthesis. It was hoped that the cell lines available would be representative of a series of early stages in B-lymphocyte differentiation analogous to the situation described for murine myeloma tumours (Andersson et al., 1974). The characteristics of the individual cell lines were found to mirror those described for B-lymphocytes in ontogeny and during mitogen stimulation (see Introduction, section 5).

A general pattern in the relative synthesis of cell surface and secretory IgM emerged from a comparison of the human cell lines. Among the lymphoma cell lines, several strong producers of surface IgM were

identified (i.e. Daudi, BJAB, Namalwa and U698M). Daudi was found to combine strong IgM expression with no detectable secretion, confirming the findings of others. This result emphasised the usefulness of Daudi cells for the investigation of sIgM-associated questions. The other lymphoma cell lines (excluding Raji) exhibited strong sIgM expression with moderate secretion of 19S IgM. Cells such as these are analogous to those which would be produced in the very early stages of mitogen-induced maturation, at the onset of active secretion. Thus, using these cell lines it may be possible to study the events which accompany the onset of polymerisation and active secretion, as well as the factors which affect the relative amounts of Ig expressed in the two pathways. In such studies, the lymphoblastoid cell lines (such as Bri-8, Tay-3, RPMI 1788) would represent a further differentiated cell type, since their expression of secretory IgM with little or no sIgM is characteristic of the stage of differentiation seen in later mitogen stimulated cultures.

As a corollary to the investigation of sIgM-secretory IgM coordination, further study of Raji cells may prove useful. These cells synthesise significant amounts of cytoplasmic IgM, yet they secrete no detectable IgM and appear to deposit only very little in the surface membrane. According to present concepts of secretion, one might postulate that Raji cells are deficient in membrane fusion and reverse pinocytosis of post-Golgi vessicles. In their Ig expression, Raji cells are similar to the 'pre-B' cells which have been described. It is fascinating to speculate that the pre-B cell to primary B cell differentiation step may be marked principally by acquisition of the mechanisms for secretion.

Although even a moderate level of secretion was found to mask the biochemical characterisation of sIgM-associated phenomena (such as the resolution of a higher molecular weight surface μ-chain), one particular cell line, BJAB, exhibited a more balanced expression, which would allow analysis of both these pathways in a single cell. Such an investigation could focus on the intracellular transport of surface versus secretory IgM where the two are distinguishable (i.e. by the size of the μ-chain). One important question would be whether sIgM and secretory IgM are compartmentalised independently or together during transport to the cell surface.

Unfortunately, no double-secreting cell lines could be reliably identified in these studies. However, the biochemical identification of an $sIgM^{+ve}$, $sIgD^{+ve}$ lymphoma cell line (U698M) presents equally exciting prospects for biochemical analysis. Since $sIgM^{+ve}$, $sIgD^{+ve}$ B-lymphocytes are common *in vivo*, it may be expected that B-lymphoma cell lines possessing this characteristic will be reported frequently in the future. Such reports have already been made for a human cell line (Spiegelberg *et al.*, 1977) and a mouse lymphoma cell line (Laskov *et al.*, 1978). The characteristics of sIgD acquisition during ontogeny, as well as its susceptibility to proteolysis, indicate a special role for this receptor isotype. Such a role may well be determined in some way by the mode of binding of sIgD to the lymphocyte membrane, and in this regard it would be valuable to compare the binding of sIgD and sIgM in a single cell line (i.e. are both or either integral membrane proteins).

The characterisation of a lymphoblastoid cell line (Bec-11) which appears to both secrete and deposit in the surface membrane IgG,

underlines another possibility which derives from the use of continuous culture cell lines. The simultaneous expression of receptor and secretory IgG has been characterised both in ontogeny and in mitogen stimulated cultures (see Introduction, section 5), but it is a relatively rare event. As such, the biochemistry of the cells involved would be extremely refractory to analysis. The availability of a cell line apparently arrested in this stage of differentiation, however, allows this problem to be circumvented. Similar to the situation with IgM synthesising cells, questions concerning the membrane binding of sIgG and coordination of secretory IgG and sIgG synthesis can be asked using Bec-11 cells. The finding described in this section that Bec-11 surface γ-chains are of higher apparent molecular weight than secretory γ-chains is consistent with the idea that surface IgG is an integral membrane protein with a C-terminal hydrophobic extra sequence inserted into the membrane.

CHAPTER II

STRUCTURAL CHARACTERISTICS AND BIOSYNTHESIS OF CELL SURFACE IgM

2.1. MOLECULAR SIZES OF CELL SURFACE AND SECRETORY μ-CHAINS

2.1.1. General Observations

During the course of investigations of the synthesis, secretion, and surface deposition of IgM by cultured human lymphoid cells, two particular features of the SDS-PAGE profiles on polyacrylamide slab gels were observed. Firstly, the small molecular weight difference between intracellular and secreted μ-chains, presumably caused by addition of the terminal carbohydrate residues of fucose and sialic acid to the latter (Swenson & Kern, 1968; Schenkein & Uhr, 1970; Zagury et al., 1970; Parkhouse & Melchers, 1971), could be consistently resolved. Secondly, the surface μ-chains labelled by lactoperoxidase radioiodination had consistently slower SDS-PAGE mobilities than either the intracellular or secreted μ-chains from secretory cells. These observations led me to perform a more detailed investigation of the SDS-PAGE mobilities of μ-chains from several nonsecretory and secretory cell lines.

2.1.2 Biosynthetic IgM samples from secretory and nonsecretory cells

Surface, intracellular and secreted IgMs of several secretory and nonsecretory lymphoid cells were biosynthetically radiolabelled. Incorporation of 35S-methionine was allowed to proceed for 1 hour in labelling medium, followed by a further 5 hours in complete medium. After the labelling period, IgM was isolated from both cell lysates and culture media by immunoprecipitation. A pattern of biosynthetic labelling emerged which was consistent with the identification of the labelled IgM as either surface, intracellular, or secretory.

Table 2.1 shows that the two nonsecretory cell lines, Raji and Daudi, synthesized less than 0.5% of their total cellular proteins as IgM (nonspecific background radioactivity corrected for). Moreover, after the 5 hour chase, almost all of the synthesized IgM was retained within the cell. This is consistent in the case of Daudi cells, with reports from other laboratories (Klein et al.,1968; Sherr & Uhr, 1971), and presumably reflects the stable association of IgM with the outer cell membrane and the lack of an active secretion mechanism in Daudi and Raji cells. The two secretory cell lines investigated, Tay-3 and 1788, synthesized considerably more of their total cellular proteins as IgM (2.5% and 5.2% respectively). Much of this IgM is secreted into the culture medium during 5 hr chase. SDS-PAGE analysis of the unreduced extracellular IgM confirmed it to be the 19S secretory form (see Fig. 1.6).

2.1.3. Comparison of the SDS-PAGE mobilities of surface, intracellular (IC) and secretory (EC) IgM μ-chains

To more accurately compare the apparent molecular weights of μ-chains obtained from both secretory and nonsecretory lymphoid cells, surface radioiodinated and biosynthetically labelled samples of IgM isolated by immunoprecipitation were reduced and analysed by SDS-PAGE on adjacent tracks of a 12.5% polyacrylamide slab gel.

As a control, to show that the radioiodination itself did not affect the mobility of the μ-chains, biosynthetically labelled Daudi cells were subjected to a lactoperoxidase catalysed surface iodination using unlabelled iodine. When the biosynthetically labelled sIgMs from the iodinated and untreated Daudi cells were analysed on

TABLE 2.1 Biosynthesis and Turnover of Ig from Nonsecretory and Secretory Lymphoid Cells after Pulse-Chase Labelling

Cell Line	Ig Class	Level of Synthesis (a) (%)	Distribution of Ig after 5 hr chase (%)	
			Intracellular	Extracellular
BJAB	IgM	1.1	77	23
Daudi	IgM	0.4	93	7
Raji	IgM	0.3	99	1
Tay-3	IgM	2.5	49	51
1788	IgM	5.2	13	87
Bec-11	IgG	2.1	26	74

(a) 35S-methionine counts/min relative to total labelled cellular proteins after 1 hour pulse labelling.

adjacent tracks of a polyacrylamide slab gel, the profiles obtained were indistinguishable (see Fig. 1.5, track(s) 5).

Figure 2.1 shows a comparison by SDS-PAGE of μ-chains from secretory and nonsecretory cells. Three forms of μ-chains are seen. Intracellular and secreted μ-chains from secretory cells and surface μ-chains from nonsecretory cells each migrate with distinctive apparent molecular weights (except Daudi). Surface μ-chains always appear larger than secreted μ-chains, which in turn appear larger than intracellular μ-chains (i.e. those from secretory cells).

Molecular size estimations of the above forms of μ-chain were made based on a semi-log plot of molecular weight versus migration. As standards, the α and σ chains of RNA polymerase (Coggins et al., 1977), immunoglobulin γ-chain, and actin were used. The apparent molecular weights were determined to be: surface μ, 85,000; secretory μ, 83,000; and intracellular μ, 81,000. Because of the uncertainty of molecular weight determinations of glycoproteins using SDS-PAGE mobilities (Melcher & Uhr, 1976) I consider these values to be useful for comparative purposes only. Thus, the surface μ-chains studied were approximately 2000 daltons larger than secretory μ-chains. This difference is in good agreement with that reported for murine splenic cell surface μ-chain versus murine secreted μ-chain (Melcher & Uhr, 1976).

2.1.4. Correlation of Biosynthesis and Surface Labelling Results

The detection of the various forms of μ-chain by SDS-PAGE, revealed a pattern characteristic of either nonsecretory or secretory cells.

Fig. 2.1

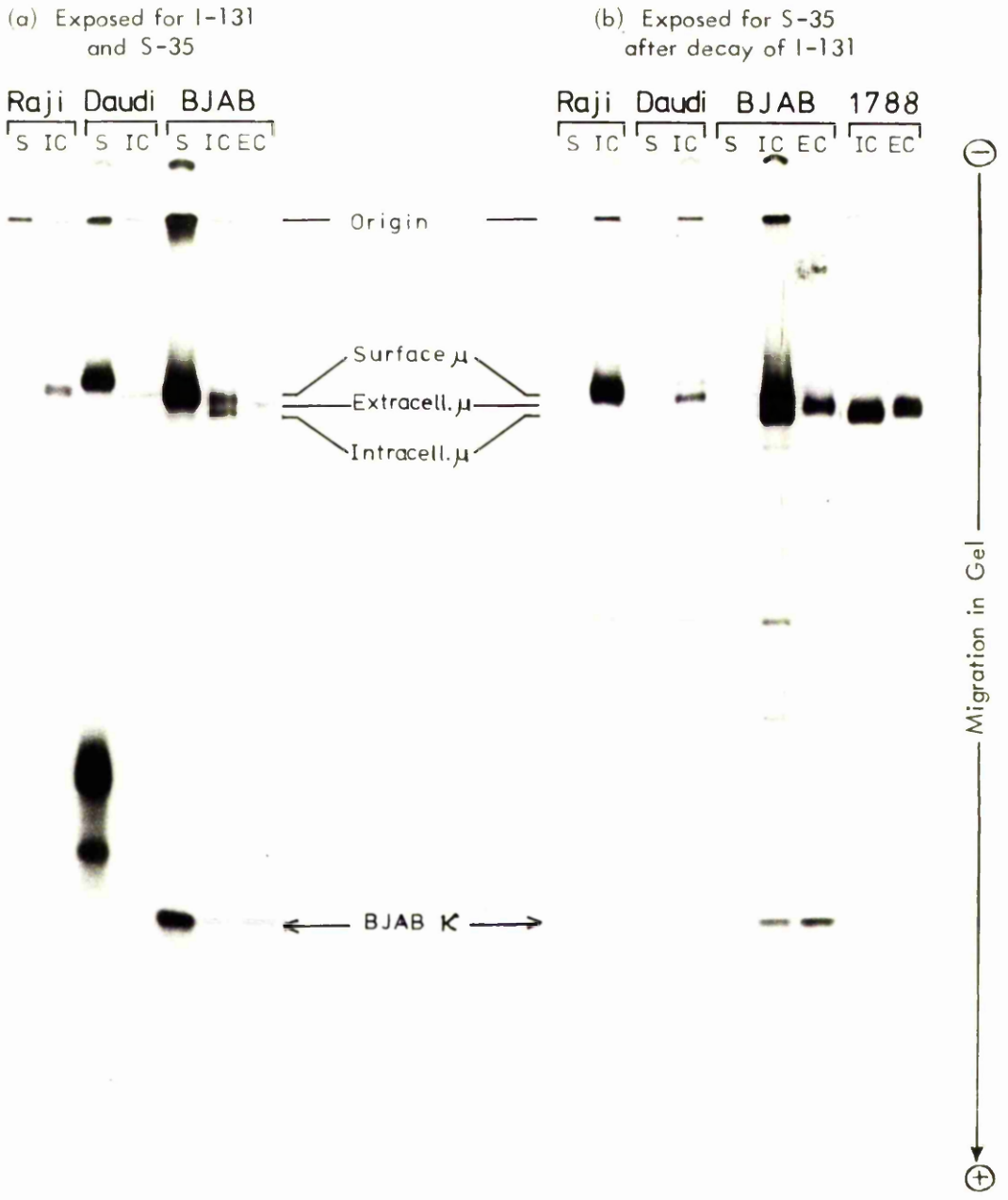
Comparison of the SDS-PAGE Mobilities of Cell Surface, Intracellular, and Secretory IgM μ-Chains

Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-131, 2 mCi) as described in Methods, section 8.2, and the cells lysed in 0.5 ml NP-40 lysis buffer containing PMSF (Methods, 8.3).

For biosynthetic labelling, 2 x 10^6 cells from log phase cultures were incubated for 1 hr in 0.4 ml labelling medium containing 100 μCi 35S-methionine. Half of the cells were removed and kept at 0°C. Complete medium (0.8 ml) was then added to the remaining cells and a further 5 hr incubation allowed (Methods, 8.1). The cells were separated from the pulse and chase media and lysed in 0.25 ml NP-40 lysis buffer as described above. The chase media was centrifuged to remove residual cells (500 x g for 10 min) and debris (30,000 x g for 30 min). Aliquots of pulse labelled cell lysate were removed for TCA precipitation.

Immunoprecipitations were carried out by addition of 20 μg RAHIgM to the radioiodinated lysates and biosynthetic chase media, and 5 μg RAHIgM to the biosynthetic pulse and chase lysates (15 min at 20°C), followed by 100 μg or 25 μg respectively of GARIgG (1 hr at 37°C, overnight at 4°C). Washed immunoprecipitates were dissolved in 200 μl 2% SDS and aliquots removed for determination of radioactivity (see Table 2.1). Aliquots of immunoprecipitate were applied in reducing sample buffer to a 12.5% polyacrylamide slab gel for discontinuous SDS-PAGE (Methods, 11). Biosynthetic IC and EC samples were derived from the 5 hr chase experiment. The gel was processed for fluorography and the fluorograms developed after a 2 hr exposure to film (a), and three months later after a 24 hr exposure to film (b). The 1788 tracks are not shown in panel (a).

(S) Surface Iodinated
(IC) Biosynthetic, Intracellular
(EC) Biosynthetic, Extracellular


(a) Exposed for I-131 and S-35
(b) Exposed for S-35 after decay of I-131
Raji
Daudi
BJAB
1788
S IC
S IC
S IC EC
S IC
S IC
S IC EC
IC EC
Origin
Surface μ
Extracell. μ
Intracell. μ
BJAB K
Migration in Gel
⊖
⊕

In nonsecretory cells, a band corresponding in mobility to the surface labelled μ-chain is detected by biosynthetic labelling. However, no band of the intracellular μ-chain size could be detected in those cells. In contrast, rapidly secreting cell lines such as 1788 show only μ-chain of the intracellular size which persisted even after a 5 hour chase. These cells did not show a band of the surface μ-chain size under the labelling conditions described. In agreement with this observation is the extremely low level of sIgM detected by lactoperoxidase radioiodination on rapidly secreting lymphoblastoid cells. Several lymphoblastoid cell lines under investigation in our laboratory conform to this pattern of IgM expression, among them Bri-8, Smi-1, Tay-3 and 1788. Thus, it appears that secretory cells retain only a small amount of their immunoglobulin product as the distinct surface immunoglobulin.

The anomalously slower mobility of Daudi sIgM has been mentioned above. Figure 2.1. shows that the biosynthetically labelled bands also reflect this anomaly, in that after the 5 hour chase period a faint band corresponding to the large surface iodinated form can be seen (see also Fig. 1.5). However, the major biosynthetically labelled band of Daudi μ comigrates with the normal surface forms from the other cell lines investigated. When a short pulse labelling is used, this latter band is the only μ-chain detected in the Daudi sIgM profile (see Figs. 2.3 and 2.4). Based on this observation I favour the hypothesis that the modification of Daudi surface μ-chains which confers an abnormally high apparent molecular weight on them is a secondary phenomenon, such as the addition of terminal sugar moieties additional to that added in the normal pathway for the expression of cell surface IgM. This phenomenon may be related to

the glycosylation of Daudi κ(see section 2.3.9) and to the presence of the novel p33 polypeptide associated with the sIgM of Daudi cells (see chapter 3).

2.1.5. Surface deposition and secretion of IgM by BJAB cells

The lymphoma cell line BJAB showed an unusual pattern of IgM synthesis, which proved useful to the present investigation. Table 2.1 shows that the synthesis of IgM in BJAB cells was intermediate in amount to that of the secretory and nonsecretory cells. In addition, a significant amount (23%) of the IgM synthesised was detected in the culture medium. This extracellular IgM was also found to be of the 19S secretory form (see Fig. 1.6). Since BJAB cells also have readily detectable surface IgM, they provide a useful model system for the biochemical analysis of surface deposition and secretion of IgM in a single cell line.

In BJAB cells the three forms of μ-chain distinguished as surface, intracellular and secretory, can be identified by their migration on SDS-PAGE with characteristic mobilities similar to their counterparts in other cell lines (Fig. 2.1). A pattern of IgM expression similar to that seen in BJAB is also found in two other lymphoma cell lines, Namalwa and U698M (see Figs. 1.3 and 1.5). The most striking difference observed between these lymphoma cells and the IgM-secreting lymphoblastoid cells such as Tay-3 and 1788 was the high level of sIgM expressed on the former cells contrasting with the barely detectable sIgM on the lymphoblastoid cells.

The lower portion of Fig. 2.1 shows the migration of BJAB κ-chain co-precipitated with the various forms of μ-chain. The κ-chains associated with intracellular secretory and surface (lactoperoxidase

radioiodinated) μ-chains were indistinguishable. This is in contrast to the situation with the μ-chains, and is evidence against structural differences of light chains regulating surface deposition or secretion of associated immunoglobulins.

2.2. CARBOXY-TERMINAL ANALYSIS OF CELL SURFACE AND SECRETORY μ-CHAINS

2.2.1 Rationale for Investigation

The previous findings that cell surface γ-chains (section 1.6.3) and μ-chains (section 2.1) are of higher apparent molecular weight than their secreted counterparts is consistent with current concepts of receptor Ig binding to the cell surface. Thus, it has been postulated (Singer, 1974; Vitetta & Uhr, 1975) that the basically hydrophilic Ig heavy chains may possess an extra hydrophobic sequence when they are sequestered in the membrane. The findings that murine surface μ-chains are of higher molecular weight than secreted μ-chains (Lisowska-Bernstein & Vassalli, 1975; Melcher & Uhr, 1976) supports this idea. In addition, the same relationship has been shown between cell surface IgD and myeloma IgD δ-chains (Spiegelberg, 1977). It has been further shown that murine sIgM μ-chains are less soluble in aqueous medium (Melcher et al., 1976) and bind more detergent (Melcher & Uhr, 1977) than μ-chains from serum IgM, consistent with an extra hydrophobic sequence in the surface form.

A widely favoured model of receptor Ig membrane binding places the extra hydrophobic sequence at the carboxy terminus of the surface

μ-chain, in order to orientate the receptor Ig with its antigen binding sites clearly exposed. Direct evidence for this model has been sought by carboxy-terminal analysis of surface μ-chains. The method used exploits the known presence of a C-terminal tyrosine residue on secretory μ-chains (Putnam et al., 1973), and the ability of the enzyme Carboxypeptidase-A to rapidly and completely remove C-terminal tyrosine (reviewed by Ambler, 1967). It is reasoned that surface μ-chains, if they possess an extra hydrophobic sequence at their C-terminal end, would not have a terminal tyrosine residue.

McIlhinney et al., (1977) have used the above approach to investigate the carboxy terminus of 3H-tyrosine labelled surface μ-chains from human (Bri-8 cell line) lymphoid cells and mouse (McPC 1748) myeloma cells. They reported significant release of tyrosine, and therefore argued against a C-terminal extra sequence. However, work already presented in this thesis on the Ig expression of Bri-8 cells (section 1.5.4) raises serious objections to these findings. Specifically, these authors have not convincingly demonstrated the μ-chains they investigated to be the surface form. In addition, their data does not provide kinetic evidence that the released tyrosines were indeed C-terminal (and not, for instance, preceded by one or more rapidly released amino acid).

In view of the criticisms of the above investigation, I felt it useful to perform a similar investigation of the surface μ-chains of Daudi and Raji lymphoma cells, and to compare the results obtained with those from the secretory μ-chains of 1788 and Bri-8 cells. One advantage of this system is that Daudi and Raji cells not only express surface IgM, but are also strictly non-secretory. Moreover,

the μ-chains from these cells can be distinguished from secretory μ-chains by their slower mobility on SDS-PAGE, thus providing additional evidence that they are indeed the surface form of μ-chain.

2.2.2. Source of Samples for Digestion

To obtain appropriately labelled μ-chains (and light chains) for C-terminal analysis, Daudi, Raji, 1788, and Bri-8 cells from log phase cultures were labelled by incorporation of 3H-tyrosine for 1 hr. The cells were separated from the labelling medium and lysed in NP-40 lysis buffer containing protease inhibitors. Labelled IgM was isolated from the cell lysates by immunoprecipitation, and the washed immunoprecipitates subjected to SDS-PAGE under reducing conditions in a 10% polyacrylamide slab gel. The gel was processed for fluorography and exposed to film, giving profiles similar to those shown in Fig. 2.3, tracks 8-10 (Bri-8, not shown, was identical to 1788). The fluorograph was then placed over the dried gel and the area of gel underlying each μ or light chain band carefully sliced out and the radiolabelled chains eluted (Methods, 15). Approximately 15-20,000 counts/min was recovered for each chain to be analysed, except 1788 μ, which yielded 60,000 counts/min and was therefore used in preliminary experiments to determine the kinetics of digestion.

2.2.3 Kinetics of Release of Carboxy-Terminal Tyrosine by Carboxypeptidase A

The secretory μ-chain from 1788 cells was expected to have a C-terminal tyrosine (Putnam et al., 1973) and so was used to establish the kinetics of release characteristic for tyrosine at the C-terminal position. A previous investigation of aldolase (Winstead & Wold,1964)

was particularly useful in this regard. This investigation clearly showed that a rapid time course and nearly 100% release of C-terminal tyrosine could be expected, even with comparatively mild digestion conditions.

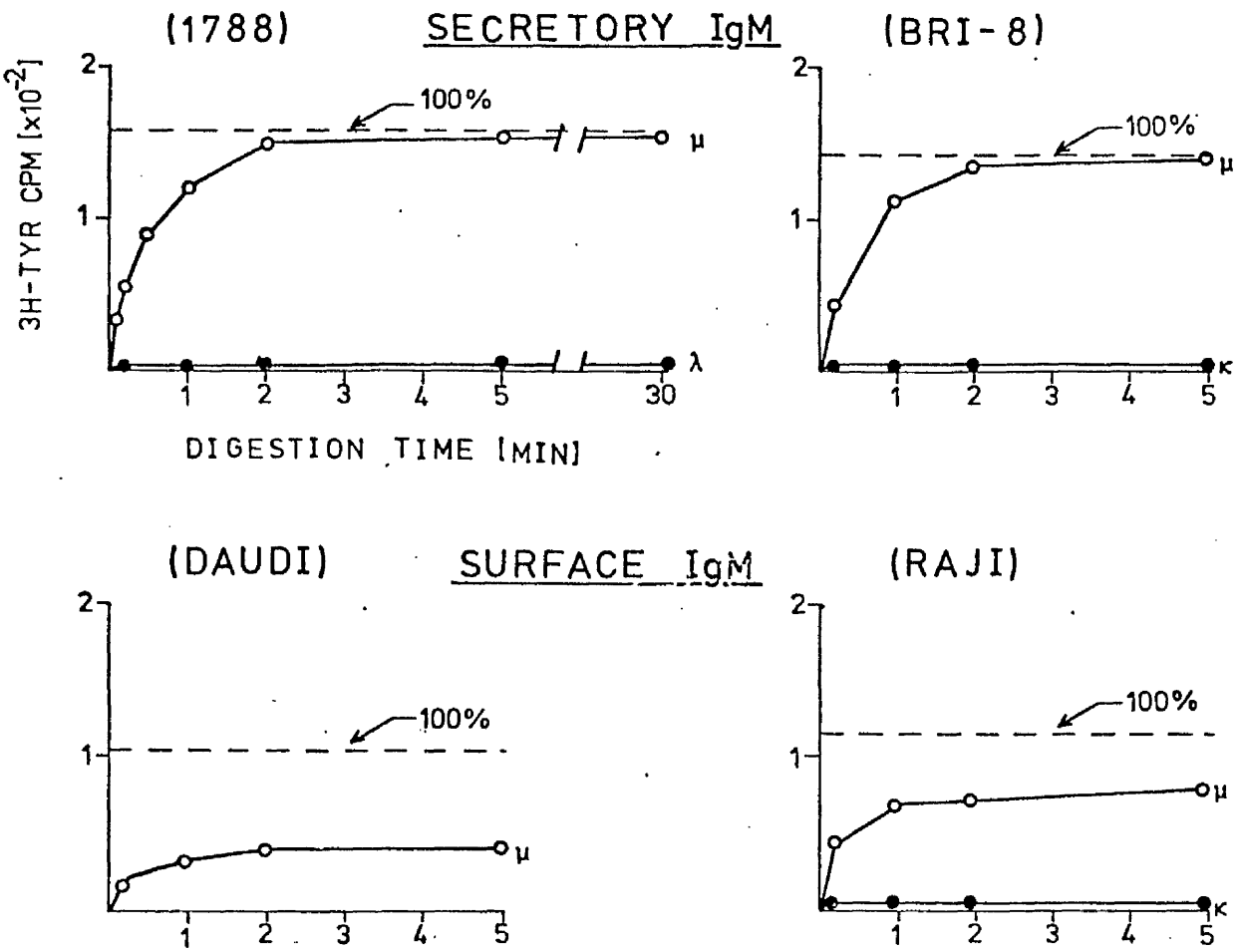
A preliminary digestion was performed on labelled 1788 μ-chain, with the parameters used in the investigation of aldolase (Winstead & Wold, 1964) as a guideline. Incubation was at 25°C, using an enzyme to substrate (carrier BSA) ratio of 1:100. The digestion was rapidly stopped at times of 0.2, 2, and 30 min by dilution of the reaction mixture into ice cold trichloroacetic acid, and the acid soluble counts/min determined. The results indicated detectable release of tyrosine after 0.2 min and maximum release after 2 or 30 min incubation. A second digestion was then performed with several additional time points. The counts/min released were consistent with those from the first digestion, and are plotted as a function of digestion time in Fig. 2.2 A. The figure shows that a plateau value was reached by 2 min, and that the release of C-terminal tyrosine approached 100%, based on a tyrosine content of 18 residues per 1788 μ-chain. This is a reasonable estimate of μ-chain tyrosine content since the μ constant region is known to contain 9 tyrosine residues (Putnam et al,, 1973) and, based on a sampling of 18 complete human V-region sequences from subgroups I, II and III (Kabat et al., 1977) μ-chains would be expected to vary in tyrosine content from 12 to 17 residues. On the other hand, taking all the known positions from partial and complete V-region sequences where tyrosine has been found (Kabat et al., 1977) a maximum figure of 23 tyrosine residues per μ-chain would be obtained.

Fig. 2.2

Release of Carboxy Terminal Tyrosine from Cell Surface and Secretory IgM μ-Chains by Carbodypeptidase Digestion

For biosynthetic labelling, 1 x 10^6 (1788, Bri-8) or 5 x 10^6 (Daudi, Raji) cells from log phase cultures were incubated for 1 hr in 0.2 ml or 1.0 ml of labelling medium containing 500 μCi/ml 3H-tyrosine. The cells were separated from the culture medium and lysed in 0.2 ml (1788, Bri-8) or 0.5 ml (Daudi, Raji) NP-40 lysis buffer containing PMSF and pepstatin-A (Methods, 8.3). Immunoprecipitates were carried out by addition of 10 μg (1788, Bri-8) or 25 μg (Daudi, Raji) RAHIgM (15 min at 20°C), followed by 40 μl or 100 μl respectively of S.aureus immunoadsorbent (overnight at 4°C). Washed immunoprecipitates were dissolved in reducing sample buffer for discontinuous SDS-PAGE in a 10% polyacrylamide slab gel (Methods, 11). The gel was processed for fluorography (Methods, 17.4) and exposed to film for 8 hrs, giving profiles similar to those shown in Fig. 2.3, tracks 8-10 (Bri-8 not shown).

The area of gel containing each μ and light chain band was sliced out and the radiolabelled chains were eluted, amidomethylated, and lyophilised as described in Methods, section 15. The lyophilised samples were then dissolved in Digestion Buffer (0.05 M $NaHCO_3$, 0.2 M LiCl), pH 8.0 - 8.2, and carboxypeptidase-A Digestion carried out at 20°C at an enzyme to substrate ratio of 1:100 (Methods, 16.3). Aliquots of the digestion mixture were removed at specified times and added to 1/3 volume of 40% ice cold TCA. After approximately 1 hr at 0°C, the TCA insoluble material was pelleted by centrifugation (8000 x g for 2 min). Released tyrosine was measured as TCA soluble radioactivity, and plotted versus digestion time. The hypothetical 100% release values represent 1/20th the total radioactivity per aliquot (except for 1788, where the released tyrosine approached 1/18th the total radioactivity).


(1788)
SECRETORY IgM
(BRI-8)
3H-TYR CPM [x10^{-2}]
100%
μ
λ
κ
DIGESTION TIME [MIN]
(DAUDI)
SURFACE IgM
(RAJI)

From the above experiment, the digestion conditions used were found to be satisfactory for following the kinetics of C-terminal tyrosine release from human μ-chains; the kinetics observed being consistent with those for aldolase (Winstead & Wold, 1964). It therefore seems appropriate to point out the possibility of misinterpreting digestion data which is not based on kinetic analysis. Thus, the previous investigation of aldolase clearly showed that substantial release of aromatic amino acids (i.e. tyrosine) several residues from the carboxy terminus occurs when the intervening amino acids are also rapidly released species (i.e. aromatic or hydrophobic).

2.2.4 Carboxypeptidase - A Digestion of Surface and Secretory μ-Chains

Having established the kinetics of release of C-terminal tyrosine from 1788 secretory μ-chains, Carboxypeptidase-A digestions were performed on Bri-8 secretory μ-chain and Raji and Daudi surface μ-chains. The results are shown in Fig. 2.2. B,C, and D.

Tyrosine was released from Bri-8 μ with kinetics and yield (100% release, based on 20 residues/chain) nearly identical to those observed with 1788 μ, consistent with the identification of both these μ-chains as secretory.

Digestion of Daudi and Raji surface μ-chains was expected to show either tyrosine release similar to that from 1788 and Bri-8 μ-chains, arguing against an extra C-terminal sequence; or alternatively, a lack of tyrosine release, arguing in favour of such an extra sequence. The results obtained were not completely consistent with either of these expectations. Tyrosine was released from the surface μ-chains with kinetics indicating a C-terminal location, but with a yield in

both cases significantly lower than expected. Figure 2.2 C and D shows the yields to be approximately 35% for Daudi and 70% for Raji μ-chains, based on the arbitrary but convenient figure of 20 tyrosine residues/chain. As evident from the preceding discussion, however, 20 tyrosine residues/chain is likely to be an overestimate. Therefore the yields determined above are, if anything, likely also to be overestimates.

2.2.5 Discussion of Surface μ-Chain Digestion Results

The digestion results described above and shown in Fig. 2.2 for both C-terminal tyrosine positive (1788 and Bri-8 μ-chains) and negative (κ and λ light chains) polypeptides provide strong evidence for the sensitivity and reproducibility of this experimental approach. In particular, the data argue strongly against nonuniform release of tyrosine, both with respect to kinetics and yield. Therefore, in considering interpretations of the results, I have assumed that the partial release of C-terminal tyrosine from the surface μ-chains is significant and must be explained.

2.2.5.1 Interpretations Against a Modified C-terminus

Several trivial explanations are possible for the low yields of released tyrosine from Daudi and Raji μ-chains, which would argue against a modified carboxy terminus. If the total radioactivity in these μ-chains was overestimated due to the presence of contaminating proteins, the data might be consistent with complete release of C-terminal tyrosine. However, the SDS-PAGE profiles (Fig. 2.3, tracks 8-10) showed no evidence of such contamination, especially in the case of Daudi μ-chain, which was better resolved and also contained more radioactivity relative to the background than Raji μ-chain, and

yet had a lower yield of released tyrosine. Another possible explanation for the apparent partial release of carboxy terminal tyrosine would be if the tyrosine content of the μ-chains was greatly underestimated. However, to conclude that there was 100% release on this basis would require 28 tyrosine residues in Raji and 57 in Daudi μ-chains - clearly inconsistent with the reported figures indicating a likely 12-17 residues/chain.

2.2.5.2 Interpretations Favouring a Modified C-terminus

In view of the above discussion, the digestion results from Daudi and Raji μ-chains appear to be most consistent with the existence of two forms of μ-chain. According to this interpretation, a form possessing a C-terminal tyrosine residue (putative secretory type μ-chain) would account for approximately 35% of Daudi and 70% of Raji μ-chains, while a form negative for C-terminal tyrosine (putative surface μ-chain) would account for the remainder in each case.

The presence of two forms of μ-chain in the Daudi and Raji cells would not be unexpected. Molecules with C-terminal tyrosine may reasonably be thought to arise in either the synthetic or degradative pathways of surface IgM expression by cleavage of a putative C-terminal extra sequence. Cleavage immediately following synthesis could divert newly synthesised μ-chains from surface expression, thereby regulating the amount of surface IgM on the cell. Such cleavage may in fact yield a μ-chain of similar structure to the secretory form; however, since Daudi and Raji cells do not secrete IgM, cleavage could not alone be sufficient for secretion.

Alternatively, cleavage of the putative extra sequence could be a signal for the degradation of the surface IgM molecule. However, the rapid labelling of C-terminal tyrosine containing μ-chains argues against this explanation.

2.2.5.3 Conclusion: Detection of a Modified C-terminus on a Portion of Daudi and Raji μ-Chains

According to the discussion outlined above, I believe these experiments provide strong evidence in favour of a modified C-terminus on a portion of Daudi and Raji μ-chains. This conclusion is in basic agreement with the recently reported findings from another laboratory using similar methods (Williams & Grey, 1978). These workers reported no C-terminal tyrosine but good yields of C-terminal valine upon carboxypeptidase digestion of Daudi μ-chains. With regard to the contradictory results of McIlhinney et al. (1977), I believe their experiments lacked the sensitivity to detect a modified C-terminus on putative surface μ-chains, especially if their preparations were contaminated with secretory type μ-chains.

Assuming that the above evidence for two forms of μ-chain in Daudi and Raji cells is correct, it remains problematic whether the forms detected are those of functional secretory μ-chains and surface μ-chains (i.e. those containing an extra hydrophobic C-terminal sequence). Two observations provide indirect evidence that this is indeed the case. First, only with the sIgM-expressing Daudi and Raji cells, and not with any of the IgM secreting cells investigated, can the clear presence of modified μ-chains be demonstrated. Second, the higher proportion of modified μ-chains from Daudi cells (70%, compared to 35% for Raji) is consistent with the greater ease of sIgM detection on Daudi cells. A more convincing demonstration that the forms detected are indeed the

putative surface and secretory μ-chains would ideally show their resolution by SDS-PAGE and, importantly, show that the form not contributing the C-terminal tyrosine is the higher molecular weight form. In this regard, a difference of about 20 amino acid residues would be expected; this being the approximate length of several hydrophobic membrane-associated sequences recently discovered, including Ig light chain precursor piece (Milstein et al., 1972; Mach et al., 1973; Burstein & Schechter, 1976), Ig heavy chain precursor piece (H. Singer & Williamson, in preparation), and the glycophorin membrane-associated region (Segrest et al., 1972). It has been demonstrated by comparative SDS-PAGE of nonglycosylated mouse 5563 γ_{2a} heavy chain and its cell-free synthesised precursor (H. Singer & Williamson, in preparation) that a difference of 19 amino acid residues, out of approximately 450, can be resolved.

Figure 2.3 (tracks 8-10) shows that the normal cellular μ-chains isolated from Daudi and Raji cells do not resolve into two bands. This observation is apparently contradictory to the evidence from the carboxypeptidase digestion, but can however be reconciled with the latter evidence in one of several ways. First, the molecular weight difference between the two forms of μ-chain may be too small to be resolved; but not necessarily too small to be consistent with a functional hydrophobic sequence on the surface μ-chains. Alternatively, variable glycosylation of the two forms of μ-chain may mask the molecular weight difference contributed by an extra hydrophobic sequence. SDS-PAGE analysis of nonglycosylated Daudi and Raji μ-chains synthesised in vivo in the presence of tunicamycin (see section 2.3.7) lends strong support to this latter explanation.

2.3 ANALYSIS OF NONGLYCOSYLATED CELL SURFACE AND SECRETORY μ-CHAINS

2.3.1 Rationale for Investigation

Carbohydrate constitutes approximately 15% of the molecular weight of human IgM, and is known to occur as either linear or branched structures attached at five sites on each μ-chain (Spragg & Clamp, 1969 Shimizu et al., 1971). The attachment of carbohydrate to μ-chains has long been thought to affect the physical properties and biological functions of IgM. Specifically, differences in the carbohydrate composition of cell surface and secretory IgM have been searched for to explain the unique cellular roles of these molecules (Melchers & Andersson, 1973). Although it is clear that glycosylation patterns alone are not sufficient to distinguish surface IgM from secretory IgM, subcellular transport of many newly synthesised proteins, including Ig, is characterised by a series of specific glycosylation events (Eylar, 1965; Melchers, 1973; Sherr & Uhr, 1971). In this way, glycosylation may indirectly determine the pathways which lead to either surface deposition or secretion of Ig. With regard to μ-chain structural differences which may adapt sIgM to the membrane environment, it has been convincingly argued on the basis of thermodynamic considerations (Singer, 1974) that carbohydrate differences alone are insufficient. However, indirect effects of carbohydrate, such as causing conformational changes in the sIgM molecule which would bring hydrophobic regions into association with the membrane, have been postulated (Vitetta & Uhr, 1975b).

The analysis of nonglycosylated μ-chains synthesised in vivo was undertaken primarily to further investigate the μ-chain structural differences previously shown to exist between surface associated and secretory IgM molecules. Three aspects were of particular interest. First, with the carbohydrate moiety removed, apparent molecular weight differences between the respective μ-chains (see section 2.1.3) could be localised to either the polypeptide portion or the carbohydrate portion of the molecules. Second, SDS-PAGE of nonglycosylated Daudi and Raji μ-chains may allow the resolution of two forms postulated from the carboxypeptidase digestion evidence, thereby testing the idea that unequal glycosylation of the two forms masks their resolution under normal circumstances (see section 2.2.5.3). Third, the processing of precursor segments (both putative N- and C-terminal) could be studied by comparative SDS-PAGE on nonglycosylated cellular chains and their cell-free synthesised counterparts (see sections 2.4.4 and 2.4.5).

In addition to the structural studies outlined above, possible effects of glycosylation in determining subcellular transport of IgM can be studied in tunicamycin-treated cells. Thus, it can be asked whether nonglycosylated μ-chains are secreted or deposited in the cell membrane with the same efficiency as normal μ-chains.

2.3.2 The Use of Tunicamycin to Inhibit Glycosylation

To obtain nonglycosylated μ-chains (and light chains) for SDS-PAGE analysis, the antibiotic tunicamycin was utilised. Tunicamycin has been shown to act by inhibiting the formation of N-acetyl-glucosamine lipid intermediates (Tkacz & Lampen, 1975; Bettinger & Young, 1975; Takatsuki et al., 1976; Lehle & Tanner, 1976) which serve as donors

for the synthesis of the core regions of N-glycosydically linked oligosaccharides (Waechter & Lennarz, 1976). Thus, treatment of cells with tunicamycin results in the synthesis of proteins deficient in asparagine linked oligosaccharides (Struck & Lennarz, 1977). Since all known μ-chain carbohydrate has been found to be asparagine linked (Shimizu et al., 1971; Putnam et al., 1973), treatment with tunicamycin should result in the synthesis of completely nonglycosylated μ-chains.

2.3.3 IgM Synthesis in Tunicamycin Treated Cells: General Effects

To investigate the biosynthesis of IgM in the presence of tunicamycin, cells were first preincubated with the antibiotic for either 2 hr or 5 hr (separate experiments). For these preincubations, tunicamycin to a final concentration of 0.5 μg/ml was added to aliquots of log phase cultures without prior harvesting of the cells. After the preincubation, the cells were harvested and biosynthetic labelling carried out (Methods, 8.1) using labelling and chase media which were also 0.5 μg/ml with respect to tunicamycin.

In one such biosynthesis experiment, Daudi, Raji, and 1788 cells were first preincubated with tunicamycin for 5 hr, after which time the cells were allowed to incorporate 35S-methionine for 1 hr. The Daudi and Raji cells were then kept at 2°C while the 1788 cells were incubated under chase conditions (Methods, 8.1) for a further 2 hr to allow for some secretion of labelled IgM to occur. NP-40 cell lysates were then prepared. Total protein synthesis was determined by TCA precipitation and IgM synthesis by immunoprecipitation, using excess RAHIgM and S. aureus immunoabsorbent to affect complete precipitation. The synthesis of total protein and of IgM in tunicamycin treated cells was compared to the synthesis in untreated cells which had been labelled similarly.

The results are summarised in Table 2.2.

Table 2.2 Synthesis of Total Protein and IgM in Tunicamycin Treated and Untreated Cells*

Cell Line	Tunicamycin Treated		Untreated	
	Protein	IgM	Protein	IgM
Daudi	22.7	0.34	31.0	0.45
Raji	38.9	0.57	35.3	0.54
1788 (IC)	55.5	1.44	56.1	1.07
1788 (EC)	-	0.29	-	1.00

*35S-methionine counts/min (x 10^{-6}), corrected for 1 x 10^{6} cells. (IC) intracellular, (EC) extracellular.

As can be seen from the data in Table 2.2, tunicamycin treatment had no measureable effect on total protein synthesis in Raji and 1788 cells, and only a marginal inhibitory effect (27%) on protein synthesis in Daudi cells. This is in agreement with the findings of Olden et al. (1978), who reported variable but generally minor inhibition of protein synthesis in chick embryo fibroblasts which had been treated with varying concentrations of tunicamycin. It is not clear whether the inhibition of protein synthesis in Daudi cells is significant, since in other biosynthetic labellings little or no inhibition was found.

Tunicamycin treatment appears to have had a differential inhibitory effect on IgM synthesis in 1788 cells. Thus in the control (untreated) culture 3.6% of the total protein synthesised was precipitated with

RAHIgM, whereas in the tunicamycin treated culture only 3.0% was precipitated. A closer examination of the data, however, indicates that the major effect of tunicamycin was the inhibition of secretion of IgM from 1788 cells (see section 2.3.5). Whereas approximately 50% of the synthesised IgM in the control cultures was secreted during the 2 hr chase, only 20% was secreted in the tunicamycin treated cultures. The buildup of intracellular IgM in the treated cultures (2.6% of the total protein compared with 1.9% in the control) presumably reflects the inhibition of secretion in these cells, and may also be an indirect cause of the overall reduction in IgM synthesis. No differential effect of tunicamycin on IgM synthesis in Daudi and Raji cells was found in this experiment.

2.3.4 SDS-PAGE of IgM from Tunicamycin Treated Cells

SDS-PAGE profiles of reduced IgM synthesised in Daudi, Raji and 1788 cells in the presence and absence of tunicamycin are shown in Fig.2.3. The results indicate that a 5 hr preincubation with tunicamycin (0.5 μg/ml) completely abrogates the synthesis of normally glycosylated μ-chains in all of the cell lines investigated. Thus the profiles obtained from the tunicamycin treated cultures (tracks 1-3) show no μ-chain bands corresponding in mobility to those obtained from the untreated cultures (tracks 8-10). Treatment of cell cultures with tunicamycin (0.5 ug/ml) for 2 hr also resulted in the complete abrogation of normally glycosylated μ-chain synthesis in Daudi and Raji cells (see Fig. 2.6). IgM from two hour tunicamycin treated 1788 cells has not been analysed by SDS-PAGE (Fig. 2.6, track 9, is a sample from 5 hr treated 1788 cells).

The μ-chains from the tunicamycin treated cells exhibited a markedly

Fig. 2.3

SDS-PAGE Analysis of Biosynthetically Labelled and Surface Radioiodinated IgM in Tunicamycin-Treated and Untreated Cells

Tunicamycin (1 mg/ml in DMSO) was added to a final concentration of 0.5 µg/ml to log phase cultures containing 1 x 10^7 cells. Parallel cultures (controls) of 1 x 10^7 cells were also maintained. The cells were incubated at 37°C and, after 5 hr, cells from the tunicamycin-treated cultures and control cultures were harvested for biosynthetic labelling. 1 x 10^6 cells were labelled for 1 hr in 0.2 ml labelling medium (with or without tunicamycin) containing 100 µCi 35S-methionine. Complete medium (0.8 ml, with or without tunicamycin) was then added and a further 2 hr incubation allowed (Methods, 8.1). The cells were separated from the culture medium and lysed in 0.2 ml NP-40 lysis buffer containing PMSF and pepstatin-A (Methods, 8.3).

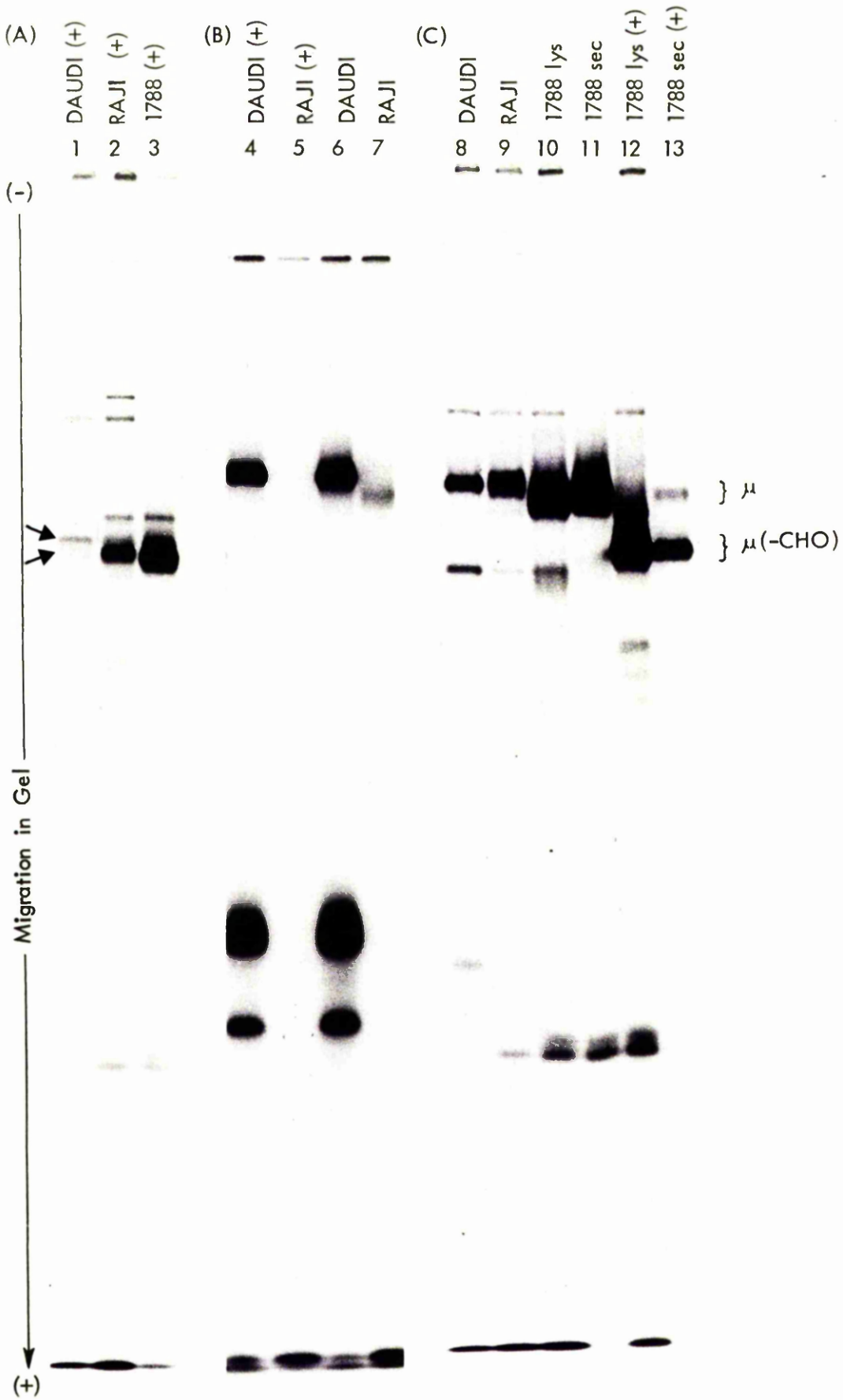
After a total of 8 hr incubation, cells from the tunicamycin treated and control cultures (Daudi and Raji only in each case) were harvested for radioiodination. Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-125, 1 mCi) as described in Methods, section 8.2, and the cells lysed in 0.5 ml NP-40 lysis buffer as descibred above.

Aliquots of cell lysate were removed for TCA precipitation, and then immunoprecipitations were carried out by addition of 10 µg RAHIgM to the biosynthetic lysates and 1788 culture medium, or 40 µg RAHIgM to the radioiodinated lysates (15 min at 20°C), followed by 25 µl or 100 µl respectively of S. aureus immunoadsorbent (overnight at 4°C). The radioactivity recovered in TCA and immunoprecipitates is given for the biosynthetic labelling in Table 2.2, and for the surface iodination in Table 2.3. Washed immunoprecipitates were dissolved in reducing sample buffer for discontinuous SDS-PAGE on a 10% polyacrylamide slab gel (Methods, 11). The gel was processed for fluorography (Methods, 17.4) and fluorograms developed after exposures of 2-24 hr.

(A) Tunicamycin-Treated Biosynthetic

(B) Surface Radioiodinated: (+) Tunicamycin-Treated
(-) Untreated

(C) Untreated Biosynthetic


(A)
DAUDI (+)
RAJI (+)
1788 (+)
1 2 3
(B)
DAUDI (+)
RAJI (+)
DAUDI
RAJI
4 5 6 7
(C)
DAUDI
RAJI
1788 lys
1788 sec
1788 lys (+)
1788 sec (+)
8 9 10 11 12 13
(-)
(+)
Migration in Gel
} μ
} μ(-CHO)

faster SDS-PAGE mobility than their counterparts from the untreated cells, (compare for example tracks 10 and 11 with tracks 12 and 13), consistent with a deficiency of glycosylation in the tunicamycin treated cells. Since tunicamycin is known to block asparagine linked glycosylation, it is reasonable to assume μ-chains from treated cells to be completely free of carbohydrate. Consistent with this assumption is the observation that μ-chains synthesised in tunicamycin treated 1788 cells have a slightly faster mobility on SDS-PAGE than their cell-free synthesised counterparts (see section 2.4.5).

2.3.5 Secretion of Nonglycosylated μ-Chains from 1788 Cells

The data given in Table 2.2 and discussed in section 2.3.3 indicated a significant inhibition of secretion of IgM from tunicamycin treated 1788 cells. It was therefore of interest to determine whether the secreted IgM consisted of nonglycosylated μ-chains or μ-chains which had been fully or partially glycosylated even in the presence of tunicamycin. Fig. 2.3, tracks 11 and 13, shows the SDS-PAGE profiles of IgM secreted from control and tunicamycin treated cells respectively. The samples used were from the same experiment described in section 2.3.3. Essentially all of the secreted μ-chain from the treated cells comigrated with nonglycosylated μ-chain (only an insignificant amount of label was detected comigrating with normal μ-chains).

The foregoing results indicated that nonglycosylated μ-chains can be secreted. However, these findings do not indicate that glycosylation is unnecessary for secretion. Thus, several secretory pathways for nonglycosylated μ-chains can be envisaged. Secretion of nonglycosylated μ-chains in monomeric form or as H_2L_2 subunits is one possibility. Alternatively, nonglycosylated μ-chains could be secreted in H_2L_2 subunits

containing one glycosylated μ-chain, or in polymeric (H_2L_2) molecules containing from 1 to 10 nonglycosylated μ-chains together with both partially and fully glycosylated 'partners'.

Since SDS-PAGE (Fig. 2.3) shows that the labelled μ-chains detected by immunoprecipitation were nonglycosylated, it follows that only non-glycosylated μ-chains were synthesised during the labelling period. This does not preclude the possibility that unlabelled μ-chains, fully or partially glycosylated were also part of the cellular μ-chain pool. To the contrary, it would be reasonable to assume, depending on the rate of action of tunicamycin and the kinetics of secretion of newly synthesised μ-chains, that a residual pool of fully and partially glycosylated μ-chains was present before the onset of labelling. Thus, some, if not all, of the nonglycosylated μ-chains detected in the culture medium could have been 'carried' out as part of H_2L_2 or $(H_2L_2)_5$ molecules containing glycosylated μ-chains as well. This situation seems more likely in the case of IgM, in that the pentameric molecule could probably contain a few nonglycosylated μ-chains without major conformational change. The amount of nonglycosylated μ-chain 'carried' out in this manner would depend on how strict the requirement for glycosylated 'partners' is, and on how many such glycosylated μ-chains were available during the labelling and chase periods. The inhibition of secretion in the tunicamycin treated cultures (20%, compared with 50% in the control cultures) indicates, according to this interpretation, that the availability of, or the requirement for 'carrier' μ-chains was somewhat but not completely limiting.

It is clear that the use of tunicamycin provides an excellent system in which to investigate the glycosylation of μ-chains, their assembly into subunits, the size and rate of depletion of the intracellular μ-chain pool,

and the glycosylation requirements for active secretion. For example, two mechanisms of IgM assembly and secretion can be proposed, based on the experiments with tunicamycin treated cells. Thus it was assumed in the preceeding discussion that glycosylated and nonglycosylated μ-chains can pair up to form pentamers which are either rapidly secreted, slowly secreted, or not secreted at all, depending on their structural similarity to normal pentameric IgM. An alternative model would propose that glycosylated and nonglycosylated μ-chains do not pair up, and that completely nonglycosylated pentamers (or monomers) can be secreted, albeit at a slower rate than normal pentamers.

To decide between these models it would be necessary to know the structure of the so-called 'carrier' units. This could be determined indirectly by SDS-PAGE of the unreduced (presumably pentameric) secretory IgM to resolve molecules containing 1,2,3,....up to 10 nonglycosylated μ-chains. This approach would probably be feasible using refinements of the available SDS-PAGE techniques for the analysis of high molecular weight molecules. The presence of partially glycosylated μ-chains in the 'carrier' units would, however, add another level of complexity to the analysis.

2.3.6 Lack of Surface Deposition of Nonglycosylated μ-Chains

The surface deposition of nonglycosylated μ-chains was investigated as part of the same experiment described in section 2.3.3. The tunicamycin treated and control cultures, from which cells were harvested for biosynthesis after 5 hr preincubation, were also the source of cells for surface iodination. Daudi and Raji cells were harvested after 8 hr preincubation with tunicamycin and cell surface proteins labelled by lactoperoxidase iodination. Cultures of untreated Daudi and Raji cells were also iodinated under similar conditions. The incorporation of 125-I

into TCA and anti-IgM precipitable material is summarised in Table 2.3.

Table 2.3 Incorporation of 125-I into Total Surface Proteins and sIgM in Tunicamycin Treated and Untreated Cells*

Cell Line	Tunicamycin Treated		Untreated	
	Total Surface Proteins	sIgM	Total Surface Proteins	sIgM
Daudi	42.3	1.07	44.9	1.31
Raji	21.8	0.20	23.3	0.35

*Counts/min (x 10^{-6}), corrected for 1 x 10^{7} cells.

No measureable inhibition of labelling of total surface proteins in tunicamycin treated cells was detected. In contrast, there was a slight inhibition of sIgM labelling after tunicamycin treatment. Thus, in the control cultures 3.0% of Daudi and 1.5% of Raji surface proteins were immunoprecipitated with RAHIgM, whereas in the tunicamycin treated cultures only 2.6% of Daudi and 0.9% of Raji surface proteins were immunoprecipitated. The significance of these differences depends on their reproducibility, which was not rigorously tested. Previous experience indicated, however, that experimental error in parallel immunoprecipitations of this sort is unlikely to lead to such differences.

It was of interest to determine whether the surface labelled IgM from tunicamycin treated cells consisted of nonglycosylated μ-chains, normal μ-chains, or both. A priori it was expected that a relatively high proportion of surface μ-chains would be glycosylated as a result of their having been synthesised before tunicamycin addition. This assumes a slow rate of turnover for cell surface IgM, which has indeed been found

to be the case for mouse splenocytes (Melchers & Andersson, 1973), although it has not been as well documented for human lymphoma-derived cells. Assuming that enough time was allowed for the surface expression of newly synthesised μ-chains, the absence of nonglycosylated μ-chains from the cell surface would indicate that glycosylation was necessary for surface deposition. In the present experiment an 8 hr preincubation with tunicamycin was allowed (the cells were shown to be synthesising only non-glycosylated μ-chains after 5 hr preincubation). It was felt that this experimental design would allow at least some surface expression of newly synthesised μ-chains to occur.

The SDS-PAGE profiles of surface IgM from tunicamycin treated and untreated Daudi and Raji cells are shown in Fig. 2.3(B). Both treated and untreated Daudi cells were found to express high levels of sIgM, consisting solely of normally glycosylated μ, p33 (see ch.3), and κ-chains (see tracks 4 and 6). To a very sensitive level of detection (i.e. compare the intensity of the μ-chain bands from Daudi and Raji cells), no nonglycosylated or partially glycosylated μ-chains were expressed on the Daudi cell surface. This was the case even though total sIgM was being measureably depleted from the Daudi cell surface, implying that normal turnover would have been replacing these molecules if suitable replacements were available. Thus, I believe this result provides strong evidence that nonglycosylated μ-chains are effectively inhibited from surface expression in Daudi cells.

Untreated Raji cells (track 7) were found to express extremely low amounts of sIgM relative to Daudi cells (track 6), consistent with previous findings (the relative amount of sIgM on Raji cells is always vastly overestimated when comparing immunoprecipitable radioactivity, as in Table 2.3). SDS-PAGE of the IgM precipitated from tunicamycin treated Raji cells (track 5) gives no detectable band of radioactivity corresponding to the mobility of

glycosylated or nonglycosylated μ-chains. This indicates, though to a much lesser sensitivity than with Daudi cells, that nonglycosylated μ-chains in Raji cells are inhibited from surface expression as well. The lack of a normal μ-chain band from the treated Raji cells suggests that these cells may have a faster rate of turnover of sIgM than Daudi cells. Thus, the tunicamycin treatment appears to have caused the modulation of sIgM from the Raji cell surface.

2.3.7 Nonglycosylated Daudi and Raji μ-Chains Resolve into Two Forms by SDS-PAGE

SDS-PAGE analysis of Raji and Daudi IgM, biosynthetically labelled in the presence of tunicamycin (5 hr preincubation), showed the nonglycosylated μ-chains to resolve into two forms. These forms, which differ in apparent molecular weight, are indicated by the arrows in Fig. 2.3, track 1. Fig. 2.6 (tracks 7 and 8) shows similar SDS-PAGE profiles obtained from 2 hr tunicamycin treated Daudi and Raji cells, in which the two forms of μ-chain are moreclearly resolved. A similar exposure of the profile from tunicamycin treated 1788 cells (Fig. 2.6, track 9) shows the μ-chain to consist of only a single band.

The existence of two forms of μ-chain in Daudi and Raji cells was predicted earlier on the basis of the C-terminal analysis performed using carboxypeptidase digestion (section 2.2). The resolution of these forms by SDS-PAGE is strong evidence in support of this earlier prediction. The ability to resolve the nonglycosylated but not the glycosylated μ-chains is consistent with the idea that variable glycosylation masks the apparent molecular weight difference of the polypeptide portions of the μ-chains (section 2.2.5.3).

As discussed earlier, if the two forms of μ-chain detected have structures

consistent with the model for secreted and cell surface molecules (the latter postulated to have an extra C-terminal sequence), the C-terminal tyrosine negative μ-chain should be the higher molecular weight form. The present findings provide indirect though strong evidence that this is indeed the case. This interpretation makes use of the observation from the carboxypeptidase digestion that Daudi cells synthesise significantly more of the C-terminal tyrosine negative μ-chain (putative surface form), and conversely, less of the C-terminal tyrosine positive μ-chain (secretory form) than Raji cells. The SDS-PAGE profiles in Figs. 2.3 and 2.6 show greater labelling of the higher molecular weight μ-chain in the case of Daudi and of the lower molecular weight μ-chain in the case of Raji cells. The higher molecular weight μ-chain can therefore be equated with the C-terminal negative form (i.e. the putative surface form), and the lower molecular weight μ-chain with the C-terminal tyrosine positive form (secretory form), as predicted from the model.

Similar results to those described above have been found from each SDS-PAGE analysis so far performed on μ-chains from tunicamycin treated cells. These experiments include those in which the cells were preincubated with tunicamycin (0.5 μg/ml) for 2, 4, or 5 hr. In all of the experiments, the relative labelling of the two forms of μ-chain in Daudi and Raji cells was similar to that shown in Figs. 2.3 and 2.6. This argues strongly against the notion that artefacts introduced during preparation or electrophoresis of the samples are responsible for the double banding pattern.

Several trivial explanations for the resolution of two forms of μ-chain from Daudi and Raji cells have not been formally ruled out. Thus, the higher molecular weight μ-chain may have on oligosaccharide subunit still present, which shows variable resistance in the two cell lines to inhibition by

tunicamycin. Alternatively, the main structural difference detected may be at the N-terminal end, caused perhaps by unequal processing of putative synthetic precursors. When viewed in the light of the C-terminal analysis, however, these alternative explanations lose much of their appeal. One important test of the hypothesis that surface μ-chains have an extra C-terminal would be to physically isolate the two forms of μ-chain and analyse each for release of C-terminal tyrosine. This experiment has been done preliminarily and the results indeed indicate release of C-terminal tyrosine from the lower molecular weight μ-chain, and not from the higher molecular weight μ-chain.

2.3.8 Apparent Molecular Weight of Nonglycosylated 1788 μ-Chains

It was previously shown (section 2.1.4) that Daudi and Raji surface μ-chains, radiolabelled with a short (1 hr) pulse, comigrated as single bands on SDS-PAGE, with a retarded mobility relative to μ-chains (intracellular or secreted) from secretory cells such as 1788. These findings are shown in Fig. 2.1, and are illustrated again in Fig. 2.3 (tracks 8-10) and Fig. 2.6 (tracks 1-3).

With the use of tunicamycin, it could be asked whether additional asparagine linked glycosylation is responsible for this retarded mobility of Daudi and Raji μ-chains. For such an experiment, it is appropriate to compare the SDS-PAGE mobility of nonglycosylated 1788 μ-chain with that of the lower molecular weight form of Daudi and Raji μ-chains. This follows from the evidence that it is the lower molecular weight form which most resembles secretory μ-chain (i.e. by C-terminal analysis, see sections 2.2 and 2.3.7).

The SDS-PAGE profiles shown in Figs. 2.3 (tracks 1-3) and 2.6 (tracks 7-9) show that nonglycosylated 1788 μ-chains migrate slightly faster than the

lower molecular weight form of either Daudi or Raji μ-chains. This finding appears to indicate that these μ-chains differ in their polypeptide portion, and not in their carbohydrate portion. Thus, the polypeptide portion of 1788 secretory μ-chain appears to be of lower molecular weight than that of the putative secretory type μ-chains from Daudi and Raji cells.

Explanations for the above finding include the possibility that Daudi and Raji μ-chains (higher and/or lower molecular weight forms) contain nonasparagine linked carbohydrate which retards their SDS-PAGE mobility relative to 1788 μ-chains. Alternatively, Daudi and Raji μ-chains may be of a subclass different from 1788 μ-chains and exhibiting a mobility on SDS-PAGE distinct from 1788 μ-chains. A third possibility is that Daudi and Raji μ-chains (both forms) retain their N-terminal precursor piece in vivo, whereas 1788 μ-chains have theirs removed. Evidence comparing the SDS-PAGE mobility of nonglycosylated in vivo-synthesised μ-chains and cell-free synthesised μ-chains (sections 2.4.4 and 2.4.5) supports this latter explanation.

2.4 CELL-FREE SYNTHESIS OF μ-CHAINS FROM CELL SURFACE AND SECRETORY IgM-PRODUCING CELLS: ANALYSIS OF PRECURSORS

2.4.1 Preparation of Polyribosomes

Messenger RNA capable of being efficiently translated in a wheat germ cell-free system was prepared by a two stage procedure. Polyribosomes were first prepared from detergent extracts of human lymphoid cells, according to the methods of either (I) Bennett, Fitzmaurice and Williamson (in preparation), or (II) T. Mosmann (personal communication). Both methods, which are modifications of published techniques, were developed specifically for use with mouse myeloma cells in tissue culture. However, both were found to be well suited for the preparation of high yields of undegraded polyribosomes from human lymphoid cells.

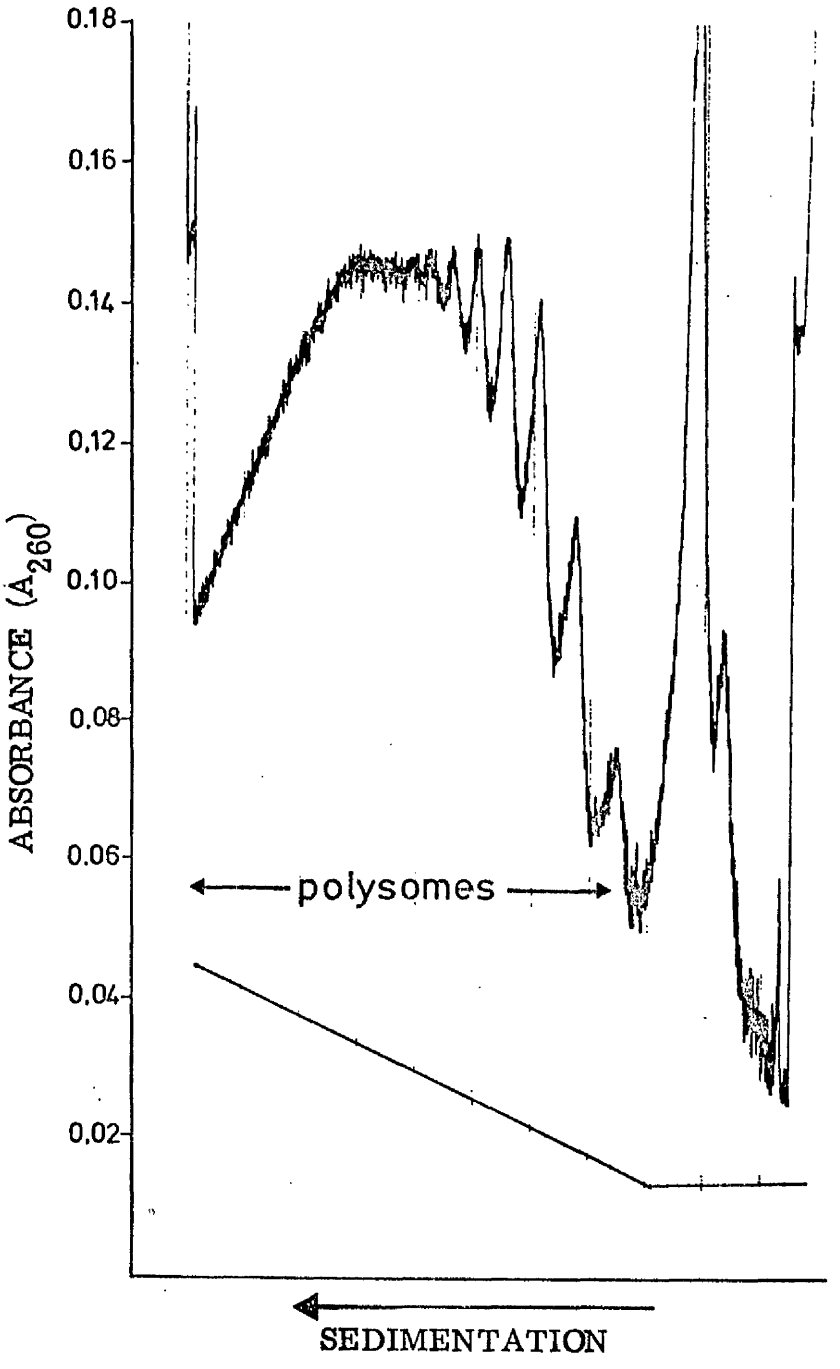
Figure 2.4 shows a typical polyribosome profile, obtained using the method of T. Mosmann. The large proportion of rapidly sedimenting material (i.e. high molecular weight polyribosomes), as well as the sharp resolution of individual peaks of up to nine ribosomal subunits, (indicated by arrows) is characteristic of an undegraded preparation. The polyribosome yield from the human lymphoid cell lines investigated was generally found to be lower than that from mouse myeloma cells, as would be expected from the less active metabolic rate of the human cells (i.e. less rough endoplasmic reticulum (RER) and less protein synthesis as measured by incorporation of labelled amino acids). Thus, the human cells yielded an average of approximately 2-3 mg (50-75 A_{260} units) polyribosomes per 1 x 10^9 log phase cells, as compared to the reported values ranging from 75-200 A_{260} units/10^9 mouse myeloma cells.

Although both methods of polyribosome preparation were suitable in most

Fig. 2.4

Sucrose Density Gradient Centrifugation of Polyribosomes Prepared from Human Lymphoid Cells

Polyribosomes were prepared according to the Modified Method of T. Mosmann (Methods, 9.3.2) from 5×10^9 log phase Daudi cells. Approximately 10 A_{260} units of polyribosomes in 100 µl of 0.25 M sucrose were layered onto linear sucrose gradients (0.5 - 1.25 M sucrose in Solution C) prepared in 12 ml polyallomer tubes to fit a Spinco SW41 rotor. The gradients were centrifuged at 4°C for 90 min at 40,000 rpm. The A_{260} profile was generated by pumping the gradient at 1 ml/min through a spectrophotometer flow cell of 1 mm path length, and recorded on a chart recorder set at 50 sec/cm with a full scale absorbence of 0.2 A_{260} units.


0.18
0.16
0.14
0,12
0.10
0,08
0.06
0,04
0,02
ABSORBANCE (A_{260})
polysomes
SEDIMENTATION

circumstances, the method of T. Mosmann (Method II) was found to be preferable because of its insensitivity to the range of RER content encountered in the human cell lines. Thus, in Method I, the amount of triton X-100 (0.04-0.1%) used in conjunction with 1 mg/ml heparin (included as an inhibitor of ribonuclease, but also acting as an ionic detergent) had to be titrated against the RER content of the cells in order to avoid nuclear lysis and consequent degradation of the polyribosomes. In Method II, however, the initial heparin concentration was tenfold lower (0.1 mg/ml) and the triton X-100 (2% w/v) was therefore the major lysing agent. Since triton X-100 does not lyse nuclei very readily, no titration was necessary and nuclear lysis rarely occurred.

2.4.2 Preparation of Messenger RNA

Messenger RNA was prepared from polyribosomes by phenol extraction and oligo dT cellulose chromatography of the resulting polysomal RNA (see Methods, section 9.4 and 9.5). The oligo dT cellulose chromatography was done by a three-step elution procedure, in which the eluting buffers contained successively lower concentrations of lithium chloride (i.e. 0.5 M, 0.1 M, and none). The method was found to be reproducible in both the quantity and quality of the messenger (i.e. poly$(A)^+$) RNA recovered. The poly $(A)^+$ fraction (eluted in salt-free buffer) was consistently 1.5-2.0% (by absorbency) of the polysomal RNA applied to the oligo dT cellulose column. The intermediate wash (eluted with 0.1 M LiCl) was 3-4% of the polysomal RNA and could be demonstrated both by PAGE and by translation capacity to consist of approximately 20% mRNA and 80% ribosomal RNA (data not shown).

In general, the quality of an mRNA preparation was judged by its ability to direct cell-free synthesis of proteins having SDS-PAGE mobilities and

relative labelling intensitites (i.e. sufficient labelling of high molceular weight proteins) resembling that of their in vivo synthesised counterparts. By this criteria, mRNA preparations were judged to be free of interfering levels of degradation. On several occasions, RNA labelled by in vivo incorporation of 3H-Uridine was also characterised by PAGE in 99% formamide. A PAGE profile of 1788 poly $(A)^+$ RNA is shown in Fig. 2.5. The RNA migrates as a heterogeneous mixture of molecular species, with sizes from greater than 28S to approximately 8S. There is no labelled material detected migrating faster than approximately 8S, indicating that no detectable degradation has occurred. As can be seen from the size of the peaks migrating with the 28S and 18S markers, very little ribosomal RNA (rRNA) is present in the preparation. It should be noted, however, that the PAGE profiles such as Fig. 2.5 necessarily give an underestimate of the rRNA present in the preparation because rRNA labels to a lower specific activity than the poly $(A)^+$ RNA under the conditions employed (i.e. 6 hr labelling, see Methods, section 9.2). Thus, in the preparation used in Fig. 2.5, the rRNA had a specific activity of 17,000 cpm/µg, whereas that of the poly $(A)^+$ RNA was 52,000 cpm/µg. These values were typical of the results obtained with other RNA preparations similarly prepared and labelled, and indicate a probable rRNA contamination of not more than 10% of the total poly $(A)^+$ preparation.

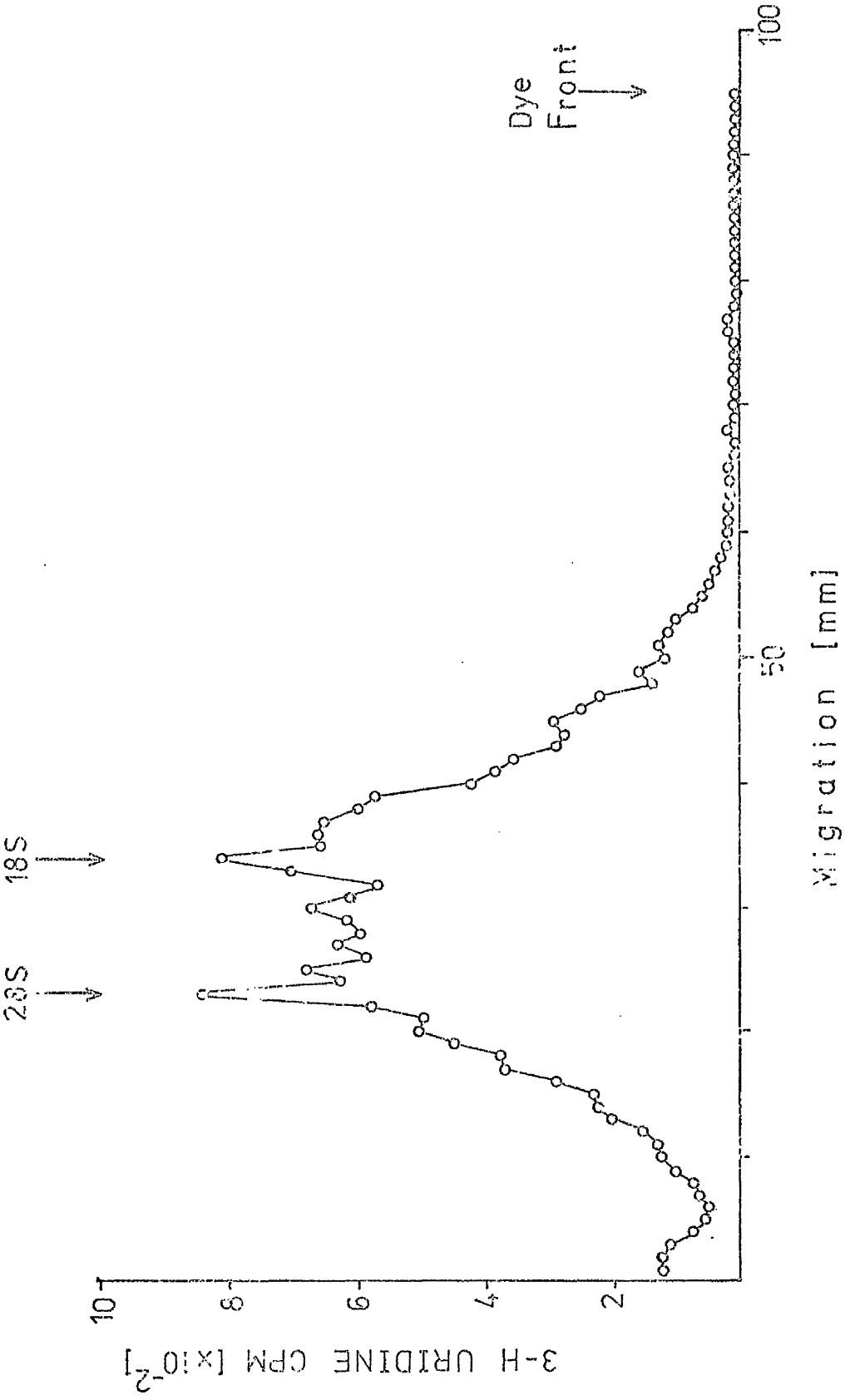
2.4.3 SDS-PAGE of Cell-Free Synthesised µ-Chains: Detection of Two Forms of µ-Precursor from Daudi and Raji Cells

Wheat germ cell-free translation of poly $(A)^+$ RNA resulted in the synthesis of proteins with a heterogeneous distribution of molecular weights as determined by SDS-pAGE. As with cellular proteins labelled in vivo, IgM heavy and light polypeptide chains could be immunoprecipitated from the bulk of the labelled cell-free synthesised proteins using specific

Fig. 2.5

Polyacrylamide Gel Electrophoresis in 99% Formamide of Poly $(A)^+$ RNA Prepared from Human Lymphoid Cells

Polyribosomes from 3H-uridine labelled (Methods, 9.2) log phase 1788 cells were prepared according to the method of Bennett, Fitzmaurice, and Williamson (Methods, 9.3.1). The polyribosomes were phenol extracted as described in Methods, section 9.4, and poly $(A)^+$ RNA prepared by oligo dT cellulose chromatography (Methods, 9.5). An aliquot of labelled poly $(A)^+$ RNA (2 µg; 100,000 cpm) was mixed with a sample of 32P labelled mouse L-cell 28S and 18S RNA (2000 cpm) and subjected to PAGE in a 3% polyacrylamide tube gel containing 99% formamide (Methods, 13). After electrophoresis, the gel was frozen and sliced at 1 mm intervals. The radioactivity in individual gel slices was determined by scintillation counting in a cocktail of 10% Protosol solubiliser in Toluene-PPO scintillation liquid. 3H radioactivity was counted in a low energy 'window' which eliminated 95% of the 32P radioactivity. The peak heights (peaks not shown) of the 32P markers were less than 500 cpm total radioactivity, as determined in the 32P 'window'. The arrows indicate the migration of the 28S and 18S markers.


3-H URIDINE CPM [x10⁻²]
10
8
6
4
2
28S
18S
Dye Front
50
100
Migration [mm]

antiserum. For such immunoprecipitations, both anti-μ antibody and anti-λ of anti-κ antiserum were utilised.

Figure 2.6 (tracks 4-6) shows the SDS-PAGE migration of Daudi, Raji and 1788 cell-free synthesised μ and light chains. Daudi and Raji μ-chains resolved into two forms, as indicated by the arrows in tracks 4 and 5. This was a consistent finding in every such SDS-PAGE of cell-free synthesised Daudi and Raji μ-chains performed, including those in which different poly $(A)^+$ RNA preparations and wheat germ extracts were used. This argues against the possibility that artefacts arising either during the preparation of the RNA, cell-free translation, or SDS-PAGE were involved. In contrast to the above findings, 1788 μ-chain (track 6) and all the light chains shown in the figure (see section 2.4.5) migrated as single bands.

2.4.4 Daudi and Raji μ-Chain Precursors (Both Forms) Comigrate with their Nonglycosylated in vivo Synthesised Counterparts

The finding that nonglycosylated Daudi and Raji μ-chains resolve into a lower and a higher molecular weight form by SDS-PAGE has been discussed previously, as has the evidence that these forms probably represent normal μ-chains and μ-chains with a C-terminal extra sequence respectively (section 2.3.7). Figure 2.6 shows that the two μ-chain forms synthesised in the cell-free system from Daudi and Raji poly $(A)^+$ RNA comigrated with the nonglycosylated forms (compare tracks 4 and 5 with tracks 7 and 8 respectively). Moreover, the relative labelling of the two cell-free synthesised forms (i.e. stronger labelling of the higher molecular weight form in Daudi, and of the lower molecular weight form in Raji cells) was similar to that of the nonglycosylated forms.

Fig. 2.6

SDS-PAGE Analysis of μ-Chains and Light Chains Synthesised in vitro in a Wheat Germ Cell-Free System and in vivo in Tunicamycin Treated and Untreated Cells

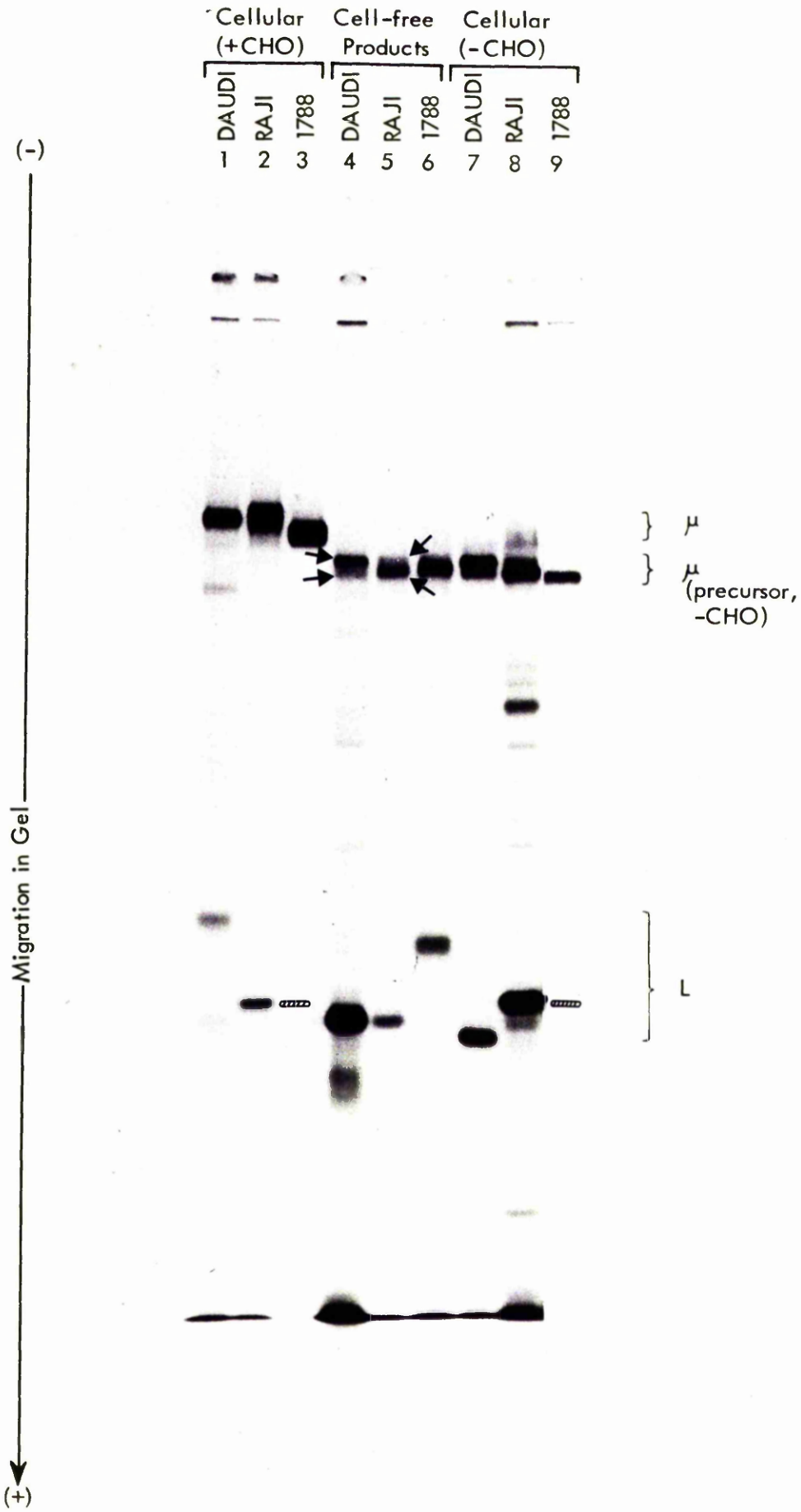
The samples shown in the figure consist of RAHIgM (10 μg) plus S. aureus immunoprecipitates from cell lysates or diluted wheat germ assays (also with anti-κ or anti-λ) prepared in 3D TKM lysis buffer containing PMSF and pepstatin-A (Methods, 8.3 and 10.4). The washed immunoprecipitates (25% of each sample) were dissolved in reducing buffer and applied to a 10% polyacrylamide slab gel for discontinuous SDS-PAGE (Methods, 11). The gel was processed for fluorography (Methods, 17.4) and the fluorogram developed after a 3-day exposure to film.

Biosynthetic samples from normal cells (tracks 1-3) were prepared by a 10 min incubation of 1×10^6 cells in 0.2 ml labelling medium containing 100 μCi 35S-methionine (Methods, 8.1).

Biosynthetic samples from tunicamycin treated (-CHO) cells (tracks 7-9) were prepared by a 4 hr incubation of 1×10^6 cells with 0.5 μg/ml tunicamycin followed by a 1 hr incubation in 35S-methionine-containing labelling medium as described above.

Wheat germ cell-free synthesised samples (tracks 4-6) were prepared by translation of 1 μg poly $(A)^+$ RNA (see Methods, 9) from each cell line in 50 μl standard wheat germ assays (Methods, 10) containing approximately 30 μCi (5 μl) 35S-methionine (sp. act. 800 Ci/mmole).

The μ and μ(precursor, -CHO) markers were derived from the migration of the samples under investigation. No independent markers were used.


Cellular (+CHO)
Cell-free Products
Cellular (-CHO)
DAUDI
RAJI
1788
DAUDI
RAJI
1788
DAUDI
RAJI
1788
1 2 3 4 5 6 7 8 9
(-)
Migration in Gel
(+)
μ
μ (precursor, -CHO)
L

2.4.5. 1788 μ-Chain Precursor Migrates with a Higher Apparent Molecular Weight that its Nonglycosylated in vivo Synthesised Counterpart

1788 μ-chain synthesised in the wheat germ cell-free system had a detectably slower SDS-PAGE mobility than nonglycosylated μ-chain from tunicamycin treated 1788 cells (Fig. 2.6, tracks 6 and 9). This can best be seen using the doublet bands from Daudi and Raji μ-chains (tracks 7 and 8) for comparison. Thus, 1788 μ-chain from the cell-free system (track 6) migrated approximately midway between the doublet bands, whereas nonglycosylated 1788 μ-chain (track 9) migrated faster than the fastest migrating member of the doublet. This finding is consistent with the presence of an N-terminal precursor piece on cell-free synthesised 1788 μ-chain. In support of this conclusion, H.Singer & Williamson (in preparation) have demonstrated by amino acid sequencing the existence of a 19 amino acid precursor piece on cell-free synthesised mouse γ_{2a} (5563 myeloma protein) heavy chain, and have further shown that this molecular weight difference is reflected in the slower SDS-PAGE mobility of the cell-free product compared with the nonglycosylated *in vivo* synthesised product.

The implications of the above findings (sections 2.4.3 - 2.4.5), concerning the comparative SDS-PAGE of normal, nonglycosylated and cell-free synthesised μ-chains, are discussed further in section 2.4.7.

2.4.6 SDS-PAGE Mobilities of Light Chains: Comparison of Precursors, Nonglycosylated and Glycosylated Products

Figure 2.6 shows the relative mobilities of Daudi, Raji and 1788 light chains synthesised in the cell-free system or *in vivo* in the presence or absence of tunicamycin (the locations of Daudi and 1788 light chains in tracks 1, 3 and 9 were determined from other SDS-PAGE analyses, and are shown here to facilitate comparison). From the mobilities of these light chains it is possible to deduce certain information concerning

their biosynthesis. Thus, Daudi and Raji κ-chains synthesised in the cell-free system (tracks 4 and 5) were found to comigrate by SDS-PAGE. Daudi κ-chain synthesised in the presence of tunicamycin (track 7) migrated faster than the cell-free product, consistent with the presence of an N-terminal precursor piece on the cell-free product which is cleaved off in vivo. Daudi κ-chain synthesised in the absence of tunicamycin (track 1) migrated slower than the nonglycosylated product, indicating the presence of asparagine linked carbohydrate.

Raji κ-chain synthesised in the presence of tunicamycin migrated slightly slower than the cell-free synthesised product, consistent with either (1) non-asparagine linked carbohydrate, or (2) non-asparagine linked carbohydrate and retention of a putative N-terminal precursor piece on Raji κ-chain. Retention of a precursor alone, without the presence of carbohydrate, is ruled out because the product from the tunicamycin treated cells appeared by SDS-PAGE to be larger and not identical in molecular weight to the cell-free product. Thus, Raji κ-chain has attached a small complement of non-asparagine linked carbohydrate. Raji κ-chain synthesised in the absence of tunicamycin (track 2) comigrated with its counterpart from tunicamycin treated cells indicating that all and not just part of the Raji κ-chain carbohydrate moiety is non-asparagine linked.

1788 λ-chain synthesised in the cell-free system (track 6) migrated markedly slower than cell-free synthesised Daudi and Raji κ-chains. The observation that Daudi and Raji κ-chain precursors comigrated argues against the hypothesis that amino acid composition differences alone are detected by Laemmli SDS-PAGE, and instead supports the hypothesis that only gross molecular weight differences (i.e. those contributed by extra

carbohydrate or extra polypeptide chain length) are detected. Thus there appears to be such a gross molecular weight difference between the κ and λ precursors investigated (and possibly between the cellular products; see Ch. 1, section 1.3.5). 1788 λ-chains synthesised *in vivo* in the presence or absence of tunicamycin (tracks 9 and 3) were found to comigrate, indicating either the absence of carbohydrate or, as in the case of Raji κ-chain, the presence of non-asparagine linked carbohydrate. The double banding pattern shown in the figure was an anomalous finding in one labelling experiment (see Fig. 2.3) which, however, does not alter the present interpretation of the results (in previous experiments it was the lower band which was detected).

In vivo synthesised λ-chain was also found to migrate *faster* than the cell-free product, again consistent with the presence of an N-terminal precursor piece on the cell-free product which is cleaved off *in vivo*. This putative λ precursor piece appears to be longer than the κ precursor piece. This conclusion is based on the smaller apparent molecular weight difference between the Daudi κ precursor and nonglycosylated product than that between the 1788 λ precursor and nonglycosylated product (compare the mobility difference in tracks 4 and 7 to that in tracks 6 and 9).

However, this conclusion assumes that the Daudi κ-chain from the tunicamycin treated cells is *completely* free of carbohydrate. If, instead, these κ-chains have tunicamycin-insensitive carbohydrate attached, this would lead to an underestimate of the size of the precursor piece.

The findings and implications discussed above can be summarised as follows. (1) Both κ and λ chains from human lymphoma and lymphoblastoid cells are probably synthesised with N-terminal precursor pieces, which are cleaved off *in vivo*. (2) Assuming that the light chains shown in

tracks 7-9 (from tunicamycin treated cells) are completely free of carbohydrate, and further assuming that the mobilities of the non-glycosylated κ and λ chains and their precursors shown in Fig. 2.6 are the same for all such κ and λ chains, it appears that both the mature polypeptide chain and the precursor piece from λ chains are probably larger than those from κ-chains. (3) Kappa chains from Daudi and Raji cells each contain carbohydrate; Daudi κ having a larger amount of asparagine linked moieties, and Raji κ having a smaller amount of non-asparagine linked moieties.

2.4.7 Conclusion: Aspects of μ-Chain Processing in Lymphoma and Lymphoblastoid Cells

The above analysis of μ-chains translated in vitro and in vivo in the presence and absence of tunicamycin reveals striking differences in μ-chain expression between the lymphoma-derived and lymphoblastoid cells, as well as offering insight into the nature of these differences. Two particular features of μ-chain processing have emerged from the data. These features are discussed below.

(i) The two forms of Daudi and Raji μ-chain are differentiated at the mRNA level. This conclusion is based on the observaton that cell-free translation of Daudi and Raji mRNA yielded both forms of μ-chain in the same relative amounts as the nonglycosylated in vivo synthesised products. This reproduction in vitro of such an in vivo labelling pattern cannot be adequately explained by artefactual effects such as processing or nonspecific proteolysis in the wheat germ system. Likewise, it is unlikely that artefacts arising during SDS-PAGE could account for the labelling pattern observed. Thus, the most compelling explanation is that the information for the synthesis of the two forms in their proper relative amounts (i.e. as in the cell) is contained in the cellular mRNA, and is not dependent on cellular translation mechanisms. The

most straightforward mechanism would be that each form of μ-chain is encoded by a separate mRNA species; however, other mechanisms are not ruled out.

(ii) Daudi and Raji μ-chain precursors (both forms) are apparently not processed in vivo, whereas 1788 μ-chain precursor is processed to a lower molecular weight form. This conclusion is based on the comigration of cell-free synthesised Daudi and Raji μ-chains with their nonglycosylated counterparts, and in contrast, the retarded mobility of cell-free synthesised 1788 μ-chain relative to its nonglycosylated counterpart. Two possibilities can be envisaged to explain the apparent lack of processing in Daudi and Raji cells. Thus, both forms of μ-chain could be synthesised without N-terminal precursor sequences, therefore requiring no processing to produce the normal cellular products. Alternatively, the μ-chains could be translated with N-terminal precursor sequences which are then left intact in the normal cellular products. Although the data discussed above is not sufficient to distinguish between these possibilities, in either case a fundamental difference in μ-chain processing would be involved. Amino-terminal sequencing of cellular Daudi and Raji μ-chains is presently being carried out in collaboration with D. Capra and his coworkers, and from such analysis it should be possible to determine whether these μ-chains have precursor-like or V region-like amino terminal sequences.

2.5. MOLECULAR EVENTS IN THE BIOSYNTHESIS OF CELL SURFACE AND SECRETORY IgM: A MODEL

The results described in this section suggest several novel features in the biosynthetic pathways of cell surface and secretory IgM in human lymphoid cell lines. It is expected that these features will

in general be similar to those involved in the in vivo surface deposition and secretion of IgM by lymphocytes. The essential features are presented below in the form of a model for IgM expression.

2.5.1. Integral Membrane Binding of Cell Surface IgM

Previous reports have determined that cell surface μ-chains are of higher molecular weight, bind more detergent, are less soluble in aqueous media, and have a modified carboxy-terminus relative to secretory μ-chains (reviewed in Introduction, section 2). The implication is that the μ-chain from sIgM has an hydrophobic carboxy-terminal peptide capable of insertion into the membrane lipid bilayer. The results presented in this section have shown that single cell lines can synthesise both normal μ-chains and μ-chains of a higher apparent molecular weight. The presence of the higher molecular weight form correlates with the cellular expression of sIgM. A modified carboxy-terminus has been detected specifically on the higher molecular weight species in two such cell lines — a prerequisite for the concept of an extra C-terminal peptide on surface μ-chains.

Thus, it appears that cell surface IgM is an integral membrane protein attached to the lipid bilayer by means of a C-terminal hydrophobic 'tail'. It would be of considerable interest to determine what type of integral membrane binding is exhibited by sIgM molecules. In a recent article, Singer (1977) has identified four classes of integral membrane protein — 1) internally (cytoplasmic) facing, partially embedded, 2) externally-facing, partially embedded, 3) trans-membrane and 4) subunit aggregates forming a transmembrane aqueous channel or 'pore'. Classes 1 and 3 are represented by cytochrome b_5 and glycophorin,

respectively. According to Singer (1977), it is not yet clear whether class 2 proteins exist. It may be that sIgM is such an integral membrane protein. Thus, the reported SDS-PAGE mobility difference between putative surface and secretory μ-chains (Melcher & Uhr, 1976; this thesis) are consistent with a 'tail' of around 15-20 amino acids in length. A tail of this size would be capable of embedding itself in the lipid bilayer, but possibly not spanning it. The finding of Walsh & Crumpton (1977) that IgM could not be radioiodinated from the cytoplasmic side of the surface membrane (using inside-out vessicles) supports this idea.

2.5.2. The Separation of sIgM and Secretory IgM Biosynthetic Pathways: Production of Distinct mRNA Species

Previous models support the idea that the biosynthetic pathways for sIgM and secretory IgM diverge only at a late stage in the intracellular transport of the molecules. Thus Uhr (1970) and Uhr & Vitetta (1973) have proposed that both forms of IgM are initially bound to microsomal membranes by their C-terminal ends. This binding is thought to facilitate glycosylation by membrane bound glycosyl transferases in the smooth ER and Golgi complex. According to their model, the newly synthesised IgM passes from the Golgi complex and into post-Golgi vessicles, where a signal is read which results in release from the membrane, by specific proteolytic cleavage, of those molecules destined for secretion. Secretion (or surface deposition, in the case of those molecules which remain membrane-bound) is accomplished by fusion of the vessicle with the plasma membrane and reverse pinocytosis.

The results presented in this thesis section suggest an alternative model. According to this model, synthesis of the μ-chain of cell

surface IgM is directed by an mRNA molecule distinct from that coding for secretory IgM μ-chain. Thus, it is proposed that the sIgM μ-chain messenger contains the sequence coding for the putative C-terminal hydrophobic peptide whereas secretory IgM μ-chain messengers do not contain such a coding sequence. The basis for this conclusion is the finding that a wheat germ cell-free system directs the in vitro synthesis of both forms of μ-chain (surface and secretory), under circumstances which preclude protein processing as a plausible explanation.

The production of distinct messenger RNA species for surface and secretory μ-chains implies either 1) the existence of different genes for these polypeptides or 2) different transcriptional or post-trascriptional processing of primary gene transcripts. Because of the increasing evidence for post-transcriptional processing of primary gene transcripts, I tend to favor alternative 2. Thus it can be envisaged that the coding sequences for μ-chain V, C, and hydrophobic 'tail' regions are all contained within the sequence of nucleotides comprising the μ-chain gene. As has recently become evident from the work of many investigators, genes need not be continuous. Thus, the μ-chain gene may be found to be interrupted by one or several non-coding sequences. The primary RNA transcript for both surface and secretory μ-chains may either contain or not contain the 'tail' region coding sequence. If the latter alternative is correct, then direct transcriptional control on the μ-chain gene would be involved. If the first alternative is correct, then removal of the 'tail' region coding sequence from secretory μ-chain messengers would presumably occur during post-transcriptional processing. The existence of large-sized heterogeneous nuclear RNA in all eucaryotic cells is generally considered as strong evidence that such post-transcriptional processing occurs. This processing is undoubtedly capable of

a high degree of specificity, however the mechanisms involved are not yet known.

Both the control of cell surface vs. secretory Ig expression, and the control of Ig isotype expression are intimately connected with B-cell maturation. A minimal hypothesis would be that the control mechanisms involved in these processes operate at the same cellular level. Ig isotype switch is clearly controlled directly at the level of the Ig H-chain genes. A minimal model would therefore propose the same direct control of surface vs. secretory Ig expression. The model proposed here is such a minimal model. Alternative concepts require that surface vs. secretory Ig expression be controlled through various complex intermediate pathways — such as the coordinated action of specific glycosylases and proteases to identify and cleave membrane bound sIgM destined for secretion; or the regulation of quantity or sIgM-binding affinity of putative 'anchor' proteins.

2.5.3. Amino Terminal Precursors

It is reasonably well established that most, if not all, proteins destined for vectorial release into the rough ER cisternae are synthesised with amino terminal precursor peptides. This has been shown to be the case for all of the several L-chain and both of the two H-chain precursors examined (see Introduction, section 3). In addition, the SDS-PAGE analysis of nonglycosylated and cell-free 1788 μ-chain suggests that it too is synthesised as a precursor. Thus it is likely that both forms of Daudi and Raji μ-chains (surface and secretory) are synthesised as precursors. One working hypothesis during the course of these investigations was that retention of the amino terminal precursor peptide might be a mechanism for membrane binding of sIgM. However, the evidence for

a carboxy terminal extra peptide removed the immediate need to postulate a role for the amino terminal precursor peptide. The comparitive SDS-PAGE analysis suggested that both the putative surface and secretory forms of μ-chain may retain their amino terminal precursors, which was difficult to reconcile with a specific role for the sIgM precursor. To test this idea directly, samples of labelled μ-chain from Daudi and Raji cells were prepared for radioactive microsequencing of the amino terminus. This sequencing work was done in collaboration with Dr. Donald Capra and his coworkers. The results revealed that both Daudi and Raji μ-chains have leucine at positions 4, 11, 18 and 20, and valines at positions 2, 5 and 12 — consistent with both of these being unblocked (V_H III) μ-chains. Since a significant portion of intracellular μ-chains from Daudi cells was always found to be of the surface form, it can be concluded that amino terminal precursor peptides are not involved in the binding of sIgM to the plasma membrane in these cells. Instead, the above data from SDS-PAGE analysis can be interpreted to indicate that precursor processing in Daudi and Raji cells is either 1) less efficient than in secretory (i.e. 1788) cells, or 2) more sensitive to tunicamycin than in secretory cells.

2.5.4. Intracellular Transport of sIgM as an Integral Membrane Protein

If cell surface IgM μ-chains are synthesised as integral membrane proteins of the class 2 type — i.e. partially embedded in the lipid bilayer and facing into the microsomal compartment, it is relevant to ask how it attains its final membrane bound position. Singer (1977) has proposed that cytoplasmic-facing partially embedded membrane proteins, such as cytochrome b_5, must insert into the membrane after completion of synthesis — without being vectorially transferred across any intracellular membranes. It is also reasoned in this article that integral membrane proteins which are vectorially transferred, but which are also of the

partially embedded type (as postulated for sIgM) may be free to reattach themselves to other areas of the intra-microsomal membrane. Provided that further evidence bears out that sIgM is a partially embedded integral membrane protein, it would be of considerable interest to determine if it is capable of detaching from and reattaching to membrane sites. Such a property of sIgM would be important for intracellular transport as well as for its putative functions in immune system interactions at the cellular level.

The apparent progression of newly synthesised cell surface and secretory Ig through the cellular compartments leading to the plasma membrane has been well documented (Introduction, sections 3 & 4). What is not completely clear, however, is whether the cellular compartments for sIg and secretory Ig are the same or independent. The findings presented in this thesis that nonglycosylated μ-chains are <u>not</u> deposited on the cell surface, but <u>are</u> secreted in significant quantities, leads to the tentative conclusion that these molecules are compartmentalised independently. The alternative view is that the two μ-chain types are compartmentalised together in post-Golgi vessicles. However, if this were the case, fusion and reverse pinocytosis which releases nonglycosylated μ-chains should also result in some surface deposition of nonglycosylated μ-chains. Clearly other explanations of the data are possible, however. One such explanation would be that nonglycosylated membrane bound μ-chains are specifically degraded within the post-Golgi vessicle. Further experiments are required to decide between the possible interpretations.

CHAPTER III

CHARACTERIZATION OF A POLYPEPTIDE (p33) IN A NOVEL COVALENT ASSOCIATION WITH DAUDI CELL SURFACE IgM

3.1. INITIAL DETECTION OF P33

3.1.1. SDS-PAGE of Daudi Surface IgM (sIgM)

Daudi surface protein p33 was first detected during a screening experiment designed to determine the cell surface Ig(sIg) expression of several lymphoma and lymphoblastoid cell lines. Cell surface proteins were labelled by lactoperoxidase catalysed radioiodination and sIg isolated from NP-40 cell lysates by immunoprecipitation with anti-IgM antiserum (μ-specific). The immunoprecipitates were reduced and alkylated and analysed by SDS-PAGE on 6% polyacrylamide gels.

The SDS-PAGE profile obtained from reduced Daudi sIg is shown in Fig.3.1(A). The label was resolved into a peak which comigrated with μ-chain, an unusually large peak of about 33,000 molecular weight (section 3.3), and a partially resolved small peak which was coincident in mobility with L-chain. The results confirmed the findings of Klein et al. (1968) that Daudi sIg was of the IgM class. These workers also characterised Daudi L-chain as being of the κ-type.

3.1.2. Nature of 33,000 Molecular Weight Peak

The nature of the SDS-PAGE profile in the L-chain region of the gel was unusual, and presented a dilemma. If the small peak of L-chain mobility represents Daudi κ-chain then the 33,000 molecular weight peak would appear to be an 'extra' component of Daudi sIgM. Alternatively, if the small peak is spurious then the 33,000 molecular weight peak would likely represent either a normal κ-chain of anomalous SDS-PAGE mobility or an abnormally large κ-chain. While it is generally accepted that spurious peaks and anomalous migration of

Fig. 3.1

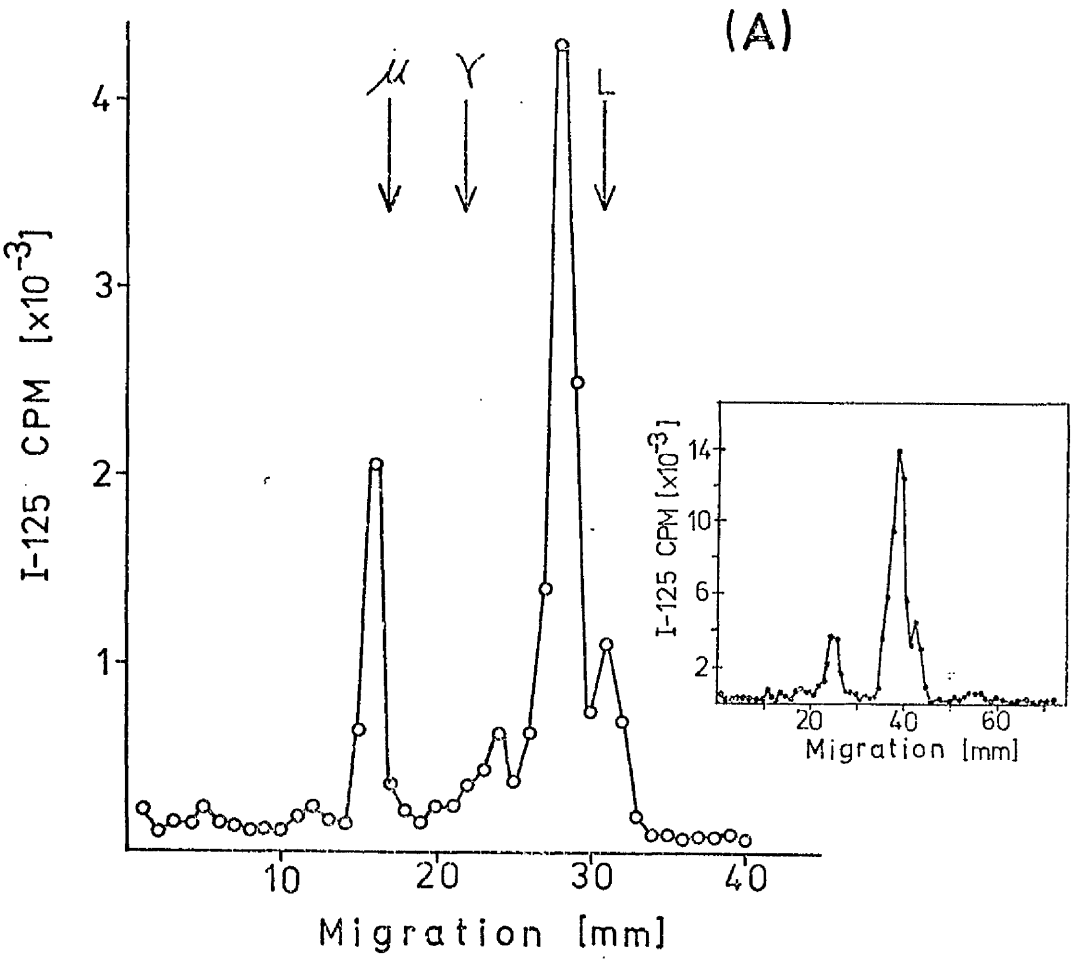
SDS-PAGE Profiles of Daudi Cell Surface and Intracellular IgM Showing Presence of p33 Peak

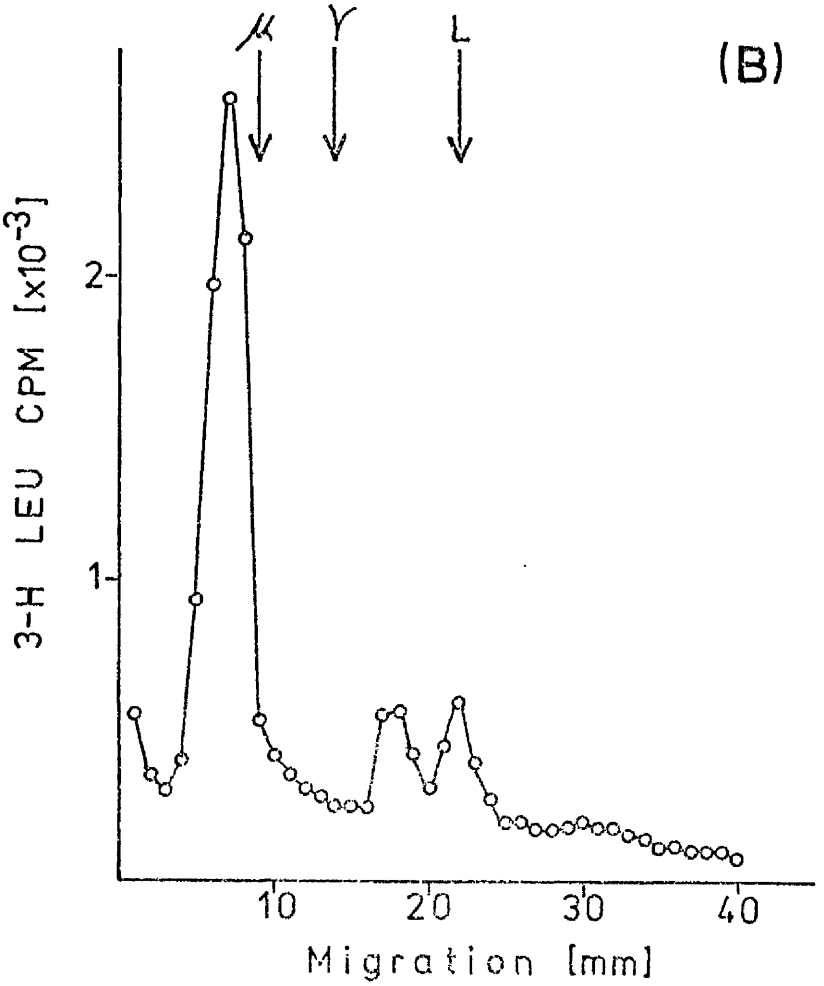
Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-125, 0.25 mCi) as described in Methods, section 8.2, and the cells lysed in 1 ml NP-40 lysis buffer containing PMSF and iodoacetate (Methods, 8.3). Immunoprecipitation from the lysate was carried out by addition of 2 µl anti-IgM (15 min at 4°C) followed by 100 µg GARIgG (15 min at 4°C).
For biosynthetic labelling, 5 x 10^6 cells from log phase cultures were incubated for 6 hr in 1 ml labelling medium containing 62.5 µCi 3H-leucine (Methods, 8.1). The cells were separated from the culture medium. Cell lysis and immunoprecipitation were carried out as described above.
Washed immunoprecipitates were dissolved in 75 µl SDS-PAGE sample buffer and half removed for reduction and alkylation (Methods, 12.4). Electrophoresis was in phosphate-buffered tube gels of either 6% polyacrylamide for the radioiodinated sample, or 10% polyacrylamide (DATD-crosslinked) for the biosynthetic sample (Methods, 12). Radioactivity in individual gel slices was determined for the biosynthetic samples by scintillation counting of the periodic acid solubilised slices (Methods, 17.2), or for the radioiodinated samples by gamma counting. Molecular weight marker µ, γ, and light chains were electrophoresed in separate 6% or 10% polyacrylamide tube gels.

(A) Cell Surface IgM (Radioiodinated)

(B) Intracellular IgM (Biosynthetic)

Inset Kennel, 1974. Anti-µ precipitate from purified membranes of surface iodinated Daudi cells, SDS-PAGE was in a 6% polyacrylamide tube gel.


(A)
μ
γ
L
I-125 CPM [x10⁻³]
4
3
2
1
10
20
30
40
Migration [mm]
I-125 CPM [x10⁻³]
14
10
6
2
20
40
60
Migration [mm]


(B)
μ
γ
L
3-H LEU CPM [x10⁻³]
2
1
10
20
30
40
Migration [mm]

proteins occur in SDS-PAGE profiles of immunoprecipitates, several arguments against this trivial explanation were apparent from the experimental observations. These arguments were:-

(i) The p33 peak was very highly labelled relative to Daudi μ-chain, whereas lactoperoxidase iodination is known to label μ-chains of sIgM preferentially over L-chains (Vitetta et al. 1971; personal observation).

(ii) The small peak exactly comigrated with the κ-chain marker and, in addition, the lesser label in this peak relative to the μ-chain peak was consistent with it being Daudi κ-chain.

(iii) No spurious peaks were seen in the SDS-PAGE profiles from the nine other cell sIgs investigated in this experiment.

(iv) Biosynthetic labelling also showed Daudi cells to have well resolved p33 and κ-chain peaks in the SDS-PAGE profile of its reduced sIgM (Section 3.2 and Fig. 3.1(B)).

These observations supported in two ways the interpretation that the p33 peak was indeed an 'extra' component of Daudi sIgM. First, it was unnecessary to identify p33 as the Daudi κ-chain because the SDS-PAGE profile contained a small peak representing an apparently normal κ-chain. Equally, it was unlikely that p33 was an abnormally large κ-chain because of its unusually high degree of labelling.

3.1.3. Comparison to Findings of Kennel (1974)

The published findings of Kennel (1974) are interesting with regard to this dilemma. He also investigated the sIgM of Daudi cells by lactoperoxidase iodination, immunoprecipitation, and SDS-PAGE of the reduced immunoprecipitates. The SDS-PAGE profile Kennel obtained of reduced Daudi sIgM isolated from pre-purified plasma membranes (Fig. 3.1(A), inset) is virtually identical to the profile I obtained. Kennel did not comment, however, on the presence of the small κ-chain peak or on the unusual labelling pattern of the 33,000 molecular weight peak, and may have overlooked the significance of these observations. In the event, he came to the erroneous conclusion that the p33 peak represented an abnormally large κ-chain.

3.2. BIOSYNTHETIC LABELLING OF p33

3.2.1. SDS-PAGE Analysis

As part of the screening experiment described in the previous section (3.1.1), cellular proteins were labelled by biosynthetic incorporation of 3H-leucine for 6 hr. Labelled cellular Ig was analysed by immunoprecipitation and SDS-PAGE on 10% polyacrylamide gels.

The SDS-PAGE profile of the reduced Daudi anti-IgM (μ-specific) immunoprecipitate (Fig. 3.1 (B)) shows the presence of three peaks of radioactivity. Besides μ-chain and κ-chain peaks, a third small peak was resolved. This peak was assumed to be the 3H-leucine labelled analogue of the p33 peak which was detected by surface iodination. This assumption was based on the similarity of the three-peak profiles obtained for Daudi sIgM by both labelling methods. In contrast, none of the other cell lines investigated showed such a profile.

The coincident migration of biosynthetically labelled and iodinated p33 has also been confirmed by SDS-PAGE on high resolution slab gels using 35S-methionine as the incorporated label (data not shown).

3.2.2. Synthesis of p33 by Daudi Cells

The biosynthetic labelling result confirmed p33 to be an actual product of Daudi cells, and not merely a serum protein from the culture medium which had become attached to the cell surface. This distinction cannot be made using lactoperoxidase catalysed iodination, although it is generally accepted that cells in long term tissue culture do not display foreign proteins on their surfaces.

3.2.3. Characteristics of Amino Acid Incorporation

In general, biosynthetic labelling of p33 was inefficient when compared with lactoperoxidase iodination. Fig. 3.1 (B) shows the amount of label incorporated into p33 to be similar to that for the κ-chain, and very much less than the label incorporated into the μ-chain of Daudi cells. This contrasts sharply with the pattern of iodinated Daudi sIgM polypeptide chains (compare Figs. 3.1 (A) and 3.1 (B)). Often in biosynthetic labelling p33 could not be detected at all by SDS-PAGE analysis on slab gels, or could be detected only with very long-exposure autoradiography. This phenomenon appears to reflect the rate of synthesis and turnover of p33, in that it was related to the labelling conditions used. Thus, incorporation of labelled amino acids for 1-3 hr was found to be better than a similar incorporation period followed by a long (6-18 hr) chase with non-labelled amino acids. This labelling pattern is consistent with that found in general for μ-chains and L-chains of sIgM and implies but does not prove that in human lymphoma cells these surface molecules

have a rather more rapid rate of turnover than that suggested by the work on mouse splenocytes (Melchers & Andersson, 1973). It has not proved possible, however, to obtain more quantitative information on the rate of synthesis and turnover of Daudi p33, principally because of its extremely low labelling efficiency.

3.3. MOLECULAR WEIGHT ESTIMATION OF p33

3.3.1. Previously Reported Value

A molecular weight estimation of 32,4000 to 34,000 $\pm$ 2,000 was given for Daudi p33 by Kennel (1974), based on SDS-PAGE on gels of 6% to 12.5% polyacrylamide and using four non-glycoprotein standards. This represents the only published information available.

3.3.2. Determination Based on SDS-PAGE Analysis

Estimates of the molecular weight of p33 which I have obtained from my own data were made from plots of log molecular weight versus migration (Fig. 3.2), based on the SDS-PAGE profiles shown in Fig. 3.1 and using μ, γ, and L-chains as standards. The molecular weights used for γ and L-chains were 55,000 and 23,000 respectively. For secretory μ-chains an apparent molecular weight of 83,000 was determined by separate SDS-PAGE analysis (Fig. 3.2, inset) using the σ, and α-chains of RNA polymerase (Coggins et al., 1977), IgG γ-chains, and actin as standards. This determination was necessary because the glycoprotein nature of the immunoglobulin μ-chain causes its SDS-PAGE mobility to be anomalously slower than expected from its known molecular weight. Using the above method, and averaging the values (32,000 and 35,000) obtained from each SDS-PAGE profile, a molecular weight of 33,500 was determined for p33.

Fig. 3.2

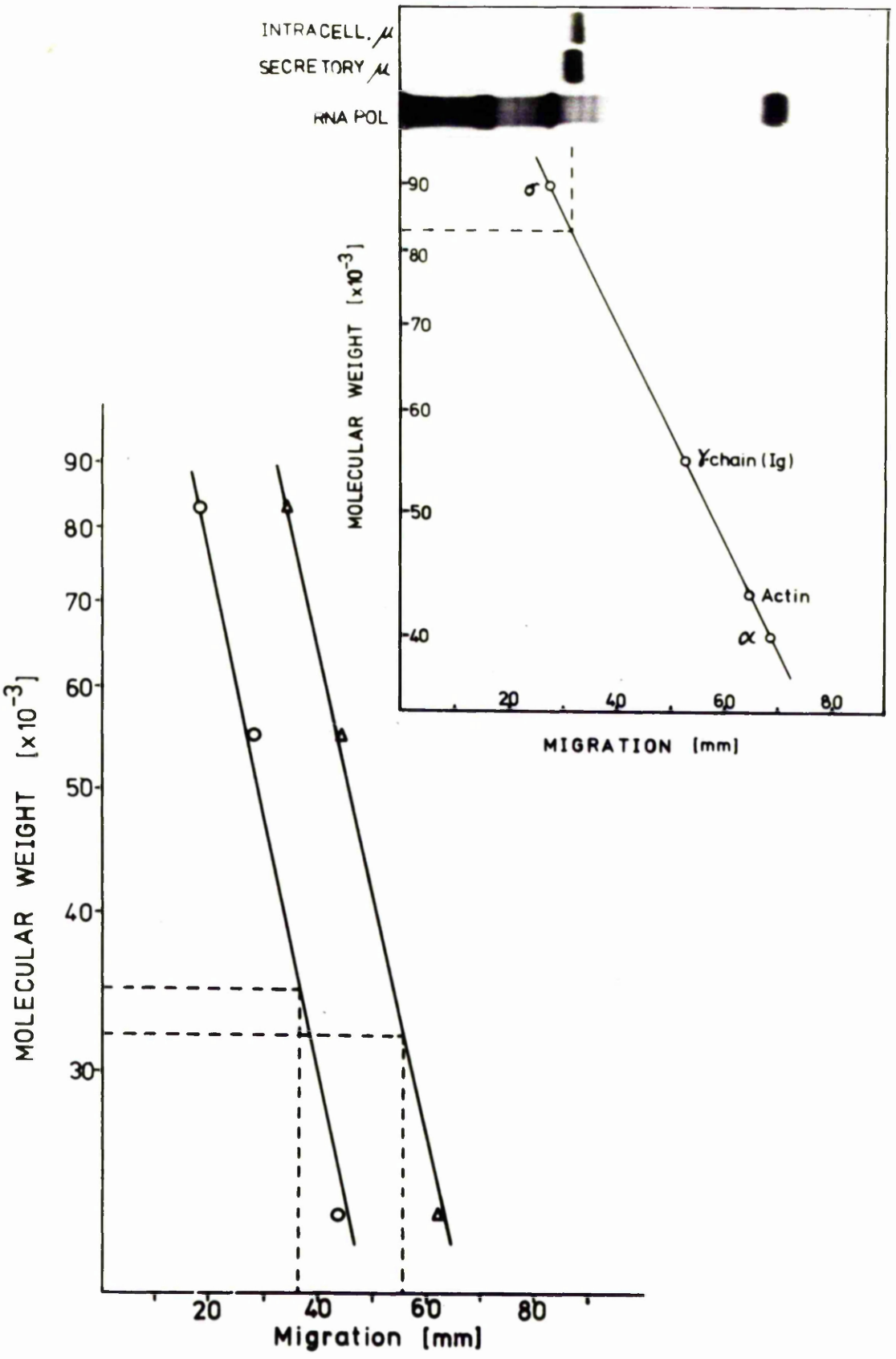
Semi Log Plot for Molecular Weight Determination of p33

The SDS-PAGE mobilities of the marker μ, γ, and light chains from Fig. 3.1 are plotted versus their molecular weights of 83,000, 55,000, and 23,000 respectively on a log scale.

(Δ———Δ) from Fig. 3.1 (A)
(O———O) from Fig. 3.1 (B)

The broken lines show the SDS-PAGE mobility and apparent molecular weight of p33 from each SDS-PAGE profile, as determined from these plots.

Inset For determination of μ-chain molecular weight.
The SDS-PAGE mobilities of the marker proteins RNA pol σ, IgG γ-chain, actin, and RNA pol α are plotted versus their molecular weights of 90,000, 55,000, 43,000 and 40,000 respectively on a log scale. The broken line shows the SDS-PAGE mobility and apparent molecular weight of secretory μ-chain as determined from this plot. All mobilities were plotted from the SDS-PAGE profile shown above obtained on a discontinuous Tris-glycine buffered slab gel of 12.5% polyacrylamide. The actin and IgG γ-chain bands are not shown.


INTRACELL. μ
SECRETORY μ
RNA POL
MOLECULAR WEIGHT [x10⁻³]
90
80
70
60
50
40
σ
γchain (Ig)
Actin
α
20
40
60
80
MIGRATION [mm]
MOLECULAR WEIGHT [x10⁻³]
90
80
70
60
50
40
30
20
40
60
80
Migration [mm]

3.3.3. Sources of Errors

The above results indicate a significant disparity in the values from the two SDS-PAGE profiles. While no clear explanation for this disparity can be given, several significant sources of error in this molecular weight determination deserve comment:-

(i) The marker μ, γ, and L-chains were, in both cases, electrophoresed on separate gels run in parallel with those loaded with the Daudi sIgM immunoprecipitates. Under these circumstances, mobility comparisons were found to be accurate to only ± 1 mm.

(ii) Molecular weight estimations of glycoproteins from SDS-PAGE on gels of different percentage acrylamide can vary considerably - higher percentage gels giving lower apparent molecular weights. This effect has been shown to be particularly pronounced in the case of μ-chains (Melcher & Uhr, 1976).

Because of the above considerations, this estimation of the molecular weight of p33 must be regarded as only approximate. However, the average value of 33,500 is in good agreement with the average of 33,000 which is reported by Kennel (1974) and so this latter value will be used for the purpose of further discussion.

3.4. RESOLUTION OF p33 BY DISCONTINUOUS SDS-PAGE ON SLAB GELS

Discontinuous pH tris-glycine slab gels proved to be vastly superior to the continuous phosphate-buffered cylindrical gels for the SDS-PAGE analysis of cell surface Ig. The discontinuous SDS-PAGE system gave the high resolution necessary to detect subtle mobility differences in

μ-chains isolated from different cell lines (Chapter 2, Section 2.1) and also resolved L-chains of sIg as fine bands of characteristic mobilities (Chapter 1, Section 1.3.5).

Tris-glycine SDS-PAGE resolved Daudi p33 as a diffuse band of radioactivity, quite distinct however from the Daudi κ-chain band (Fig. 3.3,track 6). This clear separation of the two bands, as well as their characteristic shapes on electrophoresis (p33 diffuse and κ-chain sharp) was considered to be strong evidence that p33 was a unique 'extra' component of Daudi sIgM. The p33 band was similarly seen in all subsequent experiments involving the SDS-PAGE analysis of Daudi sIgM.

3.5. TEST FOR NONSPECIFIC BINDING OF p33 TO IMMUNOPRECIPITATES

Having established that p33 was a unique component of the Daudi sIgM immunoprecipitate, it was of interest to determine if it was an integral part of Daudi sIgM or alternatively, if its presence was the result of non-antigenic binding to the immunoprecipitate.

Non-antigenic binding to immunoprecipitates was considered not merely as a spurious effect, but also as one with possible functional significance. The B-lymphocyte F_c receptor, for example, is known to bind to antigen-antibody complexes very similar to those formed during indirect immunoprecipitation. In another context, it has been suggested (Premkumar et al., 1975a, 1975b) that a type of non-antigenic binding to immunoprecipitates may also detect 'anchor' proteins which have been postulated to bind the hydrophilic C-termini of sIgs into the hydrophobic cell membrane.

Fig. 3.3

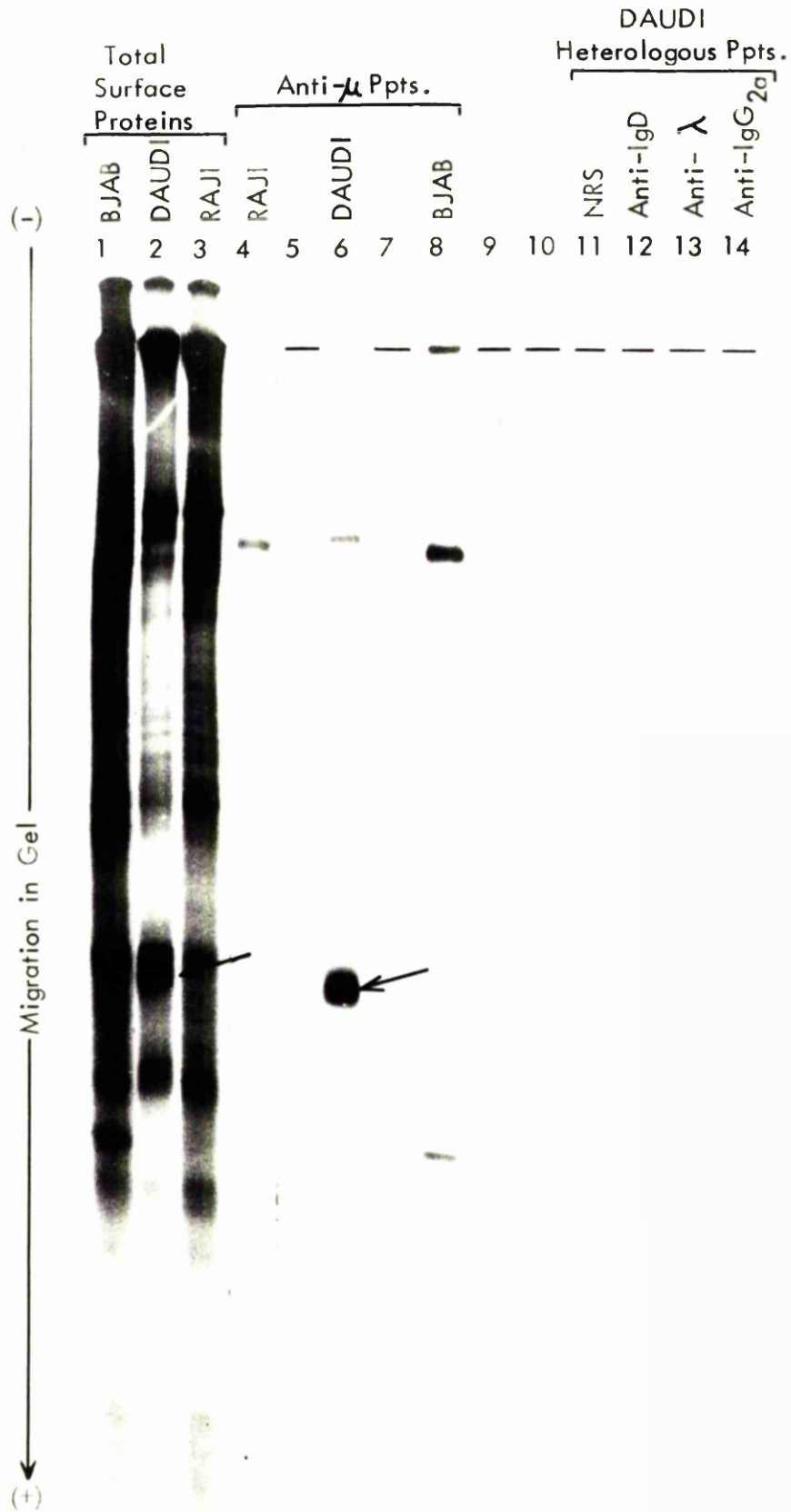
SDS-PAGE Analysis Showing p33 in Daudi Total Surface Proteins and the Specificity of p33 Immunoprecipitation

Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-131, 2 mCi) as described in Methods, section 8.2, and the cells lysed in 0.5 ml NP-40 lysis buffer containing PMSF (Methods, 8.3).. Aliquots of lysate were removed for acetone precipitation (Methods, 8.4.1) or immunoprecipitation as follows:-

Sample	Amount of Lysate	Counts/min
Total Surface Proteins		
*BJAB	5 µl (1%)	202,000
Daudi	" "	197,000
*Raji	" "	139,000
Anti-µ Precipitates		
*Raji	25 µl (5%)	5,000
Daudi	" "	34,000
BJAB	" "	35,000
Daudi Non-Specific Precipitates		
NRS	25 µl (5%)	1,250
Anti-IgD	" "	1,400
Anti-λ	" "	1,100
Anti-5563 (Mouse γ_{2a})	" "	1,550

Immunoprecipitations were carried out by addition of either 5 µg RAHIgM or 0.5 µl antiserum (15 min at 20°C) followed by 25 µg GARIgG (1 hr at 20°C, or overnight at 4°C). Washed acetone and immune precipitates were dissolved in reducing sample buffer for discontinuous SDS-PAGE in a 12.5% polyacrylamide slab gel (Methods 11). The gel was processed for fluorography (Methods 17.4) and the fluorogram developed after an exposure of 8 hr.

*These tracks were printed at higher sensitivity.


Total Surface Proteins
Anti-μ Ppts.
DAUDI Heterologous Ppts.
BJAB
DAUDI
RAJI
RAJI
DAUDI
BJAB
NRS
Anti-IgD
Anti-λ
Anti-IgG2a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
(-)
Migration in Gel
(+)

In order to test the affinity of p33 for antigen-antibody complexes, NP-40 lysates of Daudi cells labelled by lactoperoxidase iodination were subjected to indirect immunoprecipitation using either non-immune rabbit serum, or non-crossreacting rabbit antisera to human IgD, Bence-Jones lambda, or mouse IgG_{2a} myeloma protein. The results (Fig. 3.3, tracks 11-14) showed that no p33 was precipitated in these immunoprecipitates. In contrast, antibody directed against Daudi sIgMκ (μ or κ-specific) always precipitated p33 along with the μ and κ-chains (Fig. 3.3, track 6). It was possible to conclude from this data that p33 was not a nonspecific component of Daudi immunoprecipitates. Moreover, the effectiveness of both μ and κ-specific antisera strongly suggested that p33 was an integral part of the sIgM H_2L_2 complex, and not a separate antigenically related protein or fragment.

3.6. EXPRESSION OF p33 RESTRICTED TO DAUDI CELLS

3.6.1. Analysis of Total Surface Proteins from Several Lymphoma Cells

In order to determine whether p33 expression on the cell surface was unique to Daudi cells, or if only the association of p33 with Daudi sIgM was unique, total surface proteins from several lymphoma cell lines were compared.

Cell surface proteins were labelled by lactoperoxidase iodination and isolated from NP-40 cell lysates by precipitation with acetone. The labelled surface proteins were reduced and analysed by SDS-PAGE on Tris-glycine buffered slab gels of 12.5% polyacrylamide.

The SDS-PAGE profiles obtained in one such experiment from BJAB, Daudi,

and Raji-ATG cells are shown in Fig. 3.3, tracks 1-3. A comparison of these profiles with that obtained from immunoprecipitated Daudi sIgM (track 6) unambiguously identifies the p33 band amongst the total surface proteins of Daudi cells. In contrast, the profiles of Raji-ATG and BJAB surface proteins show no band of equal intensity. This observation (i.e. lack of p33) has also been extended to the U698M and Namalwa cell lines.

3.6.2. Implications of Findings

Several explanations are consistent with the above result:-

(i) p33 is unique to Daudi cells, of those cell lines tested.

(ii) p33 is expressed on the surface of other lymphoid cells but at much lower levels than on Daudi cells.

(iii) p33 is expressed on the surface of other lymphoid cells but is only exposed to surface radioiodation on Daudi cells.

Of course, alternatives (ii) and (iii) are not mutually exclusive. However, we know of no evidence to suggest that exposure to surface radioiodination is particularly variable, and therefore possibility (iii) is not considered likely. Further experiments are currently underway to prepare anti-p33 antiserum for the purpose of looking in a more sensitive way for p33 on the surface of other lymphoid cells.

3.7. INSENSITIVITY OF p33 TO CELLULAR PROTEASES

3.7.1. Rationale for Investigation of Proteolysis

The sensitivity of p33 to cellular proteases was investigated to test the hypothesis that p33 might be the result of proteolytic cleavage of Daudi sIgM μ-chains. This hypothesis would be consistent with the available data if it was postulated that some of the μ-chains within

the H_2L_2 structure were cleaved, but in such a way that the integrity of the H_2L_2 structure itself was undamaged. This would specifically explain the serological data which showed both anti-μ and anti-κ antisera to precipitate p33 equally well. In one possible mechanism, where the cleavage is postulated to occur at the Daudi μ-chain "hinge" region, two fragments of approximately p33 size could result. Upon reduction and SDS-PAGE analysis, these fragments might be expected to coelectrophorese as a broad band of radioactivity identical to the p33 band.

The recent findings that δ-chains of murine sIgD are highly sensitive to cleavage by papain (Vitetta & Uhr, 1976; Cambier et al., 1977) added impetus to the above hypothesis. Goyert et al. (1977) have also demonstrated spontaneous cleavage of human serum IgD. It has been postulated (Vitetta & Uhr, 1976) that specific proteolytic cleavage of sIgD may have a functional role in antigen-triggered lymphocyte activation. Although μ-chains are not reported to be similarly sensitive, the possibility that Daudi μ-chains were exceptional in this respect could not be ruled out a priori.

3.7.2. Experimental Observations and Conclusions

If Daudi sIgM μ-chains were sensitive to proteolytic cleavage giving rise to p33, it may be expected that inhibition of proteolysis during cell lysis and immunoprecipitation would result in decreased detection of the p33 band on SDS-PAGE analysis. Indeed, more efficient detection of sIgD in mouse spleenocytes has been reported when proteolysis was inhibited by a combination of rapid handling of cell lysates of 4°C and the use of the protease inhibitors PMSF and

iodoacetamide (Abney et al., 1976). In order to test this idea with respect to p33, data from several experiments in which iodinated Daudi sIgM was isolated under various conditions were compared.

TABLE 3.1. EFFECT OF PROTEOLYSIS ON p33 DETECTION

Experiment	Figure	Incubation Conditions	Protease Inhibitors	Ratio p33:μ
I	3.1(A)	30 min at 4°C	PMSF, IAM	*2.7
II	3.3, track 6	70 min at 20°C o/n at 4°C	PMSF	2.5
III	1.2, track 8	60 min at 20°C o/n at 4°C	PMSF	2.7
IV	1.3, track 2	75 min at 20°C o/n at 4°C	PMSF	2.4

*Estimated from peak heights

Table 3.1 summarizes the conditions used for the isolation of Daudi sIgM in four separate experiments. A visual comparison of the relevant figures for each experiment shows that no detectable change in the SDS-PAGE profile with regard to p33 resulted from short incubations on ice. Moreover, the addition of the protease inhibitors PMSF and iodo-acetamide also had no observable effect.

In a more sensitive analysis of these SDS-PAGE profiles, the ratio of radioactivity in the μ-chains and p33 bands was compared. The radioactivity in each band was determined by direct gamma counting, and the data are shown in the last column of Table 3.1. In confirmation of the visual comparison, the ratio of the counts in the μ-chain and p33 bands shows no appreciable variation with the conditions of isolation employed.

By analogy to the situation with sIgD, where the sensitivity of the δ -chain to proteolytic cleavage results in observable degradation during isolation, the above data argue against such a cleavage of sIgM being responsible for p33. However, since the above analysis applies only to cleavage during cell lysis and immunoprecipitation, the possibility of such a cleavage occurring before cell lysis was not formally excluded.

3.8. NATURE OF DAUDI sIgM - p33 ASSOCIATION

3.8.1. SDS-PAGE Analysis of Unreduced Daudi sIgM

To further the investigation of p33, it was of interest to determine whether its association with Daudi sIgM involved disulfide linkage. To this end, iodinated Daudi sIgM isolated by immunoprecipitation was subjected to SDS-PAGE analysis without prior reduction. To insure complete dissociation of non-covalent bonds, the sIgM-containing immunoprecipitate was heated at 100° for 10 min in 2% SDS-6M urea before application to the gel. Fig. 3.4(A) shows the SDS-PAGE profile obtained on a 3% polyacrylamide-0.5% agarose gel. Two peaks of radioactivity were detected - a major peak of approximately 10S (Peak I) and a minor peak of approximately 6S (Peak II). The migration of these two peaks was consistent with them being sIgM H_2L_2 and HL molecules respectively (Kennel & Lerner, 1973, have previously reported the presence of both H_2L_2 and HL molecules in unreduced sIgM-containing immunoprecipitates from the Wil-2 lymphoid cell line). In Fig. 3.4(A) no peak corresponding to 33,000 molecular weight was seen, indicating that the p33 was migrating covalently bound to either the Daudi sIgM H_2L_2 or HL peak.

Fig. 3.4

SDS-PAGE Profiles of Unreduced Daudi sIgM Demonstrating the Presence of Disulphide Bonded p33

(A) Daudi sIgM (Unreduced)

Cell surface proteins from 1 x 10^7 cells were labelled by lactoperoxidase iodination (I-125, 0.25 mCi) as described in Methods, section 8.2, and the cells lysed in 1 ml NP-40 lysis buffer containing PMSF and iodoacetate (Methods, 8.3). Immunoprecipitation from the lysate was carried out by addition of 2 µl anti-IgM (15 min at 4°C) followed by 100 µg GARIgG (15 min at 4°C). The washed immunoprecipitate was dissolved in 75 µl SDS-PAGE sample buffer, and half removed for electrophoresis in a phosphate-buffered tube gel of 3% polyacrylamide-0.5% agarose (Methods, 12). The radioactivity in individual gel slices was determined by gamma counting.

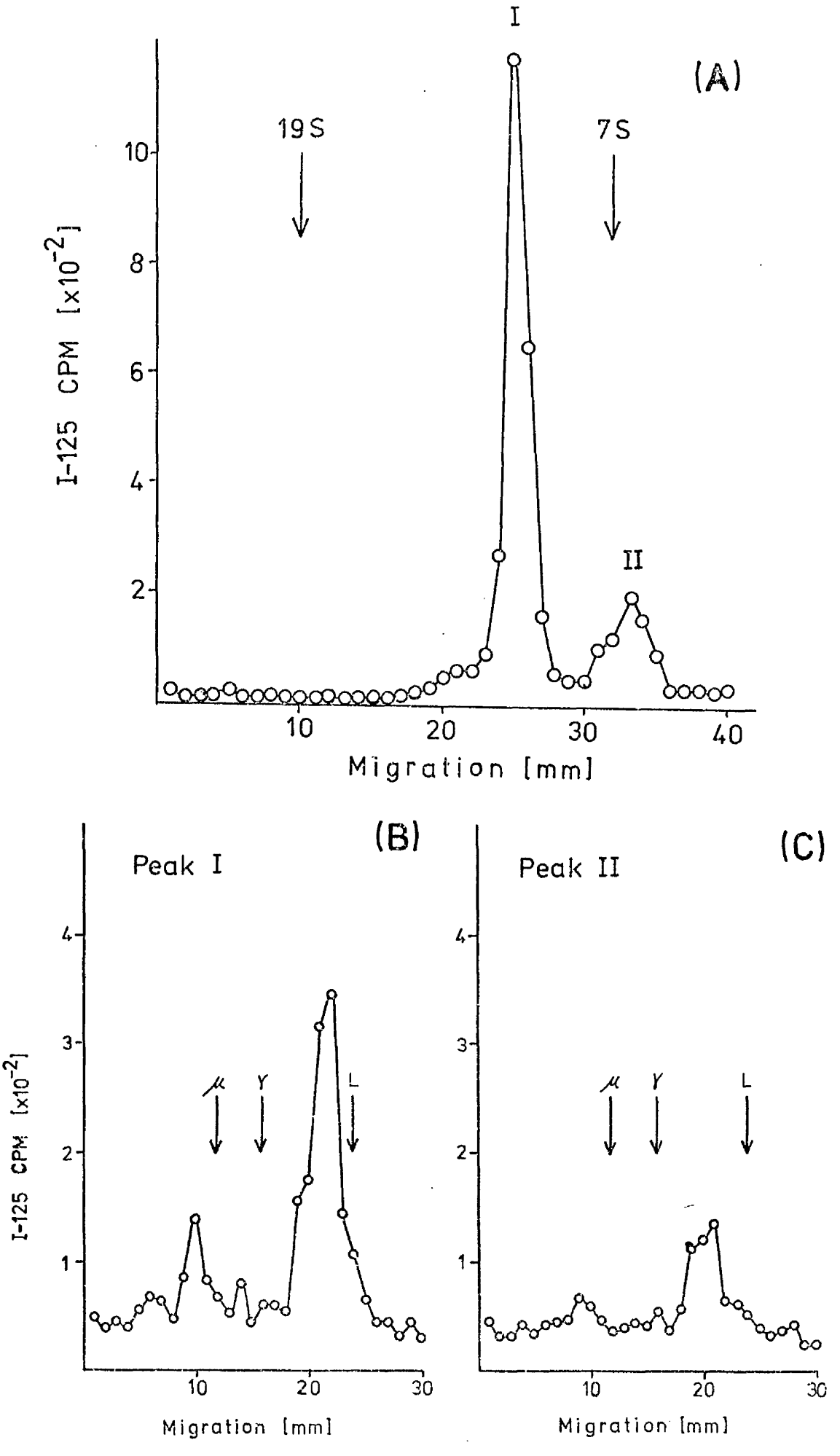
(B) Peak I (Reduced)

(C) Peak II (Reduced)

Gel slices 25 and 33 (Peak I and Peak II) shown in (A) were dried and rehydrated with SDS-PAGE sample buffer containing 50 mM DTT, and incubated in a humid chamber (30 min at 37°C). Iodoacetamide was then added to a final concentration of 125 mM (30 min at 20°C). The gel slices were then placed on top of phosphate-buffered tube gels of 6% polyacrylamide and electrophoresed (Methods, 12).

The radioactivity in individual gel slices was determined by gamma counting.

Molecular weight markers 195 IgM, 75 IgG, µ, γ, and light chains were electrophoresed in separate tube gels.


(A)
I
19S
7S
II
I-125 CPM [x10^{-2}]
Migration [mm]
(B)
Peak I
(C)
Peak II
μ
γ
L
I-125 CPM [x10^{-2}]
Migration [mm]

3.8.2. SDS-PAGE of Reduced Daudi sIgM H_2L_2 and HL Peaks

To confirm the presence of p33 in these peaks, the following experiment was performed. The slices of the gel shown in Fig. 3.4(A) corresponding to each of the two peaks (Nos. 25 and 33) were saved and the labelled material reduced and alkylated *in situ*. The slices were then placed on top of cylindrical gels of 6% polyacrylamide and electrophoresed. Figs. 3.4 (B) and (C) show the results of the electrophoresis of reduced material from peaks I and II respectively. Peak I appears to be composed of μ-chain, p33, and κ-chain in approximately the same proportions as seen in the fully reduced immunoprecipitate. This result confirmed that disulfide linkage was involved in the association of p33 with Daudi sIgM H_2L_2 molecules. Peak II also appears to be composed of μ-chain, p33, and κ-chain, suggesting that this peak represents a complex of Daudi HL plus p33. However, from this data it was not possible to characterise peak II with certainty.

3.9. MOLECULAR WEIGHT OF DAUDI sIgM-p33 COMPLEX: STOICHIOMETRY OF p33 BINDING

3.9.1. Rationale for Investigation of Molecular Weight

Previous results had established that p33 was covalently associated with Daudi sIgM H_2L_2, and also probably HL molecules, on the cell surface. If p33 was an additional component of these sIgM molecules (and not, for instance, a fragment of μ-chain), it would be expected to confer a higher molecular weight on the unreduced Daudi sIgM, and thus a slower SDS-PAGE mobility. Moreover, the extent of difference in apparent molecular weight between Daudi sIgM and other cell sIgMs would indicate the stoichiometry of p33 binding.

3.9.2. SDS-PAGE Analysis

To determine the apparent molecular weight of Daudi sIgM molecules in comparison with those of other cell lines, continuous phosphate buffered SDS-PAGE analysis was performed on the unreduced sIg molecules of cell lines Bec-11 (sIgG), BJAB (sIgM), U698M (sIgM), and Daudi (sIgM). Electrophoresis was carried out on a slab gel for accurate comparison of the bands; and polymerised BSA was used to provide molecular weight calibration in the desired range of 200,000 to 300,000 daltons.

The results of this SDS-PAGE analysis are shown in Fig. 3.5. The sIgM of cell lines BJAB, and U698M migrates as two distinct bands, suggesting H_2L_2 and HL structures respectively. Significantly, the corresponding Daudi molecules migrate with higher apparent molecular weights, which was consistent with an additional contribution to Daudi sIgM by covalently associated p33. This result was also seen in a similar SDS-PAGE analysis where the mobility of Daudi sIgM was compared with that of BJAB sIgM (data not shown).

3.9.3. Semi-Log Plot for Molecular Weight Determination

Molecular weight estimations for the sIgM molecules have been obtained from a semi-log plot of molecular weight versus migration, using the polymerised BSA as a standard (Fig. 3.6). BJAB and U698M H_2L_2 and HL molecules have apparent molecular weights of 260,000 and 142,000 respectively. These values are in close agreement with those reported by Kennel & Lerner (1973) for Wil-2 lymphoid cell sIgM.

In contrast to the above values, the Daudi sIgM molecules have apparent molecular weights of 292,000 and 165,000. These values represent an increase in molecular weight over the normal (BJAB and U698M) H_2L_2 and

Fig. 3.5

SDS-PAGE Mobilities of the Daudi $\mu_2\kappa_2$-p33 Complex and Other Cell Surface $\mu_2\kappa_2$ Molecules

Cell surface proteins from 2 x 10^7 cells were labelled by lactoperoxidase iodination (I-131, 1 mCi) as described in Methods, section 8.2, and the cells lysed in 0.5 ml NP-40 lysis buffer containing PMSF (Methods, 8.3). Immunoprecipitations were carried out by addition of 10 µg RAHIgM to 40% of each lysate, or 10 µg RAHIgG to 40% of the Bec-11 lysate (15 min at 20°C) followed by 50 µg GARIgG (1 hr at 20°C, overnight at 4°C). Washed immunoprecipitates were dissolved in 9 M urea and aliquots (one-eighth) precipitated with acetone and redissolved in SDS-PAGE sample buffer for electrophoresis in a phosphate-buffered 5% polyacrylamide slab gel (Methods, 12). The gel was stained in a solution of 0.1% w/v Coomassie Blue Dye in methanol:water:acetic acid (45:45:10), processed for autoradiography (Methods, 17.3), and the autoradiogram developed after an exposure of 8 days. The marker proteins (poly BSA) were a kind gift from Dr. Keith Whaley, Pathology, Western Infirmary, Glasgow.

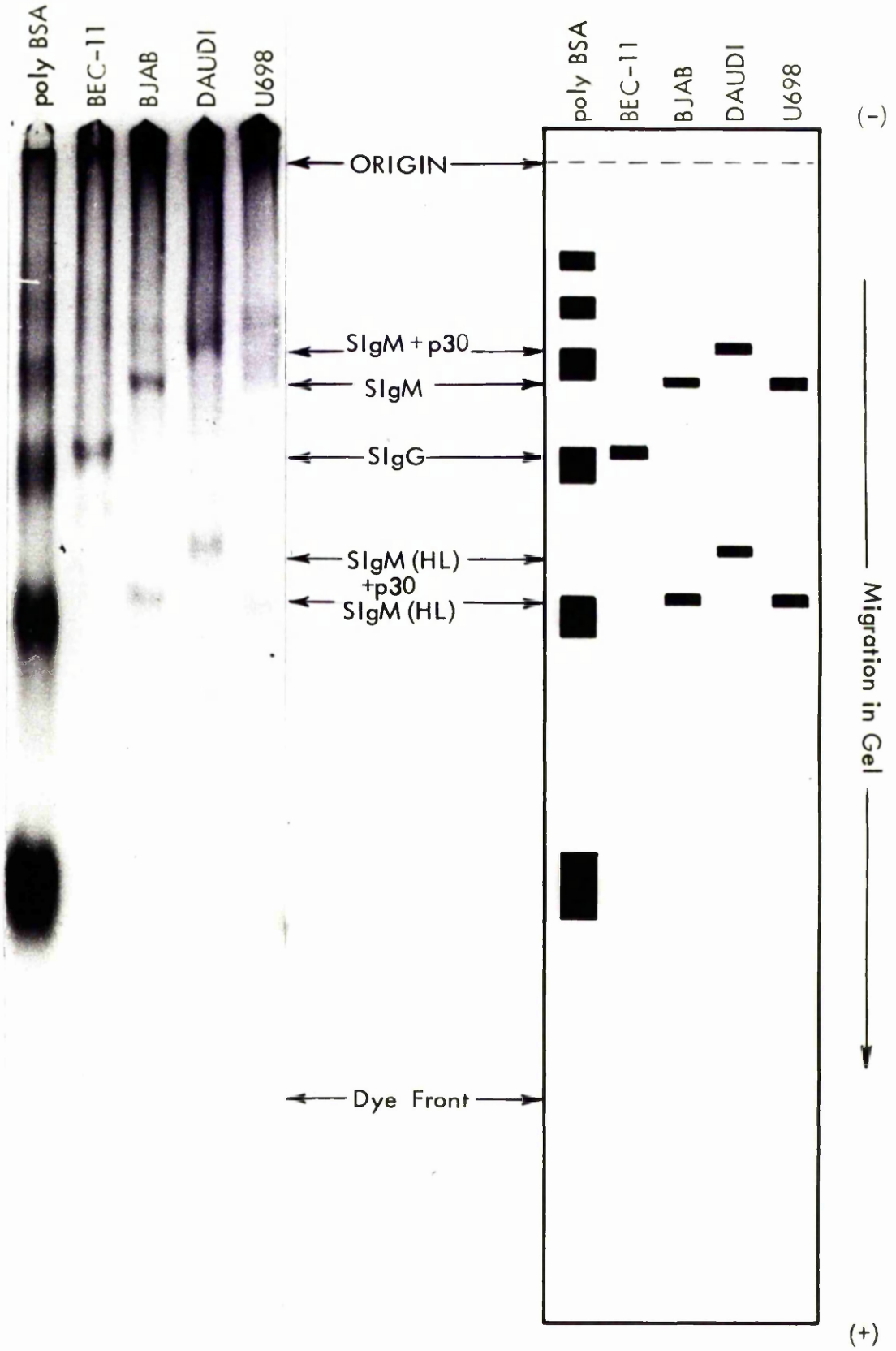
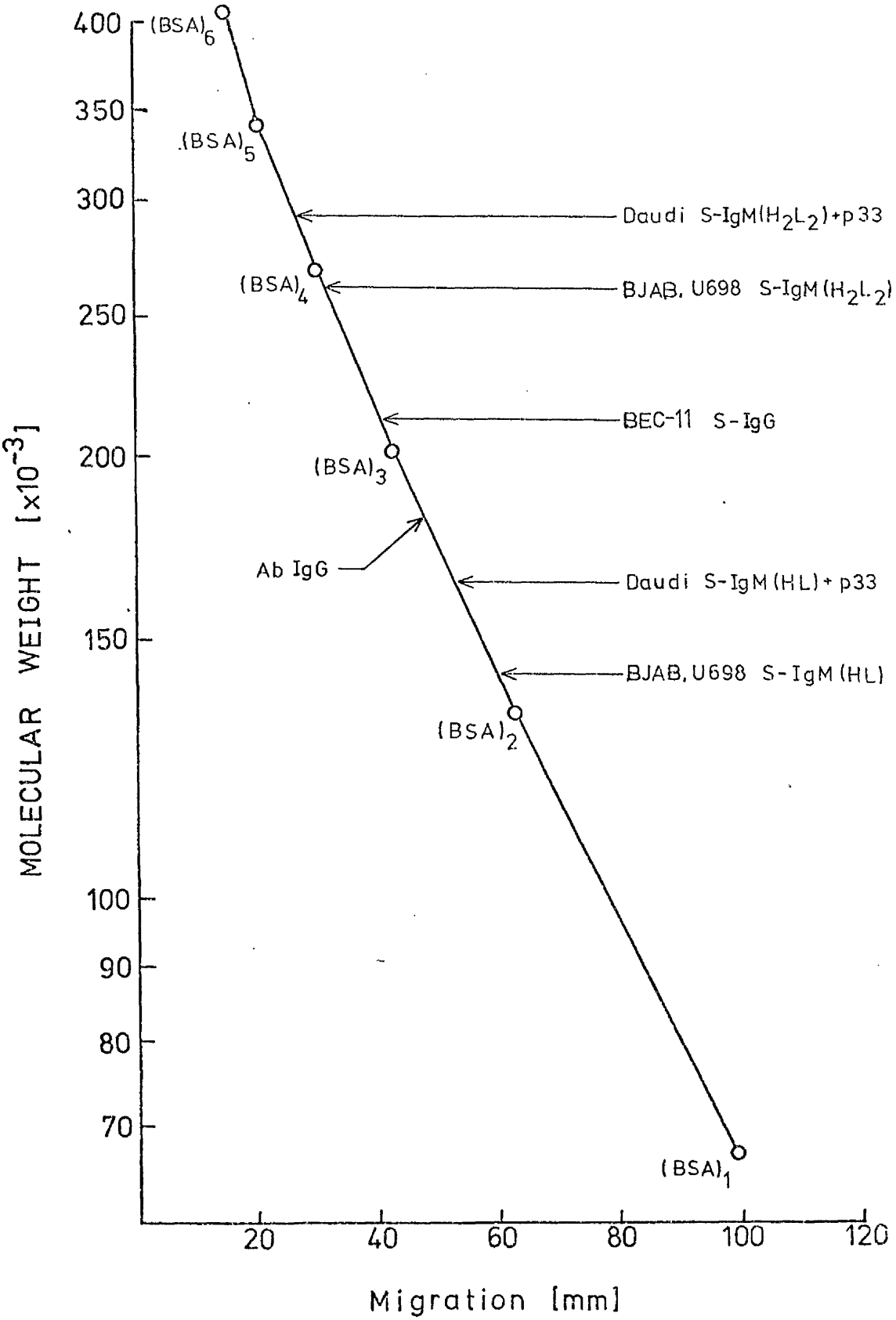

poly BSA
BEC-11
BJAB
DAUDI
U698
ORIGIN
SIgM + p30
SIgM
SIgG
SIgM (HL)
+p30
SIgM (HL)
Dye Front
(-)
(+)
Migration in Gel

Fig. 3.6

Semi Log Plot for Molecular Weight Determination of Cell Surface $\mu_2\kappa_2$ and $\mu_2\kappa_2$-p33 Moieties

The SDS-PAGE mobilities of the poly BSA marker proteins from Fig. 3.5 are plotted versus their molecular weights (BSA=67,000) on a log scale. The arrows indicate the mobilities and corresponding molecular weights of the unreduced surface Ig moieties, as determined from this plot.


400
350
300
250
200
150
100
90
80
70
MOLECULAR WEIGHT [x10^{-3}]
(BSA)$_6$
(BSA)$_5$
(BSA)$_4$
(BSA)$_3$
(BSA)$_2$
(BSA)$_1$
Daudi S-IgM(H$_2$L$_2$)+p33
BJAB, U698 S-IgM(H$_2$L$_2$)
BEC-11 S-IgG
Ab IgG
Daudi S-IgM(HL)+p33
BJAB, U698 S-IgM(HL)
20
40
60
80
100
120
Migration [mm]

HL molecules of 32,000 and 23,000 respectively. This data can serve as a basis for determining the stoichiometry of p33 binding, with the following assumptions:

(i) p33 is the only additional component of the Daudi H_2L_2 and HL molecules.

(ii) The molecular weight contribution of p33 to the H_2L_2 and HL molecules is equal, within experimental error, to its own molecular weight of 33,000.

(iii) The molecular weight difference contributed to the complexes by differences in μ and κ-chains mobilities is insignificant compared with that contributed by p33. In this regard, it should be noted that Daudi μ and κ-chains are of slightly higher apparent molecular weight than those from BJAB and U698M cells (chapter 1, Sections 1.3.4 & 1.3.6)

3.9.4. Interpretation of Results

Based on the above assumptions the data are consistent with a stoichiometry of one p33 molecule associated with both the Daudi H_2L_2 and HL bands. In the case of the HL band the calculated variance of 23,000, rather than the expected value of 33,000, can be attributed to inaccuracy in molecular weight determination. Other features of this result require comment, however. If the HL band seen on SDS-PAGE analysis arose from cleavage of H_2L_2, then the above interpretation would also predict the presence of an HL band without p33. There is no such band apparent in Fig. 3.5, although the expected low radioactivity in this band (due to the absence of highly labelled p33) may be below the limit of autoradiographic detection in this experiment.

If, on the other hand, HL-p33 exists independently on the Daudi cell surface, no further explanation is required for the absence of an HL band. The presence of HL bands in unreduced sIgM has been reported previously (Kennel & Lerner, 1973), although it is not known whether the HL molecules exist on the cell surface or are merely artefacts of isolation.

An alternative interpretation of the data presented in Figs. 3.5 and 3.6 is possible if assumption (ii) above is incorrect. Thus, if one p33 molecule <u>does not</u> contribute 33,000 daltons to the H_2L_2 and HL apparent molecular weights, the data are consistent with <u>two</u> p33 molecules associated with the H_2L_2 and <u>one</u> with the HL. This possibility can be envisaged as follows. Due to constraints on the conformation of the HL complex caused by disulfide bonding of the chains, the addition of p33 causes a non-linear increase in the apparent molecular weight of the complex. Thus, the molecular weight contribution of p33 to the HL-p33 complex could be only 23,000 daltons. The same situation would apply to the H_2L_2 complex but because it is <u>more</u> constrained by disulfide bonding the effect would be greater. Thus, <u>two</u> p33 molecules could add only 32,000 daltons to the H_2L_2 molecular weight. This hypothesis is supported by the non-linear relationship of the apparent molecular weights of the normal HL and H_2L_2 moieties - the H_2L_2 being <u>less</u> than twice the apparent molecular weight of the HL.

The above interpretations could be resolved by a further SDS-PAGE of unreduced Daudi sIgM; after which the H_2L_2 and HL bands are sliced out of the gel, reduced and re-electrophoresed. The radioactivity in the μ and p33 bands from this second gel could then be determined by direct gamma counting. If there is <u>one</u> p33 in both the H_2L_2 and HL

moieties (first interpretation), then the p33:μ ratio in the HL band should be twice that in the H_2L_2 band. If, however, there is one p33 in the HL moiety and two in the H_2L_2, then the ratios should be identical. This experiment is presently in progress.

3.10. ISOELECTRIC FOCUSING SPECTRUM AND CYSTEINE CONTENT OF p33

3.10.1. Charge-Shift Titration Experiment

Because of the discovery of its disulphide linkage to Daudi sIgM, it was of interest to determine the cysteine content of p33. It has been shown (Feinstein, 1966; Stott & Feinstein, 1973) that this determination can be done on a micro scale using the method of charge-shift titration on isoelectric focusing gels. It was felt that this technique could be adapted to the analysis of p33, and would be a useful way of further characterising the molecule. Accordingly, the following experiment was performed.

Daudi sIgM was labelled by lactoperoxidase iodination, isolated by immunoprecipitation, reduced but not alkylated, and analysed by SDS-PAGE on a Tris-glycine buffered slab gel. The profile obtained was identical to that shown in Fig. 3.3, track 6. The p33 band was then sliced out of the gel and eluted in the reduced form in buffer containing DTT and SDS. The material was then either amidomethylated or carboxymethylated as indicated in the legend to Fig. 3.7, and focused in thin polyacrylamide slab gels. Amidomethylated cysteines add no net charge, whereas carboxymethylated cysteines each add exactly one negative charge to the p33 protein. On isoelectric focusing, each negative charge added shifts the focusing spectrum one 'step' towards the anode. By counting the number of steps of shift, the

number of cysteines in the p33 protein could be directly read off. For the purpose of measuring the steps, a 'ladder' is generated. That is, all the intermediate steps of charge shift are produced by using mixtures of the carboxy- and amidomethylating agents. The results of the isoelectric focusing (Fig. 3.7) show p33 to contain three cysteine residues, as determined by the three 'steps' between homologous bands (indicated by the arrows) of the amidomethylated and carboxymethylated species. This result also rules out the hypothesis that p33 is a fragment of immunoglobulin or any Ig-like molecule, because an Ig-like molecule the size of p33 would necessarily contain two full domains and therefore at least four cysteines. Homology does exist, however, between p33 and one other known lymphocyte surface antigen. Thus, the heavy chain of human Ia antigen (p34) has also been shown to contain three cysteine residues (Snary et al., 1976; Springer et al., 1976). The possibility of a connection between p33 and human Ia-antigens will be discussed later.

3.10.2. Isoelectric Focusing Spectrum

The isoelectric spectrum of amidomethylated p33 consists of a regular pattern of five bands ranging in isoelectric points from 6.6 to 5.6. This spectrotype is consistent with the pattern of post-translational microheterogeneity resulting either from loss of amides from asparagine or glutamine side chains or the presence of variable numbers of sialic acid residues. Two observations favour the amide-loss hypothesis: i) The single charge steps introduced by carboxymethylation correspond exactly with the steps in the spectrotype of amidomethylated p33, showing that charge groups of a similar pK are involved and ii) the single charge steps between adjacent components diminish from 0.3 pH

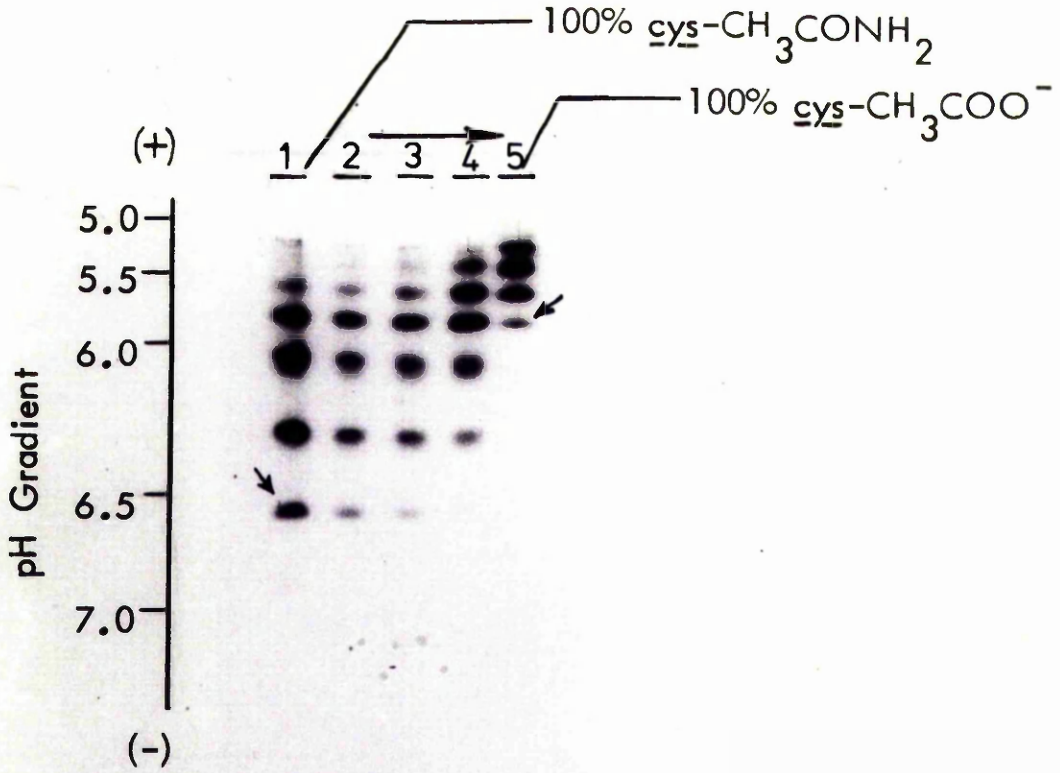
Fig. 3.7

Isoelectric Focusing Spectrum and Charge-Shift Analysis of p33

Labelled Daudi cell surface IgM was prepared as described in the legend to Fig. 3.3 and dissolved in reducing sample buffer for discontinuous SDS-PAGE in a 12.5% polyacrylamide slab gel (Methods, 11). The gel was processed for fluorography (Methods 17.4) and the fluorogram developed after an exposure of 8 hr. A profile indistinguishable from that shown in Fig. 3.3 (Daudi anti-μ) was obtained. The fluorogram was placed over the dried gel, aligned carefully, and the area of gel underlying the p33 band sliced out. (Methods, 15.1). Labelled p33 was eluted from the slice under reducing conditions (Methods 15.2) and the total recovered sample (29,000 counts/min p33, 50 μg carrier NoRIgG) divided into five tubes containing iodoacetamide and iodoacetate to yield the following final concentrations:

	1	2	3	4	5
Iodoacetamide (mM)	50	45	35	20	0
Iodoacetate (mM)	0	5	15	30	50

Alkylation was carried out for 30 min at 20°C, and the samples were then prepared for isoelectric focusing by repeated acetone precipitation and dissolution in 9 M urea (Methods, 15.4). Isoelectric focusing in a thin-layer 7.5% polyacrylamide slab gel was as described in Methods, section 14, using pH 3.5-10 Ampholine carrier ampholytes. The gel was processed for fluorography (Methods 17.4) and the fluorogram developed after an exposure of 6 days.


100% cys-CH3CONH2
100% cys-CH3COO⁻
(+)
1
2
3
4
5
5.0
5.5
6.0
6.5
7.0
pH Gradient
(-)

units between the two most basic components to 0.15 pH units between the two most acidic components; the charge groups contributing to the microheterogeneity of p33 are thus being titrated over the same pH range as the carboxymethyl groups, as would be expected for aspartic or glutamic acid side chains but contrary to expectation for sialic acid carboxyl groups. The extensive microheterogeneity seen in the p33 spectrum is consistent with this protein being a long-lived surface protein and with the fact that both old and new molecules would be labelled by surface iodination.

3.11. DISCUSSION

The results described in this section have identified and partially characterised a surface polypeptide chain of 33,000 molecular weight (p33) which is in a unique covalent association with the sIgM of Daudi cells. Earlier results from other laboratories had also suggested the presence of this component, and one specific report claimed it to be an abnormally large L-chain (Kennel, 1974). The present investigation has clarified these earlier observations. The cysteine content, size, radioiodination characteristics, SDS-PAGE behaviour, and molecular weight contribution of p33 to the Daudi $\mu_2\kappa_2$ sIgM all argue that p33 is distinct from any Ig L-chain, H-chain or fragment. In addition, the actual Daudi κ-chain has been resolved and characterised in its mature, carbohydrate-free, and precursor forms, and it appears to be normal.

The earlier findings of Kennel (1974) indicated that the p33 component could be labelled with radioactive galactose. Thus, p33 is a glyco-protein. The observation in the studies described here, that p33

appears as a diffuse band on discontinuous SDS-PAGE, suggests a heterogeneous nature of the p33 carbohydrate moieties. Microanalytical methods which can give a rough indication of the extent of polypeptide chain glycosylation (i.e. SDS-PAGE comparison of cell-free precursors and mature cellular products) can in theory be applied to the analysis of p33. However, this approach depends on the availability of a specific antiserum which will react both with the cellular and in vitro synthesised p33 polypeptide.

Other microanalytical techniques, which require only that the surface iodinated complex (p33-sIgM) be isolated and resolved into its polypeptide chain components, have been used to determine the cysteine content of the p33. The results indicated the presence of three cysteine residues, consistent with an intrachain disulphide loop and a single disulphide linkage to the sIgM. This particular configuration of disulphide linkages has not been demonstrated, however. The attachment of p33 to the sIgM of Daudi cells is most likely to a μ-chain, since μ-chains have been shown to have two free -SH groups when in intracellular μ_2L_2 subunits (Stott & Feinstein, 1973) whereas L-chains normally have no free -SH groups. Cysteine 575, which normally forms an intrasubunit disulphide bond, and cysteine 414 which forms the intersubunit bond in 19S IgM, are likely sites of p33 attachment. It cannot be stated with certainty whether one or two p33 molecules are associated with Daudi cell surface μ_2L_2, and arguments for both interpretations of the data have been presented. Thus, although the measured molecular weight contribution of p33 to the Daudi μ_2L_2 suggests the presence of only one p33 chain, caution may be necessary in interpreting molecular weights determined for covalently associated glycoproteins by SDS-PAGE.

It is tempting to consider the possibility that p33 is related to a Daudi Ia antigen heavy chain. The Ia antigens of human lymphocytes are composed of two polypeptide chains of molecular weights 33,000 and 28,000 linked non-covalently as a dimer (Snary et al., 1976; Springer et al., 1976). Thus, Daudi p33 is of similar molecular weight to Ia antigen heavy chain. In addition, the two polypeptides are both glycoproteins, they have the same number of cysteine residues, and they share similar isoelectric properties. With regard to this latter point, human Ia antigen heavy chain (carboxymethylated) has been characterised by isoelectric focusing as a poorly defined streak with its centre at about pH 5.1 (Springer et al., 1976). Another report describes a focusing spectrum for human Ia antigen heavy chain which resolves into 4 spots. Similarly, the results described in this section show p33 to resolve into 4 or 5 components, of pI 5.3 - 5.8.

Recent serological and functional evidence lends strong support to the idea that p33 may be a Daudi Ia antigen heavy chain. A xenogeneic antiserum raised against Daudi cell membranes and absorbed extensively with T-cells and EB_1 cells (a B-lymphoma cell line which fails to stimulate in mixed lymphocyte reaction (MLR)) has been found to block MLR stimulation at a dilution of 1:1000 (Steele, M., personal communication). The implication of this finding is that the antiserum has activity against Daudi Ia antigens. This antiserum was further absorbed with human IgM and whole human serum, in our laboratory, and then used to immunoprecipitate surface iodinated proteins from cell lysates of a number of sIgM synthesising lymphoma cell lines. It was found that only the Daudi cell lysate showed reactivity. Moreover, when the Daudi immunoprecipitate was analysed by SDS-PAGE, it was shown to consist of μ, κ, and p33 bands (Cushley, Singer & Williamson, unpublished).

The absence of p33 attached to the surface immunoglobulin of other Burkitt lymphoma cells rules out the idea that the complex is characteristic of the Burkitt lymphoma syndrome. It is possible that the p33-IgM complex on Daudi is related to some other unique feature of Daudi cells. Daudi cells are unusual in their failure to express products of the HL-A A, B and C loci (Fellous et al., 1975). This lack of expression has been attributed to a defect in the production by Daudi cells of ß2 microglobulin (Evrin & Nilsson, 1974) which is normally found associated with the products of the HL-A A, B and C loci. This suggests the hypothesis that the expression of the heavy chain of Ia antigen is normally linked in a balanced way with the expression of the light chain of Ia antigen, and that in Daudi cells an imbalance has arisen. Thus the p33 chain found linked to IgM on Daudi cells may represent an excess production of the Daudi Ia heavy chain relative to the light chain, leaving the excess heavy chain free to interact with the surface IgM.

Several speculations are possible as to the functional significance to the p33-IgM interaction. The significance of each possibility is enhanced if p33 is the heavy chain of a Daudi Ia antigen. One possibility is that the p33-IgM complex is characteristic of a particular stage of the differentiation of human lymphocytes. An alternative possibility is that the interaction of Daudi p33 with IgM is a unique function of the particular alloantigenic form of p33 existing on Daudi cells, and possibly of the particular antibody specificity of Daudi IgM. A third possibility is that the Daudi p33-IgM complex is indicative that a functional complex of similar nature plays a part elsewhere in antibody formation.

Viewing these functional possibilities in the light of the hypothesis that p33 is the heavy chain of Daudi Ia antigen suggests functional complementarity between the Ia heavy chains and a specific constant region domain of immunoglobulin, or alternatively, the complementarity may be between Ia heavy chain and a variable region domain of immunoglobulin. In either case the function of the interaction may be related to the role of I-region products in restricting cell-cell interactions in immune responses and in regulating responsiveness to particular antigens. Functional interaction between Ia heavy chain and immunoglobulin may normally occur not on the lymphocyte surface but on the surface of another cell type, possibly the macrophage, or in cell-cell interaction.

Further studies of the nature and function of p33 on Daudi cells and on the possibility of similar interactions occurring elsewhere should help differentiate between the various possibilities discussed above.

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