CHARACTERISATION OF HUMAN B-LYMPHOCYTE SPECIFIC ANTIGENS AND RECEPTOR IMMUNOGLOBULINS

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by

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ABBREVIATIONS

The abbreviations used in this thesis are those suggested in the Instructions To Authors of the Biochemical Journal, with the following additions:-

General:

AMW	Apparent Molecular Weight
BL	Burkitts' Lymphoma
BLCL	B-Lymphoblastoid Cell Line
BSA	Bovine Serum Albumin
CON	Control
CHO	Carbohydrate
EC, IC	Extracellular, Intracellular
FCS	Foetal Calf Serum
H-2	The Murine Major Histocompatibility Complex
HLA	The Human Major Histocompatibility Complex
LPS	Lipopolysaccharide, a B Cell Mitogen
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
Poly A^+ RNA	RNA enriched for species containing poly-adenylated 3'-ends; that is, messenger RNA
SDS	Sodium Dodecyl Sulphate
TKM	Tris-Potassium-Magnesium Buffer
TUN	Tunicamycin-Treated
3D-TKM	Three-Detergent Cocktail in TKM Buffer

Chemicals

DMSO	Dimethyl Sulphoxide
DNP	Dinitrophenol
DTT	Dithiothreitol
NAP	N-(4-Azido-2-Nitrophenyl)
PMSF	Phenylmethylsulphonyl Fluoride
PPO	2,5-Diphenyloxazole
TCA	Trichloroacetic Acid
TEMED	N,N,N′,N′-Tetramethylethylene Diamine
$F(ab)_2$	Fragment of Immunoglobulin Molecule Generated by Pepsin Digestion
GARIg	Goat Anti-Rabbit Immunoglobulin
RAHμ, RAHα, RAHε, RAHγ, RAHλ, RAHκ	Rabbit Antibody to Human μ, α, ε, γ, λ, and κ Chains
$RAH\beta_2M$	Rabbit Anti-Human β_2-Microglobulin
Ly	Membrane Marker of Murine Lymphocytes
T_H-Cell	Helper T-Lymphocyte
T_s-Cell	Suppressor T-Lymphocyte

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CHAPTER 1:

THE ROLE OF N-LINKED OLIGOSACCHARIDES IN ASSEMBLY AND TRANSPORT OF HUMAN IMMUNOGLOBULINS AND MAJOR HISTOCOMPATIBILITY COMPLEX GENE PRODUCTS

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SUMMARY

Considerable attention has recently been focused on the mechanisms which allow B-lymphocytes to effect simultaneous biosynthesis and expression of receptor and effector forms of the immunoglobulin molecule as mandated by the Clonal Selection Hypothesis. The genetic mechanisms responsible for the above phenomenon have now been established for the biosynthesis of IgM in murine lymphocytes. Also of great interest is the possible regulatory influence exerted by carbohydrate moieties on the secretion and membrane deposition of immunoglobulin molecules.

The objectives of the work described in this thesis were to attempt to ascertain whether or not human B-lymphocytes expressing IgG, IgA and IgE adopted similar genetic strategies to allow simultaneous expression of structurally distinct membrane and secretory heavy chain polypeptides. The role of N-linked oligosaccharides in secretion and membrane deposition of immunoglobulin molecules was also studied and compared with data from similar investigations on the biosynthesis of human transplantation antigens in the hope of revealing possible carbohydrate-influenced structural signals necessary for transport and expression of immunoglobulins.

The antibiotic tunicamycin was used to inhibit N-glycosylation of proteins in cells actively engaged in protein biosynthesis. SDS-PAGE analysis of specifically immunoprecipitated immunoglobulins of different classes synthesised in cells treated with tunicamycin revealed that four-chain immunoglobulin molecules were readily formed

in all cells. It is concluded that immunoglobulin assembly occurs independently of N-glycosylation of the newly-synthesised immunoglobulin chains. HLA-A,-B,-C/β_2 microglobulin complexes were also efficiently assembled in the presence of tunicamycin. Evidence is presented to support the hypothesis that 19S IgM and 11S IgA polymers are formed in cells treated with tunicamycin. No conclusive evidence was obtained with respect to incorporation of J-chain into non-glycosylated polymeric immunoglobulins.

The presence of N-linked oligosaccharides on the immunoglobulin heavy chain appeared to be mandatory for the efficient secretion of IgM and IgA from human cells synthesising these classes of immunoglobulin. By contrast non-N-glycosylated IgG and IgE molecules were efficiently secreted from IgG and IgE producing human B-lymphocytes.

Three experimental approaches were adopted to elucidate the functional role of N-linked carbohydrate side chains of immunoglobulin molecules in the efficient membrane deposition of these molecules. In one case it was found that non-N-glycosylated IgM could be demonstrated at the cell membrane of tunicamycin-treated cells: this data was shown to be incorrect by more rigorous experimental analyses. It is concluded that N-glycosylation of immunoglobulins is necessary for efficient membrane deposition of IgM and IgA. In contrast, HLA-A,-B and -C alloantigens and also HLA-DR alloantigens were detected at the membrane of tunicamycin-treated cells and, in the case of HLA-A2 and HLA-B7, the non-glycosylated molecules were alloantigenic as judged by their serological reactivity with alloantisera.

Biosynthesis of membrane and secretory forms of γ -heavy chain polypeptides was studied in the B-lymphoblastoid cell line Bec-11. Normal Bec-11 cells synthesised three intracellular forms of γ-chain of AMWs 64K, 55K and 52K and two of these chains are secreted into the culture supernatant (52K and 55K). The 64K species was presumed to be the membrane form of γ-heavy chain and this hypothesis was supported by the finding that IgG isolated from aliquots of radioiodinated Bec-11 membrane proteins contained γ-heavy chains of AMW 64K. Tunicamycin-treated Bec-11 cells contain two intracellular forms of γ-chain, 60K and 48K, and these were shown to be the non-glycosylated counterparts of the 64K and of 52K and 55K respectively. The 48K γ-chain was the sole secretory γ-chain polypeptide synthesised by Bec-11 cells and was shown to be the non-glycosylated form of both the 52K and the 55K glycosylated secretory γ-chains. Preliminary evidence that the 64K species contained hydrophobic regions not found in either 52K or 55K species was obtained from studies using a lipophilic nitrene reagent, hexanoyl-diiodo-NAP-tyramine. This reagent labelled only the 64K species which suggested that this γ-chain contained hydrophobic sequences presumably responsible for its membrane insertion.

Cell free translation of Bec-11 polyA$^+$ RNA followed by specific immunoprecipitation of the translation products and analysis by SDS-PAGE revealed two γ-chain bands. This data suggests that discrete mRNA species direct the synthesis of membrane and secretory γ-chain polypeptides.

IgA biosynthesis was investigated in cells of the Burkitt lymphoma cell line Dakiki Arosros-1. Experimental investigations

similar to those described for Bec-11 cells illustrated that Dakiki Arosros-1 cells synthesised two forms of α heavy chain, one of which was secreted and the other which was inserted into the membrane. Distinct non-glycosylated counterparts were found for each of the above mature α-chains.

When the differences in AMW of non-glycosylated membrane and secretory heavy chains of each isotype were compared an interesting observation was made. In the μ- and α-chain systems the difference was of the order of 2000-3000 daltons suggesting a similar organisation of μ- and α-chain coding sequences in the genome. In Bec-11 cells, the difference was 12,000 daltons and this suggested that the C_{γ} gene has a different structure to either Cμ- or Cα-gene system. Study of two other IgG synthesising cell lines, EB4.9 and Maija also revealed an AMW difference of 12,000 daltons. It is proposed that the Cγ gene has larger M-exon structures than those described for the human and murine Cμ genes (and postulated for the Cα gene).

A serological investigation of the unique 33,000 dalton glycoprotein (p33) found in association with the membrane IgM of the Burkitt lymphoma cell line was performed. A rabbit antiserum (serum No.52) capable of inhibiting mixed lymphocyte reactions stimulated by Daudi cells was shown to specifically recognise p33. A working hypothesis, that p33 represented an aberrant form of the HLA-DR antigen, was proposed, tested and shown to be incorrect. Rabbit antisera raised against HLA-DR antigens failed to precipitate the IgM-p33 complex, and serum No.52 failed to recognise HLA-DR allo-antigens. Furthermore, peptide maps of p33 and the HLA-DR chains

did not display any common features. Also, an antiserum to denatured HLA-A,-B,-C antigen heavy chain failed to precipitate the IgM-p33 complex. Thus, p33 was shown not to be serologically related to any of the HLA-A,-B,-C or -DR alloantigens. Carbohydrate was shown to be necessary for expression of the IgM-p33 complex at the cell membrane and also for serological reactivity of p33 with serum No.52. Tentative evidence to suggest that p33 represents a mutant C_K fragment is discussed.

INTRODUCTION

1. THEORIES OF ANTIBODY FORMATION

The selective nature of antibody formation was first alluded to by Ehrlich (1900). This early model, the 'side-chain' theory, proposed that certain cells of the body possessed receptors whose function was to recognise toxins present in foodstuffs. The toxin combined with its homologous anti-toxin and this event resulted in the excess regeneration of the anti-toxin receptor molecule. The manifestation of the excess regeneration of receptor was the appearance, in serum, of the anti-toxin.

1.1. Instructive Theories Versus Selective Theories

Ehrlich's side chain theory represents the first selective theory of antibody synthesis. The unifying principle of the selective theories is that the receptor exists prior to exposure to antigen and that antigen contact is the specific signal for the production of circulating antibody.

The finding that animals were capable of responding to non-biological antigens presented a challenge to Ehrlich's hypothesis. The observation that an animal could respond to newly synthesised chemical groups such as dinitrophenol, arsonate etc. (Landsteiner, 1946) was difficult to reconcile with the idea that the animal possessed "side-chains" capable of specific interaction with one and only one antigen. These experimental data led to the formulation of the instructive theories of antibody formation.

The instructive theorists reasoned that an animal could not possess specific receptors for each individual antigen, and

concluded that antigen must, in some manner, 'instruct' the immune system. An example of the instructive theories is provided by the Pauling model (Pauling, 1940). In this model, all antibody molecules had identical amino acid sequence but possessed different three-dimensional structures in those regions of the molecule actively responsible for antigen binding. The information for adoption of the correct conformation for the antigen-binding site was determined by antigen contact during antibody synthesis; the antigen therefore acted as a "template" for its homologous antibody. Moreover, Pauling also proposed that the conformation of the antigen binding site of the antibody after 'instruction' was stable after removal of the antigen.

The theoretical basis for an experimentally testable model of selective antibody formation was advanced in the mid to late 1950s.

Jerne (1955) proposed, first of all, that the diverse population of antibodies arose during development and also that the generation of this antibody diversity was independent of antigen. It was also postulated, inaccurately, that ingestion of immune complexes by the phagocytic cells of the system resulted in these cells producing large quantities of the ingested antibody. Although inaccurate, the Jerne proposal of a cellular involvement in antibody synthesis was developed by Talmage (1957) who proposed that it was a recognition event at the cellular level, rather than in serum, which was the critical step in the initiation of antibody synthesis.

The major conceptual advance was proposed by Burnet (1957) who fused Jerne's hypothesis and the Talmage commentary to give birth to the Clonal Selection Hypothesis (Burnet, 1959). The critical assumption in this model is that each immunocompetent lymphocyte

expressed only one receptor antibody on its cell membrane and that antibody secretion was initiated only by stimulation of the lymphocyte by specific antigen. The tenets of the Clonal Selection Model are detailed below (section 1,3).

1.2. Demise of the Instructive Theories

The proposals of instructive and selective theories of antibody formation in experimentally testable forms led to a series of experiments which resulted in the disproval of the theories from the former school of thought.

1.2.1. Negative Evidence Against Instruction

If antigen acted as a template for specific antibody formation, it would be reasonable to propose that antigen would be found in the cytoplasm of specific antibody forming cells. Studies with highly radioactively labelled flagellin failed to demonstrate the presence of this antigen within the cytoplasm of cells actively synthesising antibody (Nossal et al, 1965a,b).

1.2.2. Positive Evidence Against Instruction

The instructive theories were irrevocably refuted by the observation that denatured antibody could be renatured to an active form in the absence of antigen. Antibody was denatured in urea or in guanidine hydrochloride, and the disulphide bridges reduced; antigen binding activity was lost. Upon removal of the denaturing agent and re-oxidation of the disulphide bridges, in the absence of antigen, a significant amount of antibody activity was recovered (Haber, 1964). This experiment convincingly demonstrated that antigen does not have a role as a template during formation of specific antibody.

1.3. The Clonal Selection Theories

The Clonal Selection Theory (Burnet, 1959) and its derivatives (Mitchison, 1971) advanced the following maxims to govern antibody synthesis by B-lymphocytes:

a) the lymphocyte expresses a receptor antibody identical to that which it will later secrete;

b) only one receptor specificity is expressed by any one lymphocyte;

c) a single lymphocyte expresses only one immunoglobulin allotype (allelic exclusion), and only one light chain type;

d) antigen plays no part in the generation of antibody diversity, and the lymphocyte is pre-committed to a given antigenic specificity;

e) combination with complementary antigen is the specific signal required for differentiation of a lymphocyte to plasma cells or memory cells.

There is now overwhelming evidence to support the above tenets. The evidence has been obtained from experiments at the cellular level as well as from investigations of the molecular nature of antibody molecules and their genes.

2. THE B-LYMPHOCYTE AS THE CELLULAR SITE OF IMMUNOGLOBULIN SYNTHESIS

2.1. Antibody Synthesis is Unique to B-Lymphocytes

It is now accepted that the lymphocytes comprise two

major populations:

a) the T-lymphocytes whose development is influenced by the thymus and,

b) the B-lymphocytes whose functional differentiation depends upon processing in the avian Bursa of Fabricius (Glick et al, 1956) or in its mammalian equivalent.

Also well established is the need for co-operation between the two populations of lymphocytes for an effective antibody response (Claman et al, 1966; Mitchell and Miller, 1968; Mitchison 1967, 1969, 1971a,b; Feldmann and Basten, 1972; Katz and Benacerraf, 1972). Much evidence is available to demonstrate that B-lymphocytes are the antibody synthesising cells and that T-lymphocytes play a regulatory role. The classical experiments which illustrate this are reviewed here.

A neonatally thymectomized CBA mouse (haplotype $H\text{-}2^k$) was reconstituted at age 8 weeks with the thymus of a C57BL/6 animal (haplotype $H\text{-}2^b$): sheep red blood cells (SRBC) were administered as antigen. The spleen of the repopulated animal was removed seven days later and divided into three aliquots which were then treated with one of the following antisera plus complement:

a) anti-CBA (anti $H\text{-}2^k$);

b) anti-C57BL/6 (anti-$H\text{-}2^b$);

c) normal mouse serum.

Only the cells treated with the anti-CBA antiserum failed to make antibody suggesting:

i) endogenous host cells (i.e. B-lymphocytes) were responsible for antibody synthesis and

ii) cells from the donor (i.e. T-lymphocytes) had no effector function in the Ab formation (Mitchell and Miller, 1968).

Nossal and co-workers (Nossal et al, 1968) performed an elegant experiment employing the CBA/T6T6 mouse strain. This mutant strain of CBA mice possesses a heritable translocated chromosome marker which is easily identified in mitotic cells. (Ford, 1966.) CBA mice were irradiated and repopulated with CBA thymocytes and CBA/T6T6 bone marrow cells. In this experiment, the antibody forming cells possessed the T6T6 chromosome marker indicating that they were of bone marrow origin and not derived from the thymus. If the irradiated CBA animal was repopulated with CBA bone marrow cells and CBA/T6T6 thymocytes, then the T6T6 chromosome marker could not be found in antibody forming cells (Nossal et al, 1968). Those data suggest that the B-lymphocytes are the unique cellular site of antibody synthesis.

Many membrane markers are now defined which serologically delineate T- and B-lymphocyte populations. The most widely utilised markers are:

a) Θ or Thy-1 for T-lymphocytes (see Williams, 1976);

b) membrane immunoglobulin for B-lymphocytes (see below).

Experiments employing antibody plus complement to eliminate one or other of the lymphocyte populations serve to reinforce the classical data reviewed above.

2.2. Demonstration of Immunoglobulin on the Membrane of B-Lymphocytes.

For the tenets of the Clonal Selection Hypothesis to be upheld, antigen receptors must be demonstrated at the B-lymphocyte membrane, and these must be structurally identical to serum antibody (i.e. immunoglobulin).

2.2.1. Detection of Membrane Immunoglobulin by Anti-Immunoglobulin Reagents

Immunoglobulin was demonstrated at the B-lymphocyte membrane by use of antibody to immunoglobulin components: the antibody was labelled with either fluorescent dye or radioiodine.

Using fluorescienated anti-immunoglobulin reagents, Raff and his colleagues could easily detect immunoglobulin on B-lymphocytes (Raff et al, 1970; Raff, 1970a). Other investigators employed similar reagents to demonstrate:

a) cytoplasmic and membrane immunoglobulins of different classes in the same cell (Pernis et al, 1970) and

b) heavy and light chains were associated on the cell membrane (Takahashi et al, 1971).

Radioimmunoassay has revealed the presence of immunoglobulin on the membranes of murine (Rabellino et al, 1971) and human (Lerner et al, 1971) lymphocytes. The binding of radioiodinated anti-immunogloublin to murine lymphocytes has been directly visualised by electron microscopy used in conjunction with autoradiography (Unanue et al, 1972).

2.2.2. Antigen Binding by Lymphocytes

The technique of rosette formation has been used to illustrate the capacity of lymphocytes to bind particular antigens or antigens coupled to SRBC (McConnell et al, 1969). Radioactive antigen can also be used to detect cells with specific antigen receptors (Ada, 1970).

Passage of primed lymphocytes over a column of antigen-coated glass beads led to the depletion of specific antigen-reactive, antibody forming cells from the primed population (Wigzell and Andersson, 1969). Elution of bound cells restored the capacity to produce antibody. Thus, antigen binding B-lymphocytes are the specific precursors of antibody forming cells.

Experiments employing radioactive antigen (Ada, 1970) are useful not only for detection of antigen binding cells, but also for enumeration of the cells binding the antigen. In experiments of this type (reviewed by Warner, 1974), the number of cells capable of binding a given antigen is of the order of 10^{-5} - 10^{-6}: this figure is in good agreement with the predictions of the Clonal Selection Hypothesis.

2.2.3. Correlation of Antigen Binding Capacity and Presence of Membrane Immunoglobulin

Fluorescienated antibodies to immunoglobulins were again employed in the experiments which demonstrated that the ability to bind antigen co-capped with membrane immunoglobulin (Raff et al, 1973). This data reinforced foregoing experiments which showed that the ability of lymphocytes to bind antigen was abrogated by pre-treatment with anti-immunoglobulin reagents (McConnell et al, 1969; Greaves, 1971; Byrt and Ada, 1969; Warner et al, 1970; Warner, 1974); inhibition

of antigen binding was not observed when lymphocytes were pre-treated with anti-H-2 antisera (Hammerling and McDevitt, 1974). The data obtained from experiments using anti-immunoglobulin as an antigen-binding inhibitor are open to criticism on the grounds of steric hindrance. The data of Raff et al (1973), illustrating the co-capping of antigen-binding sites and membrane immunoglobulin, argues against the steric hindrance objections and strongly suggests that membrane immunoglobulin is the antigen receptor.

2.2.4. Direct Correlation of Membrane Immunoglobulin and Antigen Binding Capacity

Direct visualisation of antigen binding by membrane immunoglobulin was achieved in the following experiment. Membrane proteins of murine splenocytes were radioiodinated (^{131}I-iodine) by lactoperoxidase-catalysed iodination (Marchalonis et al, 1971). The radiolabelled splenocytes were then incubated with ^{125}I-radioiodinated DNP-haemoglobin. Complexes of receptor and antigen were then precipitated from cell lysates using either anti-haemoglobin or anti-immunoglobulin.

The relative amounts of the two radionuclides in the precipitate was determined (Rolley and Marchalonis, 1972). ^{131}I- and ^{125}I-radionuclides were precipitated by both anti-haemoglobuin and anti-immunoglobulin antisera. This implies that membrane immunoglobulin must have been interacting directly with DNP-haemoglobin: this is very strong evidence in favour of the hypothesis that membrane immunoglobulin is the specific antigen receptor of the B-lymphocyte.

In this experiment DNP was selected as antigen because it had been previously demonstrated that a high percentage of unimmunised

murine splenocytes (0.1 - 1.0%) would specifically bind this determinant (Lawrence et al, 1973). This system therefore overcomes the limitations normally imposed by the low precursor frequency of specific antigen binding cells.

3. BIOSYNTHESIS OF IMMUNOGLOBULINS

3.1. General Features of Immunoglobulin Structure

The expansion in the understanding of immunoglobulin structure in the early 1960s posed many interesting questions regarding the genetics of the immunoglobulins. Many of the problems presented by the elucidation of the primary structure of the immunoglobulins could be meaningfully examined by biosynthetic studies. Before discussing such experiments it is relevant to briefly review the major features of the immunoglobulin molecule: the gross structure of an immunoglobulin molecule is diagrammed in figure 1.

All immunoglobulin classes have a unit structure similar to that illustrated in figure 1. The basic immunoglobulin molecule consists of a globular protein of sedimentation coefficient 7-8S composed of two identical heavy chains and two identical light chains. (Porter, 1972, 1973; Edelman, 1973.) The heavy and light chains have several common properties as revealed by amino acid sequencing studies (Hilschmann and Craig, 1965; Milstein 1966; Baglioni et al, 1966; Putnam et al, 1966; Milstein and Pink, 1970).

a) the polypeptide chain is divisible into two regions;

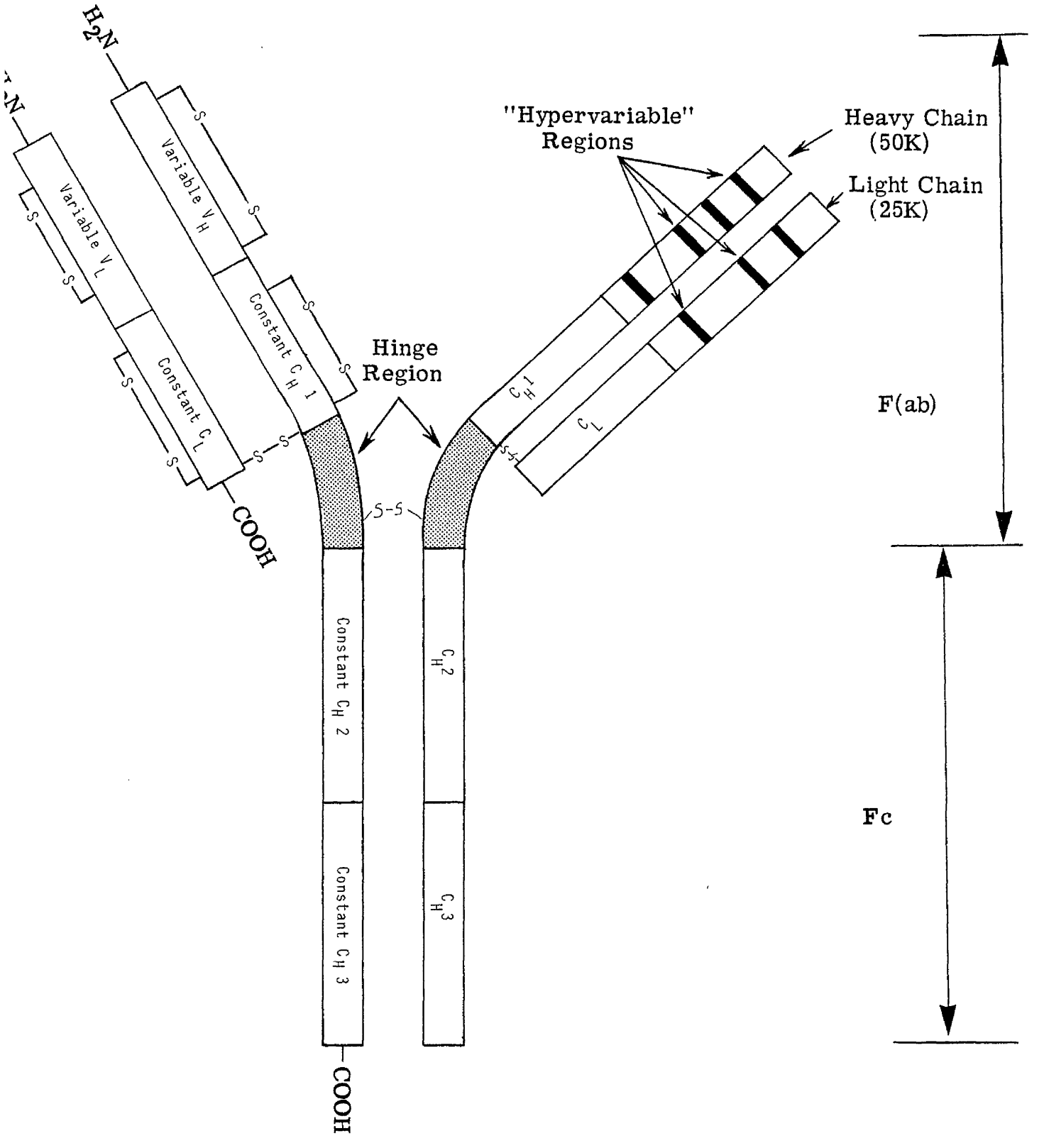
b) the N-terminal regions (residues 1 to approximately 110) exhibit great amino acid sequence variability (the

FIGURE 1

General Features of Immunoglobulin Structure

The four-chain structure of a typical IgG molecules is shown (Porter, 1972; 1973; Edelman, 1973). The hypervariable regions of amino acid sequence are shown as shaded areas, and the hinge regions as stipled areas. The molecular weights given for the heavy and light chains are approximate. The approximate locations of inter-chain and intra-chain disulphide bridges are also shown.

Fig. 1: General Features of Immunoglobulin Structure



V-region);

c) the C-terminal regions (residues 111 to the C-terminus) were constant in sequence and exhibited little amino acid sequence variability (the C-region).

All immunoglobulin sequences are in agreement with this generalisation, and it was clear from the earliest studies of immunoglobulin primary structure that sequence variability could be correlated with antibody specificity (Wu and Kabat, 1970). The genetic mechanisms underlying generation and maintainance of antibody diversity are clearly of great interest. Biosynthetic studies and investigations made possible by advances in recombinant DNA technology have contributed greatly to the solution of problems raised by the primary structure of immunoglobulins.

3.2. Synthesis of Immunoglobulin Chains.

The observation that immunoglobulin chains were composed of two easily delineable regions of amino acid sequence led to investigations into the organisation and integration of V-region and C-region genetic elements. Three possibilities could be envisaged for the integration of V- and C-region elements;

a) at the DNA level;

b) at the RNA level;

c) at the protein level.

Dreyer and Bennett (1965) had controversially postulated that two genes were responsible for encoding a single, functional, immunoglobulin chain. With the Dreyer-Bennett model in mind, the problem of the molecular level at which V-C integration occurred was

addressed by a series of experiments investigating the distribution of radiolabel in newly-synthesised immunoglobulin chains, based on the classical experiments of Naughton and Dintzis (1962).

Prior to the Dreyer-Bennett postulate experimental evidence, using the above approach, was obtained by Fleischmann (1963) and these early studies showed that Fab and Fc portions of rabbit IgG were not separate biosynthetic units (Fleischmann, 1963). Upon more detailed analysis in the same experimental system, it was demonstrated using cyanogen-bromide generated fragments of rabbit IgG that more radioactivity was located in C-terminal peptides than in N-terminal peptides (Fleischmann, 1967). This data is consistent with sequential growth of the polypeptide chain from the N-terminus to the C-terminus. Support for the hypothesis that immunoglobulin chains were synthesised from the N-terminus to the C-terminus was obtained from similar studies of murine IgG heavy chains synthesised in a murine plasmacytoma (Knopf et al, 1967). That light chains were synthesised in the same direction was evidenced from studies in a mouse myeloma synthesising only light chains (Lennox et al, 1967).

The data from the above experiments suggested a uniform gradient of radiolabel from a heavily labelled C-terminus to a lightly labelled N-terminus in both heavy and light chains of the immunoglobulin molecule. This is consistent with the synthesis of V- and C- region amino acid sequences from a single template, and is in agreement with the two gene-one polypeptide hypothesis (Dreyer and Bennett, 1965). If the above hypothesis were inaccurate and V- and C- region polypeptides were integrated at the protein level, then initial incorporation of radiolabel would be observed at

at two distinct points, each corresponding to the C-terminal region of the V- or C- region element. The hypothesis, upheld at the protein level, is further substantiated by studies of *in vitro* translation of immunoglobulin mRNA.

3.3. Sub-Cellular Site of Immunoglobulin Synthesis

There is now much evidence in the literature which demonstrates that membrane-bound polyribosomes are the intracellular site of synthesis of immunoglobulin heavy and light chains in immune lymphoid tissue and in cultured myeloma cells. A variety of experimental strategies have been adopted to demonstrate the role of polyribosomes in synthesis of immunoglobulin chains;

a) identification of nascent chains by SDS-PAGE of purified polyribosomes (Schubert and Cohn, 1968, 1970);

b) specific immunoprecipitation of polyribosomes and associated immunoglobulin chains by anti-immunoglobulin (Askonas and Williamson, 1966a; Williamson and Askonas, 1967; Schubert and Cohn, 1968; 1970; Sherr and Uhr, 1970; Cioli and Lennox, 1973);

c) electron microscopy coupled with autoradiography (Scharff and Laskov, 1970; Uhr, 1970);

d) immunoprecipitation of immunoglobulin chains synthesised by purified polyribosomes in a cell-free system (Lisowska-Bernstein *et al*, 1970) and

e) immunoprecipitation of immunoglobulin chains synthesised by a cell-free translation system programmed with mRNA purified from polyribosomes (Blobel and Dobberstein, 1975a,b).

The evidence also demonstrated that heavy and light chains were independently synthesised on separate polyribosomes (Scharff and Uhr, 1965; Askonas and Williamson, 1966a; Schubert and Cohn, 1968, 1970). These observations were entirely consistent with the genetic evidence which demonstrated non-linkage between heavy and light genes (Herzenberg et al, 1968).

Electron microscopic analysis of the polyribosomes involved in synthesis of immunoglobulin heavy and light chains in the murine plasmacytoma 5563 showed that the polyribosomes responsible for heavy chain synthesis contained 11-18 ribosomes per polyribosome, whereas those active in the synthesis of light chains contained 4-5 ribosomes per polyribosome (de Petris, 1970). These data were in agreement with estimates of the number of ribosomes per polyribosome based on sucrose density gradient profiles of polyribosomes from the same tumour cell (Askonas and Williamson, 1966a; Williamson and Askonas, 1967).

3.3.1. Nature of Polyribosomes

Polyribosomes may be placed in one of two classes based on the nature of their attachment to membranes of the rough endoplasmic reticulum (Rosbash and Penman, 1971a, 1971b). Polyribosomes can be

a) completely membrane bound, i.e. each ribosome on the messenger is tightly bound to the membrane, or

b) "dangling" when some, but not all, of the ribosomes on the messenger are membrane bound.

Investigations in myeloma cells have provided evidence that polyribosomes involved in synthesis of immunoglobulin chains are of the dangling type (Harrison et al, 1974a, 1974b). These

observations may be interpreted in terms of polyribosome-nascent chain interactions and also in terms of vectorial release of de novo synthesised immunoglobulin chains into the cisternal compartment. The role of 'dangling' polyribosomes and the interaction of these with nascent immunoglobulin chains is discussed below (section 3.4.3.) in terms of the Signal Hypothesis (Blobel and Sabatini, 1971; Milstein et al, 1972; Blobel and Dobberstein, 1975a, 1975b).

3.4. Immunoglobulin Messenger RNA and its Translation

3.4.1. Immunoglobulin mRNA Species

The Dreyer-Bennett postulate that two genes were responsible for encoding one immunoglobulin polypeptide allowed for V-C integration at DNA or RNA or protein levels. The biosynthetic experiments (section 3.2. above) illustrated that immunoglobulins were synthesised from a single growing point, and this evidence ruled out the possibility that V-C integration occurred at the protein level (Lennox et al, 1967; Knopf et al, 1967).

To explore the possibility that V-C integration occurred at the level of mRNA, the following experiment was performed. A 9-13S RNA fraction was isolated from a light chain producing tumour cell line (Stavnezer and Huang, 1971); when translated in vitro in the rabbit reticulocyte cell-free system, the light chain polypeptides obtained were almost identical to those synthesised in vivo by the tumour itself as assessed by chromatographic and electrophoretic analyses of tryptic peptides. This is compelling evidence that the light chain was synthesised from a single mRNA species which itself contained information for V- and C- regions in a single integrated macromolecule. This data suggests that

V-C integration occurred prior to biosynthesis of immunoglobulin mRNA, that is, at the DNA level.

Evidence that light chain mRNA carries the information for V- and C- regions as a contiguous unit comes from the data of Milstein and his colleagues (Milstein et al, 1974). Light chain mRNA was isolated in sufficiently pure form to allow;

a) the total length of the mRNA to be estimated as 1,250 bases (±100 bases);

b) the identification of the nucleotide sequence required to specify the V, C and precursor amino acid sequences;

c) investigation of two untranslated regions, one of 150±100 residues at the 5' end and another of 200±50 nucleotides located at the 3' end of the messenger, and

d) the finding of a 3'-poly-adenylated sequence of approximately 200 nucleotides.

These data indicated that molecular integration does not occur at the protein level or at the level of mRNA: indeed, the evidence suggests that V-C integration occurs at the DNA level.

3.4.2. Translation of Immunoglobulin Messenger RNA in Vitro

Several laboratories have used mRNA purified from immunoglobulin-synthesising cells to direct the synthesis of immunoglobulin light chains in cell-free translation systems. In all cases reported to date, it has been observed that the product synthesised in vitro is larger than the homologous product synthesised in vivo (Milstein et al, 1972; Mach et al, 1972; Tonegawa and Baldi, 1973;

Schmeckpeper et al, 1974). The in vitro synthesised product possesses an N-terminal extension of 15-20 amino acids, the "leader" or "signal" sequence, which serves to transfer the nascent chain through the rough endoplasmic reticulum membranes (Milstein et al, 1972; and section 3.4.3. below). Similar N-terminal extensions have been demonstrated for a murine α-chain (Jilka and Pestka, 1977), murine γ_{2b} heavy chain (Singer et al, 1980b) and human μ-chain (Singer et al, 1980a).

3.4.3. The Signal Hypothesis

The Signal Hypothesis (Blobel and Sabatini, 1971; Milstein et al, 1972; Blobel and Dobberstein, 1975a,b) represents an attempt to rationalise the observations of the hydrophobic signal sequences observed on many proteins and also of the attachment of polyribosomes to membranes of the rough endoplasmic reticulum into a unified model for biosynthesis of secretory and membrane proteins. The hypothesis has three basic tenets;

i) translation is initiated on free cytoplasmic mRNAs;

ii) interaction of messenger-polyribosome complexes with rough endoplasmic reticulum membranes is mediated via the signal sequence, and

iii) the signal sequence serves to effect transfer of nascent chains across rough endoplasmic reticulum membranes and into the cisternal space.

Evidence is now available to support each of the above maxims. Rosbach (1972) demonstrated that newly-synthesised mRNA could be sequestered in small cytoplasmic polyribosomes by cycloheximide: these mRNAs could be "chased" into the larger, membrane-

bound polyribosome fraction upon removal of the cycloheximide. The initial interaction between mRNA and the ribosome appears, therefore, to occur in the cytoplasm.

In immunoglobulin synthesising cells it has been demonstrated that those ribosomes which were tightly bound to the rough endoplasmic reticulum membrane were anchored via a nascent chain-membrane interaction (Harrison et al, 1974b). Polyribosomes, such as those involved in immunoglobulin synthesis, which are anchored via a nascent chain-membrane interaction can be removed from the rough endoplasmic reticulum by treatment with puromycin and KCl (Harrison et al, 1974a, b). Such treatment also removed all the immunoglobulin mRNA from the rough endoplasmic reticulum (Harrison et al, 1974b) which argues against direct binding of immunoglobulin mRNA to the rough endoplasmic reticulum membrane.

Transfer of immunoglobulin chains across membranes was demonstrated in experiments where an in vitro protein synthesis system, programmed with immunoglobulin light chain message, was supplemented with canine pancreatic microsomes (Blobel and Dobberstein, 1975a, b). At the end of the in vitro synthesis period, the newly-synthesised light chains were shown to be insensitive to exogenously added proteases which suggested that they had been transferred into the microsomal cisternae. This data implied that the information necessary for this transfer, (that is, the signal peptide), was encoded by the mRNA and was not part of the protein synthetic machinery.

Thus, it is clear that the N-terminal signal peptide of the immunoglobulin chains plays a significant role in interaction of the messenger-polyribosome complex with the membranes of the rough

endoplasmic reticulum and also in efficient transfer of proteins across rough endoplasmic reticulum membranes.

3.5. Assembly of Immunoglobulins

3.5.1. Immediate Events after Chain Synthesis

Several important events occur during or just after immunoglobulin chain synthesis and before assembly into four-chain structures. These events are

a) removal of the hydrophobic signal sequence;

b) N-glycosylation of nascent heavy chains;

c) cyclisation of N-terminal glutamine residues.

Removal of the signal sequence is believed to occur rapidly after transfer of the nascent chain across the rough endoplasmic reticulum membrane (Milstein et al, 1972; Blobel and Dobberstein, 1975a,b). The enzyme responsible for removal of the signal sequence, signal peptidase, is believed to be localised on the cisternal face of the rough endoplasmic reticulum.

The topic of glycosylation of immunoglobulin chains is dealt with in detail below (section 3.8.); there are claims that N-glycosylation of immunoglobulins is a nascent chain event (Moroz and Uhr, 1967; Sherr and Uhr, 1969) but these data may be criticised on the grounds that no special precautions were taken to prevent absorption of completed immunoglobulin chains onto the ribosomes.

Cyclisation of N-terminal glutamine residues to pyrrolidone carboxylic acid (PCA) has been observed in several immunoglobulin chains; for example human λ light chains subgroups I, II and V, some human heavy chains (see Kabat et al, 1979) and the λ-light chain of the murine plasmacytoma MOPC 104E (Appella, 1971).

Investigation of cyclisation of glutamine to PCA in a γ_{2a} synthesising murine plasmacytoma, demonstrated that cyclisation was a late event during biosynthesis, and that PCA was virtually undetectable on nascent polypeptides (Stott, 1972).

3.5.2. Order of Disulphide Bond Formation

The immunoglobulin molecule possesses several interchain and intrachain disulphide bridges. This discussion is concerned with the formation of interchain disulphide bonds, that is those between two heavy chains or between heavy and light chains. Clearly, there are two possible orders of formation of disulphide bonds;

a) heavy-heavy before heavy-light, or

b) heavy-light followed by heavy-heavy.

The order in which disulphide bridges are formed varies from one immunoglobulin class to another, but each immunoglobulin class has a characteristic pattern of disulphide bond formation (Bevan et al, 1972; Baumal and Scharff, 1973). For example, murine IgG1, IgG2a and IgA all form inter-heavy chain disulphide bridges before covalently associating with light chains (Baumal et al, 1971a; Parkhouse, 1971a); the converse situation prevails in order of disulphide bond formation in murine IgM (Parkhouse, 1971b). Human IgG molecules also form inter-heavy chain disulphide bridges first (Baumal et al, 1971b) but human IgM and rabbit IgG form disulphide-bonded heavy-light "half molecules" as their first biosynthetic intermediate (Buxbaum et al, 1971; Sutherland et al, 1970).

Several factors may influence the order in which disulphide bonds are formed:

a) non-covalent interactions;

b) intrinsic stability of the disulphide bond formed, and

c) the absolute reducing conditions in the cellular microenvironment where assembly occurs.

As regards non-covalent interactions, these forces are sufficiently strong to produce a functional antibody in the absence of disulphide bridges (see Parkhouse, 1977). These interactions are probably of great significance as a primary reaction leading to optimal conditions for disulphide bond formation (see section 3.5.3. below).

The stability of a given disulphide bond correlates well with data pertaining to susceptibility of immunoglobulin molecules to mild reducing conditions (Bevan et al, 1972). For example, treatment of murine IgG_{2a}, which assembles via a heavy chain dimer (Askonas and Williamson, 1968a), with 10mM 2-mercaptoethanol leads to formation of heavy chain dimers and free light chains (Williamson and Askonas, 1968). The data implies that the inter-heavy chain disulphide bridge is more stable than that between heavy and light chains. Similar correlations between order of formation of interchain disulphide bridges and susceptibility to mild reduction have been demonstrated for human IgG (Williamson and Askonas, 1968a), human IgA (Parkhouse et al, 1971), and murine IgM (Askonas and Parkhouse, 1971).

The absolute reducing conditions in the cellular site of immunoglobulin assembly may be expected to influence the order of

disulphide bond formation. Given that there is a differential lability of heavy-heavy and heavy-light interchain disulphide bridges, and that in each case a distinct order of disulphide bond formation is observed, it is reasonable to propose that the reducing conditions at the site of assembly would be intermediate between those required for reduction of the two types of disulphide bond (Bevan et al, 1972).

3.5.3. Non-Covalent Interactions of Immunoglobulin Chains

Due to the weakness of the forces involved, evidence for non-covalent interactions of immunoglobulin chains has been difficult to obtain. There is some evidence to support the proposals that such interactions do exist.

Non-covalently bound light chains could be demonstrated on nascent heavy chains which had been dissociated from the heavy chain synthesising polyribosomes (Shapiro et al, 1966; Schubert, 1968). Other investigators were able to demonstrate a non-covalent interaction between ^{131}I-radiolabelled light chain and polyribosomes-bound nascent heavy chains (Askonas et al, 1969).

However, it was not possible to immunoprecipitate heavy chain synthesising polyribosomes with anti-light chain antibody (Williamson and Askonas, 1967). This negative data may be explained by proposing that the antigen-antibody interaction disrupted any non-covalent interactions between the heavy and light chains.

A possible route for non-covalent interaction of immunoglobulin chains has been proposed by Bevan and his colleagues (Bevan et al, 1972). At neutral pH native heavy chains are known to form dimers with interactions occurring at the Fc and Fd regions (Björk and Tanford, 1971). An equilibrium will be established

between those heavy chains interacting at both Fc and Fd and those interacting only at Fc; this latter population will be capable of interacting with free light chains and will therefore be capable of forming four chain H_2L_2 molecules.

3.5.4. Intracellular Pools and Balanced Synthesis

In biosynthetic studies of formation of intermediates en route to the formation of H_2L_2 molecules, the first intermediate to be observed was free light chain. These newly-synthesised light chains were rapidly released from polyribosomes to form an intracellular pool of free light chains (Askonas and Williamson, 1966b). Given that the sole secretory product of plasma cells is fully assembled H_2L_2 molecules (Williamson and Askonas, 1968b; Baumal and Scharff, 1973a), it is most likely that the intracellular pool of free light chains is in precursor-product relationship with completed H_2L_2 molecules. By virtue of this precursor-product relationship, the size of the light chain pool within any given cell is maintained at a constant size (Williamson and Askonas, 1968b) although the size of the pool varies from one cell type to another (Bevan et al, 1972).

The existence of an intracellular pool of free light chains in immunoglobulin synthesising cells has been a general and consistent finding. Equally consistent has been the failure to demonstrate a corresponding intracellular pool of free heavy chains; only heavy chain dimers were readily demonstrated (Bevan et al, 1972). On the basis that immunoglobulin synthesising cells possess an intracellular pool of free light chains which is of constant size and that this pool is in precursor-product relationship with fully assembled, secreted molecules, it is reasonable to propose that the synthesis of heavy

and light chains is balanced in such a manner that the amount of heavy chain synthesised is equal to that amount which the cell requires for membrane insertion or for secretion: no excess heavy chain is synthesised. The mechanisms which control the balance of synthesis could operate at either or both transcriptional or translational levels.

3.6. Accessory Polypeptide Components of Immunoglobulins

In the polymeric immunoglobulins IgM and IgA, accessory protein chains are found in association with the completed IgM and IgA polymers. J-chain is found in both IgM and dimeric (not monomeric) IgA, and secretory IgA also acquires secretory component during externalisation.

3.6.1. J-Chain

J-chain is a glycoprotein found in covalent association with the C-terminal region of 19S IgM pentamers and 11S IgA dimers: in each case, only one J-chain molecule is found per 19S IgM or 11S IgA polymer (Chapius and Koshland, 1974). Human J-chain is a polypeptide of 129 amino acids, eight of which are cysteine (Mole *et al*, 1977). It is synthesised by B-lymphocytes and is found not only in IgM and IgA producing cells, but also in IgG-producing cells in the human (Brandtzaeg, 1974) and in the mouse (Kaji and Parkhouse, 1974, 1975; Mosmann and Baumal, 1975). The finding of J-chain in these cells probably reflects on earlier state of differentiation of these cells (i.e. as IgM producers) prior to their development into IgG-producing cells. This data supports the hypothesis that the genes encoding immunoglobulin heavy chains and J-chain are not co-ordinately controlled. This hypothesis is further supported by the finding of

normal amounts of J-chain in myeloma variant cells with suppressed heavy chain snythesis (Kaji and Parkhouse, 1974, 1975; Mosmann and Baumal, 1975). The role of J-chain in control of accuracy of polymerisation of IgM and IgA is discussed below (section 3.7.).

3.6.2. Secretory Component

In addition to one molecule of J-chain, the dimeric form of IgA found in mucous secretions, secretory IgA, contains a further unique glycoprotein of approximately 60,000 daltons molecular weight: this is secretory component (Tomasi and Grey, 1972). Secretory component is synthesised not by B-lymphocytes but by the epithelial cells in the walls of the gastrointestinal tract and airways. (Tomasi and Bunenstock, 1968; Tourville et al, 1969.)

3.7. Formation of Polymeric Immunoglobulins

IgM and IgA are found as polymeric structures in serum and secretions of higher vertebrates. In both classes, J-chain is found in association with the 19S IgM pentamer or the 11S IgA dimer, but not with the monomeric forms of these immunoglobulins. This section reviews the current status with regard to understanding of the polymerisation events and the role of J-chain in such processes.

3.7.1. Observations In Vivo

In both human and murine systems, the IgM component found in serum is the 19S pentamer. The pentamer is not readily detected in the cytoplasm of cells synthesising IgM and several groups have demonstrated that 8S IgM monomers are the precursors of 19S IgM (Parkhouse, 1971b; Parkhouse and Askonas, 1969; Buxbaum et al, 1971). Although this is the general case, there are exceptions to the rule.

For example, some human myeloma tumour cells (Buxbaum et al, 1974) and bone marrow cells from patients suffering from Waldenstroms macroglobulinaemia (Buxbaum et al, 1971) have readily detectable amounts of intracellular 19S IgM. The large weight of evidence does suggest that the predominant intracellular IgM is the 8S monomer. The data are in agreement with hypotheses suggesting that the polymerisation event and secretion of 19S IgM from B-lymphocytes are closely linked if not concommitant events.

IgA is also found in the polymeric state, but does not polymerise to a uniform extent although the dimer is the most prevalent polymer. The main cytoplasmic form of IgA is the 7S monomer (Abel and Grey, 1968; Parkhouse, 1971a; Bevan, 1971) although there are certain tumours which have small amounts of oligomer in their cytoplasm (Halpern and Coffman, 1972).

3.7.2. Observations In Vitro

Isolated intracellular 8S IgM monomers can not be polymerised in vitro to give 19S IgM pentamers unless they have been treated with a reducing agent (Askonas and Parkhouse, 1971). These data suggested that cysteine residues involved in inter-monomer disulphide bridging were reversibly blocked within the cell. Further evidence demonstrating the requirement of reducing conditions was obtained from experiments in which isolated intracellular 8S IgM was treated first with an alkylating agent and then with reducing agent. Polymerisation to 19S IgM could still be achieved from monomers treated in this fashion.

These data implicate the involvement of an enzyme mediating disulphide interchange (Fuchs et al, 1967) in the removal of the block

on the penultimate C-terminal cysteine of the 8S IgM monomer. This enzyme may also act as a control step for the final polymerisation event immediately prior to, or concomitant with, the secretion event.

3.7.3. Requirements for Polymerisation

In addition to reducing conditions and the presence of a disulphide interchange enzyme, the polymerisation of IgM and IgA seems to depend on the presence of J-chain (Inman and Mestecky, 1974; Koshland, 1975). Both IgM and IgA can be polymerised *in vitro*, from monomers prepared from the corresponding polymers by mild reduction (Della Corte and Parkhouse, 1973b). These experiments demonstrated:

a) total and accurate polymerisation;

b) IgM formed pentamers, IgA formed dimers, and

c) there were no residual monomers.

The process was critically dependent on the presence of J-chain and purified disulphide interchange enzyme. IgM, but not IgA, could be polymerised *in vitro* in the absence of enzyme provided the concentration of 8S monomer was high (Parkhouse *et al*, 1971a, 1971b; Askonas and Parkhouse, 1971). The important role of the disulphide interchange enzyme was evidenced by the finding that, in the presence of J-chain and enzyme, low concentrations of IgM and IgA subunits could be successfully polymerised *in vitro* (Della Corte and Parkhouse, 1973b).

Therefore, there appear to be three requirements for the accurate polymerisation of IgM and IgA;

a) reducing conditions,

b) an enzyme mediating disulphide interchange, and

c) J-chain.

The role of J-chain is discussed in detail below (section 3.7.4.).

3.7.4. Role of J-Chain in Immunoglobulin Polymerisation

A remarkable degree of specificity is apparent when IgM and IgA subunits are polymerised in the presence of disulphide interchange enzyme and J-chain (Della Corte and Parkhouse, 1973b). The following observations were made:

a) IgM and IgA polymerised to pentamers or dimers respectively;

b) no hybrid molecules were formed, and

c) reduced albumin or 7S IgG failed to interfere with polymerisation.

Thus, IgM and IgA subunits could not interact despite the fact that the same J-chain molecule was involved in polymerisation of both immunoglobulin classes.

Further evidence that J-chain has a critical role in accurate polymerisation of IgM comes from polymerisation studies performed *in vitro* in the absence of J-chain (Kownatski, 1973; Eskeland, 1974). Polymerisation was shown to occur, but the polymers were not 19S pentamers, but a mixture of molecular sizes of IgM.

The role of J-chain in IgM polymerisation is, however, the subject of great debate, while the above investigations strongly suggest a crucial role for J-chain in the polymerisation process, the findings of 19S IgM lacking J-chain in a human myeloma (Eskeland and Brandtzaeg, 1974) and in certain species of fish (Weinheimer *et al*, 1971), argue against J-chain being an absolute requirement for IgM polymerisation.

Furthermore, the murine plasmacytoma Y5781 has been shown to contain a large intracellular pool of 19S IgM and a portion of the pentamers in this pool apparently lack J-chain (Stott, 1976): J-chain is believed to be added to these 19S IgMs just before or during secretion. Studies in vitro of IgM polymerisation indicated that monomers would polymerise in the absence of J-chain and that the pentamers so formed were indistinguishable from normal 19S IgM as judged by electron microscopy (Feinstein, 1973). Fc fragments of μ-chain (Fcμ) were demonstrated to be capable of polymerisation to 12S pentamers (5 x Fcμ) in vitro in the absence of J-chain (Beale, 1974).

The role of J-chain in the polymerisation and secretion of IgM and IgA remains to be unequivocally proven. Biosynthetically, the evidence suggests that addition of J-chain is quite a late event. Structural investigations show that J-chain is disulphide bonded to only two of the monomeric subunits of 19S IgM (Chapius and Koshland, 1974) and to both subunits of 11S IgA (Hauptman and Tomasi, 1975) in the form of a disulphide "clasp".

3.8. Glycosylation of Immunoglobulin Chains

3.8.1. Biosynthesis of Lipid Linked Oligosaccharides

Many membrane associated and secretory proteins, including immunoglobulins, have multiple sites of addition of carbohydrate moieties. The carbohydrate prosthetic groups are added to the protein at asparagine residues (N-linked oligsaccharides) or at serine or threonine side chains (O-linked oligosaccharides). The N-linked oligosaccharides may be divided into two classes:

a) "core" or "high mannose" oligosaccharides which contain N-acetylglucosamine and variable amounts of mannose, and;

b) "complex" oligosaccharides which, in addition to N-acetylglucosamine and mannose, contains galactose, sialic acid and fucose.

In all cases where the carbohydrate is N-linked to the polypeptide chain the "bridge" sugar is N-acetylglucosamine. Indeed, both "core" and "complex" carbohydrate structures are synthesised via a common biosynthetic pathway (see figure 2).

The "core" oligosaccharides are assembled on a carrier lipid molecule, dolichol pyrophosphate, and then transferred to a specific site on the polypeptide chain. The amino acid sequence at the site of N-glycosylation is asn-x-ser or thr (Shizimu et al, 1971). The first step in the synthesis of the oligosaccharide is the formation of dolichol diphosphoryl-N-acetylglucosamine from dolichol pyrophosphate and UDP-N-acetylglucosamine (Parodi and Leloir, 1979). It is this first transfer reaction which is inhibited by the antibiotic tunicamycin (Takatsuki et al, 1975; Tkacz and Lampen, 1975; Lehle and Tanner, 1976). The oligosaccharide is then elongated by addition of a further N-acetylglucosamine moiety followed by nine mannose residues and finally three glucose residues (Li et al, 1978; Hubbard and Robbins, 1979). The internal sugars are derived from sugar nucleotides while the peripheral moeities are added from either dolichol phosphoryl mannose or dolichol phosphoryl glucose (Elbein, 1979; Parodi and Leloir, 1979). The highly branched oligosaccharide structure is then added to an asparagine residue located in the

FIGURE 2

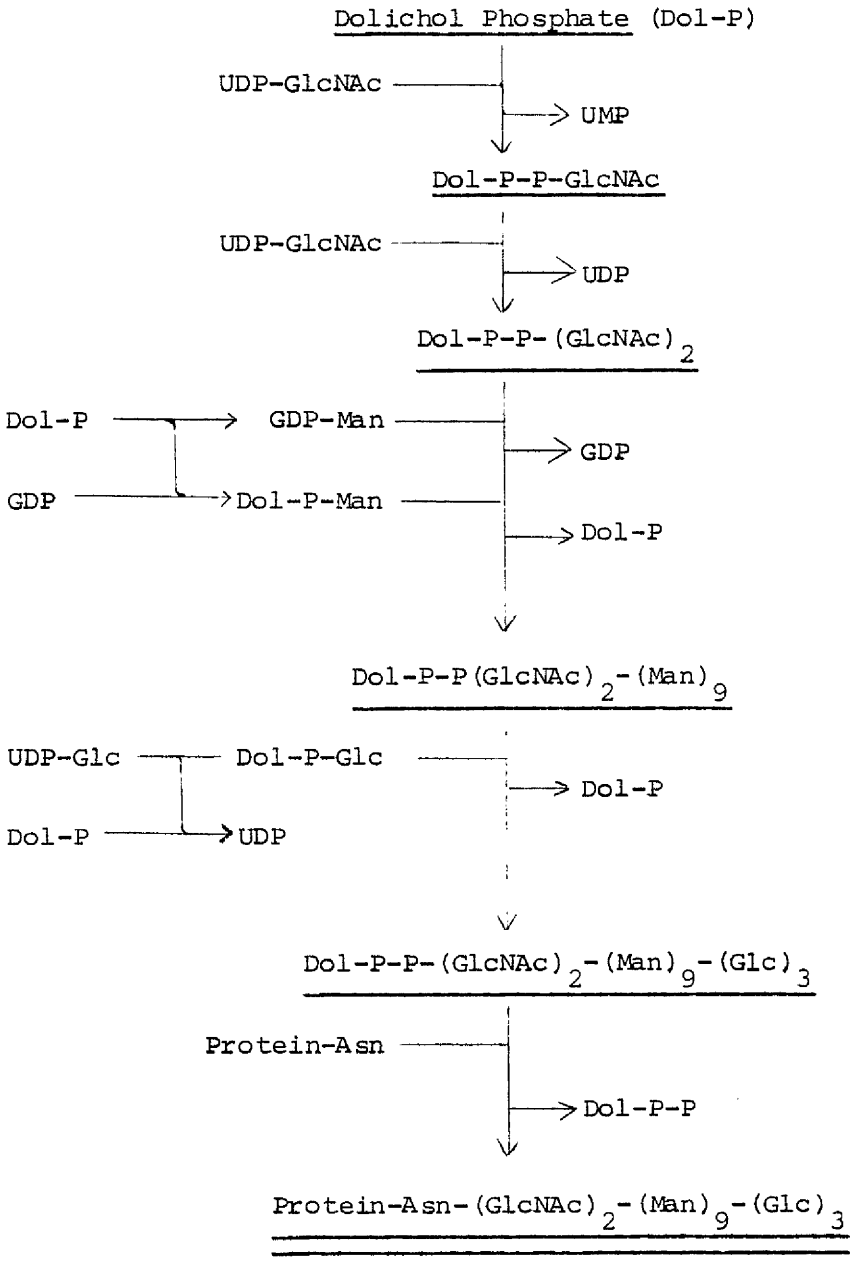
Biosynthesis of Lipid-Linked Oligosaccharides

The biosynthesis of the lipid-linked oligosaccharide moieties utilised in N-glycosylation of proteins is schematically represented opposite.

Key:-

Dol-P	-	Dolichol Phosphate
GlcNAc	-	N-acetylglucosamine
Man	-	Mannose
Glc	-	Glucose

FIGURE 2: Biosynthesis of Lipid-Linked Oligosaccharides



(Schwartz & Datema, 1980; Gibson et al, 1980; Parodi & Leloir, 1979)

asn-x-ser/thr tripeptide sequence (see figure 2).

After the oligosaccharide has been transferred to the polypeptide, during or just after synthesis of the latter on membrane bound polyribosomes, processing of the large oligosaccharide occurs to yield the "high mannose" (or core) or "complex" carbohydrate side chains found on the mature protein. Processing begins in the rough endoplasmic reticulum with the removal of the three glucose units, and continues during transport of the protein through the Golgi membranes. The formation of a complex oligosaccharide requires the removal of six mannose units followed by the stepwise addition of N-acetylglucosamine galactose, sialic acid and fucose from the appropriate nucleotide sugar. The addition of the latter sugars is performed by membrane bound glycosyl transferases. For synthesis of "high mannose" oligosaccharide side chains, a varying number of mannose residues are removed and no further sugars are added. The steps involved in processing of N-linked oligosaccharides are summarised in figure 3.

3.8.2. Glycosylation Events During Immunoglobulin Synthesis

Kinetic studies of the incorporation of defined sugar residues into newly-synthesised immunoglobulin chains illustrated that different sugars were incorporated into the molecule depending on the intracellular location of the molecule. The order in which the sugars were added appeared to be similar in rabbit lymphoid cells (Cohen and Kern, 1969), murine myeloma cells (Melchers, 1970, 1971; Choi et al, 1971; Parkhouse and Melchers, 1971; Della Corte and Parkhouse, 1973a), and in mitogen-stimulated B-lymphocytes (Melchers and Andersson, 1973) and was as follows:

a) glucosamine and mannose were added close to the time

FIGURE 3

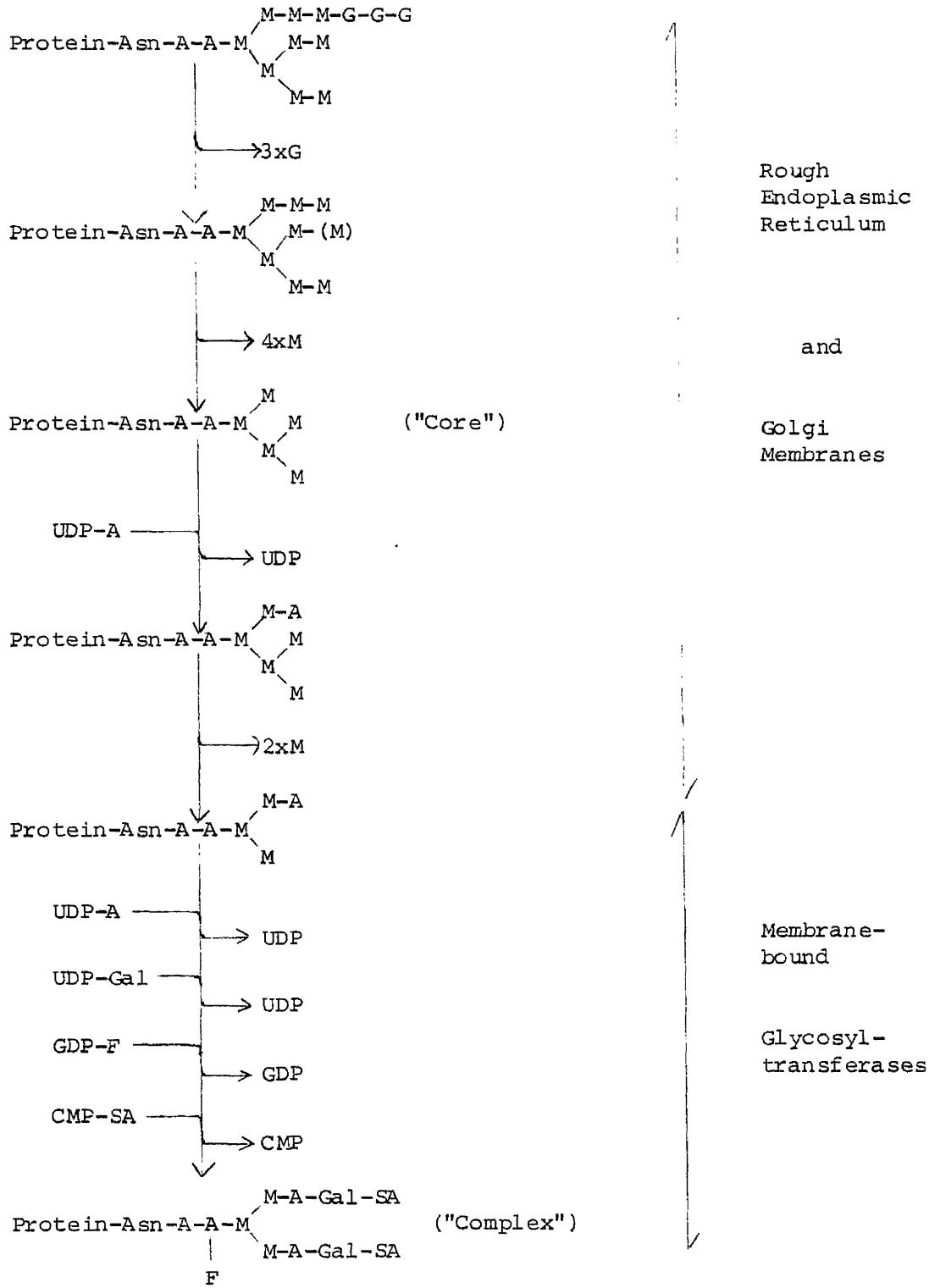
Processing of N-Linked Oligosaccharides

After addition of oligosaccharide side chains to proteins, the carbohydrate prosthetic group is modified during intracellular transport of the glycoprotein. These modification events are illustrated in the figure.

Key:-

A	-	N-acetyl glucosamine
F	-	Fucose
G	-	Glucose
Gal	-	Galactose
M	-	Mannose
SA	-	Sialic acid.

FIGURE 3: Processing of N-Linked Oligosaccharides



(Schwartz & Datema, 1980; Gibson et al, 1980; Parodi & Leloir, 1979)

of synthesis of the immunoglobulin chains (although there is some debate as to whether or not this is a nascent chain event);

b) galactose was added in the Golgi region, and

c) fucose and sialic acid were added close to, or concomitant with, secretion.

One exception to this trend was that during biosynthesis of IgM, galactose was added closer to the time of secretion.

3.8.3 Influence of Oligosaccharide Moieties on Transport of Immunoglobulins

Much controversy has surrounded the functional significance of the carbohydrate moieties of glycoproteins since it was suggested that such prosthetic groups were necessary for efficient transport of glycoproteins (Eylar, 1965). In early experiments on the IgG_I synthesising myeloma MOPC-21, it was demonstrated that addition of 2-deoxyglucose to the cells inhibited the migration of radiolabelled γ-chains from polyribosomes through the rough endoplasmic reticulum to the smooth endoplasmic reticulum (Melchers, 1973). If 2-deoxyglucose prevented only glycosylation, then this data would have been consistent with the Eylar postulate. However, 2-deoxyglucose is known to have wide effects as a metabolic inhibitor and, therefore, the data obtained from the above experiments may not be due to interference with carbohydrate-mediated processes for the control of intracellular transport of immunoglobulin chains, but may reflect interference with the energy supply required for the transport processes.

Later studies of a mutant of the MPC-11 murine plasmacytoma

which had defective glycosylation processes, revealed that IgG containing both glycosylated and non-glycosylated γ-chains was efficiently secreted from the plasmacytoma (Weitzman and Scharff, 1976).

Data from other systems is relevant. The effects of inhibition of N-glycosylation on a variety of membrane associated and secretory proteins has been investigated (reviewed by Gibson et al, 1980) and the data are summarised in table 1.

All of the experiments described in table 1 employed the antibiotic tunicamycin as the inhibitor of N-glycosylation. The antibiotic is a fermentation product of the bacterium Streptomyces lysosuperficius (Takatsuki et al, 1975) and, presumably by virtue of its possession of a glucosamine function, inhibits the first step in assembly of oligosaccharides on the dolichol carrier (Tkacz and Lampen, 1975; Lehle and Tanner, 1976; see figure 2). Inhibition of this reaction effectively inhibits synthesis of oligosaccharides which would normally be used in the process of N-glycosylation of proteins. Tunicamycin also possesses a long dolichol-like tail and a uridine moiety, and therefore has the capacity to inhibit metabolic processes other than synthesis of dolichol diphosphoryl N-acetylglucosamine, e.g. synthesis of dolichol itself.

In studies of immunoglobulinsecretionin the presence of tunicamycin, it has been demonstrated that secretion of IgM from MOPC 104E was inhibited by 81% by tunicamycin (Hickman and Kornfeld, 1978), and IgA secretion from murine plasmacytomas was inhibited by more than 60%. IgG secretion was inhibited by only 28% if the IgG synthesising cells were treated with tunicamycin (Hickman and Kornfeld, 1978). The secretion of IgE from a rat plasmacytoma was also

TABLE 1:- Effects of Tunicamycin on Protein Function

PROTEIN	EFFECT OF TUNICAMYCIN	REF.
Fibroblast Procollagen	Failure of Secretion	Olden et al, 1978
Sindbis Viral Proteins	Failure to Insert into Membrane	Schwartz et al, 1976.
Semliki Viral Proteins	Failure to Insert into Membrane	Leavitt et al, 1977.
Human Fibroblast Interferon	Secreted and Active	Mizrahi et al, 1978.
Erythrocyte Glycophorin	Inserted Normally into Membrane	Gahmberg et al, 1980.
Sindbis Viral Proteins	Increased Susceptibility to Proteolysis	Schwartz et al, 1976.

inhibited by the antibiotic (Hickman et al, 1977).

With respect to membrane insertion of immunoglobulins, it was claimed that non-glycosylated IgA could be efficiently inserted into the plasma membrane of MOPC 315 cells (Hickman and Wong-Yip, 1979). However, non-glycosylated IgM could not be detected at the membrane of Daudi human B-lymphoma cells cultured for prolonged periods in tunicamycin (Singer, 1978; this thesis). The data of Hickman and Wong-Yip (1979) is open to criticism, particularly on the grounds that no attempt was made to confirm that the presumptive membrane form of α-chain was different to the secretory α-chain made by MOPC 315 cells.

Therefore, at the outset of these studies, the situation remains unclear with regard to the importance of N-glycosylation of immunoglobulins in their secretion and membrane insertion. Since B-lymphocytes are known to be capable of synthesising structurally distinct forms of μ-chain (Singer et al, 1980; Rogers et al, 1980; Alt et al, 1980a; Bergman and Haimovich, 1978; Vassalli et al, 1979; Sidman, 1981) γ-chain (Singer and Williamson, 1980; Lifter et al, 1980; Oi et al, 1980; this thesis) and α-chains (this thesis) it is of great interest to study the comparative influence of N-linked oligosaccharide side chains in regulation of intracellular transport of membrane and secretory polypeptides of immunoglobulins of various classes.

4. STRUCTURE AND BIOGENESIS OF RECEPTOR AND EFFECTOR ANTIBODY

4.1. The Clonal Selection Paradox

The principal tenet of the Clonal Selection Hypothesis (Burnet, 1959) is that antibody forming cells express a receptor antibody which is identical to the antibody secreted by that cell upon antigen-driven clonal expansion. A paradoxical situation is therefore revealed, and the question arises; how can an hydrophilic macromolecule, such as serum immunoglobulin, exist in a functional state in the hydrophobic environment of the lipid bilayer? Two major schools of thoughtemerged to explain the paradox and the essential features of these theories were as follows:

a) immunoglobulin structure was such that it allowed the molecule to adopt different conformations and hence exist functionally in two different microenvironments, or,

b) the structures of receptor and effector (i.e. membrane and secretory) immunoglobulin were different.

The latter hypothesis has proved to be accurate with much evidence now available at protein, RNA and DNA levels which illustrates that membrane and secretory immunoglobulin heavy chain polypeptides are indeed structurally different and are discrete biosynthetic entities. The pertinent data are discussed below.

4.2. Topological Considerations for Receptor Immunoglobulins

From experiments demonstrating the capacity of B-lymphocytes to bind antigen (McConnell et al, 1969; Ada, 1970; Wigzell and Andersson, 1969; Warner, 1974) and those illustrating that the areas

of sequence responsible for antigen binding were located at the V region (at the N-terminal end) of heavy and light chains (Hilschmann and Craig, 1965; Milstein, 1966; Baglioni et al, 1966; Putnam et al, 1966; Wu and Kabat, 1970), it is clear that the N-terminal portions of membrane immunoglobulin are exposed to the extracellular environment while the amino acids at or near the C-terminus are likely to be responsible for anchorage of the immunoglobulin in the cell membrane. Recent evidence to support this notion comes from studies with lipophilic nitrenes which label only the μ-chain of membrane IgM; this is consistent with the hypothesis that the C-terminal sections of membrane IgM are buried in the lipid bilayer. Light chains, peripheral proteins, are not labelled by this reagent (Owen et al, 1980).

Studies of membrane IgM radioiodinated either in situ or in free solution (Uhr and Vitetta, 1973) showed that the ratio of radioactivity in μ-chains compared with light chains was almost the same irrespective of the iodination regime, a result which suggests that the membrane IgM is largely exposed on the cell membrane. Labelling of components buried in the lipid bilayer may have occurred due to diffusion of short-lived iodine radicals into the bilayer (Bayse and Morrison, 1971; Marchalonis and Cone, 1973). More recent studies using a similar strategy on human IgM, illustrated that the μ/L ratio for incorporation of radioiodine was significantly lower for membrane IgM than for 8S IgM monomers derived from monoclonal 19S IgM myeloma proteins (Seon and Pressman, 1979). This data suggests that a portion of the membrane IgM is buried in the lipid bilayer.

Fluorescent antibody directed against determinants located in the Fc portions of μ-chains failed to effectively stain lymphocytes bearing membrane IgM (Pernis et al, 1970; Froland and Natvig, 1972). Furthermore, it was demonstrated that the 50 amino acids at the C-terminus of membrane IgM were inaccessible to the antibody directed against Fc determinants of μ-chains (Fu and Kunkel, 1974). These data support the notion that a portion of membrane IgM is inserted into the lipid bilayer.

Data pertaining to the susceptibility of membrane IgM to proteolysis were also in accord with the above hypothesis. Membrane IgM of intact cells was shown to be less susceptible to proleolytic degradation than its secreted counterpart when treated with papain (Vitetta and Uhr, 1976), trypsin, α-chymotrypsin or pronase (Hough et al, 1977).

The large weight of evidence therefore supports the notion that a portion of the heavy chain of membrane immunoglobulin is inserted into lipid bilayer.

4.3. Evidence for Structurally Distinct Membrane and Secretory μ-Chains.

Early investigations examining the possibility that the μ-chains of membrane IgM possessed a C-terminal hydrophobic region compared the relative mobilites of radioiodinated membrane and secreted μ-chains on SDS-PAGE. The initial observations suggested that membrane μ-chain was of lower AMW than its secreted counterpart Melcher and Uhr, 1973). This was taken as evidence that binding of IgM to the cell membrane was accomplished via conformational changes, perhaps mediated by under-glycosylation of the μ-chains destined for

membrane insertion, which allowed exteriorisation of hydrophobic sequences which, in turn, would form loops capable of hydrophobic interaction with the lipid bilayer (Vitetta and Uhr, 1975b).

However, similar studies by the same and different workers came to the opposite conclusion. In the later investigations the membrane μ-chain of murine splenocytes migrated more slowly on SDS-PAGE than its secretory counterpart, suggesting a higher AMW for membrane μ-chains than for secretory μ-chains. The AMW difference between membrane and secretory μ-chains was estimated to be 1700 daltons (Lisowska-Bernstein and Vassalli, 1975; Melcher and Uhr, 1976). Similar studies in the human system also illustrated that membrane μ-chains have a higher AMW than secretory μ-chains (Singer and Williamson, 1980; Singer et al, 1980a; Williams and Grey, 1978). Human γ- and α-heavy chains seem to follow this trend (this thesis) as do chicken (Lifter et al, 1980) and murine γ-chains (Oi et al, 1980).

Studies of the hydrophobic properties of membrane and secretory μ-chains established the following;

a) removal of non-ionic detergent from cell lysates containing membrane and secretory IgM molecules led to a proportion of the molecules, the membrane IgM fraction, becoming insoluble; secretory IgM was totally soluble in the absence of the detergent (Melcher et al, 1976) and;

b) membrane IgM had a lighter bouyant density in the presence of non-ionic detergents than had secreted IgM as judged by isopycnic centrifugation in caesium

chloride gradients (Melcher and Uhr, 1977).

These data, considered in conjunction with data from SDS-PAGE analyses, are consistent with the hypothesis that membrane μ-chain possesses an extra hydrophobic sequence capable of interaction with the components of the lipid bilayer. The topological arguements advanced above (section 4.1.) would suggest that the hydrophobic peptide is located at the C-terminal region of the membrane μ-chain.

The C-terminal sequences of human membrane and secretory μ-chains in the B-cell line Daudi were analysed by carboxypeptidase digestion experiments (Williams et al, 1978; Singer et al, 1980a). The kinetics of release of tritiated tyrosine were much faster for secretory μ-chain than for membrane μ-chain clearly indicating different C-terminal structures on the two μ-chain species.

The evidence from the quoted studies are consistent with the hypothesis that membrane μ-chain possesses a C-terminal hydrophobic peptide which confers the necessary structural requirements for efficient insertion into, and stability within, the cell membrane: this extra hydrophobic peptide is absent from secretory μ-chains.

4.4. Biosynthesis of Membrane and Secretory μ-Heavy chains

Two major hypotheses can be advanced to explain the simultaneous appearance within a single B-lymphocyte of structurally distinct membrane and secretory μ-chain polypeptides. These hypotheses propose either;

a) synthesis of a single polypeptide, with appropriate post-translational modifications being invoked to generate membrane or secretory μ-chain as required, or;

b) simultaneous synthesis of structurally distinct membrane and secretory heavy chain polypeptides.

4.4.1. In Vivo Biosynthesis

Investigation of the biosynthesis of IgM in the murine B-cell line 38C-13 and in normal spleen cells demonstrated three species of μ-chain (Bergman and Haimovich, 1978). These μ-chains were designated, in order of descending AMW, as membrane μ, secretory μ and intracellular μ. Digestion of isolated μ-chains with carboxypeptidase failed to illustrate any difference at the C-termini of the three μ-chain species. The authors proposed that the polypeptides resolved represented μ-chains which were structurally differentiated for expression either in hydrophilic serum or in the hydrophobic environment of the lipid bilayer.

In the human system, several B-cell lines have been shown to synthesise two distinct μ-chain forms (Singer and Williamson, 1980). In one particular cell line, BJAB, a double labelling experiment involving a pulse-chase with ^{35}S-methionine followed by lactoperoxidase-catalysed radioiodination (^{131}I) of the cell membrane proteins of the same cells showed;

a) the higher AMW μ-chain was doubly labelled, i.e. this was the membrane μ-chain;

b) the lower AMW μ-chain was labelled only with ^{35}S and was the only μ-chain found in culture supernates; i.e. this μ-chain was structurally differentiated for expression in an aquaeous environment.

Two μ-chain forms could also be demonstrated in both control and tunicamycin-treated aliquots of the B-cell lines Daudi and Raji, and also in products of in vitro synthesis programmed by mRNA purified

from these cell lines (Singer, 1978; Singer et al, 1980a).

In contrast to the data of Bergman and Haimovich (1978), the Daudi secretory and membrane μ-chains have different C-terminal amino acid sequences as judged by carboxypeptidase digestion of isolated μ-chains (Williams et al, 1978; Singer et al, 1980). These data are consistent with hypotheses favouring a C-terminal hydrophobic extension present on membrane μ-chain but absent from secretory μ-chain.

4.4.2. In Vitro Biosynthesis

The above data strongly suggests, but does not unequivocally demonstrate, the simultaneous biosynthesis of distinct membrane and secretory μ-heavy chain polypeptides in a single B-lymphocyte. The most compelling evidence that this is indeed the case comes from experiments where purified cellular poly A^+ mRNA was used to direct synthesis of polypeptides in an in vitro translation system.

Translation of mRNA isolated from the murine plasmacytoma WEHI-321 in the rabbit reticulocyte translation system followed by immunoprecipitation of the in vitro synthesised μ-chains and analysis of these by SDS-PAGE showed that two μ-chain bands were specifically immunoprecipitable from the translation products (Alt et al, 1980). These μ-chain bands corresponded to the distinct membrane and secretory μ-chains complete with signal sequences. Since there are no reports of post-synthetic modifications in the rabbit reticulocyte system it is likely that the two polypeptides are independently synthesised, i.e. separate mRNA species encode the distinct membrane and secretory μ-heavy chains.

The same conclusion was reached by Singer and his colleagues

(Singer et al, 1980a) who performed in vitro translation of mRNA isolated from human B-lymphoma cell lines. Upon immunoprecipitation of the translation products and analysis on SDS-PAGE, two μ-chain bands could be readily demonstrated. This is again indicative of independently directed synthesis of membrane and secretory μ-heavy chain polypeptides in a single cell. Additionally, it was noted that the relative amounts of secretory and membrane μ-chain polypeptides synthesised in vitro were almost identical to the in vivo biosynthetic pattern. This data suggested a significant degree of regulation at the pre-translational level, probably at the level of RNA splicing (Singer, 1978; Singer et al, 1980a).

The data from in vivo and in vitro biosynthetic investigations are consistent with the postulate that structurally distinct membrane and secretory μ-chain polypeptides are encoded by separate mRNA species and can be simultaneously synthesised in a single cell. The next stage in the investigation is to analyse the biogenesis of the discrete mRNA species and to address the question of how many genes are required for the synthesis of membrane and secretory μ-chain polypeptides in a single immunocompetent B-lymphocyte.

4.4.3. Existence of Discrete Messenger RNA Species Encoding Membrane and Secretory μ-Heavy Chain Polypeptides

The possibility that membrane and secretory μ-chain polypeptides synthesised in vitro were derived from a single precursor was ruled out by analysis of the number of mRNA molecules detected by probing separated RNAs with a highly radioactive homologous cloned cDNA probe.

The first demonstration of multiple mRNA molecules encoding immunoglobulin heavy chains was made in the murine B-cell line 70Z/3 (Perry and Kelley, 1978). Total cytoplasmic mRNA was separated by electrophoresis in agarose gels containing methyl mercury hydroxide as a denaturing agent, and transferred to diazophenylthioether paper (Alwine et al, 1977) to form an RNA "blot". The blot was probed using cloned sequences of the constant region of the μ-chain gene and four mRNA species were visualised. These mRNAs were of length 2.1kb, 2.4kb, 2.7kb, and 3.0kb, and it was proposed that the mRNA species associated with membrane-bound polyribosomes were responsible for directing the synthesis of membrane and secretory μ-heavy chain polypeptides, whereas those mRNAs found only in the cytoplasm encoded the intracellular (Bergman and Haimovich, 1978) μ-chain polypeptide.

Similar studies were performed in the cell line WEH1-231, but in these experiments the RNA "blot" was probed with a nick-translated ^{32}P-labelled cloned cDNA probe containing the 770 bases at the 3'-end of the μ-chain mRNA (Alt et al, 1980). Two mRNA species were detected by this probe one of 2.7kb and another of 2.4kb. These data illustrate that two μ-chain mRNA species are present in WEH1-231 cells, one encoding membrane μ-chain and the other encoding secretory μ-chain, and substantiate the data obtained from in vitro translation studies.

A similar finding was made in a comparable study of μ-chain mRNA species in MOPC-104E cells (Rogers et al, 1980). Cloned cDNA probes derived from μ-chain mRNA were prepared (Calame et al, 1980) and two of these probes had the following properties (Calame et al,

1980; Rogers et al, 1980; Early et al, 1980);

a) pl04Eμl2, contained nucleotide sequences from Cμ2, Cμ3, Cμ4 and the 3'-untranslated regions of germ line μ-genes, and;

b) pl04Eμ6, contained sequences derived from Cμ2, Cμ3, Cμ4 and from the so-called M-exon (Early et al, 1980).

Probing of blots of MOPC 104E mRNA with ^{32}p-nick translated pl04μl2 detected two RNA species of 2.4kb and 2.7kb. These observations are consistent with the data of Alt et al (1980) and with in vitro translation data.

Several pieces of evidence indicate a correlation between the 2.7kb mRNA species and synthesis of membrane μ-chain polypeptides, and a similar relationship between the 2.4kb mRNA and synthesis of secretory μ-chain;

a) plasmacytomas synthesising mainly secretory μ-chains in vivo were shown to possess more 2.4kb mRNA than 2.7kb mRNA, while in plasmacytomas synthesising more membrane μ-chain than secretory μ-chain in vivo were shown to contain more 2.7kb mRNA than 2.4kb mRNA.

b) the somatic cell hybrid produced by fusion of WEHl 279 cells and MPC-11 cells synthesised only secretory μ-chain in vivo and contained only 2.4kb mRNA (Rogers et al, 1980).

The data therefore suggest that two distinct mRNA species encode membrane and secretory μ-chain polypeptides and that a 2.7kb mRNA species encodes membrane μ-chain, while a 2.4kb mRNA molecule is responsible for the synthesis of secretory μ-chain.

Direct evidence to support the hypothesis that the 2.7kb mRNA encoded the membrane μ-chain was obtained from the following experiment. A specific restriction fragment was prepared from the cDNA clone pl04Eμ6 which contained nucleotide sequences found only in regions at, and close to, the M-exon (Calame et al, 1980; Early et al, 1980). The fragment was then used to probe blots of MOPC 104E mRNA, and it was observed that the probe hybridized only to the 2.7kb mRNA (Rogers et al, 1980). This is positive evidence that the 2.7kb mRNA contains the information required for synthesis of membrane μ-chain while the 2.4kb mRNA encodes the secretory μ-chain polypeptide. The organisation of coding sequences responsible for this phenomenon are reviewed below.

5. EXPRESSION OF IMMUNOGLOBULIN GENES DURING B-LYMPHOCYTE DIFFERENTIATION

5.1. Introduction

Many studies have demonstrated the existence in adult spleen and bone marrow of a cell capable of replacing the cells of the myeloid and lymphoid systems in lethally irradiated recipients (reviewed by Scher, 1981). These pluropotential stem cells are believed to give rise not only to all cells of the B-lymphocyte lineage, but also to cells of T-lymphocyte and myeloid lineages. In early B-cell development three distinct patterns of immunoglobulin expression are described:

a) cytoplasmic but no membrane or secretory IgM (pre-B-cells;

b) cytoplasmic and membrane IgM but no secretory IgM (immature B-cells), and;

c) membrane IgM with very little cytoplasmic IgM (mature B-cells).

In later stages of differentiation, the B-lymphocyte also acquires membrane IgD before switching to expression of another immunoglobulin isotype e.g. IgG or IgA. The ontogeny of these cell types and their characteristics are now discussed.

5.2. Pre-B-Lymphocytes

Pre-B-lymphocytes are defined as cells which contain detectable cytoplasmic IgM but do not express membrane IgM. In the mouse, medium to large sized cells in fetal liver were shown to actively synthesise 8S IgM as early as 10 days gestation (Melchers et al, 1975; Melchers, 1977). Other workers have detected cells containing cytoplasmic IgM in murine fetal liver at 13 days gestation (Raff et al, 1976). Lactoperoxidase catalysed iodination of cell membrane proteins was employed in an attempt to detect membrane IgM on fetal liver pre-B-cells, and membrane IgM could be demonstrated at 13-15 days gestation (Melchers et al, 1976). Rosette formation assays also detected membrane IgM positive fetal liver pre-B-cells at 13 days gestation (Rosenberg and Parish, 1977). However, Raff et al (1976) could not demonstrate membrane IgM on their cytoplasmic IgM positive cells. The general concensus of opinion is that the cytoplasmic IgM positive cell is the precursor of the membrane IgM positive and cytoplasmic IgM positive pre-B-cell in murine fetal liver.

Evidence to support the hypothesis that the cytoplasmic IgM positive, membrane IgM negative cell is the precursor of cytoplasmic

and membrane IgM positive cells was obtained from studies in the human using anti-idiotypic antibodies raised against monoclonal IgM of patients suffering from B-cell malignancies. These antibodies were shown to react with the membrane IgM of the malignant cells and also with the cytoplasmic IgM of a small percentage of the bone marrow pre-B-cells of these patients (Kubagawa et al, 1978). Pre-B-cells also exhibit allelic exclusion (Lawton et al, 1977).

5.2.1. Distribution of Pre-B-Cells

The earliest appearance of identifiable pre-B-cells in mice is in the fetal livers at 10 days gestation (Melchers et al, 1975). Pre-B-cells are also found in murine fetal spleen and bone marrow (Marchalonis et al, 1971). In adults, pre-B-cells can be demonstrated in the bone marrow but not in lymph nodes spleen or thymus (Raff et al, 1977).

5.2.2. Heterogeneity of Pre-B-Cells

Studies of the rate of incorporation of DNA precursors into DNA was used to illustrate the heterogeneity of the pre-B-cell population. Large cytoplasmic IgM positive, membrane IgM negative cells labelled more rapidly than small cells of the same pattern of immunoglobulin expression which in turn incorporated label more rapidly than membrane IgM positive cells (Owen, et al, 1977). These data suggest that a pool of large cytoplasmic IgM positive, rapidly proliferating cells, give rise to smaller cytoplasmic IgM positive cells which in turn differentiate into membrane and cytoplasmic IgM positive B-cells.

5.2.3. Immature B-Lymphocytes

Immature B-lymphocytes are defined as cells which have readily demonstrable membrane IgM but have little or no cytoplasmic IgM.

Such cells are detectable in murine fetal liver at 16 days gestation and in fetal spleen after 16 to 17 days. These cells are also found in adult bone marrow (Scher, 1981).

Morphologically, small immature B-lymphocytes are indistinguishable from mature B-lymphocytes. However, several features allow delineation of mature and immature small B-lymphocytes. Using radioimmunoassay methods it was illustrated that immature B-cells derived from adult bone marrow had a more heterogeneous distribution of membrane immunoglobulin (Osmond and Nossal, 1974a,b), and also that neonatal B-cells have more membrane immunoglobulin than adult B-cells (Sidman and Unanue, 1975).

A more striking difference was revealed when radioiodinated membrane proteins of mature and immature B-cells were subjected to immunoprecipitation with anti-immunoglobulin reagents and the products analysed by SDS-PAGE. Both populations contained species of apparent molecular weights 63,000 daltons (μ-chain) and 22,000 daltons (light-chain), but the mature cells possessed a further component of AMW 64,000 daltons not found on immature cells: this is the δ-chain (Vitetta et al, 1975; 1977). Thus, immature B-cells possess more membrane IgM than their mature derivatives, but lack IgD which is unique to the mature B-cell population.

5.3. Mature B-lymphocytes

The mature B-lymphocyte population consists of membrane-IgM positive, membrane-IgD positive and cytoplasmic-IgM negative cells. These cells are found in the spleens of 1-2 week old mice, adult spleen and in adult bone marrow (reviewed by Scher, 1981). This population is believed to be the precursor of daughter memory cells

expressing immunoglobulin isotypes other than μ which are derived by antigen driven differentiation of the mature virgin B-lymphocyte.

5.4. Isotype Diversification

Much evidence is now available to substantiate the hypothesis that B-lymphocytes can alter their immunoglobulin isotype phenotype during differentiation without altering the idiotype which was originally expressed. The evidence comes from a variety of experimental approaches:

a) characterisation of 'double' myelomas;

b) study of idiotype expression on progeny of a single antigen binding cell, and;

c) investigation of classes of immunoglobulin produced by antibody forming cells.

Several types of 'double' myeloma syndromes have been described in which two myeloma proteins of different classes share an identical idiotype and, apparently, an idential V region amino acid sequence. 'Double' myelomas of the following types have been described:

a) IgM and IgG2 (Wang et al, 1970a,b; Fudenberg et al, 1971);

b) IgM and IgG3 (Penn et al, 1970);

c) IgM and IgA (Seon et al, 1973; Yagi and Pressman, 1973) and;

d) IgG and IgA (Sledge et al, 1976).

It is generally accepted that the explanation for the occurence of such rare myelomas was that transformation of a lymphocyte clone occured just as the lymphocyte was in the process of switching from

expression of one C_H gene to another while being committed to expression of one particular V_H gene.

In studies of idiotype expression, it was found that the progeny of a single antigen binding cell had identical idiotypes to that found on the original cell (Press and Klinman, 1973; Eichmann and Rajewsky, 1975). Furthermore, it was demonstrated that IgM and IgD expressed on the same lymphocyte bore identical idiotypes (Salsano et al, 1974; Fu et al, 1975) and had identical antigenic specificities (Pernis et al, 1974).

Immunofluorescence studies revealed cells which expressed different classes of immunoglobulin in the cytoplasm and on the cell membrane (Pernis et al, 1971). Several groups of investigators have reported that a small percentage of antibody forming cells secrete both IgM and IgG (Ivanyi and Dresser, 1970; Nordin et al, 1970; Nossal et al, 1971).

These data are indicative of isotype diversification during B-lymphocyte differentiation and argue strongly that a B-lymphocyte is capable of altering the pattern of isotype expression whilst remaining committed to the original idiotype and antigen binding specificity, i.e. to the original V_H gene. These findings are consistent with the predictions of the Clonal Selection Hypothesis.

5.5. Sequential Expression of Immunoglobulin Isotypes

The use of anti-Ig reagents to specifically inhibit immunoglobulin synthesis by B-lymphocytes has established that immunoglobulin isotypes are sequentially expressed in vivo (reviewed by Lawton and Cooper, 1974).

In the chicken it has been demonstrated that such treatment of embryonic lymphocytes resulted in:

a) depression of serum IgM;

b) depression of serum IgG, and;

c) depression of serum IgA levels,

all in later life (Kincade et al, 1970; Kincade and Cooper, 1973). The same treatment at hatch depressed only serum IgM levels: IgG and IgA levels were unaffected (Kincade et al, 1971). Similar observations were made in equivalent studies in germ-free mice (Lawton et al, 1972).

Anti-immunoglobulin reagents were also used to inhibit primary or secondary antibody responses in vitro. In the primary response, anti-μ suppressed all classes of response while anti-γ_1, anti-γ_2 or anti-α suppressed only the response of the isotype to which they were directed. However, in secondary responses, anti-μ had progressively less effect with time after priming (Pierce et al, 1972a, b). These data suggest that some membrane IgM-expressing cells are pre-committed to differentiate into cells expressing one of the other isotypes.

Two models may be invoked to explain the sequential expression of isotypes:

a) the switch model for antigen-driven isotype diversification, or;

b) the minimal receptor model for antigen independent diversification of immunoglobulin isotypes.

Both models share the concept of isotype switch during an immune response but disagree on the underlying mechanisms. The

'switch' model proposes that antigen drives diversification of receptor isotypes from a single precursor cell, while the 'minimal receptor' hypothesis suggests the existence of separate precursor populations for any given isotype which arise independently of antigenic influence. The data from *in vivo* anti-immunoglobulin treatment (Kincade *et al*, 1970, 1971; Lawton *et al*, 1972, Kincade and Cooper, 1973) supports the minimal receptor model while the *in vitro* studies using anti-immunoglobulin as an inhibitor of antibody synthesis (Pierce *et al*, 1972a,b) supports the switch model.

5.6. Current Concepts in Generation of Multiple Receptor Isotypes

The initial impetus for investigation of receptor isotype diversity came from the finding of IgD on the membranes of human (van Boxel *et al*, 1972) and murine lymphocytes (Abney and Parkhouse, 1974; Vitetta *et al*, 1975). The existence of lymphocytes in spleens of 3 day old mice expressing membrane IgG_1, IgG_2, IgG_3 and IgA isotypes (Parkhouse and Cooper, 1977; Kearney and Abney, 1978) has recently been established. These isotypes could only be demonstrated on cells which had previously been membrane IgM positive: IgD was also demonstrated on cells at this stage of development. A significant conclusion drawn from these studies was that only one of the additional isotypes could be expressed in any membrane IgM-positive cell (Kearney and Abney, 1978). Acquisition of IgD by membrane IgM positive cells was shown to be an antigen independent event and also to be independent of the switch to other receptor isotypes (Lawton and Cooper, 1974; Vitetta and Uhr, 1975).

5.6.1. *In Vitro* Stimulation of Lymphocytes with Lipopolysaccharide

As soon as immature B-cells appeared in the livers of fetal

mice, they could be induced to generate other receptor isotypes by culturing in vitro in the presence of lipopolysaccharide (LPS) (Kearney and Lawton, 1975). The expression of such isotypes was independent of proliferation but required mRNA and protein synthesis (Kearney and Abney, 1978). Upon examination of cells positive for both membrane IgG_2 and IgM, it was discovered that membrane IgM was rapidly lost from the cells at the time of maximum proliferation and, concomitantly, cytoplasmic IgG_2 became readily detectable (Kearney and Abney, 1978). Persistence of membrane and secretory IgM in cells differentiating to IgG_2 isotype expression could be promoted by inhibiting the proliferation of the differentiating cells (Kearney et al, 1977b). These data suggested that cellular proliferation was required for the loss of IgM and IgD isotypes and differentiation to single (e.g. IgG_2) isotype daughter cells.

Much information remains to be obtained regarding the biological processes responsible for the control of B-lymphocyte differentiation and isotype diversification. Recombinant DNA technology has, however, provided great insights into the molecular mechanisms which underly many of the phenomena described during B-lymphocyte differentiation. The molecular genetics of the antibody genes are detailed in the next section (section 6).

6. MOLECULAR BIOLOGY OF IMMUNOGLOBULIN GENES

The most striking feature of the primary structure of immunoglobulins is the great variability of amino acid sequences at the N-terminal regions of both heavy and light chains. Clearly this variability can be correlated with the capacity of a given antibody to specifically bind its complementary antigen. Genetic explanations must therefore be found to account for:

a) the extent and maintenance of amino acid sequence diversity of V-regions;

b) the phenomena of allelic exclusion i.e. the expression of only a single V_H and a single V_L resulting in one antibody specificity per cell and

c) the capacity of a single B-lymphocyte to switch from synthesis of one immunoglobulin isotype to another during antigen-driven clonal expansion.

6.1. The Murine Igλ Locus

Studies of the amino acid sequences of mouse myeloma λ_1 light chains demonstrated a simple pattern of variability consistent with the hypothesis that λ_1 V-region is encoded in the germ-line by a single gene (Weigert and Riblet, 1976; Gottlieb, 1980). Twelve of the eighteen λ_1 proteins sequences had identical sequences (termed the λ_O sequence) while the remainder differed by one or two amino acid substitutions restricted to the regions corresponding to the hypervariable regions of kappa chains (Weigert and Riblet, 1976).

By treatment of murine embryonic or plasmacytoma HOPC2020 DNA with restriction endonucleases and subsequent molecular cloning

of the DNA fragments generated, Tonegawa and his associates prepared cloned nucleotide sequences which contained:

a) $V_{\lambda 1}$ and $C_{\lambda 1}$ sequences in a single restriction fragment of plasmacytoma HOPC2020 DNA (Brack and Tonegawa, 1977) and;

b) $V_{\lambda 1}$ and $C_{\lambda 1}$ sequences in separate restriction fragments of embryonic DNA (Brack et al, 1978).

Furthermore, the mouse DNA appeared to contain a single $V_{\lambda 1}$ gene (Brack et al, 1978), and this data was consistent with the predictions based on amino acid sequence analysis of λ_1 polypeptides (Wiegert and Riblet, 1976; 1978).

The data of Tonegawa's group has several significant features when considered in conjunction with the known structures of immunoglobulin light chain mRNAs and polypeptides (Brack and Tonegawa, 1977; Brack et al, 1978; Bernard et al, 1978):

a) the nucleotide sequence of the $V_{\lambda 1}$ gene accounts for only 97 residues of the 110 amino acids of the V-region of the λ_1 polypeptide;

b) the remaining V-region codons (the J-segment) are found in segments of DNA close to, but not contiguous with, the C-region;

c) the $V_{\lambda 1}$ gene segment is separated at an unknown distance from the $J_{\lambda 1}/C_{\lambda 1}$ segments in embryonic DNA;

d) a somatic recombination event has joined $V_{\lambda 1}$ and $J_{\lambda 1}$ contiguously in the DNA of a λ_1 expressing plasmacytoma, and;

e) the coding sequence for the hydrophobic leader sequence is separated from the 5'-end of the V-region by a 93 base pair intervening sequence.

These data clearly illustrated that a functional λ_1 gene is composed of multiple genetic elements, each coding sequence or exon being separated from the others by an intervening sequence (intron) of non-coding DNA. The $V_{\lambda 1}$ and $C_{\lambda 1}$ exons are separate throughout the life of the B-lymphocyte but the critical event in formation of an active gene appears to be the recombination event bringing $V_{\lambda 1}$ and $J_{\lambda 1}$ exons together. In the plasmacytoma, integration of all the exons needed for a functional λ_1 polypeptide appears to occur by splicing of the primary nuclear RNA transcript, which contains leader, VJ and C exons and the appropriate introns, to give a messenger with contiguous leader, V, J and C coding sequences. This process is summarised in figure 4.

The tenets of the Dreyer-Bennett postulate are supported by the evidence obtained, not only from the study of the structure of murine λ_1 light chain genes, but also by similar studies of the murine Igκ and Igh loci (see below).

6.2. The Murine Igκ Locus

In contrast to the limited sequence variability of the λ_1 polypeptides, the amino acid sequence diversity of murine κ-chains is very extensive. Multiple germ-line V_κ genes were postulated to account for the differences in length and amino acid sequence characteristic of different groups of κ-myeloma proteins, and also because of the difficulties associated with models accounting for generation of individual V_κ gene groups from a single germ-line gene

by somatic diversification (Köhler et al, 1970). Analysis of murine V_κ amino acid sequences suggested that a minimum of 50 V_κ groups were necessary to account for the framework diversity observed in myeloma κ-chains (Weigert and Riblet, 1976).

Preparation of restriction fragments from embryonic and plasmacytoma DNA followed by electrophoretic separation and probing of the fragments with cloned V_κ and C_κ ^{32}P-cDNA probes illustrated that V_κ and C_κ exons resided in separate fragments in embryonic DNA but were in the same fragment in myeloma DNA (Hozumi and Tonegawa, 1976). These data illustrated that Igκ genetic elements were in different configuration in embryonic and plasma cell DNA.

Electron microscope analysis of cloned κ genes also demonstrated that the organisation of the coding sequences was different in embryonic and plasmacytoma DNA (Seidman et al, 1979). Nucleotide sequencing of the cloned κ-genes revealed that, as in the λ_1 situation, the V_κ gene possessed codons for only 95 of the 108 amino acids conventially assumed to represent the phenotypic V_κ-region (Seidman et al, 1978; Tonegawa et al, 1978). Further sequencing and electron microscopic analysis located the J-segments approximately 3.7 kilobases from the 5'-end of the C_κ-gene (Bernard et al, 1978; Brack et al, 1978; Seidman and Leder, 1978; Sakano et al, 1979). The J-segment contained not only the remaining 13 codons of the V_κ region but also specified the site for somatic recombination.

Molecular hybridisation studies confirmed the data obtained from analysis of the V_κ protein sequences, namely that multiple V_κ genes were present in the germ line (Seidman et al, 1978; Valbuena et al, 1978). The number of separate V_κ genes per haploid

genome has been postulated to be of the order of 300-1000 (Seidman et al, 1978; Leder et al, 1980). By integrating data obtained from sequence and electron microscopic investigations of the cloned genes a model may be proposed in which a V gene could be selected by joining it to a J-segment in close proximity to the single C-gene thus promoting transcription of an active immunoglobulin gene. It is evident from the data obtained from both Igλ and Igκ systems that the J-region is of critical importance in the generation of functional immunoglobulin genes via somatic recombination.

6.3. Molecular Structures in Somatic Recombination

6.3.1. The J-Segment

The J segment is found in Igλ, Igκ and Igh genetic loci (Leder et al, 1980). Igλ, appears to contain a single J-exon and Igh possesses 4 J segments while Igκ has 5 J segments one of which $J_{\kappa 3}$, is a pseudogene (Bernard et al, 1978; Sakano et al, 1979; Leder et al, 1980). The J-exons of J_{κ} and probably J_{H} have the following characteristics:

a) each has an unique nucleotide sequence;

b) each J segment is separated from its neighbours by approximately 300 base pairs;

c) at the 3' end of each J exon is an RNA splicing site;

d) at the 5' end of each J exon is a highly conserved DNA recombination site, and

e) in the J_{κ} system, the remaining codons of the V-region are encoded by the J-exon. (This is not the case in the J_{H} system where the D-segment, discussed below, is also responsible for encoding part of the V_{H} region of the immunoglobulin heavy chain polypeptide.)

6.3.2. Palindromic Sequences and the 12/23 Spacer Rule

All J exons sequenced to date contain a self-complementary heptanucleotide palindrome at their 5'-end (Sakano et al, 1979; Seidman et al, 1979). The sequence of the palindrome is CACTGTG. Furthermore, a nonameric nucleotide sequence, GGTTTTTGT, is located 12 ± 1 or 23 ± 1 base pairs 5' to the heptameric palindrome. Similar highly conserved nonamer and palindromic heptamer nucleotide sequences and associated 12 or 23 base pair sequences are also found at the 3'-end of the V gene. Recombination occurs only between a V gene with a 12 base pair spacer and a J segment with a 23 base pair spacer or vice versa (Early et al, 1980a; Sakano et al, 1980). The same 12/23 spacer rule also applies to V_H-D_H recombination in the Igh system. It has been speculated that the enzymes mediating V-J joining in Igκ and Igλ are the same and that such a recombinase has two functionally distinct units, one recognising the heptamer and nonamer separated by the 12 base pair spacer and the other recognising the heptamer and nonamer separated by the 23 base pair spacer (Sakano et al, 1980 ; Early et al, 1980a).

The orientation of the heptameric and nonameric sequences associated with the V- and J-genes is such that the sequences associated with the V gene are an inverted repeat of those associated with the J gene. In this orientation pairing of complementary palindromes within (rather than between) strands of DNA would favour formation of an inverted repeat stem structure (Sakano et al, 1979; Max et al, 1979). The formation of an inverted repeat stem structure would bring V and J coding sequences into close proximity and may promote activity of the recombinase necessary to effect V-J joining. The recombination event is apparently accompanied by loss of DNA between the selected

V-gene and the chosen J-gene. Precedents for the existence of inverted repeat structures and of putative recombinases are provided by studies of bacterial transposons which demonstrate formation of inverted repeat stem structures and deletion of intervening material (Cohen, 1976); also some DNA binding proteins such as the λ repressor are known to recognise palindromic sequences in the DNA (Ptashne et al, 1976). The role of inverted repeat sequences in somatic recombination is shown in figure 4.

6.3.3. Variability in the Frame of Somatic Recombination

Position 96 in the amino acid sequence of BALB/C κ light chains is known to be hypervariable (Barstad et al, 1978; Max et al, 1979). The sequences of many κ-chain proteins can not be accounted for in terms of any known J_κ nucleotide sequence unless one proposes that the frame of somatic recombination is variable. If such a mechanism were operative, cutting and splicing of the DNA at slightly different positions to form a contiguous VJ unit would result in several different amino acids being possible at position 96 in the amino acid sequence (Leder et al, 1980); Gottlieb, 1980).

Evidence for variability of crossing-over points in somatic recombination was obtained from nucleotide sequence analysis of several plasmacytoma recombinants and from amino acid sequences of myeloma proteins. The individual recombination events had the following consequences:

a) MOPC 41 (Seidman et al, 1979) and MOPC 321 (Sakano et al, 1979) both of which preserved the germ line sequence;

b) MOPC 173B deleted a single base giving rise to a cryptic gene with a nonsense J (Max et al, 1980) and

FIGURE 4

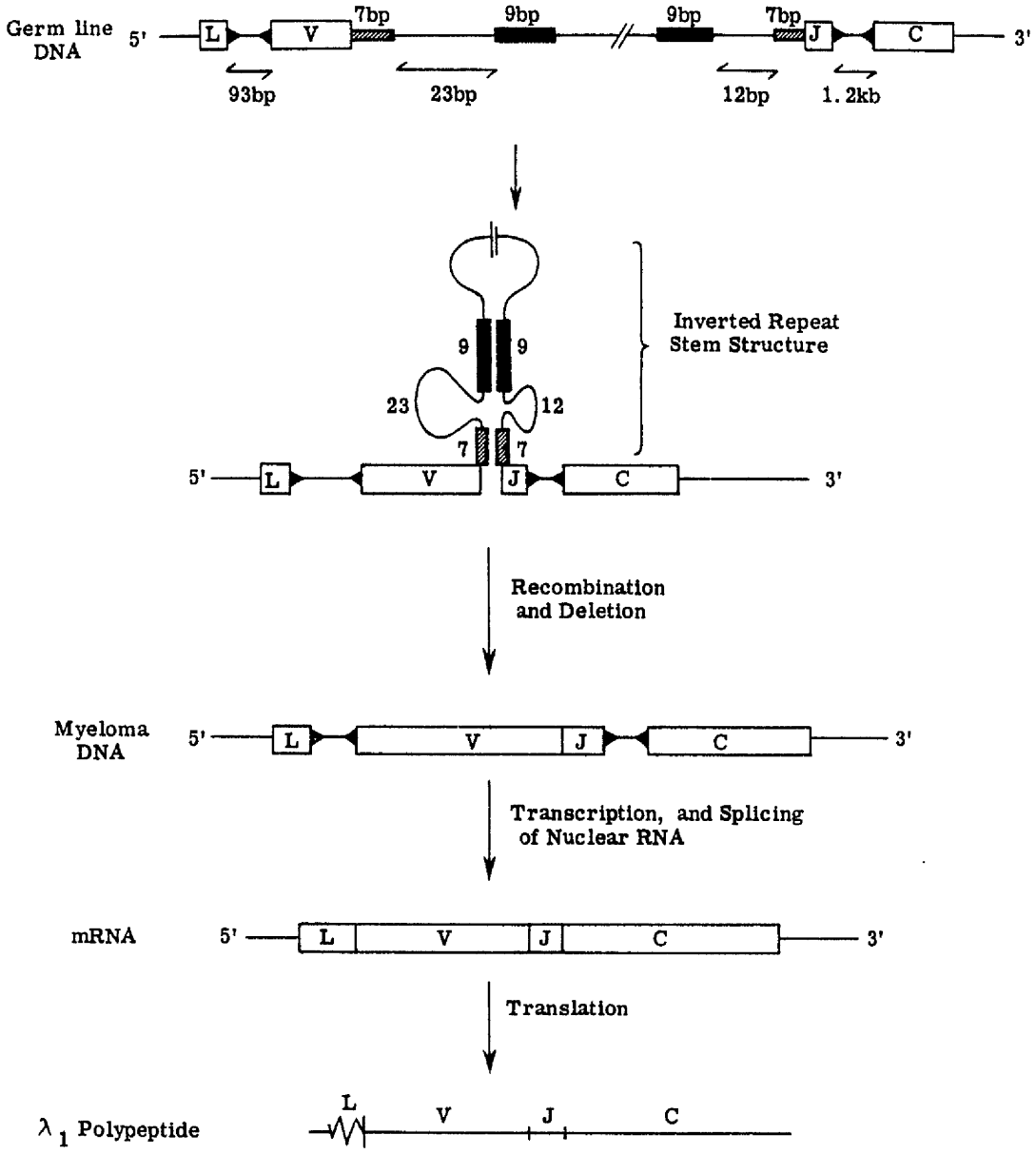
Somatic Recombination Events in Formation of Active λ_1 Immunoglobulin Genes

The molecular events involved in processing of germ line DNA encoding the λ_1 gene to yield functional mRNA and λ_1 polypeptides are illustrated. L, V, J and C-exons (open boxes) are separate in germ line DNA. Pairing of palindromic heptamers of sequence CACTGTG (shaded boxes) and pairing of nonameric sequences GGTTTTTGT (black boxes), which obey the 12/23 spacer rule, gives rise to inverted repeat stem structures (Early et al, 1980a; Leder et al, 1980). Recombination and deletion occurs to produce a contiguous VJ unit in myeloma DNA. A large nuclear RNA transcript is formed which contains L,V,J and C-exons separated by introns bounded by RNA splice sites (shaded triangles). RNA splicing yields an mRNA containing contiguous L, V, J and C-exons which is translated to yield a λ_1 polypeptide. Note that the antisense strand of DNA is not shown.

Key:-

L	-	Leader sequence
V	-	Variable region exon
J	-	Joining exon
C	-	Constant region exon
—∧—	-	Hydrophobic leader polypeptide
bp	-	base pairs
kb	-	kilobases.

Fig. 4 Somatic Recombination Events in Formation of Active λ_1 Immunoglobulin Genes



c) S107B deleted 2 codons to give an in phase but shortened J (Leder et al, 1980).

The data suggest that V-J recombination can occur at various cross-over points and may not always occur in the proper phase.

6.3.4. The $J_{\kappa 3}$ Pseudogene : A Molecular Lesion

After V -J recombination, the next major step in generation of κ-chain mRNA is the synthesis of a primary nuclear RNA transcript from which introns are removed to yield an mRNA with contiguous coding sequences for leader, V, J and C region amino acid sequences. The mechanism of removal of introns is not understood, but the consensus of opinion at present is that introns begin with the sequence GT and end in AG (Cohen ,1976) and that this is, in part, involved in recognition and removal of intron sequences.

In the J_{κ} system, four of the five J exons are followed at their 3'-ends by the GT sequence (Sakano et al, 1979; Max et al, 1979). The intron following $J_{\kappa 3}$ does not begin with GT and this may be the reason that amino acid sequences encoded by $J_{\kappa 3}$ are never observed in BALB/C κ-chains. If the GT sequence is required for removal of the intron between $J_{\kappa 3}$ and C_{κ} exons then absence of the sequence may result in failure of processing of the primary nuclear RNA transcript to yield a functional mRNA. This would be manifested as an absence of $J_{\kappa 3}$ sequences at the protein level, as has been observed. Thus the $J_{\kappa 3}$ exon has the genetic information to encode a polypeptide sequence but is not expressed due to lack of an RNA splicing signal; it is therefore a pseudogene.

6.4. The Murine Igh Locus

6.4.1. The V_H Gene : Nomenclature of Components

The generation of a functional V gene for immunoglobulin

light chains involves a single rearrangement to bring V_λ and J_λ or V_κ and J_κ coding sequences together. The generation of a V_H gene requires multiple recombination events. In addition to V- and J-gene segments, the Igh locus also possesses a third coding region associated with the V-region phenotype: this is the D_H or diversity segment (Sakano et al, 1981). Thus formation of the V-region phenotype depends on two somatic recombination events to give a contiguous V-D-J coding segment in myeloma DNA.

6.4.2. The D_H-Segment

In the plasmacytoma MOPC 141, the coding sequence for the fourteen residue peptide which comprises the third hypervariable region of the heavy chain is found neither in the germ line V_H gene nor in the J_H segment (Sakano et al, 1980). A similar finding has been reported for a α-heavy chain gene (Early et al, 1980a). It has been proposed that the third hypervariable region of the heavy chain is encoded in a segment of DNA that is distinct from V_H and J_H coding regions and that has been termed the D_H segment (Schilling et al, 1980).

Tonegawa and his associates have recently identified several putative D_H segments in murine germ line DNA (Sakano et al, 1981). One D_H segment (Q52) was shown to consist of a 10 base pair coding region flanked on both sides by heptameric and nonameric nucleotide sequences which are believed to be recognition sequences for a recombinase. The spacer separating the heptamer and nonamer is 12 base pairs long on both sides of the D_H coding region (Sakano et al, 1981). Since the length of the spacer between the complementary heptameric and

nonameric sequences associated with V_H and J_H DNA is 23 $\pm$ 1 base pairs the 12/23 spacer rule (Early et al, 1980a; Sakano et al, 1980) is upheld for recombination between V_H and D_H, and between D_H and J_H. The Q52 D_H segment was located 700 base pairs 5' to the J_{H1} nucleotide sequence in murine germ line DNA (Sakano et al, 1981).

D_H-J_H recombination events have been shown to occur in cytolytic T-cell lines (Kurosawa et al, 1981) but V_H-D_H joining has not been observed. Also, it was observed that D-J recombination could occur at different crossing-over points, thus providing a mechanism for amplification of diversity of the heavy chain gene repertoire (Kurosawa et al, 1981).

6.4.3. Structure of Heavy Chain Constant Region Genes

Light chains contain a single constant region domain which is encoded by a single C-gene exon. Heavy chains have multiple constant region domains which exhibit a significant degree of amino acid sequence homology to one another (Kabat et al, 1979). This homology suggests that each domain may be encoded by a separate exon and it is possible that the constant region exons arose from a single ancestral gene by tandem duplication.

The application of recombinant DNA technology to this problem has resulted in the elucidation of the precise genomic structure and organisation of the murine Cμ gene. In the earliest experiments R-loop mapping (Thomas et al, 1976; Kaback et al, 1979) was performed using three cloned Cμ gene segments (Calame et al, 1980) and mRNA purified from the MOPC 104E myeloma (Rogers et al, 1980). The data obtained from R-loop analysis of each Cμ clone gave similar results:

a) two to four R-loops were observed, and this was consistent with the hypothesis that the Cμ domains were encoded by separate exons (Calame et al, 1980), and

b) a further R-loop was detected in 10-15% of the mRNA-cDNA hybrids suggesting that a small exon was located some 2 kilobases 3' to the end of the Cμ4 exon.

Several experimental observations suggested that although μm and μs polypeptides were encoded by distinct mRNA molecules both messengers were derived from a single μ-chain gene;

a) restriction maps and DNA sequences of large portions of μ_m and μ_s cDNA clones were identical (Rogers et al, 1980) and

b) hybridisation of ^{32}P-labelled μ_s plasmid DNA to Southern blots of germline or embryo DNA treated with a variety of restriction endonucleases gave only the pattern of bands expected from the cloned germline Cμ gene (Davis et al, 1980; Early et al, 1980).

Analysis of the genomic organisation of Cμ gene sequences by hybridisation of ^{32}P-labelled μ_s and μ_m cDNA clones to Southern blots of restriction fragments of a germline Cμ genomic clone and by nucleotide sequencing of the various cloned fragments revealed the following (Calame et al, 1980; Early et al, 1980; Rogers et al, 1980);

a) the Cμ1, Cμ2, Cμ3, Cμ4 and M-exons are separate in germline DNA;

b) the entire Cμ4, C-terminal and 3'-untranslated regions of the μ_s cDNA clone are found as a contiguous nucleotide sequence in the genomic Cμ clone which terminates 187 nucleotides 3' to the end of the Cμ4 exon;

c) the sequences encoding the 3'-end of the μ_m-mRNA are found in two separate M-exons located 1850 base pairs 3' to the end of the Cμ4 exon;

d) the first M-exon encodes amino acids 557-595 and is separated by an intron of 118 base pairs from the second M-exon which encodes amino acids 596 and 597 and contains the termination codon;

e) the RNA splice sites at the 5' end of the first M-exon and those between the two M-exons obey the GT.....AG rule (Breathnach et al, 1978; Catterall et al, 1978) and

f) both μ_m and μ_s cDNA clones contain the putative polyadenylation signal (AATAAA) (Proudfoot and Brownlee, 1976) located 19 base pairs 5' to the terminal polyA sequence, and polyA addition to both μm and μs mRNA species appears to occur within the TCACT sequence located at the 3'-terminus of both cDNA sequences in the genomic clone.

Southern blot analysis of E.CoRI digested DNA from embryo or MOPC 104E using ^{32}P-labelled μ_m cDNA as probe revealed only a single Cμ band in each case. This rules out the possibility that μ_m and μ_s mRNAs are derived from two copies of the expressed Cμ gene, and also excludes deletions of the M-exons in one of the two putative

$C\mu$ gene copies since such an event would alter the size of the resulting $C\mu$ gene (Early et al, 1980). These data argue that μ_m and μ_s mRNA species are derived from a single nuclear RNA transcript of a single gene by alternate RNA splicing pathways. The role of RNA splicing in control of immunoglobulin gene expression is detailed below (section 7).

6.4.4. Assembly of Heavy Chain Genes and Messages

Two distinct molecular events are required for the formation of active immunoglobulin light chain genes. Firstly a somatic recombination event joins V DNA to J DNA and thus commits the B-lymphocyte to the expression of that VJ combination. Secondly, the introns separating the 3'-end of the leader sequence from the 5'-end of the V gene and the 3'-end of the J-exon from the 5'-end of the C gene are spliced out from the primary nuclear RNA transcript to yield a contiguous light chain mRNA.

The assembly of active heavy chain genes from their component parts is a much more elaborate process although DNA recombination and RNA splicing are used to generate the functional gene. At least two DNA joining events are necessary to produce a V_H gene encoding a V_H region possessing antigen binding activity. A V exon must be joined with a D exon and that D exon must be joined to a J exon. In addition any one D segment may be capable of somatic recombination with any other D segment located 3' to itself (Sakano et al, 1981; Kurosawa et al, 1981). After V-D-J joining, the cell is then faced with the choice of expressing a membrane or secretory form of the heavy chain isotype to which it is committed. This is accomplished by RNA splicing mechanisms. The intron separating the leader sequence from the V_H

gene and that separating J from the first C_H gene are removed as in the light chain system, but also spliced out from the primary transcript are the introns separating each of the C_H exons from each other. The $C_\mu 4$ exon has an unique structure in that it contains an intra-exon splice site. This splice site allows removal of the intervening RNA between $C_\mu 4$ and the M-exons: this "intron" also contains the coding sequence for the C-terminal amino acids of the secretory μ-chain (section 6.4.3. above). Alternative splicing mechanisms allows the transcriptional and processing machinery to utilise or ignore this splice site and hence give rise to mRNA molecules encoding μ_m or μ_s polypeptides respectively.

At present, only the μ-gene system has been fully characterised. In this study it was hoped to gain some insight into possible similarities between the organisation of μ-chain genes and the equivalent components of γ- and α-genes in human B-lymphocytes.

6.5. The Molecular Basis of Allelic Exclusion

An unique genetic characteristic displayed by lymphocytes is the phenomenon of allelic exclusion. Allelic exclusion is the situation where a lymphocyte expresses only one heavy (or light) chain allotype of its two chromosomal allelic alternatives. The related phenomenon of isotype exclusion will also be discussed in this section.

6.5.1. Rearrangement on a Single Chromosome

The simplest mechanism which may be invoked to explain allelic exclusion at the DNA level is to propose that for the chromosome bearing the allele to be expressed, the V_L and J_L or V_H, D_H and J_H elements are somatically recombined to give a rearranged V gene configuration while the chromosome carrying the allele to be excluded remains in the unrearranged, germ-line configuration. Clearly, the

rearranged allele can be expressed while the allele remaining in the germ-line configuration can not. Examples of such events have been reported in the literature (Brack et al, 1978; Seidman and Leder, 1978; Joho and Weissman, 1980) but seem not to be the general rule, particularly at the Igh locus.

6.5.2. Non-Productive Rearrangement and a Stochastic Model for Allelic Exclusion.

Three types of nonproductive gene rearrangements have been described for V_L-J_L or V_H-D_H-J_H joining;

a) frameshift rearrangements;

b) incomplete rearrangements, and

c) rearrangements to an abnormal DNA sequence.

DNA rearrangement requires recognition of precise nucleotide sequences, but despite the highly conserved nature of these sequences the somatic recombination event may occur at various points in the recognition sequence. Several example of frameshift rearrangements have been reported in several myelomas (see section 6.3.3. above). Frameshift rearrangements have an immunobiological significance. As discussed above (section 6.3.3.) a frameshift rearrangement mechanism can be invoked to explain the amino acid sequences of Balb/C κ myeloma proteins which can not be accounted for in terms of known V_κ and J_κ nucleotide sequences. In the case of amino acid 96, which is known to be hypervariable in Balb/C κ proteins (Barstad et al, 1978; Max et al, 1979), frameshift rearrangements increase the number of amino acids available at this position. Such variability increases the repertoire of the κ gene pool and, consequently, leads to an increase

in antibody diversity. An example of a frameshift rearrangement and its consequences is given in Figure 5.

In the heavy chain gene system, two distinct recombination events are required to generate a functional V_H gene. In some instances, only one of the two DNA joining events, V_H-D_H and D_H-J_H, will occur and this will give rise to an incomplete rearrangement. One example of such an incomplete rearrangement is the finding of a normal splenic B-cell which displays a D_H-J_H recombination but not V_H-D_H, on the unexpressed chromosome (Early and Hood, 1981). Possibly V_H-D_H joining with no D_H-J_H joining can occur also giving rise to another form of incomplete rearrangement.

The murine plasmacytoma MPC-11 synthesises a mutant κ-light chain fragment (Kuehl and Scharff, 1974). Analysis of the DNA of MPC-11 cells revealed that a V_κ gene segment unrelated to the V-gene functionally expressed by the MPC-11 cells had been joined to a CACAGTG palindrome located between J_κ and C_κ (Seidman and Leder, 1980; Choi et al, 1980). This rearrangement deletes the J_κ segments and, because this nonproductive rearrangement yields a pseudo exon with no J_κ and no RNA splice site 3' to the V_κ sequences, RNA splicing occurs between the C_κ exon and the hydrophobic leader sequence exon located 5' to the V_κ sequence thus giving an mRNA containing C_κ but no V_κ or J_κ sequences. It is not known if this type of null recombinant is mediated by specific V-J joining enzymes or if it is a more general type of recombination event. Many κ-producing plasmacytomas contain non-productively arranged κ-genes (Perry et al, 1980) and small κ-mRNA molecules of similar size to the mutant MPC-11 κ-mRNA have been observed in virally transformed cell lines (Alt et al,

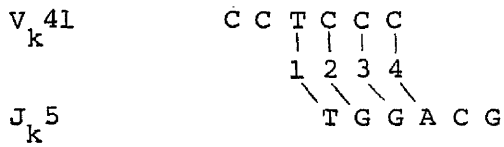
FIGURE 5

Frameshift Mutations in Generation of Antibody Diversity

V-J joining can occur in a variety of frames. The germ line sequences of V_K41 and J_K5 DNA are shown. The V_K41 sequence is that of codon 95 (proline) and the junction site: the J_K5 sequence is of the first six nucleotides (codons for typtophan and threonine). Each of four possible somatic recombinations is illustrated (numbered 1-4) and the resultant nucleotide and polypeptide sequence shown. Note that each amino acid found at position 96 has been described at the protein level (Max *et al*, 1979).

FIGURE 5: Frameshift Mutations in Generation of Antibody Diversity

Germ Line Sequences



Recombined Nucleotide and Protein Sequences

Recombination Event	1	2	3	4
Nucleotide Sequence	C C T T G G	C C T C G G	C C T C C G	C C T C C
Protein Sequence	PRO TRP	PRO ARG	PRO PRO	PRO PR

(After Max et al, 1979).

1980a). Quantitation of Southern blots of nonproductive rearrangements have illustrated that 30% of the unexpressed κ-chromosomes are in a rearranged configuration as are between 80% and 100% of unexpressed heavy chain chromosomes (Coleclough et al, 1981).

The observation that many, though not necessarily all, B-lymphocytes exhibit nonproductive rearrangements allows a probabalistic or stochastic model for allelic exclusion to be forwarded (Coleclough et al, 1981; Early and Hood, 1981). The model makes three principal predictions;

a) a non-productive rearrangement on one chromosome does not exclude an attempt to form a productive rearrangement on the second chromosome;

b) multiple rearrangements can occur on a single chromosome and

c) some cells with two functionally rearranged chromosomes should exist (though possibly at low frequency) in the population.

6.5.3 Evidence Against a Stochastic Model of Allelic Exclusion

Experimental evidence is available which suggests that allelic exclusion is not a totally random process as predicted by the stochastic model. Kuehl has performed experiments where a pre-B-cell is fused with a myeloma cell which does not produce a functional light chain. The hybrid cell was found to express a light chain presumably as a result of somatic recombination of V and J exons of the pre-B-cell genome. If, however, the myeloma fusion partner does produce a functional light chain, no new light chains were found to be expressed, presumably because functional V-J recombination is excluded.

This data raises the possibility that active suppression of further gene rearrangement may occur once a functional gene is formed.

6.5.4. Isotype Exclusion

The evidence from studies of immunoglobulin synthesis in pre-B-cells suggests that μ-chain is the first phenotypically detectable immunoglobulin component. This data indicates that V_H-D_H-J_H recombination is the first DNA joining event to occur in the immunoglobulin genes. Evidence is now available which suggests that κ genes are expressed prior to their counterparts in the Igλ family. In a study of 10 human λ-producing human B-cell lines it was observed that all C_κ genes were either deleted or rearranged (Hieter et al, 1981). Similar analysis of κ-producing human B-cell lines showed that all of the C_λ genes were in the germ line configuration (Hieter et al, 1981). The hypothesis has been advanced that once a cell has successfully joined V_H, D_H and J_H, it then attempts to join V_κ with J_κ. If this is successful then no further gene rearrangements occur but if two nonproductive V_κ-J_κ recombination events occur then the cell attempts to join V_λ with J_λ to obtain a functional V_λ gene. Thus, antibody gene families appear to be expressed in the order Igh, Igκ, and Igλ, and models of isotype exclusion must attempt to explain this sequential (i.e. nonstochastic) mode of expression.

6.6. Molecular Basis of the Heavy Chain Switch

During maturation, a clonally expanded B-lymphocyte is capable of changing the heavy chain isotype which it expresses although it retains the original idiotype and antigenic specificity (see section 4 above and Scher, 1981). It is now evident from the work of a

number of laboratories that the switch in expression from one isotype to another involves joining of an active V-D-J gene to one of the possible γ, α or ε C-region exons situated 3' to the end of the Cμ and Cδ exons. The switch is accompanied by deletion of all DNA between 3'-end of the V-D-J gene and the 5' of the switch site preceeding the selected constant region exon. This hypothesis is based on hybridisation kinetics experiments investigating the gene numbers of C_H genes in myelomas expressing different immunoglobulin classes (Honjo and Kataoka, 1978), and this has been confirmed by more recent studies (Cory and Adams, 1980). Thus, according to the arrangement of C_H genes illustrated in figure 6 (after Honjo et al, 1980) a heavy chain switch, at the phenotypic level, from IgM to IgG_{2b} would result in deletion of the exons encoding Cμ, Cδ, Cγ3, and Cγ1, but those coding for Cγ2a, Cε and Cα would remain in the genome.

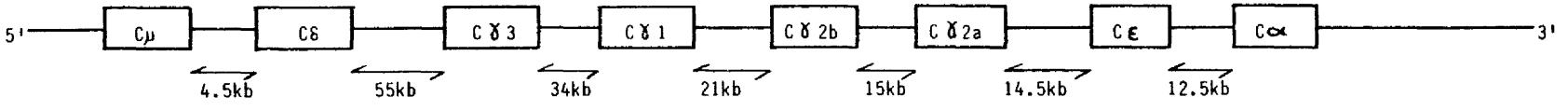
Heavy chain switching and V-J joining are disimilar events. V-J recombination is a precise event demanding recognition of heptameric and nonameric nucleotide signal sequences before the joining process can occur (Sakano et al, 1979; Seidman et al, 1979). Heavy chain class switching appears to occur at a variety of switch recombination sites which are located in the 5' flanking sequence of each C_H exon (Davis et al, 1980a,b,c; Kataoka et al, 1980; Takahashi et al, 1980). The precise nature of the signals involved in the heavy chain switch are not yet well understood, but runs of repeated nucleotide sequences are thought to identify the location of switch recombination sites. The Cα switch site appears to be located in a 30 nucleotide segment (Davis et al, 1980b,c) and the equivalent site for Cγ switching is believed to be located in a 49 nucleotide repeated

FIGURE 6

Organisation of Murine C_H Genes

The order of the constant region genes for mouse immunoglobulin heavy chains as determined by Honjo and his collaborators is shown (Honjo et al, 1980). Although shown as a continuous sequence, it should be noted that each C_H gene contains multiple exons coding for separate domains and M-exons. Approximate distances between genes are also shown. Note that distances are not drawn to scale.

Fig. 6 Organisation of Murine Heavy Chain Constant Region Genes



sequence (Honjo et al, 1980). Similar sequences have also been reported in the human Igh system (Ravetch et al, 1980). There is significant nucleotide sequence homology between μ and α switch sites but not between μ and γ switch sites or between γ and α switch sites. This suggests that μ→γ and μ→α switching mechanisms are mediated by distinct enzyme systems, both of which are, in turn, distinct from the recombinase mediating V-D, D-J and V-J joining events (Davis et al, 1980b,c).

6.6.1. Simultaneous Expression of Cμ and Cδ Genes

IgM and IgD of identical idiotype are often found on the membrane of a single B-lymphocyte (Abney and Parkhouse, 1974; Fu et al, 1975; Coffman and Cohn, 1977). The presence of identical idiotypes on the expressed IgM and IgD strongly suggests that the same V_H gene is being expressed in conjunction with both Cμ and Cδ genes and it is therefore pertinent to explore the molecular mechanisms which permit the simultaneous expression of Cμ and Cδ genes with the same V_H gene.

Several features of the organisation of Cμ and Cδ exons suggest that simultaneous expression of Cμ and Cδ genes may be accomplished via RNA processing rather than via DNA rearrangement:

a) the Cδ is located some 2.5kb 3' to the M-exons of the Cμ gene (Liu et al, 1980);

b) Cμ and Cδ genes are identically oriented (Liu et al, 1980) and

c) Cμ and Cδ can both be associated with the same V_H-C_H-J_H exon.

Given that RNA processing allows the simultaneous expression of membrane and secretory μ-chain polypeptides, (Rogers et al, 1980; Early et al, 1980), it is reasonable to propose that V_H-D_H-J_H, Cμ and Cδ exons could be contained in a single large nuclear RNA transcript which could then be appropriately processed to yield functional μ-mRNA or δ-mRNA (Liu et al, 1980). Preliminary evidence for the existence of such a transcript has been recently reported (Wall et al, 1981).

6.7. Molecular Events During B-Lymphocyte Differentiation

It is now possible to understand the molecular events at the DNA level which are responsible for the characteristic phenotypic traits which are seen during B-lymphocyte differentiation. The data also demonstrate that DNA recombination events are the decisive step in commitment of B-lymphocytes to make antibody of a given specificity.

The earliest molecular events occuring in stem cells are attempts to form a function V-D-J gene at the Igh locus. Each cell has a minimum of two attempts to form such an active gene and failure to do so results in cell wastage. A successful V-D-J recombination commits that cell to the B-lymphocyte lineage and possibly also actively suppresses further rearrangement at Igh (Early and Hood, 1981). This cell is phenotypically a pre-B-cell.

The next stage in development of the B-lymphocyte is the selection of a light chain to complement the heavy chain and so form an active antibody molecule. The cell appears to attempt to form an active V_κ-J_κ gene in preference to a V_λ-J_λ gene (Hieter et al, 1981; Coleclough et al, 1981), presumably due to the larger V_κ gene

pool which would be of selective advantage to the animal. If, however, no successful V_κ-J_κ joining is achieved the cell will attempt to rearrange its Igλ locus. Once an active light chain gene has been formed, the polypeptide synthesised and IgM located on the cell surface, the cell will be phenotypically an early B-lymphocyte. Transcription of Cμ and Cδ with a single V_H-D_H-J_H gene and expression of IgM and IgD yield an antigen sensitive, virgin B-lymphocyte.

Upon antigen contact, the lymphocyte undergoes clonal expansion to yield memory cells and plasma cells. The memory cells may express an isotype other than IgM, but carry the same idiotype as the original B-lymphocyte, and the isotype switch is accomplished by joining the expressed V_H-D_H-J_H gene to a selected C_H exon located 3' to the Cμ and Cδ exons. The switch is accomplished through recombination events controlled by signals encoded in stretches of repeated nucleotide sequence in the non-coding regions 5' to the C_H exons. Switch recombination, like V_L-J_L and V_H-D_H-J_H joining, is accomplished by deletion of intervening DNA.

The above summary illustrates the profound effect that the advent of recombinant DNA technology has had on the understanding of B-lymphocyte differentiation.

7. REGULATION OF IMMUNOGLOBULIN GENE EXPRESSION

7.1. Cellular Regulation

At the cellular level, the immune response involves the carefully controlled interplay of a variety of cell populations and

subpopulations in a dynamic, interactive system. This discussion of cellular regulation of antibody synthesis will deal only with the influences of T-helper, T-suppressor, and macrophage populations upon B-lymphocytes.

7.1.1. Role of T-lymphocytes

Early experiments examining the cell population responsible for antibody synthesis illustrated not only that B-lymphocytes were the antibody forming cells, but also that T-lymphocytes exerted a regulatory influence upon the B-lymphocytes (Introduction, section 2; Claman et al, 1966; Mitchell and Miller, 1968; Nossal et al, 1968). The influence of T-lymphocytes upon antibody synthesis by B-lymphocytes is of two kinds, help or suppression. The efforts of a great many investigators have helped to elucidate the functional and immunogenetic bases of T-lymphocyte help and suppression of immunoglobulin gene expression (see reviews by Benacerraf and Katz, 1975; Katz, 1977).

The helper T-lymphocytes (T_H-cells) have the phenotype Ly 1^+ (Cantor and Boyse, 1975a,b). The existence of T_H-cells was demonstrated in adoptive transfer experiments in which lethally-irradiated recipient mice or guinea pigs received primed T- or B- lymphocyte populations from syngeneic animals (Mitchison, 1971a,b; Katz et al, 1970). It was illustrated that both T- and B- lymphocyte populations were required before an effective antibody response could be observed in the irradiated recipient. Furthermore, the specificity of the T-lymphocyte was for the carrier moiety of a hapten-carrier conjugate and was specific for the carrier to which the T-lymphocyte was educated (Mitchison, 1971a,b; Katz et al, 1970). Immunogenetic analyses of the influence of H-2 genes upon the activity of T_H-cells

on antibody production showed that the T_H-cells and B-lymphocytes required to be identical at the I-region of the H-2 complex (Katz and Benacerraf, 1975) and, more exactly, to the I-A subregion of H-2. T_H-cells bind antigen only in the context of I-A and, unlike suppressor T-lymphocytes, cannot bind free antigen (Okumura et al, 1977; Taniguchi and Miller, 1977).

The T-suppressor (T_S) lymphocytes have the phenotype $Ly2^+3^+$ (Cantor and Boyse, 1975a,b): this is also the Ly phenotype of the cytotoxic T-lymphocyte. Adoptive transfer experiments also illustrated the existence of the T_s-lymphocyte subpopulation. It was found that T-cell depleted mice, when challenged with antigen, made antibody when repopulated with T-cells. Surprisingly, if T-lymphocytes were always present the B-lymphocytes made a significantly lower response (Gershon and Kondo, 1970). T_s-cells also require to recognise antigen in the context of the products of the H-2 region. However, T_s-cells are restricted to the H-2K or H-2D products (Gershon and Cantor, 1977). The nature of the H-2I product expressed on the cell membrane of $Ly2^+3^+$ T-lymphocytes also delineates the T_s-cells and cytotoxic T-lymphocytes: cytotoxic lymphocytes express I-A subregion products while T_s-cells express products of the I-J subregion (Murphy et al, 1976; Tada et al, 1976).

7.1.2. Role of Macrophages

The evidence that macrophages were essential in development of in vitro antibody responses was obtained at about the same period that lymphocyte interactions were shown to be important in antibody formation. Mosier (1967) showed that if murine non-immune splenocytes were separated into adherent and non-adherent populations, an

IgM response to sheep erythrocytes could not be mounted *in vitro* by either population. Remixing of the populations led to generation of an *in vitro* response comparable to that observed with unmanipulated cells. The macrophages were shown not to be precursors of the antibody forming cells (Munro and Hunter, 1970), and, furthermore, macrophages from T-cell depleted animals were also capable of supporting *in vitro* primary responses (Munro and Hunter, 1970). Macrophages are also mandatory for generation of primary IgG and IgA *in vitro* responses to sheep erythrocytes (Pierce, 1973). The X-irradiation studies of Gorczynski and his collaborators illustrated defects of macrophage function, both *in vivo* and *in vitro*, three days after irradiation which were abrogated by normal macrophages (Gorczynski *et al*, 1977). This shows good correlation between studies *in vitro* of adherent cells and *in vivo* studies of macrophage function.

Several properties of the macrophage membrane are important for the fulfillment of its antigen-presenting function. Cytophilic antibody binds to macrophage membranes via a macrophage F_c receptor (Boyden, 1964). Immune complexes also bind avidly to this receptor. Antigen-specific helper factors, which bind antigen, are cytophilic for macrophages (Feldmann, 1972). Thus, the macrophages have intrinsic mechanisms to promote acquisition of antigen in a passive manner, and to act as an 'antigen-focusing'point for efficient presentation of antigen to the lymphocytes.

As in the collaboration between lymphocytes, the interaction of macrophages with lymphocytes is subject to strict genetic restriction by the H-2 complex (Katz and Unanue, 1973). Genetic

restriction was first observed in the guinea pig where it was demonstrated that antigen-specific *in vitro* proliferative responses occurred only if the macrophages and T-lymphocytes were histocompatible. Furthermore, the response was inhibitable by antibody to the Ia-like antigens of the guinea pig lymphocyte membrane (Rosenthal and Shevach, 1973; Shevach *et al*, 1972; B.D. Schwartz *et al*, 1976). In the mouse, it was shown that anti-Ia antibodies would inhibit the *in vitro* proliferative response, and also that the restriction mapped to the I-A subregion of the H-2 complex (R.H. Schwartz *et al*, 1976, 1978). The requirement for histocompatible macrophages was also shown for the *in vitro* induction of T_H-cells and the restriction mapped to the I-A subregion of H-2 (Erb and Feldmann, 1975).

7.2. Molecular Regulation

The foregoing section reviewed the influence of T-lymphocytes upon the antibody response. In this section the role of macromolecules secreted from T-lymphocytes in regulation of the antibody response is evaluated, and the function of RNA processing of immunoglobulin gene transcripts as a control mechanism is also discussed.

7.2.1. T-Lymphocyte Factors

The earliest evidence of the ability of T-lymphocyte derived molecules to mediate T-cell help was provided by the classical double-chamber experiments of Feldmann and Basten (1972). These experiments illustrated that DNP-primed B-cells would make a good anti-DNP response when cultured in the presence of DNP-KLH and T-lymphocytes educated to KLH even though the two cell populations were separated by a cell-impermeable membrane.

Many antigen-specific soluble 'factors' have now been described (reviewed by Tada and Okumura, 1979) and these may act as helper factors or suppressor factors. Helper factors replace live T_H- cells in *in vitro* or *in vivo* T- and B-lymphocyte responses and suppressor factors replace T_S-cells in the equivalent situations. The question of genetic restriction of factors is a complex and unanswered problem (see review by Berzofsky, 1980). However, although the role of macrophages in production of helper or suppressor factors appears more critical for help than for suppression (Feldmann *et al*, 1979) the effects of both classes of factors appears to be mediated via macrophages (Howie and Feldmann, 1978).

Another similarity between helper and suppressor factors is found in their biochemical and serological properties. The factors are protein in nature (Tada and Okamura, 1979) and have AMWs of the order of 30-50K. (Recent studies have shown that the factors are glycoproteins (Webb, unpublished data). Furthermore, the factors express Ia antigenic determinants; I-A encoded determinants are found on helper factors and I-J determinants are detected on suppressor factors (Tada and Okumura, 1979). Some helper and suppressor factors appear to share V_H idiotypes with antibodies of corresponding specificity: C_H and C_L determinants are not found on helper or suppressor factors (Mozes, 1978; Germain *et al*, 1979). The Ia determinants and V_H determinants co-elute from affinity columns which is consistent with the hypothesis that the determinants are on the same glycopeptide or are within the same macromolecular complex.

Much remains to be discovered regarding the structure and mechanism of action of these factors. Particularly of interest is

the relationship of factors to the T-lymphocyte receptor. Certainly, the finding of V_H determinants upon factors and on T-cell 'receptors' (Berzofsky, 1980) arouses the suspicion that the factors may represent a secreted form of the receptor. The genomic structure and processing of T-cell receptor and factor genes then becomes of considerable interest. The mechanisms governing alternate expression of secretory and membrane forms of the immunoglobulin heavy chain polypeptides are relevant in this regard, and these processes are described in the following section.

7.2.2. RNA Splicing of Immunoglobulin Gene Transcripts

The molecular processes involved in synthesis of μ-heavy chains by B-lymphocytes is summarised in figure 7. An important event in these processes is the alternative splicing of the primary nuclear RNA transcript of the μ-chain gene to yield mRNAs for either the membrane or secretory μ-chain polypeptides. It is therefore pertinent to explore the possible molecular signals which underly this phenomenon.

The processing events which generate the immunoglobulin mRNA from the primary nuclear RNA transcript appear to occur in a specific order. The earliest post-transcriptional event appears to be polyadenylation (Gilmore-Herbert and Wall, 1978, 1979) which is followed by splicing of the transcript to remove introns (Gilmore-Herbert and Wall, 1978,1979; Perry et al, 1979). The fully processed immunoglobulin mRNA molecules are then immediately transported to the cytoplasm (Wall, 1980).

The molecular mechanisms which permit alternative expression of mRNA for the secretory or membrane form of immunoglobulin heavy

FIGURE 7.

Molecular Events in Formation of Active Immunoglobulin Heavy Chains

The formation of distinct mRNA species encoding μ_m and μ_s polypeptides from a single μ-chain gene via splicing of a single nuclear RNA transcript is diagrammed.

Key:-

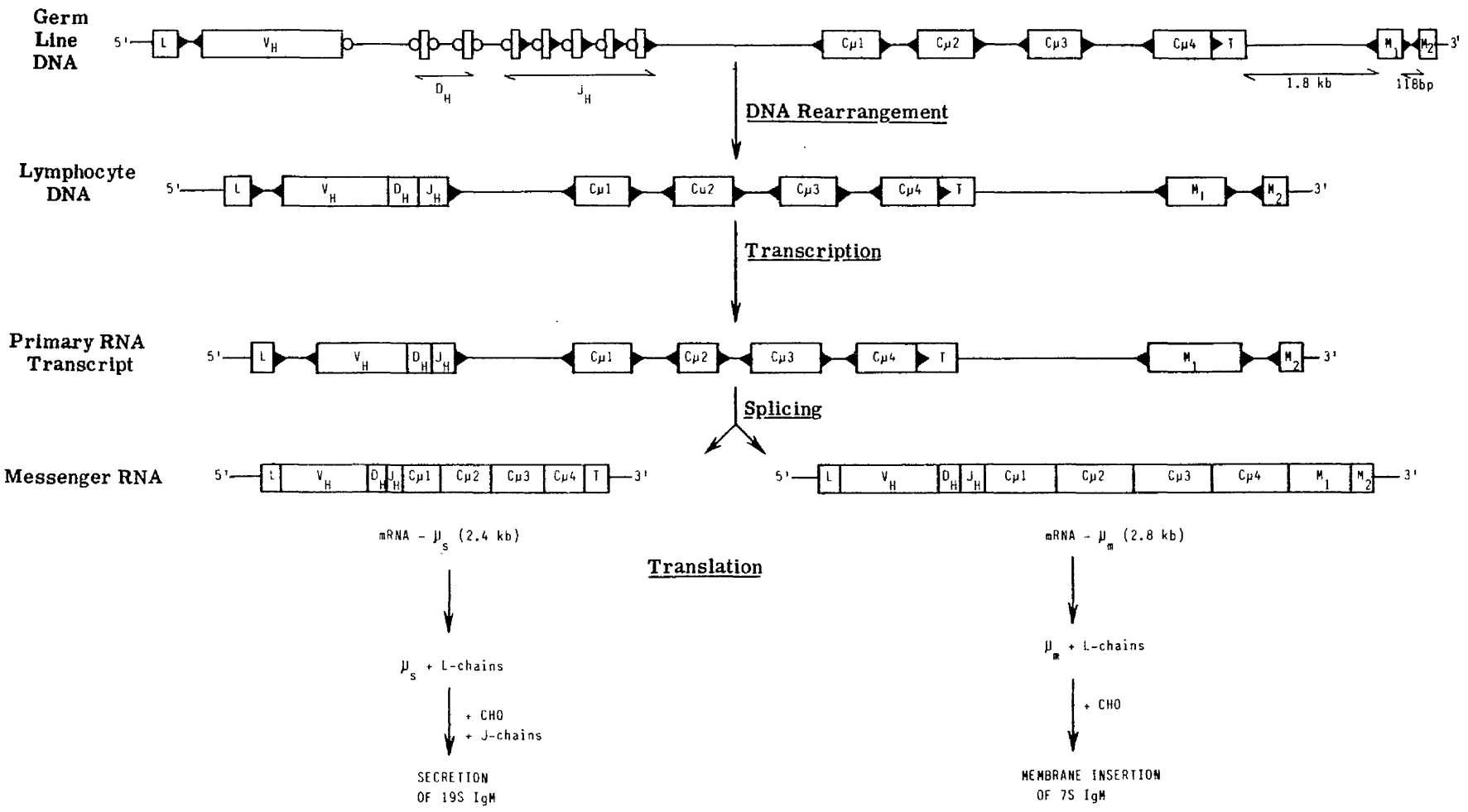
Open boxes:- exons

Open circles:- DNA recombination sites, which obey the 23/12 spacer rule (Early et al, 1980a; Sakano et al, 1980).

Shaded triangles:- RNA splice sites at intron boundaries.

L	-	hydrophobic leader sequence exon
V_H	-	V exon
D_H	-	diversity segment exon
J_H	-	J exon
C_μ	-	constant domain exon
T	-	DNA encoding the C-terminal secretory peptide of μ_s
M_1, M_2	-	membrane exons
bp	-	base pairs
kb	-	kilobases
CHO	-	carbohydrate

Fig. 7 : Molecular Events in Formation of Active Immunoglobulin Heavy Chains



chain polypeptide are not yet understood. However, an attractive model, based on polyadenylation of specific sites, has been proposed to explain this phenomenon (Rogers et al, 1980; Wall, 1981; Johnson, et al, 1981). Polyadenylation precedes removal of introns and polyadenylation of one of the two polyadenylation sites present in the Cμ gene or other C_H gene may determine which of the two possible nuclear RNA transcripts is expressed. Polyadenylation sites are located just 3' to the end of the Cμ4 exon and T-piece and also 3' to the end of the M_2 exon (see figure 7.). If polyadenylation occurs at the former site (i.e. 3' to the C 4 exon and T-piece) then only μ_s mRNA is synthesised as no information for the M-exons is present in the nuclear RNA transcript: polyadenylation of the latter site (3' to the M_2 exon) gives rise to μ_m mRNA. Thus, polyadenylation regulates the size of the primary nuclear RNA transcript and therefore the nature of the mRNA generated for export to the cytoplasm.

The alternative splicing mechanisms described above are clearly developmentally regulated: early B-lymphocytes synthesise predominantly μ_m-mRNA while plasma cells synthesise mostly μ_s-mRNA. Current theories propose that inducible soluble factors may enforce polyadenylation of the site nearest the Cμ4 exon thus switching off μ_m-mRNA synthesis and promoting transcription of nuclear RNA species containing information only for μ_s-mRNA (Wall, 1980). However, these mechanisms and the molecular mechanisms responsible for T-cell mediated control of B-lymphocyte differentiation are interesting questions which remain to be answered in the future.

MATERIALS AND METHODS

1. GENERAL MATERIALS

1.1. Cell Culture Materials

RPMI-1640 Medium powder	Gibco Biocult Ltd., Paisley, Scotland.
Foetal Calf Serum (FCS)	Flow Laboratories Ltd., Irvine, Scotland.
Penicillin	Glaxo Pharmaceuticals Ltd., England.
Streptomycin	Glaxo Pharmaceuticals Ltd., England.

Tissue culture flasks were provided by Costar Inc., Cambridge, Massachusetts. Accessory sterile plastics were obtained from Flow Laboratories Ltd.

1.2. Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, England. The specific activities of the radiochemicals used are shown below.

Amino Acids	Specific Activity
L-[2-^{3}H]-glycine	30 Ci/mmol.
L-[4,5-^{3}H]-leucine	100-150 Ci/mmol.
L-[^{35}S]-methionine	> 600 Ci/mmol.
L-[2,6^{3}H]-tyrosine	37 Ci/mmol.
Other Compounds	**Specific Activity**
^{125}I-iodine	Carrier-free
^{131}I-iodine	Carrier-free

1.3. Photographic Materials

X-Omat-H X-ray film	Kodak (U.K.) Ltd., London.
SX-80 developer	Kodak (U.K.) Ltd., London.
FX-40 X-ray liquid fixer	Kodak (U.K.) Ltd., London.
Lightproof vinyl bags	H.A. West Ltd., Edinburgh.

1.4. Fine Chemicals

The following fine chemicals were supplied by Sigma Ltd., England.

Cycloheximide

Deoxycholic acid

Dimethyl sulphoxide (DMSO)

D-L-Dithiothreitol (DTT)

Phenylmethyl sulphonyl fluoride (PMSF)

Polyoxyethylene sorbitan monopalmitate (Tween 40)

N,N,N',N' tetramethylethylene diamine (TEMED)

Tris (hydroxymethyl) aminomethane (Trizma base)

Other fine chemicals were supplied as listed below.

2,5-diphenyloxazole (PPO)	Koch-Light Laboratories, England.
Toluene (AR grade)	Koch-Light Laboratories, England.
2-mercaptoethanol	Koch-Light Laboratories, England.
Heparin	Evans Medical Ltd., Liverpool, England.
Hexanoic anhydride	Aldrich Chemical Company, England.

N,N'-methylene bis acrylamide	Eastman Kodak Co., Rochester, New York, U.S.A.
Pepstatin A	Protein Research Foundation, Osaka, Japan.
Protosol	New England Nuclear Ltd., England.
Triton X-100	Rohm and Hass Ltd., England.
Tunicamycin	Glaxo Pharmaceuticals Ltd., England.

All other reagents were "Analar" grade and were obtained from BDH Chemicals Ltd., England.

1.5. Enzymes

Deoxyribonuclease I (DNAase I)	Sigma Ltd., London.
Lactoperoxidase	Sigma Ltd., London.
Pepsin	Sigma Ltd., London.
Trypsin	Sigma Ltd., London.
Carboxypeptidase A	Worthington Biochemical Corporation, New Jersey, U.S.A.

1.6. Chromatographic Reagents

Oligo dT cellulose	Collaborative Research Inc., Waltham, Massachusetts, U.S.A.
Cyanogen-Bromide Activated Sepharose 4B	Pharmacia Ltd., London.
Sephadex G200	Pharmacia Ltd., London.
Sephadex G25	Pharmacia Ltd., London.
QAE Sephadex	Pharmacia Ltd., London.

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-Tryptophan	5.0
L-Tyrosine	28.94
L-Valine	20.0
Inorganic Salts	
$Ca(NO_3)_2.4H_2O$	100.0
KCl	400.0

TABLE 1 (cont'd)

Inorganic Salts (cont'd)	mg/litre
$MgSO_4$	48.84
NaCl	6000.0
Na_2HPO_4 (anhydrous)	800.0
Vitamins	
Biotin	0.2
D-calcium pantothenate	0.25
Choline chloride	3.0
Folic acid	1.0
i-Inositol	35.0
Nicotinamide	1.0
Para - aminobenzoic acid	1.0
Pyrodoxine hydrochloride	1.0
Riboflavin	0.2
Thiamine hydrochloride	1.0
Vitamin B_{12}	0.005
Other components	
Glucose	2000.0
Phenol red	5.0
Reduced glutathione	1.0

2000mg/litre $NaHCO_3$ was added separately to dissolved medium. The pH was adjusted to 7.0 with concentrated HCl. The media was sterilized by filtration and stored at $4^{o}C$.

2. STANDARD SOLUTIONS

2.1. Phosphate-Buffered Saline (PBS-A)

170mM NaCl

3.4mM KCl

10mM Na_2HPO_4

1.8mM KH_2PO_4 pH7.2.

2.2. Toluene - PPO Liquid Scintillant

4g PPO per litre of toluene.

2.3. Triton-Toluene - PPO Scintillant

67% (by volume) Toluene

33% (by volume) Triton X-100

4g/litre PPO

2.4. Three-Detergent Lysis Buffer (3D-TKM)

1% (w/v) Triton X-100

1% (w/v) Deoxycholic acid

0.5% (w/v) SDS

0.1M Tris-HCl pH 8.2

0.1M KCl

5mM $MgCl_2$

3. TISSUE CULTURE

3.1. Tissue Culture Medium

The composition of RPMI-1640 powdered medium is detailed in table 1. "Complete" tissue culture medium consisted of:

90% (v/v)	RPMI-1640
10% (v/v)	FCS
10^5 units/litre	Penicillin
100mg/litre	Streptomycin
2.0mM	Glutamine

The FCS was heat inactivated at 56°C for 1 hour and stored at -20°C prior to use. Penicillin and streptomycin were mixed and stored at -20°C as a 100X stock, and glutamine was also stored at -20°C as a 100X stock.

I am indebted to Mrs. Irene Gall for routine preparation of tissue culture medium.

3.2. Routine Culture

Cells were grown in complete medium as suspension cultures in Costar flasks at 37°C in a 95% O_2/5% CO_2 humid incubator and were sub-cultured every third day.

3.3. Large Scale Culture

For culture of $>10^9$ cells, 2.5 litre roller bottles were employed. The bottles containing the cultures were gassed with CO_2, sealed, and placed on a mechanical rolling device in a 37°C hot room.

3.4. Determination of Cell Viability

Cell viability was determined by adding an aliquot of cell suspension to an equal volume of trypan blue dye solution. The suspension was mixed and the numbers of live and dead cells estimated by counting in a Neubauer haemocytometer.

Trypan Blue Dye Solution

4 parts 1% (w/v) NaCl

1 part 1% (v/v) Trypan blue dye.

TABLE 2

CHARACTERISTICS OF HUMAN B-CELL LINES

CELL	TYPE[a]	Ig	MEMBRANE	SECRETION	REFERENCE
BEC-11	BLCL	IgGκ	++	++	Singer et al, 1976.
BJAB	BL	IgMκ	++	++	Menezes et al, 1973.
BR1-8	BLCL	IgMλ	-	++	Crumpton and Suary, 1976.
Dakiki Arosros I	BL	IgAλ	++	++	Steinitz and Klein, 1980.
Daudi	BL	IgMκ	++	-	Klein et al, 1968.
Raji	BL	IgMκ	-	-	Pulvertaft, 1965.
U266BL	Myeloma	IgEλ	-	++	Nillson et al, 1970.

a) BL - Burkitts Lymphoma

BLCL - B-Lymphoblastoid Cell Line.

TABLE 3

ANTIBODY PREPARATIONS

Antibody	Specificity	Source
Goat Anti-Rabbit IgG (GARIg)	Rabbit IgG	-
Rabbit Anti-Human IgM (RAHμ)	Human μ-chain	-
Rabbit Anti-Human IgG (RAHγ)	Human γ-chain	-
Rabbit Anti-Human IgA (RAHα)	Human α-chain	-
Rabbit Anti-Human IgE (RAHε)	Human ε-chain	Miles Laboratories
Rabbit Anti-Human kappa (RAHκ)	Human κ-chain	Miles Laboratories
Rabbit Anti-Human lambda (RAHλ)	Human λ-chain	Miles Laboratories
Rabbit Anti-Human β_2 microglobulin (RAH β_2M)	Human β_2M	Biogenes Ltd.
Rabbit Anti-Human MHC (No.70)	HLA-A,-B,-C and -DR Antigens	Dr. C.M. Steel, Edinburgh.
Rabbit "Anti-Daudi" (No.52)	See Results, Chapter 3.	Dr. C.M. Steel, Edinburgh.
Rabbit Anti-Human HLA-DR	HLA-DR Antigens	Dr. M.J. Crumpton, London.
Rabbit Anti-Mouse IgG	Mouse IgG	-
Monoclonal Anti-Human κ	κ-chain	Dr. R. Jefferis, Birmingham.
Monoclonal Anti-Human λ	λ-chain	Dr. R. Jefferis, Birmingham.
Monoclonal Anti-HLA-DR	HLA-DR-β-chain	Dr. R.S. Accolla, Lausanne.

(a) Where no source is given, antisera were prepared in Glasgow.

4. HUMAN LYMPHOID CELL LINES

The characteristics of the B-lymphoma and B-lymphoblastoid cell lines used in this study are presented in table 2. Cells were maintained in culture as described above (section 3.2) or stored frozen in liquid nitrogen. Regular contamination checks were performed to exclude cultures containing bacteria, fungi or mycoplasma.

5. SEROLOGICAL REAGENTS.

5.1. Immunization Schedule

Rabbits were immunized intramuscularly with purified antigen emulsified in Friends Complete Adjuvant, and received several booster injections prior to collection of immune serum. Antibody was prepared from the immune serum and was purified by isolation of IgG (see below) followed by affinity chromatography using appropriate immobilized immunoadsorbents. A similar procedure was employed in raising goat anti-rabbit IgG (GARIg) antiserum. I am indebted to Mrs. Liz Blakeley for immunizing the animals and for collection of the immune serum.

5.2. Purification of IgG

The use of purified IgG, or fragments of this molecule, is often preferred to the use of whole antiserum. The two methods used for purification of IgG are described below.

5.2.1. Ammonium Sulphate Precipitation

A known volume of antiserum was clarified by centrifugation (20,000 x g for 30 minutes) and stirred on ice. Solid ammonium

sulphate was added slowly to 33% saturation. Stirring was continued, on ice, for 30 minutes and the precipitate collected by centrifugation (10,000 x g for 30 minutes). The pellet was resuspended in PBS-A to the original sample volume, and reprecipitated with ammonium sulphate. After a third precipitation with ammonium sulphate, the pellet was dissolved in PBS-A and dialysed against the same buffer to remove all traces of ammonium sulphate. The sample was stored frozen at -20°C in aliquots.

5.2.2. QAE-Sephadex Chromatography

A column of QAE-sephadex was constructed and equilibrated with the following buffer:

0.1M Tris-HCl pH 6.5.

After extensive dialysis against this buffer and clarification by centrifugation, the serum sample was applied to the column and washed through with a large volume of buffer. Elution profiles were obtained by spectrophotometric analysis at 280nm. IgG is the major component in the first protein peak to be eluted. The IgG-containing fractions were pooled, concentrated, and dialysed against PBS-A. The samples were frozen in aliquots at -20°C.

5.3. Preparation of $F(ab)_2$ Fragments of IgG

IgG, prepared by one of the above methods (5.2.1 or 5.2.2) was dialysed against several changes of digestion buffer.

0.07M Acetate buffer pH4.0

0.05M NaCl.

5 mg of IgG was then mixed with 250µg pepsin in 1ml digestion buffer, and the mixture incubated at 37°C for 18 hours. After

digestion, the pH of the mixture was adjusted to 8.0 with 1N NaOH and then dialysed extensively against PBS-A.

$F(ab)_2$ fragments generated by pepsin digestion were isolated by gel filtration on a column of sephadex G200 equilibrated with PBS-A (Stanworth and Turner, 1978).

5.4. Affinity Chromatography

5.4.1. Preparation of Immunoadsorbents

Antigen was covalently linked to cyanogen bromide-activated sepharose 4B according to Axen et al (1967). Cyanogen bromide-activated sepharose was rinsed with ice-cold 0.1M $NaHCO_3$ and resuspended in the same buffer to give a solution of approximately 20% (w/v) sepharose. The sample to be coupled was immediately added and the mixture agitated gently for 16 hours at $4^{\circ}C$. The sepharose was then rinsed with 0.1M $NaHCO_3$ and the absorbance (A_{280}) of the eluates determined. The amount of protein coupled during the reaction was calculated from the following equation.

Coupled protein = (Total protein) - (Uncoupled Protein).

The conjugate was then rinsed overnight at $4^{\circ}C$ in 1M glycine to quench any remaining protein binding sites. The sepharose was washed extensively in PBS-A and stored at $4^{\circ}C$ in the same buffer containing 0.05% sodium azide as preservative.

4.5.2. Chromatographic Procedure

The IgG fraction of immune serum was passed over a small column of sepharose-4B-antigen. After thorough washing of the column with PBS-A, bound antibody was eluted with 0.1M acetic acid. Elution profiles were obtained by spectrophotometric analysis at 280nm and fractions containing protein were pooled and immediately

neutralized by addition of solid trizma base. The sample was dialysed extensively against PBS-A and stored in aliquots at -20°C.

5.5. Serological Precipitation

The optimum proportions, or point of maximal precipitation, for the GARIg-rabbit antibody system employed in these investigations was determined as follows. A fixed amount of GARIg was mixed with increasing amounts of rabbit serum (or IgG) in a constant reaction volume. The mixtures were incubated at 37°C for 1 hour and overnight at 4°C. The precipitates were collected by centrifugation (10,000 x g for 2 minutes), washed thrice in PBS-A and dissolved in 0.2N NaOH. The absorbance (A_{280}) of each sample was measured and the optimum proportions point determined as the sample containing most protein.

5.6. Preparation of Serum No.52

A rabbit was immunized with Daudi cell membranes coated with antiserum to EB-1 cells. The resulting immune serum was absorbed twice with pooled T-cell lines (CCRF-CEM and Molt-4) and four times with EB-1 cells. This absorbed serum was capable of blocking mixed lymphocyte reaction activation by Daudi cells (Steel et al, 1978).

6. PREPARATION OF PLASMA MEMBRANE

Cells were grown in roller bottles and harvested by centrifugation (1,000 x g for 10 minutes). The cells were washed twice with ice-cold tris-saline prior to preparation of the plasma membrane fraction according to Standring and Williams (1976).

Tris-saline

25mM Tris-HCl pH 7.4

150mM NaCl

10mM NaN_3

1mM $MgSO_4$

2mM $CaCl_2$

Washed cells were resuspended in ice-cold tris-saline at a concentration of 5 x 10^8 cells/ml, and stirred on ice. An equal volume of ice-cold 4% (w/v) Tween-40 in tris-saline was added to the suspension and the mixture stirred on ice for 1 hour. The cells were then homogenized and nuclei and undisrupted cells removed by centrifugation (3,000 x g for 10 minutes). A crude membrane extract was obtained by centrifugation of the post-nuclear supernatant at 70,000 x g for 1 hour at 2°C in the Beckman L5-65 ultracentrifuge. The crude membrane pellet was homogenized into a small volume of 10mM Tris-HCl (pH 7.4) and carefully layered onto the top of a three-layer discontinuous sucrose gradient composed of 11ml volumes of 40% (w/v) 28% (w/v) and 10% (w/v) sucrose in 10mM Tris-HCl (pH7.4). The gradients were centrifuged at 25,000 rpm for 15 hours at 2°C in the Beckman L5-65 ultracentrifuge using an SW27 rotor. Purified plasma membrane was harvested from the first interface and collected by centrifugation (100,000 x g for 2 hours).

7. SYNTHESIS AND USE OF HEXANOYL DIIODO-NAP-TYRAMINE

7.1. Radioiodination of NAP-Tyramine

The following procedures were developed by Hebdon et al

(unpublished). NAP-tyramine was a generous gift from Dr. M.J. Owen, ICRF, Lincolns Inn Fields, London. All procedures were performed in the dark.

The following mixture was used for radioiodination of NAP-tyramine.

10μM KI

100mM borate pH 9.2

200μM NAP-tyramine

2mg Chloramine - T

10μl of this mixture (i.e. 2 nanomoles of NAP-tyramine) was reacted with 1mCi radioiodine for 30 minutes at room temperature. 5μl of 10mM KI was then added and the mixture allowed to stand at room temperature for 20 minutes prior to chloroform extraction. The chloroform extracts (0.6ml total) were washed with 1% (w/v) sodium metabisulphate and thrice with water. The chloroform was removed by a stream of N_2 and the residue dried *in vacuo*.

7.2. Acylation of Diiodo-NAP-Tyramine

The dried product from 7.1 above was dissolved in 30μl of the following solvent:

0.5ml Pyridine (dried over NaOH pellets)

0.01ml Hexanoic Anhydride.

The mixture was allowed to stand at room temperature for 30 minutes, when an equal volume of water was added to destroy the anhydride. After standing for a further 20 minutes, the sample was lyophilized. The dried product was dissolved in 0.4ml water and extracted with 0.4ml chloroform. The aqueous phase was further extracted with 0.2ml chloroform and the pooled chloroform extracts

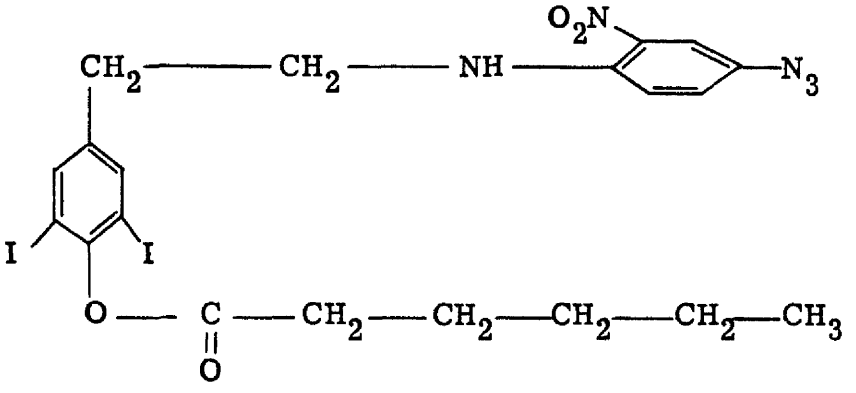
were washed with water. The chloroform was removed by a stream of N_2 and the product dried *in vacuo*. The structure of hexanoyl-diiodo-NAP-tyramine is shown in figure 1. The product was dissolved at 1μCi/μl in methanol immediately before use. The product was used within 24 hours of synthesis. A routine yield of 8-10% was achieved with the specific activity of the product being 20-25 Ci/mmol.

7.3. *Use of Hexanoyl Diiodo-NAP-Tyramine* (Owen *et al*, 1980).

Membranes were prepared as described above (section 6). Purified membrane from approximately 10^8 cells in 250μl 10mM Tris-HCl (pH 7.4) was incubated with 50μCi ^{125}I-hexanoyl diiodo-NAP-tyramine for 15 minutes at room temperature and then placed in the beam of light from a standard slide projector for 30 minutes at room temperature. The radiolabelled membrane was collected by centrifugation and dissolved in 3D-TKM for immunoprecipitation analysis.

Alternatively, 10^7 cells were harvested from log phase cultures, washed extensively in ice-cold TKM buffer (0.1M Tris-HCl (pH 8.2), 0.1M KCl, 5mM $MgCl_2$) and lysed in 90μl of ice-cold 1% (w/v) triton X-100 in TKM buffer. The nuclei were removed by centrifugation and the supernatant was incubated with 20μCi of ^{125}I-hexanoyl-diiodo-NAP-tyramine for 15 minutes at room temperature prior to exposure to light for 30 minutes at room temperature. After photolysis, the solution was treated with 10μl of a solution of 10% (w/v) deoxycholic acid and 5% (w/v) SDS in TKM buffer. Insoluble debris was removed by centrifugation (30,000 x g for 30 minutes) and the supernate used for serological analysis.

Fig. 1 Structure of Hexanoyl-Diiodo-N-(4-azido-2-nitrophenyl) Tyramine



8. RADIOLABELLING PROCEDURES

8.1. Radioiodination of Immunoglobulins and Proteins

Proteins were iodinated by the chloramine-T method (Hunter and Greenwood, 1962). All reactants were prepared in PBS-I with the exception of the radioiodine.

PBS-I

50mM Sodium phosphate pH 7.2

150mM NaCl

50µg of protein in 10µl PBS-I was placed in a chilled eppendorf polypropylene tube and the following reagents added in order.

1mCi ^{125}I

10µl freshly prepared 1mg/ml chloramine-T in PBS-I.

The mixture was mixed thoroughly and allowed to stand on ice for 3 minutes whereupon the following were added.

20µl freshly prepared 1mg/ml sodium metabisulphite in PBS-I.

10µl 100 mM KI.

Radiolabelled protein and unreacted radioiodide were separated by repeated acetone precipitation or by gel filtration on a short column of sephadex G25. The sample was stored in aliquots at -20°C.

8.2. Radioiodination of Cell Membrane Proteins

Radioiodination of cell membrane proteins was effected by lactoperoxidase-catalysed iodination (Marchalonis et al, 1971). 10^7 cells from log phase cultures of greater than 95% viability were harvested and washed twice with 25 ml aliquots of ice-cold serum-free

RPMI 1640 medium, and once with 25 ml of ice-cold PBS-I. The washed cells were transferred to an eppendorf tube, pelleted, and resuspended in 50µl of a 1mg/ml solution of lactoperoxidase in PBS-I. After incubation at 30°C for 10 minutes the following reagents were added, in order:

1-2mCi ^{125}I (or ^{131}I)

10µl freshly prepared 1:1000 H_2O_2 in PBS-I

The mixture was incubated at 30° for 3-4 minutes and 1ml ice-cold 5mM KI was then added. The radiolabelled cells were pelleted and washed extensively before cell lysates were prepared (see section 9 below).

8.3. Biosynthetic Labelling of Cellular Proteins

2 x 10^6 viable cells were harvested from log-phase cultures and washed twice in serum-free RPMI-1640 medium lacking the amino acid to be used as radiolabel (labelling medium). The cells were resuspended in 200µl of labelling medium supplemented with 200µCi of the selected radioactive amino acid and incubated at 37°C for 60-90 minutes (see individual figure legends). The radiolabelled cells were washed and cell lysates prepared.

8.3.1. "Pulse-Chase" Experiments

Cells were biosynthetically radiolabelled as described in section 8.3 above. After the labelling period, the cells were pelleted and resuspended in 0.25-0.5ml RPMI-1640 medium plus 10% (v/v) FCS and reincubated at 37°C for a further 4-6 hours. Following the reincubation period, the cells were again pelleted and the supernatant used for analysis of secreted proteins.

8.3.2. Use of Tunicamycin

In biosynthetic labelling experiments where tunicamycin was employed, an incubation period of 3 hours at 37°C was allowed prior to addition of radiolabel. Tunicamycin was present in all solutions used at a concentration of 2µg/ml. Stock solutions of 1mg/ml tunicamycin were prepared in 25mM NaOH.

9. PREPARATION OF CELL LYSATES

Radiolabelled cells were washed twice in 0.5ml of ice-cold TKM buffer prior to solubilization in 225µl of ice-cold 1% (w/v) triton X-100 in TKM buffer. The suspension was mixed thoroughly and allowed to stand on ice for 20 minutes. Nuclei were removed by centrifugation (3,000 x g for 30 minutes). Deoxycholic acid and SDS were added to the post-nuclear supernatant to final concentrations of 1% (w/v) and 0.5% (w/v) respectively. Insoluble debris was removed by high speed centrifugation (30,000 x g for 30 minutes) and the supernatant was used immediately for serological analysis, or was stored at -70°C. If stored, the high speed centrifugation step was repeated on the thawed lysate (Mosmann et al, 1979).

10. ANALYSIS OF CELLULAR PROTEINS

10.1. Total Cellular Proteins

Total cellular proteins were precipitated from an aliquot of cell lysate by addition of five volumes of acetone. The protein precipitate was collected by centrifugation, air-dried, and solubilized in SDS-PAGE loading buffer (see section 13.1 below).

10.2. Immune Precipitation

10.2.1. Indirect Immunoprecipitation

10µl of a 1:10 dilution of rabbit antiserum was added to an aliquot of cell lysate (or supernatant) and an incubation of 30 minutes at room temperature allowed. An equivalence of GARIg was added and the mixture incubated overnight at 4°C. Immune precipitates were harvested by centrifugation.

10.2.2. Fixed Staphyloccocal Immunoadsorbent

The Cowan I strain of Staphylococcus aureus is capable of binding to the F_C portions of certain immunoglobulins in immune complexes, for example IgG1, Ig2, Ig4 in humans (Kornvall and Williams, 1969), IgG2a, IgG2b and IgG3 in mice (Kornvall et al, 1970 and rabbit, IgG (Lind et al, 1970). The binding is mediated by protein A found in the cell wall of S. aureus (Forsgren and Sjoquist, 1966) and whole heat-killed formalinized organisms may be used as a convenient method of isolating immune complexes (Kessler, 1975, 1976). For immune precipitation using heat-killed formalin-fixed, S. aureus, 5µl of antiserum was added to an aliquot of cell lysate or supernatant and the mixture incubated at room temperature for 30 minutes prior to addition of 100µl of a 10% (v/v) solution of S. aureus in 3D-TKM. The mixture was incubated overnight at 4°C.

Specifically precipitated radiolabelled material was recovered by washing the bacteria three times in 1ml 3D-TKM and then resuspending in 0.2ml 2% (w/v) SDS. The bacteria were heated to 100°C for 2-3 minutes and then pelleted. Immune complexes eluted into the supernatant were recovered by acetone precipitation. The

samples were dissolved in SDS-PAGE loading buffer (see 13.1 below) or in 10M urea.

10.3. Purification of Serological Precipitates

Serological precipitates derived from aliquots of radio-iodinated cell membrane proteins were washed with three successive 1ml aliquots of ice-cold 3D-TKM prior to analysis. This method was not suitable for immune precipitates derived from biosynthetically labelled cells. For purification of such immunoprecipitates, the sample was dissolved in 50µl 3D-TKM and layered onto the top of a two-step discontinuous sucrose gradient. The gradient consisted of 300µl 1M sucrose in 3D-TKM and 300µl of 0.5M sucrose in 3D-TKM. After addition of the sample, the gradients were centrifuged at 3,000 x g for 30 minutes and the gradients rinsed with the following buffer.

50 mM Tris-HCl pH 8.8

1% (w/v) SDS

0.01% (w/v) Phenol red.

The washed immune precipitate was dissolved in SDS-PAGE loading buffer (see 13.1 below) or 10M urea. (Mosmann et al, 1978).

11. PREPARATION OF CELLULAR RIBONUCLEIC ACID

11.1. Isolation of Cytoplasmic RNA

Polyribosomes were prepared from large scale cultures of cells by the method of Mosmann et al, (1980). Ribonuclease-free solutions and baked glassware were used throughout.

11.1.1. Stock Solutions

Cell Suspension Buffer

0.88M	Sucrose
0.2M	KCl
0.05M	Tris-HCl (pH 7.4)
25mM	NaCl
5 mM	Magnesium acetate
7mM	2-Mercaptoethanol
0.1mg/ml	Cycloheximide
0.1mg/ml	Heparin.

PBS-Cycloheximide (PBS-CH)

PBS-A	(section 2.1)
0.1mM	$CaCl_2$
0.1mM	$MgCl_2$
0.1mg/ml	Cycloheximide.

Lysis Buffer

0.88M	Sucrose
0.2M	KCl
0.05M	Tris-HCl (pH 7.4)
25mM	NaCl
5mM	Magnesium Acetate
5mM	2-Mercaptoethanol
10% (w/v)	Triton X-100.

Sucrose Cushion

1.5M	Sucrose
0.05M	Tris-HCl (pH 7.4)
25mM	NaCl

5mM Magnesium acetate

7mM 2-Mercaptoethanol

0.1mg/ml Heparin.

11.1.2. Procedure

Cells were pooled and treated with 0.1mg/ml cycloheximide for 3 minutes at 37°C. The cells were collected by centrifugation and washed three times with ice-cold PBS-CH. After resuspension in four volumes of ice-cold cell suspension buffer, the cells were lysed by addition of one volume of lysis buffer. The suspension was centrifuged at 10,000 x g for 1 minute and the supernatant transferred to a fresh tube containing sufficient heparin to give a final cocentration of 0.1mg/ml. This sample was centrifuged at 10,000 x g for 15 minutes in the Sorvall SS34 rotor and the supernatant transferred to 10ml sucrose cushions and centrifuged at 60,000 rpm for 2 hours at 2°C in the 60Ti rotor on the Beckman L5-65 ultracentrifuge. The polyribosome pellet was resuspended in water and subjected to oligo-dT cellulose chromatography or stored at -70°C.

11.2. Oligo-dT Cellulose Chromatography

11.2.1. Stock Solutions

Column Buffer

0.5M LiCl

50mM Tris-HCl (pH 7.4)

1mM EDTA

0.1% SDS

Elution Buffer

10mM Tris-HCl (pH 7.4)

1mM EDTA

0.1% SDS

11.2.2. Chromatographic Procedure

Fresh or thawed polyribosomes were adjusted to 50mM Tris-HCl (pH 7.4), 2mM EDTA, 0.5% (w/v) SDS, 0.5M LiCl and heated to 37°C for 2 minutes before loading onto a small column of oligo-dT cellulose equilibrated with column buffer. Elution profiles were obtained by spectrophotometric analysis at 260nm. Poly-adenylated RNA was eluted from the column by addition of elution buffer. Fractions containing RNA were pooled and adjusted to 0.3M NaCl. RNA was precipitated by addition of 2 volumes of ethanol and incubation overnight at -20°C. The RNA pellet was dissolved in water and stored at -70°C in small aliquots.

12. IN VITRO TRANSLATION OF MESSENGER RNA

Rabbit reticulocyte lysate (Pelham and Jackson, 1976) was purchased from the Radiochemical Centre, Amersham, England, as a micrococcal nuclease-treated, messenger-dependent lysate (MDL). Translations were performed as follows; 1µg of mRNA (1mg/ml in H_2O) was added to 15µl of MDL supplemented with 20µCi L-[^{35}S]-methionine. The mixtures were incubated at 30°C for 90 minutes in sealed eppendorf tubes. The reaction was terminated by addition of 5 volumes of ice-cold 3D-TKM. Translation products were analysed by immunoprecipitation or acetone precipitation as described above (section 10.2.1, 10.2.2.).

13. POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was carried out using a discontinuous tris-glycine buffering system (Laemlli, 1970). Electrophoresis was performed on slab gels and in cylindrical disc gels.

13.1 Stock Solutions

Solution A

45% (w/v) Acrylamide

1.2% (w/v) N,N'-methylene bis acrylamide

Deionized, filtered and stored at 4°C.

Solution B

1.5M Tris-HCl (pH 8.8)

0.13% (v/v) TEMED

Filtered and stored at 4°C.

Solution C

12% (w/v) SDS

Filtered and stored at 4°C.

Complete Sample Buffer (10X)

0.65M Tris-HCl (pH 6.8)

Filtered and stored at 4°C.

Non-Reducing Loading Buffer

65mM Tris-HCl (pH 6.8)

2% (w/v) SDS

10% (w/v) Glycerol

0.01mg/ml Bromophenol blue

Stored at -20°C

Reducing Loading Buffer

As for non-reducing buffer with the addition of:

100mM DTT

Stored at -20°C

Reservoir Buffer

25mM Trizma base

192mM Glycine

0.1% (w/v) SDS

13.2 Separating Gel Preparation

Separating gels of the required percentages were prepared from stock solution according to the following table:

	Percentage Acrylamide				
	*3.5%	5%	10%	12.5%	15%
Solution A(ml)	9.3	13.3	26.6	33.3	40
Solution B(ml)	30	30	30	30	30
Solution C(ml)	1	1	1	1	1

* Agarose was incorporated to 0.5% (w/v) by heating in water to 80°C and adding to the other components.

Deionized water was added to 120ml. The solution was degassed, 120mg of ammonium persulphate added, and the solution poured immediately into the casting apparatus and allowed to polymerise.

13.3 Stacking Gel Preparation

Stacking gels were prepared from stock solutions as follows:

2.64ml Solution A

2.40ml Complete Sample Buffer (X10)

0.2ml Solution C

18.67ml Deionized Water

24µl TEMED

The solution was degassed and 24mg of ammonium persulphate added. The solution was pipetted onto the top of the separating gel and allowed to polymerize around a 26-well teflon template.

13.4 Sample Preparation and Electrophoresis Conditions

Pellets from acetone or immune precipitation were air-dried and dissolved in 20-25µl of reducing or non-reducing loading buffer as desired (see individual figure legends). Samples were heated to 100°C for 2 minutes and immediately loaded into individual wells in the stacking gel or to individual cylindrical gels. Electrophoresis was performed for 16-18 hours at 4°C at a constant current of 14mA per slab gel or 3mA per cylindrical gel. After electrophoresis, slab gels were processed for autoradiography or fluorography (see section 18.6 below) and cylindrical gels were sliced and the radioactivity in each slice determined (see section 18.4 below).

13.5. Molecular Weight Standards

The following proteins were used as molecular weight standards. The apparent molecular weight (AMW) of each protein is given in parentheses.

Carbonic Anhydrase (30K)	A gift from Dr. H.G. Nimmo
Lactate dehydrogenase (35K)	A gift from Dr. J.R. Coggins
Aldolase (40K)	A gift from Dr. J.R. Coggins
Pyruvate Kinase (57K)	A gift from Dr. J.R. Coggins
Creative Kinase (46K)	A gift from Dr.E.J. Milner-White
Ovalbumin (43.5K)	
Bovine Serum Albumin (67K).	

13.6 Recovery of Proteins from Acrylamide Gels

After electrophoresis, gels were processed for fluorography (see 18.6 below). The radioactive components were located in the gel and the area of gel excised and scraped off the backing paper. Protein was eluted by incubating gel slices in 200µl of extraction buffer at 60°C for 16 hours (Singer, 1978).

Extraction Buffer

250mM Tris-HCl pH 8.2

2% (w/v) SDS

20mM DTT

The gel slice was pelleted and the supernatant removed and carrier protein (50µg BSA) added. The supernatant was treated with five volumes of acetone and the precipitate collected by centrifugation. The pellet was dissolved in 10M urea.

14. PEPTIDE MAPPING PROCEDURES

Peptide mapping was carried out according to Barber and Crumpton (1976). Immune precipitates were reduced and components separated by SDS-PAGE. The proteins of interest were eluted from the gel as described above (section 13.6) and subjected to repeated acetone precipitation prior to lyophilization.

14.1. Digestion

The dried sample was resuspended in 100µl of 0.1M NH_4HCO_3. 20 µg of diphenyl carbomyl chloride-treated trypsin was added and the mixture incubated at 37°C for 2-3 hours. The sample was then lyophilised.

14.2. Electrophoresis

The digest was dissolved in 20 µl of water and samples to be compared were loaded on either side of the mid-line of a 20 x 20 cm thin layer cellulose plate pre-wetted with electrophoresis solvent.

Electrophoresis Solvent

Pyridine-Acetic Acid-Water (111 : 2.7 : 1,000 by volume)

pH 6.5

Electrophoresis was carried out at 3,000 V for 4-5 hours at room temperature. After completion of the electrophoresis, the plate was air-dried and subjected to chromatography.

14.3. Chromatography

Chromatography was performed perpendicular to the direction of electrophoresis in the following solvent.

Chromatography Solvent

Water - Acetic Acid - Pyridine - Butanol (143 : 50 : 143: 204 by volume).

After chromatography, the plates were dried and autoradiographs prepared.

15. TRYPTIC REMOVAL OF CELL MEMBRANE PROTEINS

Cell membrane proteins were enzymically digested according to Hickman and Wong-Yip (1979).

5×10^7 cells were harvested from culture, washed twice with serum-free RPMI-1640 medium and resuspended in the same medium at a concentration of 5×10^6 cells/ml. DNAase I was added to a concentration of 25µg/ml to prevent irreversible clumping of cells during digestion. Trypsin was added to a final concentration of 1mg/ml and the suspensions incubated at 37°C for 30 minutes with occasional mixing. At the end of the digestion period, FCS was added to 20% (v/v) and the cells washed twice in RPMI-1640 medium containing 10% (v/v) FCS. Washed cells were resuspended in RPMI-1640 medium containing 10% (v/v) FCS to a final concentration of 10^6 cells/ml: an aliquot of cells was removed for immediate analysis to assess the efficiency of the trypsin digestion. The suspensions were incubated overnight at 37°C and analysed by radioimmunoassay the following day. In experiments where tunicamycin was employed, a three-hour pre-incubation period at 37°C was allowed before trypsin-treatment; tunicamycin was present in all solutions used at 2µg/ml.

16. RADIOIMMUNOASSAY

16.1. Preparation of Microtitre Trays

Radioimmunoassay was performed in flexible polyvinyl 96-well microtitre trays. Before addition of cells or antisera, the

trays were soaked in 1mg/ml BSA for 1 hour at room temperature. The trays were rinsed twice with PBS-A and used immediately.

16.2 Preparation of Cells and Assay Procedure

Cells were harvested from culture and washed twice in serum-free RPMI-1640 medium and then once in serum-free RPMI-1640 medium containing 0.25% (w/v) gelatin (RPMI-gelatin). The cells were resuspended at 10^7 cells/ml in RPMI-gelatin and 100µl aliquots dispensed into wells of a BSA-pretreated microtitre tray; each well contained 25µl of monoclonal antibody. After incubation at room temperature for 1 hour, the cells were washed three times with RPMI-gelatin prior to addition of 30,000 cpm of ^{125}I-labelled rabbit anti-mouse Ig ($F(ab)_2$ fragments). This mixture was incubated for a further hour at room temperature and then the cells were washed four times with RPMI-gelatin. The plate was air-dried, individual wells excised and the radioactivity in each determined.

17. DETERMINATION OF RADIOACTIVITY

17.1. Counting of Aqueous Samples

Aqueous samples were counted as 1% or 10% (v/v) solutions in triton-toluene-PPO scintillant (section 2.3) in a Beckman L5 335 liquid scintillation spectrometer.

17.2. Counting of Non-Aqueous Samples

An aliquot of sample (5-10%) was spotted onto a glass fibre disc and air-dried. The disc was immersed in two successive baths of ice-cold 10% (w/v) TCA for 15 minutes and then rinsed with absolute alcohol. The disc was air-dried and placed in a scintillation vial containing 5ml toluene-PPO scintillant. The sample was

counted in a Beckman LS liquid scintillation spectrometer. The counting efficiences were:

^{3}H :- 58%

^{35}S :- 97%

17.3. Counting of Gamma Radiation

Radioactivity in samples radiolabelled with ^{125}I or ^{131}I was determined by counting in a Beckman "Biogamma" gamma counter.

17.4. Counting of Polyacrylamide Gel Slices

After electrophoresis, cylindrical gels containing samples labelled with ^{35}S or ^{3}H were sliced into 1mm slices and the individual slices treated with 0.5ml protosol at 60°C for 2 hours in tightly capped scintillation vials. 5mls of toluene-PPO scintillation fluid was then added to each vial and the samples mixed vigorously. The samples were counted in the LS-335 scintillation spectrometer.

17.5. Autoradiography of Slab Gels

After electrophoresis, gels were soaked in two successive baths of 20% (v/v) ethanol for 30 minutes and then dried under vacuum onto Whatman 3mm filter paper. The dried gel was held in contact with X-ray film between two glass plates and was kept in a light-proof bag for the appropriate exposure time. Exposure was at room temperature.

17.6. Fluorography of Slab Gels

Fluorography was performed according to Bonner and Laskey (1974). Gels were immersed in three successive baths of dimethyl sulphoxide (DMSO) for 30 minutes each. The gel was then immersed in a 20% (w/w) solution of PPO in DMSO for 40 minutes. PPO impregnating the gel was precipitated by rinsing the gel in running tap water for

1 hour. The gel was then dried onto paper under vacuum and exposed to film at -70C.

17.7. Development of Films

After exposure, autoradiographic and fluorographic images were visualized by immersing the film in DX-80 developer for 5 minutes, rinsing with water and then immersing in FX-40 fixer for 5 minutes. The film was rinsed with water for 10 minutes and allowed to air-dry prior to inspection.

RESULTS AND DISCUSSION

CHAPTER 1

THE ROLE OF N-LINKED OLIGOSACCHARIDES IN ASSEMBLY AND TRANSPORT OF HUMAN IMMUNOGLOBULINS AND MAJOR HISTOCOMPATIBILITY GENE COMPLEX PRODUCTS

1.1. OBJECTIVES OF INVESTIGATION

The function of N-linked oligosaccharides in control of biosynthesis, intracellular transport and functional integrity of glycoproteins is the subject of great controversy (reviewed by Gibson et al, 1980; see Introduction section 3.8.3). The study of immunoglobulin biosynthesis in human B-lymphoid cell lines is an attractive model system for investigation of the functional role of such prosthetic groups for several reasons:

a) antisera of high avidity and known specificity are readily prepared to component chains of immunoglobulins thus facilitating the isolation of radiochemical quantities of proteins from cells actively synthsising immunoglobulins;

b) cell lines are available which synthesise different classes of immunoglobulin and which also possess secretory and membrane forms of a given heavy chain polypeptide; this feature allows one to probe the different carbohydrate-mediated control mechanisms which may influence the expression of immunoglobulin in a particular cellular compartment; and

c) the immunoglobulin synthesised by such cell lines is homogeneous in nature, thus permitting a degree of electrophoretic resolution of component chains not possible when studying immunoglobulin isolated from peripheral blood lymphocytes.

The products of the human major histocompatibility complex (MHC), the HLA locus, are also of interest since they represent a population of molecules expressed exclusively on the cell membrane. Again, the influence of the N-linked oligosaccharides of these macromolecules on expression of these antigens may be explored, and comparisons of the role of carbohydrate side chains in expression of membrane immunoglobulin and MHC gene products are possible.

The objectives of the experiments described below were to assess the role of N-linked oligosaccharides in:

a) intracellular assembly of immunoglobulins and MHC gene products;

b) polymerisation of immunoglobulins and acquisition of J-chain;

c) secretion of immunoglobulins, and;

d) membrane localisation of immunoglobulins and MHC gene products.

The experimental approach was to treat cells with tunicamycin, an inhibitor of N-glycosylation of proteins (Tkacz and Lampen, 1975; Lehle and Tanner, 1976), and to specifically isolate radiolabelled proteins from various cellular compartments using a variety of antisera. Proteins isolated from control or tunicamycin-treated cell lysates and supernates were analysed by high resolution SDS-PAGE.

1.2. ASSEMBLY OF IMMUNOGLOBULINS

1.2.1. IgM and IgG

The B-lymphoblastoid lines Bri-8 and Bec-11 synthesise IgM

and IgG respectively (Singer, 1978; Singer and Williamson, 1980; Methods, Table 2). Aliquots of log-phase cultures of Bri-8 and Bec-11 cells were treated with tunicamycin, radiolabelled with ^{35}S-methionine, and cell lysates prepared. Aliquots of lysates derived from tunicamycin-treated or control cells were treated with rabbit antibody to human λ-light chain (Bri-8) or human κ-light chain (Bec-11) and GARIg. The immunoprecipitates were collected, washed, and reduced: material derived from control and tunicamycin-treated cells were compared on adjacent tracks of a 10% (w/v) SDS-PAGE slab gel.

Figure 1.1. illustrates the profiles of radioactive components resolved by SDS-PAGE. Glycosylated μ-chains were precipitated from control Bri-8 lysates using RAHλ, and non-glycosylated μ-chains were specifically immunoprecipitated from lysates of tunicamycin-treated Bri-8 cells by the same antibody. A similar result was obtained in Bec-11 cells. Multiple forms of glycosylated γ-chains were isolated from lysates of control Bec-11 cells using RAHκ, and the same antibody specifically immunoprecipitated non-glycosylated γ-chains from lysates of tunicamycin-treated cells. (The complex SDS-PAGE profiles obtained for Bec-11 IgG are discussed in detail in chapter 2.) Since anti-light chain antibodies were employed as the precipitating reagent in these experiments, heavy chains could be immunoprecipitated, and hence visualised by fluorographic analysis, if and only if they were associated with light chain. The data of figure 1.1. strongly suggest that the interaction of immunoglobulin μ- and γ-heavy chains with light chains during biosynthesis is not impaired by the absence of N-linked oligosaccharides. This is preliminary evidence to suggest that assembly of IgM and IgG is independent of N-glycosylation processes.

FIGURE 1.1.

Assembly of IgM and IgG

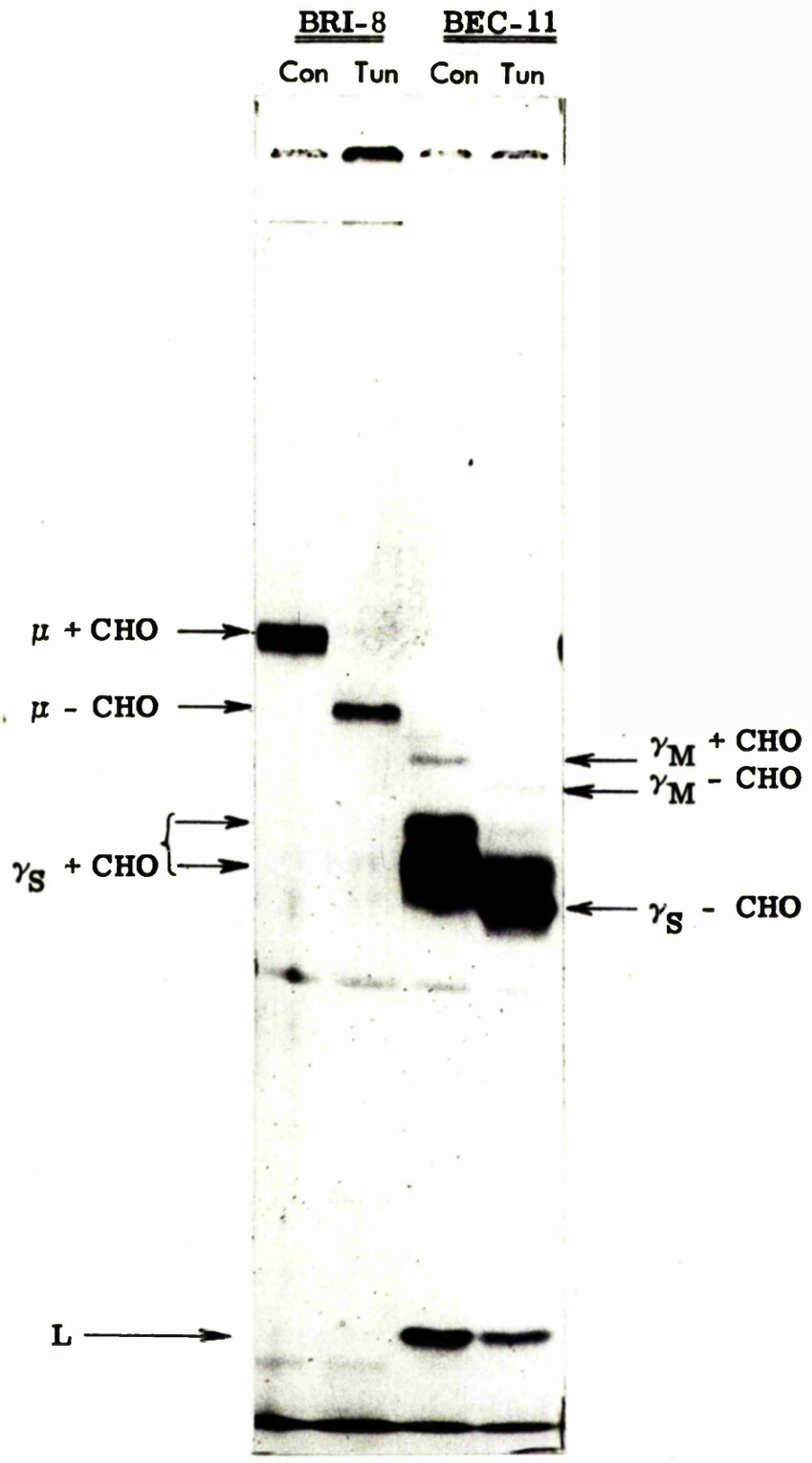
Aliquots of 2.5 x 10^6 Bri-8 and Bec-11 cells were harvested from log-phase cultures and incubated at 37°C for 90 minutes in the presence or absence of 2 µg/ml tunicamycin in complete medium. The cells were then washed twice in labelling medium and resuspended in 0.1 ml of the same medium supplemented with 200 µCi ^{35}S-methionine (Methods, 8.3.). After incubation at 37°C for 90 minutes, the labelled cells were washed twice with 1 ml aliquots of ice-cold TKM buffer and lysates prepared in 250 µl 3D-TKM (Methods, 9.).

Immunoprecipitation was carried out by treating 40 µl aliquots of lysate with 1 µl rabbit antiserum to human κ- or λ-light chain and an equivalence of GARIg (Methods, 10.2.1.). Immunoprecipitates were harvested by centrifugation and washed by pelleting through a two-step discontinuous sucrose gradient in 3D-TKM (Methods, 10.3.). Samples were dissolved in 25 µl of reducing SDS-PAGE loading buffer (Methods, 13.1.), boiled for 2 minutes and electrophoresed on a 10% (w/v) acrylamide slab gel (Methods, 13.). After electrophoresis the gel was processed for fluorography (Methods, 17.6.).

Key:-

Con - Immunoglobulin isolated from lysates of control cells

Tun - Immunoglobulin isolated from lysates of tunicamycin-treated cells.


BRI-8
BEC-11
Con
Tun
Con
Tun
μ + CHO
μ - CHO
γS + CHO
L
γM + CHO
γM - CHO
γS - CHO

The data of figure 1.1. do not, however, formally exclude the possibility that only 'half molecules' (HL) are formed or that the interaction between heavy chains may be critically dependent on the presence of N-linked carbohydrate side chains. Thus, this data does not allow any conclusions to be drawn regarding the assembly of completed 7S IgG or 8S IgM molecules. The molecular size of assembled immunoglobulins is investigated below (section 1.3.).

1.2.2. IgA and IgE

Biosynthetic assembly of IgA and IgE cultured in the presence and absence of tunicamycin was investigated using identical protocols to those employed for analysis of assembly of IgM and IgG (section 1.2.1.). The B-lymphoblastoid line Dakiki Arosros I (Steinitz and Klein, 1980) was used for the study of IgA biosynthesis, and the myeloma B-cell line U266BL (Nillson et al, 1970) was employed for analysis of IgE biosynthesis.

Figure 1.2. demonstrates that the RAHλ antiserum was capable of specifically immunoprecipitating glycosylated α-heavy chains from lysates of control Dakiki Arosros I cells and non-glycosylated α-chains from the lysates of tunicamycin-treated cells. Since RAHλ was used to precipitate the radiolabelled IgA, α-chains should be isolated only if associated with λ-light chains. As α-chains are indeed present in the SDS-PAGE profiles of reduced immunoprecipitates, this data implies that association of α-chains and light chains occurs in the absence of N-linked carbohydrate side chains.

The corresponding experiment in U266BL cells is also presented in figure 1.2. RAHλ was employed as the precipitating antibody and was capable of precipitating IgE from lysates of control and

FIGURE 1.2.

Assembly of IgA and IgE

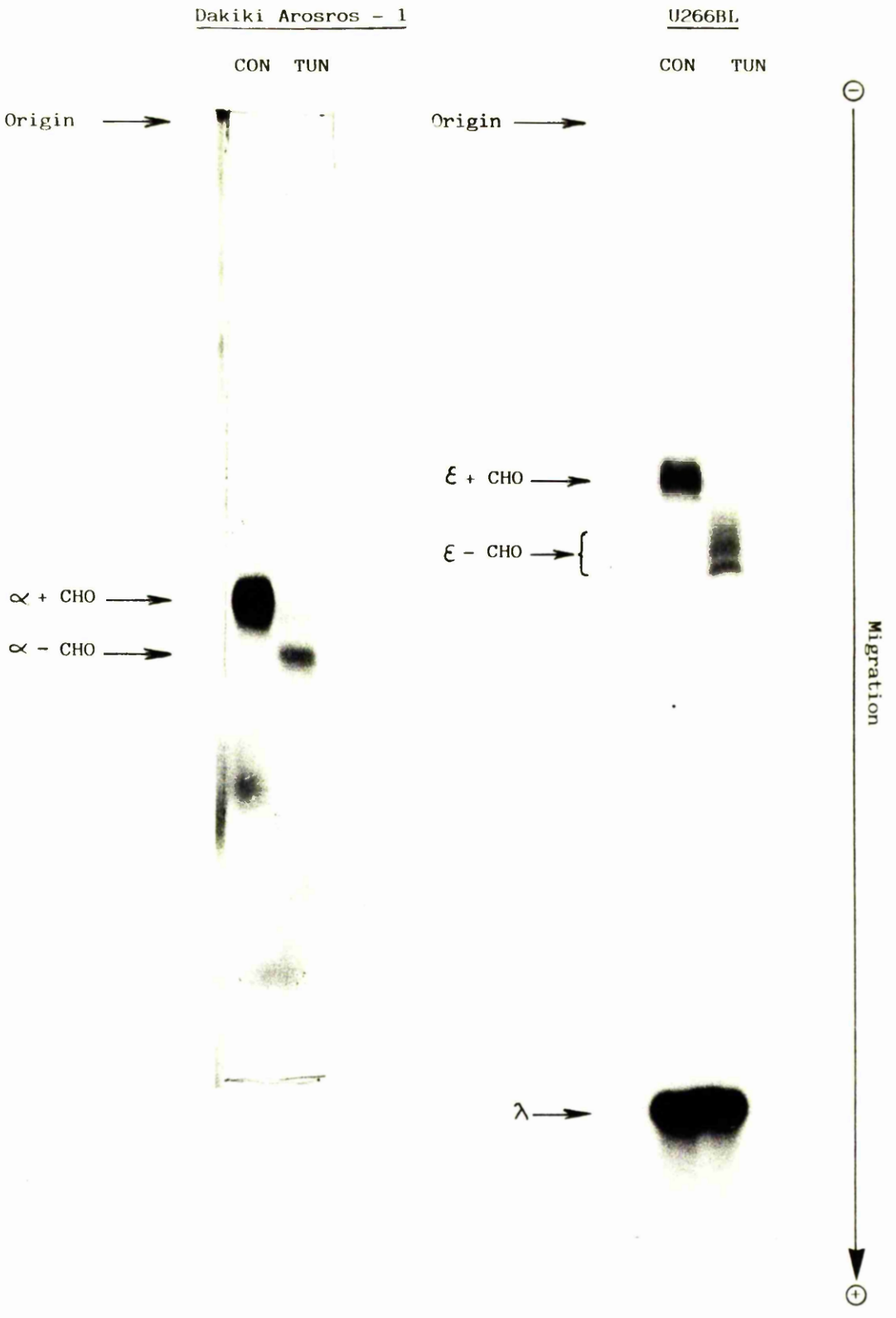
Aliquots of 2.5 x 10^6 Dakiki Arosros 1 or U266BL cells were harvested from log-phase cultures and incubated at 37°C for 90 minutes in the presence or absence of 2 µg/ml tunicamycin in complete medium. The cells were then washed twice in labelling medium and resuspended in 0.1 ml of the same medium supplemented with 200 µCi of ^{35}S-methionine (Methods, 8.3.). After incubation at 37°C for 90 minutes, the labelled cells were washed twice with 1 ml aliquots of ice-cold TKM buffer and lysates prepared in 250 µl 3D-TKM (Methods, 9.).

Immunoprecipitates were prepared by treating 30 µl aliquots of lysate with RAHλ and an equivalence of GARIg (Methods, 10.2.1.). After harvesting and purification on two-step discontinuous sucrose gradients (Methods, 10.3.) the samples were dissolved in 25 µl of SDS-PAGE loading buffer and boiled. Analysis was performed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The gel was processed for fluorography (Methods 17.6.).

Key:-

Con - Immunoglobulin isolated from lysates of control cells

Tun - Immunoglobulin isolated from lysates of tunicamycin-treated cells.


Dakiki Arosros - 1
U266BL
CON
TUN
CON
TUN
Origin
Origin
ε + CHO
ε - CHO
α + CHO
α - CHO
λ
Migration

tunicamycin-treated U266BL cells. Glycosylated ε-chains were evident in the profiles obtained for material immunoprecipitated from control cell lysates and multiple forms of non-glycosylated ε-chains were demonstrable in the corresponding profiles of tunicamycin-treated cell lysates of U266BL cells. The multiplicity of non-glycosylated ε-chains in lysates of tunicamycin-treated U266BL cells is believed to be due to use of tunicamycin at non-limiting concentrations, and that the species resolved represent intermediate forms between totally glycosylated and completely oligosaccharide free ε-chains. Once more the data infers that assembly of immunoglobulin heavy and light chains is occuring independently of N-glycosylation of the component chains.

1.2.3. Limitations

Although the above data (sections 1.2.1. and 1.2.2.) strongly suggest that immunoglobulin assembly occurs in the absence of N-glycosylation of the immunoglobulin heavy chain, direct evidence for formation of H_2L_2 macromolecules is lacking. The data illustrate that HL molecules have been formed but do not conclusively demonstrate the presence of H_2L_2 structures. In the case of IgG and IgE, the detection of 7S structures is possible by analysis of radioactive immunoglobulins on low percentage acrylamide gels under non-reducing conditions. The detection of 11S IgA and 19S IgM polymeric immunoglobulins is more difficult.

The principal intracellular forms of IgM and IgA are the 8S and 7S monomers respectively (see Introduction, section 3.7.). Formation of polymers is believed to occur immediately before secretion and therefore it is reasonable to expect 19S IgM and 11s IgA to be minority species in the cytoplasm of cells actively synthesising immunoglobulin. A second approach to the problem is to assay for the

presence of J-chain in IgM or IgA isolated from cells synthesising these immunoglobulins. However, this approach is subject to the same problems as that of assaying for 19S IgM itself; that is, J-chain addition is believed to be a late event during the intracellular processing of IgM or IgA.

1.2.4. Assembly of HLA-A,-B and -C Alloantigens

Aliquots of log phase cultures of Bjab and Bri-8 cells were treated with tunicamycin, biosynthetically radiolabelled, and cell lysates prepared: control cell lysates were also prepared. Aliquots of lysate were then treated with RAHβ_2M and S.aureus immunoadsorbent. Bound complexes were eluted from washed bacteria and analysed by SDS-PAGE.

Figure 1.3. illustrates the profiles obtained. Material specifically immunoprecipitated from lysates of control cells contained a single polypeptide of AMW 44,000 daltons; this represents the polymorphic HLA-A,-B, or -C chain. This component was also present in immunoprecipitates derived from lysates of tunicamycin-treated cells, but an additional component of AMW 42,000 daltons was also resolved in the SDS-PAGE profiles of thes immunoprecipitates. HLA-A and -B antigens contain a single N-linked oligosaccharide moiety at asparagine 86 (Parham et al, 1977). The finding of a 42,000 dalton species in association with β_2 microglobulin in tunicamycin-treated cells is consistent with the 42,000 chain being the non-glycosylated counterpart of the 44,000 dalton chain found in control cells.

The data of figure 1.3. strongly suggest that the interaction of HLA-A,-B or -C chain with β_2 microglobulin does not depend upon the N-glycosylation of asparagine 86 of the HLA-A, -B or -C chain. These

FIGURE 1.3.

Assembly of HLA-A,-B,-C - β_2 Microglobulin Complexes

2.5×10^6 Bri-8 or Bjab cells were harvested from culture, treated with tunicamycin, biosynthetically radiolabelled, and lysed as described in the legend to figure 1.2. (Methods, 8;9).

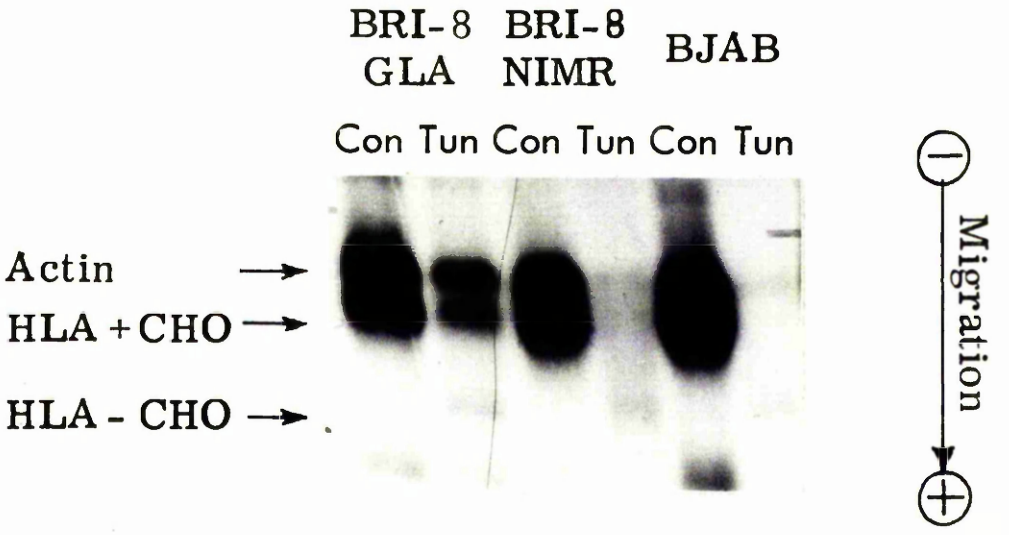
Immunoprecipitates were prepared by adding 5 µl of RAHβ_2M to 50 µl aliquots of lysate and then adding 100 µl of a 10% (v/v) suspension of the S.aureus immunoadsorbent (Methods, 10.2.2.). After overnight incubation at $4^{\circ}C$, the bacteria were washed three times with 1 ml of ice-cold 3D-TKM, and bound immune complexes eluted by boiling the organisms for 2 minutes in 100 µl 2% (w/v) SDS. The bacteria were pelleted, the supernate removed and treated with 500 µl acetone to precipitate protein. The precipitate was dissolved in 25 µl of reducing SDS-PAGE loading buffer, boiled, and analysed on a 10% (w/v) acrylamide slab gel (Methods, 13.). After electrophoresis the gel was processed for fluorography (Methods, 17.6.).

Key:-

Con - Material derived from lysates of control cells

Tun - Material derived from lysates of tunicamycin-treated cells.

Assembly of HLA-β2 Microglobulin Complexes



conclusions are based on the same arguement made above for immunoglobulin assembly; that is, since RAHβ_2M is the precipitating antibody, HLA-A,-B or -C chains should be immunoprecipitated if, and only if, they are associated with β_2 microglobulin. Since HLA-A,-B and -C chains are present in immunoprecipitates derived from either control or tunicamycin-treated cells, it is reasonable to suggest that the interaction of the 44,000 dalton chain with β_2 microglobulin does not depend on N-glycosylation of the former.

1.3. MOLECULAR SIZE OF ASSEMBLED IMMUNOGLOBULINS

1.3.1. IgG and IgE

Radiolabelled IgG was isolated, by specific immunoprecipitation, from lysates and supernates of control and tunicamycin-treated Bec-11 cells. Radiolabelled IgE was isolated from lysates of control and tunicamycin-treated U266BL cells. After washing, the immunoprecipitates were analysed on a 6% (w/v) acrylamide gel under non-reducing conditions. The data obtained are shown in figure 1.4.

Bec-11 cells are known to secrete 7S IgG (Singer and Williamson, 1979; Singer, 1978). Using biosynthetically radiolabelled IgG secreted from control Bec-11 cells as an internal marker and BSA as an independent marker, it is clear from the mobilities of material isolated from lysates of control and tunicamycin-treated Bec-11 cells that 7S IgG is the predominant species isolated. The molecular size of IgG secreted from tunicamycin-treated Bec-11 cells also appears to be 7S. The data are therefore consistent with the hypothesis that N-linked oligosaccharide moieties do not exert any influence on the assembly

of H_2L_2 IgG macromolecules.

Two major species were detected in the SDS-PAGE profiles of IgE isolated from control and tunicamycin-treated U266BL cells. The major component in both immunoprecipitates migrated with a slightly slower electrophoretic mobility than the BSA marker: its AMW was therefore of the order of 70-80K which would be consistent with the hypothesis that this species might represent an HL intermediate.

The other species present on the profiles had an electrophoretic mobility comparable to that of 7S IgG: this is believed to represent 7S IgE. Significantly, the material isolated from lysates of control U266BL cells had a higher AMW than the corresponding material in tunicamycin-treated cells. This observation supports the hypothesis that these bands represent 7S IgE since the IgE isolated from lysates of tunicamycin-treated U266BL cells would contain non-glycosylated ε-chains, of lower AMW than their glycosylated counterparts, and hence the assembled complex would be of lower AMW than 7S IgE isolated from lysates of control U266BL cells.

The data are therefore consistent with the hypothesis that N-glycosylation of immunoglobulin heavy chains is not required for efficient assembly of H_2L_2 molecules. The analysis is now extended to encompass assembly of IgM and IgA molecules to the H_2L_2 and polymer states.

1.3.2. IgM and IgA

In accord with the above hypothesis, SDS-PAGE analysis of immunoprecipitated IgM and IgA from lysates and supernates of control and tunicamycin-treated Bri-8 and Dakiki Arosros-1 cells demonstrated

FIGURE 1.4.

Molecular Size of Assembled IgG and IgE

Aliquots of 2.5 x 10^6 Bec-11 or U266BL cells were harvested from log phase cultures and treated for 3 hours at 37°C in the presence or absence of 2 µg/ml tunicamycin in complete medium. The cells were washed twice in labelling medium resuspended in 0.1 ml of the same medium supplemented with 250 µCi of ^{35}S-methionine and incubated at 37°C for 90 minutes. After labelling the Bec-11 cultures were divided into two equal aliquots. A pulse-chase was performed on one aliquot and a lysate prepared from the other; the final volume of both samples was 250 µl. Lysates were prepared from the U266BL sample (Methods, 8,3;9).

Immunoprecipitation was effected by treating 50 µl aliquots of lysate or supernate with 1 µl rabbit anti-light chain antibody, plus an equivalence of GARIg (Methods, 10.2.1.). Washed precipitates were dissolved in 25 µl non-reducing SDS-PAGE loading buffer, boiled, and loaded onto a 6% (w/v) acrylamide slab gel (Methods, 13). After electrophoresis, the gel was processed for fluorography (Methods, 17.6.).

Key:-

IC - Intracellular

EC - Extracellular

Con - Immunoglobulin isolated from lysates or supernates of control cultures.

Tun - Immunoglobulin isolated from lysates or supernates of tunicamycin-treated cultures.

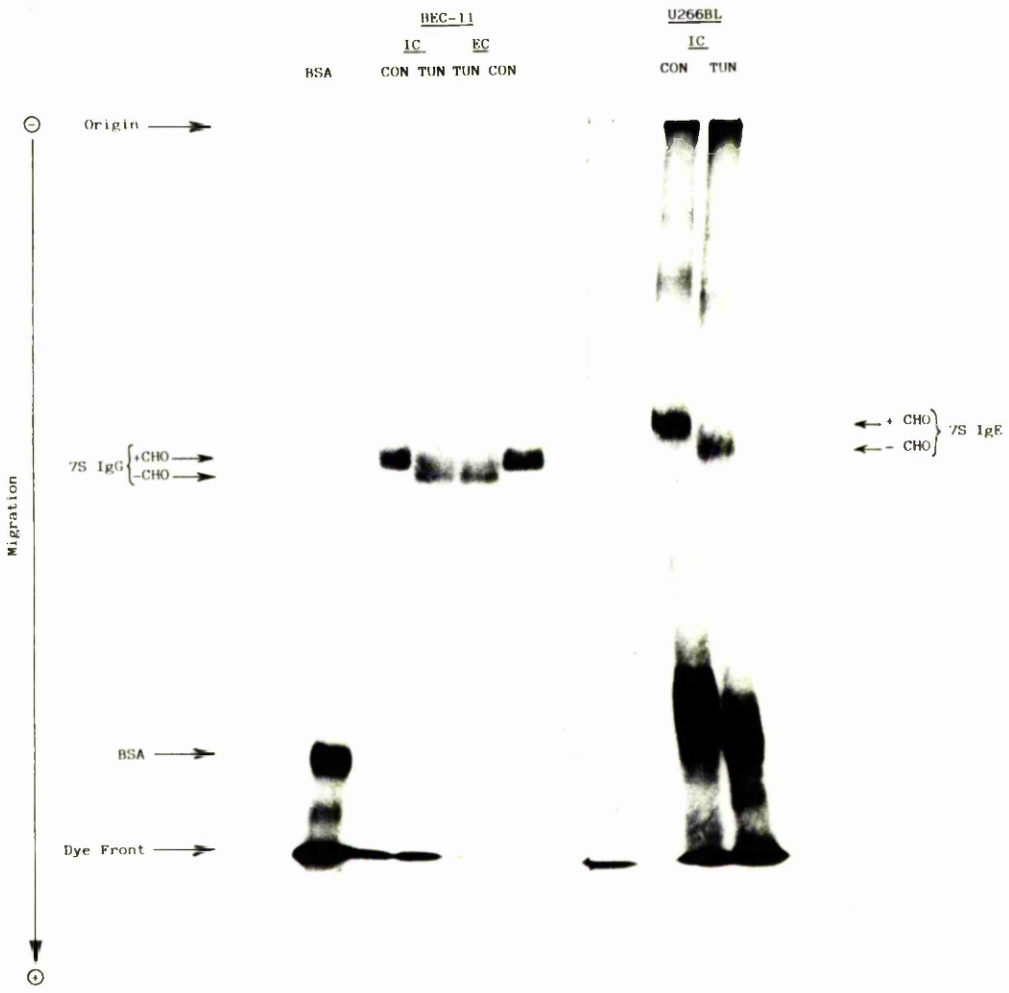

HEC-11
IC
EC
BSA
CON TUN TUN CON
U266BL
IC
CON TUN
Origin
7S IgG
+CHO
-CHO
+ CHO
- CHO
7S IgE
Migration
BSA
Dye Front

FIGURE 1.5.

Molecular Size of Assembled IgM and IgA

Aliquots of 2.5 x 10^6 Bri-8 or Dakiki Arosros-1 cells were harvested from culture and treated in the presence or absence of 2µg/ml tunicamycin for 3 hours at 37°C in complete medium prior to biosynthetic radiolabelling (Methods, 8.3.). 500 µCi of ^{3}H-leucine was used as label for Bri-8 cultures and 500 µCi ^{35}S-methionine was employed with Dakiki Arosros-1 cultures. Pulse-chase experiments were performed (Methods, 8.3.) and immunoprecipitation performed on aliquots of chase supernate or lysate.

Washed immune precipitates, generated using RAHµ or RAHα, were dissolved in non-reducing SDS-PAGE loading buffer, boiled, and analysed on a 6% (w/v) acrylamide gel (Methods, 13). The gel was processed for fluorography (Methods 17.6.). Samples derived from Bec-11 lysates (see legend to figure 1.4.) were also electrophoresed as marker proteins.

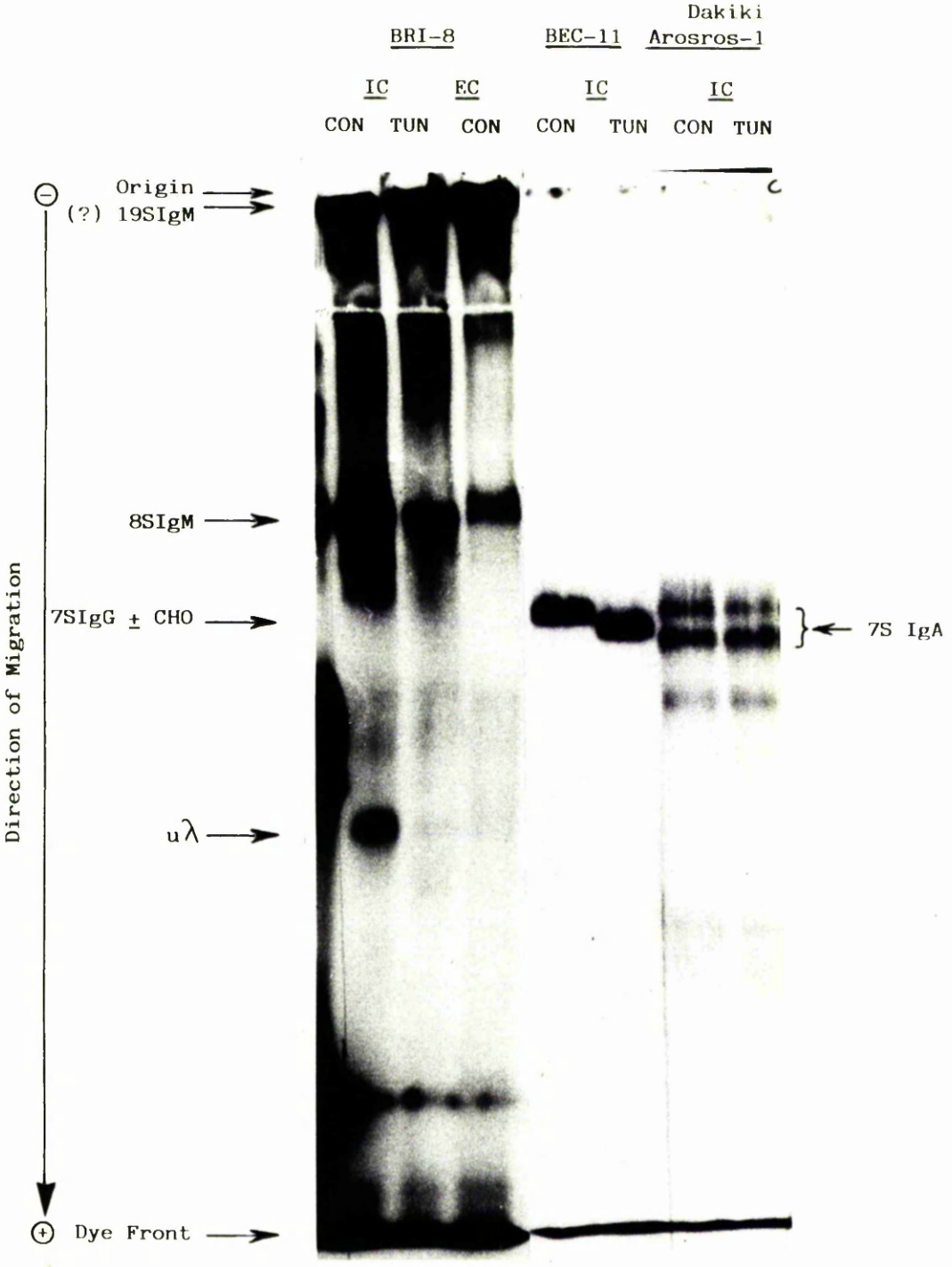
Key:-

IC - Intracellular

EC - Extracellular

Con - Immunoglobulin isolated from lysates and supernates of control cultures.

Tun - Immunoglobulin isolated from lysates and supernates of tunicamycin-treated cultures.


BRI-8
BEC-11
Dakiki Arosros-1
IC
EC
IC
IC
CON
TUN
CON
CON
TUN
CON
TUN
Origin
(?) 19SIgM
8SIgM
7SIgG ± CHO
uλ
Dye Front
7S IgA
Direction of Migration

that 8S IgM and 7S IgA structures were formed in the absence of N-glycosylation (figure 1.5.).

Material of slower electrophoretic mobility than the marker 7S IgG proteins derived from lysates of control and tunicamycin-treated Bec-11 cells was specifically immunoprecipitated from lysates and supernatants of Bri-8 cells using RAHμ and GARIg. The mobility of the isolated components is consistent with the notion that they represent 8S IgM. The 8S IgM species was present in lysates of control and tunicamycin-treated Bri-8 cells and in the supernates of control cultures. Also present in each of these profiles was material located at the origin of each track which had failed to enter the gel. This is believed to be 19S IgM. These data illustrate that lack of N-glycosylation in IgM-producing cells does not interfere with the assembly of 8s IgM monomers and tentatively indicates that 19S IgM pentamer formation also occurs independently of N-glycosylation.

Analysis of IgA assembly in Dakiki Arosros-1 cells demonstrated that material of comparable AMW to Bec-11 7S IgG could be immunoprecipitated from control and tunicamycin-treated cells. Once again these data are consistent with the hypothesis that N-glycosylation is not important in the control of assembly of complete immunoglobulin molecules. No high molecular weight material was observed in IgA-containing immunoprecipitates derived from Dakiki Arosros-1 cells and this is consistent with the interpretation that these cells are secreting monomeric IgA.

1.3.3. Assembly of J-Chain into Polymeric Immunoglobulins

In an attempt to provide more substantial data pertaining to the intracellular assembly of the 19S IgM pentamer, radiolabelled IgM was isolated from lysates and supernatants of control and tunicamycin-

treated cultures of Bri-8 cells. After reduction, the immunoprecipitates were analysed by SDS-PAGE, and the fluographic pattern of radioactive components resolved is shown in figure 1.6.

The data fail to show the presence of J-chain in either glycosylated or non-glycosylated IgM isolated from lysates of Bri-8 cells. However, the apparent absence of J-chain from glycosylated IgM isolated from Bri-8 culture supernatants suggests that conditions for the detection of J-chain itself are not optimal under these experimental conditions. In experiments studying the biosynthesis and fate of murine J-chain (Mosmann et al, 1979) ^{35}S-cystine was employed as radioactive tracer. This is a more appropriate radiolabel in experiments studying J-chain due to the large number of cysteine residues in the molecule: in the above experiments, tritiated leucine was the radioactive amino acid used.

Experiments addressing the question of integration of J-chain into polymeric immunoglobulins have been performed in the mouse. In studies of IgM polymerisation, it was found that pentamer formation and addition of J-chain to 19S IgM was not inhibited by addition of tunicamycin to a murine plasmacytoma synthesising IgM (Tartakoff and Vassalli, 1979). In the IgA-producing murine plasmacytoma MOPC 315, it was demonstrated that J-chain was covalently bound to IgA in cells treated with tunicamycin; covalently bound J-chain could also be demonstrated in IgM isolated from tunicamycin treated cells (Mosmann and Williamson, unpublished observations).

The murine data suggest that J-chain is successfully added to non-glycosylated polymeric immunoglobulins. This would indicate that the failure to find J-chain in IgM synthesised and secreted by

FIGURE 1.6.

Apparent Absence of J-Chain from Non-Glycosylated 19S IgM

Aliquots of 2.5 x 10^6 Bri-8 cells were treated with tunicamycin as described in the legend to figure 1.5. and biosynthetically labelled with 500 µCi ^{3}H-leucine: control cultures were also prepared. After a 2 hour labelling the cultures were divided into equal aliquots and a pulse-chase performed on one aliquot and a lysate prepared in 3D-TKM from the other. The final volume of each sample was 250 µl (Methods, 8,9.).

IgM was specifically immunoprecipitated from 50 µl aliquots of lysate or supernate by addition of 1 µl RAHµ plus an equivalence of GARIg (Methods 10.2.1.). The immunoprecipitates were collected by centrifugation, and washed by centrifugation through a two step discontinuous sucrose gradient in 3D-TKM (Methods, 10.3.). Samples were dissolved in 25 µl reducing SDS-PAGE loading buffer, boiled for 2 minutes, and analysed on a 15% (w/v) acrylamide slab gel. The gel was processed for fluorography (Methods, 17.6.).

The region of the gel where J-chain would be expected to migrate is indicated on the figure. β_2 microglobulin was visualised in an adjacent track on the same gel (not shown).

Key:-

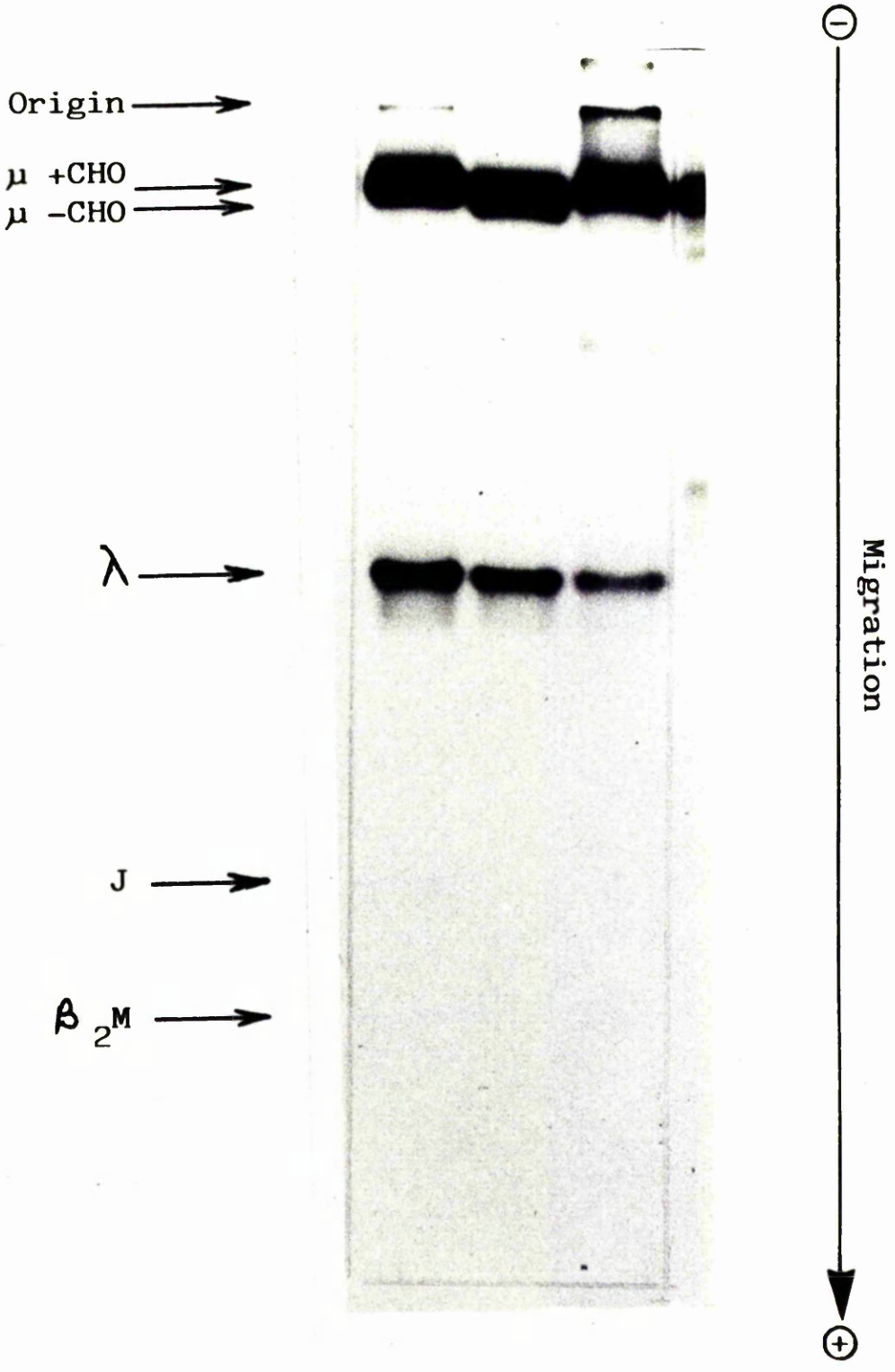
IC - Intracellular

EC - Extracellular

Con - IgM immunoprecipitated from lysates or supernates of control cultures.

Tun - IgM isolated from lysates of tunicamycin-treated cells.

BRI-8



Bri-8 cells could be due to insensitivity of fluorographic detection in the experimental system rather than absence of J-chain from the IgM pentamer, although alternative explanations are possible, (for example, lack of J-chain in Bri-8 cells or a more rigorous requirement for N-glycosylation of μ- or α-chains in human cells than in murine cells prior to J-chain addition).

1.4. SECRETION OF IMMUNOGLOBULINS

1.4.1. IgM and IgA

The efficiency of secretion of immunoglobulins from normal and tunicamycin-treated cells was investigated by isolating radio-labelled immunoglobulin from lysates and culture supernates of control and tunicamycin-treated cells by immunoprecipitation. Isolated immunoglobulins were analysed by SDS-PAGE.

The cell line Bri-8 was employed for analysis of IgM secretion in control and tunicamycin-treated cells. IgM was isolated from cell lysates prepared after a 90 minute pulse labelling and secreted IgM was immunoprecipitated from culture supernatants after a 5 hour pulse-chase. Figure 1.7. illustrates that glycosylated μ-chains were present in culture supernatants of control Bri-8 cells. The secretion of IgM from tunicamycin-treated Bri-8 cells was greatly inhibited as indicated by the almost total absence of non-glycosylated μ-chains in the supernatants of such cultures. The trace amount of non-glycosylated μ-chain found in such cell supernates was always accompanied by the presence of glycosylated μ-chains. This suggests that the presence of a small amount of carbohydrate may be sufficient

FIGURE 1.7.

Inhibition of Secretion of Non-Glycosylated IgM

2.5×10^6 Bri-8 cells were harvested from log-phase cultures and incubated in the presence or absence of 2 μg/ml tunicamycin for 3 hours at $37^{\circ}C$ in complete medium. The cells were washed twice in labelling medium and then biosynthetically labelled with 200 μCi of ^{35}S-methionine. Incorporation was allowed to proceed for 90 minutes whereupon the culture was divided into equal aliquots and a 5 hour pulse-chase performed on one aliquot and a lysate prepared from the other (Methods, 8,9.).

IgM was isolated from aliquots of lysate and supernate by specific immunoprecipitation using 1 μl RAHμ plus an equivalence of GARIg (Methods 10.2.1.). Washed immune precipitates were dissolved in 25 μl reducing SDS-PAGE loading buffer and analysed on 12.5% (w/v) acrylamide gel (Methods, 13). After electrophoresis, the gel was processed for fluorography (Methods 17.6.).

Key:-

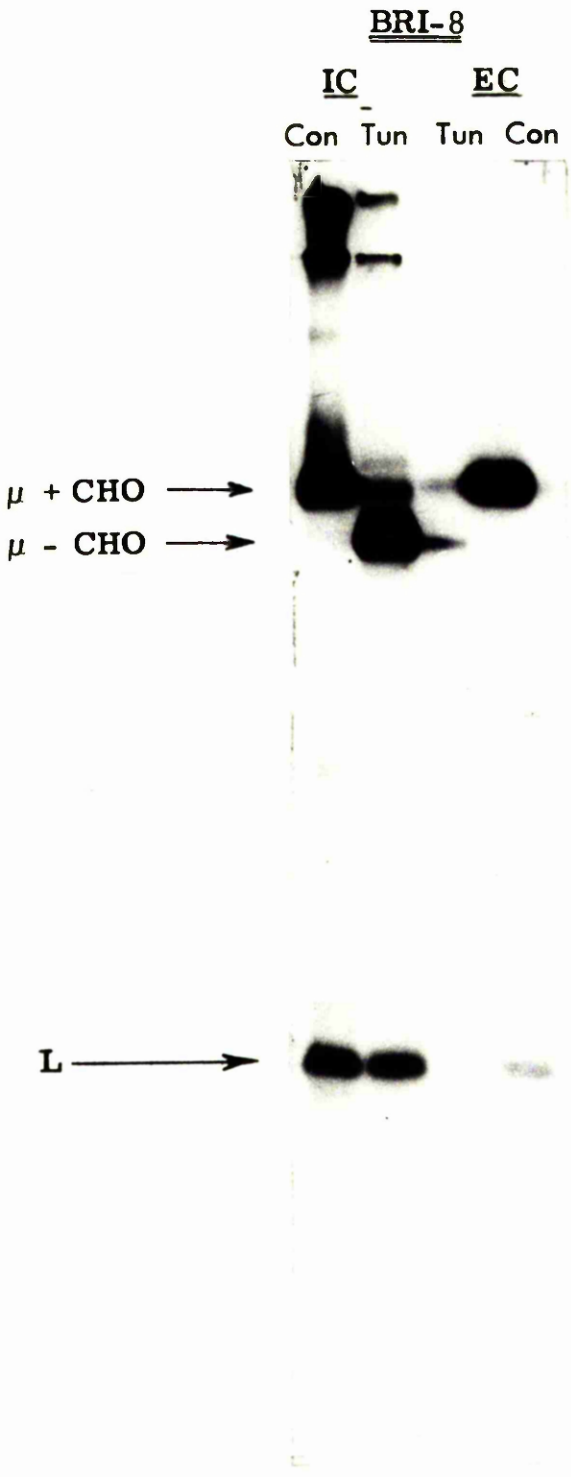
IC - Intracellular

EC - Extracellular

Con - IgM isolated from lysates and supernates of control cultures.

Tun - IgM isolated from lysates and supernates of tunicamycin-treated cells.

Inhibition of Secretion of Non-Glycosylated IgM



to enable the cell to secrete IgM. However, the secretion of IgM containing totally non-glycosylated μ-chains was never observed. This data strongly suggests that the N-linked oligosaccharide prosthetic groups of μ-chains are important in control of secretion of IgM, and may represent a mandatory structural requirement for this biosynthetic event. Similar data have been obtained for other IgM-secreting cells (e.g. Bjab and Namalva).

Figure 1.8. also shows the results obtained from a similar experiment performed on the IgA secreting B-lymphoma cell line Dakiki Arosros 1. IgA was efficiently secreted from control Dakiki Arosros 1 cells but IgA containing non-glycosylated α-chains was never isolated from culture supernates of tunicamycin-treated Dakiki Arosros 1 cells. Thus, IgA secretion seems to depend on the presence of N-linked oligosaccharide moieties on the α-chain, in the same way that N-glycosylation of α-chains appears to be mandatory for secretion of IgM.

1.4.2. IgG and IgE

Radiolabelled IgG was isolated, by specific immunoprecipitation, from lysates and supernates of control and tunicamycin-treated cultures of Bec-11 cells. The washed immune precipitates were reduced and analysed by SDS-PAGE.

Figure 1.9. demonstrates that two γ-chain polypeptides were isolated from supernates of control Bec-11 cultures but that only one γ-chain species could be immunoprecipitated from supernates of tunicamycin-treated cultures.

This data suggests that:

a) non-glycosylated IgG is efficiently secreted from tunicamycin-treated Bec-11 cells, and;

FIGURE 1.8.

Inhibition of Secretion of Non-Glycosylated IgA

Tunicamycin-treatment, biosynthetic labelling and pulse-chase treatments of Dakiki Arosros-1 cells were as described for Bri-8 cells in the legend to figure 1.7.

Aliquots of lysate and supernate were treated with 1 µl RAHα and an equivalence of GARIg (Methods 10.2.1.) to specifically immunoprecipitate IgA. Washed immunoprecipitates were dissolved in 25 µl of reducing SDS-PAGE loading buffer, boiled, and electrophoresed on a 10% (w/v) acrylamide slab gel (Methods, 13). The gel was processed for fluorography (Methods 17.6.).

Key:-

Con - IgA derived from lysates and supernates of control cultures

TM - IgA derived from lysates and supernates of tunicamycin-treated cultures.

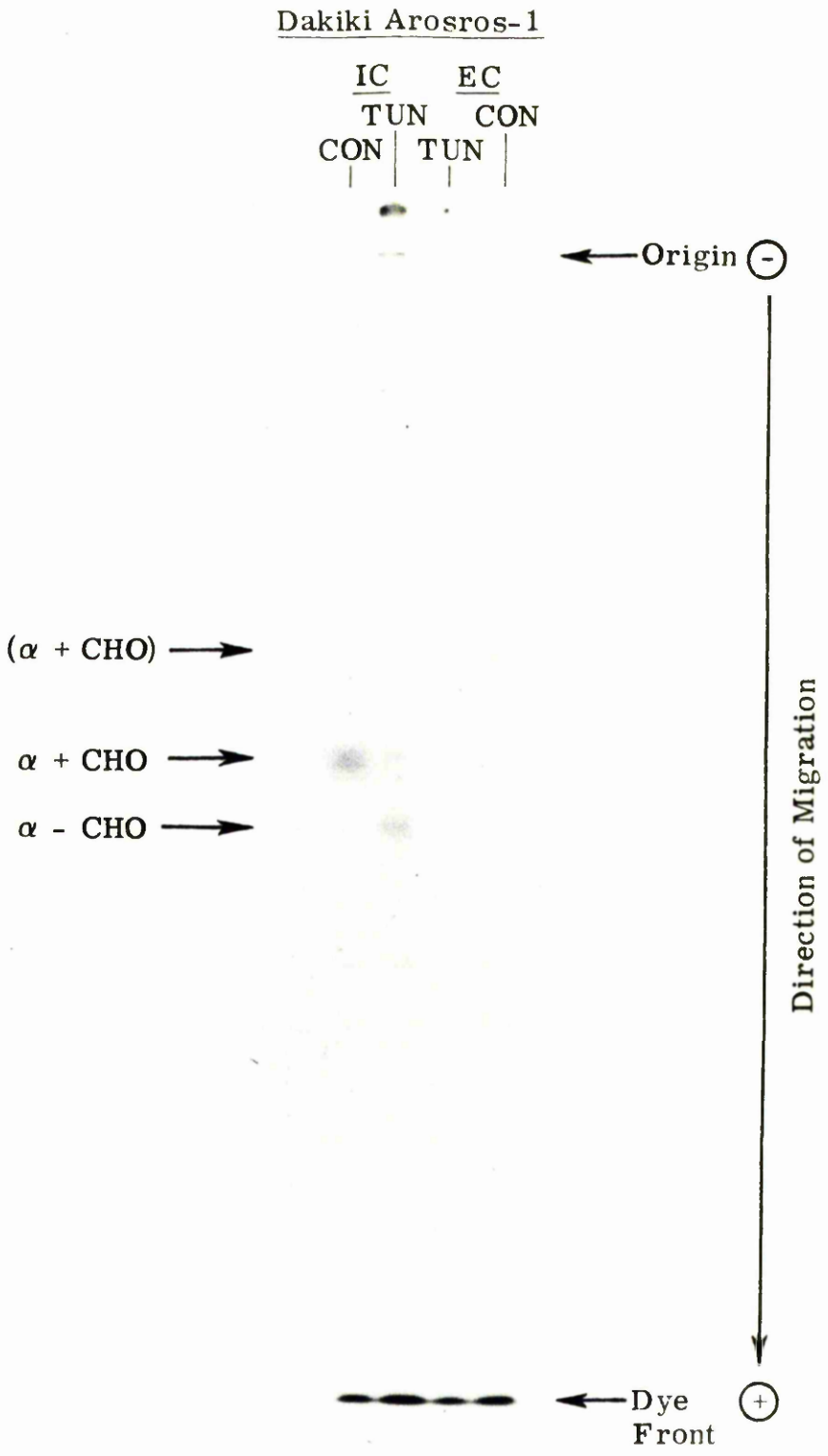

Dakiki Arosros-1
IC
EC
TUN
CON
CON
TUN
Origin
(α + CHO)
α + CHO
α - CHO
Direction of Migration
Dye
Front

FIGURE 1.9.

Secretion of Non-Glycosylated IgG

The protocols used for tunicamycin-treatment, labelling and pulse-chase of Bec-11 cells were identical to those described for Bri-8 cells in the legend to figure 1.7. (Methods, 8,9).

Immunoprecipitation was carried out on aliquots of lysate and supernate by addition of 1 µl of RAHκ plus an equivalence of GARIg (Methods 10.2.1.). Washed immunoprecipitates were dissolved in reducing SDS-PAGE loading buffer and analysed on a 10% (w/v) acrylamide slab gel (Methods, 13) and the pattern of radioactive components visualised by fluorography (Methods, 17.6.).

Key:-

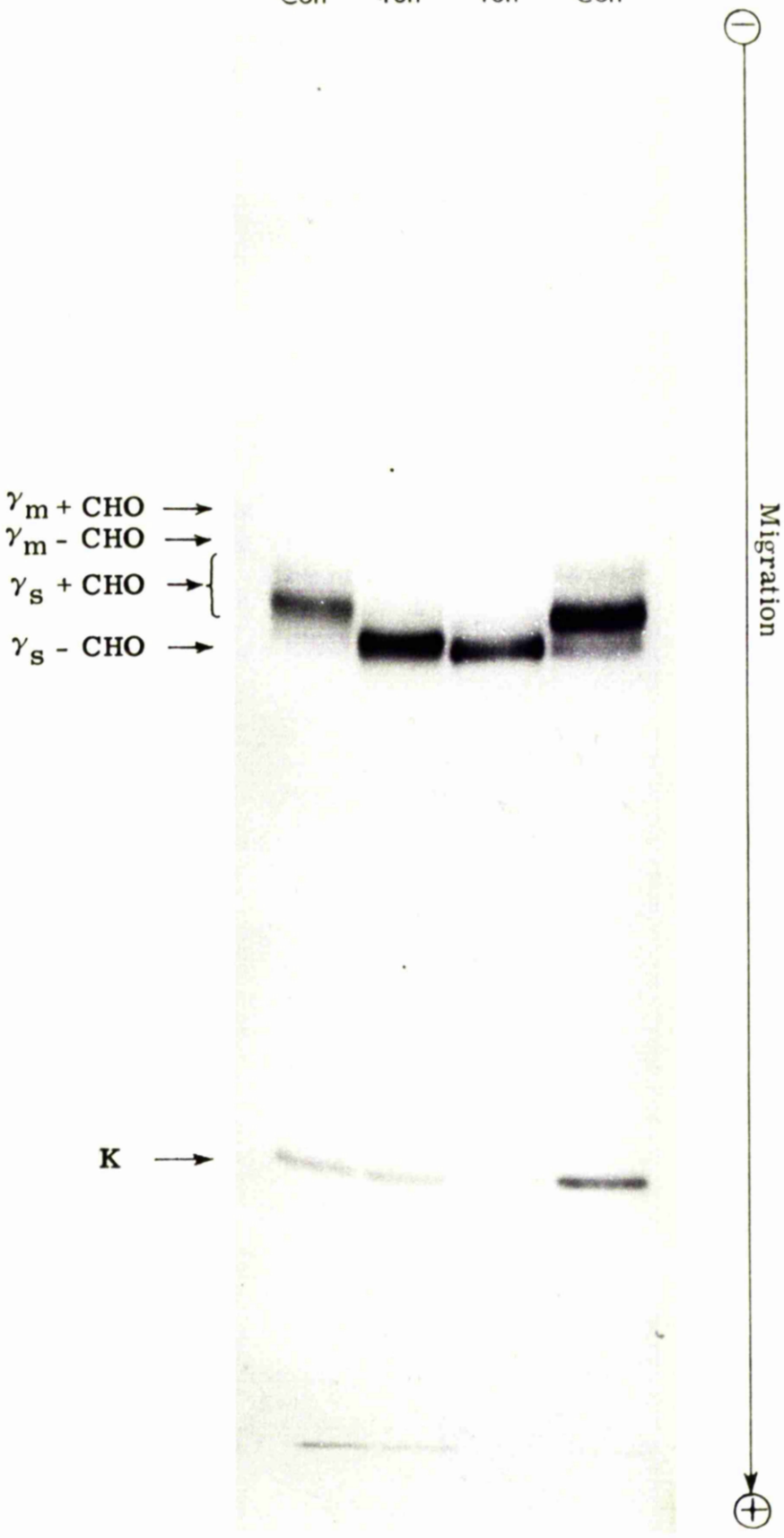
IC - Intracellular

EC - Extracellular

Con - IgG immunoprecipitated from lysates and supernates of control cultures.

Tun - IgG immunoprecipitated from lysates and supernates of tunicamycin-treated cultures.

Secretion of Non-Glycosylated IgG



b) only one secretory γ-chain polypeptide is synthesised by Bec-11 cells.

An identical experiment performed on U266BL myeloma cells suggested that IgE could be secreted from tunicamycin-treated cells (figure 1.10, panel A). However, the ε-chains specifically immunoprecipitated from culture supernates of tunicamycin-treated U266BL cells had identical electrophoretic mobilities to those isolated from supernates of control cultures. Based on the assumption that this effect was due to residual N-glycosylation of ε-chains after a 3 hour tunicamycin-treatment, the experiment was repeated but with an extended (12 hour) pre-treatment with tunicamycin prior to pulse-chase. An identical result was obtained: ε-chains isolated from supernates of control and tunicamycin-treated cultures of U266BL cells had identical electrophoretic mobilities as judged by high resolution SDS-PAGE (figure 1.10, panel B). However, in both instances the AMW of the secreted ε-chains is greater than that of the glycosylated intracellular ε-chains. This is indicative of extensive post-synthetic modification of ε-chains. One possible explanation is that the post-synthetic modification events are O-glycosylation events. Such processes would occur independently of N-glycosylation and would not be inhibited by tunicamycin. The objection to such an hypothesis is that the ε-chain secreted from tunicamycin-treated cells would still be expected to have a lower AMW than its counterpart secreted from control cells since tunicamycin-treatment would have prevented addition of N-linked oligosaccharides to the former. Therefore, equal modification of ε-chains in control and tunicamycin-treated U266BL cells would result in an AMW difference consistent with absence of

FIGURE 1.10.

Secretion of Non-Glycosylated IgE

Protocols for generation of lysates and supernates from control and tunicamycin-treated cultures of U266BL cells were identical to those described in the legend to figure 1.7. (Methods, 8,9).

IgE was isolated from aliquots of lysate or supernate by addition of 1 µl RAHε plus an equivalence of GARIg (Methods, 10.2.1.). Washed immunoprecipitates were dissolved in 25 µl of reducing SDS-PAGE loading buffer, boiled, and electrophoresed on a 10% (w/v) acrylamide gel (Methods, 13). The gel was processed for flurography (Methods 17.6.).

Panel A - 3 hour pretreatment with tunicamycin.

Panel B - 12 hour pretreatment with tunicamycin.

Key:-

IC - Intracellular

EC - Extracellular

Con - IgE derived from lysates and supernates of control cultures.

Tun - IgE derived from lysates and supernates of tunicamycin-treated cultures.

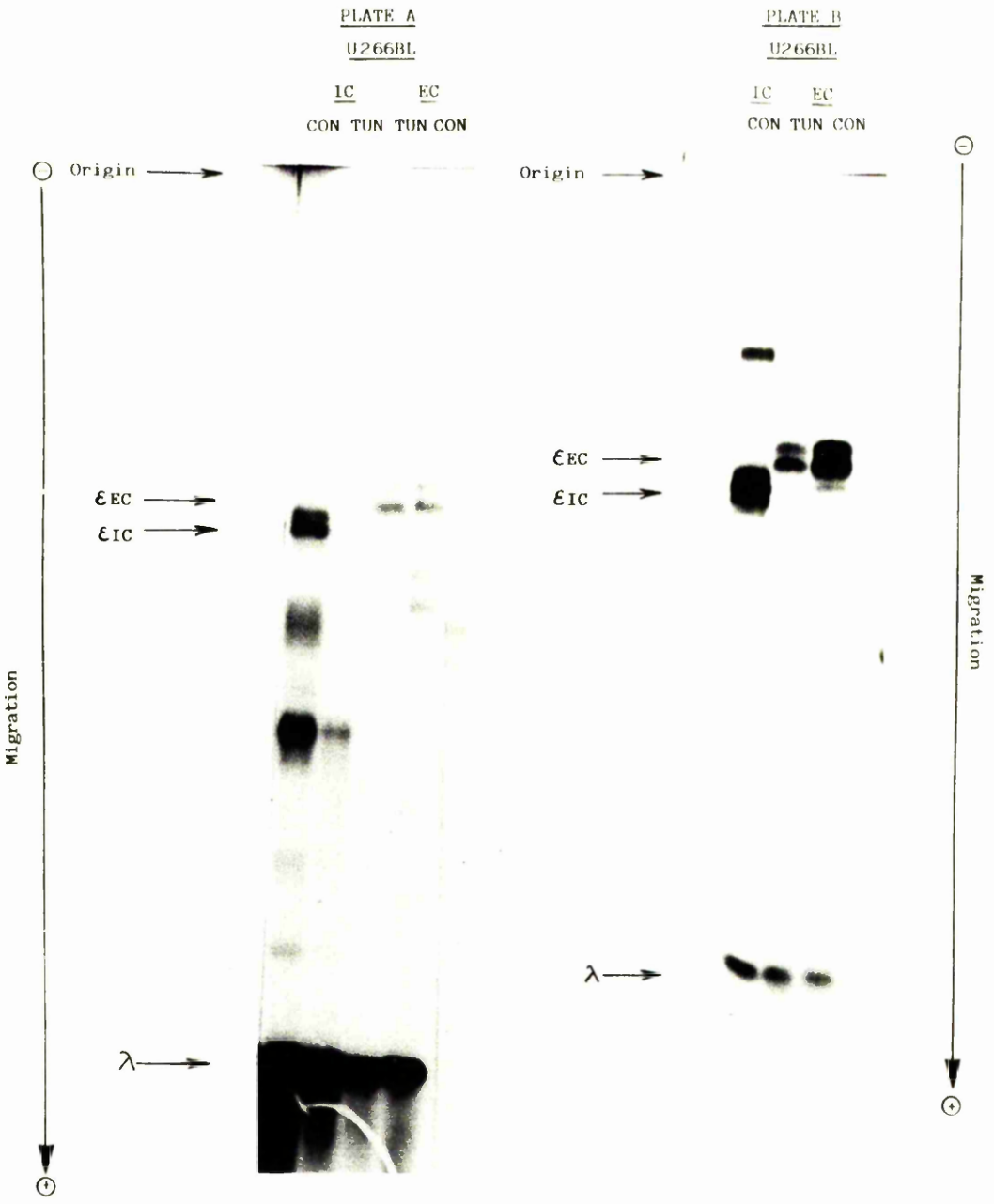


PLATE A
U266BL
IC EC
CON TUN TUN CON
Origin
εEC
εIC
λ
Migration
PLATE B
U266BL
IC EC
CON TUN CON
Origin
εEC
εIC
λ
Migration

N-linked oligosaccharides from the ε-chain secreted from the latter cells. This is clearly not the case (figure 1.10, panel B). Hence, one must propose two alternative explanations:

a) U266BL cells contain an extremely large intracellular pool of dolichol phosphates which is not depleted even after 12 hours treatment with tunicamycin, or;

b) the post-synthetic machinery, postulated to be O-glycosylation in this instance, compensates for the absence of N-linked oligosaccharides by adding carbohydrate side chains to the N-glycosylation sites themselves, or to alternative sites, to render the ε-chain "fully glycosylated".

The extent of glycosylation of ε-chains secreted from tunicamycin-treated U266BL cells is currently being investigated. The nature of the data shown in figure 1.10. renders meaningful interpretation of the data difficult. The least controversial conclusion is one which proposes that the N-linked oligosaccharide moieties of ε-chains are not critical for secretion of IgE but that other glycosylation mechanisms may operate which provide oligosaccharide side chains capable of delivering the correct signal for secretion of IgE from U266BL cells.

1.4.3. Trends in Immunoglobulin Secretion and Possible Explanations

The data presented in this section demonstrates an apparently differential function of the N-linked oligosaccharides of human immunoglobulin heavy chains in the control of active secretion of assembled immunoglobulin molecules. The presence of

N-linked oligosaccharides was apparently mandatory for the efficient secretion of IgM and IgA but non-N-glycosylated IgG and, perhaps, IgE were efficiently secreted from cells synthesising these classes of immunoglobulin.

The data are in good agreement with the results of similar studies in other species. The requirement for glycosylation of μ-chains for efficient secretion of IgM appears to be universal: treatment of murine (Hickman and Kornfeld, 1978), human (this thesis) and rabbit lymphocytes (Williamson et al, unpublished) with tunicamycin results in almost total abrogation of IgM secretion. The data presented herein has not been rigorously quantitated, but it strongly argues in favour of the notion that IgM containing only non-glycosylated μ-chains can not be secreted from B-lymphocytes. Residual secretion of IgM from tunicamycin treated murine plasmacytoma cells (Hickman and Kornfeld, 1978) may be accounted for in terms of 'mixed' molecules which contain not only non-glycosylated μ- chains, but also one (or, in the case of pentamers, several) glycosylated μ-chain(s) which serves to deliver the necessary biochemical signal for secretion of the 'mixed' molecule: high resolution SDS-PAGE analysis of the secreted product would reveal apparently efficient secretion of IgM containing non-glycosylated μ-chains. The apparent exception to the stringency of requirement for N-glycosylation of μ-chains for efficient secretion is the cell line WEHl-279 (Sibley and Wagner, 1981). However, high resolution analysis of the secreted product was not performed and the arguements regarding secretion of 'mixed' molecules may be advanced to explain this data.

The finding that non-glycosylated IgM is not efficiently secreted from B-lymphocytes is almost unanimous. Equally ubiquitous is the observation that non-glycosylated IgG is efficiently secreted from tunicamycin-treated cells synthesising this immunoglobulin isotype. These findings are made not only in the human system, but also in the rabbit (Williamson et al, unpublished) and in the mouse (Hickman and Kornfeld, 1978). However, the most elegant demonstration of the differential role of N-linked oligosaccharides in secretion of IgM and IgG came from studies of a cloned hybridoma, X63/38C-13, which synthesises and secretes both IgM and IgG. Upon tunicamycin-treatment of the hybridoma, it was found that IgG was efficiently secreted in the non-glycosylated form whereas IgM was not (Blatt and Haimovich, 1981).

Comparatively little data is currently available on the role of N-linked carbohydrate moieties on the efficiency of secretion of IgA and IgE. However, with respect to secretion of IgA, the data presented above is in agreement with findings in the murine plasmacytoma MOPC 315; that is, non-glycosylated IgA is not efficiently secreted from tunicamycin-treated cells (Hickman and Wong-Yip, 1979). In the case of IgE, however, the data herein and that published for the rat plasmacytoma are in disagreement. Treatment of the rat plasmacytoma with tunicamycin resulted in inhibition of secretion of IgE from that cell line (Hickman et al, 1977). However, the human myeloma cell line studied here, U266BL, continues to secrete IgE even after prolonged culture in tunicamycin (see figure 1.10). The paradoxical finding of ε-chains of apparently identical AMW in supernates of control and tunicamycin-treated U266BL cells has been discussed (section 1.4.2.). It may be that U266BL cells possess alternative

glycosylation mechanisms which allow for addition of carbohydrate in the absence of normal N-glycosylation mechanisms: this may be a reflection of the state of differentiation of this cell. In the absence of such alternative glycosylation pathways, secretion of non-glycosylated IgE may be inhibited as has been demonstrated in the rat (Hickman et al, 1977). This would imply that oligosaccharide moieties, irrespective of the nature of their chemical linkage to the polypeptide chain, are a prerequisite for the efficient secretion of IgE.

Examination of carboxy-terminal sequences of human immunoglobulin heavy chains reveals that μ- and α- heavy chains possess an extension of 19 amino acids at their C-termini: such extensions are absent in ε and in all γ-subclasses. The amino acid sequences of the μ- and α-heavy chain C- terminal extensions are greater than 68% homologous. The penultimate C-terminal residue is a cysteine which is involved in inter-chain disulphide bonding between the subunits of polymeric immunoglobulins and J-chain. The asparagine residue 14 residues proximal to the C-terminus is part of the asn-x-ser/thr sequence necessary for N-glycosylation and is, indeed, glycosylated (Shimizu et al, 1971). However, it is interesting to speculate that this glycosylation site is critical in efficient transport and secretion of IgM and IgA. IgG and IgE lack such a site and are efficiently secreted without N-linked carbohydrate moieties at other sites on the molecule. Several possible functions could be envisaged for N-linked oligosaccharides located at the asparagine residue noted above:

a) enhanced solubility of the IgM molecule;

b) adoption of the correct configuration for polymerisation;

c) promotion of J-chain integration via glycosylation of J-chain or μ-chain, and;

d) a signal for transport of IgM through the membranous elements of the cell.

The data of Tartakoff and Vassalli (1979) would argue against alternatives b) and c) since their data indicates that the polymerisation of murine IgM and integration of J-chain occur in tunicamycin-treated cells, which does not favour a model based on carbohydrate-mediated control of conformation(s) necessary for polymerisation.

There are reports in the literature of carbohydrate having a role in the hydrophobicity of certain glycoproteins. In the case of human fibroblast interferon, inhibit of N-glycosylation by tunicamycin treatment of the fibroblasts inhibited neither secretion of interferon nor the biological activity of the molecule. However, there was an increase in hydrophobicity of the molecule (Mizrahi et al, 1978). In the case of a large molecule, such as IgM, the presence of carbohydrate moieties may critically influence the stability of the molecule in an aquaeous environment. This arguement would be applicable in more general terms; that is, the level of glycosylation of the entire immunoglobulin molecule may be important in determining its solubility.

In early studies of the mechanisms responsible for simultaneous expression of membrane and secretory forms of IgM a model was proposed in which the carbohydrate moieties of IgM played

a critical role in controlling the expression of such molecules (Vitetta and Uhr, 1975b). Although this model has now been shown to be inaccurate, the role of carbohydrate, particularly that moiety closest to the C-terminus of the μ-chain, in control of transport and secretion of polymeric immunoglobulins may still prove to be important.

The μ-chain is synthesised on membrane-bound polyribosomes, and the nascent chain is directed through the rough endoplasmic reticulum membranes by the signal sequence (see Introduction, section 3). Glycosylation then occurs either at the level of the nascent chain (Moroz and Uhr, 1967) or during transport. A fully glycosylated secretory human μ-chain contains five N-linked carbohydrate side-chains, the two closest to the C-terminus being simple units and the remainder being complex oligosaccharides (Shimizu et al, 1971): membrane μ-chain has only four N-glycosidically linked prosthetic groups. Both μ-chain forms will be synthesised on polyribosomes and transferred through the rough endoplasmic reticulum: N-glycosylation of asparagine 563 of the secretory μ-chain may be the biochemical signal required for release of the secretory μ-chain into the cisternal space or into a secretory vesicle. This event requires a single chemical reaction: the addition of a preformed 'core' oligosaccharide to asparagine 563 from the dolichol carrier. A similar arguement, involving the N-glycosylation of asparagine 483, may be invoked as a signal for release of secretory α-chains from the polyribosomes. Formation of "non-cyclic" pentamers of IgM could still occur via the interchain disulphide bonds between adjacent $C_\mu 3$ domains (Putnam et al, 1973). The binding of J-chain is, however, more difficult to account for if a carbohydrate-mediated

signal is necessary to release the IgM from the polyribosome, since the relevant cysteine residue may be subject to steric hinderance due to its proximity to the rough endoplasmic reticulum membrane or membranes of other sub-cellular compartments.

The above hypothesis pertains only to IgM and possibly IgA secretion. Equivalent sites are absent from human IgG (the closest N-glycosylation site is 55 residues from the C-terminus) and also from IgE (no glycosylation sites in $C_\varepsilon 4$). A different explanation must therefore be found to account for the apparent failure to secrete IgE from tunicamycin-treated rat plasmacytoma cells (Hickman et al, 1977).

One possible explanation is that the amount of carbohydrate present on the rat ε-chain is related to its solubility; absence of N-linked oligosaccharides may render the molecule unstable in aquaeous solution (Hickman and Kornfeld, 1978). Such a model is also relevant to the data described in the above experiments. The human secretory immunoglobulin heavy chains have varying amounts of carbohydrate:

Heavy Chain	Number of N-linked Carbohydrate Side Chains	
μ	5	(Shimuzi et al, 1971)
α	3	(Turner, 1977)
ε	6	(Turner, 1977)
γ	1	(Turner, 1977)

According to the Hickman and Kornfeld model, which proposes that heavily-glycosylated glycoproteins are less soluble in the non-glycosylated state, human IgG would be efficiently secreted in the non-glycosylated state whereas non-glycosylated IgM, IgA or IgE

would not be efficiently secreted. The data of this section is in agreement with such a model, with the exception of the observation of the apparent secretion of non-glycosylated IgE. This anomaly may be due to large intracellular pools of dolichol phosphates in U266BL cells or O-glycosylation of the ε-chain (see section 1.4.2. above).

1.5. MEMBRANE DEPOSITION OF IMMUNOGLOBULINS AND MAJOR HISTOCOMPATABILITY COMPLEX GENE PRODUCTS

1.5.1. Experimental Approaches

Three major experimental regimes were employed to investigate the influence of N-linked carbohydrate moieties in localisation of immunoglobulins and HLA-A,-B,-C and -DR alloantigens into the cell membrane:

a) cell membrane proteins were removed by tryptic digestion and the cell allowed to synthesise antigens de novo in the presence or absence of tunicamycin. Expression of newly synthesised proteins at the cell membrane was determined by radioimmunoassay using monoclonal antibodies or human alloantisera;

b) cells were cultured for prolonged periods in tunicamycin prior to radioiodination of cell membrane proteins. Membrane proteins were isolated by immunoprecipitation and analysed by SDS-PAGE, and;

c) cells were exposed to a large amount of radioactivity for a short period and the fate of immunoglobulins

labelled during this short pulse was determined by analysis of membrane proteins by immunoprecipitation at various times during a pulse-chase in the presence of cycloheximide.

1.5.2. Radioimmunoassay

As a preliminary attempt to assess the functional role of N-linked oligosaccharides in the efficient expression of immunoglobulins and MHC gene products at the cell membrane large cultures of cells were subjected to treatment with trypsin and allowed to resynthesise and, if possible, re-express immunoglobulins and MHC gene products at the cell membrane. The experimental strategy is open to criticism on several grounds (for example, loss of cell viability, and incomplete removal of endogenous membrane proteins) but the data obtained from this approach provided tentative evidence that immunoglobulin and MHC products were expressed at the cell membrane of tunicamycin-treated cells.

The cell line Daudi was selected as the model system for the study of expression of HLA-DR alloantigens and IgM because of the lack of HLA-A,-B,-C alloantigens at the Daudi cell membrane (Jones et al, 1975) and because of the high level of membrane IgM expression on Daudi cells (Klein et al, 1968).

Analysis of the level of re-expression of HLA-DR alloantigens on the membranes of control or tunicamycin-treated cultures of Daudi cells was performed using a monoclonal antibody directed against the β-subunit of the HLA-DR alloantigen molecule and ^{125}I-rabbit anti-mouse immunoglobulin ($F(ab)_2$ fraction). The data shown in figure 1.11A. illustrate that HLA-DR alloantigens could be

FIGURE 1.11.

Membrane Deposition of Non-Glycosylated Proteins

5×10^7 cells from log phase cultures were treated with 2 µg/ml tunicamycin for 3 hours prior to treatment with trypsin in the presence of DNAse 1 (Methods, 15). Cells were washed extensively and cultured at 37°C for 16 hours at a concentration of 10^6 cells/ml of RPMI-1640 supplemented with 20% (v/v) FCS and 2 µg/ml tunicamycin. Parallel control cultures were also prepared.

For radioimmunoassay, 10^6 cells in 100 µl RPMI-gelatin were added to individual wells of a BSA-treated 96-well microtitre plate, each well containing 25 µl of the desired antiserum (Methods, 16). After a 1 hour incubation at room temperature, the cells were washed three times with RPMI-gelatin prior to addition of approximately 3×10^4 cpm of ^{125}I-rabbit anti-mouse IgG ($F(ab)_2$ fraction). After a 1 hour incubation at room temperature, the cells were washed four times with RPMI-gelatin and the plate dried. Individual wells of the plate were assayed for ^{125}I in the gamma counter (Methods 17.3.).

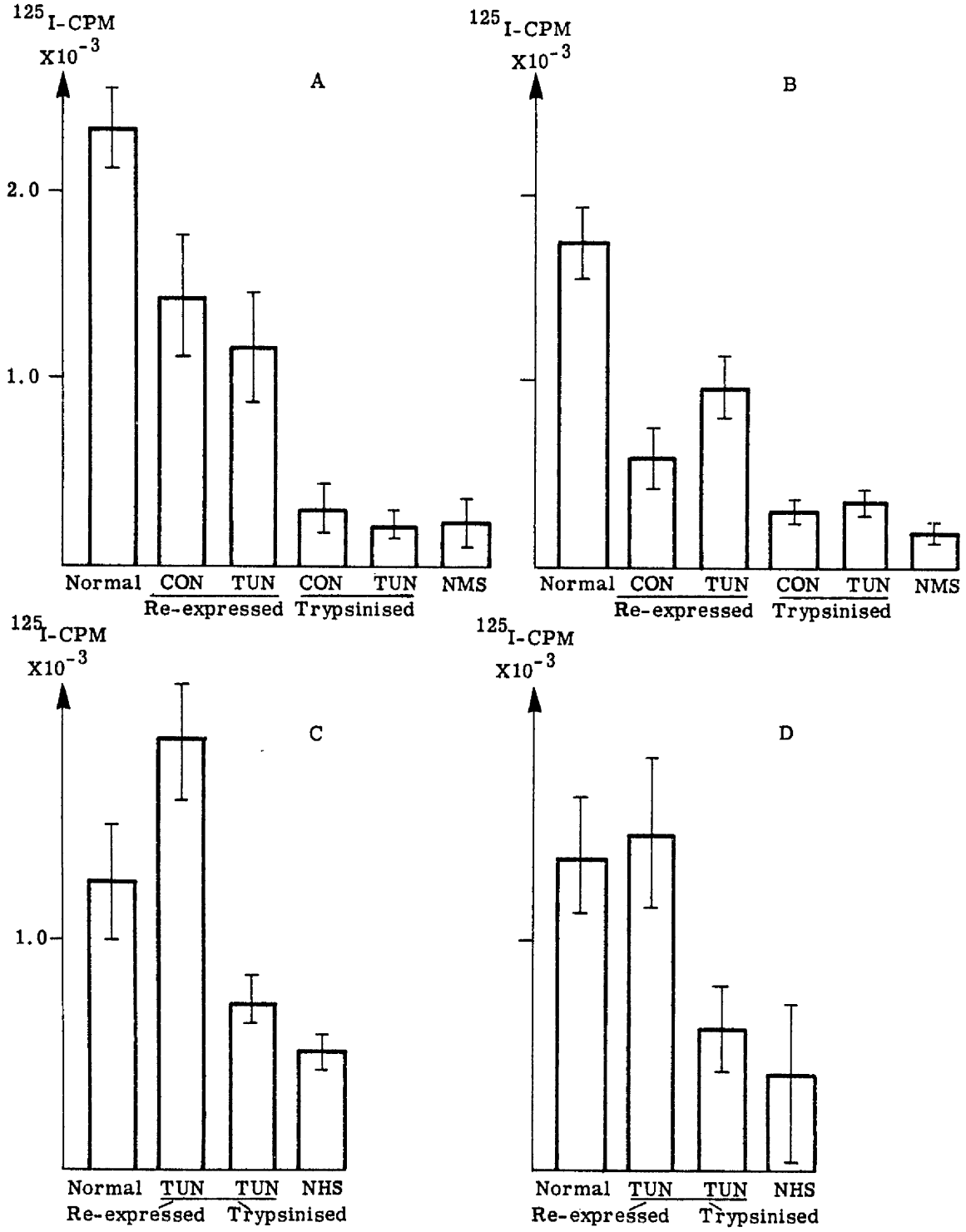
Key:-

A - Daudi cells, assayed with monoclonal anti-HLA-DR β-chain.

B - Daudi cells, assayed with monoclonal anti-κ.

C - Bri-8 cells, assayed with anti-HLA-B7 alloantiserum.

D - Mich cells, assayed with anti-HLA-A2 alloantiserum.


125I-CPM
X10-3
A
2.0
1.0
Normal
CON
TUN
CON
TUN
NMS
Re-expressed
Trypsinised
125I-CPM
X10-3
B
Normal
CON
TUN
CON
TUN
NMS
Re-expressed
Trypsinised
125I-CPM
X10-3
C
1.0
Normal
TUN
TUN
NHS
Re-expressed
Trypsinised
125I-CPM
X10-3
D
Normal
TUN
TUN
NHS
Re-expressed
Trypsinised

detected at the cell membranes of control and tunicamycin-treated cultures. The level of detectable HLA-DR alloantigens was, in each case, statistically significantly different from the level of detectable HLA-DR on cells assayed immediately after trypsin treatment ($p<0.05$) and significantly different from the normal mouse serum control ($p<0.05$). It can be inferred from these data that HLA-DR alloantigens may be expressed at the cell membrane even if they lack N-linked oligosaccharide side chains. However, the level of expression of HLA-DR on both control and tunicamycin-treated Daudi cells after trypsin treatment and re-expression was significantly different ($p<0.05$) from the amount of HLA-DR detectable on Daudi cells assayed immediately after harvesting from normal culture. This may reflect the harshness of the procedures used in this experimental regime and indicates that the cells, although viable, may be severely debilitated after exposure to trypsin and tunicamycin.

In an equivalent experiment to examine membrane IgM expression in the presence or absence of tunicamycin, a monoclonal antibody to human kappa chain was used as probe. The data from this experiment were not as conclusive as those obtained for expression of glycosylated and non-glycosylated HLA-DR antigens. A degree of re-expression on both control and tunicamycin-treated cells was evident (figure 1.11B.) and was significantly different from the normal mouse serum control ($p<0.05$) in both cases. However, the level of detectable IgM on control cells after re-expression was not significantly different from the level of IgM detectable on control cells immediately after trypsin treatment. The corresponding difference in the tunicamycin-treated culture was, however, statistically significant

($p<0.05$). In both cultures, the level of re-expressed IgM is lower than that detected on freshly harvested cells and the difference is statistically significant ($p<0.05$). Thus, the data are consistent with the suggestion that non-glycosylated IgM may be expressed at the cell membrane. However, the degree of confidence which one can have in the data is not great and the need for a more rigorous experimental strategy is clear.

The expression of non-glycosylated HLA-A,-B, and -C antigens was also investigated by this approach. Human alloantisera in combination with ^{125}I- rabbit anti-human immunoglobulins (Fab_2 fraction) were used to detect the re-expressed alloantigens. The data are summarised in figures 1.11C. and 1.11.D. Briefly, the data suggest that in tunicamycin-treated Bri-8 cells and Mich cells the HLA-B7 and HLA-A2 molecules re-expressed by these cells were alloantigenic. In each case, the levels of detectable re-expressed HLA-B7 or HLA-A2 was statistically significantly different ($p<0.05$) from the amounts detected on cells assayed immediately after trypsin digestion or the normal human serum control. These data are subject to the same faults described above in the case of IgM re-expression and must be regarded with some scepticism. However, the data are exciting from the point of view that, if correct, they suggest that the N-linked olligosaccharide moiety linked to asparagine 86 of the HLA heavy chain not only plays no role in membrane deposition of the HLA-A,-B, -C antigens, but also has no influence on the alloantigenicity of HAL-A2 or HLA-B7 molecules, at least in a serological context. These findings are in agreement with recently published data (Ploegh et al, 1981) which elegantly demonstrate the assembly, membrane

deposition and alloantigenicity of non-glycosylated HLA-A2 and HLA-B7 molecules. An interesting question does remain to be answered regarding the functional integrity of a non-glycosylated HLA-A,-B, -C alloantigen as a target molecule for a cytotoxic T-lymphocyte.

In summary, the data from this experimental approach suggest that N-linked oligosaccharides have no role in the membrane deposition of IgM, HLA-DR and alloantigenic HLA-A2 and HLA-B7 molecules. The protocols used are extremely harsh and the experiments are consequently subject to a variety of errors. In an attempt to support the above observations, two new approaches were devised and these are described below.

1.5.3. Analysis of Membrane Proteins of Cells Cultured for Prolonged Periods in the Presence of Tunicamycin

The data presented in the previous section (1.5.2.) lead to the suggestion that non-glycosylated IgM could be expressed at the cell membrane. However, the methods employed led to extensive cell damage and tended to give rise to irreproducible results. It was necessary to adopt a new approach to test the hypothesis, arising from the above data, that N-linked oligosaccharides play no functional role in efficient transport of glycoproteins to the cell membrane.

Daudi and Dakiki Arosros-1 cells were cultured for 96 hours in the presence of 1μg/ml tunicamycin. At the end of the culture period the membrane proteins were radioiodinated by lactoperoxidase-catalysed iodination and aliquots of the radiolabelled proteins subjected to immunoprecipitation with anti-κ or anti-λ antisera plus GARIg. The immune precipitates were washed, reduced and analysed by SDS-PAGE. The data are presented in figure 1.12.

FIGURE 1.12.

Absence of Non-Glycosylated IgM and IgA from Membranes of Cells Cultured for Prolonged Period in Tunicamycin

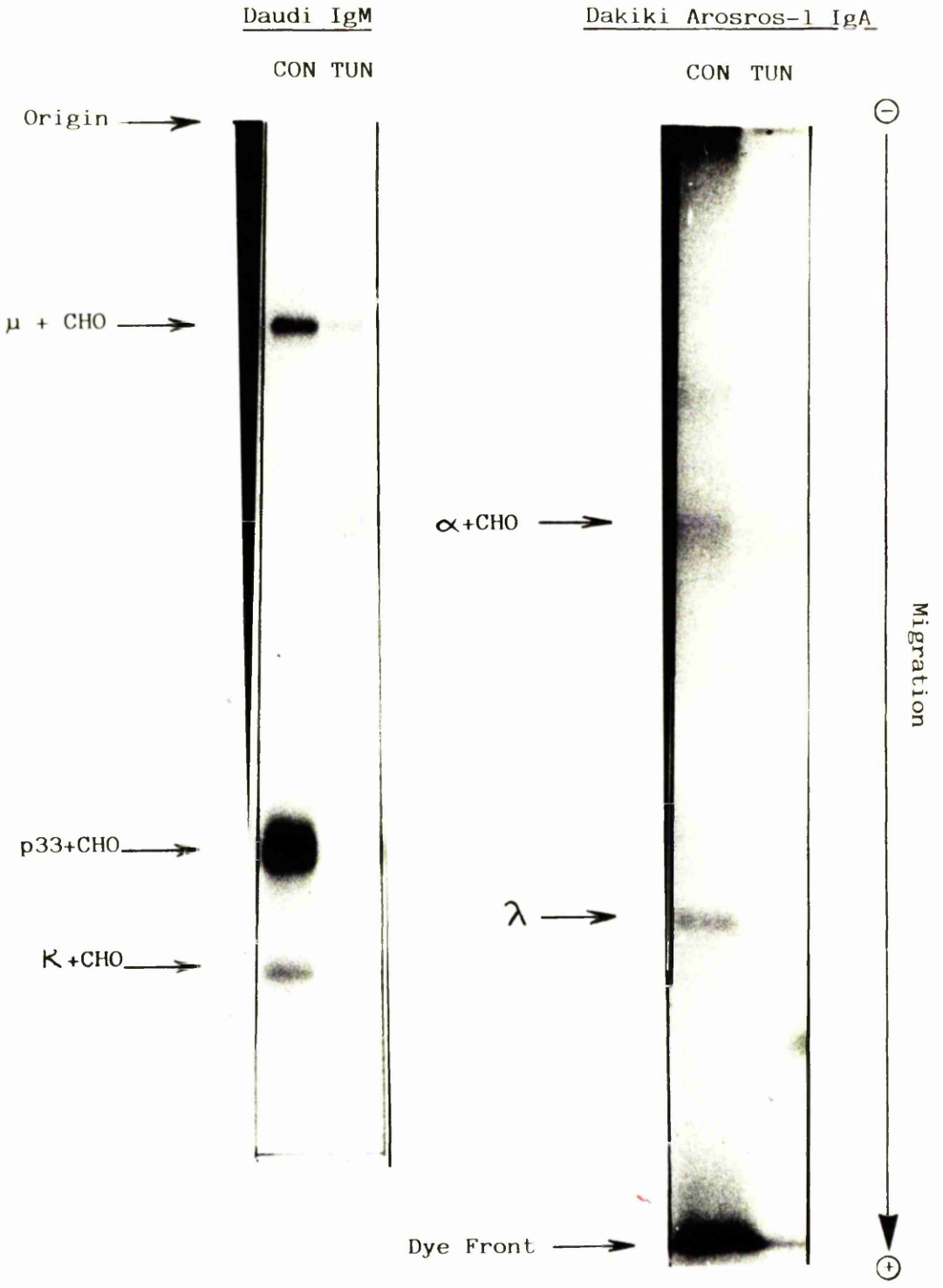
Daudi and Dakiki Arosros-1 cells were cultured in complete medium supplemented with 1 μg/ml tunicamycin for 96 hours, with a single change of medium. After culture 10^7 viable cells of each line were harvested from culture, washed twice with 25 ml aliquots of ice-cold serum-free medium and once with 25 ml ice-cold PBS-I. The membrane proteins of these cells were radioiodinated by lactoperoxidase-catalysed iodination using 1mCi of ^{131}I (Methods, 8.2.). Lysates were prepared in 250 μl 3D-TKM (Methods, 9.).

IgM and IgA were isolated by specific immunoprecipitation using 1 μl of either RAHμ or RAHα plus an equivalence of GARIg (Methods, 10.2.1.). Washed immune precipitates were dissolved in 25 μl reducing SDS-PAGE loading buffer and electrophoresed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The gel was processed for fluorography (Methods, 17.6.).

Key:-

Con - Membrane immunoglobulin of control cells.

Tun - Membrane immunoglobulin of tunicamycin-treated cells.


Daudi IgM
CON TUN
Origin
μ + CHO
p33+CHO
κ +CHO
Dakiki Arosros-1 IgA
CON TUN
α+CHO
λ
Dye Front
Migration

The SDS-PAGE profile obtained for Daudi membrane IgM was identical for material derived from either control, freshly harvested cells or from cells cultured for four days in tunicamycin; that is glycosylated μ, p33 and κ-chains were demonstrable in both profiles and non-glycosylated chains were not observed. In these experiments ^{131}I was used in a deliberate attempt to identify minor membrane components and, therefore, small quantities of non-glycosylated μ-chain, but non-glycosylated μ-chains were never observed. An identical result was obtained with the IgM producing B-lymphoma Bjab (data not illustrated).

An identical experiment was performed using the IgA producing cell line Dakiki Arosros-1. In these experiments, no non-glycosylated α-chains were observed in the SDS-PAGE fluorographic patterns of IgA immunoprecipitated from either control or tunicamycin-treated cells.

In both cell lines the only immunoglobulin components detectable on the membranes of tunicamycin-treated cells were glycosylated μ- or α-heavy chains and light chains. The amount of these components which were specifically immunoprecipitated from tunicamycin-treated cells was very low compared to the amount isolated from the freshly harvested cells. This was not unexpected as, in the design of the experiment a half life of 24 hours (Vitetta and Uhr, 1975b) was assumed for membrane immunoglobulin, so that after 96 hours only 6.25% of the original immunoglobulin would remain and any newly-synthesised material would be the predominant molecular species on the cell membrane. The finding of low amounts of glycosylated immunoglobulin components on the cell membranes of cells exposed to

tunicamycin for prolonged periods is consistent with the above assumption. The data from these experiments are inconsistent with the hypothesis that the N-linked carbohydrate moieties of immunoglobulins are not important in the membrane localisation of these proteins. The data suggest that the N-linked oligosaccharides of immunoglobulins are necessary for efficient membrane deposition of IgM and IgA. However, the possibility that the failure of the cells to insert immunoglobulins onto the cell membrane reflects a toxic effect of the tunicamycin rather than absence of N-linked carbohydrate on the immunoglobulins can not be excluded. Therefore, it is again necessary to attempt to find a less ambiguous experimental regimen to explore the role of N-glycosylation of immunoglobulins in efficient membrane insertion of these macromolecules.

1.5.4. Immune Precipitation of Biosynthetically Labelled Immunoglobulin Inserted into the Cell Membrane

The experimental protocols hitherto employed to attempt to demonstrate non-glycosylated immunoglobulin on the cell membrane have yielded conflicting results. Furthermore, these strategies necessitated harsh treatment of the cells either by trypsin digestion and/or by prolonged exposure to tunicamycin, both procedures leading to loss of large quantities of cells. In a final attempt to unequivocally answer the question "Are non-N-glycosylated immunoglobulins efficiently localised into the cell membrane?", the following protocol was adopted. Cells were exposed to tunicamycin for a short period of time prior to labelling with a large amount of radioactive amino acid for 30 minutes. Cycloheximide was then added and aliquots removed at various intervals. The cells were treated with antibody, washed extensively, and cell

FIGURE 1.13.

Inhibition of Membrane Insertion of Non-Glycosylated IgM

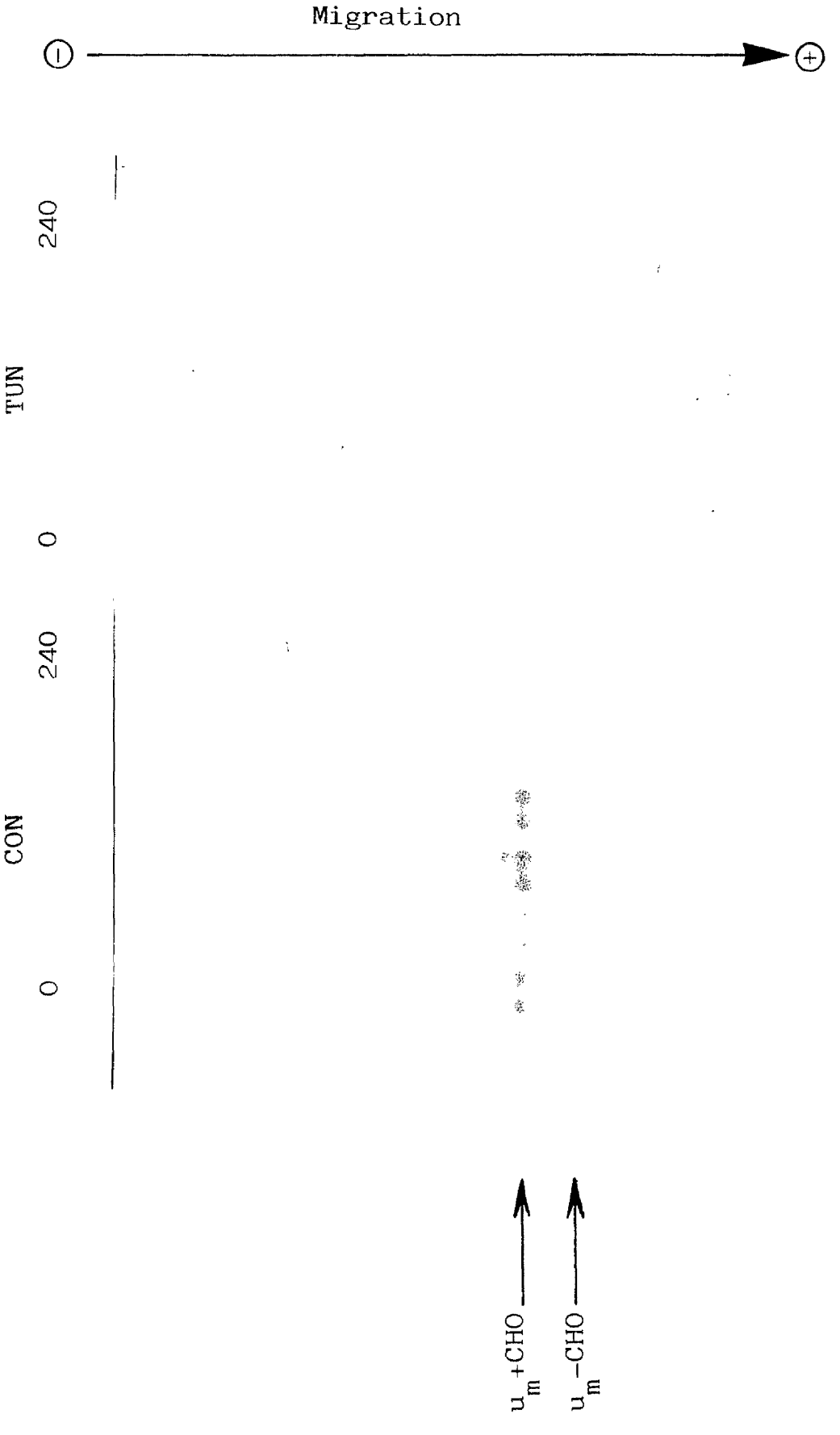
10^7 Bjab cells were treated with 2 µg/ml tunicamycin in complete medium for 3 hours at 37°C. Cells were then washed twice with labelling medium prior to resuspension in 0.1 ml of the same medium supplemented with 0.5 mCi ^{35}S-methionine. Incorporation was allowed to proceed at 37°C for 30 minutes whereupon 1 ml of complete medium containing 20 µg/ml cycloheximide was added to the culture (time 0). The culture was reincubated at 37°C, and 0.1 ml aliquots removed at time 0, 30, 60, 120, 180 and 240 minutes. Control cultures were prepared simultaneously.

The cells were washed twice in PBS containing 0.01% (w/v) azide (PBS-azide) and then treated with 25 µl of a 1:10 dilution of RAHµ in PBS-azide for 30 minutes at room temperature. The cells were washed three times with PBS-azide, and lysates prepared in 100 µl 3D-TKM (Methods, 9.). 50 µl of 10% (v/v) S.aureus in 3D-TKM was added to each lysate and the mixtures incubated overnight at 4°C. The bacteria were washed three times in 3D-TKM and bound immune complexes eluted by boiling in 2% (w/v) SDS. The bacteria were pelleted, and the supernates treated with 5 volumes of acetone to precipitate protein. The precipitates were dissolved in SDS-PAGE loading buffer, boiled, and analysed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The pattern of radioactive components was visualised by fluorography (Methods, 17.6.).

Key:

Con - Membrane IgM of control Bjab cells.

Tun - Membrane IgM of tunicamycin-treated Bjab cells.


Migration
⊖
⊕
TUN
240
0
CON
240
0
u_m +CHO
u_m −CHO

lysates prepared. S.aureus was added to immunoprecipitate the immune complexes. This approach has several attractive features:

a) the cells are not subjected to excessive handling or enzymatic treatment prior to analysis;

b) the exposure to tunicamycin is relatively short, and;

c) kinetic effects, if any, due to absence of N-linked oligosaccharides may be revealed.

Such an experiment was performed on Bjab cells, and the data is illustrated in figure 1.13. In control cells, IgM could be precipitated from the cell membrane immediately after the end of the labelling period, and was easily isolated from aliquots of cells removed throughout a 4-hour chase in the presence of cycloheximide. By contrast, no radiolabelled immunoglobulin could be immunoprecipitated from the cell membranes of tunicamycin-treated Bjab cells at any time during the chase period. This is good evidence that IgM containing non-glycosylated μ-chains is not as efficiently inserted into the cell membrane as its glycosylated counterpart. A longer chase period would be needed to confirm or refute the hypothesis that IgM containing non-glycosylated μ-chains is never localised on the cell membrane.

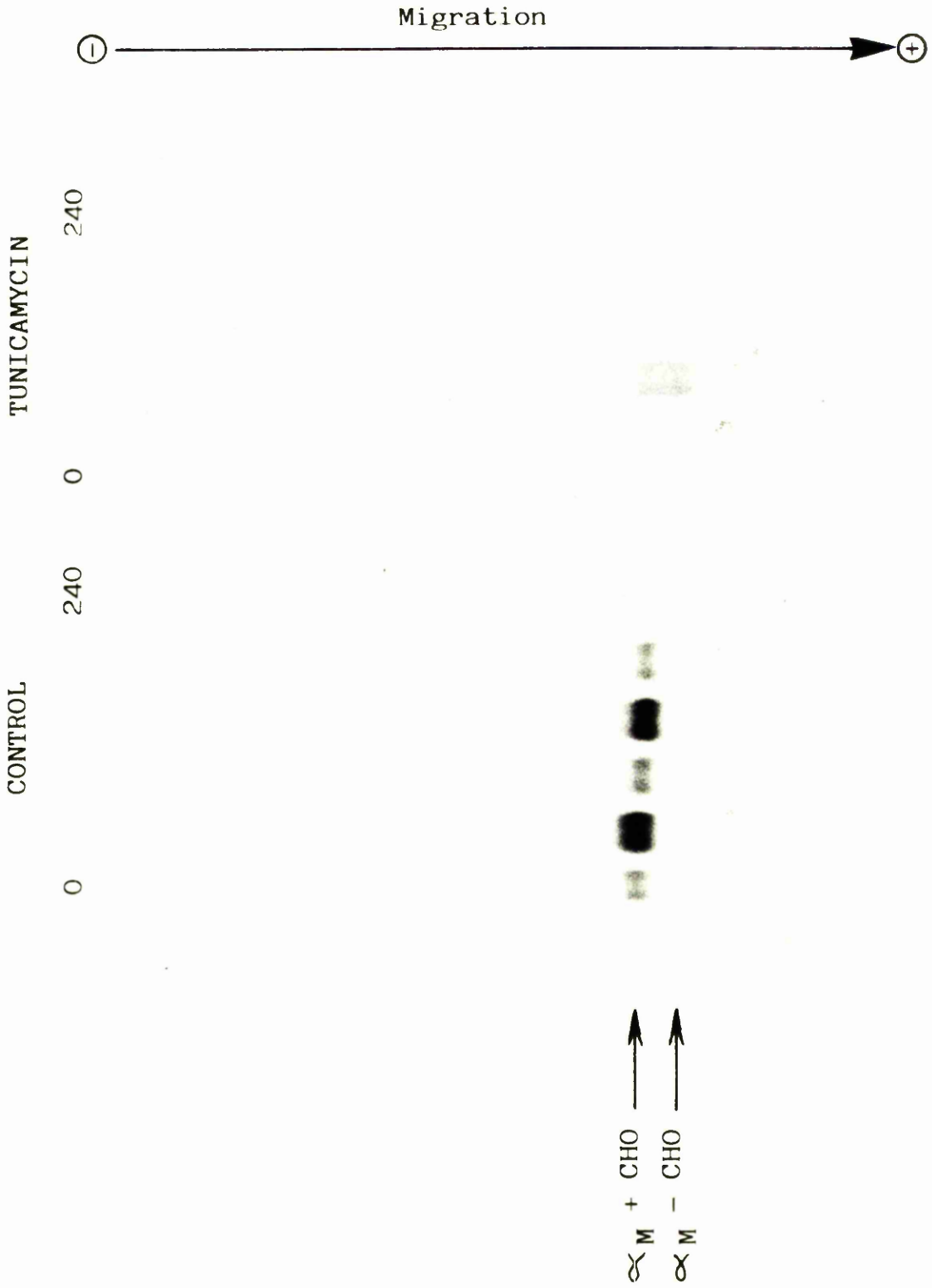
Non-glycosylated α-chains could, however, be precipitated from the membranes of tunicamycin-treated Dakiki Arosros-1 cells (figure 1.14.). In control cells, radiolabelled glycosylated α-chains were specifically immunoprecipitable from Dakiki Arosros-1 cell membranes throughout the chase period. In tunicamycin-treated Dakiki Arosros-1 cells, radiolabelled glycosylated and non-glycosylated α-chains were immunoprecipitable from cell membranes. In this

FIGURE 1.14.

Inhibition of Membrane Insertion of Non-Glycosylated IgA

10^7 Dakiki Arosros-1 cells were treated for 3 hours at $37^{\circ}C$ with 2 µg/ml tunicamycin in complete medium prior to washing in labelling medium and labelling with 500 µCi ^{35}S-methionine for 30 minutes at $37^{\circ}C$. 1 ml complete medium containing 20 µg/ml cycloheximide was then added to the culture (time 0) which was re-incubated at $37^{\circ}C$. 0.1 ml aliquots were removed at times 0,30,60, 120,180 and 240 minutes. Control cultures were prepared simultaneously.

Cells were washed twice in PBS-azide and treated with 25 µl of a 1:10 dilution of RAHα for 30 minutes at room temperature. The cells were washed thrice with PBS-azide and lysates prepared in 100 µl 3D-TKM (Methods, 9.). 50 µl of 10% (v/v) S.aureus in 3D-TKM was added to each lysate and the mixtures incubated overnight at $4^{\circ}C$. The bacteria were washed three times in 3D-TKM and bound immune complexes eluted by boiling the washed bacteria for 2 minutes in 2% (w/v) SDS. The bacteria were pelleted and the supernate removed and treated with five volumes of acetone. The resulting precipitate was dissolved in 25 µl reducing SDS-PAGE sample buffer, boiled, and analysed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The gel was processed for fluorography (Methods, 17.6.).


Migration
⊖
⊕
TUNICAMYCIN
240
0
CONTROL
240
0
+ CHO
- CHO
M
M

case it is believed that the non-glycosylated α-chain appeared on the cell membrane only by virtue of its association with a glycosylated α-chain. Therefore, by an arguement similar to that advanced for "secretion" of non-glycosylated μ-chains (section 1.4.1.), the presence of a minimal number of N-linked oligosaccharide chains on a given molecule may be sufficient to allow a 'mixed' molecule to be transported through the cell to its final destination. The notion that totally non-glycosylated IgA would not be inserted into the cell membrane could be easily tested by exposing cells to tunicamycin for a slightly longer time prior to labelling. Based on the above arguement and upon the data of figure 1.12. it is reasonable to propose that the above hypothesis is correct.

1.5.5. Comparative Role of N-Linked Oligosaccharides in Membrane Deposition of Immunoglobulins and MHC Gene Products

The N-linked oligosaccharide moieties of membrane immunoglobulins and of MHC gene products appear to have different functional significances in transport of these macromolecules to the cell membrane. The data presented here suggests that N-glycosylation of the immunoglobulin heavy chain is necessary for the efficient membrane deposition of the completed immunoglobulin, while the carbohydrate side chains appear to be unnecessary for membrane localisation of HLA-A,-B,-C and HLA-DR antigens.

As regards membrane deposition of immunoglobulins, the present findings made by radioimmunoassay of cells which had been treated with trypsin and allowed to resynthesise and re-express antigens in the presence of tunicamycin agree with those of other workers: that is, N-linked oligosaccharides of immunoglobulin heavy

chains do not influence membrane deposition of these macromolecules. However, more rigorous analysis by SDS-PAGE of specifically immunoprecipitated material derived from:

a) cells subjected to prolonged culture in tunicamycin, or;

b) immunoglobulins 'chased' onto the cell membrane of actively synthesising cells,

were at variance with published data. The data derived from the 'pulse-chase' experimental strategy is probably the most reliable of the three methods used, although the data for HLA-A,-B,-C antigen expression has been vindicated by the work of other investigators (Ploegh et al, 1981).

In the case of expression of non-glycosylated IgM, it was found that the presence of N-linked oligosaccharides on the μ-chain was required for efficient membrane insertion. The method of culturing cells for prolonged periods followed by SDS-PAGE analysis of specifically immunoprecipitated, radioiodinated membrane protein has been employed previously and yielded the same result (Singer, 1978). The length of culture period was extended in the experiments described herein from 8 hours (Singer, 1978) to 96 hours (figure 1.12). The finding of only small quantities of glycosylated IgM, and no non-glycosylated IgM, supports the findings and conclusions of Singer (1978); that is, non-glycosylated IgM is not inserted into the plasma membrane of B-lymphocytes.

One group of workers has recently claimed that non-glycosylated IgM can be demonstrated at the cell membrane of tunicamycin-treated murine plasmacytoma cells (Sibley and Wagner, 1981).

However, two major criticisms of their data are possible;

a) no pre-incubation period in tunicamycin was employed prior to allowing cells to replace antigens removed by trypsin, and;

b) no analysis, other than binding of fluorescent antibody, was performed on the immunoglobulins which were claimed to be on the membrane of the cell.

The data are, of course, also subject to the same criticisms which apply to the radioimmunoassay data reported in this thesis. The pre-incubation of cells with tunicamycin is a critical step in any experiment of the kind quoted above. Sibley and Wagner (1981) may have succeeded in removing all IgM from the membranes of the cells, but failure to exhaust endogenous dolichol phosphate pools would result in continuation of glycosylation for some time with the resultant appearance of IgM (glycosylated) at the membrane of tunicamycin-treated cells.

The pulse-chase data presented herein demonstrate that cells pre-incubated with the antibiotic, pulsed, and allowed to express immunoglobulins fail to deposit IgM on their cell surfaces. These cells have not been subjected to trypsinisation or prolonged exposure to tunicamycin, but are viable, actively-synthesising cells. The conclusion from this experiment is that non-glycosylated μ-chains are incapable of being transported onto the cell membrane.

With respect to IgA expression, prolonged culture in tunicamycin followed by high resolution SDS-PAGE analysis of membrane proteins, and pulse-chase experiments demonstrated that non-glycosylated IgA could not be found on the cell membrane of tunicamycin-treated

cells. In pulse-chase experiments, glycosylated and non-glycosylated α-chains were detected on the membrane, but this is believed to be due to incomplete exhaustion of dolichol phosphate pools within the cell and bears directly on the criticisms of the claims of other groups to be able to detect non-glycosylated IgM (Sibley and Wagner, 1981) and IgA (Hickman and Wong-Yip, 1979) on the cell membrane of tunicamycin-treated cells. It is proposed that IgA containing solely non-glycosylated α-chains would not be expressed at the cell membrane.

The data obtained herein by pulse-chase experiments may be questioned on the grounds of the length of chase period, four hours. This may be thought to be too short, giving rise to a false negative if lack of carbohydrate caused only a kinetic effect rather than a total inhibition of expression. The data from the experiments analysing the membrane proteins of cells cultured for 96 hours in tunicamycin tend to argue against these criticisms since no non-glycosylated μ- or α-chains were found in SDS-PAGE profiles of radioiodinated immunoglobulins specifically immunoprecipitated from such cells. The data is entirely consistent with the hypothesis that N-linked oligosaccharide prosthetic groups are a mandatory requirement for efficient membrane localisation of human IgM and IgA: it is proposed that this requirement will also apply to membrane expression of IgG, IgD and IgE. Experiments are in progress to test this proposal.

While the data for immunoglobulins suggests a crucial role for carbohydrate in transport of proteins to the cell membrane, the radioimmunoassay data (however controversial) suggests that the

opposite is true for gene products of the HLA complex. The data suggested that non-glycosylated HLA-A,-B and -C antigens could be efficiently expressed on the membrane of tunicamycin-treated cells and, moreover, that the HLA-A2 and HLA-B7 alloantigens were serologically alloreactive. Similar findings have been made by a more rigorous pulse-chase approach by several workers (Ploegh et al, 1981; Owen, 1981) and immunoprecipitation data supports the finding that non-glycosylated HLA-A,-B and -C antigens are serologically alloreactive (Ploegh et al, 1981). Similar experiments on murine class 1 MHC antigens (H-2K, H-2D, H-2L and H-2R) suggest that these molecules are efficiently inserted into the plasma membrane in the non-glycosylated state (M.J. Owen, personal communication; Black et al, 1981). Indeed, it appears that β_2 microglobulin is of greater importance in the efficient membrane localisation and serological activity of class 1 molecules than carbohydrate (Ploegh et al, 1979; Ploegh et al, 1981a).

The appearance of non-glycosylated HLA-A,-B,-C and H-2K molecules at the cell membrane raises interesting questions regarding the immunobiological functional capacity of these molecules. H-2K and other H-2 class 1 molecules are known to serve as target molecules for H-2-restricted cytotoxic T-lymphocytes (Doherty et al, 1976), and HLA-A,-B and -C antigens perform the corresponding function in humans (McMichael et al, 1977). The data presented herein and that of other groups (Ploegh et al, 1981) suggest that the non-glycosylated HLA-A, -B and -C antigens are serologically alloantigenically active. However, it is not clear if these molecules will be functionally active as target molecules for cytotoxic T-lymphocytes. Experiments to

test the functional integrity of non-glycosylated H-2 class 1 molecules as target molecules for killer T-cells have recently been reported. The data suggest that the efficiency of killing of virally-infected target cells by virus-specific, H-2 restricted cytotoxic T-lymphocytes was inhibited by up to 50% if the target cell expressed non-glycosylated H-2 antigens (Black et al, 1981). These data suggest that the genetic determinants recognised by B-cells (antibody) and by T-cells (killer cells) are different: the serologically defined alloantigenic determinant does not depend upon N-glycosylation whereas the expression of the determinant recognised by cytotoxic cells seems to depend on the presence of oligosaccharide side chains upon the class 1 molecule. In a different system, it was demonstrated that carbohydrate was needed on target molecules for efficient killing of targets by H-2 restricted cytotoxic T-lymphocytes specific for the sex-linked transplantation antigen H-Y (Shapiro and Erickson, 1981).

The radioimmunoassay data also suggested that non-glycosylated HLA-DR antigens were expressed at the cell membrane. This data has yet to be corroborated by pulse-chase experiments but if accurate, several interesting biological questions may be asked. Firstly, are non-glycosylated HLA-DR molecules capable of stimulating the mixed lymphocyte reaction? A similar question could be asked of murine Ia antigens if these antigens are expressed at the cell membrane in the non-glycosylated state. Secondly, one may also investigate the possibility that the serologically defined HLA-DR allospecificities are distinct from the determinants defined by cellular reactions, as has been suggested by analysis of class 1 molecules.

Available data illustrates that the N-linked oligosaccharides of glycoproteins are of different degrees of importance in intracellular transport and in expression of glycoproteins. In many cases the requirement for glycosylation of a given protein for efficient expression appears to be mandatory. N-linked oligosaccharides may serve to protect proteins from proteolytic degradation thus ensuring the efficient transport and expression of the mature molecules (Schwartz et al, 1976). An interesting question remains to be answered. That is, does the cellular machinery involved in transport and ultimate expression of glycoproteins recognise the oligosaccharide moieties themselves, or is the recognition unit a complex three-dimensional structure, controlled by the presence of the oligosaccharides, but dependent more on higher protein structure than on carbohydrate?

CHAPTER 2

BIOSYNTHESIS OF STRUCTURALLY DISTINCT MEMBRANE AND SECRETORY FORMS OF IMMUNOGLOBULIN HEAVY CHAINS IN IgG AND IgA PRODUCING HUMAN B-LYMPHOCYTES

2.1. RATIONALE FOR INVESTIGATION

The existence of structurally distinct membrane and secretory forms of μ-heavy chain in a single B-lymphocyte is now well established in murine (Melcher and Uhr, 1976; Bergman and Haimovich, 1978) and human (Singer et al, 1980(a); Singer and Williamson, 1980) lymphocytes. Studies at the RNA (Singer et al, 1980a; Rogers et al, 1980; Alt et al, 1980) and the DNA levels (Early et al, 1980) have demonstrated that the membrane and secretory μ-heavy chain polypeptides are encoded by separate mRNA species, and that the two mRNA species are generated by RNA splicing of a primary nuclear RNA transcript of a single gene.

IgM-producing cells are representative of primary or early B-cells. The Clonal Selection Hypothesis predicts that during antigen-driven B-lymphocyte differentiation, daughter cells will arise which will have altered the heavy chain isotype which they synthesise, but that the idiotype expressed will be identical to the original IgM-producing cell. Bearing in mind the precedents of the IgM system it is of interest to ask the question "do memory B-lymphocytes synthesise structurally distinct receptor and effector forms of the heavy chain isotype (γ,α or ε) to which they are committed?". Some preliminary data is available showing that membrane γ-chains have a higher AMW than their secretory counterparts in human (Singer and Williamson, 1980) mouse (Oi et al, 1980 and chicken lymphocytes (Lifter et al, 1980). In the following studies, biosynthesis of IgG and IgA were studied in established human B-cell lines.

2.2. MODEL SYSTEMS

2.2.1. IgG Biosynthesis: Bec-11 Cells

Bec-11 cells are B-lymphoblastoid cells established by *in vitro* transformation of human tonsillar lymphocytes by Epstein-Barr virus. The line was established by Searle Laboratories, High Wycombe, England. The cells express HLA-A,-B,-C antigens (Dick *et al*, 1975) and HLA-DR antigens. Although originally believed to express IgD on the cell membrane (Singer *et al*, 1976), Bec-11 cells are now known to express IgG on the cell membrane and to secrete IgG into the culture supernate: two gamma chains are found in such supernatants as judged by SDS-PAGE under reducing conditions (Singer and Williamson, 1980). The cells express a kappa light chain.

2.2.2. IgA Biosynthesis: Dakiki Arosros-1 Cells

Dakiki Arosros-1 cells were established in continuous culture by *in vitro* transformation with Epstein-Barr virus of peripheral blood or cervical lymph node lymphocytes from an African patient suffering from nasophoryngeal carcinoma (Steinitz and Klein, 1980). Dakiki Arosros-1 cells were shown to express IgA on their cell membrane with a lambda light chain. Two other clones derived at the same time, Dakiki Arosros-2 and 3 were shown to express IgM (κ) and IgA (λ) respectively on their cell membranes (Steinitz and Klein, 1980). Dakiki Arosros-1 cells were shown to secrete IgA (Results chapter 1, figure 1.8.).

2.3. IgG BIOSYNTHESIS IN BEC-11 CELLS

2.3.1. Synthesis of Multiple Gamma Chain Forms

IgG synthesised by Bec-11 cells in the presence or absence of tunicamycin was isolated from cell lysates by immunoprecipitation with RAHκ, and GARIg, reduced and analysed by SDS-PAGE; figure 2.1. shows the pattern of radioactive components detected by fluorography.

Tracks A and B show the component chains of IgG synthesised by Bec-11 cells in absence or presence of tunicamycin respectively. Three γ-chain bands predominate in track A and these correspond to glycosylated γ-chains. Track B shows one major band not present in track A together with three other minor components. A photographic enlargement of the relevant area of the fluorogram permits closer examination of the resolved components and illustrates that of the four γ-chain bands immunoprecipitable from tunicamycin-treated Bec-11 cell lysates, two are of identical electrophoretic mobility to those found in immunoprecipitates derived from lysates of control Bec-11 cells, and the remaining two γ-chain bands are unique to tunicamycin-treated Bec-11 cells. These latter two bands are postulated to be non-glycosylated forms of the γ-chain bands resolved in track A.

The data suggest that there may be two structurally distinct forms of γ-chain in Bec-11 cells. Previous data from this laboratory have demonstrated that IgG isolated from aliquots of radioiodinated cell membrane proteins of Bec-11 cells contains γ-chains of higher AMW than the γ-chains isolated from secreted IgG from Bec-11 cells (Singer and Williamson, 1980). Therefore, the slowest mobility (64K)

FIGURE 2.1.

Multiple Gamma Chain Forms in Bec-11 Cells

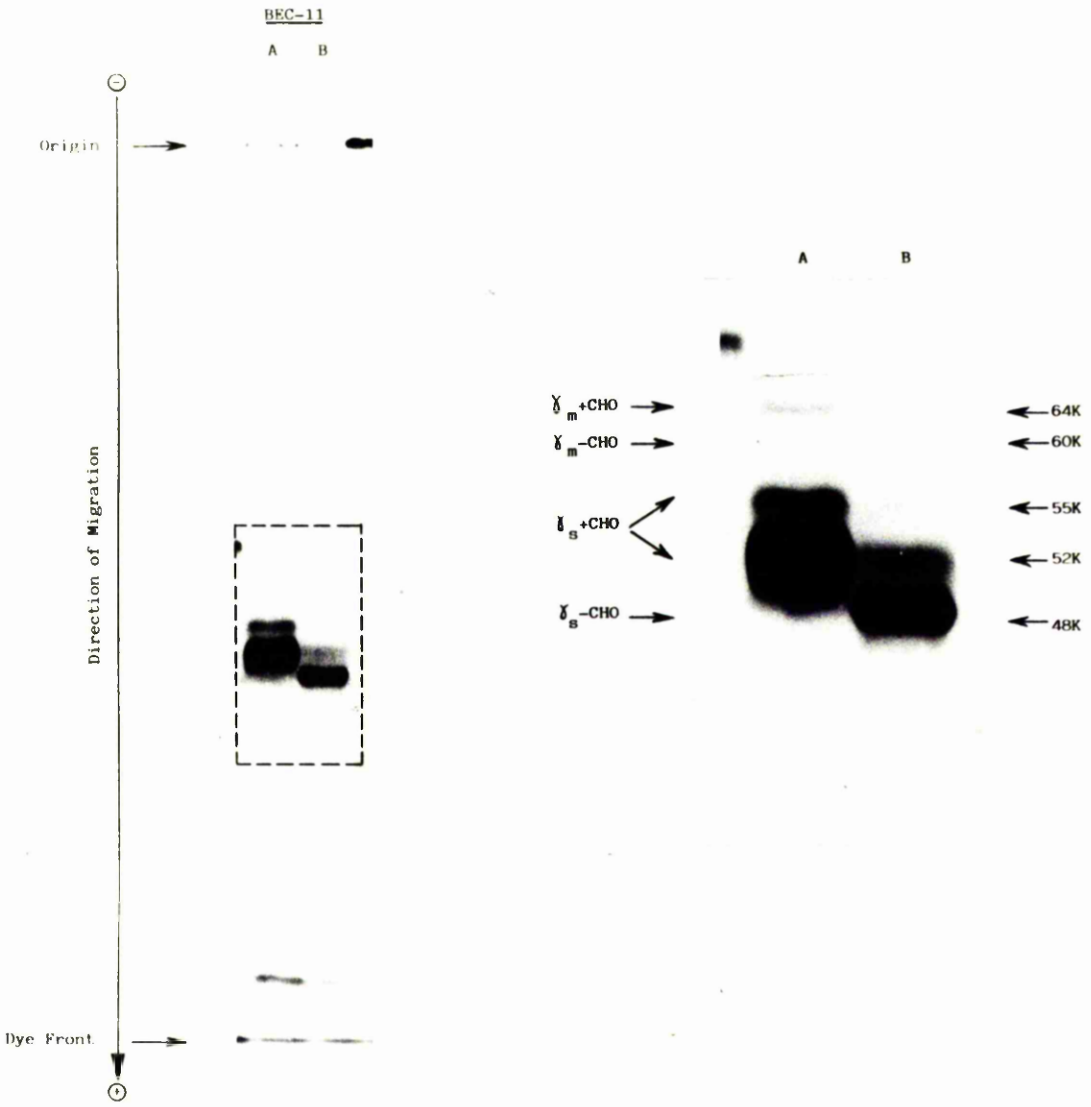
2.5×10^6 Bec-11 cells were harvested from log phase cultures and incubated at $37^{\circ}C$ for 90 minutes in complete medium containing 2 µg/ml tunicamycin. The cells were then washed twice in labelling medium and resuspended in 0.1 ml of the same medium supplemented with 200 µCi ^{35}S-methionine (Methods, 8.3.). After incubation at $37^{\circ}C$ for 90 mintues the cells were harvested, washed twice in ice-cold TKM buffer and cell lysates prepared in 250 µl 3D-TKM (Methods, 9). Control cultures were prepared in parallel.

Immunoprecipitation was carried out on 40 µl aliquots of lysate by addition of 1 µl RAHκ plus an equivalence of GARIg (Methods, 10.2.1.). Immunoprecipitates were collected by centrifugation and washed by pelleting through a two-step discontinuous sucrose gradient in 3D-TKM (Methods 10.3.). The washed precipitates were dissolved in 25 µl of reducing SDS-PAGE loading buffer, boiled, and electrophoresed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The pattern of radioactive components in the gel was detected by fluorography (Methods, 17.6.). A photographic enlargement of the area of the fluorogram containing the γ-chains is also shown.

Key:-

A - IgG immunoprecipitated from lysates of control Bec-11 cells.

B - IgG immunoprecipitated from lysates of tunicamycin-treated Bec-11 cells.


BEC-11
A
B
Origin
Direction of Migration
Dye Front
A
B
γm+CHO
γm-CHO
γs+CHO
γs-CHO
64K
60K
55K
52K
48K

band resolved in figure 2.1. track A is proposed to represent the mature, glycosylated membrane form of γ-chain (γ_m); the slowest mobility band (60K) in track B is postulated to be the non-glycosylated form of γ_m. The two faster mobility bands (55K and 52K) in track A are the secretory γ-chains (γ_s), and the fastest mobility (48K) band in track B is postulated to be the single non-glycosylated γ-chain from which both secretory forms of γ_s arise.

2.3.2. Molecular Weight Estimations

The AMW of each γ-chain form was determined relative to a set of molecular weight standards (Methods, 13.5.). These calibrations are important for two reasons:

a) the number of N-linked oligosaccharide side-chains possessed by each γ-chain form can be estimated, and;

b) the length of the C-terminal extension, postulated to effect membrane insertion of stability properties on γ_m, may also be estimated.

10% (w/v) acrylamide SDS-PAGE gels were found to give optimal resolution of γ-chain bands and, therefore, AMW values obtained from experiments on such gels are used throughout. The AMWs of each γ-chain form are given in table 2.1.

TABLE 2.1.

AMWs of Bec-11 γ-chain Polypeptides

Polypeptide	AMW
γ_m + CHO	64K
γ_s - CHO	60K
γ_s (minor) + CHO	55K
γ_s (major) + CHO	52K
γ_s - CHO	48K

The AMW estimates given are representative values and are useful only in a comparative context: they are not intended as absolute values.

AMW estiamtions were also performed on 11% (w/v) and 12.5% (w/v) acrylamide gels but resolution of the γ-chain components was inferior to that obtained with 10% gels. The data given in table 2.1. may be tentatively interpreted in terms of the number of N-linked oligosaccharide moieties added to a non-glycosylated γ-chain. The data obtained from AMW estimations on 11% (w/v) and 12.5% (w/v) acrylamide gels (not shown) were in good agreement with the interpretations drawn from the data shown in table 2.1. and discussed below.

N-linked oligosaccharides are added to glycoproteins as large pre-formed units from a lipid carrier (see Introduction, section 3.8.). Each N-linked oligosaccharide added to the polypeptide contributes 2500-3000 daltons to the AMW and subsequent processing reduces this contribution by a very small amount. Indeed,

the anomalous behavour of glycoproteins on SDS-PAGE is well documented (Melcher and Uhr, 1976) and thus it is possible to predict only the approximate number of N-linked oligosaccharide moieties present on any one peptide.

Therefore, if one N-linked oligosaccharide is assumed to contribute 3000 daltons to the AMW of a polypeptide, then the data of table 2.1. may be interpreted as follows. The AMW difference between glycosylated and non-glycosylated forms of γ_m is 4000 daltons; given the intrinsic inaccuracies in AMW calibrations and also the anomalous behaviour of glycoproteins on SDS-PAGE, this data suggests that one N-linked oligosaccharide is added to the 60K γ_m-chain to yield the fully glycosylated, mature 64Kγ_m-glycoprotein. By a similar arguement, the 52K glycosylated γ_s species could be generated from the 48K non-glycosylated chain by addition of a single N-linked oligosaccharide side chain, and the 55K γ-chain species could then be generated by addition of another carbohydrate side-chain to the 52K molecule. The finding of 52K and 55K secretory γ-chains in the cell cytoplasm and supernatant raises intriguing questions regarding the mechanisms of intracellular processing and transport of secretory IgG in Bec-11 cells, and these problems are explored below (section 2.3.5.).

The most interesting feature of this comparative study is the AMW difference between the non-glycosylated forms of γ_m- and γ_s- polypeptides. The difference, 12,000 daltons, is equivalent to an entire immunoglobulin domain and is approximately four-fold larger than the equivalent difference between γ_m- and γ_s- polypeptides. This finding has consequences for the structure and organisation of

human γ-chain genes and notably the M-exon components of these genes. The AMW difference between γ_m and γ_s of Bec-11 cells suggests a larger C-terminal extension, perhaps promoting binding to the cytoskeleton via a long cytoplasmic tail. The data herein is in agreement with observations made in the chicken and mouse IgG systems (Lifter et al, 1980; Oi et al, 1980) but represents an extension to the above work in that the comparisons were made on non-glycosylated molecules where the errors introduced by the anomalous behaviour of glycoproteins on SDS-PAGE is minimised. Clearly, it is essential to discover if a putatively larger C-terminal extension on the membrane heavy chain is a characteristic feature of 'memory' immunoglobulin isotypes and therefore related to memory B-cell activation, or if it is peculiar to IgG synthesising memory cells. Accordingly, biosynthesis and structure of IgA molecules has been investigated, and the data of this section may be discussed more meaningfully in the light of data pertaining to α-chain structure (see section 2.8. below).

The biosynthetic features of the putative γ_m- and γ_s-polypeptides are now presented below.

2.3.3. Comparison of Radioiodinated Membrane IgG and Biosynthetically Labelled IgG

The hypothesis that the 64K γ-chain band precipitated from control Bec-11 cells is the membrane γ-chain form was tested by comparing, under reducing conditions, IgG isolated from aliquots of radioiodinated Bec-11 cell membrane proteins or from aliquots of lysate of biosynthetically labelled Bec-11 cells.

FIGURE 2.2.

Comparison of Radioiodinated Membrane IgG with Biosynthetically Labelled IgG

2.5×10^6 Bec-11 cells were harvested from log phase cultures, washed twice in labelling medium and resuspended in 0.1 ml of the same medium supplemented with 200 µCi ^{35}S-methionine (Methods, 8.3.). After incubation at 37°C for 90 minutes, the cells were harvested and washed twice in ice-cold TKM buffer prior to preparation of cell lysates in 250 µl 3D-TKM (Methods, 9.).

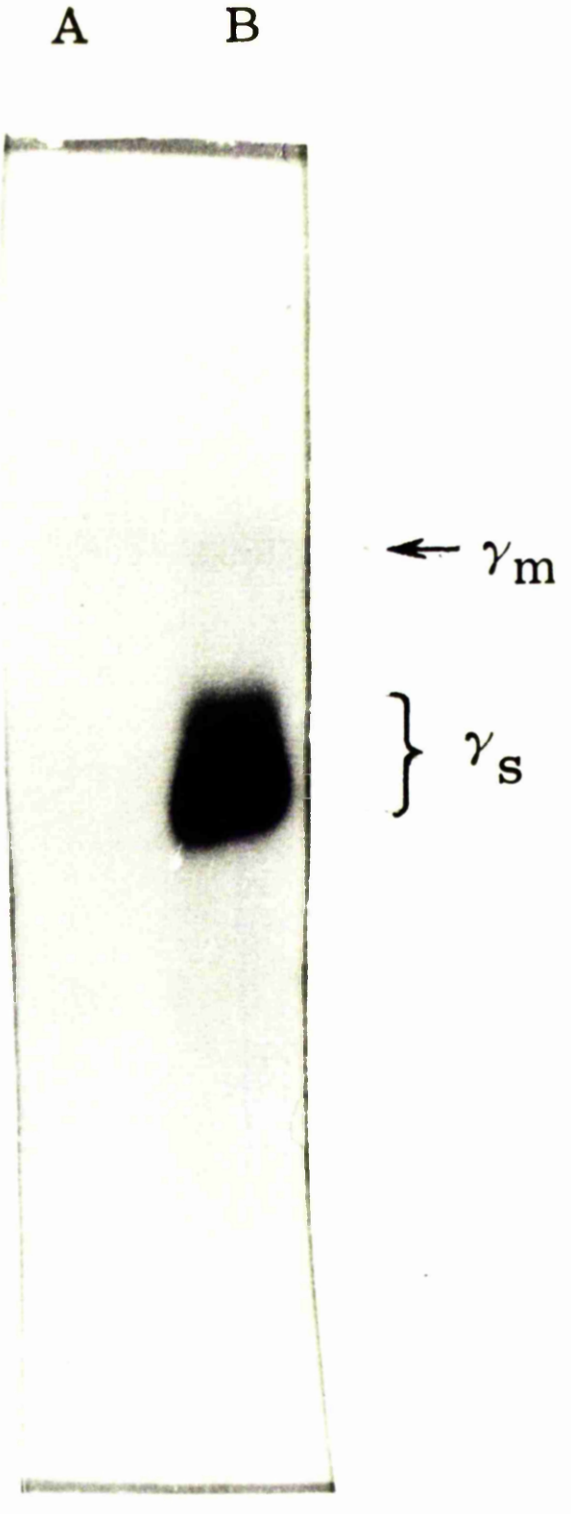
10^7 Bec-11 cells were harvested from log phase cultures of >95% viability and washed twice with 25 ml aliquots of ice-cold serum-free medium and once with 25 ml ice-cold PBS-I prior to lactoperoxidase catalysed radioiodination of membrane proteins using 2 mCi of ^{125}I (Methods, 8.2.). The labelled cells were washed extensively prior to preparation of cell lysates in 250 µl 3D-TKM (Methods, 9.).

IgG was isolated from 50 µl aliquots of lysate by immunoprecipitation with 1 µl RAH and an equivalence of GARIg (Methods, 10.2.). The washed immunoprecipitates were reduced and analysed by SDS-PAGE on a 10% (w/v) acrylamide slab gel (Methods, 13.). The gel was processed for fluorography (Methods, 17.6.).

Key:-

A - Radioiodinated Bec-11 membrane IgG.

B - Biosynthetically labelled Bec-11 intracellular IgG.


A
B
γ_m
γ_s

A single band was resolved when reduced immunoprecipitates derived from aliquots of radioiodinated Bec-11 cell membrane proteins were analysed (figure 2.2.). This band co-migrated with the 64K band resolved from identical immunoprecipitates derived from lysates of biosynthetically labelled Bec-11 cells. This observation is consistent with the hypothesis that the 64K γ-chain species is the membrane form of γ-chain.

Although only γ-chain is visible in the SDS-PAGE profile of radioiodinated Bec-11 IgG, two pieces of evidence may be cited to support the proposal that Bec-11 membrane IgG is 7S (i.e. $\gamma_2 L_2$). Firstly, the precipitating reagent was an anti-κ reagent and so the γ-chain could only be immunoprecipitated if it was associated with the κ-chain. Secondly, previous experiments have shown Bec-11 membrane IgG to have an AMW of 150,000 under non-reducing conditions (Singer and Williamson, 1979).

The data of figure 2.2. strongly suggest that the membrane form of Bec-11 γ-chain is the 64K species, consistent with the assignment made in section 2.3.1.

2.3.4. Both Glycosylated Secretory Gamma Chains Arise from a Single Non-Glycosylated γ-Chain

Two electrophoretically distinct γ-chain forms can be demonstrated in IgG secreted from Bec-11 cells (Singer and Williamson, 1980). The data of figure 2.1. suggest that both of these forms of γ-chain arise from a single non-glycosylated form. This postulate was tested experimentally by isolating IgG from culture supernatants of control and tunicamycin-treated cultures of Bec-11 cells by immunoprecipitation, and comparing the SDS-PAGE profiles

of the reduced immunoprecipitates with those of identically-prepared samples derived from lysates of control and tunicamycin-treated cells. The observation that IgG containing only non-glycosylated γ-chains is efficiently secreted from Bec-11 cells (see Results, chapter 1, figure 1.9.), allows direct comparison of the γ-chains secreted from tunicamycin-treated cells with γ-chains isolated from lysates of tunicamycin-treated cells (figure 2.3.).

Analysis of the SDS-PAGE profile of the component chains of IgG secreted from tunicamycin-treated cells (see figure 2.3.) revealed that only one non-glycosylated γ-chain is secreted. Comparison of the electrophoretic mobility of this secreted non-glycosylated γ-chain with γ-chains immunoprecipitated from cell lysates of tunicamycin-treated Bec-11 cells showed that the secreted chain is of identical electrophoretic mobility to that of the fastest mobility (48K) intracellular non-glycosylated γ-chain. The following conclusions may be drawn from this data:

a) the 48K γ-chain species detected in cell lysates of tunicamycin-treated cells is the single non-glycosylated form of the two secretory γ-chains;

b) only one potentially secretory γ-chain is synthesised in Bec-11 cells, and;

c) both γ-chains found in the supernate of Bec-11 cells are derived from this single non-glycosylated γ-chain form, and differ only in the amount of carbohydrate they possess.

These data, when considered in conjunction with the data of figure 2.2., are in agreement with the assignments made in

FIGURE 2.3.

Bec-11 Cells Synthesise a Single Secretory Gamma Chain Polypeptide

2.5×10^6 Bec-11 cells were harvested from log-phase cultures and treated for 3 hours at $37^{\circ}C$ in the presence or absence of 2 µg/ml tunicamycin in complete medium. The cells were then washed twice in labelling medium, resuspended in 0.1 ml of the same medium containing 250 µCi ^{35}S-methionine, and incubated at $37^{\circ}C$ for 90 minutes (Methods, 8.3.). After incubation, the culture was divided into two equal aliquots. A lysate was prepared in 250 µl 3D-TKM from one aliquot (Methods, 9.) and a 5-hour chase performed on the second aliquot: the final volume of the chase supernate was 250 µl (Methods, 8.3.).

IgG was isolated from aliquots of lysate and supernate by specific immunoprecipitation using 1 µl RAH plus an equivalence of GARIg (Methods, 10.2.). The washed immunoprecipitates (Methods, 10.3.) were dissolved in 25 µl of reducing SDS-PAGE loading buffer, boiled and electrophoresed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The gel was processed for fluorography after electrophoresis (Methods, 17.6.).

Key:-

IC - Intracellular

EC - Extracellular

Con - IgG isolated from lysates or supernates of control cultures.

Tun - IgG isolated from lysates or supernates of tunicamycin-treated cultures.

N.B. This figure is identical to figure 1.9.

BEC-11

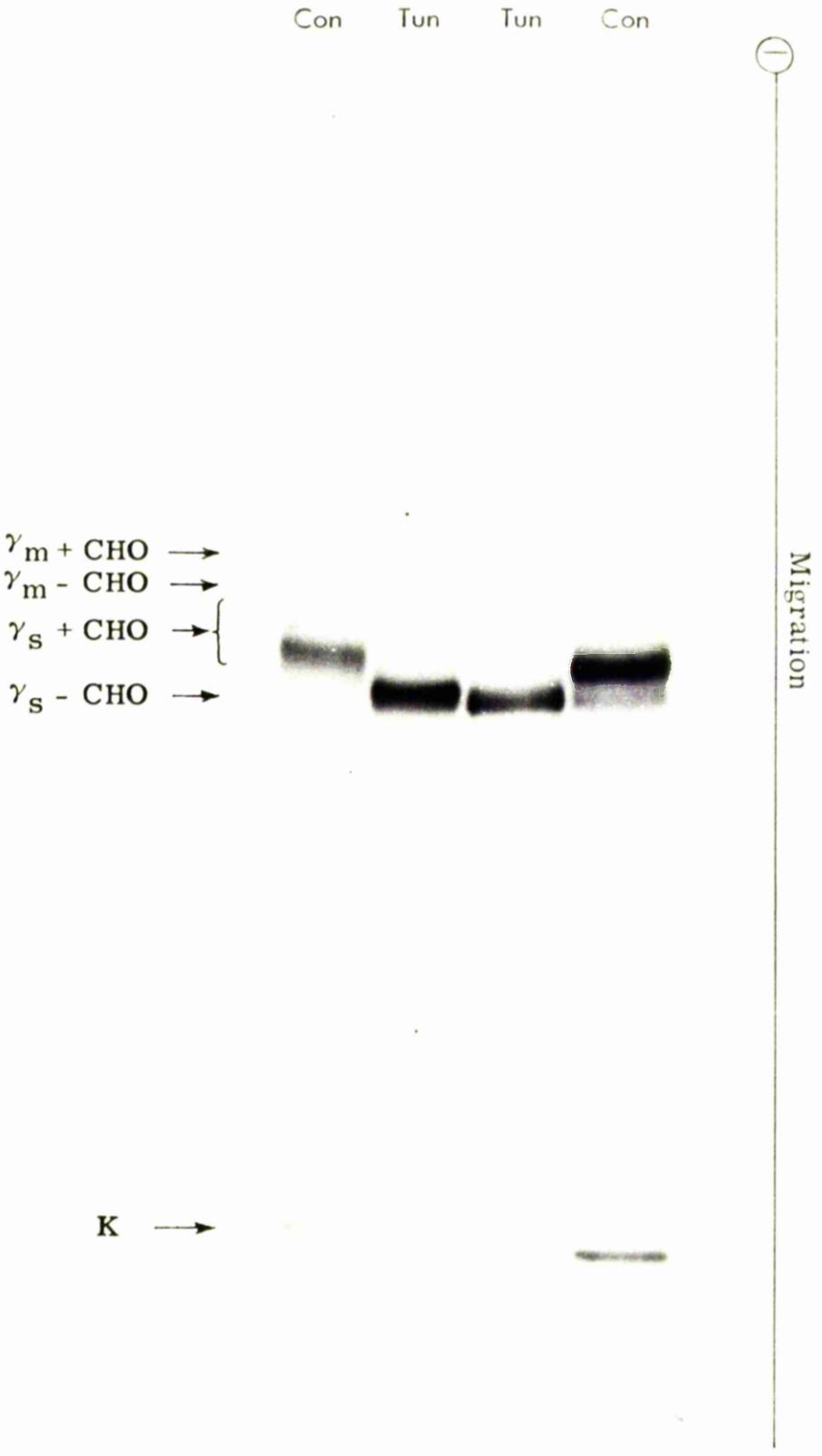

IC
EC
Con
Tun
Tun
Con
γ_m + CHO
γ_m - CHO
γ_s + CHO
γ_s - CHO
K
Migration

figure 2.1. and table 2.1. That is, the mature membrane form of γ-chain (64K) is apparently derived from a non-glycosylated γ-chain of 60,000 daltons, while both mature secretory forms (55K and 52K) arise from a single non-glycosylated γ-heavy chain polypeptide (48K).

The finding of two non-glycosylated γ-chains of markedly different AMWs in Bec-11 cells leads to the conclusion that Bec-11 cells are capable of simultaneous biosynthesis of structurally distinct membrane and secretory γ-heavy chain polypeptides.

2.3.5. Pattern of Glycosylation of Bec-11 Secretory γ-Chain

Two possible mechanisms may be invoked to account for the finding of secretory γ-chain polypeptides containing different amounts of carbohydrate in the assembled IgG molecules secreted from Bec-11 cells;

a) Bec-11 cells secrete two distinct 7S IgG* species containing identical polypeptide chains but with each individual 7S molecule differing in the extent of glycosylation of its constituent γ-chains;

b) Bec-11 cells secrete a single 7S IgG molecule which has been assymetically glycosylated during intracellular transport: that is, after initial glycosylation (conversion from 48K to 52K) one γ-chain undergoes a further glycosylation step (conversion to 55K) while the other γ-chain does not: such a precedent exists in rabbit IgG (Hinrichs and Smyth, 1970), or;

c) Bec-11 cells secrete multiple 7S forms which may be accounted for in terms of a mixture of a) and b) above.

Two experimental observations favour the first of these interpretations. As previously demonstrated (figure 2.3.), two γ-chains are observed in culture supernates of control Bec-11 cells. If the sole IgG secretory product of Bec-11 cells was an assymetically glycosylated molecules, the γ-chain bands resolved by SDS-PAGE would be expected to be of similar, if not identical, fluorographic intensity since the 7S IgG molecule would contain γ-chain polypeptides of identical radiochemical specific activity (^{35}S-methionine) which would have different AMWs due to glycosylation differences. Visual inspection of profiles of γ_s-polypeptide isolated from lysates or supernates of control cultures of Bec-11 cells (e.g. figure 2.3.) indicate that this is not the case. In both lysates and culture supernates, the 52K γ-chain species predominates over the 55K γ-chain form. This argues against the notion that assembled Bec-11 IgG is assymetrically glycosylated prior to secretion.

The similarities of <u>relative</u> intensities of the 55K band to the 52K band in lysates and supernatants of control Bec-11 cultures (i.e. the intensity of the 52K band is always greater than that of the 55K band) is inconsistent with the hypothesis that the 55K band is in a precursor-product relationship with the 52K γ-chain band or vice-versa. Indeed, the finding of the 55K γ-chain in the culture supernate is a strong arguement against this hypothesis.

In an attempt to directly demonstrate two distinct 7S IgG forms in culture supernates of normal Bec-11 cells, immunoprecipitated Bec-11 IgG was analysed on a 6% gel under non-reducing conditions (figure 3.4.).

FIGURE 2.4.

Secretion of Discrete 7S Molecules from Bec-11 Cells

Pulse-chase radiolabelling and immunoprecipitation of IgG from lysates and supernates of control and tunicamycin-treated Bec-11 cells was performed identically to the method described in figure 2.3.

Washed immunoprecipitates were dissolved in 25 µl of non-reducing SDS-PAGE loading buffer, boiled for two minutes, and electrophoresed on a 5% (w/v) acrylamide slab gel (Methods, 13.). The pattern of radioactive components in the gel was visualised by fluorography (Methods, 17.6.).

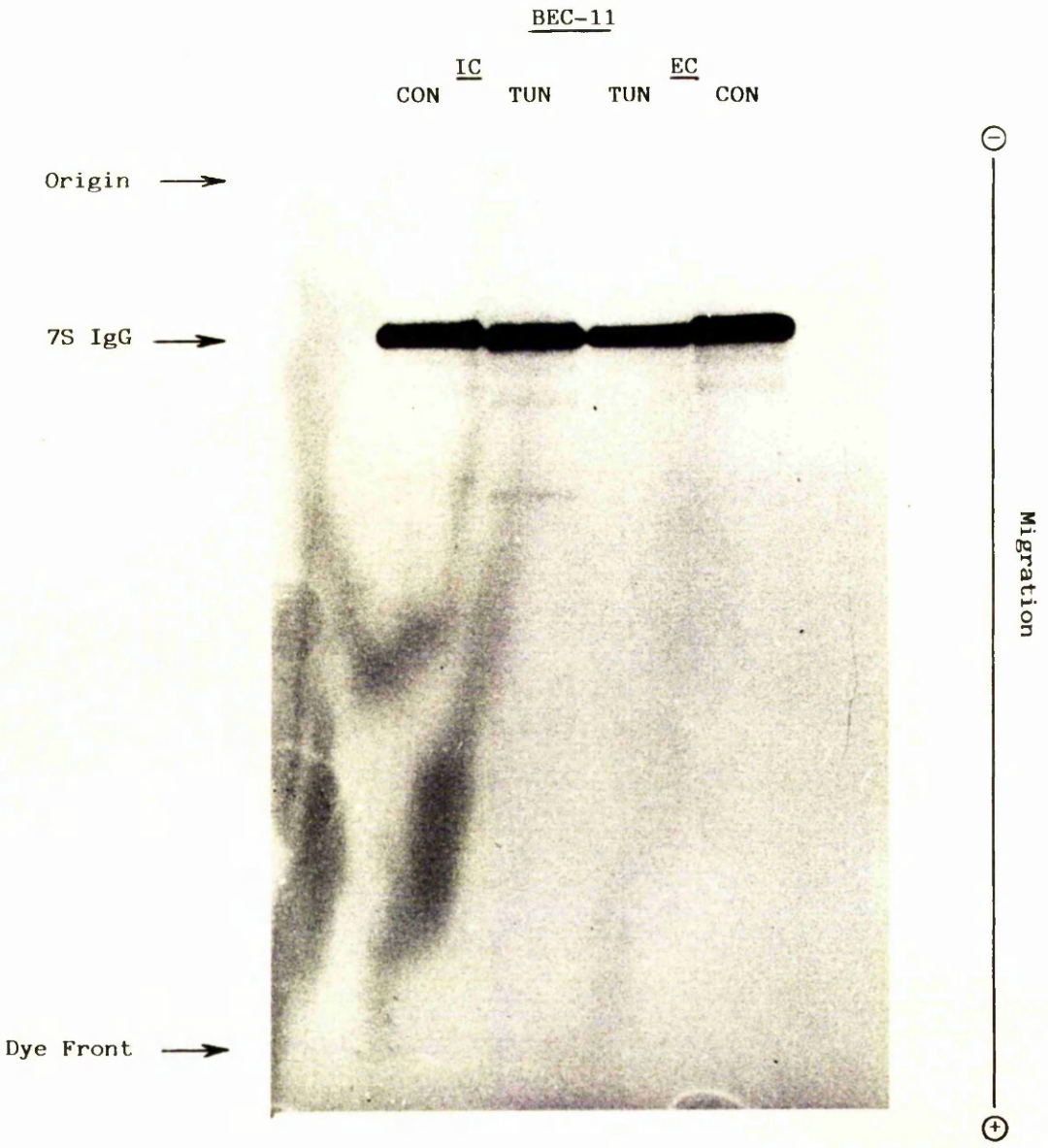
Key:-

IC - Intracellular

EC - Extracellular

Con - IgG isolated from lysates or supernates of control cultures.

Tun - IgG isolated from lysates or supernates of tunicamycin-treated cultures.


BEC-11
IC
EC
CON
TUN
TUN
CON
Origin
7S IgG
Dye Front
Migration

The SDS-PAGE profiles of IgG specifically immunoprecipitated from supernates of control and tunicamycin-treated Bec-11 cultures each have a major component in the region of the gel expected to correspond to 7S IgG. The difference in AMW between IgGs from control and tunicamycin-treated culture supernates is small at this gel percentage but is nonetheless visible in the profiles. In addition to the major IgG component, several minor bands of lower AMW are evident in the profile of IgG derived from supernates of tunicamycin-treated cultures. This is taken as stronger evidence that Bec-11 cells secrete several forms of 7S IgG molecules which differ not in their polypeptide structure, but in the relative amounts of carbohydrate they possess. The observation in the SDS-PAGE profiles of unreduced IgG of multiple 7S species argues against assymetric glycosylation as the principal explanation for the finding of two secretory γ-chain polypeptides upon analysis of secreted IgG under reducing conditions. However, it is possible that assymetrically glycosylated IgG accounts for some of the heterogeneity of the SDS-PAGE profiles observed for unreduced IgG secreted from Bec-11 cells. The data are most consistent with the proposal that Bec-11 cells secrete 7S IgG molecules which are symetrically glycosylated (i.e. containing two 55K or two 52K γ-chains) and also molecules which are assymetrically glycosylated.

2.4. EVIDENCE FOR AN HYDROPHOBIC SEQUENCE PRESENT ONLY ON MEMBRANE GAMMA CHAIN

Preliminary evidence that the membrane form of Bec-11

γ-chain possessed hydrophobic sequences not found in the secretory γ-chain polypeptide came from studies with the lipophilic nitrene, hexanyol-diiodo-NAP-tyramine. Lysates of unlabelled Bec-11 cells were prepared in triton X-100 and treated with 25 μCi of radioactive nitrene in the presence of strong light: after photolysis, the lysate was made to 3D-TKM by addition of concentrated deoxycholate and SDS. Analysis by SDS-PAGE was then performed.

Figure 2.5. compares the results of labelling whole, viable cells by lactoperoxidase-catalysed iodination or by use of the photo-activated nitrene, with the labelling of a cell lysate with hexanyol-dioodo-NAP-tyramine. The profiles shown in tracks A, B and C are those of total proteins precipitated by acetone; attempts to immuno-precipitate IgG labelled with the nitrene have so far been unsuccess-ful. However, several factors indicate that γ_m is labelled by the nitrene:

a) a band of AMW 64K is evident in the SDS-PAGE profile of lysate material radiolabelled by hexanyl-diiodo-NAP-tyramine;

b) this band is of identical electrophoretic mobility to the biosynthetically labelled 64K γ_m species (track D), and;

c) no 55K or 52K species are evident in the profiles of nitrene-labelled material, nor is there evidence of labelled κ-light chain.

These preliminary data tentatively suggest that the 64K γ_m species found in control Bec-11 cells possesses hydrophobic areas of amino acid sequence not found in the secretory form of the γ-chain.

FIGURE 2.5.

Labelling of Membrane Gamma Chain using a Radiolabelled Lipophilic Nitrene

Aliquots of 10^7 Bec-11 cells were harvested from log phase cultures of 95% viability, washed twice with 25 ml aliquots of ice-cold RPMI-1640 and once with 25 ml of ice-cold PBS-I. The cells were then resuspended in 50 µl of PBS-I and radiolabelled by either:

a) lactoperoxidase-catalysed radioiodination using 1 mCi of ^{131}I (Methods, 8.2.), or;

b) treatment with radioiodinated ^{131}I-hexanoyl-diiodo-NAP tyramine in the presence of light.

Radiolabelled cells were washed extensively and cell lysates prepared in 3D-TKM (Methods, 9.).

A lysate of 10^7 Bec-11 cells was also prepared in 1% (w/v) triton X-100 in TKM buffer and the nuclei removed. The lysate was then treated with ^{131}I-hexanoyl-diiodo-NAP-tyramine in the presence of light. After photolysis, deoxycholic acid and SDS were added to the lysate to the concentrations required to yield 3D-TKM lysis buffer (Methods, 9.).

Aliquots of lysate were treated with five volumes of acetone, centrifuged and the precipitates allowed to air dry prior to dissolution in reducing SDS-PAGE loading buffer. The samples were boiled and electrophoresed on a 10% (w/v) acrylamide slab gel (Methods, 13.). Radioactive components were detected by autoradiography (Methods, 17.5.).

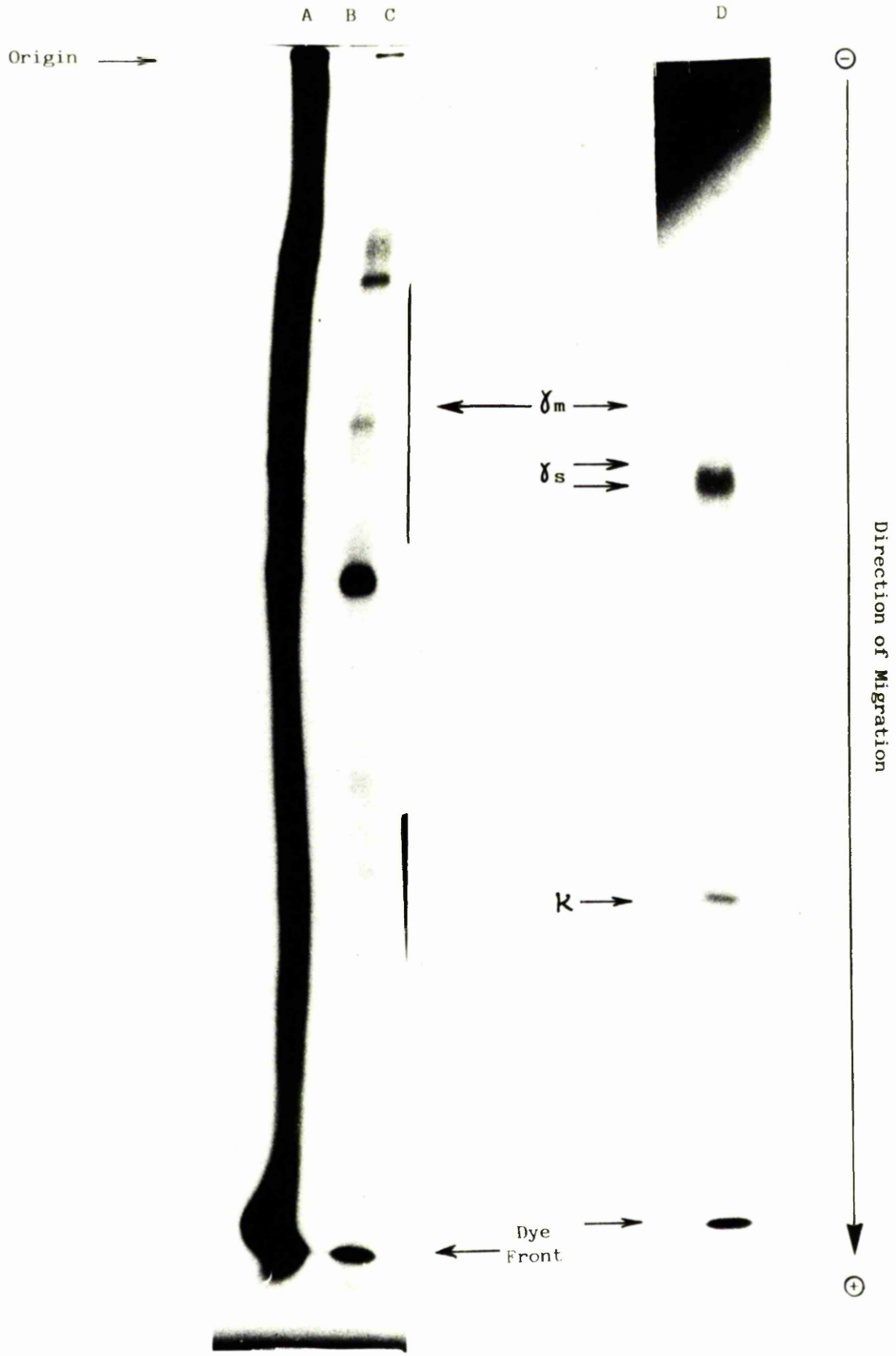
Key:-

A - Membrane proteins labelled by lactoperoxidase-catalysed radioiodination.

B - Membrane proteins labelled by photoactivated ^{131}I-hexanoyl-diiodo-NAP-tyramine.

C - Cell lysate proteins labelled by photoactivated ^{131}I-hexanoyl-diodo-NAP-tyramine.

D - Biosynthetically radiolabelled Bec-11 intracellular IgG (see legend to figure 2.2.).


A
B
C
D
Origin
γm
γs
κ
Dye
Front
Direction of Migration

Topological considerations and the known precedents for μ-chain structure lead one to propose that the putative hydrophobic sequence would be located at the C-terminus of the membrane γ-chain polypeptide.

The data of figure 2.5. can only be regarded as a preliminary indication of the hydrophobic properties of the γ_m polypeptide. A significant advance towards substantiation of these data will have been made when the material, radiolabelled with the nitrene, is successfully immunoprecipitated.

2.5. UBIQUITY OF THE SIZE DIFFERENCE BETWEEN MEMBRANE AND SECRETORY GAMMA HEAVY CHAIN POLYPEPTIDES

Analysis of the AMWs of non-glycosylated Bec-11 γ-chains demonstrated that the AMW of γ_m was 60K and the AMW of γ_s was 48K. The difference in AMW between the two γ-chain forms, 12,000 daltons, is unexpectedly large and it is, therefore, of great importance to examine the three most plausible explanations for this observation. These possibilities are:

a) the apparently large size of the C-terminal extension of membrane heavy chains is a feature of isotypes associated with B-memory cells,

b) the phenomenon is peculiar to IgG-producing cells but is not evident in IgA or IgE producing cells, and;

c) the large C-terminal extension is peculiar to one subclass of IgG.

To investigate these possibilities, pulse-chase experiments were performed on control and tunicamycin-treated cultures of

three IgG producing cell lines, Bec-11, EB4.9, and Maija. IgG was isolated from lysates and supernates derived from these cultures by specific immunoprecipitation and analysed by SDS-PAGE under reducing conditions (figure 2.6.).

Comparison of the pattern of radioactive components on the fluorogram of the SDS-PAGE profile of IgG derived from tunicamycin-treated Maija cells reveals that the difference in AMW between the γ_m and γ_s polypeptides of that cell is of the order of 12,000 daltons; that is, identical to the difference between γ_m and γ_s polypeptides of Bec-11 cells. In control Maija cells, it is interesting to note that both γ_s and γ_m species are apparently expressed as a 'doublet'; that is, two discrete glycosylated γ_s and γ_m bands. These are apparently derived from single non-glycosylated γ_s and γ_m polypeptides since only one band is resolved in each of the positions predicted for non-glycosylated γ_s and γ_m polypeptides. In the case of γ_m the fluorographic intensity of the glycosylated bands is very similar and this is taken as tentative evidence for assymetric glycosylation of γ_m polypeptides during intracellular transport of membrane IgG. It is noteworthy that Maija cells have a glycosylated κ-light chain. Since C_κ sequences do not contain N-glycosylation sites, this suggests that the V-region of Maija κ-light chains is glycosylated.

Examination of the AMWs of the γ_m and γ_s polypeptides of EB4.9 cells again suggested an AMW difference of approximately 12,000 daltons between the two polypeptides. This is in agreement with the findings made for the AMW difference between γ_m and γ_s polypeptides in Bec-11 and Maija cells. These data suggest that finding that the

FIGURE 2.6.

Ubiquity of the 12,000 Dalton AMW Difference between Membrane and Secretory Gamma Chains

2.5×10^6 cells were harvested from log phase cultures of Bec-11, EB4.9 and Maija cell lines and incubated for 3 hours at 37°C in the presence or absence of 2 µg/ml tunicamycin in complete medium. The cells were then washed twice with labelling medium, resuspended in 0.1 ml labelling medium supplemented with 250 µCi ^{35}S-methionine and incubated at 37°C for 90 minutes (Methods, 8.3.). Cell lysates were prepared in 250 µl 3D-TKM (Methods, 9). Additionally, a pulse-chase experiment was performed on EB4.9 cells.

IgG was isolated from lysates and supernates by treating aliquots of the samples with 1 µl RAHγ and an equivalence of GARIg (Methods, 10.2.1.). Washed immunoprecipitates were dissolved in 25 µl of reducing SDS-PAGE loading buffer, boiled, and analysed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The SDS-PAGE profiles were visualised by fluorography (Methods, 17.6.).

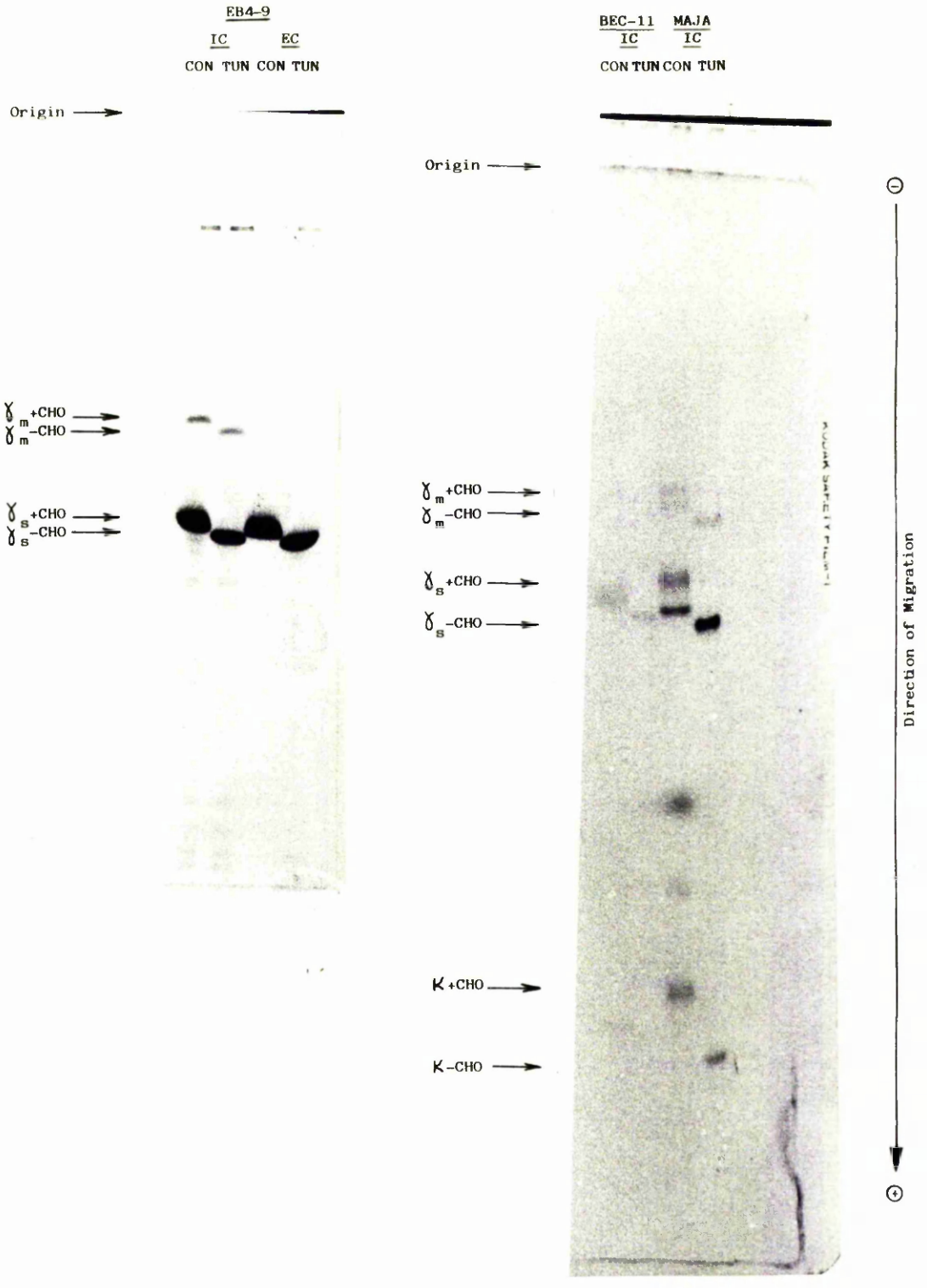
Key:-

IC - Intracellular

EC - Extracellular

Con - IgG isolated from lysates or supernates of control cultures.

Tun - IgG isolated from lysates or supernates of tunicamycin-treated cultures.


EB4-9
IC
EC
CON TUN CON TUN
Origin
γm+CHO
γm-CHO
γs+CHO
γs-CHO
BEC-11
IC
MAJA
IC
CON TUN CON TUN
Origin
γm+CHO
γm-CHO
γs+CHO
γs-CHO
K+CHO
K-CHO
Direction of Migration

large difference in AMW between γ_m and γ_s polypeptides seems to be common to IgG-producing cells. Although the possibility that it is a subclass specific phenomenon cannot be ruled out, it is felt unlikely that all of the cell lines studied expressed the same subclass of IgG. The data of section 2.7. demonstrates a difference of 2000 daltons between α_m and α_s polypeptides which refutes the hypothesis that a long C-terminal extension on the membrane heavy chain is characteristic of the immunoglobulin isotypes expressed on the memory B-lymphocytes. The data from murine and chicken IgG glycoproteins (Oi et al, 1980; Lifter et al, 1980) also demonstrate a large AMW difference between γ_m and γ_s polypeptides. These preliminary studies, together with the data of figure 2.6. would support the notion that a large C-terminal extension is common to all IgG subclasses.

The data obtained for EB4.9 IgG also substantiate two other findings originally made in Bec-11 cells;

a) non-glycosylated IgG is efficiently secreted, and;

b) the AMW of the γ_s polypeptide is approximately 48K: this is entirely consistent with the assignments made for Bec-11 γ-chains in figure 2.1.

2.6. IN VITRO TRANSLATION OF BEC-11 POLY A^+ RNA

To test the hypothesis that the syntheses of γ_m and γ_s polypeptides were directed by independent mRNA molecules, the following experiment was performed. Purified poly A^+ RNA from Bec-11 cells was

used to programme protein synthesis in the rabbit reticulocyte lysate cell-free translation system. After synthesis, the translation products were treated with RAHγ plus GARIg to specifically immunoprecipitate *in vitro* synthesised γ-chains. Purified immunoprecipitates were reduced and analysed by SDS-PAGE.

Figure 2.7. illustrates that two putative gamma chains were precipitated from cell-free translation products and that these were of slower electrophoretic mobility than their non-glycosylated counterparts synthesised *in vivo*. These molecules are believed to be the precursors of γ_m and γ_s polypeptides.

The data presented in figure 2.7. represent an initial step in testing the hypothesis that separate mRNAs encode γ_m and γ_s polypeptides. Given that there are no reports of post-synthetic modification in the rabbit reticulocyte lysate protein synthesis system, the data are consistent with the above hypothesis. Furthermore, the small AMW difference between pre-γ_m and non-glycosylated γ_m and the equivalent difference for the γ_s polypeptides, suggests that the *in vitro* synthesised γ-chains possess hydrophobic leader peptides.

The data are, however, defective in two respects. Firstly, the AMW difference between pre-γ_m and non-glycosylated γ_m is greater that the equivalent difference between *in vitro* and *in vivo* synthesised γ_s polypeptides. If γ_m and γ_s mRNA species are derived from a single gene by splicing of a single nuclear RNA transcript, then both mRNAs should contain the same exon for the leader peptide (see Introduction, figure 7.). Therefore, the AMW difference between *in vivo* and *in vitro* synthesised forms of a given γ-chain should be identical. Secondly, decisive proof that the two species postulated to represent

FIGURE 2.7.

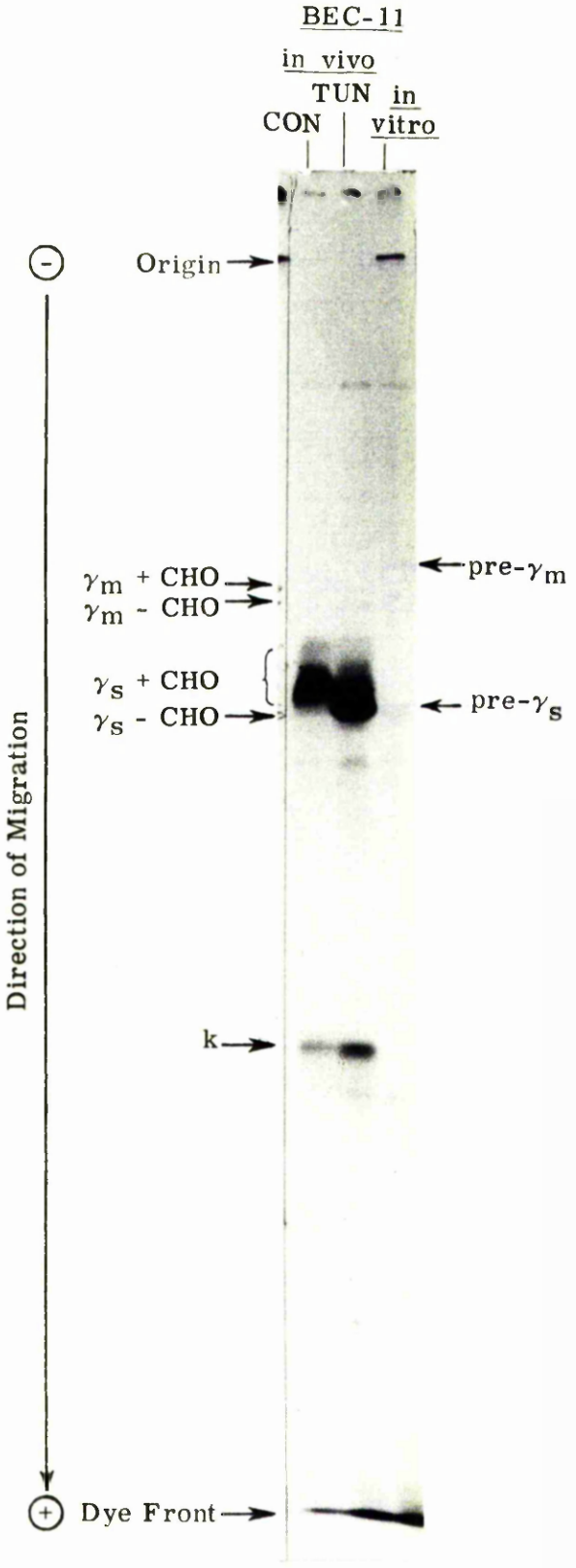
Synthesis of γ-Chains In Vivo and In Vitro

Polyribosomes were prepared from 5 x 10^8 Bec-11 cells (Methods, 11.1.) and poly A^+RNA was purified from this subcellular fraction by oligo-dT cellulose chromatography (Methods, 11.2.). The purified mRNA was resuspended in water at a concentration of 1mg/ml.

In vitro translation was performed by stimulating the rabbit reticulocyte lysate cell-free system by addition of 1-2 μg of Bec-11 poly A^+ RNA: 30 μCi of ^{35}S-methionine were also added. The mixtures were incubated at 30°C for 90 minutes whereupon the reaction was terminated by placing the tubes on ice (Methods, 12.). In vitro synthesised γ-chains were specifically immunoprecipitated by addition of 1 μl of RAHγ plus an equivalence of GARIg to the translation products. The immunoprecipitates were washed, reduced and analysed by SDS-PAGE (Methods, 13.). The pattern of radioactive components was visualised by fluorography (Methods, 17.6.).

Key:-

In Vivo	-	IgG synthesised by viable cells.
In Vitro	-	IgG synthesised in reticulocyte lysate programmed with Bec-11 poly A^+ RNA.
Con	-	IgG synthesised by control cultures of Bec-11 cells.
Tun	-	IgG synthesised by tunicamycin-treated cultures of Bec-11 cells.
pre-γ	-	Denotes γ-chain containing leader sequenc


BEC-11
in vivo
CON
TUN
in vitro
Origin
γm + CHO
γm - CHO
γs + CHO
γs - CHO
pre-γm
pre-γs
k
Dye Front
Direction of Migration

precursor γ-chains are indeed gamma chains is lacking: evidence to support this action could be obtained by 'cold competitor' experiments where unlabelled IgG would be used to inhibit immunoprecipitation of radiolabelled γ-chains synthesised *in vivo* or *in vitro*.

Experiments are in progress to substantiate the above data. Preliminary studies with the Burkitts Lymphoma EB.4 show an SDS-PAGE profile of *in vitro* synthesised, immunoprecipitated γ-chains which is identical to that of *in vitro* synthesised Bec-11 γ-chains (not shown). The data of figure 2.7. do, however, provide tentative evidence that independent species of mRNA direct the synthesis of γ_m and γ_s polypeptides.

2.7. IgA BIOSYNTHESIS IN DAKIKI AROSROS-1 CELLS

1.7.1. Alpha Chain Species in Dakiki Arosros-1 Cells

IgA was isolated from aliquots of lysates of control and tunicamycin-treated Dakiki Arosros-1 cells by specific immunoprecipitation with RAHλ and GARIg. The washed precipitates were reduced and analysed by SDS-PAGE.

The fluorogram of the above experiment (figure 2.8.) illustrates that two α-chain bands were specifically immunoprecipitable from lysates of control and tunicamycin-treated cells. The alpha chains isolated from lysates of control cells had AMWs of 55K and 53K, and these are postulated to be the membrane (α_m) and secretory (α_s) forms of the alpha chain: the AMWs of the corresponding chains derived from lysates of tunicamycin-treated cells were 52.5K (α_m) and 50.5K (α_s). The observation of two distinct α-chain species in

FIGURE 2.8.

Distinct Alpha Chain Polypeptides in Arosros-1 Cells

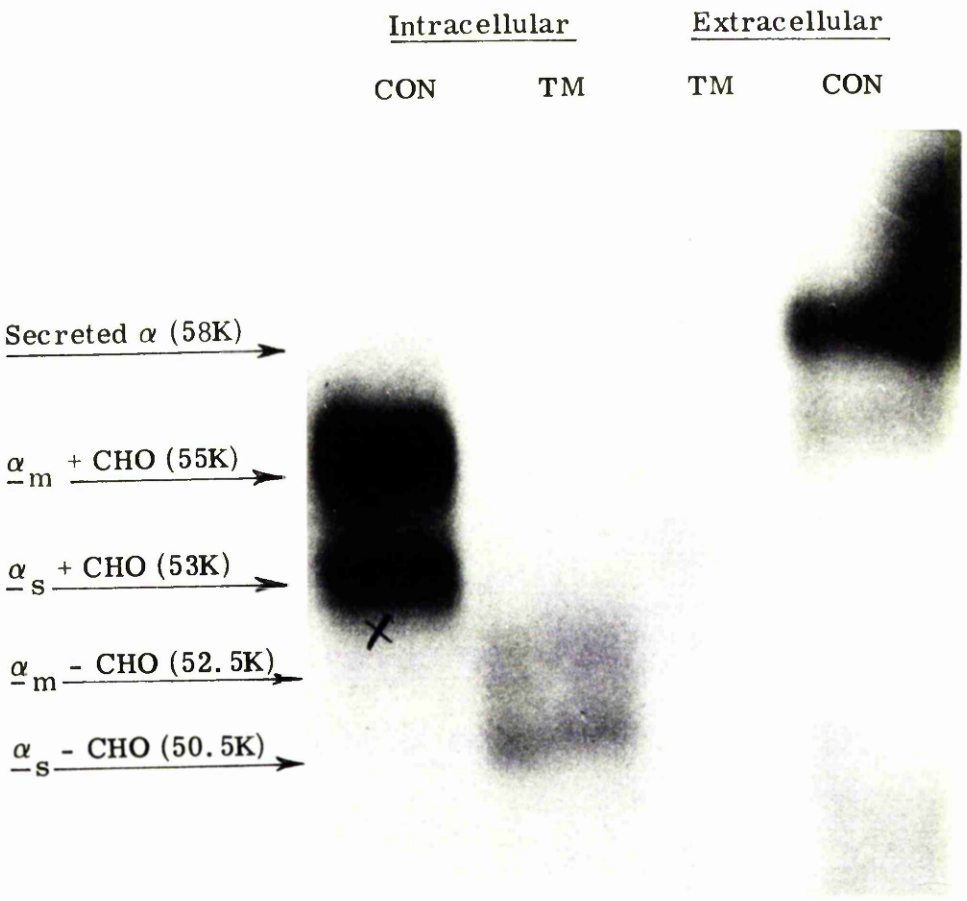
2.5×10^6 Dakiki Arosros-1 cells were harvested from log phase cultures and incubated in complete medium for 3 hours at $37^{\circ}C$ in the presence or absence of 2 µg/ml tunicamycin. The cells were washed twice with labelling medium, resuspended in 0.1 ml of labelling medium containing 250 µCi ^{35}S-methionine, and incubated at $37^{\circ}C$ for 90 minutes (Methods, 8.3.). After incubation, cultures were divided into two equal aliquots; a cell lysate was prepared in 250 µl of 3D-TKM buffer from one aliquot (Methods, 9.) and a 5-hour chase carried out upon the other aliquot (Methods, 8.3.).

IgA was specifically isolated from aliquots of lysate or pulse-chase supernate by immunoprecipitation using 1 µl RAHα and an equivalence of GARIg (Methods, 10.2.1.). Immunoprecipitates were harvested by centrifugation and washed by pelleting through a two-step discontinuous sucrose gradient in 3D-TKM (Methods, 10.3.). The washed immunoprecipitates were dissolved in 25 µl of reducing SDS-PAGE loading buffer, boiled and electrophoresed on a 10% (w/v) acrylamide slabe gel (Methods, 13.). SDS-PAGE profiles were visualised by fluorography (Methods, 17.6.).

Key:-

Con - IgA isolated from lysate or supernate of control cultures.

TM - IgA isolated from lysate or supernate of tunicamycin-treated cultures.


Intracellular
Extracellular
CON
TM
TM
CON
Secreted α (58K)
α m + CHO (55K)
α s + CHO (53K)
α m - CHO (52.5K)
α s - CHO (50.5K)

lysates of tunicamycin-treated Dakiki Arosros-1 cells leads to the proposal that these cells are capable of synthesising structurally distinct forms of α-chain: one for insertion into the lymphocyte membrane and the other for secretion into the extracellular environment.

2.7.2. Molecular Weight Estimations

The AMW of each α-chain species resolved in figure 2.8. was determined with reference to a set of standards of known molecular weight (see methods section 13.5.). 10% (w/v) acrylamide gels were found to give optimum resolution of α-chains. The AMWs are given in table 2.2. below.

TABLE 2.2.

AMWs of Dakiki Arosros-1 Alpha Chains

Alpha Chain	Apparent Molecular Weight
Membrane + carbohydrate	55K
Membrane - carbohydrate	52.5K
Secretory + carbohydrate	53K
Secretory - carbohydrate	50.5K

These figures are representative values for AMW and are not intended as absolute values. As in the equivalent study on γ-chains (table 2.1.), these values are useful in a comparative context only.

The differences in AMW between glycosylated polypeptides may again be accounted for in terms of addition of pre-formed N-linked

oligosaccharide moieties. The AMW difference of 2500 daltons between the glycosylated and non-glycosylated forms of the membrane α-chain polypeptide is consistent with the notion that a single N-linked oligosaccharide is added to the non-glycosylated α_m polypeptide during its intracellular transport to give the mature α_m polypeptide. A similar interpretation may be advanced for the explanation of the 2500 dalton difference in the AMWs of glycosylated and non-glycosylated secretory α-chains.

Comparison of the AMWs of the non-glycosylated α_m- and α_s-polypeptides reveals a difference of 2000 daltons. This is taken as preliminary evidence that the C-terminal structure of α_m- and α_s-polypeptides closely resembles that of μ_m- and μ_s- polypeptides. The data also have implications for the genomic structure and organisation of the 3'-end of the C_α gene: this is discussed in section 2.8. below. The data refute the hypothesis that a large C-terminal extension on the membrane polypeptide (i.e. a 12K difference between membrane and secretory polypeptides) is a characteristic of memory cell isotypes: this phenomenon appears to be unique to γ-producing lymphocytes.

2.8. SIMULTANEOUS BIOSYNTHESIS OF RECEPTOR AND EFFECTOR IMMUNOGLOBULINS BY SINGLE B-LYMPHOCYTES: A GENERAL PHENOMENON

The capacity of primary B-lymphocytes to effect simultaneous biosynthesis of membrane (receptor) and secretory (effector) forms of μ-chain is now established (Bergman and Haimovich, 1979;

Singer et al, 1980; Alt et al, 1980; Rogers et al, 1980; Early et al, 1980). The data of this chapter confirm previous preliminary reports that B-lymphocytes expressing other isotypes, e.g. IgG (Oi et al, 1980; Lifter et al, 1980; Singer and Williamson, 1980) are also capable of the simultaneous synthesis of membrane and secretory polypeptide forms of the immunoglobulin isotype to which that cell is committed. Thus, lymphocytes expressing IgG and IgA have been shown to synthesise distinct heavy chain polypeptides which are structurally differentiated for expression on the cell membrane or in aquaeous environments (e.g. serum). In the case of IgG biosynthesis, the experiments described above suggest that distinct mRNA species direct the synthesis of γ_m and γ_s polypeptides.

In view of the observations made during these investigations it is reasonable to propose that the genetic mechanisms which permit memory B-lymphocytes to express membrane and secretory heavy chains simultaneously is similar, if not identical, to that described for IgM producing cells (see introduction, figure 7.). Thus, it is hypothesised that after isotype switching has occurred and the V-D-J gene joined in close proximity to any selected C_H gene, transcription will occur to yield a large nuclear RNA transcript which will contain nucleotide coding sequences for:

a) the leader sequence followed by an intron separating it from;

b) the V-D-J unit which will, in turn, be separated by an intron from;

c) the C_H gene, each domain exon being separated from its neighbours by an intron, and the most 3' C-region

domain exon being separated from the M-exon(s) by an intron.

The primary nuclear RNA transcript will then undergo differential RNA splicing to yield two functional mRNA molecules; one encoding the membrane form of the heavy chain polypeptide and the other mRNA directing the synthesis of the secretory heavy chain polypeptide. After undergoing the appropriate cytoplasmic processing and being assembled with light chains, the immunoglobulin will be expressed either at the cell membrane or in serum depending on the structure of the heavy chain contained in any one molecule.

The data also reveal an interesting feature with respect to structural differences between membrane and secretory heavy chain polypeptides. In agreement with data for mouse (Oi et al, 1980) and chicken IgG glycopeptides (Lifter et al, 1980) the data herein suggest a large AMW difference between γ_m and γ_s polypeptides. The estimates herein suggest the AMW difference to be 12,000 daltons. This difference is, so far, unique to γ-chains; α_m and α_s polypeptides differ by only 2000 daltons in terms of AMW. Comparison of these AMW differences with the equivalent difference in the μ-chain system (table 2.3.) permits meaningful speculation upon the structure and genomic organisation of human C_γ and C_α genes.

TABLE 2.3.

Comparisons of AMW Differences Between Membrane and Secretory Heavy Chain Polypeptides

	Apparent Molecular Weight[1]		
	MEMBRANE	SECRETORY	DIFFERENCE
μ	68,000	65,000	3,000
α	52,500	50,500	2,000
γ	60,000	48,000	12,000

[1] Based on AMW of non-glycosylated heavy chains

From this type of comparative analysis it is suggested that the structure and organisation of the 3' end of the Cγ gene, particularly the M-exon, is different from that of the Cμ gene, while the data suggest that Cμ and Cα genes may be similarly organised.

The amino acid sequences of secretory human μ- and α- chains are very similar at their C-termini. Most worthy of note is the fact that both μ- and α- chains possess a 20 amino acid tail-piece not found of γ- or ε- heavy chains. Therefore, based on these observations at the amino acid sequence level coupled with the experimental observations herein it is proposed that the 3' end of the human Cα gene will be similar to that of the human Cμ gene (Rabbitts et al, 1981). That is, the Cα gene will possess:

a) a nucleotide sequence encoding the 20-amino acid tail piece contiguous with the 3'-end of the $C_{\alpha}3$ exon (the T-piece);

b) an intra-exon splice site within $C_\alpha 3$ conferring the ability to transcribe through to the end of the $C_\alpha T$ sequence or to splice to the M_α exons;

c) an M-exon of similar size and sequence to that described for the C_μ gene separated from the 3' end of $C_\alpha T$ by an intron of about 2kb, and;

d) a small coding sequence (the M_c exon) for the cytoplasmic tail of the α_m polypeptide, again similar to that found in the Cμ gene, and located some 120 base pairs 5' to the M_1 exon.

The proposed structures of the human Cμ, Cα, and Cγ genes are diagrammed in figure 2.10.

The large difference in AMW between γ_m and γ_s polypeptides is more difficult to explain. However, it is proposed that the 3' structure of the $C_\gamma 3$ exon will differ considerably from that of either $C_\mu 4$ or $C_\alpha 3$ in that it will not possess a large T-piece. Furthermore, the M-exons of the C_γ gene may also be different from those of C_μ and C_α, and it is predicted that the C_γ M-exons will be larger than their counterparts in C_μ or C_α.

The lack of a 20 amino acid T-piece in C_γ genes is an intriguing paradox. At the protein level, the C-terminal sequence of γ myeloma proteins has been shown to be pro-gly (see Kabat et al, 1979). However, nucleotide sequence analysis of cloned germ-line Cγ genes and cloned cDNAs copies of γ-chain mRNA of the mouse predict lysine codon at the C-terminus of γ_1, γ_{2a} and γ_{2b} polypeptides (Honjo et al, 1979; Yamawaki-Kataoka et al, 1980; Ollo et al, 1981) followed by a TGA termination codon. Comparing the nucleotide

sequence at the 3' end of the Cγ3 exon and with the equivalent sequence of $C_\mu 4$ (figure 2.9.) it is clear that the glycine codon followed by the lysine codon generates a concensus RNA splice site for the 5'-boundary of an intron (Rogers and Wall, 1980; Wall, 1980). This nucleotide sequence arrangement suggests that C_γ genes do possess an intraexonic RNA splice site to allow simultaneous expression of γ_m and γ_s polypeptides and also possess a T-piece of two codons (lys-stop).

The 2-codon T-piece has functional significance. The existence of an RNA splice site at the 3'-end of the $C_\gamma 3$ exon confers upon the cell the ability to select for expression of γ_m or γ_s polypeptides, or to express both forms simultaneously. Absence of the splice site would result in lymphocyte being capable of synthesis of γ_s polypeptides only. The presence of the intra-exonic RNA splice site permits the lymphocyte to express receptor immunoglobulin, and hence to become an immunocompetent cell.

The structure of the M exons of the C_γ genes has not yet been described. The data described in this chapter lead to the conclusion that the M-exons of C_γ will be considerably larger than those described for C_μ (Early et al, 1980) and predicted for C_α. Two major alternative structures and organisations are envisaged for the M-exons of C_γ (see figure 2.10.).

a) a large M_1-exon encoding negatively charged amino acids and hydrophobic amino acids for localisation on the outer membrane face and within the bilayer respectively; this large exon would be separated by an intron from a second exon (M_c), encoding the positively charged amino acids of the cytoplasmic level, and;

FIGURE 2.9.

Alternative RNA Splice Sites in Murine C_μ and C_γ Genes

The nucleotide sequences of germ line Cμ4 DNA and μ_m- and μ_s-RNA at the alternative RNA splice site are shown: the splice site is underlined and the exon-intron boundary is denoted by a slash.

Putative alternative RNA splice sites are illustrated in the known sequences for murine $C_\gamma 1$, $C_\gamma 2a$ and $C_\gamma 2b$ genes at the 3'-end of their $C_\gamma 3$ exons. The concensus RNA splice site for the 5'-end of an intron (Rogers and Wall, 1980) is also shown.

FIGURE 2.9.

Alternative RNA Splice Sites in Murine C_μ and C_γ Genes

Germ Line $C_\mu 4$	5' - T C C A C T G/G T A A A C C C - 3'
μ_s-mRNA	5' - T C C A C T G/G T A A A C C C - 3'
μ_m-mRNA	5' - T C C A C T G A G G G G A A G - 3'
Germ Line γ_1	5' - T C T A C T G/G T A A A T G A - 3'
Germ Line γ_{2a}	5' - A C T C C G G/G T A A A T G A - 3'
Germ Line γ_{2b}	5' - T C T C C G G/G T A A A T G A - 3'
Concensus Site	5' A G/G T A A G T A - 3'

b) a M_1-exon of similar size and composition as the equivalent exon in C_μ, separated by an intron from the M_c exon encoding a larger cytoplasmic tail than that predicted for μ_m-polypeptides (Rogers et al, 1980).

In alternative a), the M_1-exon may be divided into a number of smaller exons (represented as broken lines in figure 2.10) or it may be a single large exon.

The second genomic structure postulates a large M_c exon which would encode a long cytoplasmic extension of the γ_m polypeptide. The function of such a large cytoplasmic tail may be to promote interaction of membrane IgG with elements of the lymphocyte cytoskeleton (Flanagan and Koch, 1978).

The structures of the M-exons proposed above would conform to rules already established for RNA splicing of the primary transcript. That is, the RNA splicing sites would obey the 5'-GT....AG-3' rule (Breathnach et al, 1978; Catterall et al, 1978) and would be positioned to promote joining of:

a) the $C_\gamma 3$ and M_1 exons;

b) the M_1 and M_c exons, and;

c) the M_1 and M_1' exons if necessary.

A termination codon is predicted to be located at the 3' end of the M_c exon and two sites of poly-A addition ought to be found within the C_γ gene. One poly-A addition site should occur just 3' to the $C_\gamma 3$ exon, and the second should be located 3' to the M_c exon. It is predicted from the estimates of the AMW difference between γ_m and γ_s polypeptides and from the structure of the 3'-end of the $C_\gamma 3$

FIGURE 2.10.

Possible Structures of the 3'-end of Human Cμ, Cα and Cγ Genes

The organisation of coding DNA for each of the Cμ, Cα, and Cγ genes from the exon encoding the last domain to the exon encoding the cytoplasmic tail is illustrated: two alternative models are presented for the Cγ gene.

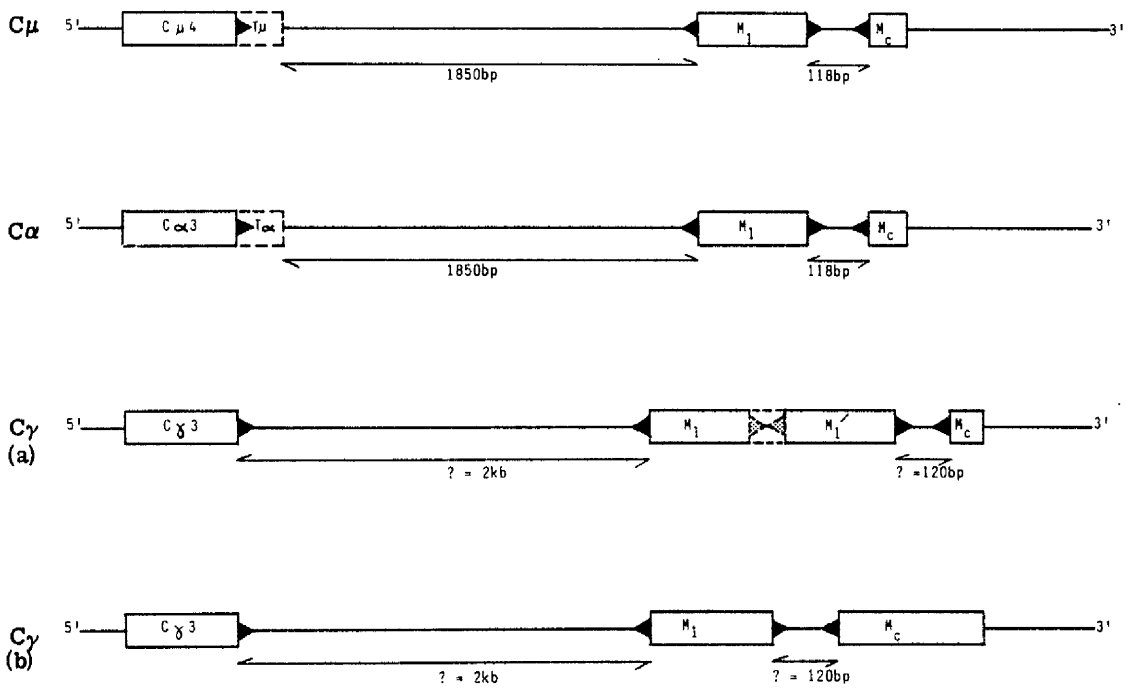
It is envisaged that Cμ and Cα will have a similar structure and organisation. A sequence encoding a secretory peptide (the T- or tail-piece) will be found contiguous with the last constant region domain exon. It is predicted that an intraexon RNA splice site will also be found in $C_\alpha 3$. The structure of the M-exons of the C_α gene will be similar to that of Cμ and the intron between $C_\alpha 3$ and M_1 will of approximately the same length as that between $C_\mu 4$ and M_1.

Data at the protein level suggest a large C-terminal extension of γ_m polypeptides. The two most plausible genetic structures which could account for these data are illustrated. In the first example the M_1' exon may represent a limited tandem repeat of M_1 which may or may not be separated from M_1 by an intron. In the second example, the M_1 exon of C_γ is proposed to be similar to those of C_μ and C_α, but M_c, which is proposed to encode the cytoplasmic tail of the γ_m polypeptide, is postulated to be extended to give rise to a large cytoplasmic protrusion. A large T-piece is not envisaged for the Cγ gene (see text).

Key:-

- T - Nucleotides encoding the 19 amino acid secretory tail of secretory μ- and α-chains.
- M_1 - First membrane exon, containing coding sequences for hydrophobic intramembrane amino acids.
- M_c - Second membrane exon, containing the coding DNA for the cytoplasmic tail of membrane immunoglobulins
- Shaded Triangle - RNA splice site.
- bp - Base pairs.

Fig. 2.10 Possible Structures of the 3-End of Human Cμ, Cα and Cγ Genes



exon (i.e. the 2-codon T-piece) that the total coding DNA found in the M_1 and M_c exons irrespective of their organisation will be in the range of 250-300 base pairs. That is, the M exons of the C_γ genes will encode 100 $\pm$ 20 amino acids.

The results of this chapter provide some evidence that cells representative of the memory B-lymphocyte population are capable of effecting simultaneous biosynthesis of structurally distinct membrane and secretory immunoglobulin heavy chain polypeptides. In agreement with the precedent established in the murine and human μ-chain systems, it appears that the messenger RNA species encoding the membrane and secretory polypeptides are distinct molecular species generated by differential RNA splicing of a large nuclear RNA transcript of a single active V_H-D_H-J_H-C_H gene. It is proposed that this pathway is common to all types of B-lymphocyte whether primary or memory, and operates irrespective of the isotype which the lymphocyte is expressing.

The biochemical differences between γ_m and γ_s polypeptides and between α_m and α_s polypeptides remain to be established. The data herein has delineated the two structural forms only on the basis of which cellular or extracellular environment a given polypeptide is found. The evidence from experiments using lipophilic nitrenes can only be regarded as tentative and more conclusive evidence, for example, from carboxypeptidase digestion of isolated radiolabelled polypeptides, is needed before it can be stated unequivocally that the structural differences between membrane and secretory γ- and α-polypeptides are located at the C-termini of these chains.

The studies of the phenotypic product of the C_γ and C_α genes have provided hints as to the genotypic structures of C_γ and C_α genes.

Based on differences in AMW between membrane and secretory polypeptides, it is suggested that the C_α gene is structurally homologous to C_μ and possess similar T- and M-exons. The large AMW difference between γ_m and γ_s polypeptides suggests a different organisation of genetic elements. C_γ is proposed to have a small T-piece (2 codons) and large M-exon structures. Although the biosynthesis of ε_m and ε_s polypeptides has not been studies (there is no detectable ε_m polypeptide in U266BL cells), it is felt that the organisation of the C_ε gene will prove to be similar to that of the C_γ gene. IgD remains unique in having a genomic structure which conforms to neither the C_μ pattern or that proposed for C_γ (Tucker et al, 1980; Maki et al, 1981).

The conclusion drawn from these investigations is that memory B-lymphocytes utilise similar genetic strategies to those employed by primary B-lymphocytes to allow simultaneous biosynthesis of structurally distinct membrane and secretory immunoglobulin heavy chain polypeptides.

2.9. NOTES ADDED IN PROOF

1. Probing of a Northern blot of Bec-11 poly A^+ RNA with a ^{32}P-nick-translated γ_4-cDNA clone reveals two reactive species: these are proposed to represent the distinct mRNAs for γ_m and γ_s polypeptides (Coupar, Cushley and Williamson, unpublished).

2. The nucleotide sequence of the murine C_α gene has been determined and has been shown to have a T-piece, contiguous with $C_\alpha 3$, encoding 20 amino acids, and also to possess an intraexonic RNA splice site (Tucker *et al*, 1981, in press).

3. *In vitro* translation of poly A^+ RNA derived from a human lymphoblastoid cell line synthesising IgA followed by specific immunoprecipitation and analysis by SDS-PAGE reveals that two distinct α-chains are synthesised *in vitro* (McCune *et al*, 1981). This is in agreement with the findings for translation of Bec-11 mRNA *in vitro*.

CHAPTER 3

IMMUNOCHEMICAL INVESTIGATION OF THE UNIQUE 33,000 DALTON GLYCOPROTEIN (P33) FOUND IN ASSOCIATION WITH MEMBRANE IMMUNOGLOBULIN M OF THE B-LYMPHOMA CELL LINE DAUDI

3.1. BACKGROUND AND OBJECTIVES

The B-lymphoma cell line Daudi (Klein et al, 1968) exhibits several features which represent a departure from the expected pattern of expression of membrane antigens by B-lymphocytes. The most notable of these features is the failure to express HLA-A,-B or -C alloantigens on the cell membrane (Jones et al, 1975) despite the fact that Daudi cells synthesise the heavy chain of HLA-A, -B and -C alloantigens in their cytoplasm (Pleogh et al, 1979). The defect in membrane insertion of these macromolecules has been shown to be due to the absence of β_2 microglobulin (Fellous et al, 1977) caused by an abnormality on chromosome fifteen which carries the gene for β_2 microglobulin (Goodfellow et al, 1975). The Daudi cell line does express the HLA-DR alloantigens (Jones et al, 1975) and consequently has found much favour as a model system for the investigation of the role of HLA-DR alloantigens in activation of human mixed lymphocyte reactions.

The membrane IgM of Daudi cells also displays an unusual feature. The unusually high AMW of the Daudi kappa light chain has been well documented (Sherr and Uhr, 1971; Kennel, 1974) and it was originally proposed that the higher AMW could be accounted for by glycosylation of the normal light chain. This anomalous component has since been shown to be a distinct molecular species from the normal kappa light chain synthesised by Daudi cells (Singer and Williamson, 1979).

Many of the biochemical properties of this novel 33,000 dalton glycoprotein (P33) have been elucidated (Singer and Williamson, 1979) and there are striking similarities between P33 and the α-chain

of the HLA-DR alloantigens particularly with regard to isoelectric point and cysteine content (see table 3.1.). Furthermore, preliminary work leading up to this study suggested that a rabbit antiserum raised against Daudi membranes and capable of blocking mixed lymphocyte reaction activation (Deane et al, 1978; Van Heyningen et al, 1981) had specificity for P33 suggesting a functional relationship between P33 and the HLA-DR alloantigens.

These investigations were designed to study the serological reactions in which P33 was involved and to attempt to elucidate its immunological identity and therefore explain its apparent role in activation of the human mixed lymphocyte reaction (MLR).

3.2. SEROLOGICAL REACTIONS

3.2.1. Reaction of an MLR-Inhibiting Antiserum with P33

Rabbit antibody to Daudi cell membrane components involved in MLR activation was prepared as described by Deane et al (1978). (See materials and methods, section 5.6.). MLR activation is a property often associated with the HLA-DR alloantigens (Van Rood et al, 1976) and since the rabbit antibody (serum No.52) has been shown to be capable of inhibiting MLR activation (Van Heyningen et al, 1981) it was reasonable to propose that serum No.52 would recognise and precipitate the HLA-DR alloantigens.

The following experiment was performed to investigate the specificity of serum No.52. Aliquots of radioiodinated cell membrane proteins were treated with one of the following antisera plus GARIg:

a) rabbit anti-human μ-chain;

b) serum No.52, and

c) serum No.52 after absorption with Bjab cells.

FIGURE 3.1.

Serum No.52 is Specific for Daudi P33

10^7 viable Daudi cells were harvested from log phase cultures, washed twice with 25 ml aliquots of ice-cold serum-free RPMI-1640 and once with 25 ml of ice-cold PBS-I. The cells were radiolabelled by lactoperoxidase-catalysed iodination using 1 mCi ^{125}I (Methods, 8.2.), washed extensively and lysates prepared in 3D-TKM (Methods, 9.).

Radioiodinated membrane proteins were then treated with 1 µl of rabbit antisera and an equivalence of GARIg (Methods 10.2.1.). Washed immune precipitates were dissolved in 25 µl of SDS-PAGE loading buffer, boiled, and electrophoresed on a 12.5% (w/v) acrylamide slab gel (Methods, 13.). The pattern of radioactive components was visualised by fluorography (Methods, 17.6.).

The precipitating reagents used were:

A - Acetone (total membrane protein fraction).

B - RAHµ

C - Serum No.52.

D - Serum No.52 after absorption against Bjab cells.


A
B
C
D
μ
p33
K
Migration

The immune precipitates were washed, reduced and analysed by SDS-PAGE. The profiles obtained are shown in figure 3.1. An aliquot of total cellular proteins is also shown (track A).

Each antiserum precipitated identical components from aliquots of radioiodinated Daudi cell membrane proteins (tracks B, C and D), that is μ- and κ-chains and P33. The observation that serum No. 52 specifically immunoprecipitates the IgM-P33 complex (track C) and not the HLA-DR alloantigens, as was expected, suggests that the antiserum recognises one of the component chains of the IgM-P33 complex.

Treatment of aliquots of radioiodinated cell membrane proteins of several B-cell lines with serum No.52 and GARIg failed to immunoprecipitate any membrane proteins. Bjab cells were the only exception; serum No.52 was capable of immunoprecipitating Bjab membrane IgM but only to an extremely limited extent (data not shown). P33 is unique to Daudi (Singer and Williamson, 1979) and is not found on Bjab cells, and therefore it is probable that some of the antibody activity of serum No.52 is directed against μ-chains, κ-chains or an interaction antigen presented by μ- and κ-chains in complex. Therefore serum No.52 was extensively absorbed against Bjab cells, and the absorbed serum used in immunoprecipitation.

Reactivity with Bjab membrane IgM was abolished (not shown), but reactivity with the Daudi membrane IgM-P33 complex was unaffected. Since anti-μ and anti-κ antibodies would have been removed by absorption with Bjab cells, precipitation of the Daudi IgM-P33 complex by serum No.52 occurs through recognition of P33; μ- and κ-chains are co-precipitated by virtue of their covalent association with P33. Thus, serum No.52 has specificity for the P33 component of the Daudi IgM-P33 complex.

The above absorption experiments strongly suggest that serum No.52 recognises the P33 component of the Daudi IgM-P33 complex. However, the experiments do not rule out the possibility that serum No.52 recognises idiotypic determinants on the V_H or V_κ sequences of Daudi membrane IgM. These idiotopes may be a function of the amino acid sequence of the protein or may be influenced by carbohydrate moieties.

3.2.2. Hypothesis: P33 is Structurally Related to the Alpha Chain of the HLA-DR Alloantigens

P33 has several features in common with the HLA-DR α-chain (see table 3.1.). The most striking similarities between P33 and HLA-DRα-chain are the identical isoelectric points (pI=5.2) and identical cysteine content (3 residues per molecule). This latter finding is particularly significant since it rules out the possibility that P33 is an extended or heavily glycosylated light chain, as proposed by Kennel (1974), since light chains have a minimum requirement for five cysteine residues to fulfil their disulphide bonding requirements. The finding that MLR activation by Daudi cells can be blocked by an anti-serum specific for P33 adds support to the notion that P33 has some functional relationship to HLA-DR α-chains. Taken together, the biochemical and functional similarities lead to the formulation of the hypothesis that P33 represents an abberant expression of the HLA-DR α-chain.

Table 3.1. Biochemical Properties of HLA-DR α-Chain and Daudi P33

	HLA-DR α[1]	P33[2]
Molecular Weight	34 ± 2	33 ± 3
Glycosylation	Yes	Yes
Isoelectric Point[3]	5.2	5.2
Cysteine Content	3	3

1. After Springer et al, 1976.
2. After Singer and Williamson, 1979.
3. The isoelectric points given are approximate since both P33 and HLA-DR α-chains give a multiple band spectrotype upon isoelectric focusing. The value given corresponds to the mean isoelectric point.

3.3. RELATIONSHIP OF P33 TO HLA-A, -B, -C AND DR ALLOANTIGENS.

3.3.1. Serological Investigations.

The first test of the hypothesis stated above (3.2.2.) was to treat aliquots of radioiodinated Daudi cell membrane proteins with antisera to products of the HLA gene complex, and to compare the nature of the isolated products with those precipitated using RAHμ and serum No.52.

Figure 3.2. demonstrates that RAHμ (track A) and serum No. 52 after extensive absorption against Bjab cells (track B) both precipitate the IgM-P33 complex: HLA-DR alloantigens were never observed in SDS-PAGE profiles of RAHμ or serum No.52 immunoprecipitates of Daudi cell membrane proteins. Two rabbit antisera to HLA-DR alloantigens, No.254 (track C) and No.70 (track D), specifically immunoprecipitated components of AMWs 34,000 daltons and 29,000 daltons; these are the α- and β-chains, respectively, of the HLA-DR alloantigens. The characteristic profile of the IgM-P33 complex was never observed upon SDS-PAGE analysis of immune precipitates generated using the antisera specific for HLA-DR. The electrophoretic mobilities of P33 and HLA-DR α-chain are distinct suggesting a difference in primary structure. These data, particularly the failure of anti-HLA-DR antisera to precipitate the IgM-P33 complex, are a strong indication that P33 has no serological or structural relationship the the HLA-DR α-chain.

Support for the latter interpretation was obtained from further serological studies employing monoclonal antibodies covalently bound to sepharose. These studies (data not illustrated) provided

FIGURE 3.2.

P33 is not Recognised by Antibody to HLA-DR Antigens

10^7 viable Daudi cells were harvested from log phase cultures and prepared for lactoperoxidase catalysed iodination as described in the legend to figure 3.1. (Methods, 8.2.). Radioiodinated cells were washed extensively prior to preparation of lysates in 3D-TKM (Methods, 9.).

Aliquots of radioiodinated membrane proteins were treated with 1 µl rabbit antisera plus an equivalence of GARIg (Methods, 10.2.). Washed immunoprecipitates were dissolved in 25 µl of reducing SDS-PAGE loading buffer and analysed on a 12.5% (w/v) acrylamide slab gel (Methods, 13.). After electrophoresis, the gel was processed for fluorography (Methods, 17.6.).

The precipitating rabbit antisera were:

A - RAHµ

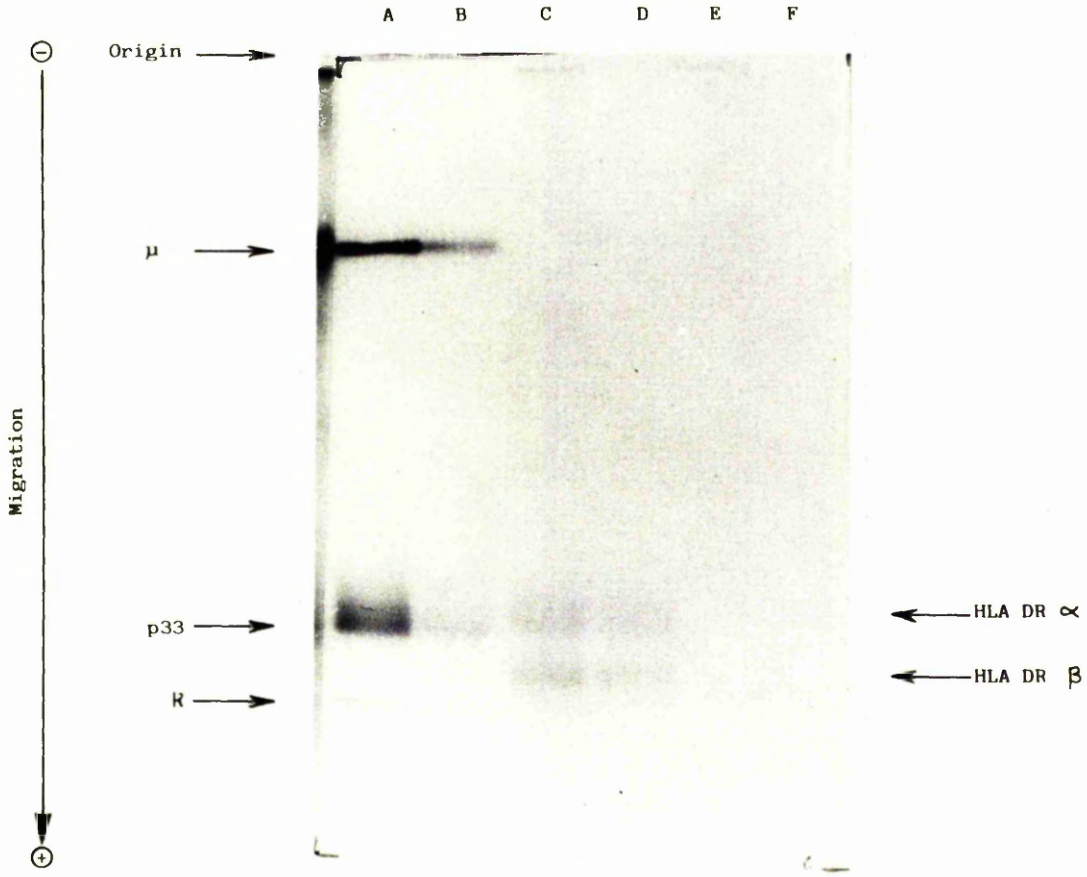
B - Serum No. 52 (absorbed against Bjab cells).

C - Serum No.254 (anti-HLA-DR).

D - Serum No. 70 (anti-HLA-A,-B,-C,-DR).

E - Anti-denatured HLA-A,-B,-C.

F - Normal rabbit serum.


A
B
C
D
E
F
Origin
μ
p33
R
Migration
HLA DR α
HLA DR β

the following information:

a) IgM-P33 was not retained on affinity columns of monoclonal anti-HLA-DR antibody, and could be immunoprecipitated from material eluting in the void volume of the column, and

b) HLA-DR antigens were retained by the affinity columns and could be subsequently eluted.

Thus, the evidence argues strongly against the hypothesis that P33 represents an abberant expression of the HLA-DR α-chain.

It has been reported that certain xenoantisera could detect HLA-A, -B and -C antigens on the Daudi cell membrane (Ostberg et al, 1975). Track E of figure 3.2. illustrates that a rabbit antiserum to denatured HLA-A, -B, -C antigen heavy chain (Tanigaki et al, 1975) failed to precipitate any material from aliquots of radioiodinated Daudi cell membrane proteins. Furthermore, no Daudi membrane proteins were retained on an affinity column of monoclonal antibody to a determinant common to HLA-A, -B, -C antigens but which was not β_2 microglobulin (data not shown). This evidence also refutes any suspicions that P33 may have been a fragment of the HLA-A, -B, or -C antigen chain.

The serological data are therefore inconsistent with the hypothesis that P33 is in any way related to HLA-A, -B, -C or -DR antigen chains.

3.3.2. Two-Dimensional Analysis of Tryptic Peptides of P33 and HLA-DR Antigens.

Tryptic digests of SDS-PAGE purified P33 and HLA-DR antigen component chains were compared by two-dimensional peptide mapping

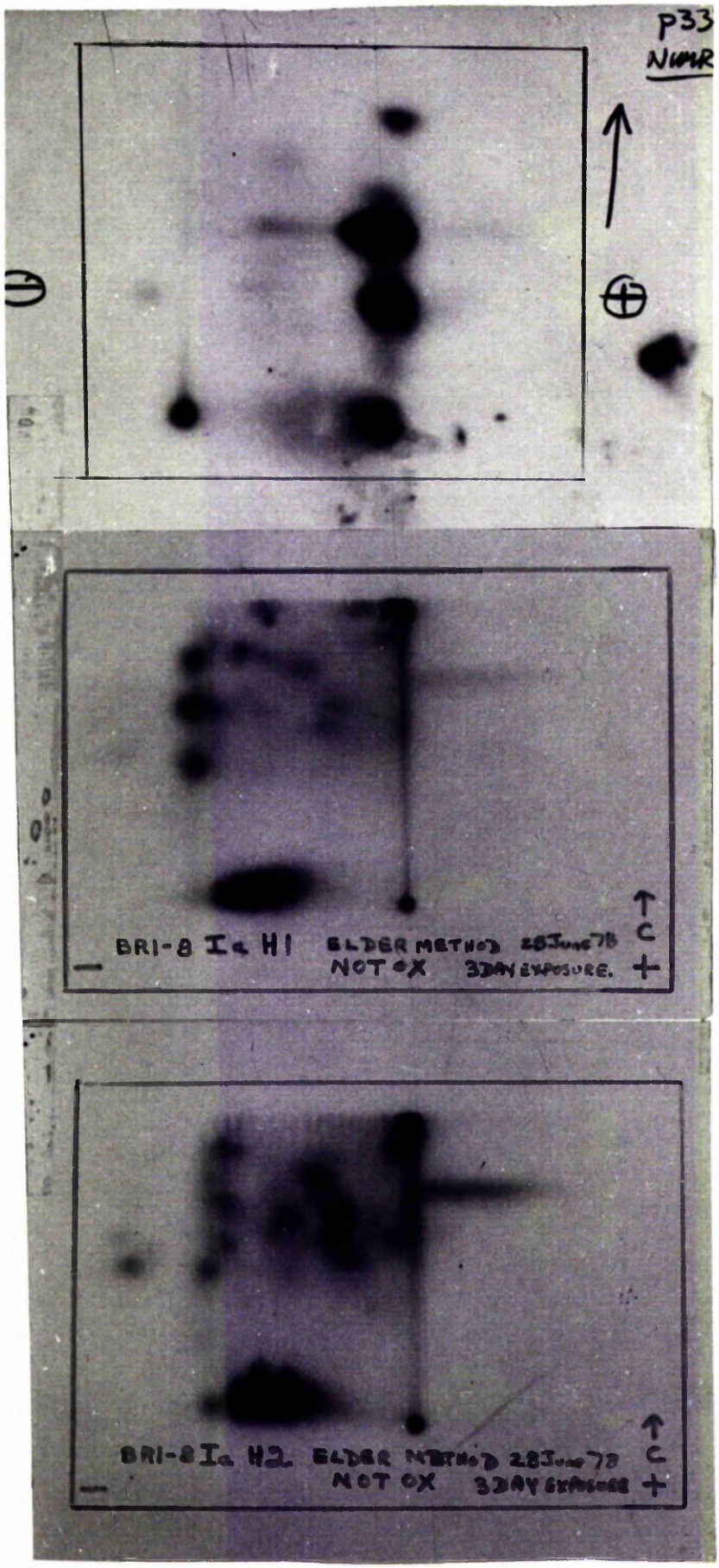
FIGURE 3.3.

Two-Dimensional Analysis of a Tryptic Digest of P33

IgM was isolated from aliquots of Daudi radioiodinated cell membrane proteins by specific immunoprecipitation using RAHμ and GARIg. The washed precipitate was reduced and electrophoresed on 12.5% (w/v) acrylamide tube gels. The gels were sliced, counted, and the slices containing p33 identified. P33 was eluted from the gel slices (Methods, 13.6.) and digested with trypsin (Methods, 14.1.).

The digest was dissolved in water and subjected to high voltage electrophoresis (Methods, 14.2.) followed by ascending chromatography (Methods, 14.3.) on a thin-layer cellulose plate. After chromatography, the plate was allowed to dry and was then autoradiographed.

Upper Plate	-	P33
Middle Plate	-	HLA-DR-α
Lower Plate	-	HLA-DR-β


P33
NMR
BRI-8 Ia H1 ELDER METHOD 28 June 78
NOT OX 3 DAY EXPOSURE.
C
BRI-8 Ia H2. ELDER METHOD 28 June 78
NOT OX 3 DAY EXPOSURE
C

(Barber and Crumpton, 1976). I am indebted to Dr. M.J. Crumpton for his assistance in carrying out this experiment during a visit to his laboratory.

The chromatographic fingerprint obtained is shown in figure 3.3. Comparison of the P33 fingerprint with either of those of the HLA-DR alloantigen chain shows no similarity. The major components of the P33 fingerprint migrate anodally while those of the HLA-DR alloantigens migrate cathodally. These data provide further circumstantial evidence that P33 has no structural relationship to HLA-DR α-chain. The differences seen are too great to be accounted for by the fact that the comparison is made between Daudi P33 and Bri-8 HLA-DR alloantigen.

The serological and chemical analyses described above provide compelling evidence that P33 is not an HLA-DR alloantigen α-chain.

3.4. ROLE OF N-LINKED OLIGOSACCHARIDES IN EXPRESSION OF DAUDI MEMBRANE IgM AND IN SEROLOGICAL ACTIVITY OF P33

3.4.1. Membrane Deposition of Daudi Membrane IgM Requires N-Glycosylation

Data presented in results chapter 1 illustrate the requirement for N-glycosylation of Daudi IgM for efficient membrane deposition of this macromolecular complex (see figure 1.12). In the SDS-PAGE profiles of IgM immunoprecipitated from tunicamycin-treated Daudi cells, only the glycosylated μ, κ and P33 components were resolved. No bands were resolved which could be proposed as candidates for non-glycosylated P33 molecule. These data also

suggest that the unusual structure of Daudi membrane IgM does not exempt this molecule from the requirement for N-linked carbohydrate side chains for efficient membrane deposition of immunoglobulins. P33, whether glycosylated or not, is probably not involved in the intracellular events leading to the membrane deposition of Daudi IgM.

3.4.2. Role of Carbohydrate in Serological Activity of P33

The role of oligosaccharide moieties in the serological activity of Daudi P33 was investigated in the following experiment. Cultures of Daudi cells, control or tunicamycin-treated, were pulsed with 500μCi ^{35}S-methionine for 10 minutes prior to a chase for 1 hour in complete medium. Aliquots were removed at frequent intervals, lysates prepared, and IgM-P33 macromolecular complexes specifically immunoprecipitated using RAHμ or serum No.52 and the S. aureus immunoabsorbent. The SDS-PAGE profiles are shown in figure 3.4. Note that in this analysis, the presence of μ-chain is taken as being indicative of:

a) IgM-P33 complex assembly, and

b) serologically active P33.

Methionine is not readily incorporated into P33 and, therefore, it was decided to invoke similar arguements to those advanced in results chapter 1 in the analysis of immunoglobulin assembly. P33 is covalently bound to the IgM molecule (Singer and Williamson, 1979) and serum No.52 has specificity for P33 (see figure 3.1.). Thus, the presence of a serologically reactive P33 moiety on Daudi IgM would result in the precipitation of μ-chains.

RAHμ was capable of immunoprecipitating IgM from the lysates

FIGURE 3.4.

Role of Carbohydrate in Serological Reactivity of P33

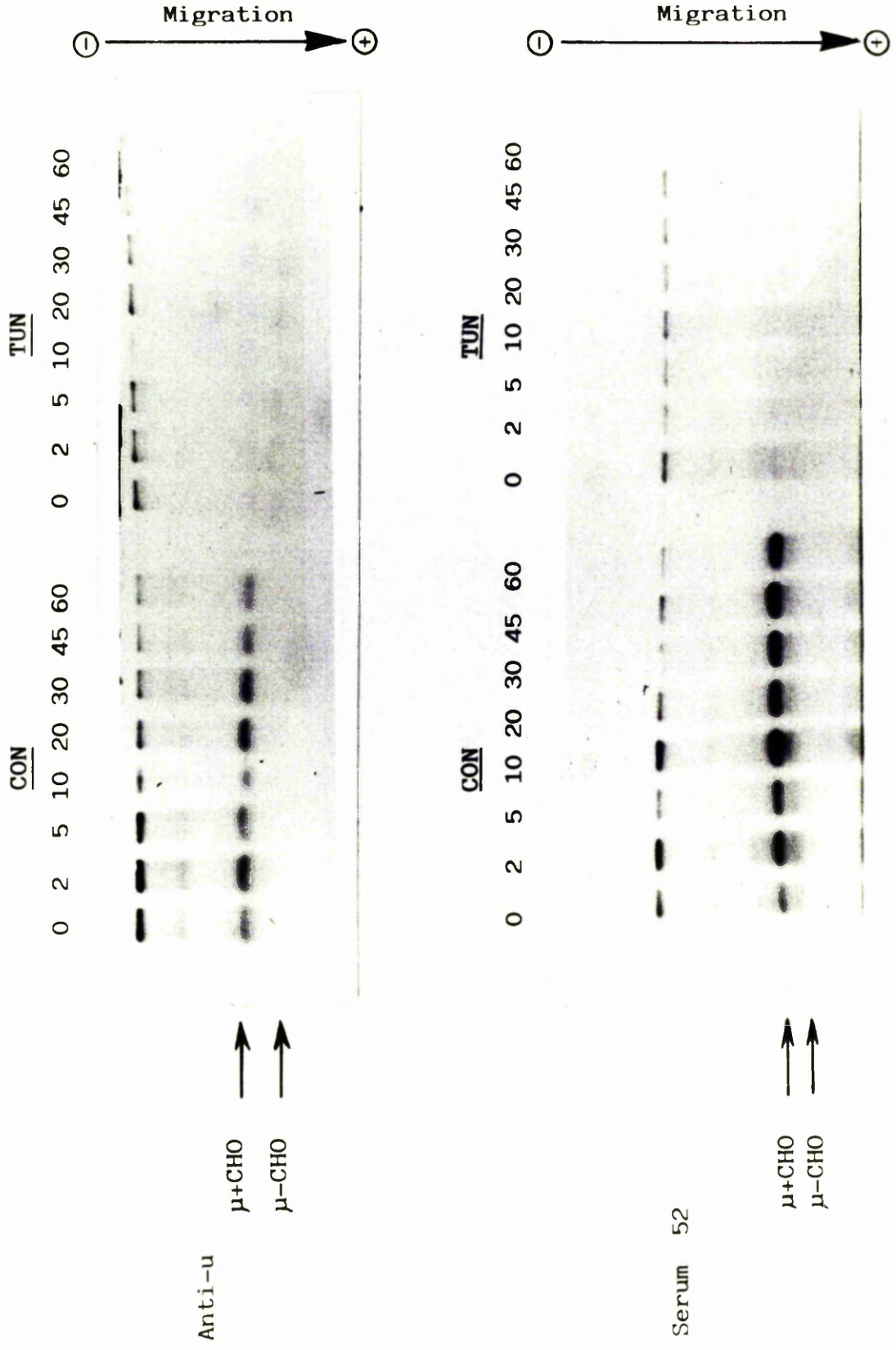
10^7 Daudi cells were treated with 2 µg/ml tunicamycin at 37°C for 3 hours in complete medium. Cells were washed twice with labelling medium and resuspended in 0.1 ml of the same medium containing 500 µCi ^{35}S-methionine: parallel control cultures were also prepared. Labelling was carried out at 37°C for 7 minutes whereupon 1 ml complete medium was added (time 0) and the culture reincubated at 37°C. 0.1 ml aliquots were removed at times 0,2,5, 10,20,30,45 and 60 minutes.

Aliquots removed at each time point were added to 100 µl ice-cold PBS-azide and washed twice with the same buffer prior to preparation of lysates in 100 µl 3D-TKM (Methods, 9.). Lysates were treated with 5 µl of either RAHµ or serum No.52 (absorbed on Bjab cells) plus 100 µl of 10% (v/v) S.aureus in 3D-TKM and incubated overnight at 4°C. The bacteria were washed three times with 3D-TKM and immune complexes eluted by boiling the organisms in 2% (w/v) SDS. The bacteria were pelleted, and proteins in the supernates were precipitated by addition of 5 volumes of acetone. The precipitates were dissolved in 25 µl of reducing SDS-PAGE sample buffer and analysed on a 10% (w/v) acrylamide slab gel (Methods, 13.). The gel was processed for fluorography (Methods, 17.6.).

Key:-

Con - Material isolated from lysates of control cultures.

Tun - Material isolated from lysates of tunicamycin-treated cultures.


Migration
⊖
⊕
CON
TUN
0 2 5 10 20 30 45 60 0 2 5 10 20 30 45 60
μ+CHO
μ−CHO
Anti-u
Migration
⊖
⊕
CON
TUN
0 2 5 10 20 30 45 60 0 2 5 10 20 30 45 60
μ+CHO
μ−CHO
Serum 52

of control and tunicamycin-treated Daudi cells (figure 3.4. upper panel). However, serum No.52 precipitated IgM from lysates of control cells only: IgM was never detected in immunoprecipitates derived from tunicamycin-treated cells (figure 3.4. lower panel). It is therefore reasonable to conclude that the N-linked oligosaccharides of P33 are of critical importance in the serological reactivity of that glycoprotein.

However, the level at which the N-linked sugar moieties exert their influence is not clear from the above experiment, and two explanations of the data are possible. Firstly, one might propose that the IgM-P33 complex is assembled but is not serologically reactive due to:

a) absence of an exclusively carbohydrate determinant recognised by serum No. 52, or

b) failure of expression of a complex 'interaction antigen' structurally influenced by oligosaccharide moieties.

A second interpretation is that P33 is not integrated into the IgM molecule in the absence of N-linked carbohydrate and the IgM complex can not be immunoprecipitated by the P33-specific serum No.52.

Preliminary experiments to explore the latter possibility suggest that assembly occurs in tunicamycin-treated Daudi cells, as evidenced by the presence of non-glycosylated μ-chains in SDS-PAGE profiles of immunoprecipitates generated from lysates of such cells using RAHκ (data not shown). These data favour the hypothesis that carbohydrate plays an important role in expression of the serological determinant recognised by serum No.52. Clearly, however, more

decisive experiments are required before this notion can be regarded as conclusive.

3.5. SPECULATION ON THE GENETIC ORIGIN AND IMMUNOLOGICAL SIGNIFICANCE OF P33

3.5.1. Role of the HLA Complex.

Serum No.52 has been demonstrated to have the capacity to inhibit MLR reactions in which Daudi cells act as the stimulator population (Deane et al, 1978; Van Heyningen et al, 1981). The evidence herein is consistent with the hypothesis that serum No.52 is specific for the P33 component of the Daudi IgM-P33 complex. The serological studies herein disprove the hypothesis that P33 is a fragment or other abberant phenotypic expression of either HLA-A, -B, -C or HLA-DR antigens. The immediately obvious conclusion from these data is that P33 is not a product of a structural gene located in the HLA complex.

An explanation must be found for the inferred ability of P33 to activate the MLR. Three possible explanations may be tentatively proposed:

a) an artefact of the antiserum;

b) recognition of P33 as antigen X in association with self-histocompatibility antigen, and

c) P33 as a glycosidically 'altered self' molecule.

The serological data described above (figure 3.1.) suggest that serum No.52 recognises P33 and not HLA-DR antigens as assessed by immunoprecipitation studies. The possibility that the antibody

responsible for the MLR-inhibition and that responsible for immunoprecipitation of the IgM-P33 complex are different, must be considered. If this proposal is correct, then a minor population of anti-HLA-DR antibodies would exist in serum No.52 which would be non-precipitating, either with GARIg (outside the optimum proportions limits of the system) or with S.aureus such that any immune complexes formed by this minor population would not be detected by the immunoprecipitation - SDS-PAGE analysis. The anti-HLA-DR antibodies would, however, be capable of inhibiting the MLR. It may be possible that the antibody population responsible for MLR inhibition recognises a non-HLA-DR, MHC-encoded MLR activatory molecule (the elusive HLA-D product).

Can the postulated ability of P33 to activate the MLR be accounted for in terms of current models of immune recognition by T-cells? A simple explanation which may be advanced in answer to this question is that in a dual-recognition model (e.g. Doherty et al, 1976; Williamson, 1980) P33 could be recognised as nominal antigen X which is recognised in association with the histocompatibility antigen of Daudi cells, HLA-DRw6 in the case of the MLR. Thus, simultaneous occupation of the:

a) V_T anti-X receptor (by P33), and

b) R anti-H receptor (by HLA-DRw6),

would result in amplification of a specific clone of T-lymphocytes, giving rise to a detectable MLR. Recognition of P33 as antigen X in association with histocompatibility antigen is also compatible with one-receptor models.

An example of an altered-self model is provided by an

hypothesis which proposes that the mechanism which results in alteration of self antigens is variation in glycosylation of the antigen (Parish et al, 1981). If such a model were representative of in vivo mechanisms, the immunological properties of P33 may be accounted for by proposing that P33 is an over-glycosylated κ-light chain or κ-light chain fragment (vide infra) and as such presents an altered-self idiotype to the T-lymphocyte in conjunction with HLA-DR which results in a proliferative response. Such a model is inconsistent with the findings of Black et al, (1981) who found that non-glycosylated H-2 class 1 molecules are less efficient target molecules for cytotoxic T-lymphocytes than their glycosylated counterparts. The Parish model is also inconsistent with findings of many groups that viral glycoproteins require N-glycosidically linked oligosaccharide groups for membrane localisation (see introduction, table 1), and it is known that cytotoxic T-lymphocytes require to "see" both antigen and histocompatibility antigen (Doherty et al, 1976).

These arguements lead to the conclusion that the role of the HLA gene complex in the structure and function of P33 is associative rather than integral or primary: that is, P33 is not a gene product or abberant gene product of the HLA supergene, but the possible MLR activatory properties of this molecule would be accountable in terms of recognition of P33 in association with histocompatibility antigen.

3.5.2. P33 as a Kappa Chain Fragment

In the original observations of Daudi IgM (Sherr and Uhr, 1971; Kennel, 1974), it was proposed tha P33 represented a highly

glycosylated κ-light chain. Charge-shift isoelectric focusing experiments demonstrated that this was not the case (Singer and Williamson, 1979). However, there are data available which suggest that P33 has κ chain determinants and the possiblity that P33 represents a heavily glycosylated κ chain fragment must therefore be considered.

Elution of P33 from SDS-PAGE gel slices followed by digestion with the enzyme endoglycosidase H1 generated a molecular species which was precipitable by anti-κ antibody and had an AMW of 15-16,000 daltons, (R.T. Kubo, personal communication). Treatment of cell-free translation products, the synthesis of which had been directed by Daudi poly A^+ RNA, with anti-κ antibody resulted in immunoprecipitation of a 28K species and of an 18K species. The 18K species was a minor product visible only upon prolonged fluorographic exposure (Singer and Williamson, unpublished observation). It is proposed that this 18K species is the primary translation product of an abberant κ-mRNA, which loses its leader sequence to generate the 15-16K species described by Kubo prior to N-glycosylation to yield the 33K glycopeptide.

3.5.3. Possible Genetic Origin of Mutant κ-mRNA

Unequivocal evidence to demonstrate that P33 is a heavily glycosylated fragment of a κ-light chain is needed. However, in view of the circumstancial evidence to support this hypothesis it is relevant to speculate on the arrangement of the κ-coding sequences involved and the possible molecular lesions which might give rise to such an unusual polypeptide. A precedent for an abberant κ-chain is provided by the plasmacytoma MPC-11 (Kuehl and Scharff, 1974). The

mutant κ-mRNA produced by this cell line contains nucleotide sequences coding for the leader sequence and C_κ sequences but lacks the corresponding information for V_κ and J_κ (Seidman and Leder, 1980; Choi et al, 1980). A similar phenomenon in Daudi would explain the observed data for the P33 protein.

The P33 molecule must contain three cysteine residues. These three residues can be easily obtained by translation only of the C_κ gene which contains 3 codons for cysteine. This proposal involves either:

a) deletion of V_κ and J_κ exons;

b) failure to transcribe V_κ and J_κ, or

c) failure to correctly process the VJ unit in the primary nuclear RNA transcript to yield a functional VJ unit in the mRNA.

These caveats are necessary because utilisation of V_κ and J_κ would require partial use of C_κ to obtain a third cysteine, and such a model requires that the P33-κ gene be a pseudogene; i.e. contains a termination codon located 3' to cysteine codon 134. While such a situation is not impossible, the use of the C_κ exon and failure to use V_κ and J_κ sequences is a more attractive model. A further piece of circumstantial evidence to support the above notion is the lack of methionine in C_κ sequences (see Kabat et al, 1979). Methionine is not incorporated into P33 in biosynthetic labelling experiments, but is incorporated in in vitro translation of Daudi poly A^+ RNA. These observations are again consistent with the hypothesis that P33 mRNA contains coding sequences for the leader sequence and C_κ but has no V_κ or J_κ sequences. Translation of such

a messenger would give rise to a translation product of AMW 16,000-17,000 daltons which after removal of the leader sequence would have an AMW of 13,000-14,000 daltons. This is consistent with findings at the protein level.

One flaw in the arguement is the lack of N-glycosylation sites in C_κ sequences (Kabat et al, 1979). However, C_κ is rich in serine (16 residues) and threonine (7 residues) which may be O-glycosylated. If O-glycosylation of P33 occurs, this would account for the failure to detect a putative non-glycosylated form of P33 in biosynthetic experiments in the presence of tunicamycin.

The molecular mechanism which may give rise to such a unique polypeptide is non-productive gene rearrangement. As has been described for MPC-11, it is proposed that a non-productive V_κ•-J_κ joining event has occurred which has deleted the J_κ sequences and their RNA splice site. Therefore, J_κ-C_κ RNA splicing can not occur and the only splicing event which the κ-nuclear RNA transcript can undergo is to splice the leader sequence immediately 5' to C_κ. This would give rise to an mRNA containing leader and C_κ exons but no V_κ or J_κ coding sequences.

The chemical characterisation and immunological identification of P33 remains incomplete. The data presented herein demonstrate that P33 is not a gene product of the HLA gene complex although an antiserum capable of inhibiting the MLR reacts specifically with P33. Glycosylation appears to be necessary for the serological reactivity of P33. The hypothesis proposed above, that P33 is a heavily glycosylated C_κ fragment, makes it unclear whether the glycosylation is necessary on P33 or on μ-chains. The hypothesis that

P33 is a product of a κ-pseudogene is interesting because, if substantiated, it will represent the first example of assembly of a defective immunoglobulin gene product into a completed and expressed immunoglobulin molecule.

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