BIOCHEMICAL AND GENETIC STUDIES OF ANTIBODY (IgM) PRODUCING CELLS

by

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SIGNED: Luz Vázquez Moreno

DEDICATION

This dissertation is dedicated to my parents, Carlos and Rosalba Vazquez for their constant support and love.

To my sisters Rosalinda, Delia and Hilda, and to my brother Carlos.

Very specially, I would like to offer this work to : Francisco, Carlos, Efrain, Karla, Alberto, Rodolfo C., Oscar, Margot, Paola and Gabriela. They are continuous inspiration and the future's hope.

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ABSTRACT

We have chosen the murine immunoglobulin M (IgM) as system to study glycoprotein biosynthesis and carbohydrate processing. Secreted IgM heavy chain (μ) has five glycosylation sites which location and structures have been determined. μ chain variable region (VH) is involved in antigen binding, while the constant region (CH) is responsible for the effector functions in which the carbohydrate plays an important role.

We have determined the carbohydrate structures present at each glycosylation site of IgM produced by a hybridoma cell line (PC 700) and its derived mutants and compared them to IgM from myeloma cell MOPC 104E. PC 700 mutants secrete altered IgM. The alterations include: deletion of one or more constant domains (mutants: 128, 313, and 562) and μ chain hyperglycosylation (mutants 21 and 38). Gene analysis indicated that deletions can arise from two different mechanisms. One of these involve a major gene change (mutant 128), while others come from base point mutations (mutants 313 and 562). Cells 21 and 38 did not appear to have μ gene insertions.

Determination of purified single glycosylation site structures show that PC 700 μ chain is processed only to

biantennary. Heavy chain protein fragmentation and carbohydrate atudies indicate that mutants 21 and 38 alterations are due to an increase in oligosaccharide processing and reduction of unprocessed atructures. There is a trend of processing going from PC 700< 21< 38. In additon, our results show how growth cell conditions can affect the carbohydrate processing without altering the determinants of m chain oligosaccharide atructures.

Studies on the IgM molecule illustrate the need for precisely define atructure-function relationships. This would allow the selection of the best antibodies for studies such as those involved in immunotherapy.

CHAPTER 1

INTRODUCTION AND RATIONALE

Glycoproteins are a complex group of macromolecules widely distributed in nature. They have been found in virtually all forms of life. Glycoproteins have been identified in extra and intracellular fluids, connective tissue and cellular membranes.

Interest in glycoproteins is due to the diverse biological functions which they perform. These include, among others, immunological protection, enzyme catalysis, hormonal control, blood clotting, surface protection, cell adhesion and molecular recognition (1). Recent work continues to support a primary role for glycoproteins in tumor metastasis (2).

The key step in the transformation of a protein into a glycoprotein is the glycosylation of the peptide chain during translation. In asparagine-linked (N-glycosidic) glycoproteins, most Asn-X-Ser/Thr sequences serve as acceptor for the same carbohydrate structure. After translation is completed, the oligosaccharides linked

to the folded protein serve as substrates for a number of glycosidases and glycosyltransferases which act in concert to modify (or process) the carbohydrate to give the final structures [see (1) for recent review]. These events occur begining in the endoplasmic reticulum (ER) and continue in the cis and trans regions of the Golgi apparatus (1,12,15).

In this section I will give a brief overview of research into the process of protein glycosylation. A more detailed discussion will be presented in the later sections in the context of my experiments.

The regulation of oligosaccharide processing is not well understood, mainly because of the complexity of the glycosylation system and the great difficulty in obtaining carbohydrate structural data. Current research is in three areas:

1) Studies on the pathway of cellular glycoproteins through the endoplasmic reticulum, and to the cell surface (3). The signal sequences on secretory, plasma membrane and lysosomal proteins initiate the common pathway through the ER-Golgi system (4). Using <u>in vitro</u> translation systems it was possible to follow the fate of a nascent secretory protein. First, as translation occurs, the nacent protein signal peptides are recognized by a signal recogniton particle (SRP) which interacts with its

receptor (SRP receptor) located at ER membrane (5, 6, and 7). Then, the protein is glycosylated as is transported across the membrane. Rothman and coworkers (9 and 10) in studies of the mechanisms for transport through the Golgi apparatus have designed a cell fusion technique which results in the formation of hybrid cytoplasms containing two distinct Golgi populations. These studies defined three sequencial compartments in the Golgi stack, where transport-coupled glycosylation was used to monitor movement of a glycoprotein between two distinct Golgi apparatus populations. Final carbohydrate processing takes place in these cell compartments.

2) Studies on the specificity of glycosidases and glycosyltransferases as determinants of carbohydrate structure. Robbins et al (11) compared the specificity of rat liver glucosidases. Glucosidase I removes only the terminal residue of the oligosaccharide precursor which contains three glucose residues. Glycosidases II and III are inactive towards the substrate for glucosidase I. High Mannose oligosaccharides arise by specific mannosidase action on various mannose residues from the oligosaccharide precursor containing nine mannose residues (1). N-acetylglucosamine transferases (GlcNAC-transferases) are required for the synthesis of complex type oligosaccharides (12). Schachter et al (13) have purified four of

these enzymes. Studies using these enzymes provided important information about steric factors involved in the control of oligosaccharide branching in glycoproteins.

3) Studies of how the system functions to regulate the carbohydrate structure on glycoproteins. This is the least understood area. Conformational protein studies and in vitro processing of glycoproteins have indicated that factors such as oligosaccharide chain accessibility and tissue substrate enzyme-specificities are involved in regulation of processing (14,15,16). This research is complementary to the two approaches described above.

My own research is an effort to further explore the capability of the in vivo glycosylation system to determine carbohydrate structures. The combination of these approaches should greatly expand our understanding of cellular mechanisms determining carbohydrate structures.

Mouse immunoglobulin M (IgM) offers many advantages as a model glycoprotein. The gene rearrangements producing the heavy chain gene have been extensively characterized (17-23). The primary amino acid sequence and the location of five asn-linked glycosylation sites are known (24, 25, and 26). The heavy chain is produced by tumor cell lines and hybridomas that represent several differentiation stages (27). Finally, Anderson, Atkinson and Grimes (26) have recently completed the studies on the carbohydrate

structures at each of the five glycosylation sites on murine IgM produced by MOPC 104E cells using 500 mHz proton nuclear magnetic resonance (H-NMR). Figure 1 shows a schematic drawing of the heavy chain (m) of IgM showing the location of glycosylated asparagine residues.

In my own experiments, I set out to study IgM produced by a hybridoma cell line PC700. This antibody is specific for phosphorylcholine. Shulman (28) have mutagenized cells and use a suicide selection method to isolate cell lines producing altered IgM. My goal was to compare the glycosylation of IgM from PC 700 with structures determinated for each glycosylation site on MOPC 104E, and determine how glycosylation is altered in variants isolated by Shulman from PC700 cells.

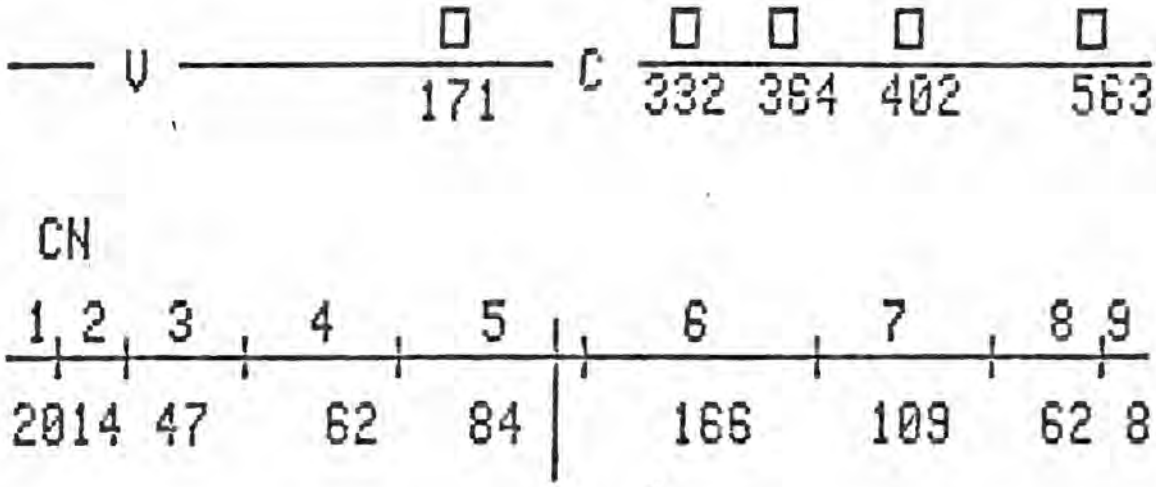


Figure 1. Schematic drawing of the heavy chain of IgM MOPC 104E. Shown are (i) carbohydrate attachment sites and corresponding asparagine residue locations within the polypeptide chain, (ii) the demarcation between constant (Cm) and variable (VH) regions, and (iii) the sites of cleavage by CNBr and fragments generated (CN1-CN9).

CHAPTER 2

LITERATURE REVIEW

Glycoproteins: Structure and Function

Glycoproteins occur in cells both in soluble and membrane bound forms, as well as in the intercellular matrix and extracellular fluids. Plants, viruses, bacteria and higher animal cells carry glycoproteins which serve a variety of functions (1).

The presence of oligosaccharide chains covalently attached to the polypeptide backbone is the feature that distinguishes glycoproteins from other proteins and determines some of their biological properties. A great deal of effort has been exerted in recent years, to relate structures of oligosaccharides to specific functions.

The body fluids are rich in glycoproteins secreted from various glands and organs. Glycoproteins of blood plasma which include transport proteins (e.g.transferrin),ceruloplasmin and immunoglubulins, and many components of complement (29,30).

Other glycoproteins are enzymes such as ribonuclease, deoxyribonuclease and a-amylase (31).

The vast majority of cell membrane integral proteins are glycosylated. There is increasing evidence suggesting that cell surface glycoproteins are involved in a number of physiological functions, including cell interactions, adhesion of cells to a substratum, and migration of cells to particular organs (32). For example, neural cell adhesion molecules or N-CAM are glycoproteins present in a variety of species and appear early in embryogenesis (32). The N-CAM molecules are postulated to be involved in the formation of cell-cell bonds as demonstrated in studies using anti-N-CAM (fab') fragments. The antibody fragments specifically block adhesion of spinal cord neurites and muscle cells, without altering the cell morphology. CAM molecules vary in their sialic acid content according to tissue and developmental stage. Edelman (33) has proposed that CAM molecules act as regulators of morphogenic movements that are essential for early induction of differentiation. Studies of cell substratum interactions have shown that specific changes in cell surface structures are associated with mammalian cell transformation. These changes include alterations in oligosaccharide structures of glycoproteins and glycolipids. The asparagine-linked oligosaccharides of transformed cells appear to be larger due to increased

terminal sialylation and/or increased branching (34,35). Dennis and Carver (2) have shown that the metastatic capacity of malignant cell lines may be closely related to the acquisition of certain surface oligosaccharides. A non-metastic mutant cell line contained a three to four fold reduction in cell surface sialic acid. Furthermore, these cells attach more readily to fibronectin and to collagen-coated surfaces than wild type cells which show high metastatic capacity. This characteristic may prevent the cells (non-metastic mutants) from escaping their primary subcutaneous site.

Types of Carbohydrates Found in Glycoproteins

There are five major types of linkages between oligosaccharide and protein. The most common on cellular glycoproteins include: N-acetylglucosaminyl-asparagine linkage or N-glycosidic, and O-glycosidic linkages. Glycoproteins containing the N-glycosydic type are the most extensively studied and are reviewed here.

The N-glycosydic-linked oligosaccharides have heterogenenous structures that usually fall into two categories: "high-mannose" and "complex". Examples of both types are given in figure 2. Diagrams (a) and (b) are complex triantennary and biantennary, while (c) is a high mannose type. Both classes have an inner core of Mannose$_3$-N-acetylglucosamine-$_2$

(Man_3-$GlcNac_2$) attached to an asparagine residue. High mannose oligosaccharides contain additional a-linked mannose residues, while complex oligosaccharides carry other external sugars such as GlcNac, galactose (Gal), Fucose (Fuc), and sialic acid (SA) (1). Complex type oligosaccharide can have several branches, with biantennary complex oligosaccharide (Figure 2b) containing two, while triantennary (figure 2a) have three. For a while it was thought that sialic acid acted as a termination signal, limiting the number of structures formed. This is now known not to be the general case, since some N-glycosidic units terminate in galactose or NAcGlc. Recently several groups have reported the presence of hybrid structures in rhodopsin, ovalbumin and some lysosomal hydrolases (36,37,38). In these structures, one branch contains outer sugars (Gal, SA, and/or Fuc) while the other contains 1 or 2 Man residues. Yamashita et al (40), working on hamster kidney cells and their polyoma transformants found an increase in tetraantennary oligosaccharides containing Gal-[1-4]-GlcNAc repeating structures in their outer chain moieties. A single glycoprotein may carry different types of oligosaccharides as has been demonstrated in VSV glycoprotein, ovalbumin, and murine IgM heavy chains (1,25,26). Furthermore, a purified glycopeptide containing a single glycosylation site can exhibit a number of related structures, called microheterogeneity (1).

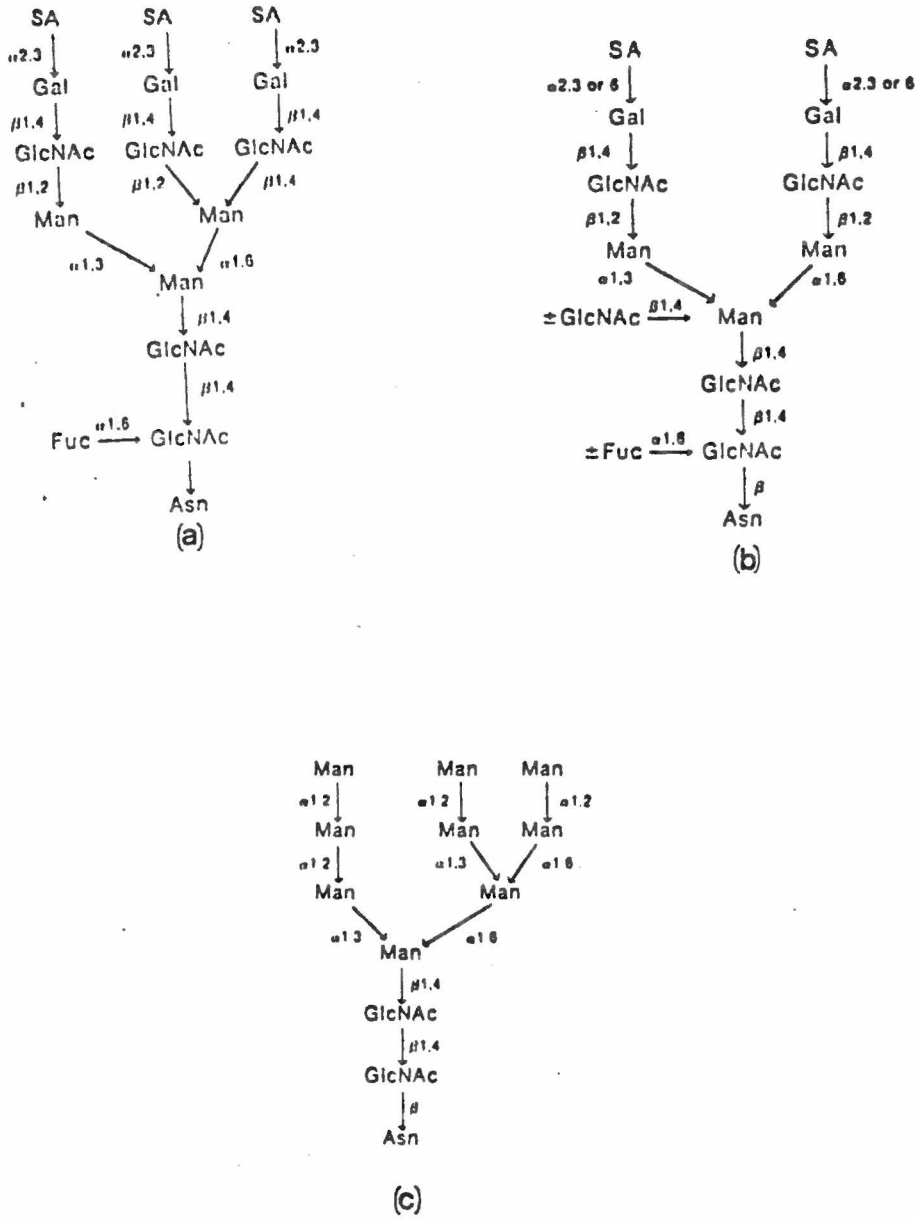


Figure 2. Common structures of asparagine-linked glycopeptides. a) Complex Triantennary, b) Complex Biantennary, and c) High Mannose. Fucose (Fuc), Mannose (Man), N-acetylglucosamine (GlcNAc), Galactose (Gal),and Sialic acid (SA).

The pathways and reactions leading to the formation of asparagine-linked carbohydrates have been described in detail (1). Regulation of how a form of glycosylation is expressed at a given protein is not understood. Chapter 4 includes a detailed description of oligosaccharide biosynthesis, assembly to the protein backbone, and some information about regulation of final structures.

Role of Carbohydrate in Glycoproteins

In recent years, the complete structure of carbohydrate units of a number of glycoproteins of well defined function have been elucidated in detail (41-45). At the same time, attention has been focused on the intriguing question of the biological functions of the carbohydrate units. One of the most important observations is the realization that sugar polymers can carry more information than polypeptides or polynucleotides (31).

Demonstrated carbohydrate function include:

a) The serological specificity of blood group heteropolysaccharide, which is dictated by specific sugars linked to a common core. Morgan and Watkins (41) demonstrated that the specificity of the major blood types is determinated by sugars. For example the difference between the blood types A and B lies in a single sugar unit. In type A the determinant is N-acetylgalactosamine, and in blood type B galactose.

b) The role of certain sugars in controling the lifetime of glycoproteins in the circulatory system of higher animals, and regulation of glycoprotein uptake by cells. Ashwell and Morell (42) have shown that many native and modified glycoproteins carrying exposed Gal, GlcNac, or Man are recognized by specific liver receptors and removed from circulation .

c) A fibroblast receptor which is able to recognize hexose phosphate is involved in the uptake of lysosomal glycoproteins (B-glucuronidase, B-hexosaminidase, and B-galactosidase). In these cells mannose-6-phosphate is the signal to direct hydrolytic enzymes from the endoplasmic reticulum to lysosomes. The existence of a receptor mediated system in cultured fibroblast was discovered by Neufeld and Ashwell (43) as a result of studies on genetic disorders of mucopolysaccharide catabolism.

d) The removal of carbohydrate from gonadotropins such as human Chorionic Gonadotropin (hCG) results in the loss of biological activity, implying that the carbohydrate is essential for hormonal action (44,45). Reconstitution experiments using deglycosylated hCG failed to stimulate hormonal activity measured by cAMP production (in vitro) and progesterone release (in vivo). In addition, deglycosylated hCG acts as a potent inhibitor of hCG activity, since removal of carbohydrate does not affect receptor binding.

e) It has recently been shown by Nose and Wigzell (46) that the carbohydrates of immunoglobulin G (IgG) are required for certain antibody function. IgG isolated from cells treated with a glycosylation inhibitor had normal antigen binding and protein A binding capabilities, but have lost their ability to activate complement, to bind to the Fc receptor, and to induce antibody-dependent cellular cytotoxicity.

Immunoglobulin M (IgM) as a System to Study Glycosylation

Immunoglobulins (Ig's) form a set of glycoproteins that have the ability to bind other molecules with a high degree of specificity. Immunoglobulins have been used to study processes such as protein synthesis and secretion, gene expression and regulation (17-27). Analysis of these functions facilitate the examination of other events, in particular glycosylation and oligosaccharide processing (25,26,28,47).

All immunoglobulin classes have the same fundamental structure, consisting of four polypeptide chains: two light (L) and two heavy (H) chains linked by interchain disulfide bonds. The light chains which are common to all Ig classes are either called Kappa (k) or Lambda (l). The heavy chains are specific to and determine the class of immunoglobulin.

In IgM the heavy chain is designated with the greek letter μ (mu).

Each chain (L or H) consists of two regions which perform totally independent functions. The variable (V) region is different in every chain that has been partially or completely sequenced (48). This region is located at the protein amino-terminal side. The carboxy-terminal or Constant (C) region carries the same sequence for each type of heavy or light chain. A variable region from L chain combines with a V region of a heavy chain to form the antigen binding site. There are two of these sites per each basic structure. Antibody effector functions which are carried out by the heavy chain C region include : Fc receptor binding and complement fixation. All Ig heavy chains carry carbohydrate moieties in the C region with their own characteristic sugar content (26,49).

The IgM molecule is the most primitive immunoglobulin in that it is the first to appear in vertebrate evolution. Moreover, it is the first immunoglobulin to appear during the ontogenic development of the immune system (50).

The IgM molecule is synthesized by cells derived from the lymphocyte series (B lymphocyte). Depending upon the stage of development, the B cell expresses μ chain at different locations, including subcellular , a surface membrane bound or secreted to the plasma. The mature B

lymphocyte contains IgM expressed as a membrane bound glycoprotein, while plasma cells actively secrete IgM molecules (19,51).

There are tumors of the B cell lineage corresponding to each of these developmental stages which permit ready analysis of the immunoglobulin expression. The B cell is represented by B cell lymphomas and the plasma cell by plasmacytomas or myeloma cells, each consisting of a clone of cells derived from a single cell. The cells produce a single homogeneous specific type of immunoglobulin molecule. Moreover, a single cell lineage can be analyzed in both stages because a B cell lymphoma carrying membrane bound IgM, upon cell fusion with the appropriated myeloma cell line will form a hybridoma, which is induced to secrete IgM (27). The induction of the murine plasmacytomas by intraperitoneal injection of mineral oil, was one of the most important discoveries of modern molecular immunology (53,54). There is a vast literature indicating the validity of using these clonally expanded immunoglobulins to study normal immunoglobulins (53). Recent findings using mouse and human lymphomas indicate that cell transformation (leading to the clonal expansion) result from an increase in the expression of a normal cellular product. The transformation may be due to the translocation of a relevant chromosome fragment carrying region which brings the gene(s) under the

the influence of highly active promoters, or next to enhancer sequences (55).

Origin of Cell Lines Producing Altered IgM

In addition to myeloma and hybridoma cell lines that produce normal IgM, it is possible to use cell lines that synthesize abnormal immunoglobulins to elucidate changes occurring in many of the phenomena related to IgM synthesis. Altered properties may include: structure of the binding site, interaction with complement, formation of polymers, size of the m Heavy chain and glycosylation (56,57).

Two general properties recommend the mouse immunoglobulin system for biochemical studies. First, it is relatively easy to isolate mutants in which Ig production is qualitatively or quantitatively altered. Second, Ig comprises a relatively large fraction of the protein made by these cells, facilitating the comparison of the mutant with the normal products. Shulman and Kohler recently described the selection of an IgM deletion mutant, and Scharff has isolated an IgG producing mutant (28,57,76). It is important to point out that these mutations are similar to those naturally occurring. Kenter et al (58) have studied two variant cell lines that produce short IgG heavy chains. The alteration is due to C-terminal deletions caused by premature termination of the translated

product. Carbohydrate analysis of variant heavy chains show differential glycosylation in oligosaccharides derived from HL (heavy and light, halfmer) and H_2L_2 (monomer) secreted forms. The heavy chain of HL form contains more carbohydrate. In addition, the parental cell line contains mainly high mannose type oligosaccharides, while variant heavy chains carry complex type as predominant species. Thus, modifications in heavy chain primary structure in these mutant cell lines lead to alteration in glycosylation patterns.

Shulman et al (57), have isolated a group of hybridoma cell lines which produce altered IgM. The suicide selection method used yield mutants which produced IgM that was unable to bind antigen or to activate complement. Further analysis of these mutants show that m chains were altered. The alterations include, among others, hyperglycosylation and deletions of fragments of heavy chains. Figure 3 shows the scheme used to obtain mutant cell lines by the suicide selection method.

Here, comparative studies of the parental and mutant cell lines are being presented. Studies compriaing genetic mapping of both types of cells, and gene product analysis of the hyperglycosylated IgM.

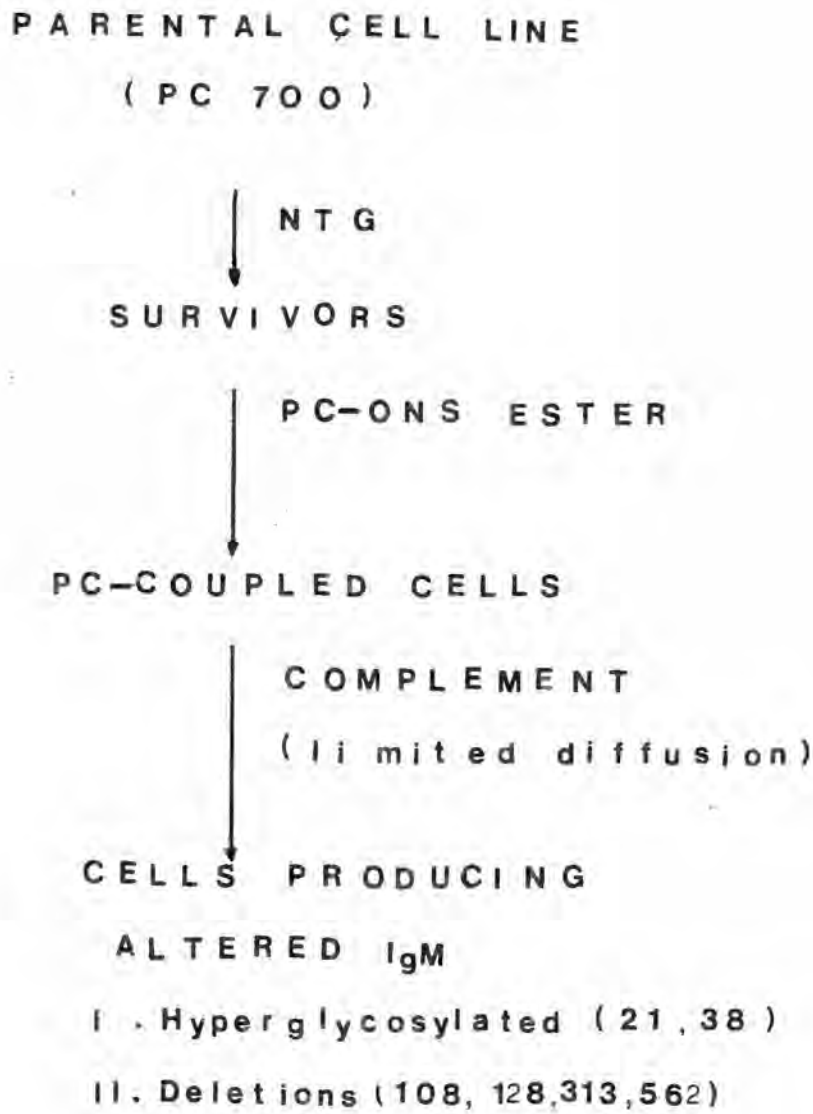


Figure 3. Scheme of "Suicide Selection Method" to obtain cells producing altered IgM (details in text). Phosphorylcholine (PC), Nitrosoguanidine (NTG), hydroxy-N-succinic ester (ONS). Mutant numbers 21 and 38 are called "hyperglycosylated". Mutants 108, 128, 313 and 562 were designed as "deletion".

CHAPTER 3

MOLECULAR BIOLOGY OF IgM MUTANTS

Introduction

Antibodies are composed of two types of polypeptide chains, light (L), and heavy (H), which are in turn divided into an N-terminal variable (V) and a C-terminal constant (C) region. In 1965 Dreyer and Bennett postulated that the antibody polypeptide was encoded by two genes that were joined together during the differentiation of antibody-producing B cells (59). This was the initial suggestion that the coding sequences for a polypeptide were separated in the genome. In addition, it implied that developmentally regulated DNA rearrangements were a fundamental element in antibody gene expression. Nucleic acid analyses which include cloning, DNA hybridization and sequencing have verified both of the suppositions and revealed that the antibody system employs two distinct types of DNA rearrangements. They include variable region formation and class switching, both of which appear to be

part of a precise program of DNA rearrangement that occur during differentiation (17-23, 60,61).

Antibody Gene Families

The Light (L) Chain

Antibody molecules are encoded by three unlinked gene families: two for light (L) chains, lambda (l) and kappa(k), and one for heavy (H) chains. The light chains are encoded by three gene segments, V(L) (variable), J(L) (joining), and C(L) (constant), which are separated in the genomes of undifferentiated cells (20). In the embryonic mouse DNA, several hundred V genes have been identified. They encode the first 95 amino acids of the light chain (kappa). The remaining portion of the variable region is encoded by the J gene a sequence located downstream from the V gene, near the constant-region gene. The J gene is repeated five times at intervals of about 300 nucleotides (62,63). During B cell differentiation a V(L) and a J(L) gene segment are joined together by DNA rearrangement to generate a V(L) region. Extra V's and J's segments (and the long non-coding sequences) between them are deleted (review 65).

The DNA rearrangement is termed variable region formation or V-J joining. Nucleic acid sequencing shows that certain features of the light chain genes have been conserved and appear to play a role as recombination

signals (64). The short gene fragments are located on the 3' side of the V genes and on the 5'side of the J genes. Each sequence has a stretch of about nine nucleotides of which a large portion are either A's or T's. The nonamer is followed by an interval of either eleven or twenty two nucleotides, then by a seven nucleotide segment containing mainly C's and G's. The nucleotides from the V gene may form a stem by complementation with those in the J gene, bringing both gene fragments together. Then by a DNA recombination mechanism, the stem forming sequences are deleted (64). The flexibility of the recombinational system explains in part the generation of antibody diversity (reviewed in 65).

During the expression of the rearranged gene in the differentiated B cell, the coding regions as well as the intervening DNA between the J (L) and C (L) gene segments are transcribed as part of a high molecular weight transcript. The intervening sequence is subsequently removed from the initial transcipt by splicing to produce the functional light chain m (66). This mRNA contains a V(L), a J(L), and a C(L) coding sequences (67).

The Heavy (H) Chain

The heavy chain gene organization was revealed using similar techniques as for the light chain (23). Studies from Hood, Tonegawa and co-workers demonstrated

that at the embryonic stage, the heavy chain locus is constituted by a large number of V(H) genes, at least ten D (diversity) genes (23,68), four functional J(H) genes and eight C(H) genes. Figure 4 is a schematic representation of the complete gene locus.

Each V, D, and J gene sequences are surrounded by recombinational signals (similar to those in the light chain). Early in B cell differentiation a complete variable region is formed by two DNA rearrangements. First, V and D genes are assembled together followed by the addition of a J gene. Hood and associates (69) found that heavy chain variable region formation follows the same mechanism as the V(L) region. The extra piece of genetic information (D gene) multiplies the combinatory possibilities of creating antigen binding sites (68).

The mouse C(H) gene locus, in undifferentiated cells, consists of eight genes. The arrangement spans close to 200 Kb of DNA (Figure 4). The sequence is Mu, Delta, Gamma 3, Gamma 1, Gamma 2b, Gamma 2a, Epsilon and Alpha (reviewed in 70). Depending on the heavy chain type, the C(H)-coding regions are composed of two to six structural domains, most of which consist of 100 amino acids that are separated by small introns (see later) (20,61,70).

Koshland et al (27), in studies with cell lines which produce different immunoglobulins demonstrated that the stage of B cell development is distinguished by the

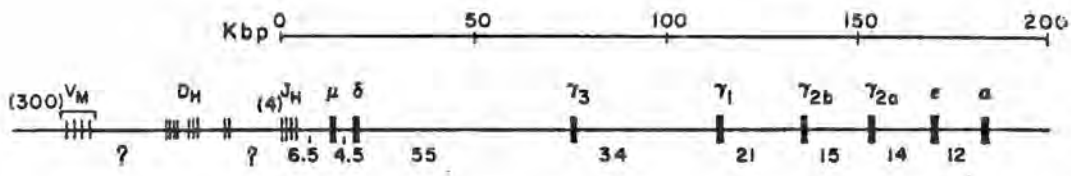


Figure 4. Schematic diagram of genomic DNA coding for the mouse immunoglobulin heavy chain genes. Coding segments represented by boxes and non-coding regions by lines. Variable region constituted by gene fragments: Variable (V), Diversity (D), Joining (J). Constant regions (greek letters): Mu (m), Delta (d), Gamma (g) 1, 2a, 2b, and 3, Epsilon (e) and Alpha (a).

type of heavy chain gene expressed. The pre-B lymphocyte makes a Mu heavy chain constant region linked to a specific variable region. The heavy chain remains inside the cell until synthesis of the light chain is completed. Light and heavy chains combine to form complete IgM molecules, which are expressed as membrane bound glycoproteins. The subsequent steps in lymphocyte maturation are antigen driven. Mature B cells synthesize IgM as a membrane bound receptor. The terminal stage of B cell differentiation is the plasma cell which is committed to synthesize and secrete large quantities of a single molecular species of antibody.

There are cell lines that represent each of the maturation stages of B lymphocyte. Lymphoma cells have properties similar to mature B cells, while myeloma and hybridoma cells resemble plasma cells (fully differentiated cells). The cell lines are clones which produce a single specific antibody. Studies on the genetics of antibody production have relied heavily on the availability of myeloma and lymphoma cell lines (27).

Several studies using myeloma cell lines indicate that IgM disappear from the cell surface followed (during maturation) by secretion of IgM, IgG, or IgA (21,71). Heavy chain genes isolated from genomic libraries show that each of these classes of immunoglobulins has a different heavy

chain constant region, but the antigen specificity or variable region remains the same. A second DNA rearrangement mediates class switching to generate expressed alpha (21) and gamma chains (21,72,73). The process is called heavy chain class switching.

Close analysis of the sequences around the germline and rearranged genes showed groups of repetitive DNA sequences (Switching segments) located about 2 kb 5'to each C(H) gene. The switching (S) segments contain several common tandemly repeated DNA sequences (S sites) (74). Each C(H) genes have specific and different S segments. The signals mediate a recombination event that joins a S site sequence to another S site located next to a downstream constant-region sequence. Constant regions which are located in between the S regions are deleted. Thus, the variable region is now next to a different constant region which in turn is going to be expressed by the cell. In summary, two distinct types of DNA rearrangements to occur: a VDJ joining and a C(H) switch. The molecular requirements and mechanism of regulation of C(H)-gene switching remains to be elucidated (17,21).

Mouse C(m) Heavy Chain Gene Organization

The IgM constant heavy chain gene, C(m), appears to play a pivotal role in B cell maturation. All

immunoglobulin secreting cells initially expressed IgM (27). Furthermore, the IgM molecule can appear successively in two forms, one bound to the B cell membrane and the other secreted.

Uhr and Vasalli (51,75) using carboxypeptidase analysis and detergent studies of the IgM protein, indicated that the heavy chain membrane bound form ends in a short sequence of hydrophobic amino acids, which anchor the antibody to the cell membrane. The secreted mu chain lacks this hydrophobic sequence. These observations originated the search for the C(m) gene organization.

Early and Cori (18,61), by extensive hybridization analysis using C(m) probes, independently determined that a single C(m) gene copy is present in both embryonic and differentiated cells (IgM producing). The DNA context in both cells is different. Gene restriction mapping show that in cells secreting IgM, C(m) is located closer to the VDJ than in undifferentiated cells. In addition, cells secreting other immunoglobulins have the C(m) gene deleted.

As many other eukaryotic genes, C(m) gene contains coding and non-coding sequences. R-loop hybridization analysis of C(m) clones and mRNA isolated from MOPC 104E cells (which mainly secrete IgM but produce some membrane bound IgM), show that about 90% of the molecules have two to four R-loops, while the rest of the molecules contained

one more hybrid loop (18,19). These results suggested two populations of mRNAs. The location and distribution of the coding, and intervening sequences was confirmed by detailed restriction mapping and sequencing (18,19,61). Early et al (18,19) conclusively determined the genomic origin of the two mRNA populations using RNA blotting and hybridization to specific cDNA clones. Both mRNA were transcribed from the same C(μ) gene which is constituted by six coding sequences, $C(\mu_1)$ to $C(\mu_6)$. Both mRNAs were identical from $C(\mu_1)$ to $C(\mu_4)$, but the membrane bound form had two extra coding sequences. Figure 5 shows the restriction map of Cμ gene containing the first four coding regions. The last two coding genes for the membrane bound end were found about 2 kb downstream $C(\mu_4)$, and code mainly for hydrophobic amino acids (18,19). Processing of nuclear RNA appears to be responsible for determining the relative levels of mu(membrane) mRNA and mu(secreted) mRNA produced in different cell types. Splicing events removing the stop codon at the end of $C(\mu_4)$ and the rest of the non-coding sequences, leads to membrane bound mRNA. Poly A tail addition to $C(\mu_4)$ forms secreted mRNA (18,22). DNA rearrangement does not play a direct role in controlling either form.

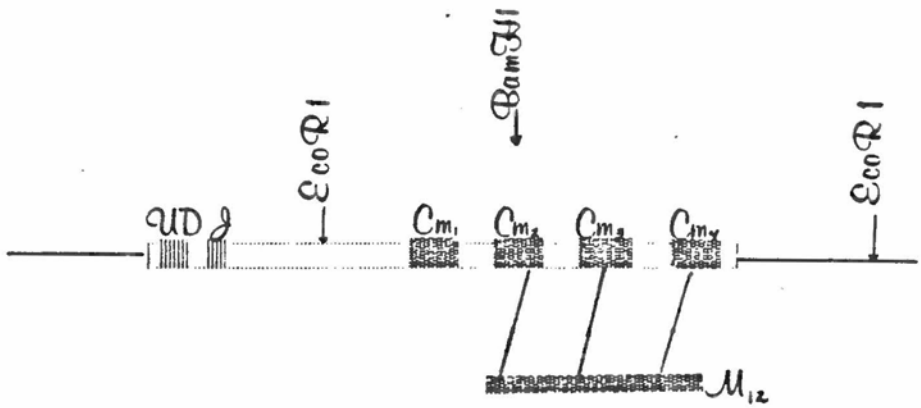


Figure 5. Restriction map of the mouse m chain gene. The Eco R1 fragment shown contains the complete C(m) gene. Bam H1 cleaves at the middle of C(m). Shaded areas are coding sequences. Lines are non-coding sequences or introns. The lower portion of the figure correspond to m12 cDNA clone used as probe for hybrization experiments.

Mutations to the C(m) Gene

Having defined the structure of immunoglobulins and its basic gene organization, several groups have began to isolate cell lines that secrete altered immunoglobulins (28,57,76). The rationale for obtaining these cell lines is to provide a means of identifying the structural basis of IgM function, and of studying the biochemistry of IgM synthesis and processing.

At the gene level, there are certain advantages for producing mutants. First, as mentioned above, only one chromosome directs the synthesis of IgM (18,19). The other chromosome is silent. Therefore, altered Ig synthesis is dominant. Second, the presence of non-coding sequences between the V region and each of the constant domain genes gives a mechanism to partially correct errors. If there is a deletion around a splice site, the processing could go on to the next site, and delete the domain (or domains) in between (18-23,61).

The isolation of cells mutated in genes for nonessential proteins, such as immunoglobulins is relatively simple. Scharff and his colleagues (76) discerned mutant clones making altered Ig by testing for antibody-dependent precipitation around colonies in agar. Kohler (28,57) designed a method of selection by which cells secreting wild type antibodies commit suicide. Figure

3. For mutant selection, cells were treated so that phosphorylcholine (PC) was covalently bound to the cell membrane,and then were grown under conditions of limited diffusion. Under these conditions cells secreting IgM bind PC on their own membrane. Thus, in the presence of complement the wild type cells are killed and mutant cells producing altered immunoglobulin survive. The success of this suicide selection was assessed by measuring the reaction with specific rabbit anti-mouse (m) serum, and by protein A plaque formation. Initial characterization of mutations affecting the IgM molecule included: partial C(m) protein deletion, polypeptide addition, and C(m) abnormal glycosylation (57,77).

In this chapter, the C(m) gene structure of mutant cell lines which secrete heavy chain with deletions and hyperglycosylated heavy chain were analyzed using the Southern method (78). Several points facilitated these studies: (a) Normal C(m) restriction enzyme mapping (figure 4), and sequencing have been published, for MOPC 104 E and other hybridoma cell lines that secrete IgM (18,20). (b) cDNA clones that code for normal C(m) gene which can be used as probes are available(figure 5) (18,19). (c) Direct comparison of mutants with the parental and wild type cell lines. The usefulness of other techniques to define genetic alterations are discussed.

Materials and Methods

Cell Lines and Culture Conditions

All cells were derived from the hybridoma cell line PC 700 which secretes IgM specific for phosphorylcholine (57). The cells were gifts of Dr. M. Shulman from Toronto, Canada. Hybridoma cells were grown as suspension cultures in Dulbecco's modified minimal essential medium (DMM) (Grand Island Biological Co., Santa Clara, California) supplemented with 15 % heat inactivated fetal calf serum, non-essential amino acids, streptomycin and penicillin at concentrations of 50 micrograms/ml and 50 units, respectively. Flasks containing cultures were incubated at 37 ^{o} in a 5% CO_2 atmosphere.

Bacterial Strain and Transformation

Escherichia coli X 1776 is a derivative strain of E. coli K12, and was donated to us by Dr. L. McReynolds. Bacteria was grown in L-Broth containing thymidine (20 mg/l) at 37 ^{o}C. Cells (1-2 x 10^{8}) were harvested from 100 ml of culture and washed in tris-saline buffer (10 mM Tris-HCl pH 7.0, 140 mM NaCl). The pellet was resuspended in 30 ml of $CaCl_2$ buffer (70mM $CaCl_2$, 10mM Tris, pH 7.0 and 140 mM NaCl) (81). The cell suspension was allowed to stand for 20 min at 0^{o}C. Calcium treated cells were harvested and resuspended in 5

ml of the same buffer. This suspension was mixed with a plasmid containing solution in a ratio 2:1 (v/v) and held at 0 °C for 20 min. After 5 min at 37 °C, aliquots of 100 microliters were spread over L-broth agar plates containing tetracycline (20 microgram/ml). Tetracycline resistant transformants were observed after incubating at 37 °C for 48 hs. Isolated colonies were tested for ampicillin sensitivity (80).

Purification and Analysis of Plasmid

Transformed bacteria cells were grown in L-broth to late exponential phase (ABSORBANCE 600 nM=0.3). An aliquot of 25 ml was then used to inoculate one litre of M-9 media supplemented with tetracycline (12 microgram/ml) and thymidine to a final concentration of 20 microgram/ml. DNA plasmid amplification was achieved by adding chloramphenicol (20 microgram/ml), after the cells reached the late exponential phase. Cells were harvested and lysed as recommended (Bethesa Research Laboratories, BRL) in order to prepare a sample for NACS chromatography (see later). Briefly, collected cells (usually 1 to 2 gms wet weight) were resuspended in 70 ml of 50 mM Tris-HCl (pH 8), 50 mM Na_2EDTA, and 15% (w/v) sucrose. Lysozyme was added to final concentration of 1mg/ml. After incubation at room temperature for 30 min, the solution was made 0.5 M with potassium acetate. Cells were then lysed by adding

sodium dodecyl sulfate (0.5%), and incubated for 30 min at 0 °C. The lysate was subjected to centrifugation for 30 min at 10Kxg. The supernatant solutions was ajusted to 0.5 M NaCl with solid NaCl and nucleic acid precipitated with 80% ethanol at - 20 °C. The dried pellet was resuspended in 4 ml of 0.05 M NaCl buffer containing 10 mM Tris-HCl (pH 7.2),1 mM Na_2EDTA (TE buffer). RNAse T1 (Sigma) was added (one unit / unit of Absorbance at 260 nm), and incubated for 15 min. The solution was extracted twice with phenol and alcohol precipitated as above. The pellet was then resuspended in 0.5M NaCl TE buffer and directly applied to a NACS 37 column. Two hundred micrograms of pBR322- m12 were recovered from one litre of M-9 media.

NACS-37 Chromatography Conditions

NACS (purchased from BRL) separations are based primarily on ion- exchange mechanisms. NACS is composed of a thin film of trialkylmethylammonium chloride covering a particulated resin. The anion exchanger interacts with the phosphate residues in nucleic acids. Once bound to NACS, the nucleic acids are eluted by increasing the salt concentration. The NACS-37 column (1.0 x 15 cm) was packed at a flow rate of 2.0 ml/min in 2.0 M NaCl containing TE (10mM Tris-HCl, 1 mM EDTA) buffer. The column was equilibrated with 100 ml of 0.5 M NaCl TE buffer.

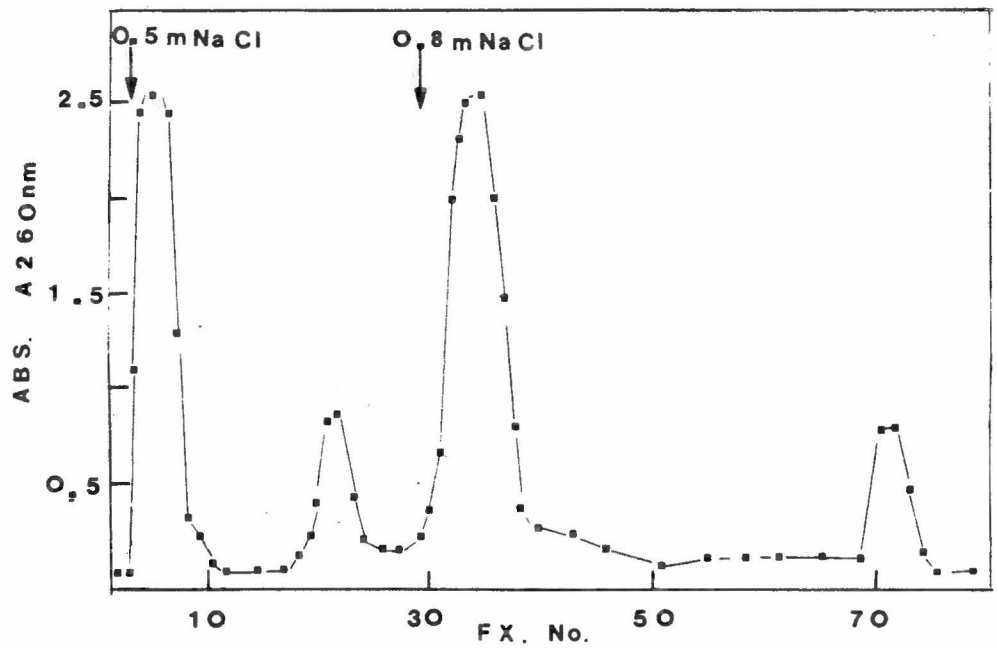


Figure 6. Column chromatographic purification of plasmid DNA. Samples were loaded on pre-equilibrated NACS-37 column (0.5 M NaCl column buffer). Fractions were collected and detected by monitoring the ABSORBANSE (ABS) at 260 nM. First peak represents non bound material. Plasmid containing fractions were eluted with 0.8 M NaCl buffer.

The sample was loaded on the column and 4 ml fractions collected. A large volume of starting buffer was used to elute non-bound material. The bound fraction was eluted with 0.8 M NaCl TE buffer. The nucleic acid containing fractions were monitored by absorbance at A 260 nm. PBR-322-m12 eluted with the high salt buffer (Figure 6). Peaks containing nucleic acids were dialyzed, concentrated by ethanol precipitation and analyzed in agarose gels [Figure 7 (a) and (b).

Cellular DNA

High molecular weight cellular DNA was prepared from cells grown in tissue culture (1 ml of packed cells) using standard procedures (83).

Briefly, the cell pellet suspended in 0.15 M NaCl, 0.1 M EDTA (pH8.0) was lysed by adding SDS to a final concentration of 1%, digested with pronase (Calbiochem) (100 micrograms/ml) and further deproteinized by extractions with phenol and then chloroform-isoamyl alcohol (24:1). Following dialysis against 0.15 M NaCl, 10mM Tris (pH 7.4), 1mM EDTA. The DNA was then digested with RNAse A (20 micrograms/ml) (SIGMA) for 1 hr at 37 °C. The solution was extracted sequencially with phenol and chloroform-isoamyl alcohol and dialyzed against 10 mM Tris

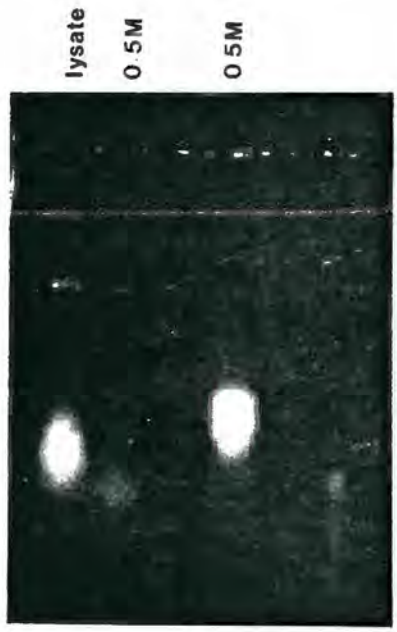
(pH 7.4), 1mM EDTA. DNA concentration was estimated by determining the absorbance at 260 nM. (absorbance at 260nm 1%= 200).

Endonuclease Digestion

Eco R1, Pst1, and Bam H1 were purchased fron New England Biolabs. DNA digestions were carried out following standard conditions provided with the enzyme. Four times the amount of enzyme needed to completely digest the bacteriophage lambda DNA was used to cleave the cellular DNA. After digestion, reaction mixtures were precipitated with 2 volumes of ethanol at -20 °C overnight. The pellets were resuspended in 10 mM Tris (pH8.0), 1 mM EDTA.

Agarose Gel Electrophoresis

Nucleic acid samples (10-20 ug for Southerns, 0.5-1.5 ug for plasmid analysis) were separated by electrophoresis in horizontal slab gels (23x14x0.4cm) containing 0.8 or 1.0 % agarose (BRL Laboratories) in Tris acetate buffer, pH 8, containing 1 mm EDTA. Samples were mixed with an equal volume of starting buffers (1x Tris acetate buffer/10% glycerol/bromophenol blue) and were run at 25-30 V for 20-24 hr (78)



7(a)

7(b)

Figure 7. Agarose gel electrophoresis of plasmid purification. Total lysate and fractions eluted from NACS 37 were analyzed. After electrophoresis gels were stained with ethidium bromide and photographed. 7a) Lysate and material that did not react with the column (mainly small RNA fragments). 7b) Lysate and column bound fractions. These fractions contained the three plasmid forms: supercoiled, open circle and linear form.

Preparation of Probes

The m_{12}-plasmid DNA used as a specific probe for m gene is shown in figure 4. Complete plasmid DNA (0.2-0.4 micrograms) was labeled by nick translation using BRL Nick Translation Kit (82) and ^{32}P-dCTP (specific activity of 3000 Ci/mmol) purchased from New England Nuclear, Boston, MA. Radioactivity incorporated was 1-2.5x 10^{8} CPM/microgram.

Transfer and Hybridization

Following agarose gel electrophoresis, staining with ethidium bromide (5 micrograms/ml) and ultraviolet-fluorescence photography, the DNA fragments were transfered to nitrocellulose filters (Bethesa Research Laboratories) by the method of Southern (78). DNA fragments were denatured for 15 min in alkali (0.1 M NaOH, 0.1 M Tris-HCl), and then neutralized using 0.3 M NaCl, 3xSSC buffer for 30 min. Transfer from 4 mm gels was carried out for 20-24hr. Nitrocellulose filters were rinsed in 2X SSC (0.3M NaCl, 0.03 sodium citrate), dried at room temperature, and heated at 80 °C under vacuum for 2 hr. Pre-hybridizations used 0.2% Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrolidine, 6x SSC for 2-4 hr at 68 °C. Hybridization reaction mixtures contained 0.1 M

NaCl, 50 mM sodium phosphate (pH 6.5), 5 mM EDTA, 0.5% SDS, 0.2 % Ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrolidine, and the denatured probe (1-2x10 ^{8} CPM). After 24-36 hr at 68 ^{o}C, the filters were washed successively at hibridization temperature, in the following buffers: 1M NaCl, 50mM Tris (pH 8.5), 2mM EDTA, 1% SDS for 1-2 hr with one change; 0.5M NaCl, 0.5 mM sodium phosphate (pH 6.5), 0.5 % SDS for 1-2 hr. Finally, the filters were rinsed with 2x SSC at room temperature and dried. Autoradiography in the absence of intensifier screens used Kodak OMAT film. Exposures varied from 1-6 hr.

Results

We used the Southern blotting technique to compare gene structures in cell lines that secrete altered IgM (78). Gene rearrangements are common in cells which produce antibodies. Embryonic and differentiated B cells have a single C (μ) gene copy formed by DNA rearrangement. Thus, it was necessary to consider whether rearrangements of the of C(μ) DNA sequences occur in the genomes of the cells producing deleted or hyperglycosylated heavy chain molecules (28,57). Deletions or additions of fragments within the expressed C(μ) gene should alter the size of restriction enzyme fragments (18,78). Separated fragments

were hybridized with a labeled C(μ) probe to identify the fragment containing the constant region gene. Figure 5 is a schematic representation of the complete heavy chain gene and the cDNA μ_{12} probe used for these experiments (18,19).

Plasmid Containing cDNA Purification

The μ_{12} cDNA clone represents about 1 kb of the constant C(μ) 3' end of the gene (Figure 1) (18).The cDNA clone which contains $C(\mu_3)$ and $C(\mu_4)$, and most of $C(\mu_2)$ constant domains is inserted in pBR_{322} plasmid at the Pst 1 site (80). pBR_{322}-μ_{12} was constructed by Early et al (18) at Lee Hood's laboratory. The plasmid was used to transform E. coli X 1776 by using the $CaCl_2$ shock technique (81) . The transformants were identified by their resistance to tetracycline (10 mg/ml). After 12-18 hs of chloramphenicol amplification, the cleared lysate was prepared as described in methods.

The μ_{12} containing fragment was isolated using a NACS 37 column (Bethesa Research Laboratories). RNA and DNA molecules react with different affinity to the column. Bound material is eluted by changing the salt concentration. The lysate was applied to a pre-equilibrated column and extensively washed with 0.5M NaCl in TE buffer until the eluate was essentially free of absorbance at 260

nM. Plasmid elution was carried out with 0.8M NaCl in TE buffer. The elution profile is shown in Figure 6. The fractions were pooled and analysed in 1% agarose gels (Figure 7). The material eluted at 0.5M NaCl contained low molecular weight RNA and DNA fragments (Figure 7a). The three forms of plasmid DNA eluted at 0.8M NaCl are supercoiled, open circle and linear form (Figure 7b). The purified plasmid was dialyzed and concentrated by lyophylization.

Analysis of Plasmid

We tested the fragment pattern after digestion with specific restriction endonucleases to show that the isolated plasmid contained the m_{12} insert. Aliquots (50 ul) of each reaction were loaded onto a 1.4% agarose gel (Figure 8). The undigested lane contains the plasmid forms. Eco R1 digest shows a single fragment of 5.2 kb with slower mobility than pBR_{322}. The Pst1 digest is comprised of several fragments, the largest contains the complete pBR_{322} (4.36 kb), and 2 small fragments each of about 0.5 kb which migrate close to the front dye. pBR_{322} fragments were used as m.w. standards to calibrate the gel. The total size of the inserted DNA was about 1 kb. It is in agreement with the sequence data obtained by Early et al

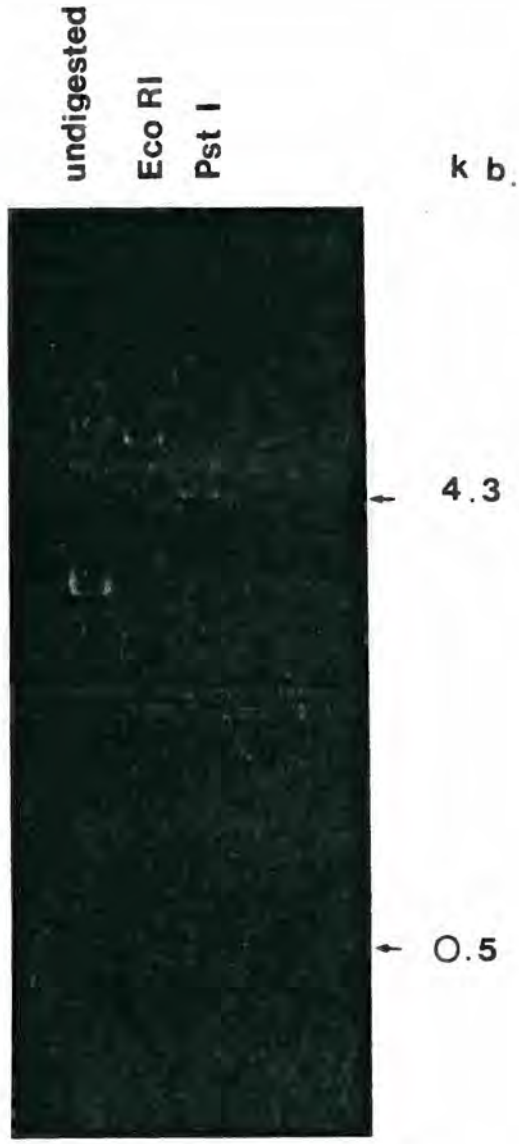


Figure 8. Agarose gel electrophoresis of restriction DNA fragments. pBR322-m12 (0.5-1.5 micrograms) were cleaved with the enzymes indicated and electrophoresed in a 1.4% agarose gel. Stained as in figure 7.

(18). The purified plasmid was then nick-translated in the presence of ^{32}P-dCTP as described in methods (82). Normally 1 x 10 ^{8} CPM were incorporated per microgram of plasmid DNA.

Identification of the Parental C(m) Gene and Comparison with Derived Mutants

Genomic DNA studies have shown that the C(m) gene is completely contained in an Eco R1 fragment (18). In normal B cells the fragment is 13.0 kb (79). Figure 5. Myeloma and some hybridoma cells present a single fragment, indicating that both chromosomes have been rearranged (60,79). The presence of non-coding regions around the constant domains make possible the deletion of complete coding regions without altering the expression of others (47). Any deletion or addition should alter the size of the Eco R1 fragment.

To test for C(m) alterations, cellular DNA was prepared from mutant cell lines 21,38,128,313,and 562, and from PC 700 (parental) and MOPC 104 E (wild type). The method used has been described elsewhere (83) . DNA from all cell lines was digested either with Eco R1 or Bam H1 enzymes, then examined by using Southern blot hybridization (78).

C(m) gene mapping and comparison with other cell lines which produce normal IgM was required to determine that the parental cell line (PC 700) has the correct gene size. PC 700 (hybridoma) and MOPC 104 E (myeloma) cellular DNAs were digested with Eco R1. Following staining, the gel was used to prepare a Southern blot which was hybridized with the C(m) probe. Both cell lines presented a single 13.0 kb restriction fragment that migrated with identical mobility (Figure 9). Thus the parental cell line appears to have retained the wild type C(m)gene.

Having defined the size of the parental cell line constant gene, it was possible to determine if these methods could detect any DNA alterations in mutant cell lines that secrete abnormal IgM. Figure 9. A single Cm Eco R1 fragment of 13.0 kb which hybridize with m_{12} was present in all cellular DNA. The lack of bands that run slower or faster than the parental cell line fragment indicated the absence of a mayor change in C(m).

To further analyze the mutants, a second restriction enzyme was chosen. Bam H1 cleaves the Cm gene at the middle of C(m_{12}) (84). Figure 5. Thus this enzyme can be used to analyze the 3' end. This fragment is 12.5 kb long. Again, all cell lines (but with the exception of 128 cell line see below) presented a single

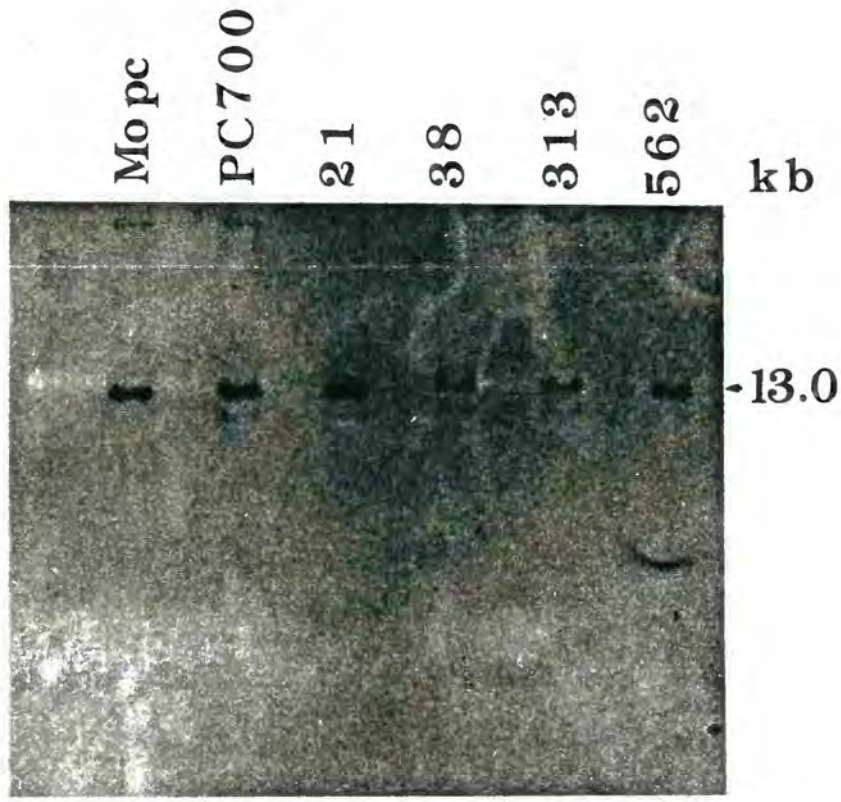


Figure 9. Southern blot from myeloma and hybridoma cells. Cellular DNAs from indicated cells were digested with Eco R1 enzyme and electrophoresed in 0.8% agarose gels. Restriction fragments were transfered to a nitrocellullose paper and hybridized with ^{32}-P-labeled pi2.

fragment as shown in Figure 10. The Southern maps do not reveal any differences in gene patterns and confirms that the C(m) 3' end of the mutant cell lines are similar to wild type cells.

Cm Domains are Deleted in
Mutant Cell Line 128

Mutant 128 cell line secretes a (m) heavy chain with reduced m.w. Preliminary data from Dr. Shulman suggested that the chain lost the constant domains: $C(m_1)$ and $C(m_2)$ (Figure 1). The rational for mapping this cell line was that the removal of the $C(m_2)$ gene fragment would delete the restriction site (Bam H1) at that domain (84). This event can either originate a new very large fragment with a m.w close to 20kd containing the V region and $C(m_3)$ and $C(m_4)$, or could create a new Bam H1 site that can generate a fragment with m.w. identical or similar to that present in the parental cell line. Figure 11 shows the Bam H1 digestion of cellular DNA from PC 700 and 128. The lack of a band running slower than PC 700 rule out the first possibility of having a single fragment containing the complete rearranged gene. A single hybridizing m_{12} Bam H1 fragment running faster than the normal C(m) indicated

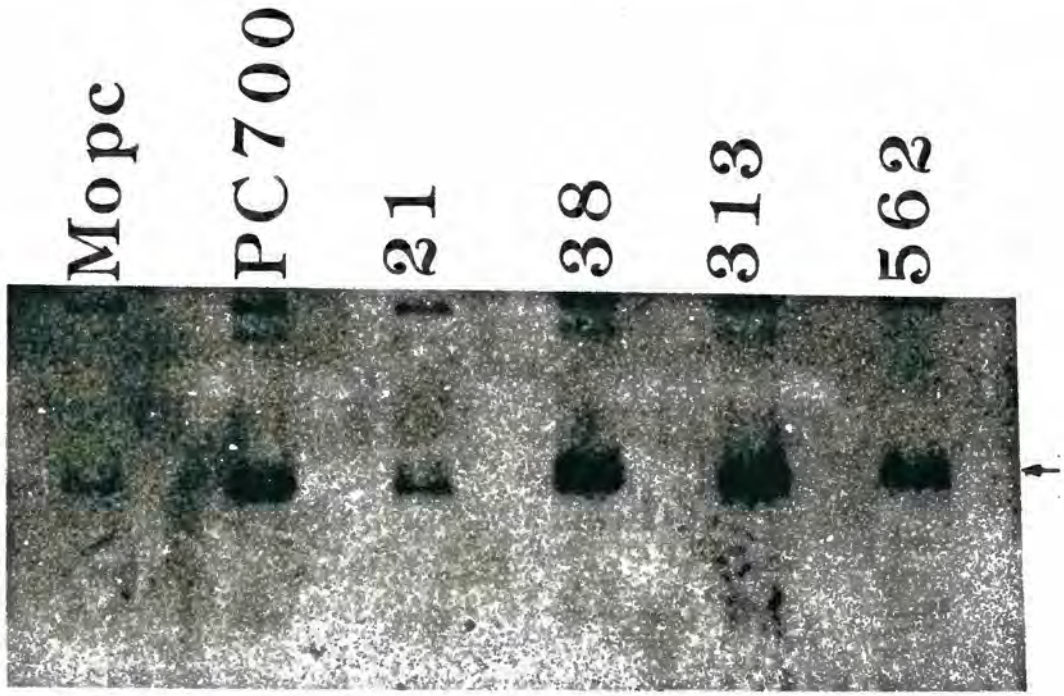


Figure 10. DNA blots from myeloma and hybridoma cells. Bam H1 digested cellular DNAs were treated as described in figure 9. Arrow indicates 12.5 kb.

(Figure 11) not only deletion at the gene's 3' end, but the formation of a new site. Dr. Shulman has now sequenced the complete region. He has shown that the removal of C(μ_1) and C(μ_2) occur by deleting an intron portion located at the 5' side of C(μ_1), and most of the intron at the 5' end of C(μ_3). The mutation leaves intact the splice site at 3' side of V region and the 5' side of C(μ_3), therefore they can be correctly spliced and expressed (77). Furthermore, the break and rejoining of introns can lead to the formation of a new restriction site . There are several potential sites where this can occur. The joining of the 5'end side of 128 deletion to the 5' side of C(μ_3) exon could originate a sequence CCTAGG which Bam H1 can cleave. This type of rejoining could form a fragment 390 bases smaller than the parental fragment which explain faster migration of the 128 band. It should be pointed out that this rejoining leaves the splice site intact and in phase.

Discussion

We have studied three types of IgM producing cells:

a)Cells which have abnormal μ polypeptide chain but do not have detectable gene alterations .

b)Cells which secrete IgM with abnormal polypeptide chains, but have have detectable alterations, and

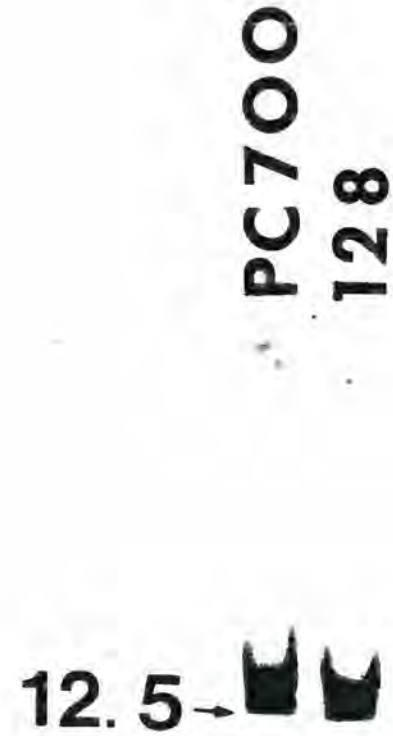


Figure 11. Southern blot from hybridomas PC 700 and 128. Bam H1 digested cellular DNAs were treated as described in figure 9. Arrow indicates 12.5 kb.

c)Cells which secrete IgM with normal polypeptide chain, no detectable gene alterations, but with hyperglycosylated mu chains.

IgM with polypeptide m chain reduced in molecular weight may arise from two different mechanisms. Mutations can lead to the formation of a stop codon which in turn can halt protein synthesis. These have normal gene arrangements (see later). On the other hand, deletions can be produced by complete removal of one or more constant gene domains (Cm gene is rearranged). The unaffected C(m) domains would be expressed because the surrounding introns contain correct splicing sequences.

Finally, the third class of mutations are due to changes in the m chain mutations are due to changes in the m chain glycosylation patterns .The characterization of mutants of this type is presented in chapter 4.

The results presented in this section illustrate that myeloma (MOPC 104E) and hybridoma cell line (PC 700) carry the same C(m) gene fragment (Figure 9). The gene products vary upon glycosylation processing. Restriction enzyme mapping and hybridization with a C (m) gene probe, show that both IgM producing B-cells contain only one functional m gene, lacking of any non-functional m gene (18,77).

Mutants cell lines producing heavy chains with reduced molecular weight were expected to have gene deletions. Preliminary studies indicated that the μ chains have lost complete constant domains (as determined by monoclonal antibody mapping) (57). Furthermore, the constant domains ($C\mu_{1-4}$) are surrounded by introns (61). Mutations leading to the inactivation or loss of a domain would not affect the expression of other domains (77). Unexpectedly, cell lines 313 and 562 presented an EcoR1 fragment with identical mobility as the normal fragment (Figure 9). Their secreted μ chains lack $C(\mu_3)$ and $C(\mu_4)$ domains, but their corresponding gene fragments were present as demonstrated by the normal size Bam H1 fragment (figure 10). RNA isolated from these cells and from the parental cell line displayed identical mobility (77). Thus the decrease in mu chain size might arise from point mutations leading to a stop codon. These kinds of alterations would not be detected with our methods, unless they fall in the enzyme restriction sites used.

On the contrary, mutant 128 originates from a different mechanism. This cell line secretes an IgM which is deficient in $C(\mu_1)$ and $C(\mu_2)$ domains (57,77). Its gene rearrangement is altered as seen in figure 11. Eco R1 fragment containing the C(μ) gene is reduced in

molecular weight (77, and my results). In addition, the RNA lacks a large fragment (650 bases) which is consistent with the protein size (77). Recently, Shulman has published DNA sequence data demonstrating that the mutant 128 Constant m gene has lost a fragment from the middle of the intron located between the J gene and Cm_1 domain to near the end of the intron in the 5' side of Cm3. The Bam Hi fragment reduced size could come from the formation of a new Bam Hi site at/or close to the rejoining site (77). The addition of two bases (A-G) at the rejoining site could originate this site. The expression of gene product (128 m chain) is consistent with the mechanism mentioned above, by which splicing proceeds by joining the nearest functional 5' and 3' splice sites. Thus it is possible to alter one or more C(m) domains without affecting the expression of the other (domains). This brings the possibility of studying the function of each immunoglobulin domain independently . There are several reports where intron structure has been altered without large effects on the level of gene expression (84,85).

Mutants 21 and 38 which heavy chains present retarded mobilities in SDS PAGE (47), with an increase in their mw ranging fron 6-8000 dalton. Both cell lines showed normal gene rearrangment which indicate that their phenotype is not due to an gene insertion, but rather a

change in their glycosylation pattern. In Chapter 4, I present data defining the types of changes occurring in their IgM molecules. Shulman and coworkers (84) have constructed a vector carrying the functionally rearranged heavy (mu) and light (Kappa) chains. A similar cloned vector has been succesfully transferred and expressed in hybridoma cells (88). Introduction of those vectors carrying mu chain to mutants that have abnormal glycosylation would give information about the cellular processing enzymes that specify mutant glycosylation patterns.

CHAPTER 4

IMMUNOGLOBULIN M CHARACTERIZATION

Introduction

Glycoprotein Synthesis

The initial steps in protein glycosylation occur during translation while the protein is passing through the endoplasmic reticulum (ER) (review 1). The sequence Asn-X-Ser/Thr serves as acceptor for a block transfer of an oligosaccharide containing $Glc_3Man_9GlcNAc_2$. The transfer requires only the three-amino acid acceptor sequence, and occurs before there is sufficient translation for extensive protein folding. The oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled on the lipid carrier dolichol pyrophosphate by the stepwise addition of sugar residues (reviewed in 89). Snider and Rogers (90) have examined the transmembrane orientation of oligosaccharide-lipid intermediates in the ER using microsomes and the lectin Concanavalin A which recognizes high mannose structures. Man_3 to $Man_5GlcNAc_2$-lipid intermediates were found at the cytoplasmic side of the microsomal membrane.

$Man_{6-9}GlcNAc_2$-lipid and
$Glc_3Man_9GlcNAc_2$-lipid compounds were found only in the ER microsomal lumen. These results support the model that oligosaccharide-lipid synthesis is completed on the ER luminal face. Mature oligosaccharides ($Glc_3Man_9GlcNAc_2$) are then transfered as a unit to asparagine residues of nascent and newly made polypeptides (91). Thus, initially all glycosylation sites receive the same carbohydrate structure. Apparently this mechanism is required since glycosylation occurs co-translationally before protein folding gives three dimensional information that processing enzymes use to determine the final carbohydrate structures (1).

Lennarz (92) utilizing denatured proteins containing potential acceptors in an in vitro glycosylation system established that a prerequisite for glycosylation is the occurrence of the acceptor -Asn- in the tripeptide sequence, Asn-X-Thr/Ser, where X can be any amino acid except for aspartic acid. However, only about 30% of the known tripeptide sites of eukaryotic proteins are glycosylated. These results initially suggested that the accessibility of the acceptor to the membrane bound transferases of the lipid linked-pathway is a critical factor for glycosylation, and would be regulated by higher orders of structure (secondary or tertiary) imposed by the primary sequence.

Delineation of glycoprotein polypeptide and oligosaccharide are required before we can begin to understand the factors which determine glycosylation (93). More recently, studies on protein conformation using the Chou Fasman technique showed that glycosylation sites are usually associated with reverse b-turns or loops (94,95). These studies support the concept that glycosylation site accessibility is a determinant of structure.

Carbohydrate Processing

Following glycosylation, a group of enzymes act by removing and adding carbohydrates in a synchronized manner leading to the final oligosaccharide structure(s) . This is called "carbohydrate processing" and occurs in the rough endoplasmic reticulum (RER) and Golgi apparatus.

After high mannose precusors ($Glc_3Man_9GlcNAc_2$) are transferred to the asparagine residues of the nascent proteins, the polypeptides are transported from RER to the Golgi apparatus. It is not known how the proteins move from one cell compartment to the other. A common hypothesis is that small vesicles bud off from a specialized region of the RER, containing a sample of the luminal contents and membrane proteins. These vesicles subsequently fuse with vesicles of the cis-(or proximal) region of the Golgi cisternae (96). Secretory and membrane bound

glycoproteins mature through this intracellular pathway at different rates. In addition, processing of oligosaccharides leads to the final carbohydrate structures (97). In several systems including vesicular stomatitis virus (VSV) infected cells (98), chick embryo fibroblast, yeast cells (1,91) and more recently in human hepatoma cells (100), the N-linked oligosaccharide processing can be divided in three phases. These include: removal of glucose residues, removal of the initial mannose residues, and synthesis of complex oligosaccharides accompanied by further removal of mannose. Removal of glucose residues from the oligosaccharide $Glc_3Man_9GlcNAC_2$ occurs soon after transfer to protein. Kinetic studies in VSV infected cells show that after 20 to 30 min the last glucose residue is removed (101). Robbins et al (102) have shown that rat liver glucosidases I,II and III are integral membrane proteins that appear to be localized at the ciaternal surface of the RER. Glucosidase I removes only the terminal residue of $Glc_3Man_9GlcNAc_2$. The second activities, glucosidase II and III can convert Glc_2 or $Glc_1Man_9GlcNAc_2$ to $Man_9GlcNAc$, but is inactive towards the glucosidase I substrate. The role of glucose in glycoprotein synthesis has been explored by Lodish and Kong (100). Deoxynorjirimycin, a specific inhibitor of a-glucosidase I and II, greatly reduced the secretion of a-1 antitrypsin and a-1 antichymotrypsin. Other glycoproteins were normally

secreted. These results were interpreted to suggest that $Man_9GlcNac_2$ oligosaccharide might be part of the recognition site for transport specific receptors for some secretory proteins.

After glucose residues are removed, the $Man_9GlcNAc_2$ intermediate is then rapidly processed in an ordered sequence. The four outer a(1-2)-linked mannose residues are removed by one or more specific a(1-2)-mannosidases. There is evidence that, at least in part, the enzymatic removal of mannose residues occurs in the rough endoplasmic reticulum. Bishoff and Kornfeld (103) have presented evidence for an a-mannosidase in the ER of rat liver. New evidence has come from recent work by Lee and Atkinson showing that the oligosaccharides of nascent chains of VSV G protein from membrane bound polysomes have already lost glucose and mannose (104). The best evidence supporting the concept that all four a(1-2) mannoses can be removed in the ER used rotavirus SA11 specific glycoproteins. The viral glycoproteins mature in the ER, and have Man_5 oligosaccharides (105). Hickman et al (106) examined the processing of IgA heavy chain in plasmacytoma cells. In these cells three of the outer mannoses were removed in the ER while the remaining mannose and further carbohydrate processing occurred just before IgA secretion. These observations explained the results

of several groups that had found partially processed glycoprotein oligosaccharides accumulating in the ER (107).

Li and Kornfeld (108) have shown that in IgM (human), VSV G protein and ovalbumin, the mannose removal is not random but rather is specific for each individual glycoprotein glycosylation site. Thus, high mannose oligosaccharides of mature glycoproteins are thought to arise by the removal of a variable number of a(1-2)-Man residues from $Man_9GlcNAc_2$. $Man_5GlcNAC$ is generally believed to be the smallest high mannose structure found in membrane bound and soluble glycoproteins. N-linked oligosaccharides that do not retain high mannose structures are converted to complex oligosaccharides in a Golgi-associated process. $Man_5GlcNAc_2$ is critical in the formation of complex oligosaccharides serving as substrate for N-acetylglucosaminyl transferase I, the first step in oligosaccharide processing. The enzyme catalyzes the reaction where a GlcNAc residue is transfered to the a(1-3)-mannose residue which is followed by the removal of 2 Man residues by mannosidase II. The $GlcNAc\text{-}Man_3$-product of these reactions can be acted on by a series of glycosyltransferases which transfer GlcNAc, Gal, sialic acid and fucose residues, to form bi-, tri-, and tetrantennary complex oligosaccharides (91). Some

examples are shown in figure 2. Recently, hybrid structures have been detected in several glycoproteins such as ovalbumin and lysosomal enzymes (37,38). These structures contain one arm processed to contain one or two Man residues, and one or more antennae containing GlcNAc, Gal(1-4)GlcNac, or sialyl-Gal b-(1-4)GlcNAc, linked to the Man a-(1-3) arm. In vitro synthesis using hen oviduct membranes show that hybrid structures appear to result from the limited action of mannosidase II, which inhibits side arm processing (12).

The involvement of cell membranes of the RER, Golgi apparatus and cell surface in glycoprotein biosynthesis are of obvious significance. Each intercompartmental transfer in the ER-Golgi system is necessarily accompanied by a sorting decision. Considerable indirect evidence suggest that clathrin-coated vesicles play a role in the sorting process (109). The Golgi apparatus is the locus where the major processing events occur. Fractionation studies have supported earlier observations that this organelle was compartmentalized. Warren and coworkers have presented electron-microscopic studies using derivatized lectins which define cis, medial and trans cisternae as compartments of the Golgi apparatus (111,112). As proteins are exported from the ER, they seem to enter the Golgi stack at one end (the cis face) and exit from the stack at the other end (the trans face).

In a different approach, Rothman and others have provide valuable information about the compartmentation of asparagine-linked oligosaccharide processing in the Golgi apparatus (110,113). Equilibrium sucrose density gradient centrifugation of membranes from chinese hamster ovary cells partially resolved two sets of enzymes. The first set contained: Mannosidase I, N-acetylglucosamine Transferase I, Mannosidase II and N-acetylglucosamine transferase II. These can be seperated from later-acting galactosyltransferase and sialyltransferase. Rothman showed that the four enzymes that transform the high mannose residue into Man3-residue containing outer GlcNAc, were found at lower densities than glucosidase I (ER located). Sialyltransferase, the last enzyme in the pathway, co-distributed with Galactosyltransferase which is restricted to the trans face of the Golgi complex (114).

Control of Carbohydrate Processing

The events occurring at the stage of processing are the least understood. The protein linked oligosaccharide must be recognized by the modifying enzymes to obtain the final structure(s). The process is further complicated due to the oligosaccharide heterogeneity. A single glycosylation site may have both high mannose and complex structures creating heterogeneity which reflects differences in the biosynthesis of the carbohydrate moiety

(1). The structure of the folded and perhaps complexed protein is likely the most important information used by these enzymes for processing (93).

Examination of the location of oligosaccharides in different glycoproteins strongly suggested that the local environment around asparagine residue plays an important role in the determination of the accessibility of enzymes that process the carbohydrate to their final form (115,116). In another approach, Hsieh, Rosner, and Robbins used endoglycosidase H (endo H) which cleaves only high mannose type oligosaccharides to probe the relative accessibility of the asparagine-linked oligosaccharide of E1 and E2 glycoproteins in intact Sindbis virus (14,15). By using virus grown in a mutant ce3 line that makes only endo H sensitive oligosaccharides on glycoproteins, they could show that sites which were more sensitive to endo H in intact virus would have been processed to complex oligosaccharides in normal cells. Sites which were relatively resistant, were generally high mannose type when virus was grown in normal cells. However, the same site could be processed to either complex or high mannose type oligosaccharides depending on the kind of cell.

Factors other than accessibility may also influence the extent of oligosaccharide processing. Williams and Lennarz using an in vitro system for glycosylation of bovine ribonuclease B showed that while bovine pancreas

Golgi membranes failed to process this enzyme in its native form, the denatured form was readily processed. In addition, the rat liver system was capable of modifying both native and denaturated ribonuclease, indicating the possibilities of tissue and/or species-specific differences in the substrate specificities of one or more enzymes (16). Finally, Pollak and Atkinson (93) in a comparison of the types of oligosaccharides with their position in the polypeptide chain, have shown that complex oligosaccharides are generally located towards the amino terminus of the polypeptide, and high mannose are located towards the carboxyl terminus . Thus, processing frequently occurs in a gradient from the N-terminus to the C-terminus for a large variety of glycoproteins. While this trend is remarkable, there are a number of exceptions. Human IgD contains a high mannose oligosaccharide at a site near the N-terminus, while two more sites further down in the chain have complex structures (117). More structural data of known glycosylation sites are required before we can understand how cells determine the final glycoprotein carbohydrate structures.

Mouse IgM Heavy Chain as a System to Study Glycosylation

IgM heavy chain (m) is 576 amino acids long and contains five glycosylation sites which are located at the

Constant region (24,25,26). Figure 1 shows an schematic diagram of the (m) chain. The exact location of all sites of glycosylation and the primary aminoacid sequence has been reported by Kehry et al (24). Glycosylated residues include Asn 171, 332, 364, 402, and 563. Human heavy chain IgM contains an identical number of glycosylation sites; however, the oligosaccharide corresponding to asparagine 364 in mouse IgM is shifted to asparagine 395 in the human (225). Mouse myeloma IgM heavy chain, derived from MOPC 104 E cells, when cleaved with cyanogen bromide breaks into nine peptide fragments (figure 1), four of which contain asparagine linked sites of glycosylation. Three glycopeptides (CN5, CN7, CN8) carry a single site including Asn 171,402, and 563 in the intact heavy chain. Another glycopeptide (CN6) contains two sites of glycosylation at Asn 332 and 364. Our laboratory has developed a complete scheme of purification steps to isolate each glycosylation site and analyze the carbohydrate structure(s) (25). All sites contain multiple oligosaccharide structures. Anderson et al (26) have determined3 by high resolution H1-NMR the major structures at each of the glycosylation sites. These structures are shown in figure 12. CN5 is completely biantennary, while CN6 and CN7 are triantennary with nearly complete sialylation of the galactose residues . The majority of the glycopeptide from CN8 is a high mannose oligosaccharide, containing surprisingly Man_3.

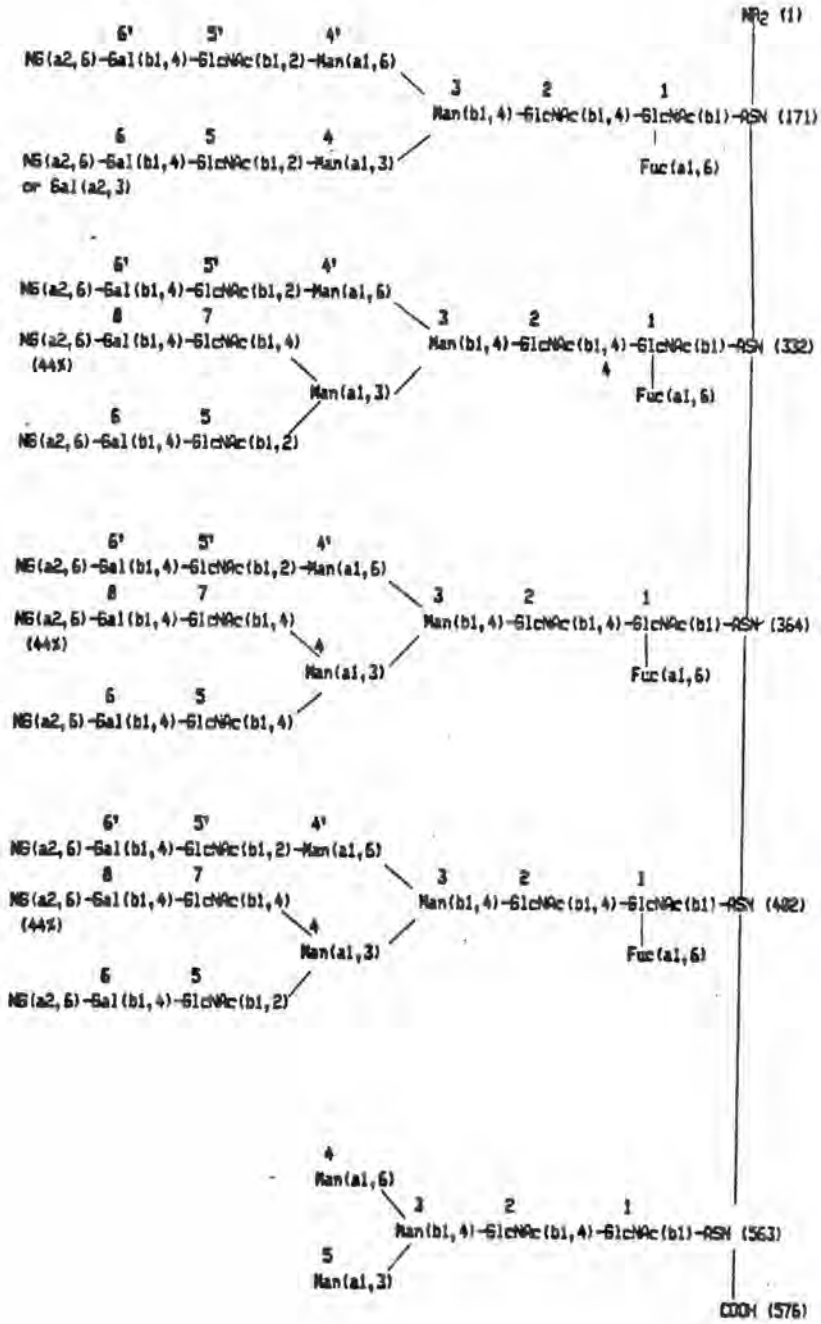


Figure 12. Summary of the major oligosaccharides on IgM from MOPC 104E. Asparagine linked carbohydrates at amino acid positions: 171 (complex biantennary), 332, 364, 402 (complex triantennary), and 563 (high mannose type).

Labeling studies of m chain implied that the glycosylation site at CN8 (nearest to the amino terminus) contained carbohydrate only part of the time. Chemical analysis which,includes amino acid and carbohydrate compositions show that Asn 563 is glycosylated only 40% of the time (118). The structure of the minor oligosaccharides found at each of the sites need to be determined. Preliminary studies indicate that each site has a unique set of heterogeneous oligosaccharides. The rules determining the relationships between structure of oligosaccharide and protein structure remain to be elucidated.

Studies using Hybridoma Cells Secreting Altered IgM

One approach to obtain information about the rules determining glycoprotein carbohydrate processing is to study mutants that secrete abnormal IgM (28,47.57). Alteration of the processing events or of the protein sequence, including changing the number of glycosylation sites and their position should allow study on the regulation of carbohydrate structures. PC700 a hybridoma cell line which secretes IgM specific for phosphoryl choline was produced by Dr. Mark Shulman while working with Dr. Kohler in Basel (28,57). A description of the method used to obtain mutant cells from PC 700 has been described (Figure 3). The alterations include hyperglycosylation

(cell lines number 21 and 38) and polypeptide deletion (cell lines number 128, 208, 313 and 562) from the μ heavy chain. The former mutants were used to analyze glycosylation.

Our initial studies of PC700 μ chain glycopeptides showed that this cell line secretes a heavy chain with oligosaccharides that are not identical to MOPC 104E μ chain. Thus, we elected to determine the structures at each glycosylation site for IgM from PC700 cells both for comparison with 104E IgM and with the product of the mutant lines 21 and 38. Results part I include biosynthetic labeling experiments to determine the overall differences between the heavy chains synthesized by the parental cell line and the mutants. Results part II contains the chemical analysis of the IgM from these cell lines.

Materials and Methods

Ascites Fluid

Hybrid F1 mice (DBA x BLAB/c) were primed by intraperitoneal injections of 0.5 ml of pristane (2,6,10,14-Tetramethylpentadecane). Cells (4×10^7) harvested from a large culture flask were resuspended in two ml of Dulbecco's Minimun Modified Media (DMM) and intraperitoneally injected into the F1 mice (DBA X BALB/c). Tumor-ascites fluid was collected after about three weeks, and after centrifugation was stored at -20 °C.

Biosynthetic Labeling of Immunoglobulin M

Hybridoma cells were washed in DMM and resuspended in 5 ml of Methionine free DMM. After 30 min at 37 °C the cells were collected by centrifugation. Cells were resuspended in 1-2ml of Methionine free DMM supplemented with 2% fetal bovine serum and ^{35}S-Met (800 Ci/mmol) (New England Nuclear) to a final concentration of 10 microCu/ml. The cultures were incubated at 37°C for 6-8hr in a 5% CO_2 atmosphere. Media lacking radiolabel was added every 2 hr to maintain the pH at 7.4. For carbohydrate labeling, D-[2-$^{(3)}$-H] Mannose (18.4 Ci/mmol) and L-[^{3}-H] Fucose (15 Ci/mmol) (New England Nuclear) were added to 100 microCu/ml final concentration in complete DMM. Cells were used as source of subcellular heavy chains (mi), and the supernatant for secreted IgM (ms). Cells were washed twice with 10 ml aliquots of PBS (10 mM phosphate buffer saline), and lysed with detergent containing buffer (0.14 MNaCl, 0.1M Tris-HCl pH 8.0, 0.5% Triton X, and 0.05 M paramethylsulfonic acid). The solution was stored at 4 °C overnight followed by centrifugation to remove insoluble cellular debris.

Immunoprecipitation

Extra-or intracellular (mi and ms) material was immunoprecipitated with (20 microliters) rabbit anti-mouse

(μ chain specific). After overnight incubation at 4 °C, an aliquot (40 microlitres) of protein A as a 10% suspension of Staphylococcus aureus (Pansorb, Calbiochem) was added and incubated for 1 hr at low temperature. Protein A-immunocomplex was collected by centrifugation and the pellet washed twice with lysis buffer. IgM was eluted from the complex by resuspending the pellet in 1% SDS buffer (0.1M Tris-HCl pH 8.0) and boiling for five min.

Endoglycosidase H (endo H) Assay Conditions

Endoglycosydase H reactions were carried out essentially as described by Tkacz (119). Endo H was a gift from Dr. P. Atkinson. Samples were resuspended to a final volume of 0.5 ml containing 0.1% SDS, 0.1M citrate/phosphate buffer pH 5.5, and 10-15 microunits of enzyme (4.01 U/ml). Reaction mixtures were incubated at 37 oC for 12hr. Three volumes of cold acetone were added to stop the enzyme reaction and precipitate the proteins. Endo H treated proteins were washed twice with cold acetone, air dried and resuspended in electrophoresis buffer (see later).

SDS-Gel Electrophoresis

IgM was analyzed in slab sodium dodecyl sulfate (SDS) gels using the Laemmli system (120). The samples were prepared by diluting 1:1 with 2-fold sample buffer (60 mM

Tris-HCl pH 6.8, 2% SDS , 10% glycerol) containing b-mercaptoethanol (unless otherwise specified) as reducing agent and boiling for five min. Reducing gels contained an 8% separating gel and a 4% stacking gel. In non-reducing conditions a gradient of 4-20% acrylamide was used with a 3.5 % stacking gel. The gradient maker was purchased from BRL. Electrophoresis was at 40 V for 16-22 hr, until the tracking dye had reached the bottom of the gel. Separated proteins were fixed by soaking the gel in 10% TCA for 1 hr. ^{35}-S-Met-proteins were directly visualized by radioautography. The sensitivity of the autoradiogram was increased by placing the gel in 50ml of Enhance (New England Nuclear). Dried gels were covered with X-OMAT film (Kodak) and stored at -60 ^{o}C for 10-15 days. Gels containing unlabeled proteins were stained for 1 hr at 37 ^{o}C with a 1.2% Coomassie blue solution and destained overnight in 50 % methanol, 10% acetic acid solution.

Protein Immunoblotting

Immunodetection of IgM was performed as described. (121). After electrophoresis, gels were placed in a transferring cassette on top of a nitrocellulose membrane (Millipore 0.45 micron HAWP) and covered with blotting paper. The complete cassette was then positioned in the transfer unit (Hoffer) containing four litres of tank buffer (20mM tris-base, 150 mM glycine, 20% methanol).

Electroelution was carried out with 60-80 V for 22 hr. After transfer, the membrane was incubated with tris-saline buffer (0.9% NaCl, 10 mM Tris-HCl pH 7.4) containing 5% bovine albumin (Sigma). The filter was transfered to a solution containing 4% rabbit anti-mouse (m chain specific) IgG (Kappel) and incubated overnight at room temperature. The nitrocellulose membrane was then washed with 200 ml of tris-saline buffer containing 0.05 % NP40 (2 times) and rinsed with tris-saline buffer. The immunocomplex was detected by autoradiography after incubation with ^{125}I-protein A (2-5 x10 ^{6}CPM/ ml) for 30 min. The nitrocellulose paper was covered with a X- ray film, and exposed for 24-36 hr using intensifying screens.

Enzyme Linked Immunoassay (ELISA)

Phosphorylcholine-bovine serum albumin (PC-BSA) was prepared in our laboratory (122). Diluted PC-BSA was dispensed in aliquots (50 microliters) over 96-well plates (Dynatech, Alexandria, Virginia) and incubated for 2 hr. After rinsing with PBS (phosphate buffer solution), PBS-1% BSA was used to block the plates. Aliquots containing IgM (50-100 microliters) were added and incubated for 1.5 hr at 37 ^{o}C. Non-reacting material was removed by rinsing with PBS. Add fifty microliters/well of affinity purified antibody to mouse IgM covalently coupled to alkaline phosphatase (Kirkegaard) was added and the reactions

incubated for 1.5 hr at 37°. The wells were rinsed with PBS and an aliquot of enzyme substrate (p-nitro-phenol phosphate, 1mg/ml) (Sigma), dissolved in diethanolamine buffer (pH 9.8) added. Incubations were for 1 hr at 25 °C. Enzyme linked immunocomplex was detected by absorbance at 405 nM.

Pronase Digestion

Immunoprecipitated subcellular and secreted chains were resuspended in one ml of 0.1M Tris-HCl (pH 7.8) buffer containing 0.01mM CaCl, 0.01 % SDS, and pre-digested pronase (Calbiochem) (ref: modified from Personen (123). Pronase was added daily for 3 days to a final concentration of 0.5mg/ml. Incubation was carried out at 37 °C under toluene to inhibit bacterial growth. The pronase digested material was then boiled for five minutes to destroy enzyme activity.

Fractionation of Radioactive Labeled Glycopeptides

Pronase glycopeptides were fractionated by gel filtration using a Biogel P-6 column (Biorad Laboratories) (1.2x135 cm) equilibrated with 0.2M NH_4HCO_3, pH 8.0. Dextran blue and phenol red were used as internal standards for Vo (fx 33-35, 1ml/fraction) and Vi (fx 83-85) respectively. In addition, the column was calibrated with MOPC 104E CN6 pronase glycopeptide (Figure 18) which

contains mainly triantennary and some high mannose structures as determined by 1H-NMR (26). One ml fractions were collected and aliquots monitored for radioactivity or carbohydrate content by the phenol sulfuric acid assay (127). Radioactive glycopeptides were detected by adding three ml of liquifluor containing cocktail (New England Nuclear) and counting in a scintillation counter (Beckmann).

Lectin Affinity Chromatography

Concanavalin A (Con A) chromatography of pronase glycopeptides was conducted essentially as described (124). A five ml column (0.9x 7cm) of Con A-sepharose (Pharmacia Fine Chemicals) was pre-equilibrated using 50 ml of Con A buffer (0.15M NaCl, 0.01 M Tris, pH 8.0, 1 mM CaCl2, 1mM MgCl2 and 0.05%NP40) with a flow rate of about 20 ml/h. Pronase glycopeptides were dissolved in Con A buffer (0.5-1.0 ml) and directly applied to the column. Material that did not interact with Con A was with eluted starting buffer (20-30 ml). Retained glycopeptides were eluted with the same volume of 10 mM a-methyl glucoside, followed by 30 ml of 100 mM methyl mannoside. Fractions (one ml) were collected and aliquots used to determine radioactive content as described above.

Ascites Fluid Fractionation

Ascites fluid (20 ml) was precipitated twice with 50% ammonium sulfate and applied to a column of ACA 22 Ultragel (LKB) (100 x 2.5 cm). Elution buffer contained 20 mM Tris Borate, 0.15 M NaCl, 0.02% NaN_3, pH 8.3 . Fractions (fx) (100 drops/fx) were collected and protein content determined by absorbance at 280nM. IgM purification was followed by enzyme linked immunoassay and SDS-PAGE immunoblotting. Peaks containing monomeric IgM (Figures 21 and 22) were rechromatographed under the same conditions.

Isolation and Purification of Heavy chains

Monomeric IgM was concentrated by ammonium sulfate precipitation (50%) and resuspended in 0.25 M Tris-HCl pH 8.2, 6M guanidine-HCl. Dithiothreitol (DTT, purchased from Sigma) was added to 10 mM (final concentration) to reduce disulfide bonds. After a four hr incubation at room temperature, iodoacetamide (Sigma) was used to alkylate the sulfide free bonds (25 mM final concentration). Reduced proteins were immediately loaded in a pre-equilibrated ACA 34 Ultragel column (100 x 2.5 cm). Fractions of 100 drops were collected using 3 M guanidine-HCl, 0.2M NH_4HCO_3 as elution buffer.

Proteins were detected by absorbance at 280 nM. Peaks were pooled and analyzed by SDS-PAGE. The ma containing material was rechromatographed over the same column using above mentioned conditions.

Preparation and Fractionation of m Chain Glycopeptides

Purified heavy chains (about 4-6 mg for mutant cell lines 21 and 38) were dissolved in 70% formic acid (re-distilled), followed by the addition cyanogen bromide (CNBr) (4 mg/ mg of protein) and incubated at 4 °C for 22 hr. The solution was lyophilized and resuspended in 6 M guanidine-HCl buffer followed by the addition of an equal volume of 0.2 M NH_4HCO_3. The cyanogen bromide glycopeptides were resolved by gel filtration over an ACA 54 column (150 x 1.5 cm) using the same elution conditions as for ACA 34. Fractions corresponding to individual CNBr glycopeptide fragments were pooled, dialysed and concentrated for chemical analysis (25).

Carbohydrate Analysis

Glycopeptides were hydrolyzed according to the method of Grimes and Greegor (125) using 2 N trifluoroacetic acid. Neutral and amino sugars (including 2-deoxyglucose as internal standard) were converted to their correponding alditol acetates by reduction and

acetylation with 0.3 N $NaBH_4$ and acetic anhydride. The volatile alditols were resuspended in acetone and injected into a gas chromatograph. Gas liquid chromatography was performed on a Hewlett packard model 5700 equiped with a flame ionization detector using a glass column (6 feet x 1/8 inch) packed with 3% OV225 on Supelcoport (Sulpelco, Inc. Bellafonte, PA) . Sialic acid was hydrolyzed from glycoproteins or glycopeptides by dissolving the dialyzed sample in 0.1 N H_2SO_4 and incubating at 80 ^oC for one hr. Sialic acid content was quantitated by the thiobarbituric acid method (126).

Phenol Sulfuric Assay

Carbohydrate content was also determined by the phenol sulfuric assay as described by Dubois et al (127). Samples were dissolved in water (0.4 ml) and treated with (10 microliters) 80% phenol reagent (80% solution) followed by rapid addition of one ml of concentrated sulfuric acid . After 30 min at room temperature, the absorbancy at 480 nM was used to calculate the sugar concentration. Mannose and Galactose were used as standards.

Results Part I

Studies of IgM Produced by Cell Lines PC700, 21 and 38

Shulman et al. isolated cell lines 21 and 38 from mutagenized PC700 cells. Both lines synthesize immnoglobulin M (IgM) which heavy chain (ms) is retarded on SDS gel electrophoresis relative to the ms chain from PC700. It was estimated that these ms chains had increased apparent molecular weights of 6000 to 8000. IgM produced in the presence of tunicamycin (a glycosylation inhibitor) results in the synthesis of secreted m chains that co-migrate with wild-type m, thus suggesting that the m chains of 21 and 38 were normal in their polypeptide chain, but abnormally glycosylated (57).

Analysis of m Chains by SDS-PAGE

In order to characterize the changes occurring in both cell lines, secreted and intracellular m chains were biosynthetically labeled using 35-S methionine. Labeled chains were immunoprecipitated using anti-m antibody, and tested for sensitivity to Endoglycosidase H (endo H). Endo H hydrolyzes the glycosidic bond of the

N,N'-diacetylchitobiose core structure N-linked to asparagine, removing the bulk of the glycan moiety. The enzyme is specific for high-mannose type oligosaccharides, and will not hydrolyze complex oligosaccharides (129). Figure 13 shows the analysis by SDS-PAGE (reduced conditions) of the intracellular m chains from mutant and wild type cells. In the absence of Endo-H, heavy chains from the mutant cells had the same mobility as the parental cell line (PC 700) (Figure 13 -Endo H). Endo H treated chains run with faster mobilities (due to the loss of carbohydrate), and the mutants and parental cells produced IgM with similar susceptibility to the enzyme. These results indicate that inside the cell, mutant and wild type products have similar or identical levels of carbohydrate processing. This experiment implies that hyperglycosylation is not caused by the presence of additional glycosylation sites on IgM from the mutant lines.

Secreted heavy chains were analyzed as described before (figure 14). PC700, 21 and 38 synthesized chains that migrate with different mobilities, with heavy chains from the mutants migrating slower than PC700. At the same time, secreted IgM from 21 and 38 is much less sensitive to Endo H. The migration of Endo-H treated 21 and 38 secreted m products also demonstrated heterogeneity in processing as shown by the multiplicity of bands.

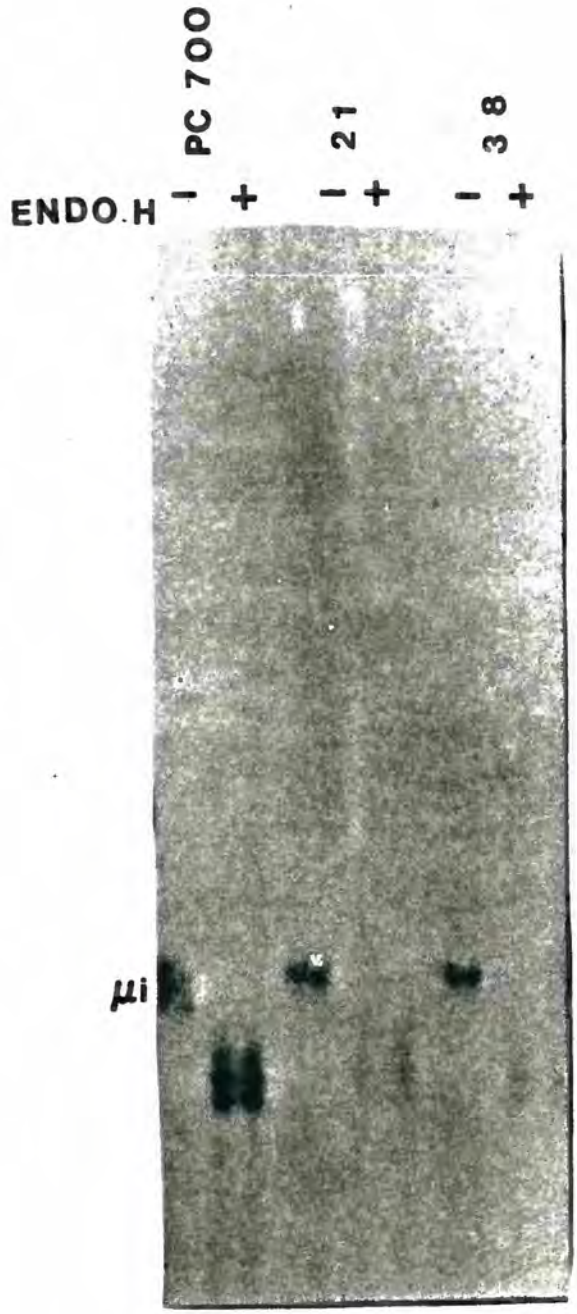


Figure 13. Intracellular (mi) heavy chains of PC 700, 21 and 38 mutants. Radiolabeled intracellular preparations were immunoprecipitated with rabbit anti-mouse IgM. The precipitate was solubilized and treated or not with endo H. Products analyzed by SDS-PAGE under reducing conditions.

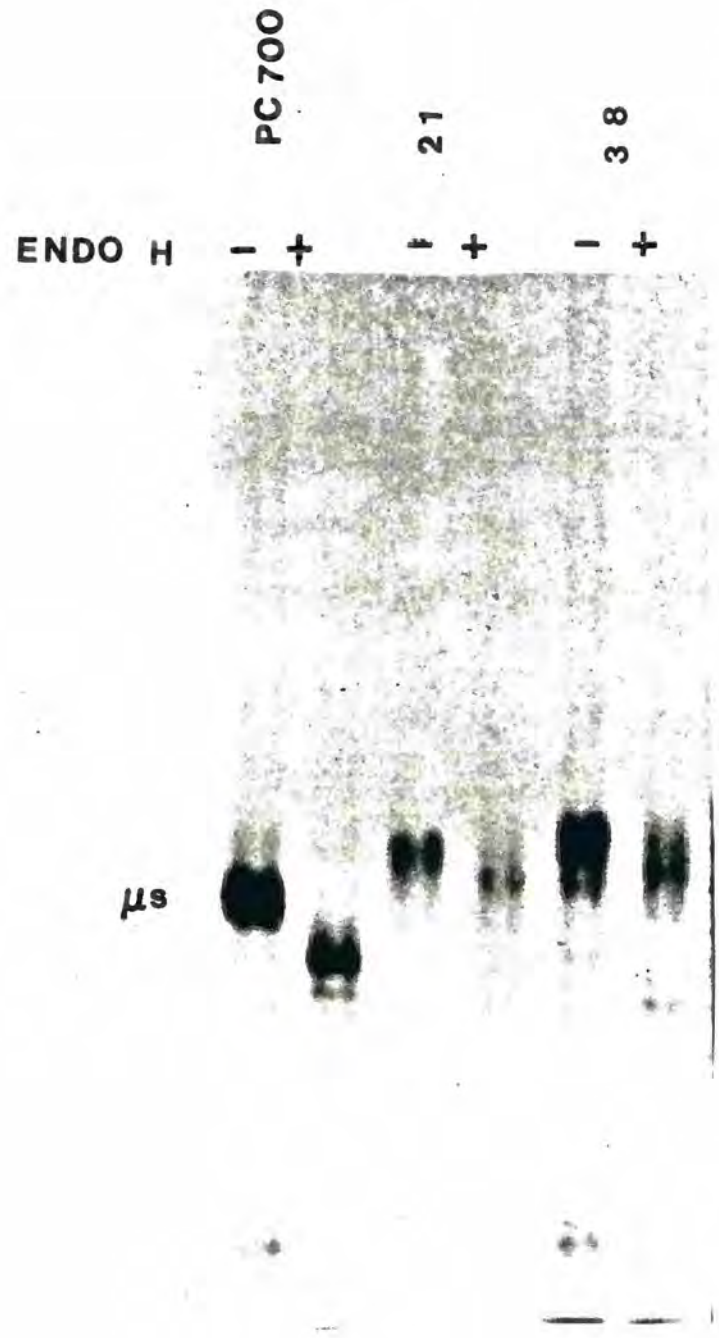


Figure 14. Secreted (μs) heavy chains from PC 700, 21 and 38 mutants. Radiolabeled secreted IgM was analyzed as described in figure 13.

Fractionation of Pronase Glycopeptides by
Affinity Chromatography

In order to determine the type of structures carried by each individual μ chain, we labeled secreted IgM from 21, 38, and PC700 cells by incubating cells in the presence of ^{3}H mannose for 4 to 8 hrs. Secreted μ chains were immunoprecipitated using anti-μ antibody and pronase treated as described in methods. The pronase glycopeptides were directly applied to lectin affinity columns. Concanavalin A is a plant lectin that has the ability to distinguish between different types of oligosaccharide structures (124,130). Tetra and triantennary complex structures, and fully processed biantennary oligosaccharides are not bound by the lectin and may therefore be separated from partially processed biantennary complex and high mannose structures. The later two structures are subsequently eluted with the addition of 10 mM α-methyl-glucoside and 0.5 M α-methyl-D-mannoside respectively. Figure 15 shows the fractionation of the types of structures produced by pronase treatment of the secreted IgM from each cell line. Tri and tetraantennary asn-linked and fully sialylated biantennary oligosaccharides are contained in the fraction I. Fraction II includes partially processed biantennary type

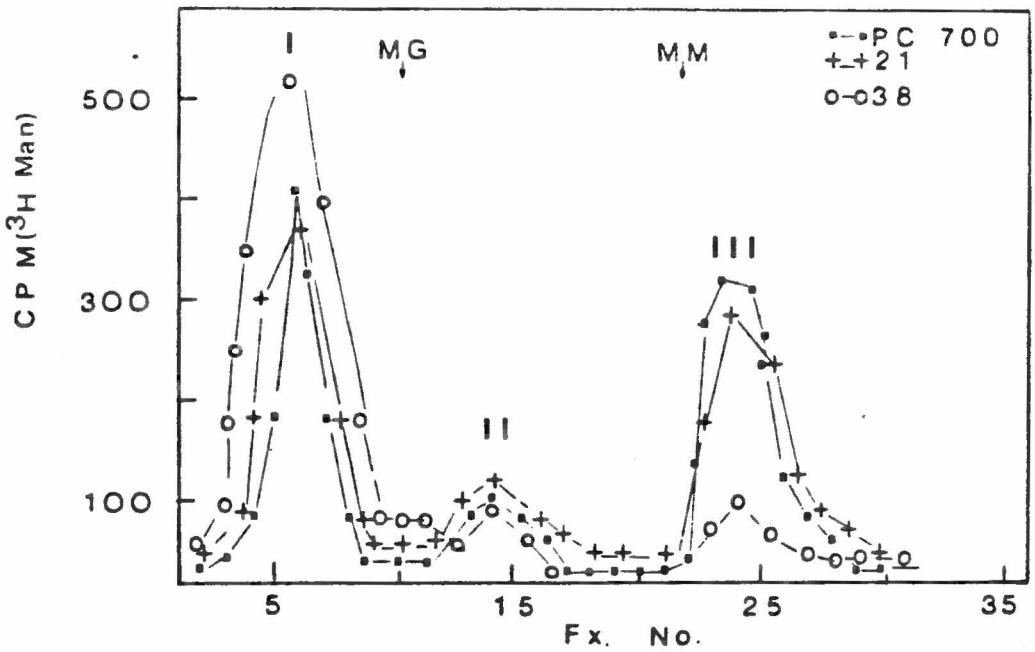


Figure 15. Concanavalin A-sepharose elution of ^{3}H-Man glycopeptides. Pronase treated glycopeptides from PC700, 21 and 38 m chains were fractionated by affinity chromatography. Arrows indicate start of elution with buffer containing 10 mM a-Methyl-glucoside and 0.1 M a-Methyl-mannoside. Fraction size was 1 ml.

Table I. Percentages of Mannose labeled glycopeptides fractionated by Concanavalin A.

	%			
Cells	FxI	FxII	FxIII	COMPLEX
MOPC 104E	36.0	46.0	15.5	81.5
PC 700	53.5	21.0	25.5	74.5
21	47.0	35.0	18.0	82.0
38	72.0	22.0	6.0	96.0

All exprimenta in 0.5% NP40.
The major peaks fron Con A affinity chromatography were pooled. FxI was the material that did not interact with the column. FxII and FxIII bind to Con A with differential affinities. Percentages calculated from the total applied radioactivity.

oligosaccharides. Fraction III includes the majority of high mannose containing glycopeptides. The percentage of each fraction are compared for the mutant lines, PC700, and MOPC 104E in the data of Table I. MOPC 104E cells produce IgM with a heavy chain that contains 81 % complex and 15 % high-mannose oligosaccharides. In SDS-PAGE MOPC 104E chains run with identical mobility as the product from 21 cells. mIgM from 38 has slower mobility, and appears to be the most processed to complex oligosaccharides (Table I). These studies confirm the preliminary data suggesting that processing of carbohydrates is quite different for the mutant lines (57). In addition, it is evident that the hybridoma cells and MOPC 104E differ in the oligosaccharides of IgM.

To further characterize the type of structures present at each of the heavy chains (both secreted and intracellular), cells were incubated with ^{3}H-Mannose and ^{3}H-Fucose, and IgM immunoprecipitated and treated with pronase. The glycopeptides were fractionated by Bio-gel P-6 chromatography. Fractionation is based on molecular weight, but generally triantennary structures are separated from high mannose type. The Bio-Gel P-6 column was calibrated using purified pronase glycopeptides derived from MOPC 104E fraction CN6. The CN6 structure has been determined by proton-NMR to be a fully sialylated triantennary oligosaccharide (26).

Intracellular ^{3}H-Man labeled heavy chains (mi) were isolated from detergent extracted cells. mi from PC700, 21 and 38 cells (Figure 16 a, b, and c, closed circles) gave pronase glycopeptides with similar elution profiles. The included peak migrates between the triantennary and high mannose markers. IgM from PC 700, 21 and 38 is a mixture that appears to include oligosaccharides with varying amounts of partially processed and unprocessed oligosaccharides. The subcellular form of IgG (52) and other cell lines (hybridoma), generally have only high-mannose oligosaccharides. The analysis of IgM in their secreted (ms) form shows a totally different picture (figure 16 a, b, and c, open circles). There is a change in the glycopeptide profile for IgM from PC700. About 50 % of the glycopeptides now elute closer to the complex triantennary marker, suggesting that the carbohydrate moieties have been further processed prior to secretion. Glycopeptides from 21 ms give an elution profile which is similar to the parental cell line, but with an increase in the higher molecular weight fractions (Figure 16b, open cicles). Mutant 38 gave the largest shift, consistant with the suggestion that the oligosaccharides for this cell line are the most processed. The majority of the glycopeptides are running as triaantennary or with higher molecular weight (Figure 16c, open circles).

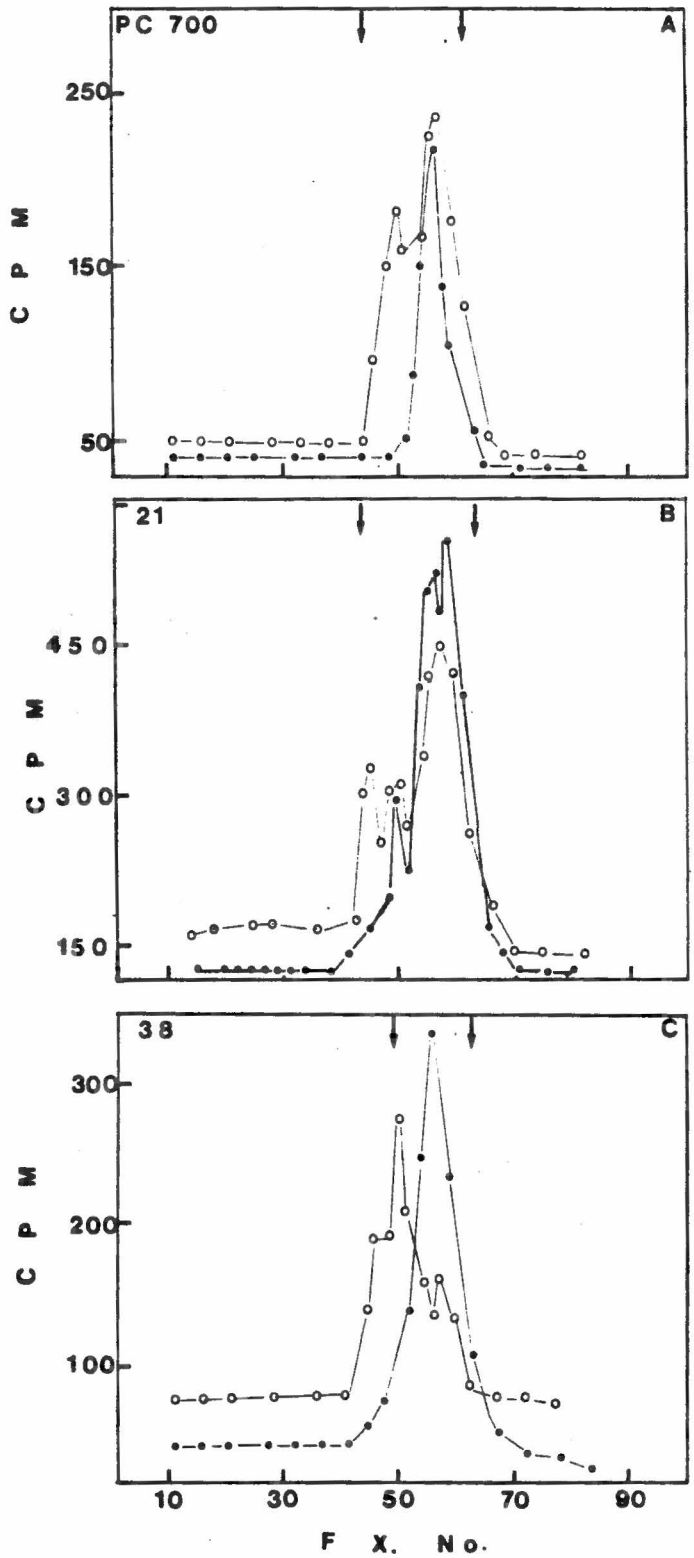


Figure 16. Gel filtration of ^{3}H-Mannose glycopeptides.

Summarizing the above results, the three cell lines synthesize heavy chains which inside the cell give similar glycopeptides. Upon secretion, the processing which we presume is in the Trans Golgi results in the carbohydrate differences for the mutant lines. These data also confirmed the low concentration of high-mannose type oligosaccharides and the resistance to Endo-H enzymatic activity for IgM from the mutants.

Analysis of Complex Glycopeptides

Tritiated fucose was used to metabolically label ms and mi. Fucose is found in N-glycosidically linked oligosaccharides on complex structures, and not on oligomannoside structures. From the studies on MOPC 104E, fucose is linked to the most internal GlcNAc residue, i.e. the residue attached to asparagine (1). The addition of fucose to the core $Man_3GlcNAc_2Asn$ is a late Golgi-localized process occuring along with the enzyme steps leading to complex fully processed oligosaccharides.

Fucose labeled pronase glycopeptides were isolated as described for the mannose labeled glycopeptides and fractionated by Bio-Gel P-6 gel filtration. In all cell lines the intracellular glycopeptides eluted in two major peaks (Figure 17 a, b, c; closed circles).

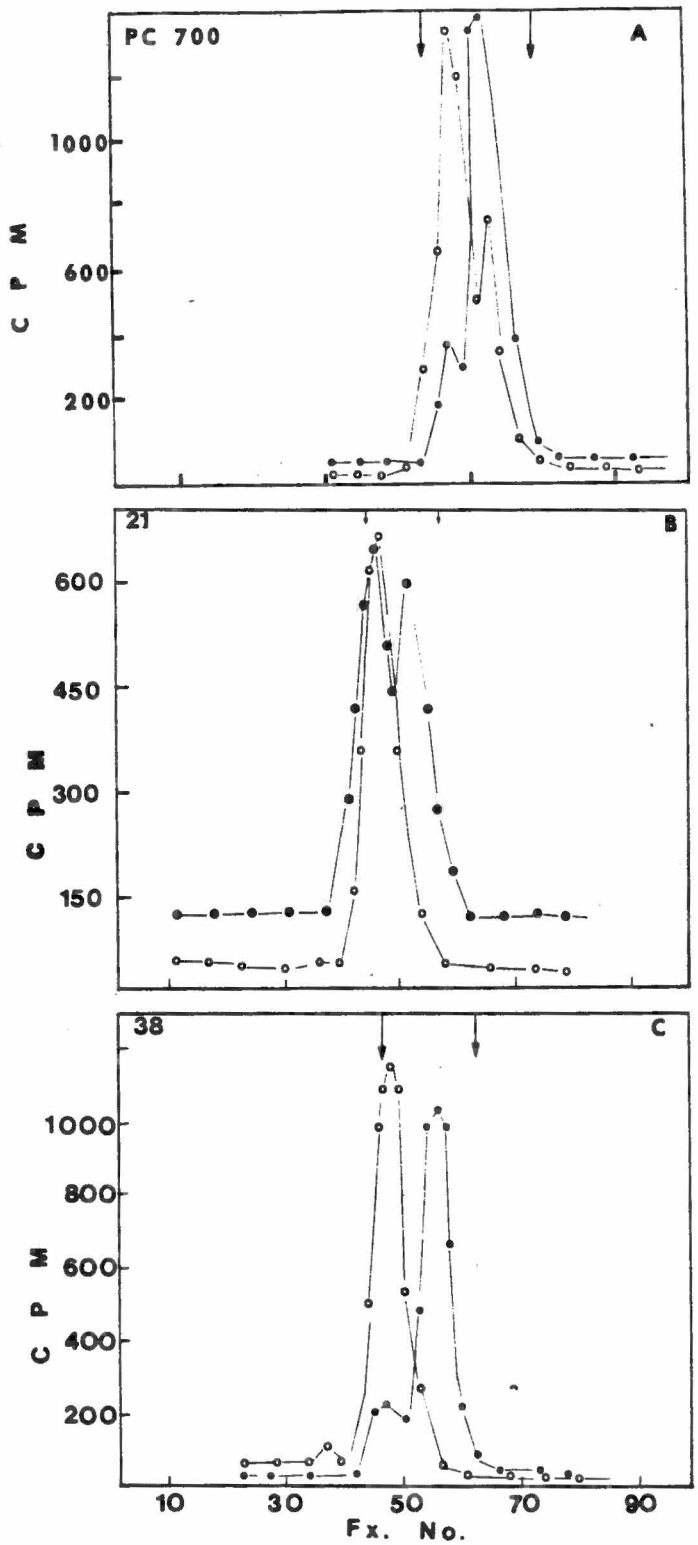


Figure 17. Gel filtration of ^{3}H-Fucose glycopeptides.

In PC700 mi, about 20 % of the label has been incorporated into the fraction with higher molecular weight, with the remainder in the retarded fraction (Figure 17a). Glycopeptides from IgM secreted by 38 cells showed a similar pattern with 24% and 76 % of the radioactivity respectively in these fractions . (Figure 17 c). Mutant 21 mi pronase glycopeptides showed an elution profile with the radioactivity symmetrically distributed in both peaks (Figure 17 b). Fucose labeled pronase glycopeptides derived from ms heavy chains demonstrate the trend of processing in each cell line. In PC 700, 38 % of the radioactivity is eluted as in the intracellular form, while the majority of the glycopeptides are now with the more processed fraction (Figure 17 a; open circles). In both mutants,(figure 17 b,and c ; open circles) secreted ms forms incorporated radioactivity only in glycopeptides eluted ahead (higher mw) than the triantennary marker. No structures with the low intracellular mw where found. Table II presents a summary of glycopeptide distribution determined by the fucose labeling.

Table II. ^{3}H Fucose labeled glycopeptides from intracellular and secreted μ chains.

Cells	μ chain	- % - Fx I	FxII
PC 700	μi	20.0	80.0
	μs	62.0	38.0
21	μi	55.0	45.0
	μs	100.0*	----
38	μi	24.0	76.0
	μs	100.0*	----

*Total radioactivity incorporated.
Heavy chains (μi and μs) were biosynthetically labeled with ^{3}H-Fucose.
Immunoprecipitated chains were then pronase treated and glycopeptides fractionated by Bio-Gel P-6 filtration (figure 17). FxI is the fraction that elutes with or close to the triantennary mw marker. FxII eluted with the high mannose marker.

Results Part II

Chemical Studies on Carbohydrate from IgM

Structure determination of the carbohydrates at each glycosylation site is required to fully understand the changes occurring in 21 and 38 m chains. Chemical characterization needs large amounts of material which can not be obtained from tissue culture media. Ascites fluid which can contain miligram amounts of antibodies was chosen as a source of IgM.

The approach taken was to first purify IgM from 21, 38 and PC 700 ascites fluid, then isolate the heavy chains from the light, and finally separate and determine the oligosaccharide structures at each glycosylation site.

Preliminary studies in our laboratory indicated that mutations were not the only origin of variation in carbohydrate structure. We have observed that IgM from cells grown in tissue culture can differ dramatically from the IgM that the same cells secrete into ascites fluid. As an example, we compared glycopeptide profiles for CN6, a cyanogen bromide cleavage product from IgM produced by MOPC 104E cells. The structure of the two oligosaccharides in this fragment from ascites fluid IgM has been characterized by 500 mHz NMR (26). The oligosaccharide chains are mainly

triantennary type, with nearly complete sialylation.

Mixtures of purified pronase treated CN6 derived from cells labeled in tissue culture with ^{3}H mannose and ascites fluid (detected by phenol sulfuric acid assay since the fragment from ascites fluid is present in large excess) were fractionated over a P-6 column (Figure 18). By this crude comparison it can be seen that the phenol sulfuric acid assay shows much more material in the larger peak. The radioactivity is found in higher amounts in the peak corresponding to high mannose oligosaccharides. As stated above, we have observed that the ascites fluid IgM can have nearly complete triantennary oligosaccharides while the tissue culture product contain only high mannose oligosaccharides. Another example of changes in processing depending on the source of IgM comes from comparisons of Endo H sensitivity. Partially purified mu heavy chains from MOPC 104E, PC 700 and 21 grown as ascites fluid were incubated in the presence or absence of Endo H and examined by immunoblotting using anti-m IgG antibody and ^{125}-I-Protein A. Figure 19 is the autoradiogram obtained. In contrast to the products from cells in culture where PC700 is very sensitive and the 21 m chain is partially resistant to Endo H (Figure 14), ascites derived

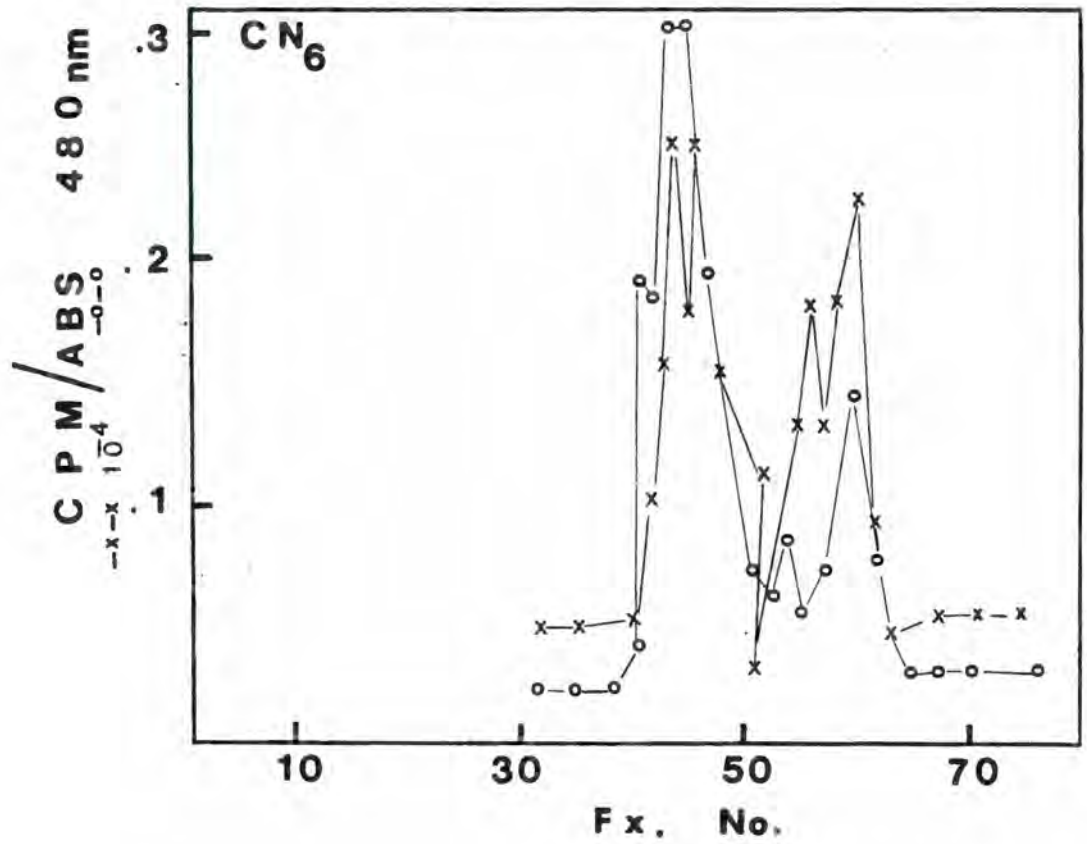


Figure 18. P-6 gel filtration of MOPC 104E cyanogen bromide fragments. Mixtures of purified pronase treated CN6 derived from cells labeled in tissue culture (CPM) and ascites fluid (ABS 480 nm) were fractionated over a Biogel P-6 column. Fractions of 1 ml were collected. Radioactivity and carbohydrate content determinated from identical aliquots.

Figure 19. Electrophoretic blotting of IgM heavy chains from MOPC 104E, PC700, and 21. Proteins from ascites fluid grown cells were ammonium sulfate precipitated and separated on an SDS-polyacrylamide gel. Proteins were blotted and μ chains immunodetected using rabbit anti-μ antibody. The blots were treated with ^{125}I- labeled protein A and autoradiographed as described in "materials and methods" (121).

chains are completely resistant to this enzyme. However, the differences in mobilities between PC700 and 21 are found regardless of whether IgM is prepared from cultured cells or ascites fluid. The processing differences are always in the direction that cultured cells have more high mannose and partially processed products. It may be that these structures are precursors to the products present in ascites fluid.

Purification of IgM Heavy Chain from Ascites Fluid

Our major goal was to determine how the carbohydrate from IgM produced by mutants 21 and 38 differed from PC 700. Since IgM from PC 700 cells appears to be glycosylated different than the IgM from MOPC 104E, we set out to do complete carbohydrate on PC 700 as well. Our objective was to determine the nature of the processing changes found in PC700, 21 and 38 relative to the IgM from MPC104 E cells. The molecular alterations in IgM chains may be due to several reasons including extra sialic acid residues, and or branching. To fully determine the changes occurring in the secreted (ms) chains, it was necessary to compare the processing at each glycosylation site for both the parental and mutant lines.

The carbohydrate chemical analyses require large amounts of starting material. These amounts can only be obtained from ascites fluid. The approach taken was to purify IgM from ascites fluid which contain miligram amounts of antibody (see later, table III), isolate m heavy chains from IgM, and finally determine the sugar composition for the complete m chain molecule and for each glycosylation site. While ascites fluid contains IgM with more processed oligosaccharides, the differences in SDS gel electrophoresis rates that define hyperglycosylation are maintained. It is critical that all of the comparisons use IgM produced from cells grown under identical conditions.

Fractionation of Ascites Fluid by Gel Filtration Chromatography

Dr. Darrel R. Anderson carried out the purification and fractionation of the IgM from PC 700 cells. In parallel, I purified IgM from 21 and 38 cells. We both were involved in the structural studies. The purification and column elution profiles steps were identical for 21 and 38, thus only part of the data using ascites fluid from mice with tumors carried by line 38 are included. Ammonium sulfate precipitated proteins from ascites fluid were resuspended in Tris-Borate buffer (see methods), and directly applied to ACA 22 Ultragel column. Proteins are

sorted based on their size within the column range of 100 to 1200 kd. IgM is composed of m-heavy and k-light chains which can be polymerized to various extents. Monomers have two light and two heavy chains (m_2K_2) joined by disulfide links. Pentameric IgM contains five of such monomers joined by m-m disulfide bonds, and one J protein (mw 10Kd). Due to differences in their mw, the forms can be purified and independently studied. In addition, antibodies secreted by these cells lines have the ability to bind phosphorylcholine (PC).

The PC specific antibody was followed by an Enzyme Linked Immunoassay (ELISA) (Figure 20, open circles). In this technique plastic wells are coated with PC-albumin, and incubated with a source of anti-PC IgM followed by phosphatase coupled goat anti-mouse IgM (57). p-Nitrophenyl phosphate is added to quantitate the immuno-complex (measured absorbance at 405 nM). Proteins were followed by absorbance at 280 nM (Figure 20, closed circles). Figure 20 shows the elution profile obtained from mutant 21 ascites fluid. Four major protein peaks were detected, with only two giving a strong ELISA response (PC-specific), eluting with the monomer (8S) marker. The second ELISA positive fraction is running with the PC 700 pentamer (19S) marker, and has a low protein content. Mutant 38 an gave identical elution profile and is shown in Figure 21. To confirm the

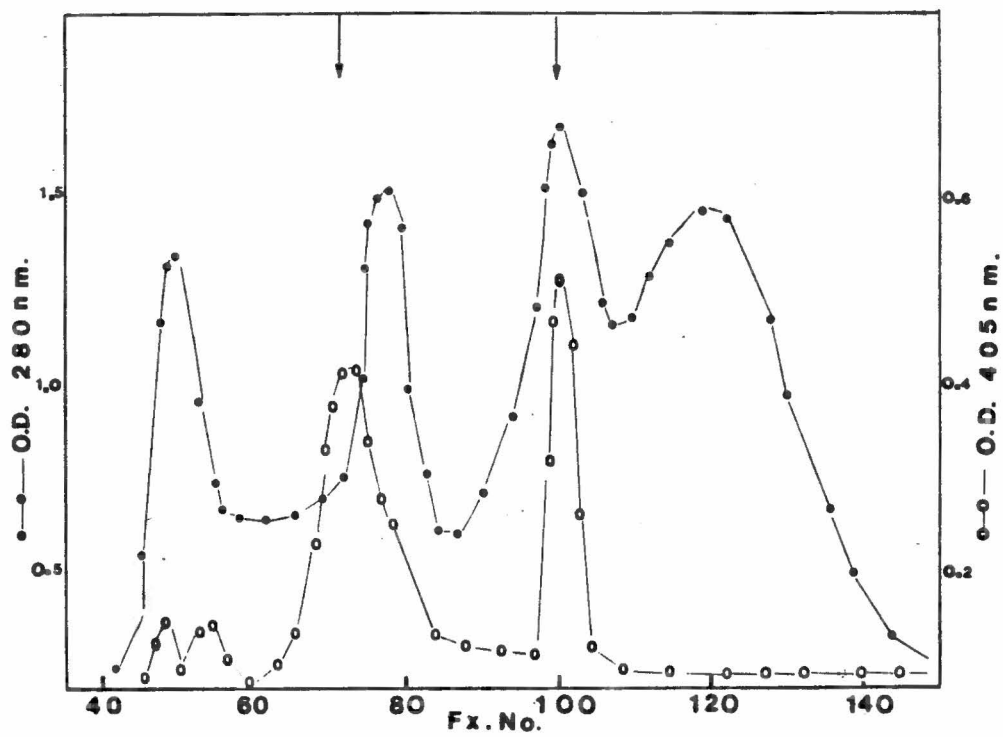


Figure 20. Gel filtration chromatography of proteins from mutant 21 ascites fluid. Cells were intraperitoneally injected into mice, and ascites tumors drawn off. Fluid used as source of IgM. After ammonium sulfate precipitation, proteins were resuspended in Tris-Borate buffer (pH 8.3) and separated by gel filtration over an ACA 22 column. Proteins were followed by ABSORBANCE (Optical density) at 280nm (closed circles). Igm containing fractions determinated by enzyme linked immunoassay (ELISA, open circles). Arrows correspond to pentameric and monomeric IgM. Dimer form eluted between the two markers.

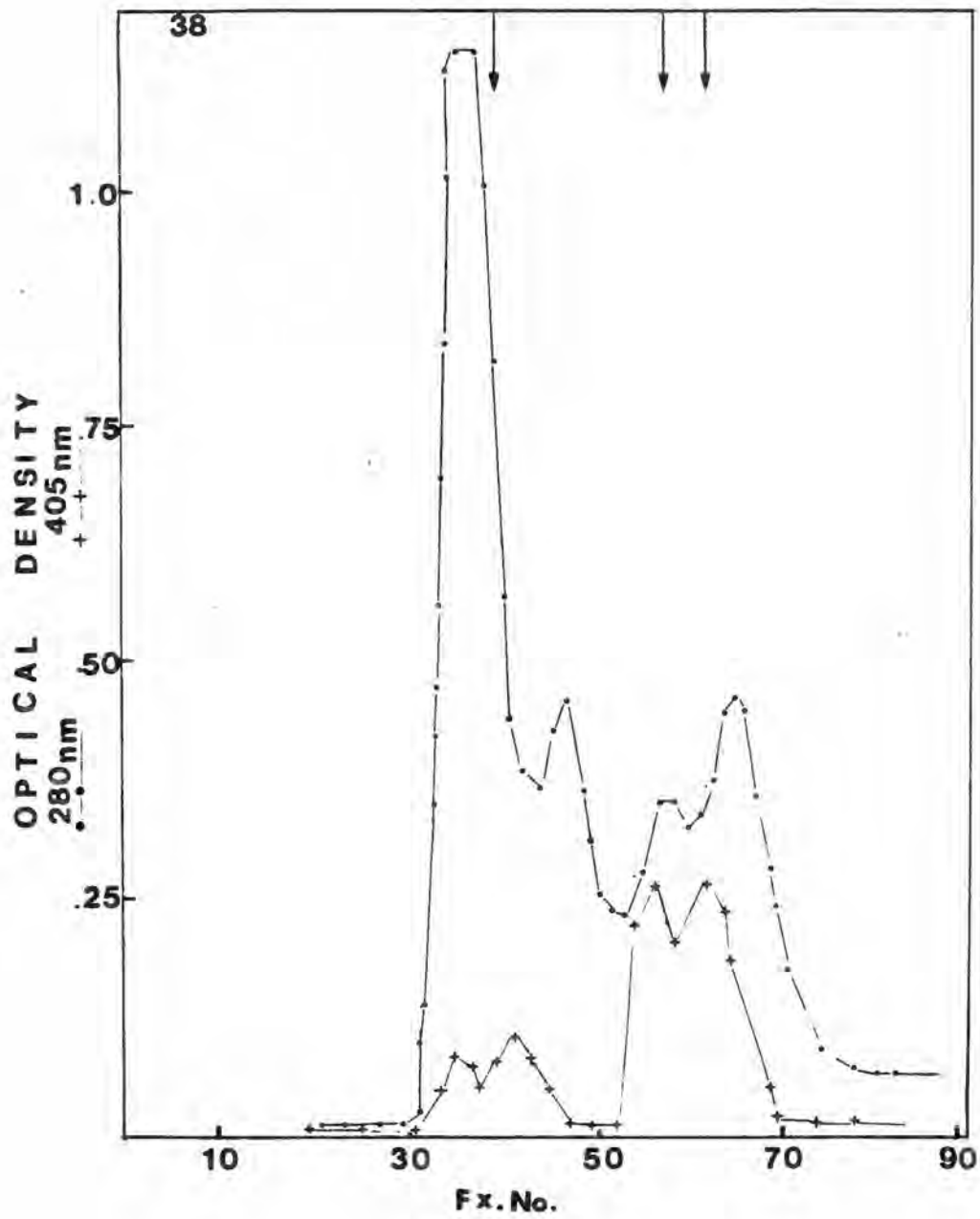


Figure 21. Gel filtration chromatography of proteins from mutant 38 ascites fluid. Fractionation and methods of detection as described in figure 20. The arrows indicate the points were pentamer, dimer and monomer IgM eluted (from PC700 cells).

IgM is detected by Coomassie blue stain (Figure 22) or by protein immunoblotting (Figure 23) (121). Both detection methods, indicate that only some of the stained proteins reacted with the anti-m antibody. Other proteins detected by Coomassie blue are mainly serum components. All fractions contained a band that migrated as pentamer. In addition, the unreduced IgM of mutants had two other bands with dimer and monomer mobilities respectively. The latter comes from the region with highest PC specific antibody and protein content (fraction 100). Fractions containing IgM were pooled and rechromatographed over the same column to increase the purification of each population. Some of the other forms detected on the gel include half-mer (HL chains) and unlinked m chains. PC700 and MOPC 104E (IgM producer) pentamers were used as markers.

Table III summarizes the estimated total PC specific IgM contained in 20 ml of ascites fluid. Cell line PC 700 secretes 200 mg of IgM while lines 21 and 38 have 4-5 times less IgM in ascites fluid. The distribution of their polymerized forms also differs. Mutants secrete 30-fold less pentamer and twice the amount of monomer than normal. The IgM from the mutants exhibits regular PC-binding (57), and ELISA absolute units drop following their antibody content. The reduction in IgM content in

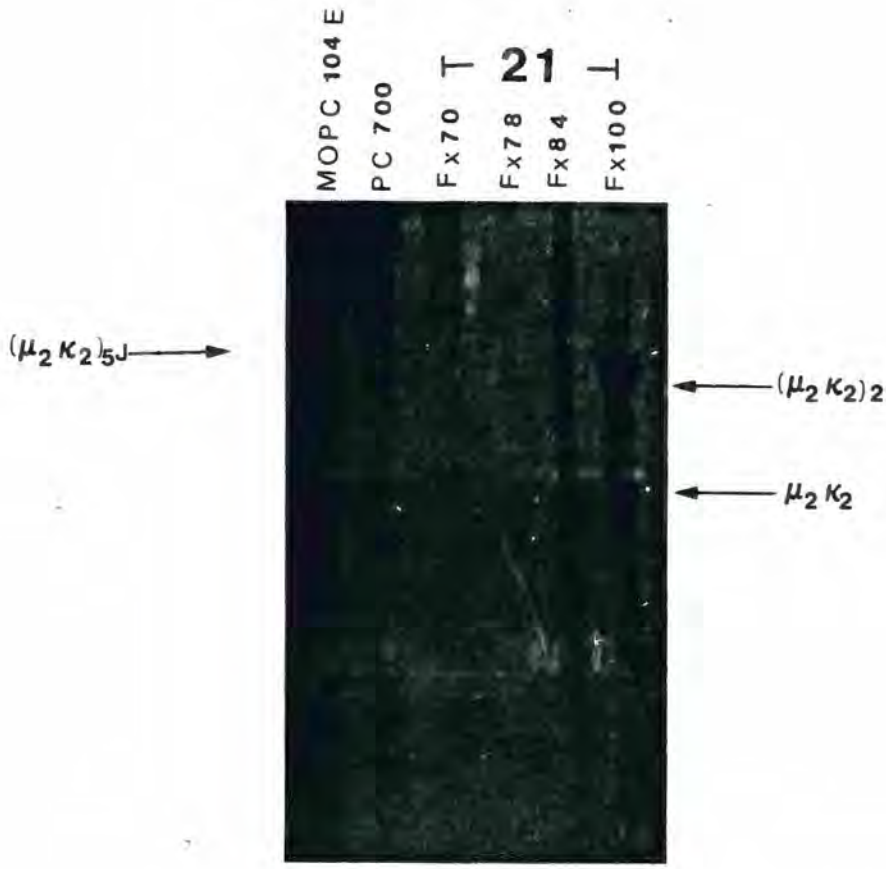


Figure 22. SDS-PAGE analysis of fractionated mutant 21 ascites fluid. Aliquots from ACA 22 gel filtration fractions were electrophoresed through a 4-20% polyacrylamide gel under nonreducing conditions. Separated proteins stained with Coomassie blue. Pentamer IgM from PC 700 and MOPC 104E used as markers. Migration of pentamer, dimer and monomer forms are indicated by the arrows

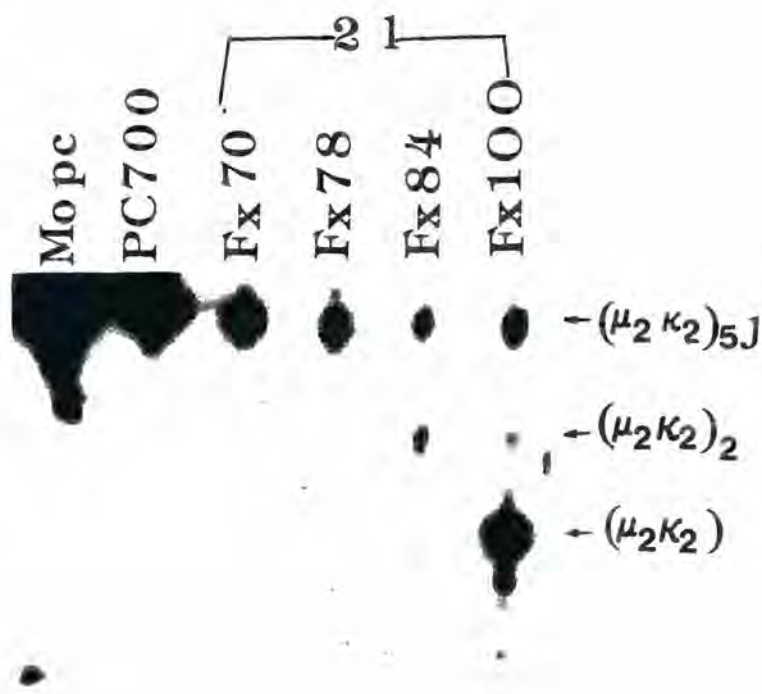


Figure 23. Protein immunoblotting of fractionated mutant 21 ascites fluid. Conditons used were as described in figure 22. After electrophoresis, the proteins were transfered to a nitrocellulose paper by electroelution. IgM polymer forms were immunodetected using anti-m chain antiserum and ^{125}-I protein A.

Table III. Comparison of IgM found in ascites fluid.

Cells	Polymer form	IgM mg/20 ml of ascites	Elisa U/50ug of protein
PC 700			
	Pentamer	180-190	0.74
	Monomer	20-21	0.26
	Total	200-211	
21			
	Pentamer	6- 7	0.11
	Monomer	40-46	0.20
	Total	46-53	
38			
	Pentamer	2- 4	0.05
	Monomer	35-40	0.22
	Total	37-44	

Ascites fluid was ammonium sulfate precipitated and fractionated over ACA 22 agarose gel. Peaks containing the various IgM pooled and analyzed. Elisa determination as described in materials and methods.

ascites fluid from 21 and 38 cells could come from either low protein synthesis or an increase in degradation of altered heavy chain. It is not known whether the differences in secreted forms (pentamer vs monomer) are due to changes in glycosylation. We have evidence that the rates of synthesis of IgM from PC 700, 21, and 38 are comparable when cells are grown in tissue culture.

Isolation and Purification of Immunoglobulin Heavy Chains

Mixtures containing reduced and alkylated monomers (8S) from each cell line were applied to ACA 34 Ultragel to separate the heavy chain from other polypeptides (light chain). Elution buffers contained 3 M guanidine to keep ms in solution. Three major protein peaks (absorbance at 280 nM) were found (Figure 24). The excluded peak represents a large mw glycoprotein from mouse serum. The second and largest peak contains the heavy chain, while the last peak has small proteins including the light chain. Figure 25 is the SDS-PAGE analysis of fraction I and II. Proteins were stained with Coomasie blue. Fraction II has two bands, one running slower (as expected for ms from 21) and a second protein with faster mobility than PC700. Both proteins react with anti-m antibody (data not shown). The later appears to be an unglycosylated form of the heavy chain.

Heavy chains were rechromatographed over the same column. No further attempts were made to separate both chains. Mutant 38 gave an identical pattern through the purification steps. Partially purified IgM from PC 700 and MOPC 104 E were used as mw markers. We obtained 7-10 mg of purified heavy chain per 20 ml. of ascites fluid. Aliquots containing 40-60 micrograms of μ chains were analyzed by immunoblotting as described above (121). Figure 26 is the autoradiogram showing the three cell lines. 21 and 38 products migrate with slower mobilities than the parental cell line IgM.

Total Sugar Composition of μ Heavy Chains

Purified μ heavy chains from PC 700, 21 and 38 cell lines were used to determine sugar compositions by gas liquid chromatography (GLC). GLC allows separation, identification and quantitation of all (but sialic acid) monosaccharides in a single procedure (44). The glycoprotein is hydrolyzed with acid and the sugars are then reduced and acetylated to their corresponding alditol acetates (118,125). The volatile alditol acetates were separated and quantitated using 2-deoxy-D-glucose as an internal standard. Table IV show the composition (in molar ratio) of the intact molecules. Mannose values were standarized to 3 moles. There is an increase in the number

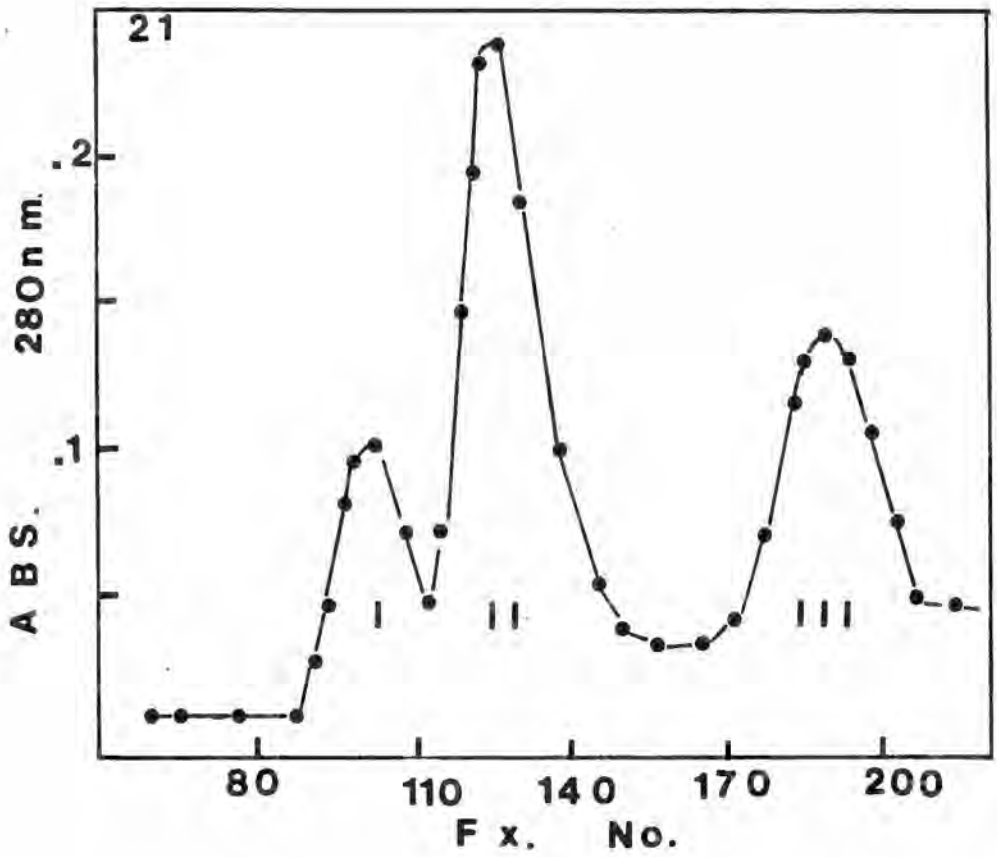


Figure 24. Purification of IgM heavy chain secreted from 21 cells. Reduced and alkylated monomer IgM were applied to ACA 34 Ultragel. Heavy chains separated from other proteins and eluted in fraction II. Fraction I contained aggregated material and fraction III was mainly light chain. Elution buffers contained 3 M guanidine HCl to keep ms in solution. Proteins detected by ABS 280nm.

Figure 25. SDS-PAGE analysis of purified 21 κ chain. Fractions I and II from ACA 34 gel filtration chromatography were pooled, dialyzed and electrophoresed over an 8% polyacrylamide gel (under reducing conditions).

Figure 26. Autoradiography of immunodetected PC 700, 21, and 38 μ chains. Purified heavy chains were separated in a SDS-PAGE gel, and analyzed by protein blotting as described in "materials and methods". 21 and 38 cell products migrate slower than PC 700 μ chain.

of moles of NAcGlc in both mutants 21 and 38. Fucose and galactose content follows the same trend with 21 m heavy chain being larger than 38. The Gal values were elevated due to the leaking of this sugar from our gel filtration columns. The ratio NAcGlc/Man increases in 21 and 38 m chains, again indicative of further processing. The cell line 38 produces IgM with the highest ratio which agrees with the possibility that triantennary structures (3 moles of man/5 moles of NAcGlc) are been produced. The 21 m chain presented a lower molar ratio (3 moles of man/4.3 moles of NAcGlc) which is indicative of mixtures of bi and triantennary structures.

IgM purified from MOPC 104E, PC 700, and mutants 21 and 38 were treated under mild conditions to release the sialic acid. This sugar was then determined by thiobarbituric acid method (126). Table V is a summary of the sialic acid content/mg of protein. PC 700 cell product has the lowest amount of sialic acid while the heavy chains from the two mutants have sialic acid concentrations similar to IgM from MOPC 104E. These results are in agreement with H-NMR data of MOPC 104E and PC 700 which have shown that the latter cell line carries mainly biantennary structures and a large amount of unprocessed oligosaccharides (see later).

Table IV. Carbohydrate composition of complete IgM m chains.

Cells	Molar Ratio			
	Fuc	Man	Gal*	NAcGlc
PC 700	0.35	3.00	3.10	2.20
21	2.60	3.00	7.00	4.30
38	1.50	3.00	6.00	5.40

* The galactose values are high due to bleed off from the ACA ultragel columns.
Purifed IgM heavy chains from ascites fluid were hydrolyzed and used to prepare alditol acetates (125). Gas liquid chromatography performed as deacribed in Materials and Methods. Deoxy-2-glucose used as internal standard. Mannose values standarized to 3.00 moles.

Table V. Sialic acid levels in IgM heavy chains.

Cells	mg/mg of protein	
MOPC 104E	4.8	+/-0.40
PC700	3.5	+/-0.25
21	5.0	+/-0.10
38	4.5	+/-0.20

*calculated from 2-4 experiments. Heavy chains from IgM secreted from myeloma and hybridoma cells were hydrolyzed under mild acid conditions. Sialic acid content determinated by the thiobarbituric acid assay (126). Protein determinations were performed by the method of Lowry (8). +/- indicate standard deviations.

Isolation and Characterization of
m Chain Glycopeptides

The location of the five glycosylation sites and primary amino acid sequence of normal m heavy chain have been previously described (figure 12) (24). In addition, the complete structures at each site has been determined for IgM MOPC 104 E (myeloma) (26) and are shown in figure 12.

IgM purified from PC 700, 21, and 38 were cleaved with cyanogen bromide into nine fragments. The fragments were resolved on Ultragel ACA 54 (figure 27) and followed by absorbance at 280 nm. The column was pre-equilibrated in 3 M guanidine buffer and standarized with MOPC 104 E m chain cyanogen bromide fragments. In general both mutants (figure 28) and parental cell m chains glycopeptides gave very similar elution profiles. The largest peak (mw=18,105, calculated from proton-NMR obtained structures) designated as CN6 contains the two oligosaccharides located at asparagine residues 332 and 364. CN5, CN7, and CN8 (with mw=12,230, 9,430, and 6,815) have one glycosylation site and eluted as expected (25). The peaks were pooled and used for chemical analysis. In CN8, from the ms synthesized by PC700 and 21, the peptide content was very low but there was sufficient carbohydrate to proceed for carbohydrate.

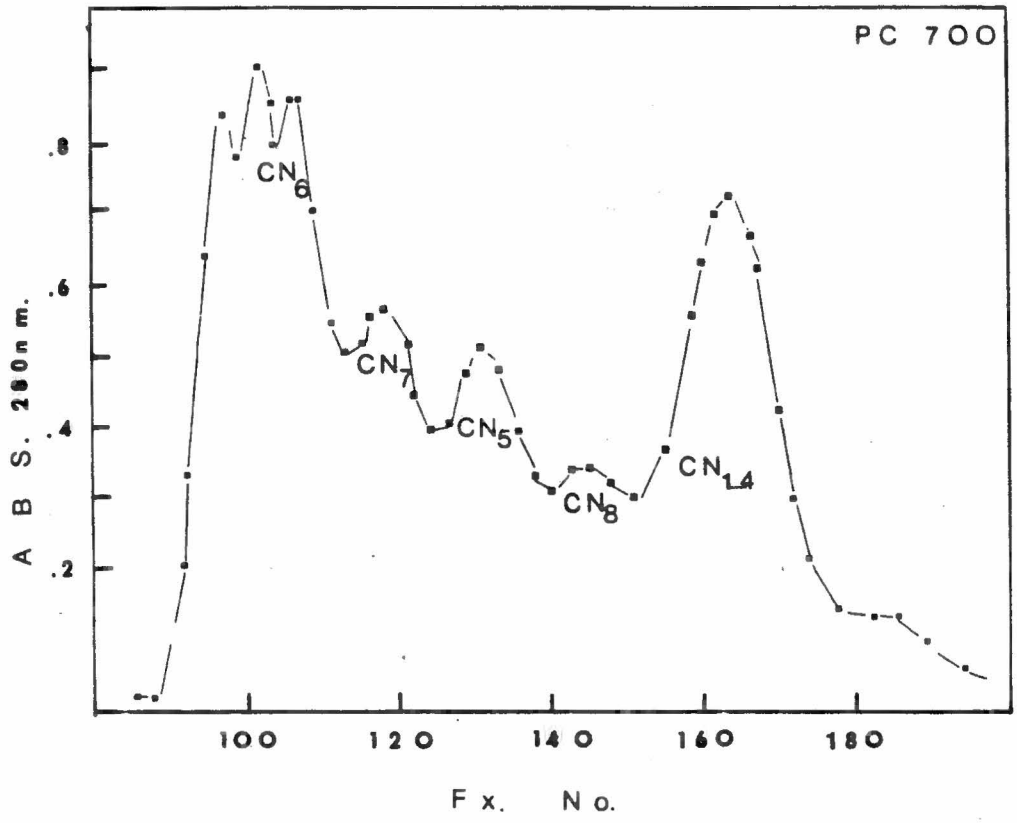


Figure 27. Fractionation of PC 700 m chain cyanogen bromide glycopeptides by gel filtration. The cleavage mixture was applied to an ACA 54 column, and eluted with 3 M guanidine HCl buffer. Fractions (1 ml) were collected and protein detected by absorbance (ABS) at 280 nm. Four peaks corresponding to glycopeptides CN6, CN7, CN5, and CN8 were collected and pooled.

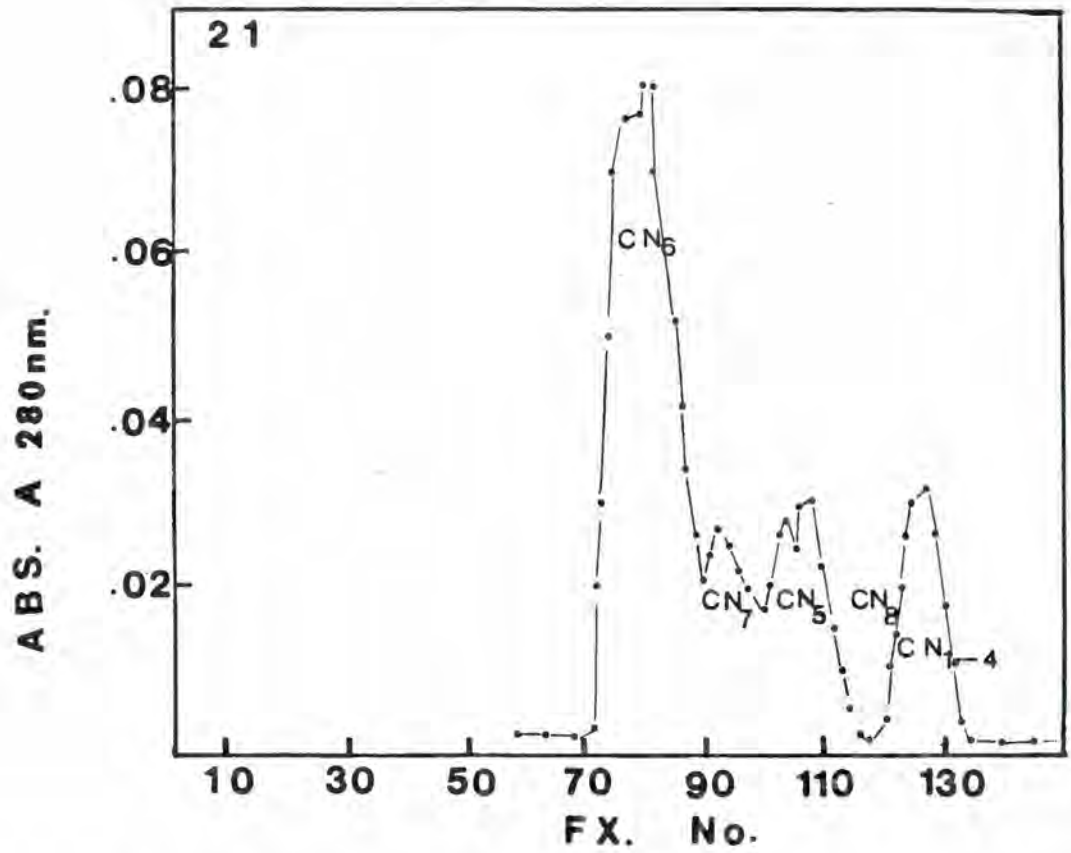


Figure 28. Fractionation of 21 m chain cyanogen bromide glycopeptides by gel filtration. Conditions used as described in figure 27. Fractions (1.3 ml) were collected and protein detected by absorbance (ABS) at 280 nm. Four peaks corresponding to glycopeptides CN6, CN7, CN5, and CN8 were collected and pooled. The agarose column was standarized with CNBr fragments from MOPC 104 E and PC700 m chains.

Anderson and Grimes (25) showed by amino acid compositions that these methods of purification gave peptides free of contamination from adjacent glycosylation sites. The fact that both parental and mutant lines secrete heavy chains with identical cyanogen fragmentation patterns, confirm the proper location of methionine residues. It is very likely that no other protein alterations have taken place. This again argues that the retarded mobilities of mutants heavy chains are due to oligosaccharide side chains.

Fractionation of m PC700 Glycopeptides

From MOPC 104E studies, we found that each cyanogen bromide fragment is a mixture of the same polypeptide containing different but closely related carbohydrate structures (26). Peptides containing the glycosylation sites were treated with pronase and further fractionated on P-6 Biogel. The profiles of glycopeptides on P-6 were assayed by their carbohydrate content using the phenol sulfuric acid reaction, and are shown in figure 29. The shaded areas were collected as the major oligosaccharide species, and carbohydrate compositions determined by gas-liquid chromatography.

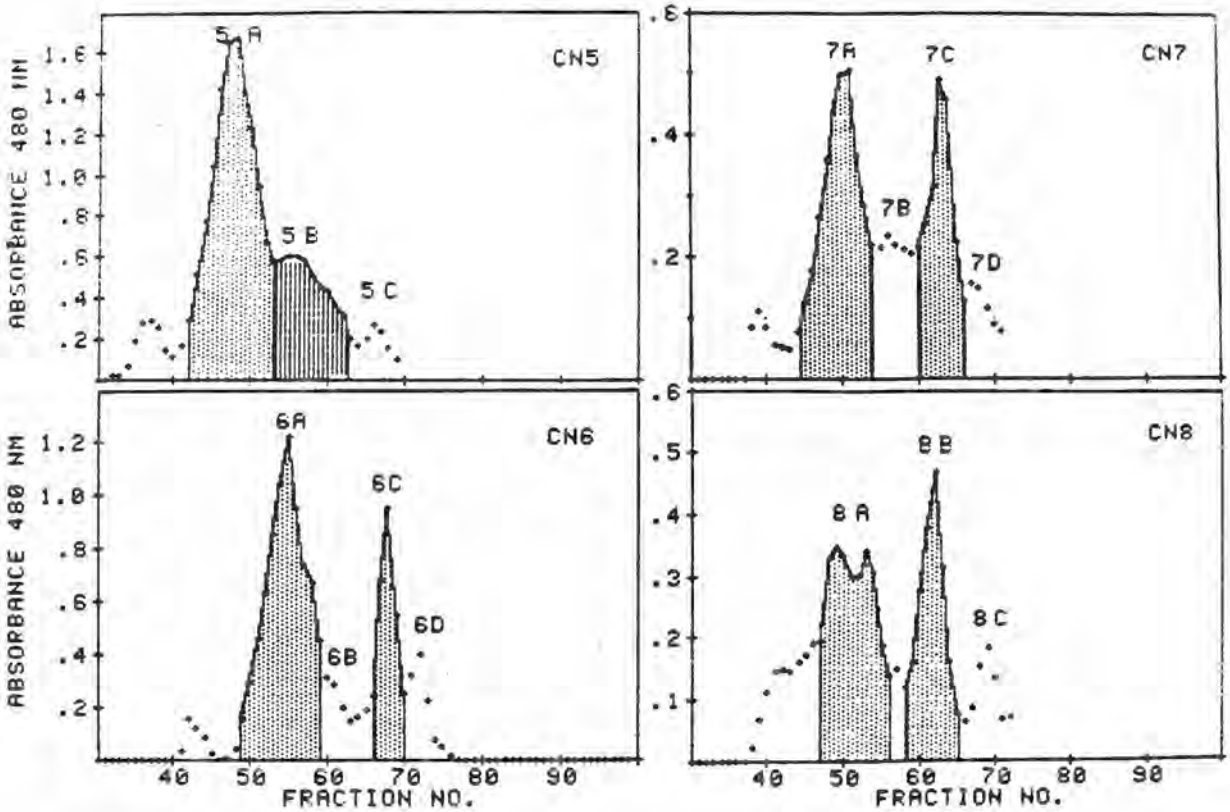


Figure 29. P-6 gel filtration of PC 700 a chain glycopeptides. Cyanogen bromide pronase treated fragments: CN5, CN6, CN7 and CN8, fractionated into several peaks. The shaded areas were collected as major oligosaccharide species. Carbohydrate compositions were obtained from both major and minor components.

Carbohydrate Compositions

All four PC 700 CN pronase glycopeptides fractionate into several peaks with compositions shown in data of table VI. In all cases there is a small portion of the total glycosylated material eluting ahead of the major peaks. By composition this peaks contains only galactose of a high molecular weight. As noted before the agarose columns release small quantities of high mw galactose containing material, and are separated from the IgM glycopeptides on the Biogel P-6 column.

CN5 pronased glycopeptide has a major peak, CN5A with a composition corresponding to a biantennary, fully sialylated structure. The second large fraction CN5B contains major monosaccharides Man and NAcGlc with only trace of galactose and fucose. This suggest a small amount of high mannose at this site. CN5C appear to be an incomplete processed structure with nearly identical moles of mannose and glucosamine. Galactose is detected in small amounts.

CN6A has the composition of a fully sialylated biantennary structure. CN6D complex structure is very similar in composition to fraction CN6A, but contain less sialic acid. 6B contains more moles of man/ mole of peptide than those found in complex structures, though it carries

Table VI. Carbohydrate compositions of P-6 purified pronase treated cyanogen bromide glycopeptides isolated from PC700 IgM heavy chain.

	Moles of Carbohydrate / Mole of asparagine				
	Fuc	Man	Gal	NAcGlc	SA
CN5A	1.2	2.8	2.6	5.2	2.3
s.d.	0.2	0.3	0.2	0.4	0.01
CN5B	0.2	2.5	0.7	1.7	0.4
s.d.	0.06	0.1	0.2	0.2	0.3
CN5C	0.6	0.3	0.7	0.1	---
s.d.	0.1	0.07	0.06	0.01	---
CN6A	1.0	2.5	2.8	3.3	2.1
s.d.	0.01	0.2	0.02	0.03	0.1
CN6B	1.7	5.3	2.2	2.5	0.4
s.d.	0.2	0.1	0.9	0.3	0.1
CN6C	0.0	3.4	0.5	1.7	0.3
s.d.	0.0	0.6	0.1	0.1	0.01
CN6D	0.5	2.1	2.2	4.3	0.2
s.d.	0.1	0.3	0.4	0.6	0.1
CN7A	1.0	3.5	3.3	2.2	3.6
s.d.	0.2	0.1	0.4	0.3	0.1
CN7B	0.6	3.4	1.0	1.9	0.4
s.d.	0.1	0.1	0.1	0.3	0.1
CN7C	0.0	1.8	0.4	1.3	0.0
s.d.	0.0	0.1	0.1	0.2	0.0
CN7D	0.7	5.1	0.4	1.3	0.0
s.d.	0.1	1.2	0.1	0.4	0.0
CN8A	0.3	1.6	2.6	2.0	1.3
s.d.	0.1	0.04	0.1	0.1	0.1
CN8B	0.1	7.5	1.2	1.7	0.6
s.d.	0.07	0.3	0.2	0.6	0.1
CN8C	0.6	2.1	0.2	1.7	0.0
s.d.	0.1	0.1	0.07	0.02	0.0

s.d. standard deviation. 3-5 different determinations.

galactose and fucose which is indicative of a hybrid structure. Fraction CN6C has a high mannose type composition.

CN7 (A,B,C) appear to have complex structures. 7A is fully completed (contains sialic acid and fucose) while the rest of molecules appear to be partially processed. 7D composition resemble a high mannose type oligosaccharide. CN8 has a complex type oligosaccharide at fraction 8A, while B and C are high mannose type by composition.

Cyanogen bromide fragments isolated from 21 ms chain were directly used for obtaining carbohydrate compositions. We were unable to purify material for pronase treatment and subsequent P-6 fractionation. Table VII shows the values obtained from each glycosylated fragment. Galactose values were very large (not shown) due to the contamination from the column supports. Thus only fucose, mannose and N-acetylglucosamine were determined. Total CN5 and CN6 have complex fucosylated complex structures. On the other hand, CN7 and CN8 appear to have mixtures of high mannose and complex oligosaccharides. These results are consistent with the labeling experiments and total compositions from the intact chain. We estimated that the purification scheme would have to be scaled up by a factor of 100 to have sufficient material for complete composition determinations and proton-NMR characterization.

TABLE VII. Carbohydrate compositions of 21 m chain cyanogen bromide glycopeptides.

	Fucose	Mannose	N-acetylglucosamine
		(molar ratios)	
CN5	**	3.0	4.0
CN6	**	3.0	2.5
CN7	--	5.2	2.0
CN8	--	3.6	2.0

** present but not quantitated.
--absent.
Cyanogen bromide fragments isolated from 21 m chain were used to determine sugar compositions by GLC. The glycopeptides were reduced and acetylated to their corresponding alditol acetates. 2-deoxy-D-glucose- included as internal standard.

Discussion

The general pathway of asparagine-linked oligosaccharide assembly and processing has recently been reviewed (1,13). Following the transfer of $Glc_3Man_9GlcNAc_2$- from dolichol diphosphate to nascent proteins (89,90), the glucose raidues are rapidly removed by glucosidases located in the endoplasmic reticulum (ER) (11,13). Oligosaccharide processing begins at the ER and continues through the Golgi apparatus catalyzed by a series of highly specific glycosidases and glycosyltransferases to yield the various classes of N-glycosyl oligosaccharides. The branches (antennae) that occur in N-glycosyl oligosaccharides are usually initiated by the incorporation of N-acetylglucosamine to a $Man_5GlcNac_2$-Asn core. In fact, branches can be initiated on this core in at least seven different ways, leading to the formation of multiple complex oligosaccharide structures (13).

Regulation of carbohydrate processing is not well understood. We do not know the protein structure and cellular glycosylation systems interact to determine a particular set of carbohydrate structures at individual

glycosylation sites . This thesis is an initial step towards learning how carbohydrate structures are determined.

IgM heavy chain from mouse myeloma and hybridoma cell lines provide a basic model by which mechanisms of glycosylation processing can be studied. Our laboratory has recently purified the heavy chain from IgM produced by MOPC 104E, isolated individual glycosylation sites and determined major carbohydrates using proton nuclear magnetic resonance (26).

Studies presented here define the differences between the glycosylation of IgM from MOPC 104E, a hybridoma PC700, and two mutants from PC700 (21 and 38) that synthesize hyperglycosylated IgM (57). The analyses include products from tissue culture and ascites grown cells.

Carbohydrate Studies of m Chains from Cells in Tissue Culture

Hyperglycosylation in mutant cell lines (21 and 38) products can be demonstrated by incubating cells with ^{35}S- Methionine. The secreted immunoprecipitated ms chains have retarded migration when compared to the parental cell line product (PC 700). Shulman et al, originally suggested that the difference was due to

hyperglycosylation since tunicamycin abolishes the differences (57).

Endo H was used to probe m chain oligosaccharide structures. The analysis revealed that intracellular processing of (mi) chains from PC700, 21 and 38 cells was not completed until the time of secretion. This susceptibility of the intracellular m chains has been described for a number of secreted glycoproteins including IgA, and human chorionic gonadotropin (45,98). The appearance of several bands upon treatment indicated that some of the glycosylation sites are partially processed as was confirmed by the sugar labeling experiments. The fact that the mi chains have the same mobilities in the absence of Endo H is one piece of evidence that hyperglycosylation is not due to an increase in the number of glycosylation sites. This conclusion is consistent with the genetic studies we described in chapter 3.

In contrast to the intracellular m chains, secreted products from 21 and 38 are much less sensitive to Endo H which is consistent with the presence of predominantly complex type oligosaccharide units. This was confirmed by analysing the structures found at each of the IgM heavy chains secreted from the mutants and parental cells. ^{3}H-mannose was used to radioactively label both complex and high mannose structures. 38 ms ^{3}H-mannose

glycopeptides presented the largest amount of complex type structures, followed by 21 ms and PC 700 glycopeptides. The high-mannose content is reversed. 38 m chain contains the least amount, then 21, and PC700 ms with the largest amount (Table I). Both 21 and 38 ms are more similar to MOPC 104 E IgM than to PC700 ms. In addition to containing more complex type oligosaccharides, there is a change to more processed structures (branched) in the ms glycopeptides from the mutant cells, as shown by their gel filtration behavior (figure 18). On the other hand, the subcellular mi chain ^{3}]H-mannose glycopeptides from all three different cells had very similar types of structures (figure 16). The retarded mobilities of the ms heavy chains from 21 and 38 cells are related to the type of glycosylation patterns that they carry. This is a combination of a reduced amount of high mannose type oligosaccharides concomitant with an increase in processing of their complex structures.

Closer examination of the complex oligosaccharides (tritiated-fucose labeling) carried by the secreted m chains showed again that this increase was caused by branching. There is a gradient of processing of complex structures in IgM produced by these three cell lines. PC700 IgM having the least processed oligosaccharide structures, followed by 21 and 38 cell lines (figure 17). PC700m chain

has processed structures, but these never reach the size (branching) of those present in 21 and 38 ma chains (figures 16 and 17). Note that when comparing glycopeptides, differences in molecular weight could be due to the polypeptide or carbohydrate. In all our experiments, the glycopeptides contain identical polypeptide sequences. Another problem could be that the same peptides with different carbohydrate chains could have different pronase susceptibility. Anderson and Grimes (25) showed that this doesn't occur for glycopeptides from IgM by determining amino acid compositions of glycopeptides having high mannose and complex oligosaccharides. The difference in column elutions are thus due to carbohydrates.

We do not know the significance of the mutant cell line alterations. The fact that there are three cells that secrete an antibody with the same antigen specificity, same polypeptide chain but different glycosylation brings the possibility of studying cellular mechanisms regulating oligosaccharide processing and the biological role of carbohydrates on antibodies.

The functions of most complex carbohydrates remain unknown, but there are evidence that the branching of N-glycosyl oligosaccharides on the cell surface may play a role in oncogenic transformation. Takasaki et al (34) found that transformation of BHK cells by polyoma virus led to a

reduction of biantennary oligosaccharides, and an increase in tetrantennary structures. Studies on mammalian lectins have shown that oligosaccharide chains are involved in the inter-and intracellular transport of glycoproteins. Ashwell (42) has extensively characterized the Gal and GalNac-specific lectin present in hepatocytes. Furthermore, Baezinger (29) has demonstrated that the rat hepatocyte system can bind galactose residues of biantennary or triantennary structures, but only the latter can be endocytosed. Nose and Wigzell (46) have demonstrated that oligosaccharide side chains play an important role in the biological functions of immunoglobulins. They prepared monoclonal trinitrophenol-specific antibodies (IgG2b), normally glycosylated or depleted of asparagine linked carbohydrates by treating the hybridoma cells with tunicamycin. The carbohydrate-deficient antibodies have no detectable differences in the antigen binding capacity. However, loss of carbohydrate had drastic consequences on antibody effector functions. Protein A binding ability was unaltered (this feature requires integrity of constant domains CH2 and CH3) but the aglycosyl antibodies have lost the ability to bind Fc receptors on macrophages. The mouse macrophage Fc receptors recognizes sites on the CH2 domain (52). The absence of carbohydrates in antibodies lead to impaired complement activation and antibody-dependent

cellular cytotoxicity. Furthermore, antigen-antibody complexes from such carbohydrate-deficient antibodies failed to be eliminated rapidly from circulation.

It is important to define the role which specific carbohydrate structures play in above antibody functions. Hybridoma cells PC 700,21 and 38 which secrete antibodies with carbohydrate differences can be used to define the IgM carbohydrate involvement and structural requirements for the constant domain functions.

m Chains from Ascites Grown
Cells are Fully processed

Our studies using MOPC 104E, showed that chemical characterization required levels of IgM found only in ascites fluid. Our original hypothesis had been that labeled IgM from cells in culture and in ascites fluid would have the same carbohydrates. In fact, we originally added labeled IgM from cultured cells to ascites fluid to follow IgM and glycopeptide purification. From these studies, it quickly became evident that processing was quite different for IgM from cultured cells and ascites fluid. For example, cyanogen bromide fragment CN6 contains two glycosylation sites (sites 332 and 364) (26) with structures that have been determined by H-NMR for IgM from ascites fluid (26). The two glycosylation sites contain

triantennary oligosaccharides that are nearly completely sialylated and some high mannose structures. When we compared CN6 glycopeptides from IgM grown in ascites fluid with tissue culture cell, we could show that the latter was processed less (figure 18).

Furthermore, complete mu chains derived from ascites grown MOPC 104E, PC700 and 21 cells were completely resistant to Endo H digestion (figure 19). This is in contrast to our studies on labeled IgM from tissue culture (figure 14). Under these conditions, PC700 mu chain were completely sensitive to endo H, while 21 chains were partially resistant to the same enzymatic digestion. IgM mu chain secreted from each of the cells have a characteristic rate of migration in SDS gels (from either type growing of conditions). PC700 product migrated with faster mobility than 21, 38 and MOPC 104 E chains (figures 19 and 26). Parental and mutant cell heavy chains retained their phenotype of differential migration regardless of whether IgM is prepared from ascites fluid or tissue cultured media. Thus the determinants for their characteristic carbohydrate processing is present in each cell. The fact that processing occurs in all three cell lines suggests that PC700, 21 and 38 have a specific set of enzymes for defining their final carbohydrate structures. The products obtained from tissue cultured cells are likely to be intermediates of those obtained from ascites fluid.

IgM Polymeric Forms Secreted by Mutant Cells

Both MOPC 104E and PC 700 ascites grown cells secrete large amounts of pentameric IgM (25,26,57). PC700 derived hyperglycosylated mutants (21 and 38 cells) produced IgM with altered carbohydrate. Mutants secreted IgM mainly as monomers (H_2L_2) (figure 20 and 23) (57 and my data), although this form is not specific for 21 and 38 cells. PC 700 also secretes monomers. The isolated m chain from monomers secreted by PC 700 does not exhibit hyperglycosylation, showing that hyperglycosylation is not simply due to the polymeric nature of the antibody. Pentamer formation requires joining protein (J) (40). It is not known if mutant cells 21 and 38 lack, or have reduced amounts of this protein. In addition, it could be that hyperglycosylation is reducing pentamer formation.

The amounts of IgM obtained from mutant cells grown in ascites fluid are greatly reduced (table III). This can not be attributed to changes in the rate of synthesis, which estimated from our labeling studies of cells in tissue culture conditions are similar for these cells. On the other hand, the type of structures carried by the heavy chains can affect their circulation in vivo (46). It is possible that the mutant secreted IgM is removed from the ascites fluid by specific receptor systems for the oligosaccharides.

The variations in glycosylation and polymerization forms observed in IgM secreted from 21 and 38 cells do not affect the antigen combining site, since their antibodies react well with phosphoryl-choline (measured by ELISA). One or more of the Fc functions appear to be altered, because these mutants were selected by their insensitivity to antibody dependent complement mediated cytotoxicity (57).

Carbohydrate Structures in Complete μs Chains

Total sugar compositions of purified μ chains from PC 700, 21 and 38 cells provided additional evidence for the changes between the immunoglobulins produced by these cells (Table IV). PC 700 μ chain has a NAcGlc/Man molar ratio (2.2/3.0) indicative of mainly biantennary structures. Mutant μ chains presented increasing NAcGlc/Man ratios (4.2/3.0, 5.3/3.0). The 21 cell product appears to have a mixture of bi and triantennary structures, while 38 μ chain has mainly triantennary complex oligosaccharides. Sialic acid, added last to the oligosaccharide chain, is also increased for 21 and 38 cells relative to PC 700 (Table V). The hyperglycosylation difference is caused by the processing steps leading to complex oligosaccharides. A trend in branching is shown going from PC 700<21<MOPC 104E<38 μ chains. PC 700 like other hybridoma cells apparently retains a number of high mannose oligosaccharides, and yet mutants derived from this line

process further to complex oligosaccharides. Mutants make mainly triantennary oligosaccharides instead of the predominant biantennary found for PC 700. The terminology used by Shulman is not correct. PC 700 is defined as normal, and the mutants as hyperglycosylated. In fact, by carbohydrate composition, the cell lines 21 and 38 may be more like MOPC 104 E in processing and PC 700 abnormal (see below).

Carbohydrate differences in Myeloma and Hybridoma IgM

Carbohydrate compositions obtained from purified single glycosylation sites demonstrated the major differences between MOPC 104E, PC700 and 21 m chain. Anderson et al (26) have determined the major structures at MOPC 104 E m chain. The same procedure was followed to characterize hybridoma cell products. CN5 from MOPC 104E m chain carries a single glycosylation site in which the major oligosaccharide structure is biantennary. The next three sites (from CN6 and CN7) have triantennary structures, while CN8 has a high mannose structure as major component. Proton-NMR (250 mHZ) spectra taken of the major PC 700 structures confirm the carbohydrate structures proposed from the composition data. Results presented here showed that PC 700 m chain have biantennary structures in four glycosylation sites (CN5, CN6 and CN7) and a combination of high mannose and complex type

oligosaccharides at CN8 (Table VI). In addition each site contained minor components which appear to be processing intermediates. Note that each site carries a particular set of structures which in turn are different from those seen in other sites. The finding of only biantennary structures at PC 700 ms is the most remarkable difference. The presence of only biantennary structures, and reduced processing observed in tissue culture cause the increased mobility of IgM from PC 700 cells.

21 and 38 m chains are fragmented by CNBr into peptides identical to PC 700 and MOPC 104 E. 21 m chain appears to have three complex sites located on CN5 and CN6, with all of them biantennary type. CN7 and CN8 have mixtures of complex and high mannose oligosaccharides. The reduced amounts of IgM obtained from mutant ascites, prevented further analyses. This points out some great difficulties in gaining structural information from glycoproteins.

All four m chains (MOPC 104E, PC700, 21 and 38) should have the same constant region. Thus the difference in IgM carbohydrate processing appears to derive from the cellular mechanisms for glycosylation. The antibody changes could result from the availability of certain glycosyltransferases required to initiate the branching synthetic pathway, or from the competition of two or more enzymes for a common intermediate which may be reduced in

PC 700. Schachter et al (13) have partially purified several glycosyl-transferases which are involved in the control of oligosaccharide branching. We do not know if the enzyme for triantennary structure synthesis is missing or ineffective in PC 700 cells. Dr. M. Shulman has designed an approach to define the molecular basis of glycosylation in 21 and 38 m chains. He has constructed a transfection vector which contains the heavy and light chains for a 2,4,6-trinitrophenyl-specific antibody (87,88). The intention is to transfect PC700,21,and 38 and see whether the specific antibody is glycosylated differently. If the normal gene leads to a hyperglycosylated product when transfected into either mutant cells, then the defect is probably at the cellular level. The same vectors could be used for site specific mutagenesis, which will allow the study of the relationship between protein sequence and glycosylation.

IgG studies have shown that the carbohydrates play an important role in the heavy chain constant region functions (46). PC 700, 21 and 38 produce IgM with the same antigen specificity but major carbohydrate differences. The differences expressed by the carbohydrates can be used to define their biological functions. It is important to note that all the known immunoglobulin carbohydrate structures have been derived from studies on antibodies synthesized by tumor cell lines. We assumed that MOPC 104E IgM represented

the normal glycosylation pattern and PC 700 was abnormally glycoaylated. On the other hand, PC 700 IgM binds complement while 21 and 38 secreted antibodies do not. In this function, PC 700 appears to carry normal carbohydrate. In fact antibody produced by cells at various differentiation stages could have different carbohydrates for different functions. This brings up the importance of using antibodies of similar specificities but with different carbohydrates in biological assays of antibody effector functions including: ability to bind Fc receptors, complement activation, antibody- dependent cellular cytotoxicity, and antibody-antigen clearance and compartmentalization (i.e. some types of antibodies circulate in lymph, others only in plasma). Definition of the important functions of carbohydrates of antibodies would allow the design of monoclonal antibodies with appropriate carbohydrate for specific functions (i.e. antibodies directed for tumor therapy might change in effector functions depending on their carbohydrate).

In summary, I have presented genetic and chemical analysis of several cell lines which synthesize IgM. Cells : MOPC 104E, PC 700, 21 and 38 have the same Cm gene arrangement and secrete IgM with identical constant polypeptide chain region. Their gene products differ in polymeric form of secretion and glycosylation. Myeloma cells (MOPC 104E) can process m chain oligosaccharides to

triantennary structures, while PC700 only to biantennary. Mutants derived from PC700 cells (21 and 38) secrete IgM with triantennary structures. These appear to originate from mutations in the cellular glycosylation-processing machinery. Heavy chains have an increase in carbohydrate processing in the order PC700 < 21 < MOPC 104E < 38. We have shown that hybridomas can have major changes in carbohydrate processing. This observation could have important implications on the use of monoclonal antibodies. Finally, the availability of PC-specific monoclonal antibodies with differences in processing could allow us to determine the functions of antibody carbphydrate. It may become possible in the future, to design antibodies with carbohydrates that determine effector functions.

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