The University of Manitoba

Studies of the N-linked Oligosaccharides of Fcε Receptors

by

Eileen L. LaCroix

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the

Degree of Master of Science

Department of Immunology

Winnipeg, Manitoba

August, 1991

ISBN 0-315-76832-0

STUDIES OF THE N-LINKED OLIGOSACCHARIDES OF $Fc\varepsilon$ RECEPTORS

BY

EILEEN L. LACROIX

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

to Dan

TABLE OF CONTENTS

Acknowledgements

I am indebted to Dr. Arnold Froese for sharing his wisdom with me and for his guidance over the years.

I am also indebted to Ms. Karol McNeill for her excellent technical assistance.

Thank-you to Ms. Kathy Risk for her assistance and patience during the typing and printing of this thesis.

ABSTRACT

This study was undertaken to investigate the nature and microheterogeneity of the carbohydrate moiety of the Fc receptors of RBL-CA10 and RBL-CA10.7 cells. Treatment using the glycosylation processing inhibitors, castanospermine, 1-deoxymannojirimycin, and swainsonine resulted in a decrease of relative molecular mass (M_r) of both the α-chain of the high affinity receptor for IgE, FcϵRI(α), and the low affinity receptor for IgE, FcϵR_L. Exposure to 1-deoxymannojirimycin had the greatest effect on the M_r, while castanospermine seemed to lead to a decreased cell surface expression of FcϵRI. Both receptors are fairly resistant to endoglycosidase H when reduced as their M_r decreased by only ~2 kDa. This suggests that both receptors are composed primarily of complex oligosaccharides with a single high mannose N-glycosylation site. FcϵR's become sensitive to endoglycosidase H if first exposed to 1-deoxymannojirimycin indicating that formation of complex structures had been inhibited by the treatment. When FcϵR's were reduced and hydrolyzed by N-glycanase, the M_r values for FcϵRI(α) and FcϵR_L decreased to 28 and 36 kDa respectively. In the case of FcϵRI(α) this implies the presence of only a small amount of O-linked oligosaccharides.

LIST OF FIGURES AND TABLES

LIST OF TABLES

CHAPTER 1

CHAPTER 1

MAST CELLS

Mast cells and basophils have classically been viewed as the chemical storehouses of bioactive substances responsible for immediate hypersensitivity reactions, inflammation, and various pathologic responses. Cross-linking of the high affinity receptor for IgE, known as FcϵRI, on the surface via IgE and specific antigen, anti-FcϵRI antibodies and/or the stimulation of these cells with complement components, neuropeptides, venom components, or a variety of other basic compounds (Gordon *et al.*, 1990) initiates the rapid cascade of biochemical processes terminating with the deposition of the mast cells' granule contents into the immediate cell environment.

Recent findings about mast cells have elevated their importance not only as the effector cell in allergic reactions, but also a variety of inflammatory disorders (Mican and Metcalfe, 1990), and as one of the possible links between the immune and nervous system due to their high incidence among nerve cells (Theoharides *et al.*, 1990). Distinct phenotypic differences have been found to exist in mast cells resident in the various tissues of the body. This mast cell phenotypic heterogeneity is likely reflective of their functional heterogeneity. Given this, researchers are forced to re-ask the question of the role of mast cells and

basophils in health and disease, with the ultimate goal being the development of strategies to better manage allergy and inflammatory diseases.

Discovery

The first to document the presence of mast cells in fixed tissue late in the 1800's are collectively: Recklinghausen, Kuline, Koelliker, Friedlander, Cohnheim, Rollett, Schobe and Flemming (Michels, 1938). Ehrlich, during his studies of fixed connective tissue, identified the unique ability of these cells to be stained using metachromatic dyes. He subsequently proposed the name "mast cell" (mast - food) for the cells and "blood mast cells" or "basophile" for a metachromaticly staining circulating leukocyte (Riley, 1954). A flurry of studies began to investigate the biological significance of such cells. Michel's review, arriving in 1938 after a fifteen year compilation, listed twenty-five hypotheses for mast cell function. Some of these functions included: carriers of oxygen, fat storage, secretion, phagocytosis, and defence mechanisms. A paper was simultaneously published by Wilander demonstrating that heparin was the metachromatic component of the mast cell granule (Padawer, 1963). Ehrlich had not assigned any function to these cells suffice to say they appeared as a "well fed" cell of the connective tissue (Riley, 1954).

Relationship between Mast Cells and Basophils

Mast cells and basophils both have metachromaticly staining granules enclosing an array of bioactive mediators, high affinity receptors to bind IgE and both cells play the role as effector cells in allergic and inflammatory responses. Distinguishing features of the two cell types lie in their ontogeny, morphology, and tissue distribution.

The precursor of murine mast cells is considered to be a pluripotential haematopoietic stem cell because of its origin in the bone marrow and ability to differentiate into more than one type of blood cell (Kitamura *et al.*, 1978; Hasthorpe, 1980). This, however, has been determined conclusively for mice only, primarily because of the availability of a genetically mast cell-deficient mutant, $WBBGF_1$-W/W^v (Nakano *et al.*, 1987) which develops mature mast cells when given bone marrow from their congenic littermates ($WBB6F_1$-+/+) (Kitamura *et al.*, 1978).

The immature precursor of mast cells, lacking granules, migrates from the blood to the connective tissue of nerves, lymphatic and blood vessels, skin, peritoneum and mucosal tissues of the respiratory and intestinal tracts (Metcalfe *et al.*, 1979; Wilhelm *et al.*, 1978; Galli *et al.*, 1984). It is within the microenvironment of these tissues that proliferation, maturation, as well as granulation take place. Evidence for the microenvironmental influence on mast cell development is provided in the murine model of genetically mast cell-deficient $WCB6F_1$-Sl/Sl^d mice. These

mice, unlike the $WBB6F_1$-W/W^v mutants, do not develop mast cells when given $WCB6F_1$-+/+ mast cell precursors (Kitamura and Go, 1979). The bone marrow of the $WCB6F_1$-$S1/S1^d$ mice can form mast cells if injected into the W/W^v mice. Therefore the mutation in the mice preventing the formation of mature mast cells differs, and in the $WCB6F_1$-$S1/S1^d$ mice it is due to defects in the microenvironment in which maturation takes place (Galli and Lichtenstein, 1988).

In contrast to mast cells, basophils originate and complete their maturation within the bone marrow (Galli, 1984). Basophils are found circulating in the blood stream at numbers estimated to be 0.5% of leukocytes and 0.3% of nucleated marrow cells but they are not resident in tissues (Galli and Lichtenstein, 1988). In contrast to mast cells which are the *in situ* effector cells of various tissues, basophils are viewed as the free moving mobile effector cells recruited to a particular immunological or inflammatory site only during IgE-dependent reactions, inflammatory and/or pathologic responses (Galli, 1984).

Mast cells and basophils can be distinguished morphologically from one another. Both cell types have cytoplasmic granules, but in basophils they are large and sparse whereas in mast cells they are numerous and small (Dvorak *et al.*, 1983). Basophils have segmented nuclei, aggregates of cytoplasmic glycogen and thick processes, whereas mast cells have round nuclei, no stored glycogen, and uniform thin processes (Galli, 1990). The cytoplasma of the mast cell has dispersed lipid bodies, absent in

basophils, which led many to believe originally that the cells played a role in fat storage and/or metabolism.

It has been suggested that circulating precursors of mast cells may, perhaps, be basophils similar to the circulating monocyte precursors of macrophages, this however has little supporting evidence (Denburg *et al.*, 1986). Generally it is believed that although mast cells and basophils possess many similarities in structure and function, they represent the mature products of distinct ontological pathways. In fact, evidence suggests mast cells may be more closely related to eosinophils than to neutrophils (Denburg *et al.*, 1986).

Mast Cell Heterogeneity

Recently, mast cells have been identified in rodents and humans with separate, unique phenotypic characteristics. These cells have been characterized extensively biochemically, morphologically, and immunologically. The majority of these studies were carried out in the rat. Original studies were done *in situ* on mast cells present in various fixed tissue and eventually studies began on cells isolated from tissue using newly developed techniques which would allow yields of mast cells with high purity. *In vitro* cell culture studies of immortalized cells or short-lived mast cell cultures were established from precursors in conditioned media.

Maximow in 1906 may have been the first to observe that mast cells in the rat intestine differed from other mast cells found in the rat. Difficulties with proper fixation of the tissue made it difficult to study such cells. The existance of these cells in the intestine was demonstrated by Enerbäck's (1966) studies showing that mast cells in mucosal tissue could be stained with tolidine blue following fixation in aldehyde-acetate-Carnoy solutions. They could not be stained if fixed in aldehyde alone.

The discrete anatomical distributions of the two types of mast cells in the rat formed the basis for their classification (Enerbäck, 1974). Those mast cells found in the submucosa, connective tissue and serosal cavity, or peritoneum were considered connective tissue mast cells, "CTMC" or "typical" whereas those of mucosal tissue such as the lamina propria of intestine were mucosal mast cells, "MMC" or "atypical" (Enerbäck, 1974; 1986). Morphologically, CTMC appeared large and contained more granules than those of mucosal tissue. In following with the larger size of CTMC granules, their histamine content, estimated to be 15 pg/cell, may be as much as 10-fold greater than MMC at 1-2 pg/cell.

The diffuse distribution of mast cells in connective and mucosal tissue made it difficult to collect adequate amounts of cells for studies. Collagenase digestion of intestinal tissue (Befus *et al.*, 1979; 1984) followed by sedimentation using a discontinuous Percoll gradient facilitated mucosal mast cell isolation to near purity (Lee *et al.*,

1985a). Studies were carried out to investigate potential markers of CTMC and MMC phenotype to facilitate isolation and analysis. Differences in the proteoglycans of the granules of CTMC and MMC provided a useful phenotypic marker. The granules of CTMC from rat peritoneum and rat serosal mast cells were found to contain heparin as the predominant glycosaminoglycan through studies of [^{35}S]-sulphate incorporation into [^{35}S]-heparin (Yurt *et al.*, 1977). MMC granules, upon *in situ* microspectrophotometric examination, showed the presence of a less sulphated glycosaminoglycan, predominately a chondroitin sulphate di-B proteoglycan (Tas and Berndsen, 1977). The glycosaminoglycans of both MMC and CTMC readily stain blue with either tolidine blue or alcian blue, but unlike CTMC, MMC fail to counterstain with safranin O. (Stevens *et al.*, 1986; Enerbäck *et al.*, 1985). Both dyes have differential affinities for the glycosaminoglycans of the granules depending on the degree of sulphate substitution (Mayrhofer, 1980). Therefore the alcian blue of stained granules of CTMC can be displaced by safranin O but stained MMC granules cannot and CTMC and MMC stain typically alcian blue + / safranin O + and alcian blue + / safranin O - respectively.

Another phenotypic marker is the granule neutral proteases of CTMC and MMC. Rat mast cell protease I (RMCP I) predominates in CTMC, and RMCP II in MMC (Woodbury *et al.*, 1978). The development of specific antibodies against the two significantly homologous but non-cross reacting proteases allowed immunohistochemical localization of RMCP I and II in various rat tissue (Gibson and Miller, 1986). Both MMC and CTMC cells have receptors

which bind IgE on their surface. CTMC isolated from the peritoneum have 3 x 10^5 Fcϵ/cell and MMC approximately ten fold less at 3.6 +/- 2.3 x 10^4 Fcϵ /cell (Lee *et al.*, 1985b).

Studying mast cell genesis was problematic because precursors lacked the distinguishing metachromatic granules. Much of the *in vitro* studies involved growth of mast cells from various rat tissues with or without accessory cells and/or growth factors. Four different tissues were examined and found to have mast cell precursors: rat mast cells developed from cultured rat thymus cells grown on fibroblast monolayers (Ishizaka *et al.*, 1976); rat peritoneal fluid (Padawar and Gordon, 1955); small mononuclear cells in mast cell depleted peritoneal fluid (Czarnetzki *et al.*, 1979); and rat bone marrow (Haig *et al.*, 1984). The differentiation of the mast cell is controlled by various growth factors and cell-cell contact between the mast cell and host tissues (Kitamura, 1989). *In vitro*, cells of mouse bone marrow origin, or from single progenitor cells in semi-solid methylcellulose cultures (Nakahata *et al.*, 1981), can differentiate into mast cells when cultured with IL-3 (also called: multipotential colony-stimulating factor, Multi-CSF; or panspecific hematopoietin, PSF) (Rennick *et al.*, 1985). IL-4 (also called: B cell stimulation factor 1, BSF-1) can also act synergistically with IL-3, but not alone, to promote the maturation of IL-3 dependent mouse mast cell cultures *in vitro* (Hamaguchi *et al.*, 1987). Also factors derived from immune mesenteric lymph nodes (IMLN) of *Nippostrongylus brasiliensis* (Nb) infected rats can stimulate the development of mast cells from precursors

(Ishizaka *et al.*, 1977; Haig *et al.*, 1982). Nb-infection *in vivo* characteristically induces extensive MMC hyperplasia in tissues and can increase mast cell numbers by approximately five times the original amount (Jarrett and Miller, 1982).

The mast cells generated *in vitro* from precursors have phenotypic characteristics similar to MMC and contain RMCP II. CTMC such as rat serosal mast cells can be maintained *in vitro* for an extended period only if cocultured with metabolically active accessory cells such as mouse skin-derived 3T3 fibroblasts (Levi-Shaffer *et al.*, 1985). This technique of cell coculture was first developed by Ginsburg (1963). Attempts to culture CTMC without exogenous growth factors or accessory cells have given transient growth at best (Czarnetzski *et al.*, 1979), although long term culture of factor independent rat peritoneal mast cells has been established in this lab (Chan *et al.*, 1988). Characterization of these cells, however, showed the expression of a MMC phenotype ie., low histamine content, alcian blue + / safranin O -, and contained RMCP II despite being derived from CTMC (Chan *et al.*, 1990). When hybrid mast cells (HRMC) were produced by fusion of rat peritoneal mast cells and RBL cells, the majority of cells had the MMC phenotype. Some cells showed the CTMC phenotype which, however disppeared even though the cells were partially cloned (Zheng *et al.*, 1991).

A useful model for mast cell studies has been the rat basophilic leukemia cell. These cells were originally identified in Wistar albino rats

following treatment with the leukaemogen β-chloroethylamine (Eccleston *et al.*, 1973), and can be considered MMC-like (Seldin *et al.*, 1985). Their characteristics resemble *in vitro* derived cells from bone marrow cultured mast cell precursors (Enerbäck *et al.*, 1985). Their staining properties are alcian blue +/ safranin O -, with small limited granules, low histamine content, chondroitin sulphate di-B proteoglycans, and RMCP II (Seldin *et al.*, 1985).

Discrete populations of mast cells exist within one species as defined by mediator content, sensitivity to cytokines, and activation stimuli but not all *in situ* rat and mouse mast cells can fit into the defined categories of CTMC and MMC (Galli, 1990). In addition, the defining characteristics of a mast cell population in one species, may not apply to a corresponding population in another species. However, many working in the field feel that knowledge of mast cell biology at this time does not justify a change of nomenclature (Bienenstock *et al.*, 1989). To complicate matters further, an ever growing list of distinct mechanisms can influence mast cell phenotype. We are now seeing *in vivo* experiments with genetically mast cell-deficient ($WBBGF_1$-W/W_v) mice where phenotypic changes occur converting the BMCMC (bone marrow-derived cultured mast cells) phenotypically resembling MMC, to CTMC (Nakano *et al.*, 1985; Otsu *et al.*, 1987). Similarly, CTMC to MMC-like changes have been documented (Kanakura *et al.*, 1988; Sonoda *et al.*, 1986).

Mast cell phenotype can be influenced by such things as: factors affect-

ing the process of mast cell maturation/differentiation (Galli, 1990); changes accociated with functional activation (Galli *et al.*, 1984); microenvironmental influences such as acquiring molecules from neighboring cells (Padawar, 1978); cytokines and specific immunological processes, for example, the MMC type may expand during "T-cell dependent" immune responses such as infection with Nb (Mayrhofer and Fisher, 1979) or *Trichinella spiralis* (Ruitenberg and Elgersma, 1976), whereas the CTMC population, considered more "T-cell independent", are found in normal numbers in athymic mice (Aldenborg and Enerbäck, 1985).

IMMUNOGLOBULIN E

The role of immunoglobulin is to recognize foreign antigen as part of an adaptive immune response. Immunoglobulins, by virtue of their structural relatedness, are the founding members of the immunoglobulin supergene family (Hunkapiller and Hood, 1986). Monomerically, all immunoglobulins are composed of four polypeptides: two light and two heavy chains, each contributing to the globular, quaternary structure. Immunoglobulins are divided into five classes, as well as various subclasses, based on their heavy chains: μ, γ, α, δ, and ϵ. Each of the polypeptides has variable and constant domains depending on the extent of sequence conservation. IgG, IgD, IgA have four such domains while both IgM and IgE possess an additional domain analogous to the hinge region of IgG.

IgE was the last of all the immunoglobulin isotypes to be chemically and structurally delineated although its activity as the reaginic antibody was well known. Part of the reason for its delayed detection was its low quantity in serum. IgE contributes less than 1/100 of one percent of total circulating serum immunoglobulins. In contrast, IgG contributes 75 - 85% (Hunkapiller and Hood, 1986). The discovery of IgE secreting tumors in humans and rodents, such as the rat immunocytoma IR-162, allowed for the detailed structural and functional analysis of IgE (Bazin *et al.*, 1974). Of the five IgE heavy chain domains, V_H, Cϵ1, Cϵ2, Cϵ3, and Cϵ4, the domains which were originally implicated in harbouring the FcϵRI binding site are Cϵ3, and Cϵ4 (Dorrington and Bennich, 1973). However,

more recent studies have pointed more strongly toward the Cε2 and/or the Cε2/Cε3 junction as being responsible for the binding of FcεRI (Perez-Montfort and Metzger, 1983; Helm *et al.*, 1988).

All immunoglobulins are glycosylated primarily on the heavy chain within the hinge and second constant domain although some oligosaccharide has been found present in variable and CH1 and CH3 domains (Hasemann and Capra, 1989). The percentage contribution of carbohydrate to the overall molecular weight ranges from 3 - 13% . Variation in carbohydrate composition and location on different heavy chains exists. It is believed that carbohydrate is important in immunoglobulin folding, transport, and turnover. Any more direct roles which may correspond to variation in oligosaccharide within heavy chain classes have not been identified. IgE contains the most oligosaccharide by weight of all the immunoglobulins. Rat IgE has nine glycosylation sites two in each of Cε1, Cε2, Cε4, and three in Cε3 as determined by its cDNA (Hellman *et al.*, 1982). Human IgE has six sites of which one in Cε1, and the two in Cε2 are homologous to equivalent sites in rat IgE (Hellman *et al.*, 1982). Detailed carbohydrate compositional analysis is only available for the two human IgE myeloma proteins (Baenziger, 1978). Both contain complex-type and high mannose structures and no O-linked oligosaccharide was detected. Oligosaccharide is not believed to play a direct role in FcεR binding since non-glycosylated rat IgE myeloma protein from tunicamycin treated IR-162 retains the ability to bind to RBL-1 cells (Kulczycki and Vallina, 1981).

RECEPTORS FOR IGE ON MAST CELLS AND BASOPHILS

Two receptors exist on the surface of mast cells and basophils which bind to the Fc portion of IgE and can be separated based on their affinities and differences in relative mobility and thus relative molecular mass (M_r). These are: FcϵRI, formerly referred to as "R", which binds IgE with high affinity and has a M_r of 45 kDa; and FcϵR_L, formerly "FcϵRII" or "H", with an M_r of 55 kDa, and where the subscript letter "L" designates the receptor as a low affinity receptor for IgE.

Ishizaka *et al.*, (1970) first demonstrated that the Fc fragment but not $F(ab')_2$ fragment of human myeloma IgE binds to human basophils and this binding can be inhibited by anti-IgE-Fc. It was then demonstrated *in vivo* that IgE injected intravenously into monkeys would bind to mast cells and could be detected by radiolabelled anti-IgE (Tomioka and Ishizaka, 1971). The ability of IgE to bind mast cells promoted investigations into its involvement in the release of mediators in immediate hypersensitivity (Ishizaka and Ishizaka, 1971). Many of the subsequent investigations were carried out in rats because of the identification of basophilic leukemia in Wistar albino rats. This allowed long term culture of these cells for various studies.

Affinity

Ishizaka first determined that the interaction between IgE and its recep-

tor on human basophils is one of high affinity with an estimated average association constant (K) of 0.5-1 x 10^9 M^{-1} where:

$$K = \frac{\text{(IgE-receptor complexes)}}{\text{(serum IgE)(free receptor)}}$$

The binding to normal rat peritoneal mast cells (RMC) and rat basophilic leukocytes (RBL) was comparable where K = 1.04-1.09 x 10^9 M^{-1} (Conrad *et al.*, 1975). A slow dissociation rate constant (k_{-1}) for the interaction of IgE with RBL-1 was an attributing factor for the high affinity binding where: the forward rate constant (k_1) is 9.6 x 10^4 $M^{-1}sec^{-1}$ and k_{-1} = 1.6 x 10^{-5} sec^{-1} at 37°C resulting in $K = k_1/k_{-1} = 6 \times 10^9$ M^{-1} (Kulczycki and Metzger, 1974). The specific association constant of $Fc\epsilon R_L$ has not been determined. Attempts were made in this laboratory to obtain a binding constant, but proved unsuccessful presumably because of the low affinity of the interaction of this receptor with IgE.

Isolation Techniques

A number of important properties of $Fc\epsilon RI$ and $Fc\epsilon R_L$ were exploited in the development of isolation techniques. These include: (1) the ease of receptor extraction from the cell membranes using mild detergents; (2) ability to tag the receptor using [^{125}I]-iodine; (3) the characteristic high binding affinity of $Fc\epsilon RI$ versus $Fc\epsilon R_L$'s low affinity for IgE provid-

ing both a mechanism for isolation and purification.

Bach and Brashler (1973) showed that lysed or sonicated rat peritoneal mast cells retained the ability to bind IgE. Recovery of the trichloroacetic acid precipitable ^{125}I-IgE in the supernatant of RBL-1 which eluted in the void volume of a Sepharose 6B column, ahead of the volume appropriate for unbound ^{125}I-IgE, indicated that cells also shed particles which indeed contain active IgE-binding receptors (Carson *et al.*, 1975).

The first studies conducted in which a receptor binding IgE was isolated were by Conrad *et al.* (1976). Since the receptor was found to be associated with the plasma membrane, and did not require an intact cell for IgE binding, disruption of the membrane using a 0.5% solution of the nonionic detergent, Nonidet P-40 (NP-40) was possible. Incubation with ^{125}I-IgE before or after membrane disruption created soluble ^{125}I-IgE-receptor complexes separable from free ^{125}I-IgE by gel chromatography on Agarose A 1.5 m (Conrad *et al.*, 1976).

Isolation became more refined with the development of the technique of cell surface iodination and receptor "sandwich" immunoprecipitation (Conrad and Froese, 1976), or using an $(NH_4)_2SO_4$ precipitation assay (Rossi *et al.*, 1977). Labelling of the proteins occurs with the aid of the enzyme lactoperoxidase, which forms a lactoperoxidase-^{125}I complex through an intermediate complex of lactoperoxidase-O with hydrogen peroxide (H_2O_2) (Tsai *et al.*, 1973). This complex will iodinate only proteins with tyrosine groups exposed to the surface (Tsai *et al.*, 1973). The

complex is presumably too large to permeate the cell and therefore no labelling of plasma proteins occurs. Addition of non-iodinated rat monoclonal IgE (IR-162), rabbit anti-rat ϵ-chain, and goat anti-rabbit immunoglobulin creates a sandwich immunoprecipitating the ^{125}I-labelled receptor. Precipitates can be collected by centrifugation, re-dissolved in sodium dodecyl sulphate and then separated by polyacrylamide gel electrophoresis (SDS-PAGE). Receptor immunoprecipitation was also possible using the $F(ab)_2'$ or Fab fragment of IgG anti-receptor antiserum against RBL-1 surface determinants developed by immunization with whole cells or IgE-receptor complexes (Isersky *et al.*, 1977; Conrad *et al.*, 1978)

A higher degree of purity was achieved when NP-40 solubilized receptors were isolated using the technique of affinity chromatography (Conrad and Froese, 1978a). This procedure involves the covalent coupling of the amino groups of a protein to an insoluble matrix, such as Sepharose. Cyanogen bromide (CNBr) reacts with the hydroxyl groups of Sepharose forming cyclic and acyclic imidocarbonates which react with the amino groups of the ligand, in this case, IgE. Receptors could then be eluted from the column using 3 M potassium thiocyanate (KSCN) (Conrad and Froese, 1978a) or 0.5 M acetic acid with 1% NP-40 (Kulczycki and Parker, 1979). The recovery of biologically active receptor following removal of KSCN or neutralization of eluting acid allowed for repetive chromatography to increase receptor purity (Kulcyzcki *et al.*, 1979). Haptenated IgE and corresponding anti-hapten-Sepharose were useful for receptor isolation because elution could be done with 0.01 M hapten, eliminating the need for

denaturation of the receptor. Haptens utilized included DNP-IgE and anti-DNP-Sepharose (Conrad and Froese 1978b), or phenylarsonate, anti-phenylarsonate- Sepharose (Kanellopoulos *et al.*, 1979).

Using techniques such as these, an additional RBL surface component of 55 kDa was resolved (Conrad and Froese, 1978a). Inhibition of the binding of this component to IgE-Sepharose with free IgE was not as effective as that of FcϵRI. This component was not found in immunoprecipitated complexes of IgE, anti-IgE, or DNP-IgE, anti-DNP-Sepharose (Conrad and Froese, 1978b). This component, designated FcϵR_L, as well as FcϵRI were found on normal mast cells of six different rat strains when isolations were carried out using IgE-Sepharose (Froese, 1980). The M_r of the two receptors as well as their relative expression varied in RBL cell lines maintained in different laboratories (Froese *et al.*, 1981). RBL_{StL} (St. Louis) had a receptor pattern which closely resembled RBL_{WPG}, but with more FcϵR_L than FcϵRI. RBL_{BALT} (Baltimore), RBL_{NIH} (National Institute of Health) and RBL-2H3 had a single broad peak of overlapping M_r for both receptors. FcϵR_L appears to be more highly immunogenic than FcϵRI and anti-receptor antibodies could be obtained that reacted only with FcϵR_L (Froese *et al.*, 1982).

Some of the binding characteristic of FcϵR_L were found to differ from FcϵRI. Both receptors were found to cross react with IgG but FcϵR_L appeared to bind this immunoglobulin with a somewhat higher affinity than FcϵRI (Kepron *et al.*, 1982). Binding preference to immunoglobulin is of

the order of IgE>>IgG_{2a}>IgG_1>IgG_{2b} for FcϵRI and IgE>IgG_{2b}>IgG_1>IgG_{2a} for FcϵR_L (Kepron *et al.*, 1988).

FcϵRI and FcϵR_L may be differentially influenced by culture conditions. Rat tissue cultured mast cells (RCMC), which are growth factor independent continuously growing lines previously characterized in this lab (Chan *et al.*, 1988; 1990), were shown to carry one or both receptors on their surface. As length of time in culture of various RCMC clones increased so did the expression of FcϵR_L (Hu *et al.*, 1990). The change in expression of FcϵRI varied, depending on the clone of cultured mast cells used.

Structure and Cloning

When Kulczycki published his review (1980) on the "Structure of the IgE Receptor", little, in fact, was actually known about the receptor - certainly in comparison to our knowledge today. At this time the receptor was known to be a glycoprotein (Kulczycki *et al.*, 1976), appeared to be encorporated into the plasma membrane, and could be freed from cells with nonionic detergents (Conrad *et al.*, 1976; Rossi *et al.*, 1977). The denatured receptor exhibited a molecular weight of 45,000 - 55,000 by SDS-PAGE (Conrad and Froese, 1976; 1978).

The possibility that the receptor binding IgE could indeed exist as more

than one polypeptide was proposed by Holowka (1980) and coworkers with their finding of a polypeptide of 30-35 kDa existing in a 1:1 stoichiometry with the receptor (Holowka *et al.*, 1980). The protein could be detected by both crosslinking studies and biosynthetic encorporation of [^{3}H]-amino acids but not by surface iodination (Holowka *et al.*, 1980). The polypeptide is found within the plasma membrane and can be detected by the photolysable hydrophobic reagent: 5-iodonaphthyl-1-azide (INA) (Holowaka *et al.*, 1981). This polypeptide is referred to as the "β-chain", a partner to the 50 kDa IgE binding "α-chain". The β-chain is a nonglycosylated protein, it dissociates easily from the α-chain in detergent solutions but there appears to be no free exchange of α-, and β-subunits as with the MHC class I α-chain and β_2-microglobulin (Holowka and Metzger, 1982).

Rivney *et al.*, (1982) showed that phospholipids are important to the stabilization of α- and β-subunits, and if an appropriate phospholipid detergent ratio is used, copurification of both subunits is possible. In these experiments they first detected the presence of a third polypeptide of relative molecular mass of 20 kDa, which is also receptor associated. It was later characterized as a disulfide linked dimer of polypeptides of 10 kDa (Perez-Montfort *et al.*, 1983). It is a nonglycosylated membrane associated protein, which could be phosphorylated within the cytoplasm (Perez-Montfort *et al.*, 1983). This polypeptide designated as the γ-subunit, along with the β-chain, could be iodinated on the cytoplasmic side of the plasma membrane (Holowka and Baird, 1984).

By this time it was clearly established that all previous studies using the isolated IgE binding receptor had, in fact, dealt solely with the α-subunit of a tetrameric protein receptor. The cDNA coding for the α-subunit and derived from RBL-mRNA coded for a 250 amino acid polypeptide. This included a 23-residue signal peptide, 180-residue extracellular portion, 20-residue transmembrane segment, and 27-residue cytoplasmic portion (Kinet *et al.*, 1987). The processed molecule has a predicted molecular weight of 26,104 (Kinet *et al.*, 1987). The α-subunit is a member of the immunoglobulin supergene family and has a sequence homology of 49% with coding regions of murine FcγRIII (FcγR-α) gene, and 32% at the amino acid level. Both receptors possess two extracellular domains which implies that they may have a common ancestral origin. No sequence similarity was found to exist between FcϵRI and CD23 (FcϵRII).

The cDNA of the β-subunit codes for a 243 residue protein. It is predicted, based on the location of the hydrophobic amino acids, to have four transmembrane regions with both COOH- and NH_2-terminal residues located cytoplasmically (Kinet *et al.*, 1988). The predicted molecular weight of the protein is 27,000 (Kinet *et al.*, 1988).

The cDNA of the γ-subunit codes for a 68-residue protein including a 18-residue signal peptide with a predicted molecular weight for the processed protein of 7,139 (Blank *et al.*, 1989). The final isolation and cloning of the cDNA for the γ-subunit facilitated expression in transfected COS 7 cells of the complete receptor for IgE since surface

expression of the rodent FcεRI receptor was found to require coordinate biosynthesis of all three subunits: α, β, and γ (Blank *et al.*, 1989). Expression of the human IgE receptor requires α-, β- and/or γ-subunits and therefore the β-subunit is not required for expression and binding to IgE (Hakim *et al.*, 1990). The expression of FcεRI is also possible when FcεRI(γ) is substituted for CD3 ζ (zeta) from the T cell receptor for antigen and MHC (Howard *et al.*, 1990). FcεRI(γ) and CD3 ζ are highly homologous within the areas of the transmembrane and COOH-terminal portions allowing for this sort of receptor isotypy (Howard *et al.*, 1990). In terms of FcεRI(α), the transmembrane and cytoplasmic portions appear to be vital for interaction with β- and γ-subunits and for subsequent expression. Site-directed mutagenesis of these areas caused decreased or abrogated expression of FcεRI *in vitro* in transfected cells (Blank *et al.*, 1990). Expression was normal for chimeric FcεRI(α) constructed of the a extracellular portion with the remaining polypeptide replaced by the p55 IL-2 receptor transmembrane and cytoplasmic portions (Hakimi *et al.*, 1990).

Once expression of the rodent receptor was achieved, it became imperative to establish a model which would fit the structure of the proteins at the amino acid level and decide if the proteins can in fact be considered subunits or "receptor-associated" proteins. The difficulty isolating the tetramer intact in detergent solution and the need for phospholipids to stabilize β and γ_2 with α, argued against a subunit model for the receptor (Rivney *et al.*, 1982; Kinet *et al.*, 1985). Strong evidence, however, sup-

ported a more integral relationship between the proteins of the FcεRI: (1) all three proteins are synthesized and catabolized together, γ associates last with α, and β directly following translation (Quarto *et al.*, 1985); (2) expression, at least in rodents, is not possible without all three subunits; (3) on occassion, β and γ have been found di-sulphide linked to one another (Quarto and Metzger, 1986) and will dissociate from α together as a single unit (Rivera *et al.*, 1988).

71K

A minor receptor exists on the surface of rat basophilic leukemia cells named 71K. It has been shown previously to be a disulphide-linked dimer of the α-subunit of the high affinity receptor for IgE, FcεRI, linked to a polypeptide chain of unknown identity (Roth *et al.*, 1986). The molecular weight of the receptor has been determined to be 71 kDa by SDS-PAGE analysis, the unidentified disulphide-linked molecule has an M_r of 40 kDa which is poorly surface iodinated and therefore difficult to detect (LaCroix *et al.*, to be published). 71K can be isolated as IgE, anti-IgE receptor complexes, or using IgE-Sepharose. The 71K receptor is present on RBL cells which are infected with the organism *Mycoplasma hyorhinis* (Chan *et al.*, 1986). De-contamination of the cell culture corresponds with a disappearance of the 71K receptor and re-infection re-establishes this receptor within 24 hours (Chan *et al., 1986).* Mycoplasmas are the smallest living independent organism and have no cell

wall. They are a common contaminant of tissue culture and have been demonstrated to have a very wide range of immunological effects on cells. They are mitogenic towards B cells and T cells (Stanbridge *et al.*, 1981; Butler *et al.*, 1984; Proust *et al.*, 1985). Evidence now suggests that *M. hyorhinis* may exert its effects through the carbohydrate portion of FcϵRI(α) (LaCroix *et al.*, to be published).

OLIGOSACCHARIDE BIOSYNTHESIS AND PROCESSING

The purpose of this section is to briefly discuss the major pathways responsible for the biosynthesis and processing of the various types of oligosaccharides since a large number of reviews are available (Elbein, 1987; Hirschberg and Snider, 1987; Pfeffer and Rothman, 1987; Kornfeld and Kornfeld, 1985; Schachter *et al.*, 1983; Hubbard and Ivatt, 1981).

Few can deny that carbohydrate plays an underlying role in a multitude of processes including cell-cell communication, ligand-receptor binding, various microorganism - host relationships, protein targetting, malignancy and malignant transformation, as well as phagocytosis. Unfortunately their importance is all too often underestimated especially in this age of molecular biology where protein production can be done in "host" cells transfected with the appropriate cDNA and where glycosylation enzymes may be nonexistant or different from those by which the protein is normally processed. Only limited generalizations about the actual role of the oligosaccharides of glycoproteins can be made because of dramatic differences from one protein to another. Most will agree that the carbohydrate component of glycoproteins is not superfluous (as evidenced by the selective pressure to maintain it) but rather its complexity and importance in biological functions is just beginning to be unravelled.

Traditionally oligosaccharides have been defined on the basis of their attachment to the protein (Figure 1.1). Therefore, in such cases

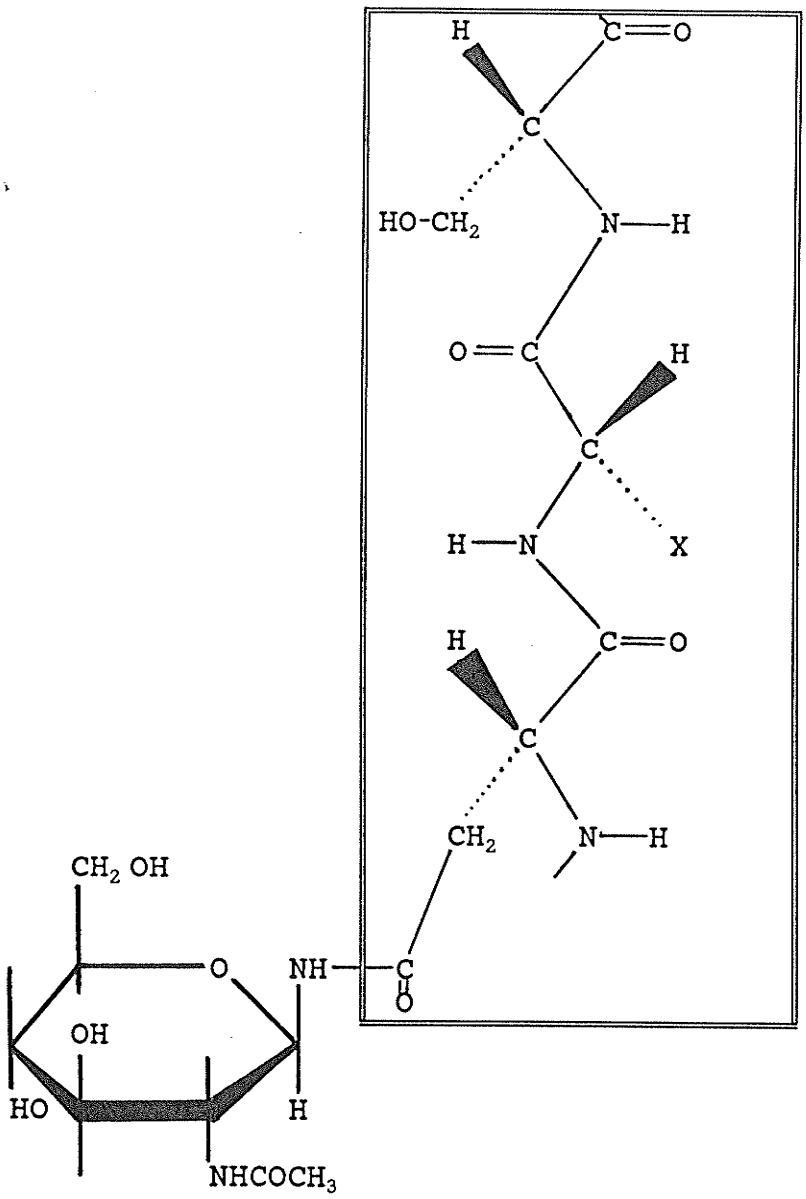


Figure 1.1 The N-glycosidic bond between N-acetyl-D-glucosamine of the core oligosaccharide and asparagine of the growing polypeptide (boxed).

"N-linked" oligosaccharides are formed from a N-glycosidic linkage between the amino acid asparagine (Asn) and carbohydrate residue, N-acetyl-D-glucosamine (GlcNac). "O-linked" oligosaccharides may be formed from any one of three O-glycosidic linkages between serine (ser) or threonine (thr) and N-acetyl-D-galactosamine (GalNac); serine (ser) and xylose (xyl); and/or hyluronic acid (hyl) and galactose (Schachter *et al.*, 1983).

N-linked oligosaccharides can be further classified according to the nature and quantity of carbohydrate residues forming branches outside of the common pentasaccharide core structure of Manα1-3(Manα1-6)Manβ1-4GlcNacβ1-4GlcNac-Asn (Figure 1.2). The high mannose-type oligocaccharide typically has two to six mannose residues attached to the core. Complex-type oligosaccharides contain sialyl lactosamine sequences (GlcNacβ1-4Galα2-6SA) and usually have other substituents like α1-6 fucose and β1-4 N-acetyl-D-glucosamine bound within the core region. Most complex-type oligosaccharides have 2, 3, or 4 outer branches making them bi-, tri-, or tetraantenary respectively (Kornfeld and Kornfeld, 1985). Hybrid-type oligosaccharides are named as such because they possess characteristics of both complex and high mannose oligosaccharides. They possess one high mannose and one complex branch in addition to a bisecting β1-4 linked N-acetylglucosamine.

The core structure of all oligosaccharide structures is identical because they all arise from the identical lipid-linked oligosaccharide formed as a

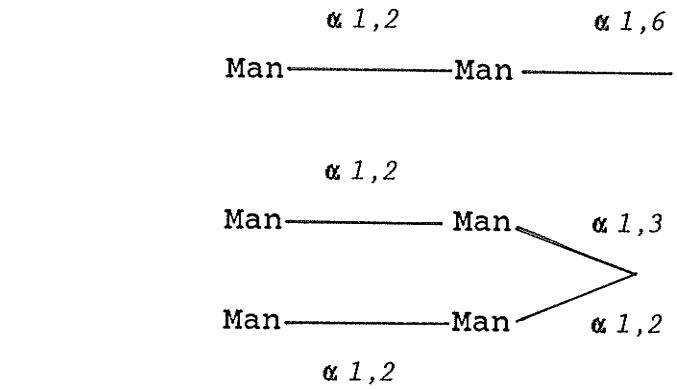
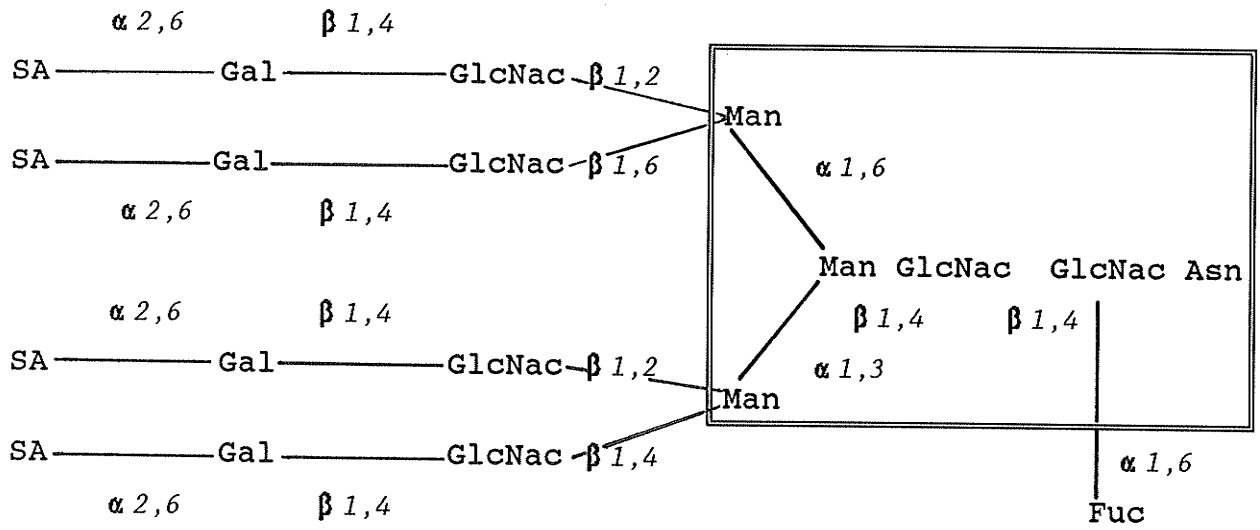
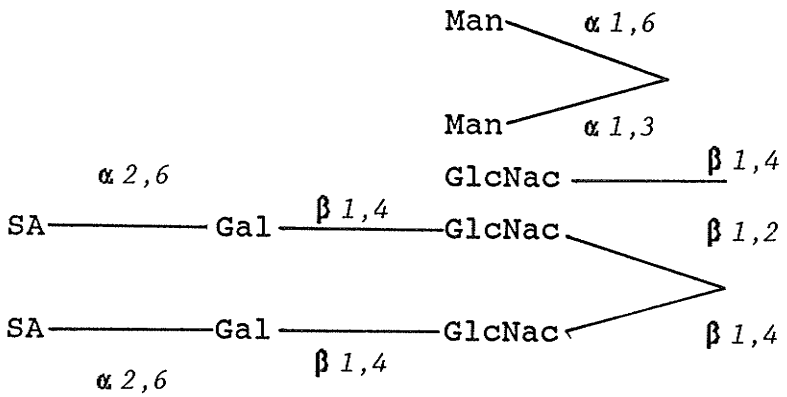


Figure 1.2
Common pentasaccharide core structure (boxed) and structures of the three oligosaccharide classes





product of the dolichol phosphate cycle. Dolichyl-P-P serves as the lipid carrier of the product $Glc_3Man_9GlcNac_2$ formed from carbohydrate residues transferred from their nucleotide or lipid linked derivatives. Tunicamycin, the antibiotic used commonly to block the N-linked glycosylation of proteins, acts at the very first step of the lipid pathway to block the transfer of GlcNac-1-P to dolichyl-P (Kornfeld and Kornfeld, 1985).

Several studies have indicated that the three terminal glucose residues play a role facilitating the transfer of the newly constructed oligosaccharide to the polypeptide (Trimble *et al.*, 1980; Murphy and Spiro, 1981; Lehle and Bause, 1984). They probably do not, however, present an absolute requirement for oligosaccharide transfer. The number of mannose residues has no significant influence on glycosylation (Kornfeld and Kornfeld, 1985).

Attachment of the oligosaccharide to protein occurs cotranslationally across the membrane of the rough endoplasmic reticulum (RER) following the synthesis of the appropriate peptide acceptor. The acceptor sequence is Asn-X-Ser/Thr where X is not proline or aspartic acid but can be any other amino acid. Several other factors come into play when considering the successful transfer of oligosaccharide from lipid to protein. It appears that not only is it necessary for the tripeptide signal to be present, but it also must achieve a "favorable conformation" (Kornfeld and Kornfeld, 1985). Since a polypeptide in the process of translation is also

undergoing secondary and tertiary structure formation, a "favorable conformation" would be one allowing maximum accessibility to oligosacchayltransferase as well good hydrogen bond formation. For example, studies by Bause (1983) showed that β-turns or loops in the polypeptide favored glycosylation and the formation of hydrogen bonds between amide of asparagine and the oxygen of serine or threonine also promoted a glycosylation reaction (Bause and Legler, 1981). Few potential tripeptide signals may actually achieve this favorable conformation. Some studies indicate as little as one third of all the asparagine signal sites are, in fact, glycosylated (Kronquist and Lennarz, 1978). The level of glycosylation has also been shown to be influenced by the availability of dolichyl-P (Carson *et al.*, 1981) of oligosaccharyltransferase which is the enzyme responsible for the transfer of oligosaccharide to protein (Oda-Tamailt *et al.*, 1985).

The processing of oligosaccharides to complex, high mannose, or hybrid-type structures involves the action of a complex series of enzymes the activity of each of which is dependent on the action of the preceeding enzyme in the sequence (Figure 1.3). The enzymes which will be emphasized in particular are those which are susceptible to inhibition by the substances castansopermine, 1-deoxymannojirimycin, and swainsonine. The structural and chemical properties of these will be discussed in the next section.

The processing of N-linked oligosaccharides occurs in both the RER and Golgi complex membranes. Following the action of

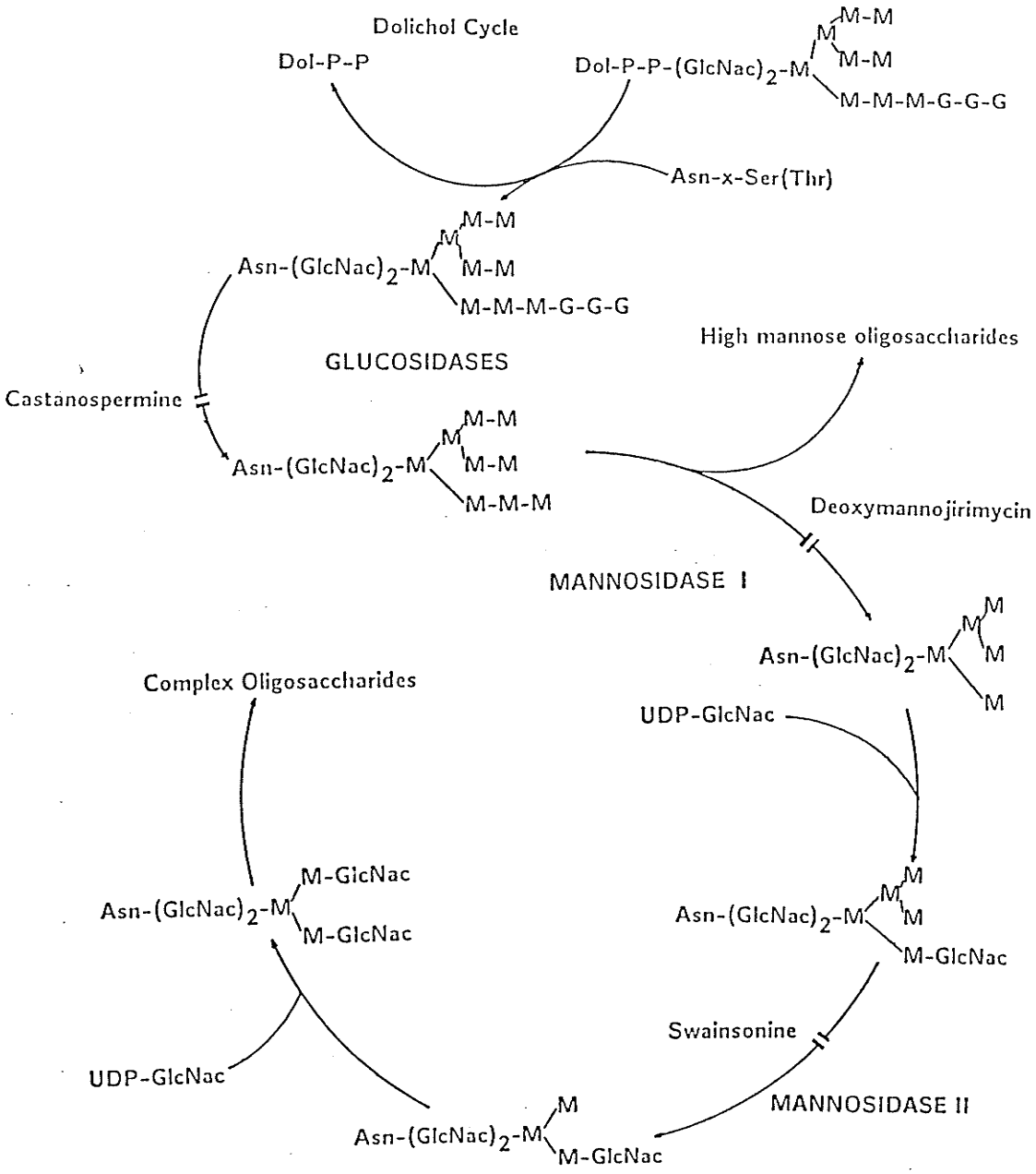


Figure 1.3

Pathway for the processing of N-linked oligosaccharides starting from the dolichol cycle (top) and action of oligosaccharyl transferase to transfer fourteen unit oligosaccharide from the lipid carrier to asparagine residue of polypeptide. Arrows indicate unidirectional flow of processed oligosaccharide structures.

oligosaccharyltransferase, α1-2-glucosidase I removes the single most terminal α1-2-linked glucose residue as shown in Figure 1.3. Most probably, there are several different enzymes that can process the glucose, and can therefore be viewed as a set than a singular enzyme. This action permits the next enzyme set, α-glucosidase II, to remove the subsequent two terminal α1-3 glucose residues. Both sets of glucose removing enzymes are inhibited by castanospermine. Failure to remove the glucose residues blocks the action of the proceeding processing enzymes in the series.

The removal of the three glucose residues signals the action of the next set of enzymes: the mannosidases (Figure 1.3). A single α1-2 mannose residue is removed by the ER α1-2 mannosidase prior to export of the glycoprotein from the RER to cis Golgi. Once in the Golgi apparatus, the Golgi α-mannosidase I acts to sequentially remove the remaining three α1-2 linked mannose residues. Cells grown in the presence of 1-deoxymannojirimycin fail to achieve this removal and the oligosaccharide becomes frozen in a Man_{8-9} structure since further processing is blocked.

The glycoproteins of normal, untreated cells, proceed through vesicle transport from the cis to medial Golgi where the α-mannosidase II enzymes are located. Following the addition of GlcNac by GlcNac transferase I to the α1-3 mannosyl arm, α-mannosidase II cleaves the α1-6 and α1-3 mannose residues from the mannosyl α1-6 arm (Figure 1.3). It is the action of α-mannosidase II within the medial Golgi membranes which is stopped by the inhibitor swainsonine. Failure to remove the two mannose residues from the α1-6 mannosyl arm prevents any further processing of this branch only.

The α1-3 mannosyl arm can be processed as normal, thus creating the new hyrid type structure. As with the glucosidases, the α-mannosidase II most probably represents a number of different enzymes with the same processing ability. Some of the processing steps which would normally occur after the action of mannosidase II include: addition of GlcNac (GlcNac transferase II) to α1-6 mannosyl arm; additions of fucose and/or galactose by their respective transferases and finally the terminal addition of sialic acid by the enzyme sialyltransferase.

CARBOHYDRATE STRUCTURE OF Fc RECEPTORS

Frequently, when studying glycoproteins, four approaches are used to investigate structure and function of oligosaccharide including: (1) biosynthetic labelling of glycoproteins using radiolabelled carbohydrate precursors; (2) destruction or elimination of the sugar moiety using periodate or glycosidases; (3) preventing attachment of sugars with inhibitors, or most recently (4) modification of oligosaccharide structure using inhibitors.

The presence of carbohydrate residues on the receptors for IgE on RBL and mast cells was initially suggested by their SDS-PAGE analysis. The appearance of the bands is broad and dispersed with molecular weight changes corresponding to changes in gel porosity. The suggestion that the FcϵR's were glycoproteins was confirmed by various biosynthetic labelling studies. Kulczycki *et al.*, (1976) using [^{14}C]-carbohydrate precursor encorporation into the receptors of RBL-1 cells (isolated by IgE complexes and therefore FcϵRI) found 46% of [^{14}C]-radioactivity was N-acetylglucosamine; 9% N-acetyl-galactosamine; and as much as 45% sialic acid. In addition, [^{3}H]-fucose and [^{3}H]-galactose were found to be incorporated into FcϵRI(α), FcϵR$_L$ and 71K (Helm and Froese, 1981a). Lectin-binding studies indicated some subtle differences in the carbohydrate composition of the three IgE receptors. FcϵRI(α) and 71K, but not FcϵR$_L$, bound winged pea- and gorse-lectin (Helm and Froese, 1981b). Castor bean-, wheat germ agglutinin- and lentil-lectin-Sepharoses bound all

three receptors however the affinity for $Fc\epsilon R_L$ appeared lower (Helm *et al.*, 1979; Helm and Froese, 1981b).

It has, therefore, become apparent that $Fc\epsilon RI(\alpha)$, $Fc\epsilon R_L$ and 71K are associated to varying degrees with fucose, mannnose, galactose and N-acetylglucosamine. The total carbohydrate composition of $Fc\epsilon RI(\alpha)$, has been estimated to be 30% (Kanellopoulos *et al.*, 1980). The carbohydrate composition established by chemical methods approximates a total contribution to M_r of 16.2 kDa with 40 residues/mol. galactose; 27 residues/mol. mannose; 27 residues/mol. glucosamine; and 7 residues/mol. fucose (Kanellopoulos *et al.*, 1980). No sialic acid residues were detected by these authors, although others confirmed its presence (Pecoud *et al.*, 1981). The extent of glycosylation of $Fc\epsilon R_L$ and 71K has not been established.

As with many glycoproteins, determining the functional importance of the carbohydrate moieties is often difficult. Non-glycosylated $Fc\epsilon RI(\alpha)$ and $Fc\epsilon R_L$ from basophils or mast cells cultured in the presence of tunicamycin still can bind IgE (Pecoud *et al.*, 1981). Conversely, non-glycosylated IgE from IR-162 cells treated with tunicamycin also exhibit unaltered $Fc\epsilon R$ binding abilities (Kulczycki and Vallina, 1981). Despite this, various studies have indicated that carbohydrate plays a role in receptor expression and perhaps an indirect role in the receptor function and interaction with IgE.

Treatment of RBL-1 with tunicamycin causes a dramatic decrease from 2.36 x 10^5 receptors/cell to 8.9 x 10^4 receptors/cell (Pecoud *et al.*, 1981). Protein synthesis is also inhibited by tunicamycin in a dose dependent manner (Hempstead *et al.*, 1981). Expression of FcϵR_L appeared to be less effected than FcϵRI. Gaveriaux and Loor (1987) reported that RBL cells cultured in the presence of either glucosidase inhibitors deoxynojirimycin or castanospermine exhibit a decreased capacity to bind monoclonal IgE. Carbohydrate may also play a role in histamine release. Treatment of rat peritoneal cells with sialidase, removing terminal sialic acid residues, at low cell/enzyme ratios enhanced the release of histamine whereas high ratios showed a sharp dose-dependent inhibition (Bach and Brashler, 1973). In addition, similar treatment with neuraminidase of RBL-1 or rat peritoneal cells resulted in a 62% increase in affinity of IgE and a corresponding increase in the forward rate constant (k_1) from 3.22 x 10^4 l mol^{-1} sec^{-1} to 7.61 x 10^4 l mol^{-1} sec^{-1} (Pecoud *et al.*, 1981).

FcϵRI, of at least some of the RBL cell lines may contain some O-linked oligosaccharides in addition to N-linked ones. A dramatic decrease in molecular weight of 28% was reported for the α-subunit of RBL-1 upon treatment with α-D-N-acetylgalactosaminyloligosaccharidase which removes O-linked sugars (Goetze *et al.*, 1981). Digestion of FcϵRI(α) with the enzymes, endoglycosidase D, endoglycosidase H and neuraminidase reportedly caused only a small decrease in molecular weight for each (Goetze *et al.*, 1981).

Murine mast cells have on their surface Fcγ receptors which are also

heavily glycosylated in a way similar to FcϵRI and FcϵR_L called FcγRII and FcγRIII (Unkeless *et al.*, 1988). These receptors were first characterized in murine thioglycollate-stimulated peritoneal macrophages, and macrophage-like cell lines, p388D_1, J774.2, IC-21 and /or FC-1 (Unkeless and Eisen, 1975; Walker, 1976; Heusser *et al.*, 1977; Diamond *et al.*, 1978). FcγRII (previously named FcγR-β) has two gene products due to alternate mRNA splicing which are designated FcγRII-1 (FcγR-β-1) and FcγRII-2 (FcγR-β-2) (Ravetch *et al.*, 1986).

The murine mastocytoma P815 has both the FcγRII and FcγRIII genes (Benhamou *et al.*, 1990). SDS-PAGE analysis of P815 showed a broad dispersed band of 45-65 kDa and following deglycosylation, three bands of 38 kDa (FcγRII-1 gene product), 32 kDa (FcγRII-2 gene product), and 29 kDa (FcγRIII gene product) (Benhamou *et al.*, 1990). Murine BMMC have genes for FcγRIII and FcγRII-1 showing a dispersed band of 55-65 kDa and two bands of 38 kDa (core protein of FcγRII-1) and 29 kDa (core protein of FcγRIII) after deglycosylation. (Benhamou *et al.*, 1990).

Studies in other labs of purified FcγRII from J774 revealed a molecule with an affinity for Con A (Mellman and Unkeless, 1980), and an M_r of 60 kDa which dropped to 37 kDa following removal of N-linked oligosaccharides with endoglycosidase F (Green *et al.*, 1985). This receptor has as many as four asparagine-linked oligosaccharides, at least three of which are complex, endoglycosidase H resistant. The presence of O-linked oligosaccharides was not detected (Green *et al.*, 1985).

INHIBITORS OF OLIGOSACCHARIDE PROCESSING

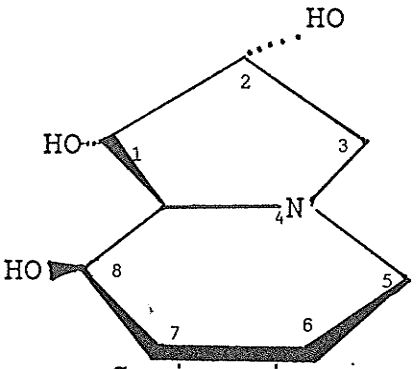
Swainsonine

Swainsonine [(1S,2R,8R,8αR)-1,2,8 trihydroxyoctahydroindolizidine)] is an indolizidine alkaloid first isolated from the Australian plant *Swainsona canescens* (Colegate *et al.*, 1979). It has more recently been extracted from the spotted locoweed *Astragalus lentiginosus* resident in the south-western United States (Molyneux & James, 1982; Davis *et al.*, 1984) and it was also found to be produced by the fungi: *Rhizoctonia leguminicola* (Schneider *et al.*, 1982), and *Metarhizium anisopliae* (Hino *et al.*, 1985). Swainsonine can now also be synthesized chemically (Suami *et al.*, 1985; Yasuda *et al.*, 1984). Two isomers are available, glc-swainsonine [(1S, 2S, 8R, 8αR)-trihydroxyindolizidine] and ido-swainsonine [(1S, 2S, 8S, 8R)-trihydroxyindolizidine] which have different specificities (Elbein *et al.*, 1987).

Swainsonine is an effective inhibitor of both lysosomal α-mannosidase and jack bean α-mannosidase (Kang & Elbein, 1983) but it has no inhibitory effect on α-glucosidase, β-galactosidases, hexosaminidase, or β-glucuronidase (Dorling *et al* 1980). Swainsonine became the first agent isolated that had the ability to inhibit the processing enzymes of N-linked oligosaccharides. In Madin-Darby canine kidney (MDCK) cells and Chinese Hamster Ovary (CHO) cells treated with swainsonine, there was an

increase in high mannose oligosaccharides with susceptibility to endoglycosidase H digestion and overall decrease of two [^{3}H]-mannose and six [^{3}H]-glucosamine residues incorporated into complex oligosaccharides (Elbein *et al.*, 1981). The cells, overall, showed changes in affinity for lectins as [^{3}H]-concanavalin A binding doubled and the amount of [^{3}H]-wheat germ agglutinin bound decreased substantially.

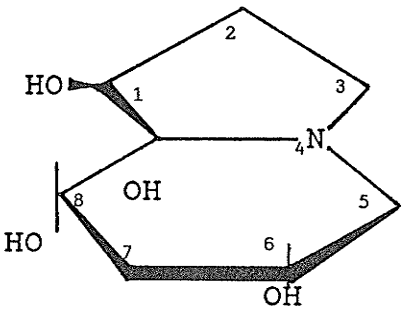
Studies using rat Golgi mannosidases showed swainsonine had the ability to inhibit mannosidase II but it was not effective against mannosidases IA or IB (Tulsiani *et al.*, 1982). The major accumulating oligosaccharide is a hybrid type structure. The cytoplasmic α-mannosidase or the membrane-bound rough endoplasmic reticulum α-mannosidase, which only removes a specific mannose residue from certain glycoproteins, are not inhibited by swainsonine (Bischoff & Kornfeld, 1986). The inhibition by swainsonine of α-mannosidase is characterized as being specific, site-directed, and somewhat reversible (Dorling *et al.*, 1980) and it is competitive (Kang & Elbein, 1983). The binding to the enzymes mannosidase II and lysosomal α-mannosidase isolated from the liver was found to be bimodal and dependent on inhibitor concentration and incubation time (Tulsiani *et al.*, 1985). One mode, dominant for mannosidase II, is largely irreversible whereas the other mode, prevalent for lysosomal α-mannosidase, is easily reversible and concentration and time independent (Tulsiani *et al.*, 1985). It is suggested that swainsonine can bind mannosidases effectively because of the presence of nitrogen in its ring instead of oxygen (Figure 1.4). In addition the similarity of its cationic center and the stereochemistry



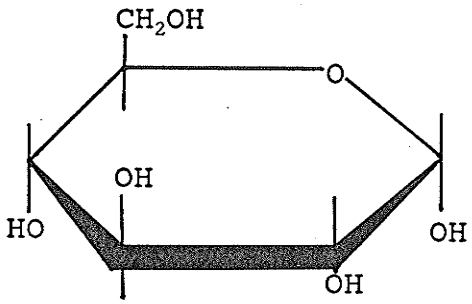
Swainsonine

Figure 1.4

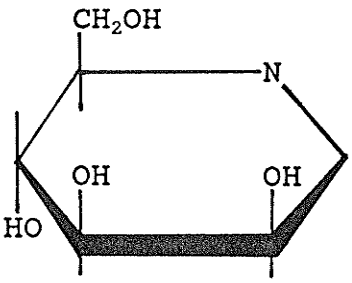
Structure of the glycosylation processing inhibitors: swainsonine; castanospermine; 1-deoxymannojirimycin; and their sugar analogues D-mannose and D-glucose.



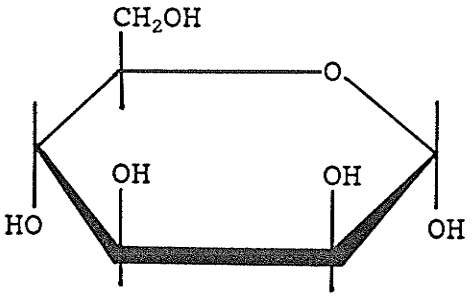
Castanospermine



D-glucose



1-Deoxymannojirimycin



D-mannose

of hydroxyl groups at positions 1, 2, and 8 to the mannosyl ion are believed to be responsible for its ability to act as an inhibitor (Dorling *et al.*, 1980). Swainsonine has been found to be the causative agent of a disease in livestock resembling the lysosomal storage disease α-mannosidosis (Dorling *et al.*, 1978; Warren *et al.*, 1988; Molyneux & James, 1982).

Swainsonine does not cause any noticeable affects on mammalian cell growth, size, shape, and it is noncytotoxic at high concentrations (Elbein *et al.*, 1983). Thus, swainsonine became a useful agent for studying carbohydrate function where previously studies using the antibiotic tunicamycin were difficult to interpret because of its complete inhibition of N-linked glycosylation. The conversion by swainsonine of complex to hybrid structures was demonstrated in many glycoprotein systems without significant changes in function or structure of glycoproteins. In general, glycoproteins formed in the presence of swainsonine had decreased molecular weight, and became sensitive to endoglycosidase H but otherwise they did behave identically to normal untreated glycoproteins. Human skin fibroblasts (Tulsiani & Touster, 1983); fibronectin (Arumughar & Tanzer 1983); secretion of the plasma proteinase inhibitor α_1-antitrypsin (Gross *et al.*, 1990); H2-DK histocompatability antigens from macrophages (Le & Doyle, 1985); and von Willebrand protein in epithelial cells (Wagner *et al.*, 1985) were uneffected by incubation *in vitro* with swainsonine. Receptor function was also tested: insertion and function of the insulin receptor (Duronio *et al.*, 1986); epidermal growth factor re-

ceptor (Soderquist & Carpenter, 1984); and asialoglycoprotein receptor (Breitfeld *et al.*, 1984) all appeared normal. In viral systems, swainsonine has no effect on the infectivity or hemagglutination titer of influenza virus in primary calf kidney cultures (Elbein *et al.*, 1982) or on the protein synthesis of vesicular stomatitis virus (Kang & Elbein, 1983). Minor effects were seen involving the biosynthesis of hormones. Lutropin, with two noncovalently linked subunits α and β, accumulated intracellularly, but synthesis and combination of subunits was not affected (Hattori *et al.*, 1988). Secretion upon stimulation with gonadotrophin-releasing hormone, (GnRh), was similar to control cells (Hattori *et al.*, 1988). The secretion of thyroglobulin from porcine thyroid cells (Franc *et al.*, 1986; Ross *et al.*, 1985) was uneffected. Swainsonine was found to cause an increase of secretion of glycoproteins from the human hepatoma cells Hep-G2 (Yeo *et al.*, 1985). This may be due to the decrease in the lag time of glycoproteins travelling within the Golgi. Swainsonine can inhibit the proteolysis of surface glycoproteins but not of nonglycosylated proteins which have been endocytosed (Winkler & Segal, 1984). Since lysosomes have no endoglycosidases (Pierce *et al.*, 1979; Tachibana *et al.*, 1982), digestion is dependent on the sequential actions of exoglycosidases such as α-mannosidases.

Castanospermine

Castanospermine, (1S,6S,7R,8R,8αR)-1,6,7,8,-tetrahydroxyoctahydroindoli-

zine obtained its name from the seeds of the Australian legume *Castanospermum australe* from which it was extracted (Hohenschutz *et al.*, 1981). Since castanospermine exhibits a strong structural resemblance to swainsonine as shown in Figure 1.4, it was tested against a number of commercially available glycosidases to determine if it, like swainsonine, has any inhibitory effects (Saul *et al.*, 1983). Castanospermine was found to effectively inhibit almond emulsin β-glucosidases, β-xylosidase, fibroblast and white blood cell β-glucosidase and lysosomal α-glucosidase and β-glucocerebrosidase from fibroblast extract. It is inactive against yeast α-glucosidase, jack bean α-mannosidase, liver β-glucuronidase, liver β-galactosidase, coffee bean α-galactosidase, kidney β-N-acetylhexosaminidase, or epididymis α-L-fucosidase (Saul *et al.*, 1983).

The inhibition by castanospermine can be classified according to the site of its action. The first site is the Golgi apparatus, the location of the neutral glucosidases responsible for the first step of N-linked oligosaccharide processing, the glucose removal. Pan *et al.*, (1983) first demonstrated inhibition of glucosidase I in Madin-Darby canine kidney (MDCK) cells. When these cells were infected with influenza virus, the major oligosaccharide accumulating in the virus was found to be $Glc_3Man_7GlcNac_2$ (Pan *et al.*, 1983). In addition, the cell surface properties changed upon castanospermine treatment as indicated by increased binding of [^{3}H]-Concanavalin A. Castanospermine does not cause any inhibition of protein synthesis, (determined by [^{3}H]-leucine or [^{14}C]-alanine encorporation), or virus release (Pan *et al.*, 1983). Interestingly,

complete inhibition of complex chain formation is not evident even at extremely high alkaloid concentrations. Glucosidase I in cultured soybean cells is also inhibited by castanospermine causing accumulating of the same major oligosaccharide (Hori *et al.*, 1984). Other neutral glucosidases shown to be inhibited are in human hepatoma cells (Sasak *et al.*, 1985); mouse fibroblasts (Repp *et al.*, 1985); rat hepatocytes (Gross *et al.*, 1986); baby hamster kidney cells (Foddy and Hughes, 1988); human monocytes (Grutes *et al.*, 1987) and lymphocytes (Arakaki *et al.*, 1987; Asakawa *et al.*, 1988); and murine hybridoma (Rothman *et al.*, 1989).

The second site of castanospermine inhibition is within the lysosomes where acid glycosidases are actively involved in glycogen metabolism. When castanospermine was injected into rats, severe weight loss and diarrhea resulted. Levels of cytoplasmic glycogen decreased and lysosomes were filled with dense granular material identified as glycogen particles (Saul *et al.*, 1985). Symptoms could be alleviated when animals were fed a high glucose diet. Purified lysosomal amyloglucosidase, an exo-1,4-α-glucosidase, was also shown to be inhibited by castanospermine (Saul *et al.*, 1984; Chambers and Elbein, 1986).

Finally, the third site of active inhibition by castanospermine is the site of certain intestinal disaccharidases. Castanospermine was found to be a very powerful inhibitor of sucrase isolated from a human enterocyte cell line (Trugnan *et al* 1986); mouse intestinal brush border (Scofield *et al.*, 1986) as well as sucrase-isomaltase complexes isolated from the rat

small intestine (Danzin and Ehrhard, 1987). Prolonged inhibition of the intestinal digestive enzymes was also seen when rats were administered castanospermine (Rhinehart *er al.*, 1987).

Studies by Saul *et al.*, (1984) demonstrated that the mechanism of inhibition of amyloglucosidase and almond emulsin β-glucosidase is by reversible, competitive inhibition. Inhibition of glycohydrolases, including the intestinal disaccharidase, sucrase appears to be by a semi-reversible non-competitive type (Trugnan *et al.*, 1986, Rhinehart *et al.*, 1990). Ellmers *et al.*, (1987) found that the apparent irreversibility of the enzyme inhibition was due to the "tight binding" of castanospermine and the competitive nature was dependent on the lack of preincubation of castanospermine with the lysosomal α-glucosidase. Chambers & Elbein (1986) clarified the differential modes of inhibition by demonstrating the high affinity of castanospermine for both the maltose and isomaltose binding sites of lysosomal α-1,4-glucosidase and the low affinity for the site catalyzing hydrolysis of glycogen which would be a noncompetitive inhibition.

Deoxymannojirimycin

Deoxymannojirimycin is the mannose analogue of the glucosidase I inhibitor, deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol) (Lalegerie and Legler, 1982; Peyrieras *et al.*, 1983; Hettkamp *et al.*, 1982) which

in turn is the deoxy derivative of the antibiotic nojirimycin (5-amino-5-deoxy-D-glucopyranose) (Inouye *et al.*, 1968) (Figure 1.5). Deoxymannojirimycin has been chemically synthesized and has been found to be an effective inhibitor of α-D-mannosidases (Legler & Julich, 1984). Deoxymannojirimycin blocks the conversion of high mannose to complex type oligosaccharides in a cell culture of hybridoma cells producing IgM and IgD with no inhibitory effects on secretion (Fuhrman *et al.*, 1984). Conversion of complex to endoglucosaminidase H sensitive mannose residues also occurs in the case of influenza viral glycoproteins grown in MDCK cells (Elbein *et al.*, 1984).

Immunomodulatory Effects of Inhibitors

The specific role of carbohydrate in the immune system remains somewhat elusive. Polysaccharide antigens, by virtue of their repeating epitopes can cause multivalent cross-linking of B cell surface immunoglobulin and can therefore induce a T-independent immune response. Pure polysaccharide cannot induce T cell immunity. It may be their hydrophilicity that prevents their binding to the class II major histocombatibility complex (MHC).

Interest in glycoprotein processing inhibitors as potential immunomodulators began when it was found that swainsonine treatment of cells could enhance the immune response in mixed murine lymphocyte culture

and restore the Con A stimulated proliferative response in cultures that had been immunosuppressed by the serum of sarcoma 180 tumor bearing mice (Hino *et al.*, 1985).

The potential use of processing inhibitors as agents against tumor metastasis and invasion was also investigated. When swainsonine was added to the growth media of B16-F10 murine melanoma cells for 24 hours prior to injection in syngeneic C57BL/6 mice, it inhibited pulmonary colonization (Humphries *et al.*, 1986). Castanospermine and tunicamycin were also found to inhibit metastasis by at least 80% indicating that blockage of N-linked oligosaccharide completely or at some stage of processing was effective. This indicated that preventing sialylated complex oligosaccharide chain formation was critical in blocking metastasis (Humphries *et al.*, 1986). Effective inhibition of lung colonization by B16-F10 melanoma cells could also be further reduced by the addition of 2.5 μg/ml swainsonine to the drinking water of mice (Dennis *et al.*, 1986). In another study loss of the transformed phenotype of NIH-3T3 cells transfected with v-ras occurred following swainsonine treatment (DeSantis *et al.*, 1987). This seemed to indicate that glycoproteins with fully processed oligosaccharides are necessary for malignant phenotypic maintenance (DeSantis *et al.*, 1987).

Evidence exists to suggest tumor regression may not only be limited to alteration of the tumor glycoproteins but also through modulation of both the natural and humoral immunity of the host. Swainsonine was found to

cause an increase in total active NK activity by an increase in total spleen cell number. The antimetastatic ability of swainsonine is abolished when assays are carried out in homozygous C57BL/6 $^{bg/bg}$ beige mice or anti-asialo GM_1 antibody or cyclophosphamide treated C57BL/6 mice, all lacking natural killer (NK) cell activity (Humphries *et al.*, 1988). Swainsonine also augments IL-2-induced lymphokine-activited killer (LAK) cell induction as well as increases the cytolytic ability of the LAK cells by 2 to 3-fold against targets *in vitro* (Bowlin *et al.*, 1989). Cytotoxic capability of human large granular lymphocytes (LGL) against a NK-resistant colon carcinoma cell line was improved by a 36 hour preincubation with swainsonine (Yagita & Saksela, 1990). Increased lymphocyte proliferation occurred in the *in vitro* mitogenic assays when cells were treated with swainsonine (White *et al.*, 1988; Myc *et al.*, 1989). The increased proliferation may be due to increases in interleukin-2 production as swainsonine treated human lymphocytes showed increases or both IL-2 receptor and IL-2 production following mitogen stimulation (Bowlin & Sunkara, 1988).

Helper T cell clones showed enhanced proliferation to antigen and Con A when treated with swainsonine but inhibited responses to both antigen and mitogen when treated with castanospermine (Wall *et al.*, 1988). Either of the treatments altered antigen recognition. The quantitative expression of IL-2R, accessory molecules L3T4, LFA-1 and CD-4 molecules was essentially unchanged by any of the inhibitor treatments, although functional activity of IL-2R was not examined (Wall *et al.*, 1988).

CHAPTER 2

CHAPTER 2

INTRODUCTION

The aim of these studies was to investigate the nature and microheterogeneity of the carbohydrate moiety of Fcϵ receptors of RBL-CA10 and RBL-CA10.7 using two main approaches: (1) modulate the oligosaccharide units present on the receptors to increase uniformity and use resulting changes as a measure of existing microheterogeneity; (2) remove the oligosaccharide units partially or completely with enzymes of different specificities. In addition, the information obtained was used to shed light onto the interaction between *M. hyorhinis* and rat basophilic leukemia (RBL) cells which causes the induction of a new receptor for IgE (Chan *et al.*, 1986). Important to these studies are the similarities or differences between FcϵRI and FcϵR_L oligosaccharide composition.

It has been shown that microheterogeneity may exist for both FcϵRI and FcϵR_L based on their diffuse banding patterns on SDS-PAGE (Kulzycki *et al.*, 1976). Oligosaccharides interact with SDS resulting in altered mobility of the protein in low porosity gels (Ferguson, 1964; Weber and Osborne, 1975). Therefore within one receptor preparation distinct oligosaccharide populations will cause the disparate movement of the protein to which it is attached. The role of oligosaccharide on FcϵR_L has not been determined as yet and although oligosaccharide is not absolutely

necessary for binding of FcεRI to IgE it has been shown to have an influence on binding affinity (Bach and Brashler, 1973).

MATERIALS AND METHODS

Buffer Composition

(1) phosphate-buffered saline (PBS): 0.01 M PO_4^{3-}, 0.14 M NaCl, pH 7.4.

(2) PBS/BSA: PBS containing 0.05% bovine serum albumin (BSA).

(3) PBS/NP-40 wash buffer: PBS containing 0.1% Nonidet P-40 (NP-40).

(4) PBS/NP-40 lysis buffer: PBS containing 0.5% Nonidet P-40 (NO-40).

(5) SDS elution buffer: 0.0625 M Tris-(hydroxymethyl)aminomethane (Tris), 4% sodium dodecyl sulphate (SDS), 10% glycerol, 0.005% bromophenol blue as the tracking dye, pH 6.8.

(6) Reducing SDS elution buffer: as above with 5% 2-mercaptoethanol.

(7) N-glycanase SDS elution buffer: 0.22M Tris-(hydroxymethyl)amino-methane (Tris)-PO_4^{3-}, 0.35% sodium dodecyl sulphate (SDS), 1.4% 2-mercaptoethanol, pH 8.6.

(8) N-glycanase NP-40 dilution buffer: 0.22 M Tris-(hydroxymethylamino-methane (Tris)-PO_4^{3-}, containing 2.5% Nonidet P-40 (NP-40).

(9) Endoglycosidase H dialysis buffer: 0.05 M PO_4^{3-}, 0.5 M phenylmethyl sulfonylflouride (PMSF: Sigma Chemical Co., St. Louis, MO), pH 6.0.

Cells

RBL-CA10.7 and RBL-CA10 used in this study have been described previously (Chan *et al.*, 1986). RBL-CA10 is a mycoplasma infected cell line used in these studies to further characterize the infection induced receptor, 71K. RBL-2H3 used in this study is a subline of RBL as described previously (Froese *et al.*, 1982a). The hybrid rat mast cell line, HRMC 9, and a cloned line, HRMC 5.1.6, were prepared by the fusion of rat peritoneal mast cells (RPMC) with two different sublines of RBL cells (Zheng *et al.*, 1991). All cells were maintained in Eagle's basal essential medium with Earle's salts (Difco laboratories, Detroit, MI). The medium was supplemented with 15% fetal calf serum (FCS), non-essential amino acids, TC vitamins, dextrose, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Burlington, Ontario). Cells were maintained in culture at 37°C in a humidified 3% CO_2 atmosphere. Harvesting of cells was achieved by gentle mechanical stripping using a bent pasteur pipet. Passage of cells constituted the transfer of approximately 1 ml of RBL cells at confluence in medium to 5 ml of fresh culture media in a new Falcon culture flack (Becton Dickinson Labware, Oxnard, CA) at approximately 1-3 x 10^5 cells/ml. HRMC 9 passage required a 10 minute incubation with 0.25% Trypsin-EDTA at room temperature (Flow Laboratories, McLean, VA) for removal of adherent cells. Cells were mechanically stripped, transferred to a 10 ml tube and centrifuged 1,000 g for 10 minutes. The pellet was washed twice with culture medium and 1 ml was added to fresh culture medium in the culture flask.

N-glycosidase Processing Inhibitors

RBL-CA10, or RBL-CA10.7 were passaged at a density of 3 x 10^5 cells/ml and incubated overnight to allow cell adherence. Spent culture medium was then aspirated and replaced by 5 ml fresh medium containing either 0.25 mM 1-deoxymannojirimycin (DMJ; Sigma Chemical Co., St. Louis, Mo.), 2.6 μM swainsonine (SW), 0.10 mM castanospermine (CN) (both from Boehringer Mannheim Biochemica (Canada) Ltd., Laval, PQ) or normal medium as the control (Spearman *et al.*, 1987). Cells were cultured in the presence of inhibitors for 48 hours prior to receptor labelling. The reversibility of the inhibitor effects was tested by transferring 1.5 x 10^6 inhibitor treated cells to a new flask containing normal medium, incubating them 48 hours and subsequently labelling them with [^{125}I]-iodine.

Mycoplasmal Infection of RBL-CA10.7 Cells

A cell concentration of 5 x 10^6 cells/ml RBL-CA10.7 was obtained and 3 ml were transferred to a new flask. Cell free supernatant containing mycoplasma was prepared from a highly confluent culture of RBL-CA10. The supernatant of the confluent culture was centrifuged 10 minutes, at 1,000 g, then filtered through a 0.46 μm pore size filter (Chan, 1988). A volume of 4 ml of supernatant was added per ml of RBL-CA10.7 cells. Infection was allowed to proceed 24 hours under normal culture condition prior to cell labelling.

IgE, Anti-IgE, & Anti-FcϵR_L Preparation

Monoclonal IgE was obtained from the ascites fluid of Lou/M/Wsl rats bearing the IR-162 immunocytoma (Bazin *et al.*, 1974). Purification was carried out as described previously (Conrad *et al.*, 1975). Ascites fluid was collected and immunoglobulins precipitated using $(NH_4)_2SO_4$ to 50% saturation. The precipitate was dissolved and dialyzed against 0.2 M Tris-HCl, 0.15 M NaCl, 0.5% NaN_3, pH 8.0 overnight at 4°C.

Purification of IgE was achieved using Ultrogel AcA 34 (LKB Produktor, Bromma, SW) instead of Bio-Gel P-300. The second peak, referred to as the "B" peak was pooled and concentrated. IgE concentration was determined using an extinction coefficient of $E^{1\%}/_{280}$=13.6 and stored at -70°C. This B peak IgE was used for all IgE-Sepharose conjugates. For experiments using free IgE for differential isolation of FcϵRI(α), it was purified one step further by preparative isoelectric focussing at 600 v for 48 hours using 1% carrier ampholytes of pH 4.0 to 6.5 (LKB Produktor, Bromma, SW) in a 0 to 40% sucrose gradient. IgE at pH 5.9 was collected and dialyzed against PBS, then stored at -70°C. Monospecific horse anti-rat IgE was a gift from Dr. K. A. Kelly and was prepared as described earlier (Kelly *el al.*, 1979). Anti-FcϵR_L was prepared by immunizing rabbits with purified FcϵR_L as described (Chan *et al.*, 1990). This antiserum interacts with FcϵR_L only (Chan, 1988).

Cell Iodination and Solubilization

Cells were harvested from confluent cultures and washed three times with PBS. Cells numbering 0.25 - 1 x 10^7 were suspended in 0.5 ml PBS. To this, 10 μl of lactoperoxidase (116 IU/ml) (Calbiochem, San Diego, CA) and 0.03% H_2O_2/PBS were alternately added three times within 1 minute intervals (Conrad and Froese, 1976). The iodination reaction was terminated by adding the reaction mixture to cold PBS/BSA. The mixture was centrifugated for 10 minutes, at 1,000 rpm and the pellet resuspended in 1 ml PBS/BSA and incubated 45 minutes at 37°C with gentle mixing. This solution was then layered over 4 ml FCS and centrifuged. A final wash using PBS/BSA was carried out and the pellet then resuspended in 200 μl PBS/NP-40 lysis buffer for 1 x 10^7 cell equivalents. Lysis occurred for a 20 minute period at 4°C with shaking. Following this, a 4 minute centrifugation at 12,000 g removed all the insoluble, particulate material. The supernatant containing soluble receptor was used in all subsequent differential isolations. In experiments where entire surface labelled components are analyzed, 2 μl of sample supernatant representing 1 x 10^5 cell equivalents, was added to the elution buffer and loaded onto the gel.

Affinity Chromatography

(1) Preparation of Conjugates

Conjugates of IgE-Sepharose (Conrad and Froese, 1978b), horse anti-rat

IgE-Sepharose (Conrad *et al.*, 1975) were prepared by the cyanogen bromide coupling procedure (Cuatrecasas and Anfinsen, 1971). Sepharose CL-4B (Pharmacia, Uppsala, Sw) was washed and resuspended in distilled water. Acetonitrile was used to dissolve 40 mg CNBr/ml Sepharose. Upon addition to the washed Sepharose, the pH was kept in the range of pH 9-11 using 0.5 N NaOH. The activated Sepharose was washed alternately with 0.05 M carbonate buffer, pH 10 and PBS pH 7.4. Protein was added at 2.5 mg/ml for horse anti-rat IgE and 5 mg/ml IgE. After overnight mixing at 4°C, the conjugates were quenched with 1 M ethanolamine in 0.2 M carbonate buffer with 0.1 M NaCl, pH 8.0. The final wash was with PBS and subsequent storage of Sepharose was in PBS/0.1% NaN_3 at 4°C. Protein A-Sepharose was purchased as a powder (Pharmacia, Uppsula Sweden) and resuspended in PBS/0.1% NaN_3 for storage at 4°C.

(2) Differential Isolation of Receptors

A packed volume of 200 μl of IgE-Sepharose per 1 x 10^7 cell equivalents was used to isolate all three NP-40 solubilized FcϵRI(α), FcϵR_L, and 71K receptors (Chan, 1988). Solubilized FcϵRI(α) and 71K from the same number of cell equivalents were also isolated as receptor-IgE complexes by preincubation with 20 μg IgE, followed by the addition of 400 μl packed volume of horse anti-rat IgE-Sepharose (Chan, 1988). FcϵR_L cannot be isolated by this technique due to its lower binding affinity for IgE, and this therefore provides a method for differential isolation. FcϵR_L was

isolated separately using 200 μg anti-FcϵR_L and 100 μl packed volume Protein A-Sepharose for each 1 x 10^7 cell equivalents.

In all cases, the protein coupled Sepharose was prewashed with PBS/NP-40 wash buffer three times and again three times after 2 hours or overnight incubation with the receptor preparation. The Sepharose was equilibrated with 0.0625 M Tris-HCL pH 6.8 prior to receptor elution with 200 μl SDS elution buffer per 1 x 10^7 cell equivalents. The Sepharose, with elution buffer, was then boiled for 90 seconds.

Endoglycosidase Treatment of Isolated Receptors

(1) N-glycanase

The procedure for N-glycanase was developed primarily on the manufacturers instructions and with reference to Plummer *et al.*, (1984); and Elder and Alexander, (1982). Receptors (1 x 10^7 cell equivalents) bound to affinity gels were eluted by boiling in 200 μl of N-glycanase SDS elution buffer containing 0.35% SDS, and 1.4% 2-mercaptoethanol (Eastman Kodax Co. Rochester, NY) according to manufacturers instructions. The Sepharose was centrifuged briefly and 93 μl of the eluate was transferred to 2-3 ml propylene tubes to which an equal volume of N-glycanase dilution buffer with 2.5% NP-40 was added, giving a final NP-40 concentration of 1.16% before N-glycanase addition. A volume of 189 μl of this mixture was added

to a 3 ml propylene tube containing 10 μl of 1,10 phenanthroline hydrate (10 mM; Sigma Chemical Co.,) followed by the addition of 1 U N-glycanase (peptide: N-glycosidase F, peptide-N^4 [N-acetyl-β-glucosaminyl] asparagine amidase; Genzyme Co. Boston, MA) yielding a final enzyme concentration of 5 U/ml as the reaction volume was adjusted to 200 μl. Enzyme concentrations of N-glycanase in the range of 1-10 U/ml were suggested by the manufacturer and tested in the present study. A final concentration of 5 U/ml was considered to be ideal within the acceptable working concentration range. The mixture was incubated for 18 hours at 37°C on a rotory shaker at 100 rpm. A volume of 100 μl reducing elution buffer with 6% SDS was added and a 100 μl aliquot was loaded onto the gel for SDS-PAGE analysis.

(2) Endoglycosidase H

The procedure for endoglycosidase H digestion was developed using the manufacturers information sheet and with reference to the Gross *et al.*, (1983) procedure. Endoglycosidase H hydrolyzes N-linked oligosaccharides of the high mannose type (Kobata, 1979; Trimble and Maley, 1984). For endoglycosidase H treatment, receptors from 1 x 10^7 cell equivalents bound to the affinity gel were eluted by the addition of 500 μl 3 M KSCN for 30 minutes at 4°C. The Sepharose was then briefly centrifuged and the eluate containing the receptors was transferred to dialysis tubing (12,000 exclusion pore size) and dialyzed extensively overnight with one buffer

change against 1.5 L of 50 mM phosphate buffer containing 0.5 M phenylmethylsulfonyl fluoride added immediately before the dialysis. Endoglycosidase H (Endo H; Endo-β-N-acetylglucosaminidase H (Boehringer Mannheim Biochemica (Canada) Ltd., Laval, PQ) was used to cleave high mannose and hybrid oligosaccharides. Endoglycosidase H, assayed by manufacturer and determined to be free of proteases, α and β-glucosidase, β-galactosidase, α-mannosidase, β-N-acetylhexosaminidase and α-L-fucosidases, was used at a working concentration of 0.025 U/ml as recommended by the manufacturer. Higher concentrations of 0.075 U/ml, and 0.15 U/ml were also tested and did not cause any detectable differences with the exception of swainsonine treated cells (see results section). Samples were removed from the dialysis tubing and 492 μl were transferred to Eppendorf tubes containing 5 μl of 2% SDS (final concentration 0.02%), and 3.52 μl of 2-mercaptoethanol (final concentration 0.1 M), and boiled for 90 seconds. A sample volume of 195 μl was treated with 5 μl of endoglycosidase H (final concentration 0.025 U/ml) or 5 μl of 50 mM phosphate buffer as control. Samples were incubated for 16 hours at 37°C with shaking (100 rpm). The reaction was terminated by addition of 100 μl reducing elution buffer containing 6% SDS and boiling for 90 seconds.

Polyacrylamide Gel Analysis and Autoradiography

SDS-PAGE on 10% gels was performed in the Tris-buffered system described by Laemmli (1970), using the following low molecular weight standards:

phosphorylase B (97,400); BSA (66,200); ovalbumin (42,700); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500) and lysozyme (14,400) (Biorad Laboratories, Missisauga, ON). The standards (2 μg) were reduced and boiled in sample elution buffer. Gels were stained using Coomassie Blue R-250 (Eastman Kodak Co., Rochester, NY). Relative mobility (R_f) was calculated by dividing the protein migration by the tracking dye migration. The R_f values obtained were plotted against the logarithm of the molecular weight using the Sigmaplot Scientific Graph System (Jandel Scientific, Sausalito, CA). This standard curve was used to calculate the molecular weights of the unknowns (see Appendix A).

The gels were dried with heating under vaccuum for 2 hours in a Hoeffer slab gel drier and were then exposed to hypersensitized Kodak X-omat AR film, -70°C (Laskey and Mills, 1977), using a Cronex Lighting Plus intensifying screen (Swanstrom and Shank, 1978).

RESULTS

Treatment of Cells with Glycosylation Processing Inhibitors

Treatment of the cell lines RBL-CA10.7 and RBL-CA10 with either of the glycosylation processing inhibitors castanospermine, 1-deoxymannojirimycin, or swainsonine resulted in a decrease in relative molecular mass of the receptors for IgE as detected by SDS-PAGE analysis and shown in Figure 2.1. In the RBL-CA10.7 control, FcϵR_L and FcϵRI(α) have M_r values of 55 and 45 kDa respectively. The mycoplasma infected RBL-CA10 control exhibits an extra band in the area of 71 kDa which is referred to as "71K". The changes in M_r observed following the inhibitor treatment appeared to be smallest with castanospermine and greatest when 1-deoxymannojirimycin was used. The effect of the processing inhibitors on the major receptor bands was the same for either RBL-CA10.7 or RBL-CA10 cells. This is assuming that after inhibitor treatment the relative positions of the two Fcϵ receptors remained roughly the same. Cell culture in the presence of castanospermine drastically reduced the surface expression of FcϵRI(α) for both cell lines which was not the case when the other two inhibitors were used. Cells treated with 1-deoxymannojirimycin and swainsonine generally exhibited narrower bands than control cells, at least as far as RBL-CA10.7 is concerned, while the receptor bands of castanospermine treated cells remained fairly broad. Figure 2.2 shows the SDS-PAGE analysis of the entire surface labelled components including major receptor bands of cells

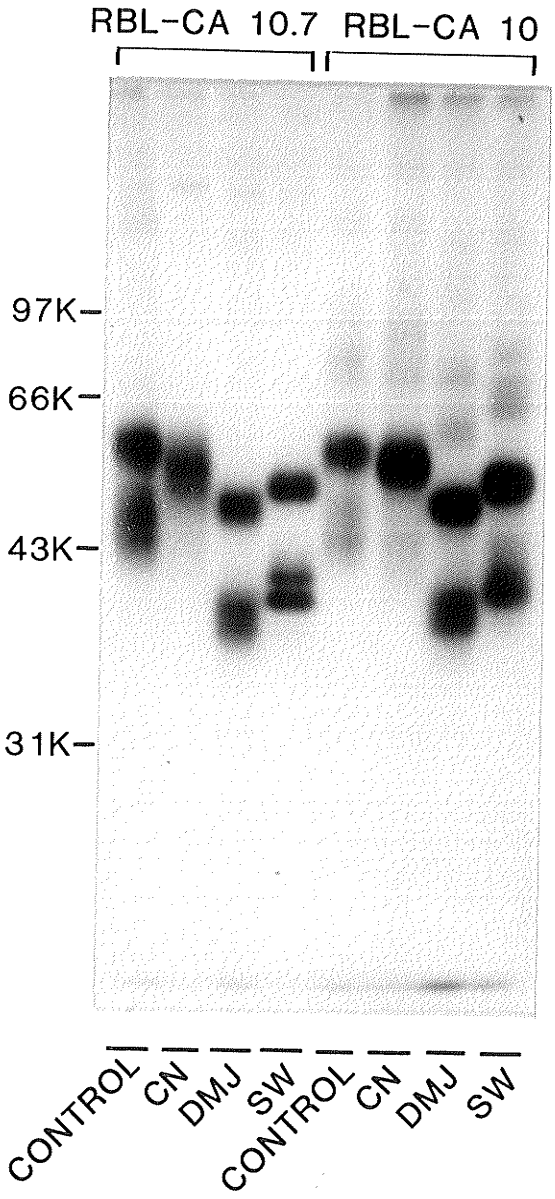


Figure 2.1 SDS-PAGE analysis of [^{125}-I]-iodine labelled receptors isolated by IgE-Sepharose from cell lines RBL-CA10.7 and RBL-CA10, as control (untreated) or following a 48 hour incubation with 0.10 mM castanospermine (CN), 0.25 mM 1-deoxymannojirimycin (DMJ), or 2.6 μM swainsonine (SW).

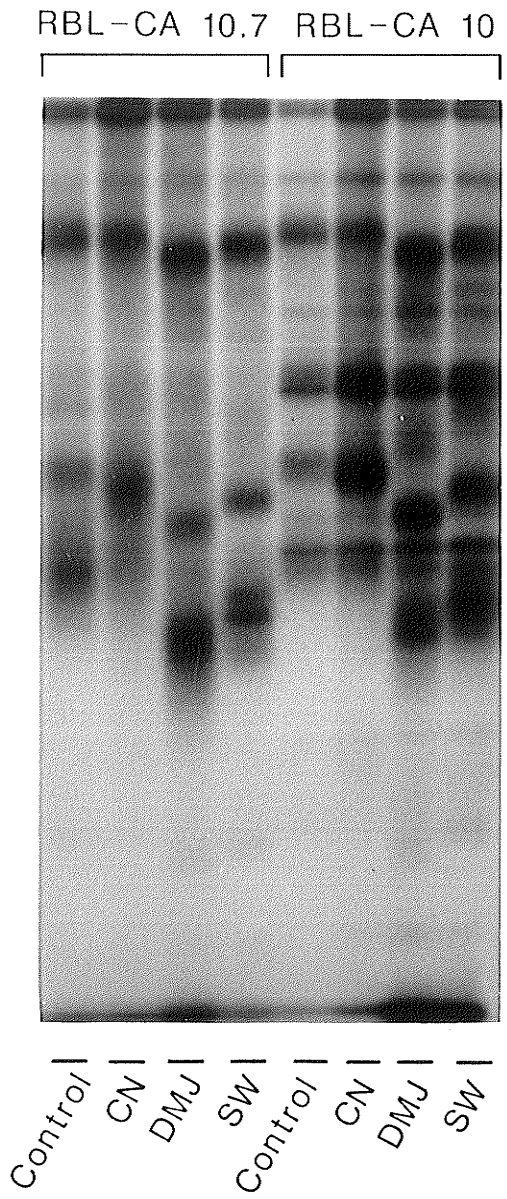


Figure 2.2 SDS-PAGE analysis of the changes following a 48 hour incubation with 0.10 mM castanospermine (CN), 0.25 mM 1-deoxymannojirimycin (DMJ), or 2.6 μM swainsonine (SW) on the entire surface [^{125}I]-iodine labelled components following NP-40 solubilization of either RBL-CA10 and RBL-CA10.7 cells.

treated with the inhibitors. It is evident that the other cell surface labelled components are also glycoproteins and that their mobility can also be affected by culture of the cells with the various processing inhibitors. Therefore, it appears that changes are not restricted to the major receptor bands. Despite the broad scope of the modulation of the cellular glycoproteins, the inhibitors had no detectable effects on the growth or appearance of the cells in culture. In addition, the ability of the glycosylation inhibitors to alter M_r of the FcϵR's was completely reversible following their removal from media. The autoradiograph of the SDS-PAGE gel in Figure 2.3 indeed shows that receptor profiles for both RBL-CA10.7 and RBL-CA10, which had been grown in the presence of inhibitors followed by growth in normal medium, are identical to those of control cells which were not treated with inhibitors.

Changes detected in receptor M_r were not dependent on the inhibitor concentration. Castanospermine concentrations of 0.03, 0.05, 0.10 or 0.20 mM produced identical changes in receptor mobility for RBL-CA10 as analyzed by SDS-PAGE (Figure 2.4). Higher concentrations of castanospermine, however, did appear to enhance the decrease in receptor expression observed previously. Receptors from cells treated with 0.06, 0.13, 0.25 or 0.50 mM 1-deoxymannojirimycin demonstrated only very slight changes in M_r by SDS-PAGE (Figure 2.5). Treatment of cells with 0.33, 0.65, 1.3, 2.6, or 5.2 μM swainsonine also did not cause significant changes in M_r.

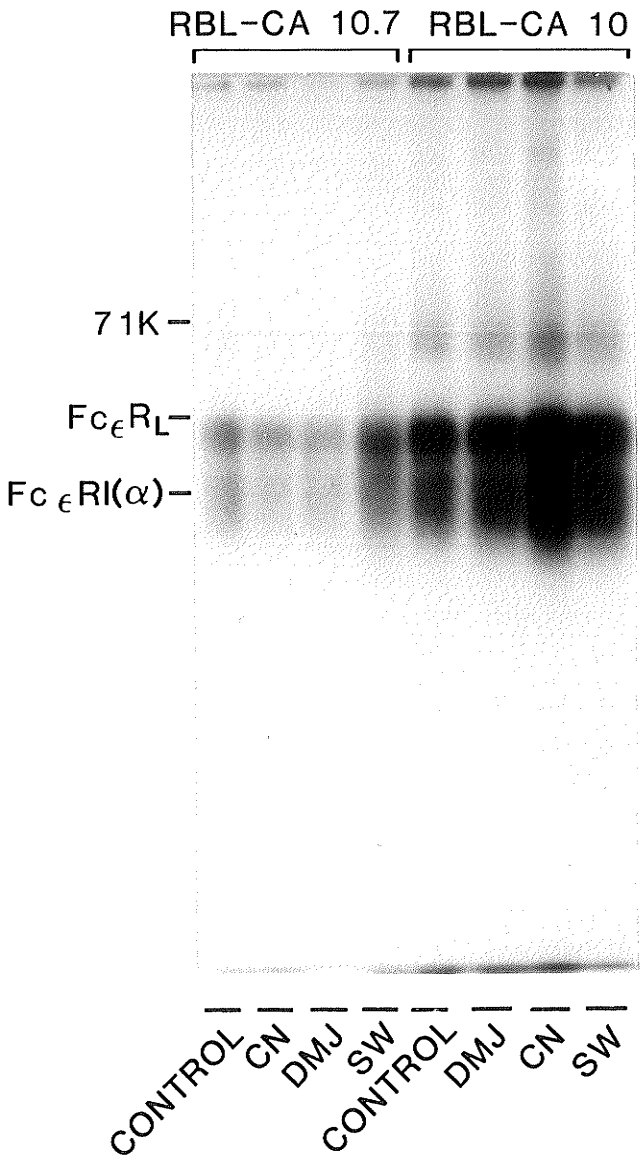


Figure 2.3 SDS-PAGE analysis showing reversibility of the effects of 0.10 mM castanospermine (CN), 0.25 mM 1-deoxymannojirimycin (DMJ), or 2.6 μM swainsonine (SW) on RBL-CA10.7 or RBL-CA10. Following a 48 hour incubation with inhibitors, 1.5 x 10^6 cells were transfered to a new flask containing normal media, again incubated 48 hours, then [^{125}I]-iodine labelled and isolated using IgE-Sepharose.

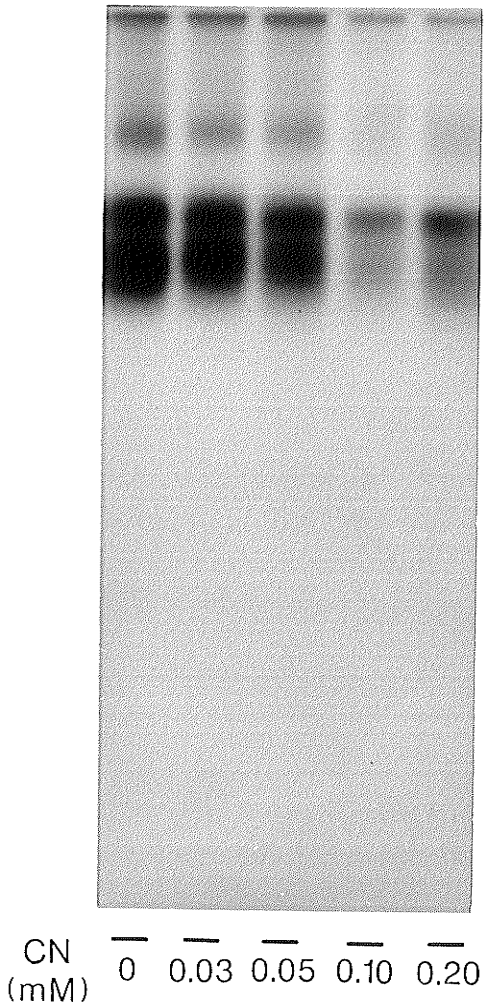


Figure 2.4 SDS-PAGE analysis of [^{125}I]-iodine labelled receptors isolated by IgE-Sepharose from RBL-CA10 cells following no treatment or treatment with 0.03, 0.05, 0.10, or 0.20 mM castanospermine (CN).

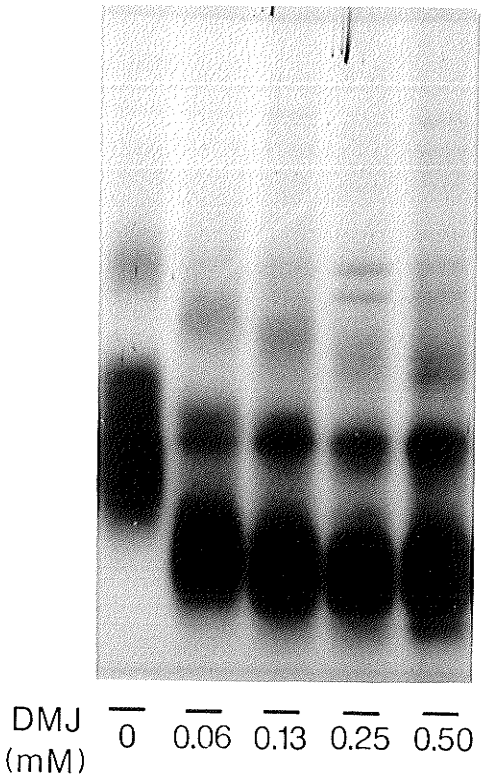


Figure 2.5. SDS-PAGE analysis of [^{125}I]-iodine labelled receptors from RBL-CA10 cells isolated by IgE-Sepharose following incubation in 0, 0.06, 0.13, 0.25, 0.50 mM 1-deoxymannojirimycin for 48 hours.

Differential Isolation of FcεR's

Since IgE-Sepharose isolates both FcεRI(α) and $FcεR_L$, differential receptor isolation from RBL-CA10 was carried out in order to identify which altered bands correspond to which FcεR (Figure 2.6). It is apparent from these results that all these receptors are affected by the treatment and that their rank in terms of apparent M_r remains the same after treatment. The reduction in the M_r of FcεRI(α) appears to be more pronounced than that of $FcεR_L$. The calculated M_r and change in M_r relative to control of two independent experiments are shown in Table 2.1. Some variability did exist in the calculated M_r from one experiment to another perhaps due to slight changes in running conditions. Treatment of RBL cells with castanospermine decreased the M_r of FcεRI(α) and $FcεR_L$ by ~2 kDa and ~1 kDa respectively. 1-Deoxymannojirimycin caused reduction of the M_r of FcεRI(α) by ~5 kDa to 42 kda and ~7 kDa to 38 kDa and that of $FcεR_L$ by ~6 and ~5 kDa to 49 kDa in respective experiments. Inhibition by swainsonine resulted in decreases of M_r for FcεRI(α) of ~6 kDa in experiment 1 and ~4 kDa in experiment 2. $FcεR_L$ decreased by ~3 kDa and ~2 kDa in respective experiments producing a band in the area of 52 kDa. There was an indication 1-deoxymannojirimycin and swainsonine treated FcεRI(α) appears as a doublet on SDS-PAGE (Figure 2.6; DMJ lane 1, 2, and SW lane 1, 2). All values in Table 2.1 represent the average M_r of the doublet in the sample in which it appeared. It is also apparent from Figure 2.6 that the receptor loss upon growing cells in the presence of castanospermine can be attributed primarily to FcεRI(α) since only a faint band appears

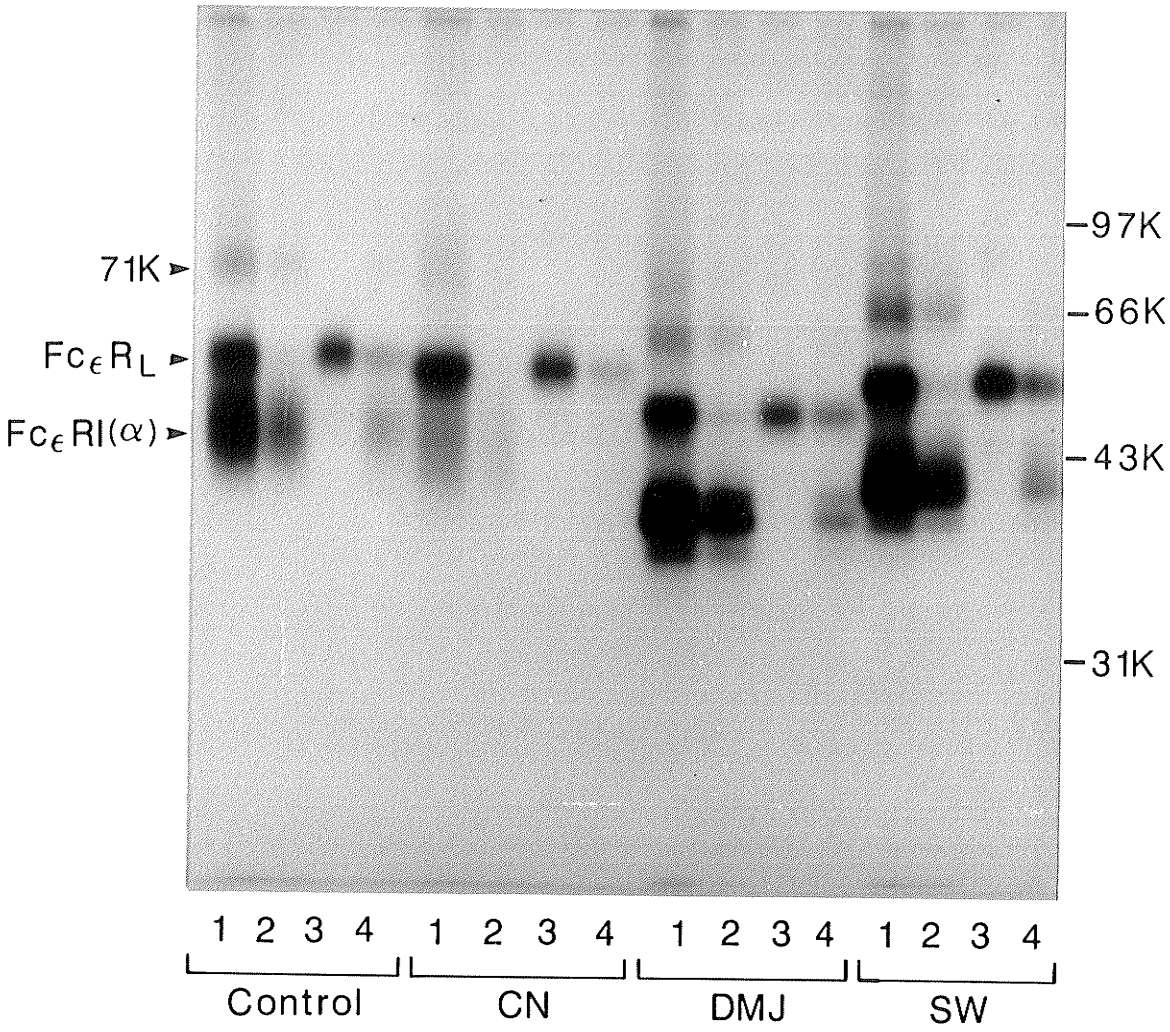


Figure 2.6 SDS-PAGE analysis of [^{125}I]-iodine labelled receptors from RBL-CA10 cells isolated by: (1) IgE-Sepharose; (2) IgE, anti-IgE-Sepharose; (3) anti-FcϵR_L, Protein A-Sepharose; or (4) IgE-Sepharose of effluent from (2) following no incubation or incubation for 48 hours with 0.10 mM castanospermine (CN), 0.25 mM 1-deoxymannojirimycin (DMJ) or 2.6 μM swainsonine (SW).

Table 2.1 Relative molecular mass (M_r) and change in relative molecular mass (ΔMr) (shaded) of $F\varepsilon R_L$ and $F\varepsilon RI(\alpha)$ following treatment with processing inhibitors (values for experiment number 2 were derived from SDS-PAGE markers on a separate gel similar to those shown in Figure 2.1 from which the experiment number 1 values were obtained).

		M_r ΔM_r (kDa)					
		Glycosylation Processing Inhibitor					
Experiment Number		None	Castano-permine		Deoxyman-nojirimycin		Swainsonine	
1	$F\varepsilon R_L$	55	54	1	49	6	52	3
	$F\varepsilon RI(\alpha)$	47	45	2	42	5	41	6
2	$F\varepsilon R_L$	54	53	1	49	5	52	2
	$F\varepsilon RI(\alpha)$	45	43	2	38	7	41	4

in the preparation isolated by means of IgE and anti-IgE. However, the $Fc\epsilon R_L$ band (as detected by anti-$Fc\epsilon R_L$) is quite strong. It should also be noted that 71K was also affected by the inhibitors. The changes in its M_r roughly paralled those of the other $Fc\epsilon R$'s.

Mycoplasma Infection of Inhibitor Treated Cells

Due to the possible association between mycoplasma infection and cellular glycoproteins, the potential alterations in infection and subsequent 71K induction following modulation of oligosaccharides with the processing inhibitors was investigated. RBL-CA10.7 cells were infected with mycoplasma following pretreatment with either of the three processing inhibitors castanospermine, 1-deoxymannojirimycin, or swainsonine. In all cases the induction and appearance of the 71K receptor on SDS-PAGE coincided with the chronically infected RBL-CA10 (Figure 2.7). As expected, the 71K band of RBL-CA10.7 was not as prominent as in the case of the chronically infected RBL-CA10 due to the latter's long term established infection.

Endoglycosidase Digestion of Receptor Glycoproteins

In order to examine effects of inhibitors on the oligosaccharide portion of the receptors, sensitivity to the endoglycosidases endoglycosidase H and N-glycanase was examined. The relative resistance or sensitivity of

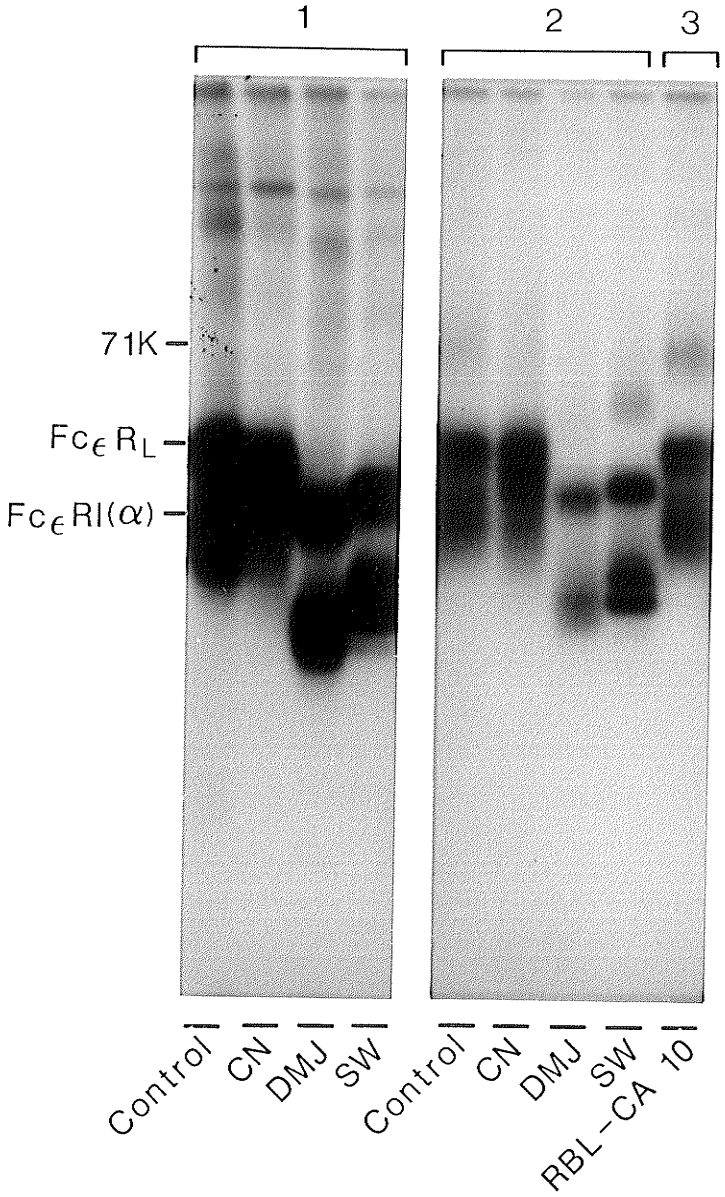


Figure 2.7 RBL-CA10.7 incubated 48 hours with or without either 0.10 mM castanospermine (CN), 0.25 mM 1-deoxymannojirimycin (DMJ), or 2.6 μM swainsonine (SW) (1), followed by a 24 hour incubation with mycoplasma supernatant (2). Also shown is chronically infected RBL-CA10 as a control for the location of 71K (3). Receptors from both cells were [^{125}I]-iodine labelled, isolated by IgE-Sepharose and analyzed by SDS-PAGE.

oligosaccharide structures to the endoglycosidases is shown in Figure 2.8. Endoglycosidase H has a substrate specificity for high mannose, N-linked, oligosaccharides. The enzyme cleaves at the chitobiose unit of the inner core between Man β1-4 GlcNac provided the N-linked oligosaccharide possesses at least three mannose residues and the α1-6 mannose arm has at least one other mannose attached to it (Maley *et al.*, 1989). Hybrid oligosaccharides are also suitable substrates and readily hydrolyzed. Complex oligosaccharides are completely resistant to hydrolysis and fucose in the core is believed to be one of the rate limiting factors (Tarentino & Maley, 1975).

N-glycanase has a much broader specificity than endoglycosidase H as it has the ability to cleave high mannose as well as complex oligosaccharides, provided both the amino and carboxyl groups of the asparagine residues are peptide bonded to another amino acid (Figure 2.8) (Tarentino *et al.*, 1985).

The enzymatic hydrolysis of denatured FcϵR$_L$ and FcϵRI(α) under reducing conditions using endoglycosidase H resulted in only a slight decreased M_r for each receptor (Figure 2.9). In each of two independent experiments, the M_r of either receptor decreased by only 2 kDa as calculated from the low molecular weight standards (Table 2.2). Considering the high content of carbohydrate known to be present on FcϵRI(α), it as well as FcϵR$_L$, appear virtually resistant to this enzyme. However, following processing inhibitor treatment of the RBL-CA10 cells, all FcϵR's were converted, in

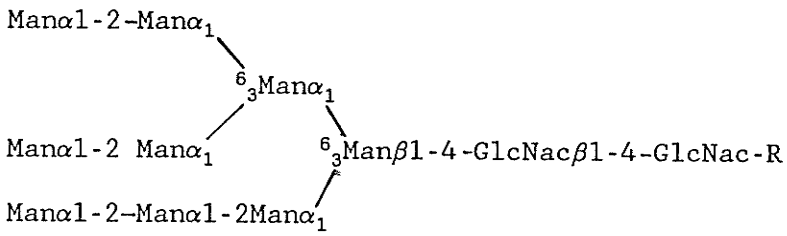
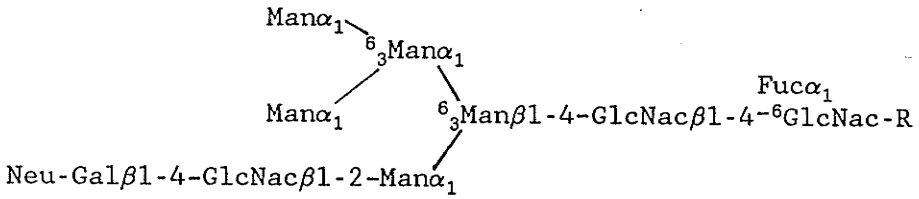
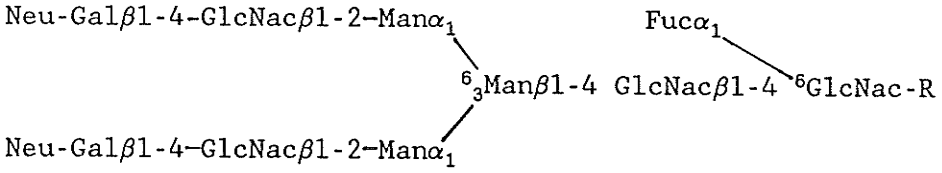
Structure	Sensitivity Endo-H	N-gly
1. High mannose		
	+	+
2. Hybrid		
	+	+
3. Complex		
	-	+

Figure 2.8

Oligosaccharide structure and relative sensitivity to endoglycosidase digestion by endoglycosidase H (endo H) or N-glycanase (N-gly).

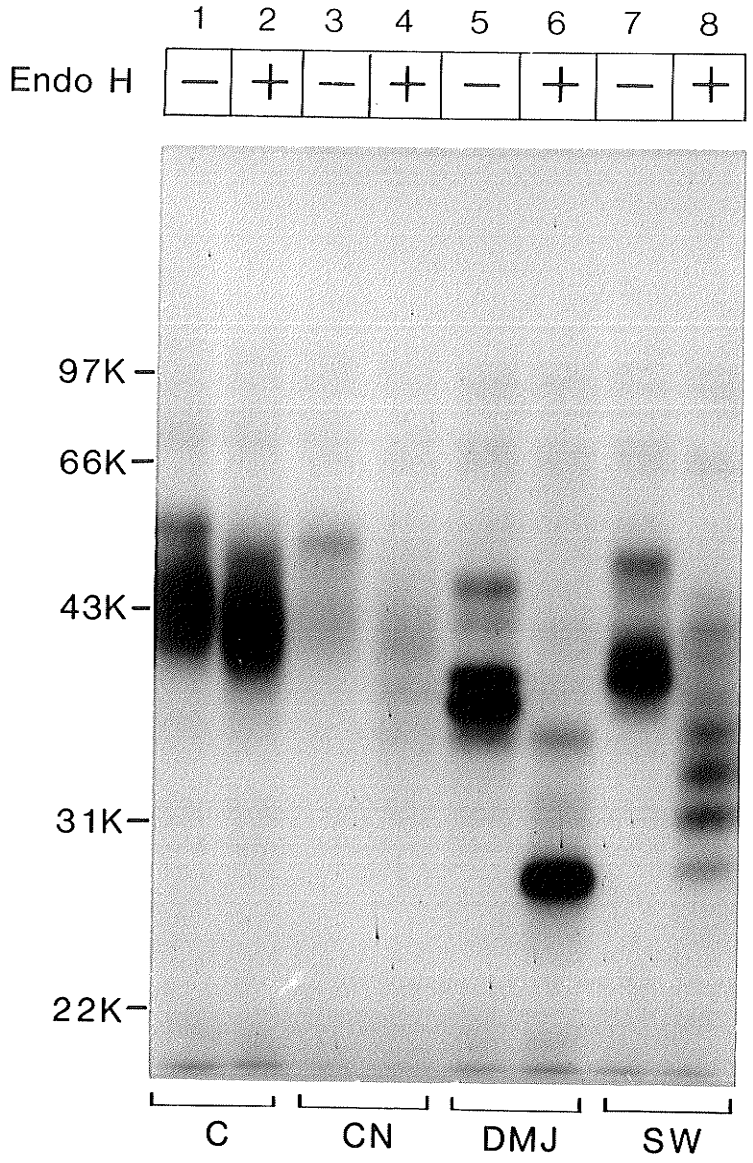


Figure 2.9 SDS-PAGE analysis of [^{125}I]-iodine labelled receptors, isolated with IgE-Sepharose, and digested with 0.025 U/ml endoglycosidase H, following a 48 hour incubation of RBL-CA10 cells with no inhibitor (C, control) or with 0.10 mM castanospermine (CN); 0.25 mM 1-deoxymannojirimycin (DMJ); or 2.6 μM swainsonine (SW).

Table 2.2 Effect of endoglycosidase H digestion on the relative molecular mass (M_r) and resulting change in M_r (ΔM_r) (shaded) of processing inhibitor treated or untreated Fcε receptors (values for experiment number 2 calculated from SDS-PAGE markers such as those shown in Figure 2.9 from which the experiment number 1 values were obtained).

Exp't. No.		M_R ΔM_R (kDa)							
		Glycosylation Processing Inhibitor							
		Control (no endo H)	None		Castano-spermine		Deoxymann-ojirimycin		Swainsonine	
1	FcεR$_L$	55	53	2	44	11	36	19	45	10
	FcεRI(α)	47	45	2	40	7	28	19	37-28	10-19
2	FcεR$_L$	54	52	2	45	9	38	16	42	12
	FcεRI(α)	45	43	2	38	7	29	16	38-29	7-16

various degrees, to enzyme sensitive oligosaccharide structures. Two bands were produced by enzymatic cleavage of 1-deoxymannojirimycin treated receptors (Figure 2.9, lane 6). The band with an approximate M_r of 37 kDa was identified as $Fc\epsilon R_L$ since it was isolated by anti-$Fc\epsilon R_L$ in a separate experiment (Figure 2.10). This experiment helped to identify the 28.5 kDa band of Figure 2.9, lane 6 as $Fc\epsilon RI(\alpha)$. The changes in the M_r of $Fc\epsilon RI(\alpha)$ and $Fc\epsilon R_L$ were virtually identical being 19 kDa in one experiment and 16 kDa in another (see also Table 2.2). Receptors from swainsonine treated cells were differentially resistant to endoglycosidase H and produced a multiple banding pattern ranging in M_r from 37 kDa - 28 kDa for both receptors. The banding pattern showed more bands in the lower M_r range as the concentration of endoglycosidase H increased (Figure 2.11). An increase in the concentration of endoglycosidase H had no effect on receptor profiles of receptors from untreated, castanospermine or 1-deoxymannojirimycin treated cells. A similar multiple banding pattern was also occasionally seen in some experiments of 1-deoxymannojirimycin treated cells (Figure 2.10) for unknown reasons. Cells treated with castanospermine, once again, yielded low receptor recovery and although endoglycosidase H caused some reduction of M_r, this was not nearly as pronounced as in the case of 1-deoxymannojirimycin and swainsonine treated cells.

N-glycanase exhibits no substrate specificity and can cleave all N-linked oligosaccharides regardless of structure (Figure 2.8) (Tarentino *et al.*, 1985). Both $Fc\epsilon R_L$ and $Fc\epsilon RI(\alpha)$ of RBL-CA10 are sensitive to hydrolysis by

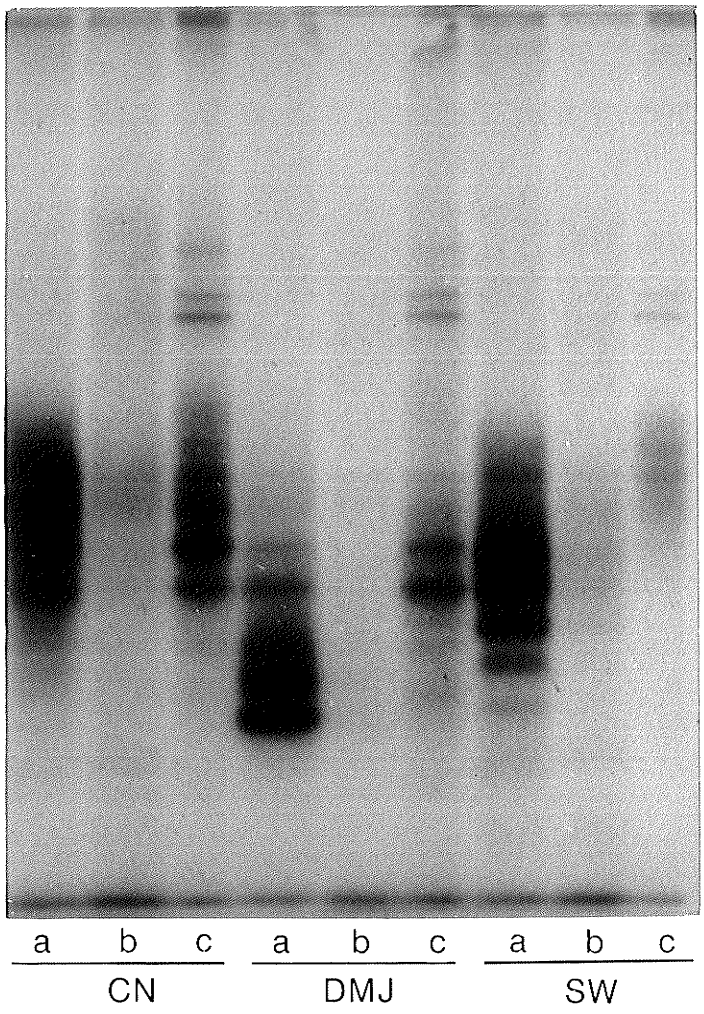


Figure 2.10 Differential isolation of 0.10 mM castanospermine (CN), 0.25 mM 1-deoxymannojirimycin (DMJ) or 2.6 μM swainsonine (SW) treated receptors from RBL-CA10, labelled with [^{125}I]-iodine, isolated by (a) IgE-Sepharose; (b) IgE, anti-IgE-Sepharose; (c) anti-FcϵR_L, Protein A-Sepharose, digested with 0.025 U/ml endoglycosidase H, and analyzed by SDS-PAGE.

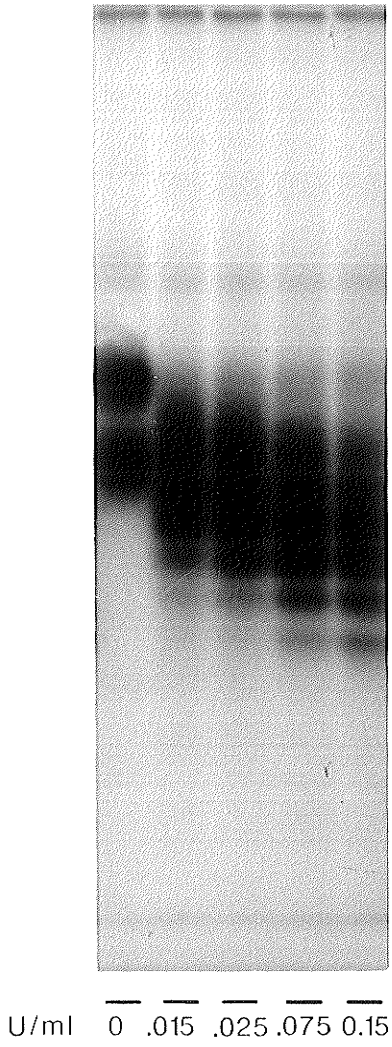


Figure 2.11 [^{125}I]-iodine labelled receptors from RBL-CA10 cells incubated 48 hours with 2.6 μM swainsonine (SW) isolated using IgE-Sepharose then digested with 0, 0.025, 0.075, or 0.15 U/ml endoglycosidase H and analyzed by SDS-PAGE.

N-glycanase as shown in differential binding experiments (Figure 2.12). Indeed the fragments produced were practically identical in M_r to those previously found when receptors from 1-deoxymannojirimycin treated cells were subjected to endoglycosidase H hydrolysis. The M_r values of 34 kDa and 37.9 in two separate experiments were obtained for $Fc\epsilon R_L$, the M_r 28 and 27.2 kDa for $Fc\epsilon RI(\alpha)$ were calculated as shown in Table 2.3. These receptors are believed to be completely free of N-linked oligosaccharides and their molecular weight decreased by as much as 24.8 kDa. On this basis a percent composition of N-linked oligosaccharide can be calculated from the difference in control M_r and M_r following N-glycanase treatment. As shown in Table 2.3, the numbers are quite large with an experimental average of 43% for $Fc\epsilon RI(\alpha)$ and 38% for $Fc\epsilon R_L$. No alteration in the receptor M_r were seen at various N-glycanase concentrations (data not shown).

Receptors isolated from HRMC 9 and HRMC 5.1.6 were also digested with N-glycanase and analyzed by SDS-PAGE (Figure 2.12). These lines were selected because of differences in their $Fc\epsilon R$ in M_r from those of RBL-CA10 or RBL-CA10.7 cells. Thus, $Fc\epsilon RI(\alpha)$ and $Fc\epsilon R_L$ of HRMC 9 have somewhat higher M_r, while those of HRMC 5.1.6 are slightly lower than those of RBL-CA10 (Table 2.4). The M_r of $Fc\epsilon RI(\alpha)$ following deglycosylation was 28 kDa for each hybrid mast cell line. The M_r values for $Fc\epsilon R_L$ were surprisingly different. Deglycosylated $Fc\epsilon R_L$ of HRMC 9 yielded a M_r of ~43 kDa compared to ~38 kDa for HRMC 5.1.6 and RBL-CA10 (Table 2.4).

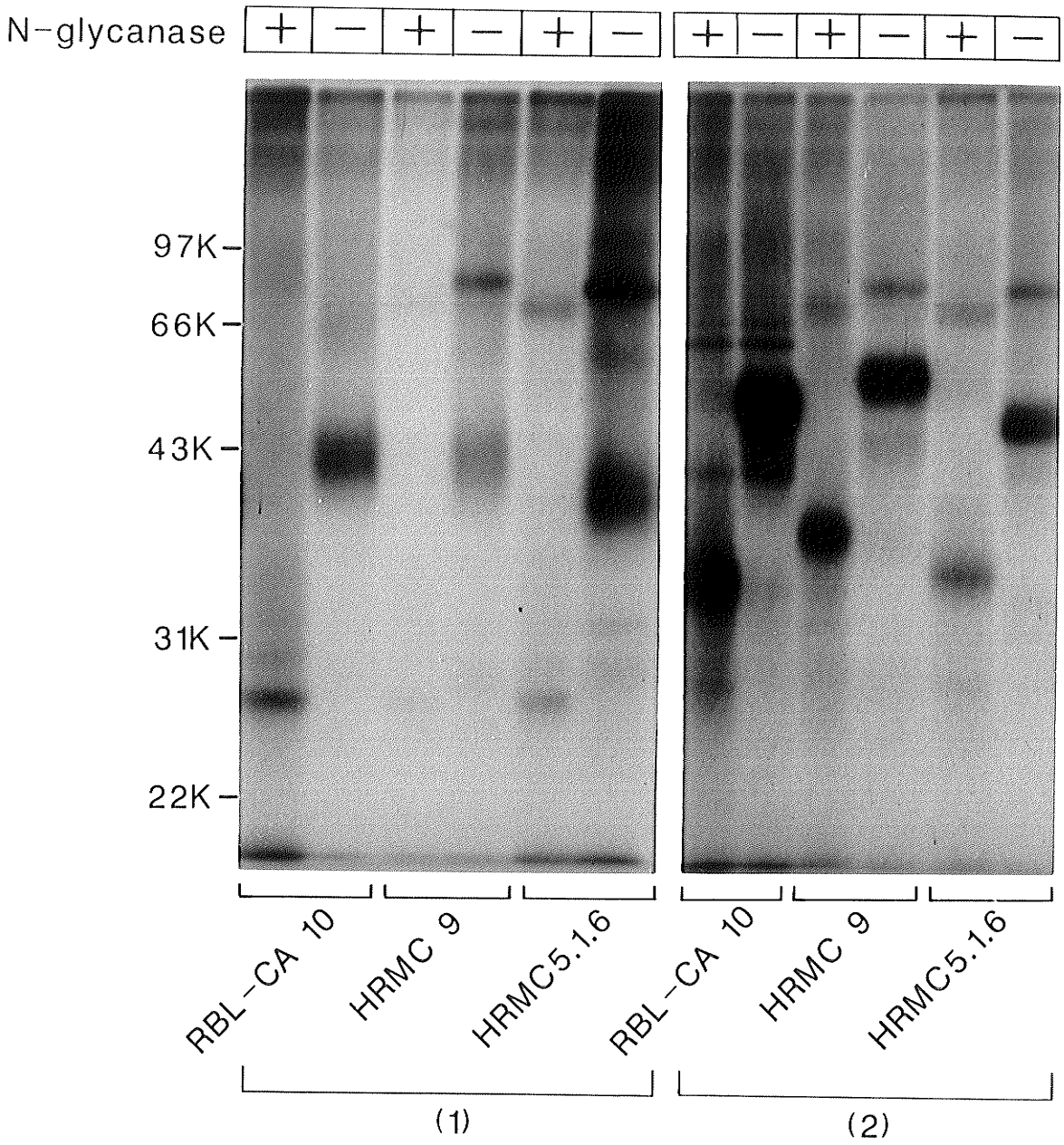


Figure 2.12 Isolation of [^{125}I]-iodine labelled receptors using (1) IgE, anti-IgE-Sepharose and (2) anti-FcϵR_L, Protein A-Sepharose from cell lines RBL-CA10, HRMC 9, HRMC 5.1.6 followed by digestion with 5 U/ml N-glycanase and analysis by SDS-PAGE.

Table 2.3 Relative molecular mass (M_r), change in relative molecular mass (ΔM_r) (shaded) and percent carbohydrate composition of $Fc\varepsilon R_L$ and $Fc\varepsilon RI(\alpha)$ of RBL-CA10 following digestion with N-glycanase (values for experiment 2 are calculated from SDS-PAGE standards identical to those used for the Figure 2.12 autoradiograph to obtain experiment 1 values).

Experiment Number		Control	N-glycanase		% Carbohydrate Composition
1	$Fc\varepsilon R_L$	56.8	34.0	22.8	40.1
	$Fc\varepsilon RI(\alpha)$	45.0	28.0	17.0	37.8
2	$Fc\varepsilon R_L$	60.0	37.9	22.1	36.8
	$Fc\varepsilon RI(\alpha)$	52.0	27.2	24.8	47.7

Table 2.4 Relative molecular mass (M_r), change in relative molecular mass (ΔM_r) (shaded) of FcεR_L and FcεRI(α) from HRMC 9 and HRMC 5.1.6 following digestion with N-glycanase (values are calculated from the SDS-PAGE standards of the autoradiograph shown in Figure 2.12).

		M_r		ΔM_r
Cell Line		Control	N-glycanase	
HRMC9	FcεR_L	64.0	43.0	21.0
	FcεRI(α)	55.0	28.0	27.0
HRMC 5.1.6	FcεR_L	58.2	38.0	20.2
	FcεRI(α)	47.0	28.0	19.0

DISCUSSION

The biosynthesis of N-linked oligosaccharides involves the transfer of the oligosaccharide $Glc_3Man_9GlcNac_2$ from dolichyl pyrophosphate to protein and its subsequent processing to yield high mannose, hybrid or complex structures with the same inner core (Hubbard & Ivatt, 1981; Schachter *et al.*, 1982, Kornfeld & Kornfeld, 1985). The biosynthetic factors which determine whether an oligosaccharide remains high mannose or becomes processed to hybrid or complex structure are not known with certainty. However, some probable factors that influence their processing are the position of glycosylation site in the amino acid sequence (Pollack & Atkinson, 1983); conformation of polypeptide backbone; host, species and tissue dependent factors for example the concentration of glycosyltransferases and glucosidases; specific neighboring amino acids, and quaternary structure. Glycosylation processing inhibitors act by blocking the action of the various glycosidases which function to remove the terminal carbohydrate residues from N-linked oligosaccharides (Elbein, 1987).

1-Deoxymannojirimycin blocks the complete processing of oligosaccharides to complex structures by inhibiting α-D-mannosidase I activity (Legler and Julich, 1984). Swainsonine also blocks complex oligosaccharide chain formation by inhibition of mannosidase II (Tulsiani *et al.*, 1982). Treatment of RBL-CA10 and RBL-CA10.7 with these processing inhibitors caused the M_r of both $Fc\epsilon R_L$ and F to decrease but the changes brought about by these inhibitors were not identical (Table 2.1). There may be several reasons for

the decrease in M_r of 1-deoxymannojirimycin and swainsonine treated cells. The endoglycosidase H resistance of the Fcϵ receptors suggests that they are primarily composed of complex oligosaccharide. 1-Deoxymannojirimycin treatment would then represent a conversion of the N-linked oligosaccharides of the Fcϵ receptors of RBL-CA10 and RBL-CA10.7 from complex structures to high mannose structures. Changes in M_r coinciding with such an oligosaccharide structure conversion can be calculated. Assuming the naturally existing complex carbohydrate structures contain fucose, are sialylated, nonbisected, and very highly branched i.e., tetraantennary (found naturally occuring on many glycoproteins such as on ovotransferrin (Dorland *et al.*, 1979)), or long poly-N-acetyllactosamine chains such as those found on erythrocytes (Fukuda *et al.*, 1979)), it would minimally be composed of: 6 N-acetyl- glucosamine residues (0.221 kDa each); 4 sialic acid residues (0.309 kDa each) ; 3 mannose residues (0.180 kDa each); and 1 fucose residue (0.180 kDa). This therefore would yield 14 carbohydrate residues per glycosylation site, and contributing a total molecular mass from the sum of the residue number multiplied by the residue molecular mass: (6 x 0.221 kDa) + (4 x 0.309 kDa) + (3 x 0.180 kDa) + (1 x 0.180 kDa) = 3.282 kDa/N-glycosylation site. The total molecular weight of the carbohydrate that contributes to the entire FcϵRI(α) molecule is calculated by multiplying this value by seven which represents the maximum number of potential glycosylation sites. Therefore 7 x 3.282 kDa/site = 22.974 kDa/mole FcϵRI(α). Similarly, the contribution per mole of FcϵRI(α) of a high mannose structure can be calculated. The final values must be calculated as a range defined by the

number of mannose residues present since high mannose oligosaccharides containing from five to nine mannose residues have often been found to occur naturally (Hubbard and Ivatt, 1981). The smallest high mannose strucutre would contain 7 residues, these are: 5 mannose residues (0.180 kDa each); 2 N-acetylglucosamine residues (0.221 kDa each). The maximum size would be 11 residues, including: 9 mannose residues (0.180 kDa each); 2 N-acetylglucosamine residues (0.221 kDa each). The range as total molecular mass (kDa)/mole FcϵRI(α) would therefore be: 7{(5 x 0.180 kDa) + (2 x 0.221 kDa)} to 7{(9 x 0.180 kDa) + (2 x 0.221 kDa)} which is 9.394 kDa/mole to 14.434 kDa/mole. Therefore total molecular mass (kDa)/mole FcϵRI(α) is 9.394 kDa/mole to 14.434 kDa/mole. Similar calculations cannot be carried out for FcϵR_L since the number of potential N-glycosylation sites on FcϵR_L is not known. In addition, since the number of the seven potential sites on FcϵRI(α) which are in fact glycosylated is not known, these values must be regarded simply as good estimates of the total carbohydrate composition.

Using the calculated values, the difference in molecular mass (M) resulting from a conversion of a complex structure to a high mannose structure equals the difference of the final total kDa/mole FcϵRI(α) of high mannose oligosaccharide and initial total kDa/mole FcϵRI(α) of complex oligosaccharide:

Final M (kDa/mole) - Initial M (kDa/mole)	=	Δ M (kDa/mole)
(14.434 kDa/mole - 22.974 kDa/mole)	=	-8,540 kDa/mole
(9.394 kDa/mole - 22.974 kDa/mole)	=	-13,580 kDa/mole

Therefore it is predicted that with a conversion from complex to high mannose, the total molecular mass of carbohydrate on FcεRI(α) would decrease from 8.540 to 13.580 kDa/mole. The Δ M_r detected by SDS-PAGE for 1-deoxymannojirimycin was determined to be 5-7 kDa.

These calculations can also be made for swainsonine. Swainsonine treatment results in a conversion from complex to hybrid structures rather than high mannose as with 1-deoxymannojirimycin. A hybrid structure which is triantennary, nonbisected, sialylated and contained fucose would be composed of 12 residues: 5 mannose residues (0.180 kDa each); 4 N-acetylglucosamine residues (0.221 kDa each), 2 sialic acid residues (0.309 kDa each); and 1 fucose residue (0.180 kDa). Therefore the total molecular mass of the oligosaccharide is: 7 {(5 x 0.180 kDa) + (4 x 0.221 kDa) + (2 x 0.309 kDa) + (1 x 0.180 kDa)} = 18.074 kDa/mole FcεRI(α). The predicted change in molecular mass resulting from the conversion of complex to hybrid structure following the treatment with swainsonine is calculated as: 18.074 kDa/mole - 22.974 kDa/mole = -4.900 kDa/mole. Therefore a conversion such as this would result in a decrease in the molecular mass of FcεRI(α) by 4.900 kDa. The Δ M_r determined by SDS-PAGE for swainsonine treated FcεRI(α) is a decrease of 4-6 kDa/mole.

Similar changes in carbohydrate content can also be calculated to predict the action of castanospermine. This inhibitor acts on the glycosidases blocking their glucose removing activities and thereby preventing the

formation of hybrid and complex oligosaccharide. Following treatment with castanospermine, it is expected that one to three glucose residues will be present on the oligosaccharide. This is because the first two glucose residues are removed by one set of glycosidases and the third is removed by another set of glycosidases. Each set may have different sensitivity to inhibition by the castanospermine. Although a pathway may exist in some cells in which one or two mannose residues are removed from an oligosaccharide retaining one or more glucose residues (Hubbard and Robbin, 1979; Kornfeld *et al.*, 1978), it is believed to be minor and for the purposes of these calculations will be ignored.

The structure resulting from castanospermine treatment would contain 12 - 14 residues composing: 9 mannose residues (0.180 kDa each); 1-3 glucose residues (0.180 kDa each); and 2 N-acetylglucosamine residues (0.221 kDa each). The total molecular mass/mole FcϵRI(α) is 7 {(9 x 0.180) + (2 x 0.221) + (1 x 0.180 kDa)} to 7 {(9 x 0.180) + (2 x 0.221) + (3 x 0.180 kDa)} which equals 15.694 kDa/mole to 18.214 kDa/mole. The predicted change in molecular mass resulting from castanospermine treatment is: 18.214 kDa/mole - 22.974 kDa/mole to 15.694 kDa/mole - 22.974 which equals the range of -4.760 kDa/mole to -7.280 kDa/mole. The decrease in M_r as determined by SDS-PAGE from castanospermine treated cells is 2 kDa/mole.

The results which were actually obtained did not yield the predicted values exactly but they nevertheless followed a trend. The largest change observed was for 1-deoxymannojirimycin treated FcϵRI(α) of 5-7 kDa fol-

lowed by swainsonine with 4-6 kDa and finally castanospermine with a decrease of 2 kDa. The changes in M_r, determined on the basis of SDS-PAGE, are overall slightly smaller than the calculated values. This may be for several reasons. Not all seven potential N-glycosylation sites may , in fact, be glycosylated as mentioned earlier. This would result in a slight overestimate of the true amount of oligosaccharide present. The total carbohydrate composition if six sites are glycosylated with complex oligosaccharide and one site high mannose (endoglycosidase H sensitive) would be 45.1%. At first glance, the calculated carbohydrate compositions from N-glycanase treatment, in the range of 37.8% - 47.7% (Table 2.3) would perhaps argue against not all sites being glycosylated but it must be kept in mind that this range is, most likely, too high because of the interaction of the oligosaccharide with the polyacrylamide gel (Ferguson, 1964; Weber & Osborn, 1975).

It must also be mentioned that the inhibitor treatment itself may have resulted in the failure to glycosylate the polypeptide at positions normally occupied by an oligosaccharide chain. This could potentially occur if inadequate glycosylation or converted oligosaccharide chains influenced the secondary, tertiary or quaternary folding of the receptor in such a way to cause a site to become inaccessable to glycosyltransferases.

The magnitude of the decrease in M_r following inhibitor treatment, as well as any discrepancies between calculated and actual values, may be a reflection of the relative efficiency of the inhibitor enzymes at altering

the oligosaccharide structure at each of the various positions along the polypeptide chain. Indeed, oligosaccharides at different glycosylation sites may have been affected differently and some may not have been altered at all.

Considering the large number of potential glycosylation sites present on the α subunit of FcϵRI, and all the possible oligosaccharide structures which may be present, it is difficult to determine the precise explanation for the decrease in M_r.

Reports exist in the literature of a decrease in molecular weight of glycoproteins containing complex oligosaccharides following treatment with 1-deoxymannojirimycin, swainsonine or castanospermine. A decrease in the ϵ-chain M_r was observed when a murine IgE monoclonal antibody (anti-β-lactoglobulin) producing hybridoma was treated with castanospermine or swainsonine (Granato and Neeser, 1987). The oligosaccharide chain of the monoclonal has been characterized as complex-type with terminal galactose residues (Montreuil, 1980). Two forms of α_1-antitrypsin exist, a 51 kDa intracellular form and 56 kDa secreted form. The 56 kDa secreted form has complex oligosaccharides and is endoglycosidase resistant (Gross *et al.*, 1990). When cells are treated with swainsonine only the 56 kDa secreted form shows a decrease in M_r, the 51 kDa form with high mannose oligosaccharides has the same M_r. Similarly, a decrease in M_r for endoglycosidase H resistant m chains from 1-deoxymannojirimycin treated hybridoma cells was reported (Fuhrmann *et al.*, 1984).

As pointed out above, the changes in M_r were smallest for castanospermine treated cells, they are also the most difficult to interpret since castanospermine treatment caused decreased receptor expression, particularly for FcϵRI(α). Expression of the receptors following treatment with 1-deoxymannojirimycin or swainsonine was normal. Carbohydrate has been shown to play a role in Fcϵ receptor expression. The antibiotic tunicamycin which blocks formation of N-acetylglucosamine - lipid intermediates thereby preventing its transfer to the polypeptide. It causes the decrease in expression of Fcϵ receptor by as much as 67% (Pecoud *et al.*, 1981). Intact IgE secretion from IR-162 immunocytoma cells is also completely blocked by tunicamycin treatment with protein accumulation in the RER (Hickman *et al.*, 1977). Hickman and coworkers (1977) proposed several hypotheses for the blockage of protein secretion and synthesis by tunicamycin. One of these is that the lack of carbohydrate causes aggregation and altered mobility of the protein due to its modulated physicochemical properties. Using the three different processing inhibitors, it was possible to determine at which step the expression is restored since castanospermine inhibits the first set of processing enzymes, the glucosidases (Saul *et al.*, 1983). It therefore appears that it is not simply because of the lack of carbohydrate that receptor expression is blocked. The possibility exists that the FcϵRI(α) seen on the cell surface are actually a subpopulation of receptors unaltered by the castanospermine. The one to three residues of glucose present on the receptors which have been altered by castanospermine, may have prevented their expression therefore accounting for the observed decrease in

expression of FcϵRI(α). It has been shown that the presence of the terminal glucose may alter the normal pathway that the receptor takes within the cell, since glucose is directly involved in the cellular trafficking of some receptors (Parodi *et al.*, 1983). However, in another study of the murine monoclonal antibody IgE, secretion was not blocked by treatment of the hybridoma's with castanospermine (Granato and Neeser, 1987) nor was expression of FcϵR_L blocked by castanospermine. Therefore the glucose on the receptors may be interfering with the association of the α, β or γ_2 subunits. Formation of this complex is mandatory for insertion of this receptor into the membrane (Blank *et al.*, 1989).

The decreased recovery of FcϵRI(α) upon treatment of cells with castanospermine could, of course, also be explained by a drastic decrease in the affinity of this receptor for IgE. However, the binding of FcϵRI(α) to IgE appears to be less carbohydrate dependent than the IgG-Fcγ receptor interaction. Thus, carbohydrate deficient monoclonal IgG cannot activate complement nor bind to Fcγ receptors of macrophage and induce cellular cytotoxicity (Nose and Wigzell, 1983) while non-glycosylated IgE and nonglycosylated FcϵRI(α) are capable of interacting with their normal counterparts, i.e. normal FcϵRI(α) and IgE respectively (Kulczycki and Vallina *et al.*, 1981; Pecoud *et al.*, 1981). Moreover, as pointed out above, changes in the carbohydrate of FcϵRI(α) due to 1-deoxymannojirimycin or swainsonine treatment of cells had no affect on the binding of this Fcϵ receptor to IgE. Despite this, the possibility that castanospermine treatment abrogated the binding of FcϵRI(α) to IgE

cannot be completely eliminated. Gaveriaux and Loor (1987) reported that RBL cells cultured in the presence of either glucosidase inhibitors deoxynojirimycin or castanospermine exhibit a decreased capacity to bind monoclonal IgE. However, decreased receptor expression would have produced the same effect.

The discussion so far has focussed on FcϵRI(α) since the number of glycosylation sites are known and it was therefore possible to make some estimates about the potential changes in ologosaccharide due to treatment of RBL cells with processing inhibitors. A similar analysis of FcϵR_L was not possible since the amino acid sequence of this molecule is unknown.

The changes induced in this receptor were, in fact, very similar to those seen in the case of FcϵRI(α), only that the decreases were somewhat smaller. Once again 1-deoxymannojirimycin induced the most pronounced delta M_r, followed by swainsonine and castanospermine. It would, therefore appear reasonable to assume that the type of oligosaccharides present on this receptor resemble those on FcϵRI(α).

The results obtained with FcϵR from castanospermine treated cells are difficult to interpret since, as pointed out before, the majority of receptors, particularly FcϵRI(α), may not have been expressed on the cell surface.

The carbohydrate moiety of FcϵRI(α) has been implicated in the induc-

tion of the 71K receptor by mycoplasma since carbohydrates have been identified as target structures for mycoplasmas (Bredt *et al.*, 1981; Kahane *et al.*, 1981). The fact the 71K band did not disappear following treatment of infected RBL-CA10 cells and did appear when RBL-CA10.7 was infected with mycoplasma after treatment with the processing inhibitors, would suggest that the infection of the cells in general and the induction of 71K in particular by mycoplasma is probably not critically dependent on a precise carbohydrate structure or composition. Alternatively the processing inhibitors may have left critical oligosaccharide structures intact, so as to still allow interaction with mycoplasma. Despite decreases in receptor expression by castanospermine treatment, 71K could still be induced.

Endoglycosidase H has been used widely to probe the relative accessibility of high mannose units of various glycoproteins. Endoglycosidase H cleaves from the polypeptide chain, oligosaccharides possessing high mannose, or hybrid structures only and cannot remove complex oligosaccharides. An inverse relationship has been postulated between extent of oligosaccharide processing and susceptibility to endoglycosidase H cleavage (Hsieh *et al.*, 1983; Trimble *et al.*, 1983; Natowicz *et al.*, 1982). That is to say, it is believed that if the oligosaccharide is not hindered by factors such as tertiary protein folding or noncovalently associated subunits it will be readily processed to a complex carbohydrate structure which is endoglycosidase H resistant.

Thus, to determine the nature of the carbohydrate chains present on the Fcϵ receptors and what changes had been induced by the inhibitors, they were treated with endoglycosidase H. We found that FcϵRI(α) and FcϵR_L are similar in their resistance to endoglycosidase H (Table 2.2). The reduction in M_r by ~2 kDa for both, would be equivalent to the molecular weight of a single high mannose or hybrid structure. Therefore both receptors may have one site occupied by a high mannose oligosaccharide structure and the remaining sites are complex, perhaps, with an α1-6 fucose linked to the core. Endoglycosidase H treatment of 1-deoxymannojirimycin and swainsonine treated cells produced dramatic changes in the M_r of both receptors. This result indicates that this processing inhibitor had significantly, if not completely, inhibited the processing to complex oligosaccharides on both receptors. Treatment of cells with swainsonine was not as effective at inhibiting complex oligosaccharide processing since multiple banding of FcϵR_L and FcϵRI(α) was observed following endoglycosidase H treatment. Although the band on SDS-PAGE, following inhibitor treatment, is narrower than control untreated cells, it is evident from the endoglycosidase H results that the microheterogeneity has been decreased but not eliminated. Multiple bands were produced following endoglycosidase H treatment which indicates that the starting population was not homogeneous and some microvariability was indeed present. The heterogenous population therefore exhibited differential susceptibility to endoglycosidase H creating distinct narrow bands.

Appearance of the doublet bands of FcϵRI(α) and not FcϵR_L upon SDS-PAGE

analysis following inhibitor treatment of cells (Figures 2.1 and 2.3) indicates that FcϵRI(α) may exhibit more N-linked microheterogeneity than FcϵR_L. This microheterogeneity may be site specific or polypeptide specific. Variation which exists within one polypeptide from one of its N-glycosylation sites to another constitutes a site specific microheterogeneity. For example, the first Asn-X-Ser(Thr) tripeptide, relative to the -NH_2 terminal of polypeptide, may carry an oligosaccharide which is predominately a fully processed, complex structure, while at the sixth Asn-X-Ser(Thr) site a high mannose structure may predominate, and the seventh Asn-X-Ser(Thr) site may remain nonglycosylated the majority of the time. It appears that the determining factors may be, at least partially, due to the primary, secondary, and even tertiary structure of the polypeptide since a favorable conformation must be attained (Kornfeld and Kornfeld, 1985). Polypeptide microheterogeneity arises from the failure of the glycosylation processing enzymes of the cell to treat each polypeptide molecule in the same manner despite each having an identical primary sequence. This results in molecules which, by virtue of differences in their oligosaccharide composition, are unique. In addition, the distribution of these molecules may shift at different times because of changes in the cell, such as the levels of oligosaccharyltransferase (Carson *et al.*, 1981), or changes in the cells growth environment.

In order to determine if the fragments produced from endoglycosidase H hydrolysis of 1-deoxymannojirimycin treated RBL cells were free of

N-linked oligosaccharides and to determine the total of N-linked carbohdrate present, another enzymatic digestion was carried out using N-glycanase. N-glycanase can cleave all types of N-linked oligosaccharide from the polypeptide regardless of structure. This enzyme was found to reduce the M_r of both receptors to about the same degree as a combination of endoglycosidase H hydrolysis and DMJ treatment of cells. In experiment number 2 (Table 2.3) the Δ M_r due to N-glycanase treatment was significantly greater than that obtained as a result of 1-deoxymannorjirimycin and endoglycosidase H treatment (Table 2.2). However, in this experiment, all the M_r for the glycosylated forms of the receptors were considerably higher than those obtained by others in this laboratory (Conrad and Froese 1978a). This would indicate that 1-deoxymannojirimycin and endoglycosidase treatment give rise to Fcϵ receptors practically free of N-linked oligosaccharide.

In a previous study, two bands of 38 kDa and 33 kDa were reported following tunicamycin treatment of RBL cells and isolation of receptors was by means of IgE-Sepharose (Hempstead *et al.*, 1981). The 38 kDa band was believed to be FcϵRI(α) devoid of N-linked oligosaccharides. Based on our results (Table 2.3) and considering that the cell RBL_{StL} used for these experiments may have expressed little FcϵRI and much FcϵR_L (Froese *et al.*, 1981), we believe this band was most likely deglycosylated FcϵR_L. It is interesting to note that N-glycanase treated FcϵRI(α) and FcϵR_L yield rather narrow bands upon SDS-PAGE analysis, compared to the native receptors. As stated above, this represents further evidence that the broad

receptor bands are indicative of carbohydrate microheterogeneity.

The results obtained with N-glycanase indicate $Fc\epsilon R_L$ was found to be associated with 38% N-linked oligosaccharide while that of $Fc\epsilon RI(\alpha)$ was calculated to be composed of 43% (Table 2.3). Both $Fc\epsilon R_L$ and $Fc\epsilon RI(\alpha)$ may be composed primarily of complex oligosaccharides differing in the extent of branching. As little as 3% O-linked carbohydrate may be present as determined by the difference in M_r of the 27.5 kDa N-glycanase treated $Fc\epsilon RI(\alpha)$ and its 26,104 molecular weight predicted by the amino acid sequence for the RBL cell line (Kinet *et al.*, 1987), and postulated to be the same for other RBL and mast cell lines on the basis of Northern blot analysis (Chan *et al.*, 1990). It should, be noted, as mentioned above, that the percentage of carbohydrate associated with the two $Fc\epsilon R$ is probably in reality somewhat lower than the values given above because the M_r of the fully glycosylated receptor is in all probability somewhat overestimated since glycoproteins of high carbohydrate content are known to yield M_r values which are too high when analyzed by SDS-PAGE in gels of low porosity (Ferguson, 1964; Weber & Osborn, 1975).

The first endoglycosidase studies done on $Fc\epsilon RI(\alpha)$ utilized endoglycosidase D, endoglycosidase H and neuraminidase (Goetze *et al.*, 1981). Each reportedly caused a small decrease in molecular weight, but the exact amount was not determined. A dramatic decrease in molecular weight of 32% to 36 kDa was reported for the α-subunit of $Fc\epsilon RI$ upon treatment with α-D-N-acetyl- galactosaminyloligosaccharidase which removes

O-linked sugars. The presence of contaminating proteases was not ruled out in those experiments and no protease inhibitors were included in the assay. It is evident that our results are not in agreement with such a high content of O-linked carbohydrate. It should, however, be pointed out that the RBL cells used by these authors, had been reported to have a FcϵRI(α) of 54 kDa when analyzed by highly cross-linked gels (Goetze *et al.*, 1981) compared to one of 45 kDa established for cells related to the ones used in this study (Conrad & Froese, 1978a). The higher M_r of the former could potentially be due to O-linked sugars.

There is strong reason to believe, based on these results and others obtained previously in this laboratory, that FcϵR_L may in fact be related to FcγRII-1 found recently on the murine mastocytoma P815 (Benhamou *et al.*, 1990) and previously characterized on the macrophage line, J774 (Mellman and Unkeless, 1980; Green *et al., 1985)*. SDS-PAGE analysis showed a broad dispersed band in the range of 55-65 kDa, and when deglycosylated, the core protein of FcγRII-1 had M_r of 38 kDa (Benhamou *et al.*, 1990; Green *et al.*, 1985) identical to that of deglycosylated FcϵR_L. FcγRII-1 had at least four N-linked oligosaccharides, of which most are endoglycosidase H resistant (Green *et al.*, 1985). In addition, FcϵR_L also has been found to bind with IgG (Kepron *et al.*, 1982; 1988). Binding of IgE to FcγRII-1 has never been tested for. Determination of the relatedness of these receptors cannot not be determined until FcϵR_L sequencing information is available.

Some microheterogeneity at the level of O-linked oligosaccharides may also exist. The deglycosylated FcϵR$_L$ of HRMC 9 exhibited a M_r of ~43 kDa compared to ~38 kDa for the same FcϵR of the related cloned line HRMC5.1.6 (Figure 2.12 and Table 2.4). Thus, the higher M_r of intact FcϵR$_L$ of RCMC9 may actually be a consequence of a higher percentage of O-linked carbohydrates. This also indicates that altered receptor profiles seen in the various HRMC clones are likely due to difference in the glycosylation machinery of the cells. Microheterogeneity of the 38 kDa receptor band in the study by Hempstead *et al.*, (1981), was still found after cell culture with tunicamycin. Isoelectric focussing of the deglycosylated receptor generated four molecular species in the pH range of 7.2 to 7.5. This may be due to variable O-glycosylation of the receptors. However, since Hempstead *et al.*, (1981), as indicated before, had isolated the receptors from tunicamycin treated cells by means of IgE-Sepharose which isolates both FcϵRI(α) and FcϵR$_L$, the receptor species detected by IEF may have been of FcϵRI(α) origin, FcϵR$_L$ origin, or both.

FcϵRI belongs to a family of receptors including the acetylcholine and β-adrenergic receptors each with seven hydrophobic membrane spanning domains and interacting with G proteins (Herskowitz & Marsh, 1987; Stryer & Bourne, 1986). Most of the receptors are glycosylated at 1-7 sites with the exception of bovine rhodopsin and hamster β-adrenergic receptor. A role for these oligosaccharides has been proposed by Merlie *et al.*, (1982) for the acetylcholine receptor. They proposed that subunit glycosylation is critical for receptor subunit assembly and subsequent receptor

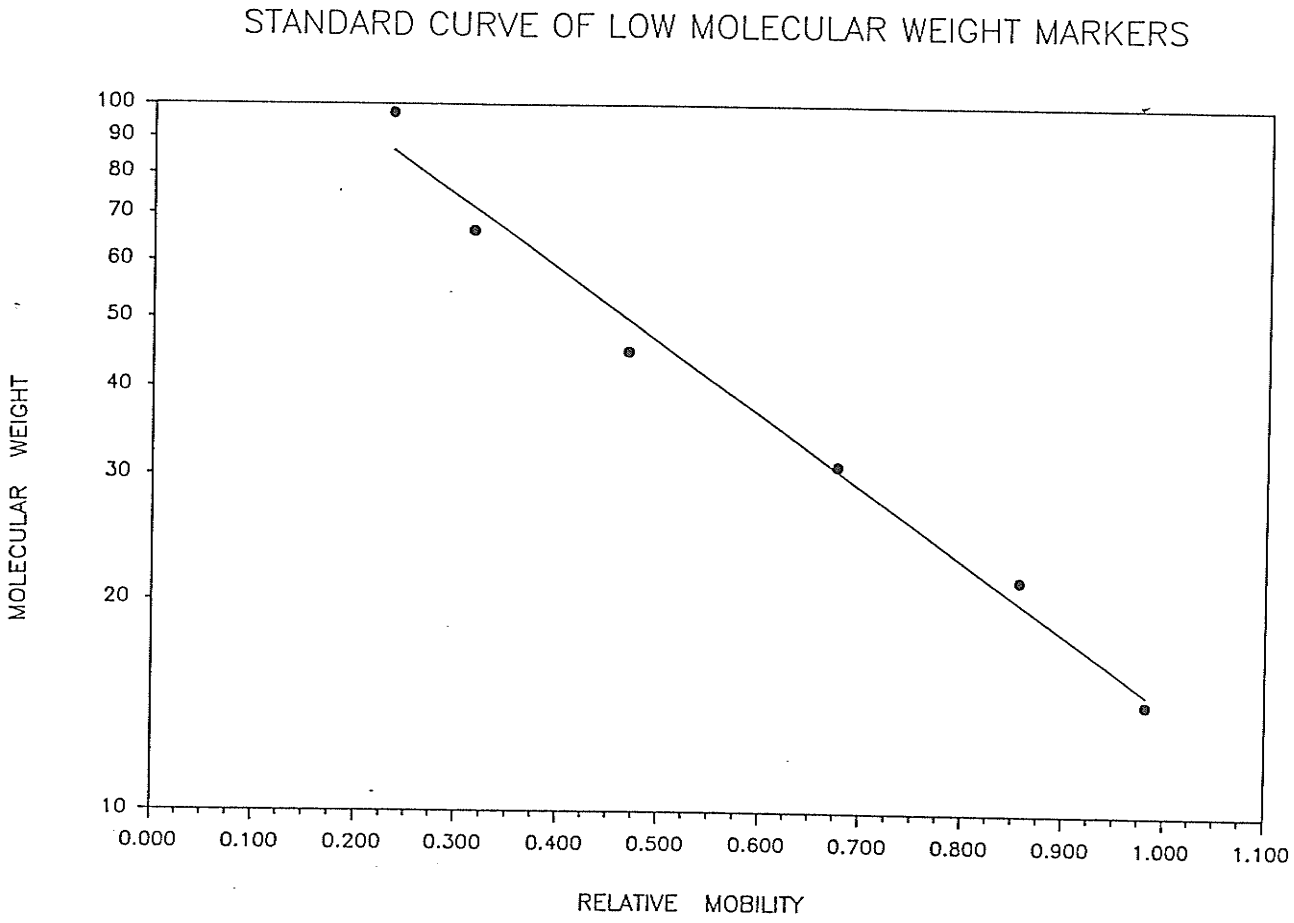
expression. Rands *et al.*, (1990) also found that glycosylation was important for correct trafficking as well as complete coupling of the endogenous adenyl cyclase system of the β-adrenergic receptor. It will therefore not be surprising if, in the future, new roles for the carbohydrate component of these receptors are found which have not been determined to date.

In summary, this study has shown that when RBL-CA10 and RBL-CA10.7 are cultured in the presence of the glycosylation inhibitors castanospermine, 1-deoxymannojirimycin, or swainsonine, the M_r of FcϵRI(α) and FcϵR_L is decreased in a manner which is consistent with the majority of oligosaccharide being of a complex structure. The complex nature of the oligosaccharide is also confirmed by the relative endoglycosidase H resistance of the Fcϵ receptors. The small decrease in M_r brought about by this enzyme when cells were not treated with inhibitors, suggests that both receptors are associated with only one high mannose oligosaccharide.

Digestion of native Fcϵ receptors with N-glycanase and Fcϵ receptors from 1-deoxymannojirimycin treated cells with endoglycanase H yielded M_r values of 28 and 36 kDa for FcϵRI(α) and FcϵR_L respectively. The values suggested that the maximum N-linked oligosaccharide composition of the two receptors is about 40%. The fact that the 71K receptor could be induced by mycoplasma on RBL-CA 10.7 cells which had been pretreated with either of the processing inhibitors does suggest that if, in general, the mycoplasma exerts its action by reacting with the carbohydrate moieties of

$Fc\epsilon RI(\alpha)$, the carbohydrate structure does not critically depend on a precise carbohydrate structure.

APPENDIX A Graph: Molecular Weight versus Relative Mobility for the calculation of M_r.



REFERENCES

Aldenborg, F., & Enerbäck, L. (1985). *Int. Arch. Allergy Appl. Immunol.* 78: 277-282.

Arakaki, R.F., Hedo, J.A., Collier, E., & Gorden, P. (1987). *J. Biol. Chem.* 262: 11886-11892.

Arumugham, R.G., & Tanzer, M.L. (1983). *J. Biol. Chem.* 258: 11883-11889.

Asakawa, K., Hedo, J.A., Gorden, P., & Shizume, K. (1988). *Acta Endocrinologica (Copenh)* 119: 517-524.

Ashman, R.I., Jarloe, D.L., Conrad, D.H., & Huff, T.F. (1991). *J. Immunol.* 146: 211-216.

Bach, M.K., Bloch, K.J., & Austen, K.F. (1971). *J. Exp. Med.* 133: 772-784

Bach, M.K., & Brashler, J.R. (1973). *J. Immunol.* 110: 1599-1608.

Baenziger, J.U. (1978). In *Immediate Hypersensitivity Modern Concepts and Developments.* Immunology series Vol. 7. Bach, M.K. ed. Marcel Dekker, Inc. New York and Basel. p.37-46.

Bause, E. (1983). *Biochem. J.* 209: 331-336.

Bause, E., & Legler, G. (1981). *Biochem. J.* 195: 639-644.

Bazin, H., Querijean, P., Berckers, A., Heremano, J.F., & Dessy, F. (1974). *Immunology* 20: 713-717.

Befus, A.D., & Bienenstock, J. (1979). *Immunology* 38: 95-101.

Befus, A.D., Pearce, F.L., Gauldie, J., Horsewood, P., & Bienenstock, J. (1982). *J. Immunol.* 128: 2475-2480.

Befus, A.D., Pearce, F.L., Gauldie, J., Horsewood, P., Goodacre, R.L., Cole, F., Heatly, R.V., & Bienenstock, J. (1979). In *The Mast Cell - Its Role in Health and Disease.* Pepys, J. & Edwards, A.M. eds. Pitman Medical Publishing Co. Ltd., Kent England. p 702-709.

Benhamou, M., Bonnerot, C., Fridman, W.H., & Daeron, M. (1990). *J. Immunol.* 144: 3071-3077.

Bienenstock, J., Tomioka, M., Matsuda, H., Stead, R.H., Quinonez, G., Simon, G.T., Coughlin, M.D., & Denburg, J.A. (1987). *Int. Archs. Allergy appl. Immunol.* 82: 238-243.

Bienenstock, J., Tomioka, M., Stead, R.H., Ernst, P., Jordana, M., Gauldie, J., Dolovich, J., & Denburg, J. (1987). *Am. Rev. Respir. Dis.* 135: S5-S8.

Bischoff, J., & Kornfeld, R. (1986). *J. Biol. Chem.* 261: 4758-4765.

Blank, U., Ra, C., Miller, L., White, K., Metzger, H., & Kinet, J.-P. (1989). *Nature* 337: 187-189.

Bolton, A.E., & Hunter, W.M. (1973). *Biochem. J.* 133: 529-539.

Bowlin, T.L., McKown, B.J., Kang, M.S., & Sunkara, P.S. (1989). *Cancer Res.* 49: 4109-4113.

Bowlin, T.L., & Sunkara, P.S. (1988). *Biochem. Biophys. Res. Commun.* 151: 859-864.

Bredt, W., Feldner, J., & Kahane, I. (1981). *Israel J. Med. Sci.* 17: 586-588

Breitfeld, P.P., Rup, D., & Schwartz, A.L. (1984). *J. Biol. Chem.* 259: 10414-10421.

Carson, D.A., Kulczycki, A., Jr., & Metzger, H. (1975). *J. Immunol.* 114: 158-160.

Carson, D.D., Earles, B.J., & Lennarz, W.J. (1981). *J. Biol. Chem.* 256: 1152-1157.

Chambers, J.P., & Elbein, A.D. (1986). *Enzyme* 35: 53-56.

Chan, B.M.C., McNeill, K., Berczi, I., & Froese, A. (1986). *Eur. J. Immunol.* 16: 1319-1324.

Chan, B.M.C., McNeill, K., & Froese, A. (1988). *Immunol. Lett.* 18: 37-42.

Chan, B.M.C., Ph. D. Thesis, University of Manitoba, Winnipeg, Manitoba, Canada (1988).

Chan, B.M.C., Ra, C., Hu, W., McNeill, K., Miller, H.R.P., Befus, D., Kinet, J.-P., & Froese, A. (1990). *Eur. J. Immunol.* 20: 185-193.

Colegate, S.M., Dorling, P.R., & Huxtable, C.R. (1979). *Aust. J. Chem.* 32: 2257-2264.

Conrad, D.H., & Froese, A. (1976). *J. Immunol.* 116: 319-326.

Conrad, D.H., & Froese, A. (1978a). *J. Immunol.* 120: 429-437.

Conrad, D.H., & Froese, A. (1978b). *Immunochem.* 15: 283-288.

Conrad, D.H., Bazin, H., Sehon, A.H., & Froese, A. (1975). *J. Immunol.* 114: 1688-1691.

Conrad, D.H., Berczi, I., & Froese, A. (1976). *Immunochem.* 13: 329-332.

Conrad, D.H., Froese, A., Ishizaka, T., & Ishizaka, K. (1978): *J. Immunol.* 120: 507-512.

Conrad, D.H., Helm, R.M., Yiu, S.H., Froese, A., Ishizaka, T., & Ishizaka, K. (1979). *J. Immunol.* 122: 2238-2244.

Crews, F.T., Morita, Y., McGivney, A., Hirata, F., Siraganian, R.P., & Axelrod J. (1981). *Arch. Biochem. Biophys.* 212: 561-571.

Czarnetzki, B.M., Hannich, D., & Niedorf, H. (1979). *Immunobiol.* 156: 470-476.

Daëron, M., Bonnerot, C., Latour, S., Benhamou, M., & Fridman, W.H. (1990). *Eur. J. Immunol.* 20: 897-901.

Danzin, C., & Ehrhard, A. (1987). *Arch. Biochem. Biophys.* 257: 472-475.

Datema, R., Romero, P.A., Legler, C., & Schwarz, R.T. (1982). *Proc. Natl. Acad. Sci. USA* 79: 6787-2385.

Davis, D., Schwarz, P., Hernandez, T., Mitchell, M., Warnock, B., & Elbein, A.D. (1984). *Plant Physiol.* 76: 972-975.

Dayton, E.T., Pharr, P., Ogawa, M., Serafin, W.E., Austen, K.F., Levi-Schaffer, F., & Stevens, R.L. (1988) *Proc. Natl. Acad. Sci.* 85: 569-572.

Debray, H., Quin, Z., Palannoy, P., Montreul, J., Dus, D., Radikowski, C., Christensen, B., & Kieler, J. (1986). *Int. J. Cancer.* 37: 607-611.

Denburg, J.A., Befus, A.D., & Bienenstock, J. (1980). *Immunology* 41: 195-202.

Denburg, J.A. (1990). *Springer Sem. Immunopath.* 12: 401-414.

Denburg, J.A., Tanno, Y., & Bienenstock, J. (1986) In *Mast Cell Differentiation and Heterogeneity*. Befus, A.D., Bienenstock, J., & Denburg, J.A., eds. pp. 71. New York: Raven. 426 pp.

Dennis, J.W. (1986). *Cancer Res.* 46: 5131-5136.

DeSantis, R., Santee, U.V., & Glick, M.C. (1987). *Biochem Biophys. Res. Commun.* 142: 348-353.

Dohlman, H.G., Caron, M.G., & Lefkowitz, R.J. (1987). *Biochem.* 26: 2657-2664.

Dorland, L., Haverkamp, J., Vliegenthart, J.F.G., Spik, G., Fournet, B., & Montreuil, J. (1979). *Eur. J. Biochem.* 100: 569-571.

Dorling, P.R., Huxtable, C.R., & Colegate, S.M. (1980). *Biochem. J.* 191: 649-651.

Dorling, P.R., Huxtable, C.R., Vogel, P. (1978). *Neuropathol. Appl. Neurobiol.* 4: 285-295.

Dorrington, K.H., & Bennich, H. (1973). *J. Biol. Chem.* 248: 8378-8384.

Duronio, V., Jacobs, S., & Cuatrecasas, P. (1986). *J. Biol. Chem.* 261: 970-975.

Dvorak, A.M., Galli, S.J., Schulman, E.S., Lichtenstein, L.M., & Dvorak, H.F. (1983). *Fed. Proc.* 42: 2510-2515.

Eccleston, E., Leonard, B.J., Lowe, J.S., & Welford, H.J. (1973). *Nature New Biol.* 244: 73-76.

Elbein, A.D. (1987). *Ann. Rev. Biochem.* 56: 497-634.

Elbein, A.D., Dorling, P.R., Vosbeck, K., & Horisberger, M. (1982). *J. Biol. Chem.* 257: 1573-1576.

Elbein, A.D., Legler, G., Tlusty, A., McDowell, W., & Schwarz, R. (1984). *Arch. Biochem. Biophys.* 235: 579-588.

Elbein, A.D., Pan, Y.T., Solf, R., & Vosbeck, K. (1983). *J. Cell. Phys.* 115: 265-275.

Elbein, A.D., Solf, R., Dorling, P.R., & Vosbeck, K. (1981). *Proc. Natl. Acad. Sci. USA* 78: 7393-7397.

Elbein, A.D., Szumilo, T., Sanford, B.A., Sharpless, K.B., & Adams, C. (1987). *Biochemistry* 26: 2502-2510.

Elder, J.H., & Alexander, S. (1982). *Proc. Natl. Acad. Sci.* 79: 4540-4544.

Ellmers, B.R., Rhinehart, B.L., & Robinson, K.M. (1987). *Biochem. Pharmac.* 36: 2381-2385.

Enerbäck, L. (1966). *Acta Path. et Microbiol. Scandinav.* 66: 289-302.

Enerbäck, L. (1974). *Histochemistry* 42: 301-303.

Enerbäck, L. (1986). In *Mast Cell Differentiation and Heterogeneity.* Befus, A.D., Bienenstock, J., & Denburg, J.A., eds. pp. 1-26. New York: Raven. 426 pp.

Enerbäck, L., Kolset, S.O., Kusche, M., Hjerpe, A., & Lindahl, U. (1985). *Biochem. J.* 227: 661-668.

Enerbäck, L. (1981). *Monogr. Allergy* 17: 222- 232

Ferguson, K.A. (1964). *Metabolism* 13: 985-1002

Foddy, L., & Hughes, R.C. (1988). *Eur. J. Biochem.* 175: 291-299.

Franc, J.L., Houseplan, S., Fayet, G., & Bauchilloux, S. (1986). *Eur. J. Biochem.* 157: 225-232.

Froese, A. (1980). *J. Immunol.* 125: 981-987.

Froese, A., Helm, R.M., Conrad, D.H., Isersky, C., & Ishizaka, T. (1982). *Immunology* 46: 117-123.

Froese, A., Helm, R.M., Conrad, D.H., Isersky, C., Ishizaka, T., & Kulczycki, A. Jr. (1981). *Immunol.* 46: 107-116.

Fujimaki, H., Lee, T.D.G., Swieter, M., Saito, A., Tamaoki, T., & Befus, A.D. (1988). *J. Immunol. Methods* 114: 219-225.

Fukuda, M., Fukuda, M.N., & Hakomori, S. (1979). *J. Biol. Chem.* 254: 8201-8207.

Fuhrman, U., Bause, E., Legler, G., & Ploegh, H. (1984). *Nature* 307: 755-758.

Galli, S.J., Dvorak, A.M., & Dvorak, H.F. (1984). *Prog. Allergy* 34: 1-83.

Galli, S.J. (1990). *Lab Invest.* 62: 5-33.

Gaveriaux, P.R., & Loor, F. (1987). *Int. Archs. Allergy appl. Immunol.* 84: 85-92.

Gibson, S., & Miller, H.R.P. (1986). *Immunology* 58: 101-104.

Gilbert, H.S., & Ornstein, L. (1975). *Blood* 46: 279-286

Ginsberg, H. (1963). *Ann NY Acad. Sci.* 103: 20-39.

Ginsberg, H., Ben-Shahai, D., & Ben-David, E. (1982). *Immunology* 45: 371-380.

Ginsberg, H., & Lagunoff, D. (1967). *J. Cell. Biol.* 35: 685-697

Goetze, A., Kanellopoulos, J., Rice, D., & Metzger, H. (1981). *Biochem.* 20: 6341-6349.

Gordon, J.R., Bard, P.R., & Galli, S.J. (1990). *Immunol. Today* 11: 458-464.

Gordon, J.R., & Galli, S.J. (1990). *Nature* 346: 274-276.

Granato, D.A., and Neeser, J.-R. (1987). *Mol. Immunol.* 24: 849-855.

Green, S.A., Plutner, H., & Mellman, I. (1985). *J. Biol. Chem.* 260: 9867-9874.

Gross, V., Tran-Thi, T.-A., Schwarz, R.T., Elbein, A.D., Decker, K., & Heinrich, P.C. (1986). *Biochem J.* 236: 853-860.

Gross, V., von Berg, D., Kreuzkamp, J., Ganter, U., Bauer, J., Wurtemberger, G., Schulz-Haotari, C., Beeser, H., & Gerok, W. (1990). *Biol. Chem. Hoppe-Seyler* 371: 231-238.

Gruters, R.A., Neifjes, J.J., Tersmette, M., de Goede, R.E.Y., Tulp, A., Huisman, H.G., Miedema, F., & Ploegh, H.L. (1987). *Nature* 330: 74-77.

Haig, D.M., Jarrett, E.E.E., & Tas, J. (1984). *Immunology* 51: 643-651.

Haig, D.M., McKee, T.A., Jarrett, E.E.E., Woodbury, R., & Miller, H.R.P. (1982). *Nature* 300: 188-190.

Haig, D.M., McMenamin, C., Gunneberg, C., Woodbury, R., & Jarrett, E.E.E. (1983). *Proc. Natl. Acad. Sci.* 80: 4499-4503.

Halper, J., & Metzger, H. (1976). *Immunochem.* 13: 907-913.

Hamaguchi, Y., Kanakura, Y., Fujita, J., Takeda, S.-I., Nakano, T., Tarui, S., Honjo, T., & Kitamura, Y. (1987) *J. Exp. Med.* 165: 268-273.

Hasemann, C.A., & Capra, J.D. (1989). In *Fundamental Immunology* 2nd ed. Paul, W.E. ed. Raven Press. New York. p. 209-233.

Hasthorpe, S. (1980). *J. Cell. Physiol.* 105: 379-384.

Hattori, M.-A., Ozawa, K., & Wakabayashi, K. (1988a). *Mol. Cell. Endocrin.* 59: 1-11.

Hattori, M.-A., Ozawa, K., & Wakabayashi, K. (1988b). *Acta Endocrinologica (Copenh)* 119: 132-138.

Helm, B., March, P., Vercelli, D., Padlan, E., Gould, H., & Geha, R. (1988). *Nature* 331: 180-183

Helm, R.M., & Froese, A. (1981a). *Immunol.* 42: 629-636.

Helm, R.M., & Froese, A. (1981b). *Int. Archs. Allergy appl. Immun.* 65: 81-84.

Hellman, L., Pettersson, U., Engstrom, A. Karlsson, T., & Bennich, H. (1982). *Nuc. Acids Res.* 10: 6041-6049.

Hempstead, B.L., Parker, C.W., & Kulczycki, A., Jr. (1981). *J. Biol. Chem.* 256: 10717-10723.

Hempstead, B.L., Parker, C.W., & Kulczycki, A., Jr. (1979). *J. Immunol.* 123: 2283-2291.

Hettkamp, H., Bause, E., & Legler, G. (1982). *Biosci. Rep.* 2: 899-906.

Hickman, S., Kulczycki, A., Jr., Lynch, R.G., and Kornfeld, S. (1977). *J. Biol. Chem.* 252: 4402-4408.

Hino, M., Nakayama, O., Tsurumi, Y., Adachi, K., Shibata, T., Terano, H., Kohsaka, M., Aoki, H., & Imanaka, H. (1985). *J. Antibiotics* 38: 926-935.

Hirschberg, C.B., & Snider, M.D. (1987). *Ann. Rev. Biochem.* 56: 63-87.

Hohenschutz, L.D., Bell, E.A., Jewiss, P.J., Leworthy, D.P., Pryce, R.J., Arnold, E., & Clardy, J. (1981). *Phytochemistry* 20: 811-814.

Holowka, D., & Baird, B. (1984). *J. Biol. Chem.* 259: 3720-3728.

Holowka, D., Gitler, C., Bercovici, T., & Metzger, H. (1981). *Nature* 289: 806-808.

Holowka, D., Hartmann, H., Kanellopoulos, J.M., & Metzger, H. (1980). *J. Rec. Res.* 1: 41-68.

Holowka, D., & Metzger, H. (1982). *Mol. Immunol.* 19: 219-227.

Hori, H., Pan, Y.T., Molyneux, R.J., & Elbein, A.D. (1984). *Arch. Biochem. Biophys.* 228: 525-533.

Hsieh, P., Rosner, M.R., & Robbins, P.W. (1983). *J. Biol. Chem.* 258: 2555-2561.

Hu, W., Chan, B.M.C., & Froese, A. (1990). *Int. Arch. Allergy Appl. Immunol.* 92: 301-305.

Hubbard, S.C., & Ivatt, R.J. (1981). *Ann. Rev. Biochem.* 50: 555-583.

Hubbard, S.C., & Robbin, P.W. (1979). *J. Biol. Chem.* 254: 4568-4576.

Humphries, M. J., Matsumoto, K., White, S.L., Molyneux, R. J., & Olden, K. (1988). *Cancer Res.* 48: 1410-1415.

Humphries, M.J., Matsumoto, K., White, S., & Olden, K. (1986a). *Proc. Natl. Acad. Sci. USA* 83: 1752-1756.

Humphries, M.J., Matsumoto, K., White, S., & Olden, K. (1986b). *Cancer Res.* 46: 4109-4113.

Hunkapiller, T., & Hood, L. (1986). *Nature* 323: 15-16.

Inouye, S., Tsurrioka, T., Ito, T., & Niida, T. (1968). *Tetrahedron* 23: 2125-2144.

Isersky, C., Mendoza, G.R., & Metzger, H. (1977). *J. Immunol.* 119: 123-130.

Ishizaka, K., & Ishizaka, T. (1971). *Ann. N. Y. Acad. Sci* 190: 443-456.

Ishizaka, K., Tomoioka, H., & Ishizaka, T. (1970). *J. Immunol.* 105: 1459-1467.

Ishizaka, T., Adachi, T., Chang, T.-H., & Ishizaka, K. (1977). *J. Immunol.* 118: 211-217.

Ishizaka, T., Okadana, H. Mauser, L.E., & Ishizaka, K. (1976). *J. Immunol.* 116: 747-754.

Ishizaka, T., Soto, C. S., & Ishizaka, K. (1973). *J. Immunol.* 111: 500-511.

Irana, A.-M.A., & Schwartz, L.B. (1989). *Clin. Exp. Allergy* 19: 143-155.

Jarrett, E.E.E., & Haig, D.M. (1984). *Immunol. Today* 5: 115-119.

Jarrett, E.E.E., & Miller, H.R.P. (1982). *Prog. Allergy* 31: 178-233.

Kagey-Sobotka, A., Dembo, M., Goldstein, B., Metzger, H., & Lichtenstein, L.M. (1981). *J. Immunol.* 127: 2285-2291.

Kahane, I., Pnini, S., Banai, M., Baseman, J.B., Casell, G.H., & Bredt, W. (1981). *Israel J. Med. Sci.* 17: 589-592

Kanakura, Y., Thompson, H.L., Nakano, T., Yamamura, T., Asai, H., Kitamura, Y., Metcalfe, D.D., & Galli, S.J. (1988). *Blood* 72: 877-885.

Kanellopoulos, J.M., Liu, T.-Y., Poy, G., & Metzger, H. (1980). *J. Biol. Chem.* 255: 9060-9066.

Kanellopoulos, J.M., Rossi, G., & Metzger, H. (1979). *J. Biol. Chem.* 254: 7691-7697.

Kang, M.S., & Elbein, A.D. (1983a). *Plant Physiol.* 71: 551-554.

Kang, M.S., & Elbein, A.D. (1983b). *J. Virol.* 46: 60-69.

Katz, H.R., Arm, J.P., Benson, A.C., & Austen, K.F (1990). *J. Immunol.* 145: 3412-3417.

Katz, H.R., Austen, K.F., Caterson, B., & Stevens, R. L. (1986). *J. Biol. Chem.* 261: 13393-13396.

Kelly, K.A., Sehon, A.H., & Bazin, H. (1979). *Mol. Immunol.* 16: 597-607

Kepron, M.R., Bazin, H., & Froese, A. (1988). *Mol. Immunol.* 25: 599-609.

Kepron, M.R., Conrad, D.H., & Froese, A. (1982). *Mol. Immunol.* 19: 1631-1639.

Kinet, J.-P., Blank, U., Ra, C., White, K., Metzger, H., & Kochan, J. (1988). *Proc. Natl. Acad. Sci. USA* 85: 6483-6487.

Kinet, J.-P., Metzger, H., Hakimi, J., & Kochan, J. (1987). *Biochem* 26: 4605-4610.

Kinet, J.- P., Quarto, R., Perez-Montfort, R., & Metzger, H. (1985). *Biochem.* 24: 7342-7348.

Kirshenbaum, A.S., Goff, J.P., Dreskin, S.C., Irani, A.-M., Schwartz, L.B., & Metcalfe, D.D. (1989). *J. Immunol* 142: 2424-2429

Kitamura, Y. (1989). *Ann. Rev. Immunol.* 7: 59-76.

Kitamura, Y., & Fujita, J. (1989). *Bioessays* 10: 193-196.

Kitamura, Y., & Go, S. (1979). *Blood* 53: 492-497.

Kitamura, Y., Go, S., & Hatanaka, S. (1978). *Blood* 52: 447-452.

Kitamura, Y., Yokoyama, M., Matsuda, H., Ohno, T., & Mori, K.J. (1981). *Nature* 291: 159-160

Kobata, A. (1979). *Anal. Biochem.* 100: 1-14.

Kornfeld, R., & Kornfeld, S. (1985). *Ann. Rev. Biochem.* 54: 631-664.

Kornfeld, S., Li, E., & Tabas, I. (1978). *J. Biol. Chem.* 253: 7771-7778.

Kronquist, K.E., & Lennarz, W.J. (1978). *J. Supramol. Struct.* 8: 51-65.

Kulczycki, A., Jr., Hempstead, B.L., Hofmann, S.L., Wood, E.W., & Parker, C.W. (1979). *J. Biol. Chem.* 254: 3194-4000.

Kulczycki, A., Jr., Isersky, C., & Metzger, H. (1974). *J. Exp. Med.* 139: 600-616

Kulczycki, A.,Jr. (1980). *J. Reticuloendothelial Soc.* 28: 29S-34S.

Kulczycki, A., Jr., McNearney, T.A., & Parker, C.W. (1976). *J. Immunol.* 117: 661-665

Kulczycki, A., Jr., & Metzger, H. (1974). *J. Exp. Med.* 140: 1676-1695.

Kulczycki, A., Jr., & Parker, C.W. (1979). *J. Biol. Chem.* 254: 3187-3193.

Kulczycki, A., Jr., & Vallina, V. L. (1981). *Mol. Immunol.* 18: 723-731.

Kumar, N., & Metzger, H. (1982). *Mol. Immunol.* 19: 1561-1567.

Laemmli, U.K. (1970). *Nature* 227: 680-682.

Lalegerie, P., Legler, G., & Yon, J. (1982). *Biochimie* 64: 977-1000.

Laskey, R.A., Mills, A.D. (1977). *FEBS Lett.* 82: 314-317.

Le, A.Y., & Doyle, D. (1985). *Biochemistry* 24: 6238-6245.

Leary, A.G., & Ogawa, M. (1984). *Blood* 64: 78-83

Lee, T.D.G., Shanahan, F., Miller, H.R.P., Bienenstock, J., & Befus, A.D. (1985a). *Immunology* 55: 721-728.

Lee, T.D.G., Sterk, A., Ishizaka, T., Bienenstock, J., & Befus, A.D. (1985b). *Immunology* 55: 363-366.

Lee, T.D.G., Swieter, M., Bienenstock, J., & Befus, A.D. (1985). *Clin. Immunol. Rev.* 4: 143-199.

Legler, G., & Julich, E. (1984). *Carbohydr. Res.* 128: 61-72.

Lehle, L., & Bause, E. (1984). *Biochem. Biophys. Acta.* 799: 246-251.

Levi-Schaffer, F., Austen, K.F., Caulfield, J.P., Hein, A., Bloes, W.F., & Stevens, R.L. (1985). *J. Immunol.* 135: 3454-3462.

Lo, T.N., Saul, W.F., & Beaven, M.A. (1985). *Pharmacologist* 27: 184-186

Lui, F.- T., & Orida, N. (1984). *J. Biol. Chem.* 259: 10649-10652.

Maley, F., Trimble, R.B., Tarentino, A.L., & Plummer, T.H. (1989). *Anal. Biochem.* 180: 195-204.

Mayrhofer, G., & Fisher, R. (1979). *Immunology* 37: 145-155.

Mayrhofer, G. (1980). *Histochem. J.* 12: 513-526.

Mellman, I.S., & Unkeless, J.C. (1980). *J. Exp. Med.* 152: 1048-1069.

Metcalfe, D.D., Kaliner, M., & Donlon, M.A. (1981). *CRC Crit. Rev. Immunol.* 2: 23-74.

Metzer, H., Alcaraz, G., Hohman, R., Kinet, J.-P., Pribluda, V., & Quarto, R. (1986). *Ann. Rev. Immunol.* 4: 419-470.

Metzger, H., Rivney, B., Henkart, M., Kanner, B., Kinet, J.-P., & Perez-Montfort, R. (1984). *Mol. Immunol.* 21: 1167-1173.

Mican, J.M., & Metcalfe, D.D. (1990). *J. Allergy Clin. Immunol.* 86: 677-683.

Michels, N.A. (1938). In *Handbook of Hematology*. Vol. 1. Downey, H. ed. London. p232-372.

Miller, H.R.P., & Walshaw, R. (1972). *Am. J. Pathol.* 69: 195-206.

Moller, G., & Konig, W. (1980). *Immunology* 41: 605-615.

Molyneux, R.J., & James, L.F. (1982). *Science* 216: 190-191.

Montreuil, J. (1980). *Adv. Carbohyd. Chem. Biochem.* 37: 157-223.

Morita, Y., & Siraganian, R.P. (1981). *J. Immunol.* 127: 1339-1344.

Murphy, L.A., & Spiro, R.G. (1981). *J. Biol. Chem.* 256: 7487-7494.

Nakano, T., Kanakura, Y., Nakahata, T., Matsuda, H., & Kitamura, Y. (1987). *Fed. Proc.* 46: 1920-1923.

Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitamura, Y., & Galli, S.J. (1985). *J. Exp. Med.* 162: 1025-1043.

Nakano, T., Spicer, S.S., Cantey, J.R., & Ogawa, M. (1981). *Blood* 60: 352-361

Natowicz, M., Baenziger, J.U., & Sly, W.S.J. (1982). *Biol. Chem.* 257: 4412-4420.

Nose, M., and Wigzell, H. (1983). *Proc. Natl. Acad. Sci. U.S.A.* 80: 6632-6636.

Oda-Tamai, S., Kato, S., Hara, S., & Akamatsu, N. (1985). *J. Biol. Chem.* 260: 57-63.

Otsu, K., Nakano, T., Kanakura, Y., Asai, H., Katz, H.R., Austen, K.F., Stevens, R.L., Galli, S.J., & Kitamura, Y. (1987). *J. Exp. Med.* 165: 615-627.

Padawer, J. (1963). *Ann. N.Y. Acad. Sci.* 103: 441-492.

Padawer, J., & Gordon, A.S. (1955). *Proc. Soc. Exp. Biol. Med.* 88: 29-31.

Padawer, J. (1979). In *Immediate Hypersensitivity*, Vol. 7. Bach, M.D. ed. p. 301-367. New York, Marcel Decker. 848 pp.

Pan, Y.T., Hori, H., Saul, R., Sanford, BA., Molyneux, R.J., & Elbein, A.D. (1983). *Biochemistry* 22: 3976-3984.

Parodi, A.J., Mendelzon, D.H., &. Lederkremer, G.Z. (1983). *J. Biol. Chem.* 258: 8260-8265.

Pearce, F.L. (1988). *Agents and Actions* 23: 125-128.

Pearce, F.L., Ali, H., Barrett, K.E., Befus, A.D., Bienenstock, J., Brostoff, J., Ennis, M., Flint, K.C., Hudspith, B., Johnson, N.M., Leung, K.B.P., & Peachell, P.T. (1985). *Int. Archs. Allergy appl. Immun.* 77: 274-276.

Pecoud, A.R., Ruddy, S., & Conrad, D.H. (1981). *J. Immunol.* 126: 1624-1629.

Peters, S.P. (1990). *J. Allergy Clin. Immunol.* 86: 642-646.

Perez-Montfort, R., Fewtrell, C., & Metzger, H. (1983). *Biochem.* 22: 5733-5737.

Perez-Montfort, R., Kinet, J.- P., & Metzger, H. (1983). *Biochem.* 22: 5722-5728.

Perez-Montfort, R., & Metzger, H. (1982). *Mol. Immunol.* 19: 1113-1117.

Peyrieras, N. *et al.*, (1983). *EMBO J.* 2: 899-906.

Pfeffer, S.R., & Rothman, J.E. (1987). *Ann. Rev. Biochem.* 56: 829-852.

Pierce, R.J., Spik, G., & Montreuil, J. (1979). *Biochem. J.* 180: 673-676.

Plaut, M., Pierce, J. H., Watson, C.J., Hanley-Hyde, J., Nordan, R.P., & Paul W.E. (1989). *Nature* 339: 64-67.

Plummer, T.H., Jr., Elder, J.H., Alexander, S., Phelan, A.W., & Tarentino, A.L. (1984). *J. Biol. Chem.* 259: 10700-10704.

Pollack, L., & Atkinson, P.H. (1983). *J. Cell. Biol.* 97: 293-300.

Quarto, R., Kinet, J.-P., & Metzger, H. (1985). *Mol. Immunol.* 22: 1045-1059.

Ravetch, J.V., Luster, A.D., Weinshank, R., Kochan, J., Pavlovec, A., Portnoy, D.A., Hulmes, J., Pan, Y.-C. E., & Unkeless, J.C. (1986). *Science* 234: 718-725.

Rennick, D., Lee, F.D., Yokota, T., Arai, K.-I., Cantor, H., & Nobel, G.J. (1985). *J. Immunol.* 134: 910-914.

Repp, R., Tamura, T., Boschek, C.B., Wege, H., Schwarz, R.T., & Niemann, H. (1985). *J. Biol. Chem.* 260: 15873-15879.

Rhinehart, B.L., Robinson, K.M., King, C.-H. R., & Liu, P.S. (1990). *Biochem. Pharmac.* 39: 1537-1543.

Riley, J.F. (1954). *Lancet* 1: 841-843.

Rivera, J., Kinet, J.-P., Kim, J., Pucillo, C., & Metzger, H. (1988). *Mol. Immunol.* 25: 647-661.

Rivnay, B., Wank, S.A., Poy, G., & Metzger, H. (1982). *Biochem.* 21: 6922-6927.

Rossi, G., Newman, S.A., & Metzger, H. (1977). *J. Biol. Chem.* 252: 704-711.

Roth, P.A., Rao, M., & Froese, A. (1986). *Immunol.* 58: 671-676.

Rothman, R.J., Perussia, B., Herlyn, D., & Warren, L. (1989). *Mol. Immunol.* 26: 1113-1123.

Ruitenberg, E.J., & Elgersma, A. (1976). *Nature* 264: 258-260.

Sasak, V.W., Ordovos, J.M., Elbien, A.D., & Berninger, R.W. (1985). *Biochem. J.* 232: 759-766.

Saul, R., Chambers, J.P., Molyneux, R.J., & Elbein, A.D. (1983). *Arch. Biochem. Biophys.* 221: 593-597.

Saul, R., Ghidoni, J.J., Molyneux, R.J., & Elbein, A.D. (1985). *Proc. Natl. Acad. Sci. USA.* 82: 93-97.

Saul, R., Molyneux, R.J., & Elbein A.D. (1984). *Archs. Biochem. Biophys.* 230: 668-675.

Schachter, H., Narasimhan, S., Gleeson, P., & Vella, G. (1983). *Can. J. Biochem. Cell Biol.* 61: 1049-1066.

Schneider, M.J., Ungemach, F.S., Broquist, H.P., & Harris, T.M. (1982). *J. Am. Chem. Soc.* 104: 6863-6864.

Segal, D.M., Sharrow, S.O., Jones, J.F., & Siraganian, R.P. (1981). *J. Immunol.* 126: 138-145.

Seldin, D.C., Adelman, S., Austen, K.F., Stevens, R.L., Hein, A., Caulfield, J.P., & Woodbury, R.G. (1985). *Proc. Natl. Acad. Sci.* 82: 3871-3875.

Shanahan, F., Lee, T.D.G., Denburg, J.A., Bienenstock, J., & Befus, A.D. (1986). *Immunology* 57: 455-459.

Shimiza, A., Tepler, I., Benfey, P.N., Berenstein, E.H., Siraganian, R.P., & Leder, P. (1988). *Proc. Natl. Acad. Sci. USA* 85: 1907-1911.

Singer, S.J., Maher, P.A., & Yaffer, M.P. (1987). *Proc. Natl. Acad. Sci. USA.* 84: 1960-1964.

Siraganian, R.P., Hook, W.A., & Levine, B.B. (1975). *Immunochem.* 12: 149-157.

Steinman, R.M., & Cohn, Z.A. (1972). *J. Cell. Biol.* 55: 616-634.

Sterk, A.R., & Ishizaka, T. (1982). *J. Immunol.* 128: 838-843.

Soderquist, A.M., & Carpenter, G. (1984). *J. Biol. Chem.* 259: 12586-12594.

Sonoda, S., Sonoda, T., Nakano, T., Kanayama, Y., Kanakura, Y., Asai, H., Yonezawa, T., & Kitamura, Y. (1986). *J. Immunol.* 137: 1319-1322.

Spearman, M. A., Jamieson, J.C., & Wright, J.A. (1987). *Exp. Cell Res.* 168: 116-126.

Stevens, R.L., & Austen, K.F. (1989). *Immunol. Today* 10: 381-386.

Stevens, R.L., Lee, T.D.G., Seldin, D.C., Austen, K.F., Befus, A.D., & Bienenstock, J. (1986). *J. Immunol.* 137: 291-295.

Suami, T., Tadano, K. I., & Iimura, Y. (1985). *Carbohydr. Res.* 136: 67-75.

Swanstrom, R., Shank, P.R. (1978). *Anal. Biochem.* 86: 184-188.

Swieter, M., Chan, B.M.C., Lee, T.D.G., Rimmer, C.J., Froese, A., & Befus, A.D. (1989). *Int. Arch. Allergy appl. Immun.* 88: 200-202.

Tachibana, Y., Yamashita, K., & Kobata, A. (1982). *Arch. Biochem. Biophys.* 214: 199-210.

Tarentino, A.L., Gomez, C.M.,& Plummer, T.H. (1985). *Biochem.* 24: 4665-4671.

Tarentino, A.L., & Maley, F. (1975). *Biochem. Biophys. Res. Commun.* 67: 55-62.

Tas, J., & Berndsen, R.G. (1977). *J. Hist. Cyto.* 25: 1058-1062.

Theoharides, T.C. (1990). *Life Sciences* 46: 607-617.

Tomioka, H., & Ishizaka, K. (1971). *J. Immunol.* 107: 971-978.

Trimble, R.B., Byrd, J.C., & Maley, F. (1980). *J. Biol. Chem.* 255: 11892-11895.

Trimble, R.B., & Maley, F. (1984). *Anal. Biochem.* 141: 515-522.

Trimble, R.B., Maley, F., & Chu, F.K. (1983). *J. Biol. Chem.* 258: 2562-2567.

Trugnan, G.M., Rousset, M., & Zweibaum, A. (1986). *Fedn. Eur. Biochem. Soc. Lett.* 195: 28-32.

Tsai, C.-M., Huang, C.-C., & Canellakis, E.S. (1973). *Biochim. Biophys. Acta* 332: 47-58.

Tulsiani, D.R.P., Broquist, H.P., James, L.F., & Touster, O. (1984). *Arch. Biochem. Biophys.* 232: 76-85.

Tulsiani, D.R.P., Broquist, H.P., & Touster, O. (1985). *Arch. Biochem. Biophys.* 236: 427-434.

Tulsiani, D.R.P., Harris, T.M., & Touster, O. (1982). *J. Biol. Chem.* 257: 7936-7939.

Tulsiani, D.R.P., Skudlarek, M.D., & Orgebin-Crist, M.-C. (1990). *Biol. Reprod.* 43: 130-138.

Tulsiani, D.R.P., & Touster, O. (1983). *J. Biol. Chem.* 258: 7578-7585.

Unkeless, J.C. (1977). *J. Exp. Med.* 145: 931-947.

Unkeless, J.C. (1979). *J. Exp. Med.* 150: 580-596.

Unkeless, J.C., & Eisen, H.N. (1975). *J. Exp. Med.* 142: 1520-1533.

Unkeless, J.C., Scigliano, E., & Freedman, V.H. (1988). *Ann. Rev. Immunol.* 6:251-281.

Wagner, D.D., Mayadas, T., Urban-Pickering, M., Llwis, B.H., & Marder, V.J. (1985). *J. Cell Biol.* 101: 112-120.

Walker, W.S. (1976). *J. Immunol.* 116: 911-914.

Wall, K.A., Pierce, J.D., & Elbein, A.D. (1988). *Proc. Natl. Acad. Sci. USA* 85: 5644-5648.

Warren, C.D., Daniel, P.F., Bugge, B., Evans, J.E., James, L.F., & Jeanloz, R.W. (1988). *J. Biol. Chem.* 265: 15041-15049.

Wasserman, S.I. (1990). *J. Allergy Clin. Immunol.* 86: 590-593.

Weber, K., & Osborn, M. (1975). *In* The Proteins. Vol. I. edited by H. Neurath & R.L. Hill. Academic Press, New York. p. 179.

Wilhelm, D.L., Yong, L.C.J., & Watkins, S.G. (1978). *Agents Action* 8: 146-152.

Winkler, J.R., & Segal, H. L. (1984). *J. Biol. Chem.* 259: 1958-1962.

Woodbury, R.G., Gruzenski, G.M., & Lagunoff, D. (1978). *Proc. Natl. Acad. Sci.* 77: 2785-2790.

Yagita, M., & Saksela, E. (1990). *Scand. J. Immunol.* 31: 275-282.

Yasuda, N., Tsutsumi, H., & Takayi, T. (1984). *Chem. Lett.* 1984: 1201-1204.

Yeo, T.-K., Yeo, K.-T., Parent, J.B., & Olden, K. (1985). *J. Biol. Chem.* 260: 2565-2569.

Yurt, R.W., Leid, R.W., Jr., Austen, K.F., & Silbert, J.E. (1977). *J. Biol. Chem.* 252: 518-521.

Zheng, Y., Chan, B.M.C., Rector, E., Berczi, S., & Froese, A. (1991). *Exp. Cell Res.* 194: 301-309.

Zucker-Franklin, D., Grusky, G., Hirayama, N., & Schnipper, E. (1981). *Blood* 58: 544-551.