Review

My career as an immunoglycobiologist

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Abstract: The research program of my laboratory included three major topics: the structures and immunology of human carbohydrate blood group and glycosphingolipid antigens; the tissue distribution, subcellular localization and biosynthesis of glycosphingolipids; and the structural basis of the binding of carbohydrates by antibodies and lectins.

Keywords: glycosphingolipids, immunochemistry, cellular localization, biosynthesis

Although my primary research training was in immunology, my studies of human blood group and other carbohydrate antigens led me to investigate the biochemistry and functions of glycoconjugates, especially glycosphingolipids (GSLs). Rather than providing a chronological account I will summarize my research in several related but distinct areas: the structures and immunology of carbohydrate antigens; the tissue distribution, expression and biosynthesis of GSLs; and mechanisms of antibody- and lectin-carbohydrate interactions.

Training in immunology

I was fortunate to spend my obligatory time in military service (1957–1959) in an immunochemistry research laboratory at Walter Reed Army Medical Center in Washington, DC. The program of the laboratory, which was directed by Dr. Elmer L. Becker, concerned biochemical mechanisms of acute hypersensitivity. Only four components of complement were known at that time. Dr. Becker had recently discovered that the first component (C1), subsequently identified as subunit C1q, was a protease proenzyme that was activated by contact with antigen-antibody complexes. I demonstrated that C1 could also be activated in the absence of antigen by aggregated immunoglobulin molecules

that mimicked immune complexes.[1] In addition, I participated in a study that demonstrated that autoantibodies in the sera of patients with systemic lupus erythematosus bound to a nucleoprotein antigen.[2]

After completing my training in Internal Medicine I did a postdoctoral fellowship in immunology in the laboratory of Dr. Elvin A Kabat in the Dept. of Microbiology at Columbia University College of Physicians and Surgeons. The major interest of the laboratory was in determining the structures of the blood group ABH carbohydrate antigens. Enzymes in a culture filtrate of *Clostridium tertium* destroyed the antigenic activity of the blood group A antigen but did not affect the B or H antigens. N-acetylgalactosamine had been identified as the N-terminal sugar of the A antigen, so it was assumed that the *Cl. tertium* enzyme was a galactosaminidase. However, I demonstrated that the partially purified enzyme was not a hydrolase. It inactivated the A antigen by deacetylation of N-acetylgalactosamine.[3] I also used partially purified *Cl. tertium* enzymes to provide the first evidence that the human erythrocyte I antigen, the target of cold agglutinin autoantibodies, was a carbohydrate structure that contained terminal galactose and N-acetylglucosamine residues.[4]

Immunological studies

Antisera to ABH blood group glycoproteins. In 1963 I joined the faculty of Albert Einstein College of Medicine and established my own laboratory. I decided to continue studying glycoprotein blood group antigens and to initiate a project concerning erythrocyte glycosphingolipids (GSLs). Analysis of the structures that carried H and Lewis antigens was

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Abbreviations: GSL: glycosphingolipids; Glc: D-glucose; Gal: D-galactose; GlcNAc: N-acetyl-D-glucosamine; GalNAc: N-acetyl-D-galactosamine; NeuAc: N-acetyl-neuraminic acid; Fuc: L-fucose.

Table 1.   Structures of neutral glycosphingolipids

Structure	Name
Glcβ1-Cer	GlcCer
Galβ1-4Glcβ1-Cer	LacCer
Galβ1-4(Fucα1-3)Glcβ1-Cer	3-FL
Galα1-4Galβ1-4Glcβ1-Cer	Gb3Cer, CTH, P^k
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer	Gb4Cer, globoside, P antigen
Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer	Galactosyl globoside
Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	IV^{4}Galα-nLc4Cer, P1 antigen
GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer	IV^{4}GalNAcα-Gb4Cer, Forssman
Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	nLc4Cer, paragloboside
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-Cer	H type I
Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	H type II
Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-Cer	Le^a
Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-Cer	Le^b

hindered by the lack of strong, specific antisera that could be used for hemagglutination or immunoprecipitation. Since rabbits responded very poorly to carbohydrate immunogens, Dr. Arthur Grollman and I immunized goats with mucin glycoproteins. We obtained antisera to Le^a and Le^b that exhibited specific immunoprecipitating and hemagglutinating properties.[5),6)] We used the anti-Le^a antiserum to identify an alpha-4-L-fucosyltransferase enzyme in human milk as the probable product of the Lewis^a blood group gene.[7)] The Lewis antisera were used subsequently by Drs. Kabat and Sen-itiroh Hakomori in their studies of the structure of Lewis antigens.

Glycosphingolipid antigens

Globoside and Forssman.   Many animal sera contain naturally-occuring "heterophile" antibodies that agglutinate sheep erythrocytes. These antibodies bind to the Forssman GSL (Table 1) in which a terminal nonreducing N-acetylgalactosamine residue is linked alpha1-3 to globoside. The Forssman antigen occurs in many animal tissues and in microbial organisms. Paroxysmal cold hemoglobinuria (PCH) is an autoimmune hemolytic anemia in which episodes of intravascular hemolysis occur when patients are exposed to a cold environment. The autoantibodies attach to erythrocytes most effectively at 4°C and lyse cells in the presence of complement when the temperature is raised to 20°–37°C.

We analyzed the specificity of four human sera that caused PCH. The hemolytic activity of all sera could be inhibited by globoside, and two of the sera were inhibited more effectively by Forssman than globoside.[8)] To further analyze the immune response to globoside and Forssman antigens we immunized rabbits with globoside. Rabbits whose preimmune sera contained anti-Forssman produced Forssman-specific antibodies.[9)] Rabbits whose preimmune sera did not contain anti-Forssmam produced antibodies specific for globoside and antibodies that reacted well with both globoside and Forssman antigens.

Most anti-carbohydrate antibodies, such as antibodies to blood group A and B antigens, are directed against the terminal nonreducing sugar sequence. Antibodies specific for globoside or Forssman fall into that category. Antibodies that crossreacted with globoside and Forssman represent a different type of specificity: they bind to the internal tetrasaccharide structure of globoside present in Forssman GSL. Paroxysmal cold hemoglobinuria occurs in patients with syphilis and following viral infections or vaccination. In those patients production of anti-globoside/Forssman antibodies is probably a response to crossreactive bacterial or animal antigens.

Galactosylgloboside (SSEA-3 antigen).   Following the development of monoclonal antibody technology mice were immunized with a variety of tissues and cell lines to develop antibodies that recognized developmental and tumor-specific antigens. Many monoclonal antibodies raised against murine teratocarcinoma cells and a number of human carcinomas and leukemic cells reacted with the sugar sequence 3-fucosyllactosamine (3-FL), (Table 1). Surprisingly, a panel of mAbs raised against a complex GSL that contained a terminal 3-FL sequence did not bind to terminal 3-FL structures. The Abs bound to an internal structure, galactosylgloboside, also known as the developmentally regulated antigen SSEA-3.[10)]

Table 2.   Structures of gangliosides

Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer	asialoGM1
NeuAcα2-3Galβ1-4Glcβ1-Cer	GM3
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	IV³NeuAcnLc4Cer
	Sialylparagloboside
GalNAcβ1-4(NeuAcα2-3) Galβ1-4Glcβ1-Cer	GM2
Galβ1-3GalNAcβ1-4(NeuAcα2-3) Galβ1-4Glcβ1-Cer	GM1
NeuAcα2-3 Galβ1-3GalNAcβ1-4(NeuAcα2-3) Galβ1-4Glcβ1-Cer	GD1a
Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3) Galβ1-4Glcβ1-Cer	GD1b
NeuAcα2-3 Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3) Galβ1-4Glcβ1-Cer	GT1b

Gangliosides. Gangliosides, sialic acid-containing GSLs (Table 2), occur in highest concentration in the central nervous system, where they are localized mainly in microsomes and in the plasma membrane of synaptosomes. We wished to prepare anti-ganglioside antibodies for studies of their subcellular localization and functions. Gangliosides are weak immunogens, but we succeeded in producing rabbit antibodies to ganglioside GM1 and asialo GM1 bound to carrier proteins. Immunization with asialo G_{M1} conjugates yielded IgG antibodies that bound only to that GSL and IgM antibodies that cross-reacted with gangliosides GM1 and GD1b, which contain the same terminal nonreducing disaccharide as asialo G_{M1}.[11] Antisera to GM1 contained IgG and IgM antibodies that crossreacted extensively with asialo GM1 and GD1b, and some specific antibodies to GM1.

For studies of the role of autoimmunity to gangliosides in the pathogenesis of neurological disorders we obtained four IgM prepared murine monoclonal antibodies against GM1 by coupling GM1 to bovine serum albumin. In contrast to the polyclonal antibodies studied previously, the antibodies bound strongly to GM1 and very weakly to asialo GM1.[12] Three of the antibodies were encoded by the same heavy and light chain variable region genes, and the genes exhibited very little somatic mutation. The lack of somatic mutation suggested that the relative unresponsiveness of mice to ganglioside antgens is regulatory, *i.e.*, probably due to inactivation of self-reactive B cells.

To obtain additional monoclonal anti-ganglioside antibodies, we selected a murine immunoglobulin phage display library. We obtained monoclonal antibody Fab fragment clone 10 that bound to asialo GM1 but not to GM1 or GD1b.[13] To analyze the contribution of the heavy and light chain variable regions to the antigen-binding properties of clone 10,

we paired the heavy chain Fd fragment with random light chains. We obtained an Fab antibody containing a different light chain than clone 10 but with the same specicifity and affinity.[13] By mutagenesis of the third hypervariable regions of the light and heavy chains we obtained three antibodies with 3–4 times increased avidity for antigen. A molecular model of the antibody displayed a shallow surface pocket that might be the antigen-binding site.

To analyze the fine antigen-binding specificities of clone 10 and its light and heavy chain mutants we selected a phage display linear heptapeptide library. Peptides with the same motif, KL/VWQXXX, were selected by clone 10 and its two heavy chain mutants. In contrast, a clone 10 light chain mutant selected an entirely different peptide, TFGLQSL.[14] These data demonstrated differences in the fine specificity of Abs that bound only to asialo GM1.

Human anti-ganglioside antibodies. Several studies reported the occurrence of antibodies to gangliosides in the sera of patients with multiple sclerosis, and in patients with systemic lupus erythematosus (SLE) who had involvement of their nervous system, but not in other conditions that affect the nervous system. In order to evaluate the possible role of the antibodies in the pathophysiology of those diseases we measured anti-GSL antibodies in the sera of patients with immunologic and non-immunologic diseases of the nervous system.

Antibodies against ganglioside GM1 and asialo GM1 occurred in a majority of patients with multiple sclerosis, SLE, cerebrovascular accidents and following cranial trauma.[15] Most normal individuals had little or no anti-GSL antibodies. No correlation was observed between the presence of anti-GSL antibodies and neuropsychiatric symptoms in patients with SLE. These data indicated that the anti-GSL antibodies may be a response to inflammation of the central nervous system rather than a cause of

damage. We noted also that a liposome lysis assay was much more sensitive than a solid-phase ELISA immunoassay for detecting anti-GSL antibodies.

Human monoclonal anti-ganglioside antibodies. Antibodies to ganglioside GM1 and asialo GM1 were reported in the sera of patients with motor neuron disease, neuropathies and Guillain-Barre syndrome, in addition to multiple sclerosis and SLE. To investigate the specificity, structure and genetic origin of these autoantibodies we used Epstein-Barr virus (EBV) to immortalize B lymphocytes from the peripheral blood of four patients with motor neuron diseases and two normal individuals. B cells secreting antibodies against GM1 and asialo GM1 were cloned and the variable regions of their light (V_L) and heavy (V_H) chains were sequenced. The antibodies were of the IgG and IgM isotypes and they were encoded by a variety of V_H and V_L genes.[16] The antibodies exhibited extensive somatic mutation and the CDR3 segments of the H chain variable regions were unusually long. Low titer antibodies against asialo GM1 occur in most normal individuals, and the genes encoding them may undergo somatic mutation in the course of antigen-driven B cell proliferation and maturation.

Monoclonal human anti-carbohydrate antibodies. Human immunoglobulin V_H4 genes encode a number of autoantibodies against carbohydrate determinants, including the erythrocyte I and i antigens and beta-galactosyl antigens. The V_H4 gene family was thought to contain 10 germline genes and to display very little polymorphism. We analyzed V_H4 germ line genes from two unrelated individuals. We identified nine new germ line genes that exhibited only 89–96% similarity to V_H4 genes identified previously.[17] The V_H4 genes displayed distinctive nucleotide motifs that accounted for most of the differences between them. The data suggest that diversity in the V_H4 gene family arose from acquisition of blocks of nucleotides rather than by point mutations. The V_H4 gene family was larger and more polymorphic than appreciated previously.

Mechanisms of binding of carbohydrates by lectins and antibodies

Concanavalin A. The observation that an antibody binds to a specific sequence of sugars raises questions concerning the molecular details of the interaction: how many atoms of the oligosaccharide are in contact with the antibody, and what is the nature of the bonds? As a model system we decided to study lectin-sugar interactions. Drs. C. F. Brewer,

H. Sternlicht and A. P. Grollman and I used ^{13}C NMR to analyze the binding of ^{13}C alpha- and beta-methyl-D-glucopyranosides to the lectin Concanavalin A (Con A). We found that alpha-methyl-D-glucopyranoside bound to the protein in a C1 chair conformation.[18)–20)] The 3- and 4-carbons were closest to a manganese ion in Con A at a mean distance of 10 angstroms. Con A preferentially binds the alpha anomer and the two anomers were bound in different conformations. The affinity constants, K_a, of both anomers decreased by a factor of 2 per 10° increase in temperature. The off-rate, k_{-1}, also increased by a factor of 2 per 10° increase in temperature but the on-rate was unaffected by increasing temperature.

Antibodies against 3-fucosyl lactosamine (3-FL). Many mAbs raised against normal and cancerous human tissues are directed against the terminal trisaccharide of the 3-FL antigenic determinant. Idiotypes are unique amino acid sequences in the variable regions of antibodies. To study the structural and genetic diversity of anti-3-FL antibodies we produced mAbs against a mouse anti-3-FL monoclonal antibody. We obtained two anti-idiotypic antibodies that bound to 50% of a panel of 20 anti-3-FL antibodies.[21] The anti-idiotypic antibodies completely inhibited the binding of anti-3-FL antibodies to a GSL bearing the 3-FL structure. That suggested that the idiotypic determinants (idiotopes) are related to the anti-3-FL binding site. The idiotypic determinant was localized to the heavy chains of three anti-3-FL antibodies.

To obtain insight into the remarkable immunogenicity of the 3-FL determinant in mice, we examined the occurrence of anti-3-FL antibodies and crossreactive idiotopes in the sera of unimmunized and immunized mice.[22] All sera of unimmunized BALB/c mice, and sera from 5/8 other strains of mice, contained IgM antibodies against 3-FL and, to a lesser extent, against other GSLs. Approximately 25% of BALB/c antibodies to 3-FL expressed the crossreactive idiotope described previously. Titers of anti-3-FL antibodies rose after immunization of BALB/c mice and 2/3 other strains that had naturally-occurring antibodies, but not in two strains that lacked natural antibodies. These data suggested that the immune response to certain carbohydrate antigens is genetically regulated and dependent on the presence of the Lyb-5 subset of B lymphocytes.

The presence of a crossreactive idiotope on the heavy chains of many monoclonal antibodies against

3-FL suggested that their variable region structures might be very similar. The variable (V) regions of the heavy (H) chains of three antibodies against 3-FL were all encoded by the same gene segments: V_H441, D_{Q52} and J_H4.[23] The light (L) chain V regions were all encoded by V_K24B and J_K1 germ line genes. The V_H regions of antibodies against the carbohydrate antigens galactan and levan are also encoded by V_H441, but there are large differences between these antibodies in the sequences and length of their third hypervariable regions, CDR3. The use of the same V_H441 gene from the smallest murine V_H gene family to encode antibodies against three different carbohydrate antgens suggests that the protein encoded by V_H441 posesses structural features that make it particularly well adapted for binding carbohydrate epitopes.

Antibodies against carbohydrate antigens occur naturally in mammalian sera and are believed to arise by stimulation from environmental antigens, particularly by microbial organisms that colonize the gastrointestinal tract. Stimulation of mice by bacterial antigens can be prevented by raising them germ-free and feeding them a chemically-defined, ultra-filtered diet (GF-CD). We examined serum immunoglobulin levels and titers of several anti-carbohydrate antibodies in the sera of GF-CD mice.[24] Serum IgM levels were normal but IgG and IgA levels were approximately 5% of conventionally reared littermates. Antibodies against levan and dextran were lower in GF-CD than in conventional mice. The levels of anti-3-FL antibodies and the crossreactive idiotope were comparable to those in conventional animals. Natural antibodies against 3-FL, but not levan or galactan, arise in the absence of stimulation by bacterial antigens.

Many murine monoclonal Abs bind to the 3-FL antigen, and to galactosylgloboside (SSEA-3). The V_H domains of both panels of Abs are encoded by the V_H441 gene. To obtain insight into the structural basis of the specificity of these antibodies we carried out site-directed mutagenesis. The CDR3 region of the low affinity anti-3-FL antibody PMN6 was replaced by the CDR3 of the higher affinity Ab PM81. The affinity of the chimeric Ab was increased when its heavy chain was paired with the light chain of PM81.[25] To evaluate the contribution of somatic mutations to the binding of galactosylgloboside by mAb 3A9 we replaced its variable region, which contains three somatic mutations with the V_H441 germ line sequence. The chimeric Ab bound galactosylgloboside as well as the original 3A9, which

indicated that the mutations did not increase affinity for the antigen.

The galactan-binding myeloma antibody J539 is encoded by the same V_H and V_k genes as mAb 3A9. We created computer models of the F_v regions of PM81 and 3A9 and compared them with the crystal structure of J539. The surfaces of 3A9 and J539 have shallow pockets that are potential antibody binding sites. In contrast, the F_v surface of PM81 contains a large cleft rather than a pocket.[25] These data provided insight into how the same V_H gene segment can be used to encode antibodies of different specificities.

The 3-FL antigen is present on the surface of human myeloid cells, and the anti-3-FL mAb PM81 is used to purge autologous bone marrow of leukemic cells before bone marrow reconstitution. This antibody has low affinity which limits its potential use for immunodiagnosis and immunotherapy. The antibody is encoded by V_H441 and V_K24B. To ascertain if we could obtain higher affinity Abs we used a murine immunoglobulin phage display library that contained random light chains and Fd fragments enriched in V_H441 domains. We obtained antibody clones 23 and 24 whose affinity for antigen was more than 100-fold higher than PM81.[26] The V_H regions of both antibodies were encoded by V_H441 but they contained different light chains than PM81. Analysis of the binding of the two clones and PM81 to synthetic glycolipids indicated that they had different fine specificities.

Glycosphingolipid blood group antigens

Lewis. The Le^a and Le^b antigens are present in mucous secretions and plasma, as well as on erythrocytes. The erythrocyte Lewis antigens were known to be acquired from plasma and not synthesized *in situ*. Although Hakomori had isolated a GSL with Le^b activity from erythrocytes, it was generally assumed that the plasma and erythrocyte antigens were glycoproteins. We demonstrated that the Lewis antigens were present in the lipoprotein fraction of plasma and absent from lipoprotein-depleted plasma.[27] Moreover, Lewis antigens could be transferred to Lewis-negative erythrocytes by incubation with plasma lipoproteins or by semipurified erythrocyte GSLs, but not by incubation with glycoproteins. Several animal erythrocyte blood group antigens are also acquired from plasma, and we found that some of those antigens were also GSLs.

As a model system to analyze the mechanism by which GSLs were transferred from lipoproteins to

Table 3. The P blood group system

Phenotype	Frequency	Antigens on erythrocytes	Antibodies in serum
P_1	75%	P_1, P	None
P_2	25%	P	Anti-P_1
p	Very rare	None	Anti-P_1, P, P^k
P^k	Very rare	P_1, P^k	Anti-P

cells, we studied the transfer of GSLs between lipoproteins. We synthesized a series of fluorescent glucocerebrosides that contained pyrene-labeled fatty acids containing 3–11 methylene units. The glucocerebrosides were incorporated into model and native high-density lipoproteins. Transfer occurred by passage of monomers through the aqueous phase.[28] The rate of transfer was accelerated by a partially purified plasma lipid exchange protein.

Norwalk virus receptor. Norwalk virus is a common cause of acute gastroenteritis. As a model system to identify the viral receptors in human tissues, we studied the binding of recombinant Norwalk virus-like particles (VLPs) to human type O erythrocytes and to synthetic carbohydrate antigens. The blood group H type 2 antigen (Table 1) is the receptor for the VLPs on type O erythrocytes.[29] The VLPs also bound to synthetic Le^b and H type 1 antigens. The data indicate the carbohydrate antigens in the gastrointestinal tract are a factor in the pathogenesis of Norwalk virus-induced gastroenteritis.

P blood group system. Analysis of this blood group system was a major project of the laboratory for a decade. The human erythrocyte P blood group system consists of three antigens, P_1, P, P^k, and p, and four phenotypes (Table 3). The P_1 and P antigens are expressed on most erythrocytes but the P^k antigen is detectable only on erythrocytes of the rare P^k_1 or P^k_2 phenotypes that lack detectable P antigen. Erythrocytes of the rare p phenotype lack expression of all three antigens.

We identified the P_1, P and P^k antigens as glycosphingolipids (Table 1). The P^k antigen is globotriaosyl ceramide, which is the biosynthetic precursor of the P antigen, globotetraosyl ceramide (globoside), the most abundant GSL of erythrocytes.[30)–33)] P_1 was a newly identified GSL that contains a terminal nonreducing alpha-galactosyl residue attached to lactoneotetraosyl ceramide. The P^k and P antigens are not biosynthetic precursors of P_1.

The P^k phenotype results from an inability to synthesize globoside from globotriaosyl ceramide, whose concentration is increased in P^k erythrocytes. The P^k antigen of normal erythrocytes is "cryptic", *i.e.*, without protease treatment it reacts very weakly or not all with anti-P^k antibodies. The p phenotype results from an inability to synthesize globotriaosyl ceramide from lactosyl ceramide. Erythrocytes of the p erythrocytes contain no detectable globotriaosyl ceramide or globoside, and their content of lactosyl ceramide was increased 2.5–3 fold. Erythrocytes from a genetic variant with weak expression of the P antigen contained only 30–40% of normal levels of globotriaosyl ceramide and 25% of globoside.[34] In addition, the cells contained increased amounts of gangliosides, primarily sialosyllneoactotetraosyl ceramide and, to a lesser extent, G_{M3}.

In addition to information about the chemistry and genetics of the P system, several interesting general points emerged from these studies.[35] Human blood groups were regarded as classic allotypic systems in which multiple alleles at a single locus encode a series of antigens. That generalization is true for many proteins, and for the blood group *A and B genes*, but not for the *P, H* and *Lewis* genes, which encode glycosyl transferases that act in a sequential manner on common substrates. Globotriaosyl ceramide and the P_1 GSL contain a terminal nonreducing alpha-galactosyl residue linked $1{\rightarrow}4$ to a beta-galactosyl residue (Table 1). Subsequent studies from another laboratory revealed that a single transferase synthesizes both structures. The absence of detectable P_1 antigen in the P phenotype results from decreased activity of the alpha-galactosyl transferase caused by a promoter polymorphism in the gene that encodes the enzyme.

Another surprising observation was that marked abnormalities in the GSL content of P^k and p erythrocytes, especially the absence of globoside, the most abundant GSL, did not appear to affect the structure or function of the cells.

Tissue distribution and immunological expression of glycosphingolipids

Gangliosides. Purified rabbit Abs to GM1, that also bound GD1b, were used to study ganglioside localization in cerebellar cortex. Using an immunoperoxidase technique, staining was observed in the granular layer of rat cerebellum, but not in the molecular layer or in Purkinje cells. Cerebellar tissue from mice, cows and humans displayed a similar pattern of staining.[36]

We used affinity purified rabbit antibodies against GM1, that also bound to GD1b and asialo GM1, to study the expression of those GSLs in mouse thymocytes and lymphocytes. The antibodies produced cell surface staining of thymocytes and T lymphocytes but not B lymphocytes.[37] The staining was due primarily to crossreacting antibodies to GD1b and asialo GM1 rather than to GM1.

To extend this study of murine lymphocytes we prepared monospecific Abs against GM1 and asialo GM1. Anti-asialo GM1 reacted with lymph node T lymphocytes but not wih thymocytes.[38] In contrast, anti-GM1 Abs reacted with most thymocytes and lymph node cells. In summary, anti-asialo GM1 Abs react primarily with mature T cells, whereas anti-GM1 Abs react with thymocytes and mature T cells.

We analyzed the reactivity of purified rabbit Abs to GSLs with mouse embryonal carcinoma cells (ECC) and preimplantation mouse embryos. Antibodies to globotetraosylceramide bound to 2 to 4 cell embryos and reached a peak of staining with morulae.[39] In contrast, Forssman Abs bound to late morulae and most intensely to early blastocysts. Chemical analyses revealed that globotetraosylceramide is the most abundant GSL of ECC, and that there is a poor correlation between the cellular concentration of GSLs and their reactivity with Abs. That observation demonstrates again the importance of cell surface topography in modulating the immunoreactivity of cell surface GSLs.

Our immunological studies demonstrated that Abs against GSLs could be used to identify subpopulations of murine T and B lymphocytes. In parallel with our studies of the immunological expression of human lymphocyte GSLs we analyzed the GSL content of lymphocytes from different human tissues. The major neutral GSLs of tonsil lymphocytes are glucosyl ceramide, lactosyl ceramide, trihexasoyl ceramide and globoside.[40] Compared to tonsil cells, thymocytes and peripheral blood lymphocytes contain more glucosyl and lactosyl ceramide, only trace amounts of trihexaosyl ceramide and globoside, and larger amounts of more complex neutral GSLs. Peripheral blood lymphocytes contained more total gangliosides and more complex gangliosides than thymocytes and tonsil lymphocytes. The differences in GSL content might be related to differences in lymphocyte subpopulations: B cells comprise 50% of tonsil cells, 10% of PBL and 0–2% of thymocytes.

Paragloboside (neolactotetraosyl ceramide) is a biosynthetic precursor of the ABH and P1 blood group antigens and of some gangliosides. We analyzed the reactivity of affinity-purified Abs against paragloboside with human erythrocytes and lymphocytes. The weak hemagglutinating activity of the antibodies against human erythrocytes was enhanced by treatment of erythrocytes with papain or neuraminidase.[41] The antibodies also reacted with about 60% of human peripheral blood B lymphocytes and 5–10% of T cells.

Monoclonal antibodies to lymphocyte cell surface antigens play an important role in studying lymphocyte differentiation and functional subspecialization. Monoclonal antibody HJ6 bound to a subpopulation of human tonsillar germinal center cells but not to peripheral blood lymphocytes; its target antigen was not identified. We demonstrated that HJ6 bound specifically to globotetraosylceramide and Forssman GSL.[42] Chemical analysis of the GSLs of HJ6-enriched cell populations revealed the presence of globotetraosylceramide but not Forssman GSL. Like other antibodies studied previously, HJ6 binds strongly to both GSLs. Globotetraosylceramide appears to be a marker of activated B lymphocytes.

Human erythrocytes. Cellular GSL composition and cell surface expression changes during differentiation and neoplastic transformation. Using high-performance liquid chromatography and a fluorescence-activated cell sorter we analyzed human fetal, umbilical cord and adult erythrocytes. Adult cells reacted most strongly with anti-globoside, less strongly with Abs against lactotriaosyl ceramide and paragloboside and very weakly with anti-lactosyl ceramide.[43] The immunological reactivity of umbilical cord erythrocytes was more variable. The intensity of staining with anti-globoside varied considerably among samples. Antibodies against lactosyl ceramide and trihexaosyl ceramide reacted moe strongly than with adult cells. In umbilical cord red cells there was a surprising lack of correlation between the immunological reactivity of GSLs and their concentration in the cells. These findings demonstrate alterations in the cell surface phenotype that accompany differentiation from the fetal to the adult state.

Although the most abundant gangliosides of erythrocytes have been characterized, less abundant gangliosides that possess interesting immunological and biological properties have not been identified. We identified four monosialogangliosides not previously known to be in erythroctes, including G_{M1} and G_{M2}, and six disialogangliosides, including GD3, GD1a and GD1b.[44] Two monosialogangliosides were

newly identified receptors for the human anti-p and anti-Gd cold agglutinins. One disialoganglioside contained a novel sugar sequence in which two sialic acid residues were linked to different hydroxyl groups of the same galactose residue.

K562 cells are an undifferentiated erythroid cell line that was obtained from a patient with chronic myeloid leukemia. The GSL composition of K562 cells differs considerably from that of adult and umbilical cord erythrocytes. The most abundant neutral GSLs are glucosyl and galactosyl ceramides, and globoside, the most abundant GSL of mature erythrocytes, was not detected.[45] Gangliosides containing N-acetylgalactosamine were more abundant than those containing N-acetylglucosamine. The molar ratio of neutral GSLs/gangliosides was $1:1$ in K562 cells, compared to $15:1$ in mature erythrocytes. These data suggest that GSLs may be useful cell surface markers of erythroleukemias.

Subcellular localization and biosynthesis of glycophingolipids

When we started this series of experiments in 1967 it was unclear whether GSLs were present in all cells and how they were distributed among cellular organelles. It was not feasible at that time to use chemical techniques for that purpose because of the low concentration of GSLs and the difficulty of obtaining purified preparations of organelles. The goals of the studies described below were to use Abs to determine the sub-cellular localization of GSLs, their immunological expression, and their use as markers for functionally-specialized subpopulations of cells.

We used rabbit antibodies to lactosyl ceramide and globoside to study their distribution in human tissues by immunofluorescence. In the human kidney globoside was detected in the plasma membrane and cytoplasm of epithelial cells of the proximal convoluted tubule.[46] Globoside was not detected in liver, skin, myocardium or in articular cartilage. In the spleen both GSLs were observed only in the plasma membrane of reticuloendothelial cells of the red pulp. This was the first demonstration of the intracellular occurrence of GSLs.

Endothelial cell (EC) surface receptors play an important role in the immune response. Their receptors modulate adhesion and extravasation of leucocytes and the receptors are modulated by cytokines. Although cell surface proteins of ECs had been characterized little was known about their GSLs. The major neutral GSLs of cultured human umbilical vein endothelial cells (HUVECs) are lactosylceramide, globotriaosylcermide and globotetraosyl ceramide.[47] Labeling with galactose oxidase/NaB3H4 revealed that neutral GSLs that contained three or more sugars were accessible on the cell surface. The major gangliosides were G_{M3} and sialosylparagloboside.

Endothelial cells alter their expression of cell surface molecules, such as major histocompatibility antigens, in response to activation by interleukin-1 (IL-1) and interferon-gamma (IFN-gamma). We investigated changes in the GSL composition and cell surface expression of GSL of HUVECs in response to cytokine activation. IL-1 increased total cells GSL content but did not alter GSL composition or cell surface binding of anti-carbohydrate Abs.[48] IFN-gamma treatment resulted in a small change in GSL composition but greatly increased cell surface expression of gangliosides, especially GM3, and decreased expression of neutral GSLs. The total cell content of globoside was decreased by 30% but its cell surface expression was decreased 3–4 fold. The decreased cell surface expression was a result of an increase in intracellular globoside and decreased surface accessibility, which could be increased by treatment of cells with neuraminidase or trypsin. Intracellular globoside was associated with filamentous structures. The data suggested that modulation of cell surface GSLs by IFN-gamma might play a role in the adhesive and receptor activities of activated endothelial cells.

We noted previously that globoside and G_{M3} ganglioside were present intracellularly in HUVECs. We demonstrated by light and electron microscopy that both GSLs were associated intracellularly with vimentin intermediate filaments, but not with actin or tubulin.[49] Both GSLs remained associated with vimentin after perinuclear collapse of the filaments caused by colcemide. IFN-gamma treatment decreased the ratio of cell surface to filamentous globoside but had the opposite effect on the distribution of GM3. These obsevations suggested that intermediate filaments may play a role in the intracellular transport of GSLs.

To determine the generality of our observations that globoside colocalizes intracellularly with intermediate filaments, and that its localization is modulated by IFN-gamma, we analyzed a variety of cell types. Globoside colocalized intracellularly with intermediate filaments in a number of cell types: with vimentin in human and mouse fibroblasts, with desmin in smooth muscle cells, with keratin in

keratinocytes, and with glial fibrillary acidic protein in glial cells.[50] Globoside colocalization was detected only with vimentin in HeLa and MDCK cells which contain separate vimentin and keratin networks. G_{M3} also colocalized with vimentin in human fibroblasts. The prevalence of the association of globoside with intermediate filament networks suggested that they may participate in transport or sorting of GSLs.

Although early studies of the subcellular localization of GSLs demonstrated their abundance on the cell surface subsequent work revealed their presence also in intracellular organelles. To extend our observation on the co-localization of some GSLs with IFs we examined the distribution of these compounds in a variety of cells. We found that the subcellular loclization of GSLs varies depending on the GSL structure and cell type. Galactosyl ceramide localized primarily to the plasma membrane of HaCaT H-3 keratinocytes but was predominantly intracellular in other epithelial cells.[51] Moreover, within a single cell type GSLs exhibited different patterns of cellular localization. In HepG2 cells lactosylceramide was associated with small vesicles whereas globotetraosylceramide co-localized with intermediate filaments. The varying localization patterns of GSLs indicated the existence of mechanisms that regulate their sorting, and raises interesting questions about their functions in different organelles.

Our previous observations on the co-localization of GSLs with intermediate filaments led us to analyze the role of the filaments in the biosynthesis and intracellular transport of GSLs. Cells with (vim+) and without (vim−) filaments were cloned from the human adrenal carcinoma cell line SW13. The vim+ cells synthesized several GSLs more rapidly than vim− cells, but the differences did not result from differences in the levels of glycosyltransferases between the cells.[52] To determine whether the presence of a vimentin network was responsible for the differences in biosynthesis, mouse vimentin cDNA was transfected into the SW13 vim− cells. Transfected cells that expressed a vimentin network demonstrated a twofold or greater increase in the biosynthesis of GSLs.

We noted previously that decreased incorporation of radiolabeled sugars into GSLs in cells lacking a vimentin network. Sugars can be incorporated into GSLs in three metabolic pathways (Fig. 1): pathway 1 - by addition to ceramide synthesized *de novo* by acylation of sphinganine; pathway 2 - addition to ceramide synthesized from recycled sphingosine; and pathway 3 - by addition to GSLs that recycle from
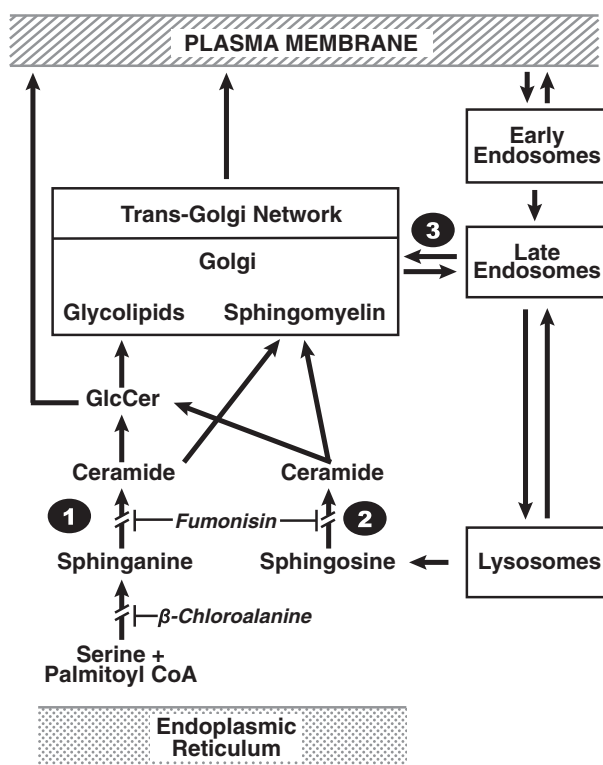

Fig. 1. Pathways for the biosynthesis and recycling of sphingolipids. *De novo* synthesis of dihydroceramide and ceramide take place in pathway 1, which is inhibited by β-chloroalanine. In pathway 2, sphingosine and sphinganine produced by hydrolysis of sphingolipids are reutilized for synthesis of sphingolipids. Fumonisin B1 inhibits pathways 1 and 2. In pathway 3, native or partially hydrolyzed glycosphingolipids recycle through the Golgi apparatus.

endosomes to the Golgi apparatus. The contribution of the pathways was analyzed by use of beta-chloroalanine, which inhibits the *de novo* synthesis of sphinganine (pathway 1), and fumonisin B1, which inhibits the acylation of sphinganine and sphingosine (pathways 2 and 3). In both vim+ and vim− cells only 20–40% of sugar incorporation resulted from *de novo* synthesis (pathway 1), and 60–80% took place in the recycling pathways 2 and 3.[53] The data indicate that in SW13 cells incorporation of sugars into GSLs occurred predominantly in recycling pathways rather than by *de novo* biosynthesis.

SW13 cells are transformed and it is possible that vim− cells have other abnormalities in addition to the lack of a vimentin IF network. We obtained primary embryonic fibroblast cells from mice in which expression of the vimentin IF gene was abolished by homologous recombination. The vim−

embryonic fibroblasts incorporated less sugar into GSLs than control vim+ embryonic fibroblasts.[54] Using two inhibitors of ceramide synthesis, beta-chloroalanine and fumonisin B1, the defect in synthesis was found to be in recycling pathway 2, and not in *de novo* synthesis. In addition, chloroquine, which inhibits the hydrolysis of sphingolipids in endosomes and lysosomes, markedly decreased the incorporation of sugars into GSLs larger than glucosylceramide. The defect in GSL in vim− cells probably results from impaired intracellular transport of GSLs and sphingoid bases between the endosomal/lysosomal pathway and the Golgi apparatus and endoplasmic reticulum. The role of intermediate filaments in this process may be in binding proteins that participate in transport processes and/or by contributing to the organization of subcellular organelles.

To extend our observations on pathways of incorporation of sugars into GSLs in SW13 cells we studied four additional cell lines. Approximately 90% of sugar incorporation took place in recyling pathway 2 in human foreskin fibroblasts and NB41A3 neuroblastoma cells, and 60% in C6 glioma cells.[55] In contrast, 50–90% of sugar incorporation occurred in *de novo* pathway 1 in C2C12 myoblasts. The C2C12 cells divide more rapidly and synthesize 10–14 times as much GSLs as the other cells lines. We suggested that in slowly-dividing cells GSLs are synthesized predominantly from sphingoid bases salvaged from the hydrolytic pathway, and that in rapidly-dividing cells the need for increased synthesis is met by upregulating the *de novo* pathway.

Reflections

Scientific research is an activity that requires full commitment of one's time and energy. In addition to the daily activities of directing a laboratory, one must develop long term plans for development of projects, try to remain abreast of the scientific literature, and to submit grant proposals. It is an all-consuming way of life that leaves little time for reflection or maintaining a perspective on one's career.

Writing this memoir over a decade over closing my laboratory stimulated me to think about how my research developed and branched out unexpectedly from immunology into biochemistry and cell biology. I wondered whether my range of interests represented a lack of focus and discipline, and if I would have been more productive if I concentrated my research in one field? There is no answer to that question, but I realized that what I did was determined by my character and temperament. Dr. Elmer Becker, my first research mentor, was fond of quoting the Greek philosopher Heraclitus who said, "A man's character is his fate."

What attracted me to research was my interest in many areas of science and medicine, and the stimulation of trying to solve problems. My studies of carbohydrate antigens led me to consider how I could use immunological techniques to study the biological properties of glycolipids and glycoproteins. For better or for worse, I pursued my interests across disciplinary boundaries.

I realized also how my life has been enriched by friendships with scientists from many countries, and by the opportunity to learn about other cultures. Although the field of glycolipid chemistry was small in the United States in the 1960s and 1970s, it was a major topic in Japan. As a consequence, I had the opportunity to visit Japan frequently, and the privilege of developing friendships and collaborations with many Japanese biochemists, including Profs. Tamio Yamakawa, Akira Kobata, Toshiaki Osawa, Tamotsu Taketomi, and Keizo Inoue. I was also fortunate to have talented young Japanese postdoctoral fellows spend time in my laboratory, including the late Masaharu Naiki, Akemi Suzuki, Tamao Endo and Masato Umeda. Although many details of my research have faded, the memories of my explorations of Japanese culture and friendships remain strong, and are a source of enduring comfort and pleasure.

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Profile

Donald M. Marcus was born in New York in 1930. He is a graduate of Princeton University, and Columbia University College of Physicians and Surgeons, where he also received training in Internal Medicine. He began his training in immunology at the Walter Reed Army Medical Center in the laboratory of Dr. Elmer Becker, and subsequently was a postdoctoral fellow in the laboratory of Dr. Elvin Kabat in the Department of Microbiology and Immunology at Columbia. In 1963 he joined the Department of Medicine of Albert Einstein College of Medicine, and in 1980 joined the faculty of Baylor College of Medicine, where he is now Professor of Medicine and Immunology Emeritus. The research program of his laboratory included three major topics: the structures and immunology of human carbohydrate blood group and glycosphingolipid antigens; the tissue distribution, subcellular localization and biosynthesis of glycosphingolipids; and the structural basis of the binding of carbohydrates by antibodies and lectins. He has served on grant review groups of National Institutes of Health and of private foundations, and on the editorial boards of scientific and medical journals. For his studies of the immunochemistry of blood groups, he received the Karl Landsteiner Memorial Award of the American Association of Blood Banks and the Philip Levine Award of the American Society of Clinical Pathologists. He was elected to membership in the American Society for Clinical Investigation and the Association of American Physicians. In 1990 he received an award of appreciation from the Tokyo Society of Medical Sciences and the Faculty of Medicine of the University of Tokyo.